Sex differences in the intestinal transcriptome and spatial patterns of lipid uptake capacity and intestinal lipid-metabolic gene expression in *Fabp2* gene-ablated mice conditioned on low or high fat diets

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Contribution of Authors

Manuscript 1: Michael G. Sugiyama was involved in the conception, writing and editing of the manuscript. Luis B. Agellon was involved in the conception, editing, and submission of the manuscript for publication.

Manuscript 2: Michael G. Sugiyama developed the analytical approach, validated the analytical approach, analyzed the data and wrote the manuscript. Luc Hobson developed and validated the analytical approach. Al. B. Agellon was involved in designing the experiment and processing of the microarray chips. Luis B. Agellon was involved in all of the above aspects of the manuscript including submission of the manuscript for publication.

Manuscript 3: Michael G. Sugiyama analyzed the lipid uptake data, conducted the high-fat diet feeding studies for measurement of intestinal gene expression/lipid flux/IPGTT, analyzed all data, and wrote the manuscript. Dr. Alan. B.R. Thomson, Laurie Drozdowski and colleagues carried out the lipid uptake study, and wrote the corresponding section in the methods section. Dr. Luis Agellon was involved in all of the above aspects of the manuscript.

Abstract

The mammalian small intestine expresses three members of the fatty acid binding protein family; Fabp1, Fabp2, and Fabp6 that differ in their spatial expression pattern and ligand binding preference. In vitro studies using recombinant FABPs suggest an important role in lipid uptake and intestinal lipid metabolism, although the in vivo functions remain unclear. Mice genetically altered to lack Fabp2 exhibit a sex-dimorphic response to high fat diet feeding featuring weight gain and fatty liver in males but not females, suggesting that Fabp2 is involved in a sex-dimorphic intestinal metabolic program. We developed a technique for the analysis of sex differences in transcriptome data and applied it to a study of the intestinal transcriptome of chow-fed *Fabp2*$^{-/-}$ mice. This analysis revealed lipid metabolic pathways that are differentially regulated in male and female *Fabp2*$^{-/-}$ mice. We next conducted a high fat diet feeding study for the purpose of analyzing the effects of Fabp2 loss on lipid uptake capacity and spatial expression patterns of lipid metabolic genes identified from the transcriptome experiment. Females lacking Fabp2 exhibited an increased capacity for lipid uptake and an increased abundance of Fabp1 mRNA in the proximal intestine. Loss of Fabp2 also caused an increased excretion of total lipids in females compared to males. This suggests that females might be protected from the adverse effects of Fabp2 loss by directing excess lipids towards excretion pathways through an intracellular, Fabp1-mediated mechanism.

Résumé

Trois membres de la famille des protéines liantes d'acides gras sont exprimés dans le petit intestin des mammifères, soit le Fabp1, Fabp2 et Fabp6. Ils diffèrent par leur structuration expressive spatiale et leur affinité de ligand. Des études in vitro par recombinaison génétique suggèrent que les FABPs jouent un rôle important dans l'assimilation et le métabolisme intestinal de lipide mais leurs fonctions in vivo restent incertaines par contre. Des souris déficientes de Fabp2 par manipulations génétiques répondent différemment selon leur sexe lorsque soumissent à une diète riche en lipide de manière que les mâles subissent un gain de poids et acquièrent une hyperlipidémie du foie qui sont absents chez les femelles suggérant que le fabp2 est impliqué dans un programme métabolique intestinal dimorphe basé sur le sexe. Nous avons élaboré une technique d'analyse de données de transcriptome pour déterminer la différence sexuelle du transcriptome intestinal de la souris Fabp2$^{-/-}$ nourri de moulé. Cette analyse révéla que la régularisation de la voie métabolique de lipides des souris mâles diffère des femelles. Nous avons poursuivis avec une étude utilisant une diète à forte teneur en lipide dans le but d'analyser l'effet sur la capacité d'assimilation de lipide et la structuration expressive spatiale des gènes du métabolisme des lipides, identifiés par l'analyse de transcriptome précédente, par la perte de Fabp2. Les femelles déficientes de Fabp2 démontrent une augmentation de la capacité d'assimiler les lipides ainsi qu'une abondance d'ARNm de Fabp1 dans l'intestin proximal. La perte de Fabp2 cause aussi une augmentation de l'excrétion total de lipide chez les femelles lorsque comparées aux mâles. Ces observations suggèrent que les femelles semblent être protégées des effets négatifs de la perte de Fabp2 en utilisant une voie intracellulaire d'excrétion des lipides excédents. Ce mécanisme est médié par Fabp1.

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Introduction

In the current epoch of health research, nutrition has emerged as an important modifier of disease risk and an attractive candidate for disease prevention and treatment strategies. It is now recognized that the whole body metabolic program is regulated by nutrients and xenobiotics that control the expression of genes involved in their assimilation, transport, and metabolism. Understanding the interaction between nutrients and genes at the molecular level is at the confluence of research in many disciplines of the life sciences including physiology, molecular biology, genetics, and nutrition. As work in these fields progress towards the ultimate goal of delineating metabolic disease processes it is important to consider the non-modifiable factors that influence metabolism and disease. Genetic sex is a prime non-modifiable factor that has an underlying influence on all aspects of metabolism and metabolic disease risk, although until recently its effects have been largely ignored, leading to an incomplete understanding of metabolism.

Studying the impact of sex on metabolism superimposes a layer of complexity and multiplies the financial burden of experiments. For these reasons, molecular biology and nutrition research have tended to be biased towards a particular sex, with results often being extrapolated to the other sex. Recent advancements in chip-based array technologies have made it possible to examine the effects of sex differences in nutrient metabolism on the expression of genes involved in entire metabolic pathways. This provides a comprehensive, molecular insight into the interaction between nutrients and genes in the regulation of metabolic and disease processes.

Fatty acid binding proteins (FABPs) are a class of proteins that have a putative role facilitating nutrient-gene interactions by delivering their lipid soluble ligands to the nucleus for the control of gene expression [1]. The mammalian small intestine expresses three members of the FABP family, Fabp1, Fabp2, and Fabp6 that differ in their ligand preference and spatial expression pattern along the length of the small intestine [2]. We previously reported that mice lacking the intestine-specific fatty acid binding protein 2 (Fabp2) exhibited a sex-dimorphic

response to high fat diet feeding featuring weight gain and fatty liver in males but not females [3-4]. Since FABPs are responsible for the transport of fat, it is possible that fatty acid trafficking through the enterocyte is regulated in a sex-specific manner, although this has yet to be assessed from the level of fatty acid entry into the enterocyte. In the following studies we provide evidence for sex differences in the intestinal lipid metabolic program and lipid uptake capacity in mice lacking Fabp2. We demonstrate that loss of Fabp2 leads to a sex-dimorphic defect in lipid metabolism featuring impaired glucose tolerance in males but not females, clinical features that have been observed in carriers of FABP2 mutations in human populations [5-6]. Finally, we discuss how our observed sex differences in the intestinal metabolic program and lipid uptake capacity could potentially lead to the sex-dimorphic Fabp2$^{-/-}$ phenotype.

Manuscript 1: Literature Review

Sex differences in lipid metabolism and metabolic disease risk

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Abstract

The ability of nutrients to regulate specific metabolic pathways is often overshadowed by their role in basic sustenance. Consequently, the mechanisms whereby these nutrients protect against or promote a variety of acquired metabolic syndromes remains poorly understood. Premenopausal women are generally protected from the adverse effects of obesity despite having a greater proportion of body fat than men. Menopause is often associated with a transformation in body fat morphology and a gradual increase in the susceptibility to metabolic complications, eventually reaching the point where women and men are at equal risk. These phenomena are not explained solely by changes in food preference or nutrient intake suggesting an important role for the sex hormones in regulating the metabolic fate of nutrients and protecting against metabolic disease pathophysiology. Here we discuss how differences in the acquisition, trafficking, and subceullular metabolism of fats and other lipid soluble nutrients in major organ systems can create overt, sex-specific phenotypes, modulate metabolic disease risk and contribute to the rise in obesity in the modern, sedentary climate. Identifying the molecular mechanisms underpinning sex differences in fat metabolism requires the unravelling of the interactions among sex chromosome effects, the hormonal milieu and diet composition. Understanding the mechanisms that give rise to sex differences in metabolism will help to rationalize treatment strategies for the management of sex-specific metabolic disease risk factors.

Introduction

With obesity and its associated metabolic complications reaching epidemic proportions, there has never been a more critical time for collaboration among researchers working in different sectors for the design of prevention and treatment strategies for diseases of excess. It has been well established that consuming a high fat, western-type diet is associated with insulin resistance and other metabolic complications [7] whereas consuming a Mediterranean-type diet comprised primarily of mono and polyunsaturated fats is associated with improved health, longevity, and cardio-protection [8]. Calorie-dense foods, such as those high in fat and/or sugar, are typical of the "western-type" diet and contribute to the development of acquired metabolic syndromes in the modern sedentary climate. There has been a strong impetus in the health community in recent years for the replacement of poor quality foods with those having health promoting properties in addition to their role as an energy source. This comes from the observation that nutrients, such as cholesterol, fatty acids, sugars and vitamins are highly bioactive molecules involved in the regulation of key physiological and cellular processes. Certain fats and lipid soluble nutrients, such as arachadonic acid and vitamin A, can only be obtained through dietary means and are essential in most mammals. Many high fat foods are now being engineered as vectors for the delivery of lipid soluble nutrients and xenobiotics, such as the fortification of margarine with phytosterols and vitamin D [9]. However, even though functional foods and nutraceuticals hold the promise of contributing to the fight against obesity, the research community must remain cautious in recommending these natural health products to the general population.

The spectrum of health research has shifted its focus to defining the role of nutrients in optimizing health and preventing disease and this approach requires a sophisticated assessment of the factors that influence whole body nutrient metabolism. Importantly, there are several non-modifiable factors such as sex, genetic makeup and age that have overriding effects on nutrient metabolism and metabolic disease risk (Table 1). Sex is the most easily identifiable of these non-modifiable factors, yet a large amount of our knowledge of human metabolism

has been garnered from experiments done on only one sex, with results being extrapolated to the other sex. A consequence of this sex bias is an incomplete understanding of nutrient metabolism. Fortunately, the research community has begun to address this issue in recent years by including both sexes in experimental designs, or making sex specific claims with regard to experimental results.

Sex differences in lipid metabolism, and the corresponding differences in disease risk, cannot be ascribed solely to the effects of estrogens or any of the other sex-related hormones. There are numerous other factors that influence the complex interactions among hormones, nutrients, and genes in metabolism (Fig. 1). Uncovering the mechanisms that lead to sex differences in lipid metabolism will expand the scope of metabolic research and enhance our understanding of the evolution of acquired metabolic disorders. An awareness of sex differences in lipid metabolism will also lead to the development of novel therapies which target specific metabolic pathways for the management of metabolic disease risk factors.

Sex and gender

It is important to make a distinction in terminology between sex and gender. Sex differences are those that are the result of the chromosomal sex of the organism, such as XX (female) and XY (male) in humans (Fig. 2). Such differences are intrinsically female or male, and persist despite the influence of the hormonal background and other environmental factors. On the other hand, gender differences arise from a combination of sex chromosome, sex hormone and epigenetic interactions with the environment, and are typically representative of a given life stage. Isolating the effects of sex differences from gender differences is inherently difficult because of the large number of confounding variables, and it has been argued that such differences may not be clinically relevant as gender differences [10]. It should be noted that estrogens and androgens are not exclusive to females and males respectively, although these effects receive far less attention in metabolic research. For these reasons, gender is not an ideal term for discussing metabolic differences between males and females. For the purpose of this review, a sex dimorphism refers to any difference between

male and female organisms, while not necessarily taking into account the modifiable factors that influence both gender and metabolism.

Sex differences in the incidence of metabolic disease

In addition to morphological changes in fat distribution, menopause also brings about a transition in metabolic disease risk (Table 1). Premenopausal women are generally protected from developing cardiovascular disease and stroke, and there is a nearly 10 year difference in the average age of onset in men and women [11-12]. After menopause, the risk of developing metabolic diseases rises dramatically, eventually putting women at the same level of risk for cardiovascular disease-related morbidity and mortality as men [12]. Whether the reduction in estrogen signalling following menopause is causative, or if the increased incidence of metabolic diseases in postmenopausal women is simply a natural part of the aging process remains to be determined. The use of hormone replacement therapy to counteract the symptoms of menopause has so far yielded conflicting results with regard to metabolic disease risk. Certain studies have found that supplemental hormones can prevent the accumulation of fat that accompanies menopause [13]. In contrast, data from the recent Women's Health Initiative study suggests that hormone replacement increases the risk of developing heart disease [14]. It is possible that the female metabolic program is finely tuned to maximize the protective abilities of the current hormonal environment. In this sense, an assault of supraphysiological levels of hormones might disrupt the fine balance in whole body metabolism, leading to a dysregulation in the handling of lipids and cholesterol. It should be noted however that premenopausal women are not protected from all diseases related to lipid metabolism. The incidence of cholesterol gallstones and cholestasis is greater in premenopausal women than men, and the pathophysiology of these conditions remains poorly understood [15].

Sex chromosome complement and metabolic disease

A significant amount of information on the effects of chromosomal sex on lipid metabolism in humans has been accumulated from studies on individuals with Turner syndrome (TS) (Fig. 2). A large scale retrospective analysis of

morbidity found that women with TS are at greater risk for the development of acquired metabolic syndromes, namely diabetes, atherosclerosis, ischemic heart disease, and hypertension [16]. These data are supported in large part by morphological and biochemical findings. Women with TS display decreased lean body mass, increased fat mass and increased leptin compared with age matched XX women [17]. Analysis of blood lipid profiles in women with TS and karyotypically normal women with premature ovarian failure, reveals that TS individuals have significantly elevated triglycerides (TG) and low-density lipoprotein cholesterol compared to premature ovarian failure individuals, indicating that the sex chromosome complement affects blood lipids irrespective of gonadal hormone secretions [18]. Taken together these studies demonstrate an increase in the number of risk factors for cardiovascular diseases in women with only one X chromosome.

It is important to make the distinction between aneuploidy of the sex chromosomes and X chromosome inactivation. In women with a normal XX chromosome complement, epigenetic silencing of one of the X chromosomes in embryogenesis prevents cells from having 2 functional alleles of X-linked genes [19]. In TS, each cell has only one allele, thus the normal inactivation process does not take place. In a process known as dosage compensation [20], X-inactivation and upregulation of certain genes on the active chromosome leads to a balance in gene expression between men and women (Fig. 2). It is likely that some genes are able to escape epigenetic transcriptional silencing mechanisms, enabling women to have 2 functional alleles for certain X-linked genes [20]. A qualitative assessment of genes that escape X-inactivation has revealed extraordinary heterogeneity in gene expression patterns [21], and it remains to be determined whether genes that escape X-inactivation protect premature ovarian failure women from the morbidities faced by TS individuals.

Central effects of the sex and satiety hormones

A common misconception has been that regional differences in liver, intestine, adipose, and muscle lipid metabolism are solely responsible for male/female differences in fat distribution, substrate utilization, energy

expenditure and metabolic disease risk. It is becoming apparent that the central nervous system responds to changes in the nutritional and hormonal status, and plays a critical role in modifying not only whole body lipid metabolism, but also feeding behaviour. Food is a powerful stimulus that elicits both physiological and emotional responses and alters feeding behaviour, thereby determining the nutrient milieu in the body (Fig. 3). Major organs involved in the metabolic processing of nutrients respond to changes in nutrient status by releasing hormones that regulate feeding behaviour and nutrient metabolism [22-24]. Brain lesion and stimulation studies have identified several key nuclei of the hypothalamus as master regulators of food intake and energy homeostasis [23]. Leptin stimulation of the satiety centres of the hypothalamus enhances production of the anorexigenic peptide, proopiomelanocortin, and suppresses the production of the orexigenic peptide neuropeptide Y (Table 2) [25]. Estrogen receptor α (ERα, encoded by the *NR3A1* gene) in the brain is crucial for the maintenance of energy homeostasis in rodents. Mice of both sexes deficient in ERα, fed a normal chow diet, display marked increases in white adipose depot size and weight, attributed to decreased whole body energy expenditure [26]. RNA interference-mediated repression of ERα in the ventromedial hypothalamic nucleus of female mice and rats leads to the development of classical indices of metabolic syndrome, including obesity and elevated blood glucose, as well as hyperphagia and reduced energy expenditure [27]. These findings persist after the administration of exogenous estrogen, suggesting that regulation of energy homeostasis by estrogen is confined to the ventromedial hypothalamic nucleus [27]. This phenomenon has only been described in the context of female rodents, and it remains to be determined whether ERα signalling in the ventromedial hypothalamic nucleus is essential for energy homeostasis in males. Interestingly, the XbaI A→G polymorphism in the gene encoding ERα is linked with a higher body mass index (BMI) in premenopausal women, but not postmenopausal women or men in two populations [28-29].

The colocalization of estrogen and leptin receptors in the satiety centres of the hypothalamus suggests the possibility for a functional overlap in the control of

food intake and energy homeostasis [25]. Leptin therapy successfully reverses hypothalamic amenorrhea in women [30] and hypogonadism in morbidly obese subjects [31] implying that leptin signalling is vital in the neuroendocrine arm of the reproductive axis. Although leptin levels are generally considered to be proportional to adipose tissue mass, women display higher circulating leptin concentrations compared with men at comparable levels of adiposity [32]. This is likely due to the fact that women have a greater percent of subcutaneous adipose tissue, which contains a greater abundance of leptin mRNA than visceral adipose tissue [33]. Leptin synthesis in the adipose tissue is directly regulated by estrogens, as plasma leptin concentrations are higher in premenopausal women compared with postmenopausal women or men [34]. Also, leptin levels peak midway through the luteal phase of the menstrual cycle, corresponding with the peak in estrogen and progesterone synthesis by the corpus luteum [34]. In the hypothalamus, estrogen deficiency brought on by ovarectomy, leads to conditions that favour hyperphagia. Ovarectomized rats gain significantly more weight than sham-operated controls, due in large part to increased neuropeptide Y in the paraventricular nucleus [35]. In the ovarectomized animals, leptin concentrations increase in a linear fashion while leptin receptor mRNA remain unchanged in both ovarectomized and intact groups, suggesting that leptin insensitivity in the hypothalamus contributes to the weight gain [35]. There are disparities between human studies in which a decline in leptin is attributable to menopause [34], and animal studies where leptin levels are increased following ovarectomy [35]. One cause of the observed disparity might lie in the timing of measurements. A recently proposed model of the central and peripheral effects of leptin and estrogens provides a potential resolution to this disparity [36]. In this model, a high concentration of estrogens in premenopausal women leads to greater subcutaneous adipose tissue volume, which in turn, increases leptin production. Leptin and estrogens act on their respective receptors in the hypothalamus to increase sympathetic nervous system activity and prevent the expansion of the visceral adipocyte pool. In postmenopausal women and men, the lack of estrogen signalling leads to decreased sympathetic nervous system activity, greater visceral

adipocyte hypertrophy and hyperplasia compared with subcutaneous adipocytes, decreased insulin sensitivity, and less leptin production, ultimately resulting in a vicious cycle that predisposes these individuals to metabolic disorders [36]. Thus, according to the model, the progressive loss of estrogen signalling that occurs during menopause would lead to changes in fat morphology and leptin concentrations, as observed in human studies [32]. Since the reduction in hormonal activity in menopause is gradual, not a sudden event as in the case of ovarectomy, experimental models based on removal of gonads do not accurately recapitulate the gradual changes in the hormonal and nutritional milieu.

The influence of androgens on leptin signalling in the hypothalamus remains a largely unexplored area. Rats lacking the leptin receptor display reduced testosterone and proopiomelanocortin levels when compared with wild-type controls [37]. However another study that compared the abundance of resistin, leptin and adiponectin mRNAs in adipose tissue of castrated and sham-operated male rats found no changes whereas ovarectomized female rats had elevated resistin and decreased leptin and adiponectin mRNA abundance [38]. It is possible that the importance of androgen signalling in the hypothalamus is secondary to its ability to act as a substrate for estrogen synthesis. Aromatase (a key enzyme involved in the biosynthesis of estrogens; encoded by the *Cyp19* gene) activity in the appetite centres of the hypothalamus of male rats is greater than in females, with castration leading to a feminization of aromatase activity levels [39]. One of the current limitations of research on androgen effects is that past studies have overwhelmingly focused on exercise, muscle accretion, and male sexual maturation. Likewise, the effects of estrogens are largely studied in relation to fertility and female sexual maturation. As a result, very little is known about the effects of estrogens in men and androgens in women in terms of their roles in metabolism.

Sex differences in gastrointestinal tract function and lipid absorption

The transit of dietary fat from the intestine to adipose tissue might be regulated in a sex dimorphic manner [40]. Fat absorption in the small intestine is a highly efficient process that is mediated by apical protein channels and

transporters such as FATP4 and CD36, which in turn are regulated by fats (Fig. 4) [41]. The fatty acid binding proteins (FABP1, 2, and 6 encoded by the *Fabp1*, *Fabp2* and *Fabp6* genes respectively) of the intestine are small abundant cytosolic proteins that are believed to function as lipid chaperones within the enterocyte by facilitating the movement of lipids to various intracellular organelles for metabolism [1]. FABP1 and FABP6 are present in higher and lower levels in the proximal and distal portions of the intestine, respectively. FABP2 is found exclusively throughout the small intestine, but with greater abundance in the distal half of the organ [2]. Ablation of the *Fabp2* gene in mice does not cause lipid malabsorption [4]. However, feeding male *Fabp2*$^{-/-}$ mice with high-fat diets reveals a distinct sex dimorphic phenotype. Both male and female FABP2-deficient mice exhibit modest weight gain in response to the diet but male mice are highly susceptible to development of fatty liver, while females remain protected [3]. Development of fatty liver in male *Fabp2*$^{-/-}$ mice may be due to increased release of fatty acids into the portal circulation causing hepatic lipid overload. On the other hand, the observed protection from fatty liver in female *Fabp2*$^{-/-}$ mice may indicate the existence of compensatory mechanisms in the female intestine that prevent the liver from becoming overloaded with fat.

Although the FABPs of the intestine play a specialized role in the uptake and metabolism of diet derived lipids and lipid soluble nutrients from the diet, the ubiquitous nature of FABPs in other tissues suggests that they possess a broader range of functions in the context of intracellular lipid trafficking and whole body lipid homeostasis. FABPs might function as subcellular gatekeepers controlling the metabolic fate of free fatty acids (FFA) by shuttling them to organelles (Fig. 4). FABP1 is also found in the liver and kidneys, and preferentially binds long-chain polyunsaturated fatty acids [42]. Mice lacking FABP1 display mildly impaired fat absorption on a high-fat diet, significantly decreased hepatic β-oxidation, and weight gain that is exacerbated in females [43]. These changes were likely secondary to sex-specific differences in the mRNA abundance of genes involved in mitochondrial and peroxisomal fat oxidation [43]. The rate of fatty acid incorporation into TG in cultured hepatocytes is almost twice as high in

female rats compared with male rats [44]. Thus, differences in FABP1 and FABP2 cellular function might explain the observed sex differences. If FABP1 is a greater contributor to TG synthesis in females, and loss of FABP1 leads to a deficiency in β-oxidation, then females might shuttle more lipids towards storage pathways. On the other hand, if FABP2 is a more substantial contributor to TG synthesis in male mice, then the loss of FABP2 would lead to increased fatty acid delivery to the liver and eventually cause hepatic lipid overload. A recent study examining mice lacking FABP1 or FABP2 found no changes in the mRNA abundance of genes involved in lipid synthesis and oxidation compared with wild-type, although there was an increase in NPC1L1 and CD36 mRNA in the FABP1 and FABP2 mice respectively [45]. Since this experiment was performed in only one sex it remains to be determined whether the loss of FABP1 or FABP2 can lead to sex-specific changes in the genes involved in lipid transport and metabolism. Mechanistic differences in the transfer of FFA to the plasma membrane might also contribute to the sex dimorphic phenotype. FFA transfer from FABP2 involves a collisional interaction between the protein and the plasma membrane, whereas FFA transfer from FABP1 occurs through aqueous diffusion [46]. An assessment of dietary fat metabolism in mice lacking FABP2 will provide insight into the involvement of FABPs in lipid transit across the enterocyte.

A single nucleotide polymorphism at codon 54 of the human *FABP2* gene, which results in the substitution of threonine for alanine (A54T), has been linked to the development of insulin resistance and dyslipidemia in certain populations [47]. Primary and immortal cell lines carrying the A54T variant display variations in lipid output, with the mutation increasing TG output in the form of chylomicrons [47-48]. However, there is a lack of consensus on the association of this variation with altered metabolic condition in various populations. In a Japanese-American population, the A54T variant was associated with increased serum TG in men, and increased serum cholesterol in women [49], but a recent meta-analysis on the association of the FABP2 polymorphism with BMI found no correlations between genotype and sex [50]. The controversy is likely due to the

fact that the exact function of FABPs in metabolism of lipids remains elusive, highlighting the need for additional effort to gain a better understanding of the nature of these ubiquitous proteins.

Sex differences in the intracellular trafficking of lipids

Based on the sex dimorphic response to loss of either FABP1 or FABP2 and the proposed function of FABPs in the trafficking of lipids from the cytoplasm to the organelles, it is likely that genetic sex plays an even greater role in determining the metabolic fate of intracellular lipids. Few studies provide direct evidence to support a sex dimorphism in intracellular trafficking, largely because of the fact that most biochemical analyses of intracellular lipid trafficking are performed in cell lines derived from one sex. A study using hepatocytes isolated from young adolescent male and female rats found a 65% increased rate of diffusion of lipids in the cytoplasm of female hepatocytes, which might be attributed to sex differences in FABP1 [51]. A recent study observed sex differences in the mobilization of lipids from sex–segregated embryonic rat primary cortical neuron cells under conditions of nutrient deprivation to induce autophagy [52]. In these cells, nutrient deprivation causes the accumulation of autophagy markers and more rapid cell death in neuronal cells obtained from males. In contrast, female cells are much more resistant to autophagy and death, and are characterized by the accumulation of TG, FFA, and neutral lipid droplets in the cytoplasm. Interestingly, pretreatment of cells with $_{L}$-carnitine enhances survival in both male and female cells and abolishes the sex difference in response to nutrient deprivation [52]. This suggests that there might be fundamental differences in the ability of males and females to mobilize and oxidize intracellular lipids under conditions of stress, and that these differences are programmed within the genetic sex of the organism to function without the aid of the male/female hormonal background. In support of the concept that males and females differ in intracellular lipid trafficking, a transcriptome analysis of human skeletal muscle found increased mRNA abundance of genes involved in β-oxidation including Peroxisome proliferator-activated receptor δ (PPARδ), peroxisome-proliferator-activated receptor-γ coactivator 1-α, uncoupling protein

2, and acetyl-CoA acyltransferase 2 [53]. Subsequent analysis of male and female skeletal muscle revealed an increased abundance of proteins involved in β-oxidation in females [54]. Taken together these studies suggest that the fate of a lipid entering a cell is subject to underlying sex differences in intracellular lipid trafficking and metabolism. Based on the limited evidence available it appears that part of the sex dimorphism in intacellular lipid trafficking is regulated at the level of β-oxidation, and might rely on differences in the ability of FABPs to transport their ligands to organelles (Fig. 4).

Sex differences in cholesterol absorption

A wide variation in sex related differences in cholesterol absorption have been reported among different mouse strains, suggesting that the same might be true of human populations. In CD-1 mice fed a cholesterol-enriched diet, the absorption of cholesterol is significantly greater in female mice [55]. Estrogen treatment and age are associated with a significant increase in cholesterol absorption in gonadectomized male and female AKR and C57 mice [56]. This is likely attributable to increased levels of the intestinal transporters NPC1L1, ABCG5, and ABCG8, since estrogen treatment of gonadectomized mice enhances the expression of ABCG5 and ABCG8 in the jejunum and ileum, while NPC1L1 is increased in the duodenum and jejunum compared with mice that did not receive the gonadectomy [56]. These results might seem paradoxical when taken in the context of cardiovascular disease risk in men and women. If estrogens and age enhance cholesterol absorption independently then it would be expected that older premenopausal women would not be at an elevated risk of developing hypercholesterolemia and atherosclerosis compared to younger premenopausal women. Since the risk of developing cardiovascular diseases increases dramatically after menopause, it is likely that the role of estrogens in cholesterol absorption has minimal contribution on sex differences in cardiovascular disease risk. In support of this notion, dual stable isotopes analysis of cholesterol absorption in humans found no difference in cholesterol absorption between men and women [57]. However the study used subjects with ages ranging from 17 to

80 years, increasing the likelihood that any sex differences might be masked by age-related confounders [57].

Sex differences in the cardiac function and cardiovascular disease

The heart is another organ central to lipid metabolism (Fig. 3) and sex differences in the incidence of cardiovascular diseases [12] suggest that there are differences in the cardiac response to nutrients and stress. The cardiovascular system is particularly resistant to injury but continuous insults of excess nutrients and other acquired metabolic disorders such as diabetes eventually overwhelm the heart's adaptive mechanisms, leading to cardiovascular diseases. Cardipvascular dysfunction ultimately leads to endoplasmic reticulum stress and chronic cardiac ischemia/hypoxia results in an induction of the unfolded protein response pathways [58]. Interestingly, multiple mild ischemia-reperfusion events protect against acute ischemia, suggesting that ER stress pathways are an adaptive response by the heart to prevent cardiovascular trauma [58]. Sex differences in cardiomyocyte function have been reported in normal and diabetic mice. Notably, cardiomyocytes from female mice display enhanced antioxidant capacity, higher AKT phosphorylation and lower c-Jun phosphorylation, which are associated with differences in cardiomyocyte contractile properties and Ca^{2+} homeostasis [59]. Rats consuming a control diet or diets enriched with conjugated linoleic acid display sex differences in the abundance of several mRNAs involved in Ca^{2+} release and reuptake. This response was further modulated when conjugated linoleic acid was provided in the form of a fatty acid or TG [60]. In another study, mice lacking PPARα and overexpressing lipoprotein lipase display cardiac abnormalities including acute cardiopulmonary congestion and premature death in males but not females [61]. This finding might be attributable to an increased availability of FFA in female mice lacking peroxisome proliferator-activated receptors (PPARs) [61]. Clearly sex differences in the cardiac response to nutrient availability and stress are important contributors to cardiac function and the pathophysiology of cardiovascular diseases.

Sex differences in nutrient-gene interactions and the control of gene expression

There is now firm evidence demonstrating the fundamental role of nutrients and xenobiotics in the maintenance of cellular function. This awareness has opened avenues for the development of functional foods, nutraceuticals and drugs aimed at controlling metabolic function. Cholesterol and fatty acids are important regulators of whole body lipid homeostasis as they or their metabolic derivatives modulate the activities of several transcription factors involved in regulating gene expression. A classic example of this type of interaction is the repression of the sterol regulatory element-binding protein (SREBP) transcription factor activity by cholesterol [62]. Unlike the SREBP transcription factor, the nuclear receptor (NR) superfamily of transcription factors to which the PPARs, liver X receptors (LXRs) and farnesoid X receptor (FXR) belong to are ligand-activated and regulate the expression of genes involved in many aspects of nutrient and energy metabolism. The ability to produce synthetic NR ligands has led to the hope of developing new therapies for dyslipidemias and lipid storage disorders [63-64]. However it is apparent that many of the NRs have competing, synergistic or opposing effects on metabolism that, along with the documented sex differences in NR function, make the development of NR based therapies a challenging enterprise. As foods contain a complex mixture of NR ligands (Table 3), an assessment of nutrient-gene interactions is vital in the engineering of nutraceuticals and drugs that target specific metabolic pathways for disease prevention and treatment (Fig. 5). In depth reviews of NR structure and function are described elsewhere [63, 65]. The present review highlights the impact of sex on the function of SREBPs, PPARs, LXRs, and FXR and their involvement in nutrient metabolism and in the development of obesity and metabolic diseases.

Sterol regulatory element binding proteins

The regulation of cholesterol homeostasis is well studied and serves as an important model for studying nutrient-gene interactions. Cholesterol synthesis is regulated by end-product inhibition through the SREBP (SREBP-1a, -1c, -2) family of transcription factors. When cellular cholesterol is high, INSIG and

SCAP proteins form a complex that confines SREBP to the ER, thereby preventing the stimulatory effect of SREBP on the expression of the cholesterol biosynthetic machinery [62]. When cellular cholesterol is low, SREBP is shuttled to the nucleus via the Golgi in a 2-step proteolytic activation process, where it activates the expression of genes involved in cholesterol biosynthesis [62]. Although SREBPs are most commonly associated with cholesterol homeostasis, the SREBP-1c isoform plays an integral role in the regulation of genes involved in TG synthesis. Thus, SREBPs represent potential targets for the management of hyperlipidemia, and gender differences in SREBP function could lead to different metabolic outcomes. Persistent environmental pollutants have been linked to the increasing incidence of metabolic diseases and rats exposed to lead nitrate, a common pollutant, manifest a sex dimorphic response in SREBP-2, HMG-CoA reductase, and hepatic cholesterol concentration [66]. SREBP-2 mRNA levels peaked 6 hours following treatment in males, but 36 hours following treatment in females. Similarly, HMG-CoA reductase levels peaked at 3 hours in males and 12 hours in females, and corresponded to an earlier rise in hepatic cholesterol concentration in males. The delayed activation in females might confer a survival advantage by allowing the liver time to compensate for increasing cellular cholesterol by increasing bile acid synthesis or limiting cholesterol reuptake into the enterohepatic circulation [66]. SREBP-1c mRNA abundance is also greater in female rat liver than in males [67]. However at the level of protein, female rats appear to have significantly less abundance of both unprocessed and processed SREBP-2, and greater levels of both INSIG-1 and INSIG-2 than male rats [68]. It may be that estrogens act to limit cholesterol production in the cell, since male rats treated with estrogen experience a chronological decrease in SREBP-2 and HMG-CoA reductase protein [68]. The effects of estrogens on lowering SREBP-2 protein might contribute to the protection of women from hypercholesterolemia before menopause but interestingly, plasma TG is a better predictor of metabolic risk in women [69]. SREBP transcription is positively regulated by LXR and its ligands, leading to an upregulation of TG and cholesterol synthetic genes [70-71]. Conversely, SREBP is negatively regulated by PPAR and its ligands, leading to a

downregulation of both SREBP-1 and SREBP-2 targets [72-73]. Based on these findings, sex related differences in SREBP function may be secondary to sex differences in NR function.

Peroxisome proliferator-activated receptors

PPARs are a class of NRs with 3 distinct isoforms (α, β/δ, and γ) that play a central role in lipid oxidation [74]. Unsaturated fatty acids are known to bind PPARs [75] and activation of PPARs might contribute to disease prevention in populations that consume Mediterranean-type diets, which are rich in unsaturated fats [8]. PPARα mRNA and protein are more abundant in the liver of male mice than in female mice, although no differences are observed in skeletal muscle between the sexes [76]. Castration reduces PPARα mRNA levels in male mice to a level comparable with that of female mice [76]. PPARα deficiency in male mice is lethal in 100% of animals treated with a carnitine palmitoyl transferase I inhibitor, whereas only 75% of female mice tolerate the drug. Conditioning of male PPARα mice with β-estradiol prior to treatment confers resistance to the drug, suggesting an important role for estrogens in the regulation of PPAR signalling pathways [77]. Hepatic PPAR activation leads to female-specific PPARα sumoylation and downregulation of genes involved in steroid metabolism and protects against estrogen-induced cholestasis [78]. The involvement of PPARs in regulating lipid metabolism has been the impetus for the development of PPAR-targeted hypolipidemic drugs that target PPAR pathways. Fibrates are a class of amphipathic compounds used as a current first-line treatment for hypertriglyceridemia and hypercholesterolemia. Early studies demonstrated the efficacy of fibrates in improving whole body lipid parameters in male rodents [79] but now it is clear that there is sexual dimorphism in the response to fibrate treatment. In a model of diet induced obesity, male mice treated with fenofibrate experienced reductions in body weight, serum cholesterol and TG, and white adipose tissue mass. Female mice fed the same diet and treated with fenofibrate also exhibited reductions in serum TG, but without reductions in body weight, adipose tissue mass, and cholesterol [80]. Acyl CoA oxidase, a PPARα target gene and the first enzyme in the β-oxidation pathway, was significantly

upregulated in only the male mice treated with fenofibrate [80]. However it is unclear whether the amelioration of whole body lipid parameters in male mice was simply due to the fact that they gained more weight on the high fat diet. Finally, another study examined the ability of estrogen and fenofibrate to modulate whole body lipids when given separately or in conjunction in ovarectomized mice [81]. Fenofibrate or estrogen treatment prevented weight gain on a high fat diet but concomitant treatment negates the effects of fenofibrate on lipids and PPAR target gene expression. Estrogen did not interfere with the binding of the PPAR:RXR heterodimer to its response element, suggesting that the role of estrogens in PPAR gene regulation occurs outside the realm of DNA binding [81].

A recent review of sex differences in nuclear receptor-regulated liver metabolic pathways has suggested that sex differences hepatic NR networks are responsible for sex-specific metabolism and disease risk [82]. Notably, sex dimorphic cross-talk between ER and PPAR seems to protect the female liver from injury by modulating the expression of genes involved in lipid metabolism and xenobiotic detoxification. If sex differences in NR networks are at the confluence of metabolic and disease pathways in the liver, analysis of NR function in the other major organ systems involved in nutrient and xenobiotic metabolism will provide insight into organ and sex-specific pathologies.

Liver X receptors

The liver X receptors (LXRα and LXRβ) are oxysterol-activated NRs plays a central role in the steroid metabolism and disposal of body cholesterol [63]. LXR agonists have been suggested as potential agents for the management of hypercholesterolemia and atherosclerosis. One side effect of LXR agonists in the treatment of atherosclerosis is a substantial increase in the expression of lipogenic genes that leads to the elevation of serum TG [70, 83]. This is the result of LXR-dependent stimulation of the gene for SREBP-1C, which in turn, upregulates the genes for steroyl CoA desaturase 1, acetyl CoA carboxylase, and fatty acid synthase [70-71]. This has important sex-specific implications for the

treatment of hyperlipidemia since serum TG concentration appears to be a better predictor of coronary heart disease in women than in men [69].

There are now studies that have observed noteworthy sex differences in LXR function in the development of atherosclerosis. In 2 mouse models of atherosclerosis, *Apoe*$^{-/-}$ mice (lacks apolipoprotein E, ApoE) and *Ldlr*$^{-/-}$ mice (lacks low-density lipoprotein receptor), there is a differential effect of LXR agonism on aortic lesion area. Treatment with the non-steroidal LXR agonist GW3965 decreases aortic lesion size in high-fat fed *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice, but the decrease is more pronounced in male mice [84]. Transgenic overexpression of LXR in macrophages leads to the upregulation of cholesterol transporters ABCA1 and ABCG1, and concomitant reduction in atherosclerotic lesion size but only in male mice [85]. These findings are consistent with the increased susceptibility of female mice to diet-induced atherosclerosis [86], and point to sex differences in LXR-mediated regulation of gene expression. Gene expression profiling of visceral white adipose tissue of ovarectomized mice treated with estrogen reveals an overall decrease in the expression of lipogenic genes including those encoding LXRα, SREBP-1c, and ApoE [87]. Furthermore, the promoter of the gene for LXRα is repressed in the presence of estrogen and ERα, suggesting a possible explanation for the repression of LXR target genes [87]. Recently, the LXR ligand 27-hydroxycholesterol has been shown to inhibit estrogen signalling through competitive inhibition of ERα and ERβ [88]. It is clear that LXR and ER signalling pathways work in concert with, or oppose one another, leading sex-specific modifications in lipid metabolism.

LXRs are also involved in integrating cholesterol homeostasis with the biosynthesis and degradation of sex steroid hormones. LXRs (especially the β isoform) are expressed in luteinized granulosa cells, which are ovarian cells responsible for the biosynthesis of female hormones [89]. The importance of LXRs in steroidogenesis is illustrated by mice lacking both LXRα and LXRβ. Mice lacking both these proteins display classic signs of ovarian hyperstimulation disorder. These mice are hyperresponsive to gonadotropins, have enlarged ovaries, and increased production of estrogens [90]. The increased estrogen

synthesis observed in these mice likely results from the lack of LXR mediated stimulation of cellular cholesterol efflux. Consistent with this idea, treating human luteinized granolosa cells with a LXR agonist reduces progesterone biosynthesis and increases the expression of genes involved in cholesterol efflux and reverse cholesterol transport, including ABCA1, ABCG1, and ApoE, and phospholipid transfer protein [89]. Similarly, LXR is fundamentally involved in male fertility and testosterone synthesis. Male mice lacking both LXRα and LXRβ become infertile much earlier than wild-type male mice and this is correlated with a decreased concentration of testosterone in the testes, which is partially attenuated by treatment with a LXR agonist [91]. Mice lacking a functional *Cyp19* gene (encodes aromatase) display serious metabolic abnormalities and marked tissue specific sex differences in cholesterol levels [92-93]. When fed a cholesterol depleted diet, female *Cyp19*$^{-/-}$ mice display elevated serum cholesterol levels, whereas male mice accumulate hepatic cholesterol [92]. Female mice lacking *Cyp19* display adipocyte hypertrophy and hyperplasia, which is prevented by cholesterol feeding, indicating a possible interplay in LXR function and sex hormones on the modelling of female body fat distribution patterns [94]. In contrast, estrogen replacement prevents the hepatic lipid accumulation that normally features *Cyp19*$^{-/-}$ in male mice, suggesting that the male liver might require estrogens and LXR signalling to maintain proper function [95]. These findings suggest that LXRs may have evolved to serve as key regulators of lipid homeostasis and sex steroid hormone biosynthesis, supporting the concept that sex differentiation and fertility are intimately linked through nutrient metabolism.

Farnesoid X receptor

The farnesoid X receptor (FXR, mainly the α isoform) is a NR that is activated by bile acids and regulates key genes in fat, sterol and carbohydrate metabolism. The inhibition of bile acid synthesis is mediated by FXR via an indirect mechanism involving another NR known as the short heterodimeric partner (SHP). FXR activation has an overriding inhibitory effect over LXR in regulating the expression of the gene for cholesterol 7α-hydroxylase (CYP7A1), the rate-controlling enzyme of the bile acid biosynthetic pathway [96-97].

Differential regulation of FXR by its ligands might also account for sex differences in the bile acid pool size [55] through the expression of genes involved in bile acid homeostasis. Guggulsterone, a phytosterol from the guggul plant, has been found to act as a FXR antagonist [98] and is being evaluated as a possible natural health product for the management of dyslipidemia [99]. In addition to being a FXR antagonist, guggulsterone might also modulate the function of other NRs such as the pregnane X receptor and the ERs [100].

Activation of FXR by a synthetic agonist prevents atherosclerotic lesion formation in both male and female *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice [101]. In *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice that are also lacking SHP, male mice remain protected from diet-induced atherosclerosis with FXR agonist treatment, whereas female mice remain susceptible [101]. Notably, the male mice treated with the FXR agonist display reductions in non-high density lipoprotein cholesterol and CYP7A1 mRNA abundance, features that are absent in the female mice. The functional redundancy in FXR signalling in male mice lacking SHP might offer a mechanistic explanation for the decreased susceptibility of male mice to diet-induced atherosclerosis.

As mentioned previously, gallstone disease and cholestasis are predominately observed in women [15]. The concentrations of bile acids, phospholipids, and cholesterol (the major lipid constituents of bile) must be kept within a very narrow range in order to maintain the solubility of cholesterol in the bile. In addition female mice have a larger bile acid pool size than male mice [55]. Elevated biliary cholesterol concentration favours the development of cholesterol gallstones [102]. Male mice lacking FXR and fed a lithogenic diet develop cholesterol gallstones and are characterized by an increase in the hydrophobicity of the bile acid pool and decreased phospholipid transporter mRNA expression compared with wild-type mice [103]. Inclusion of the FXR ligand GW4064 in the lithogenic diet prevents gallstone formation but increases the bile acid and phospholipid content of the bile in wildtype mice only [103], suggesting that FXR-targeted therapy might be a viable option for the prevention of cholesterol gallstones. Given their central role in governing cholesterol and bile acid

homeostasis, sex differences in FXR-regulated pathways might contribute to the relative protection from gallstones and cholestasis in men. Single nucleotide polymorphisms in the promoter of the human gene for FXR are associated with sex differences in gallstone disease risk in some populations but not others, indicating the involvement of additional factors [104]. Transgenic expression of activated LXRα protects female mice from hepatoxicity caused by lithocholic acid treatment or bile duct-ligation [105]. Of note is the differential regulation of the gene for SULT2A9, a bile acid detoxifying enzyme, in male and female mice expressing the activated LXRα [105].

Sex differences in metabolism: Fertility and the evolution of obesity

One of the challenges facing population-wide evolutionary theories of human obesity is that they often cannot explain common patterns of disease risk within subsets of the population, such as the relative protection of premenopausal women from certain metabolic diseases [11-12]. Premenopausal women face unparalleled fluctuations in metabolic requirements (age of menses, pregnancy, and lactation) that would favour an enhanced ability to store and metabolize lipids during periods of food insecurity [36, 106]. Concurrent with this hypothesis, insulin stimulates lipogenesis in pregnant rats until late gestation, whereupon lipolytic pathways are upregulated, presumably to meet the high metabolic demand of lactation [107]. It is likely that leptin and estrogen signalling are coupled to modify central and peripheral lipid metabolism in order to maintain reproductive viability. The ability of leptin to normalize menses in amenorrheic women is an example of the relationship between adipose tissue and fertility [25]. It has been observed that obese mothers are more likely to give birth to daughters who themselves become obese and experience reproductive complications [106]. In utero epigenetic programming of genes involved in lipid storage represents a plausible mechanism by which parental fat storage capacity contributes to the development of acquired metabolic disorders in the next generation [108]. Female offspring of high fat-fed male rats display impaired glucose tolerance and insulin secretion, indicating that epigenetic inheritance of disease risk factors depend on both the parental origin and the sex of the offspring [109]. The ability of nutrients

to alter epigenetic inheritance and disease risk is an emerging field of research and has been best illustrated in the case of folate, whereby folate status is linked to DNA methylation patterns and colorectal cancer susceptibility [110]. When these observations are considered, it becomes apparent that parental nutrition and epigenetic programming are important predictors of reproductive success and the evolution of obesity and acquired metabolic syndromes in modern populations (Fig. 6). Due to the significant sex differences in the metabolic costs of reproduction, it is not possible to explain the current obesity epidemic with a sex-neutral evolutionary hypothesis. Understanding the paradoxes associated with sex differences in metabolism and metabolic disease risk provides the framework for maximizing the efficacy of therapeutic interventions aimed at disease prevention and requires an appreciation of the modifiable and non-modifiable factors that control metabolism (Fig. 1). Since these factors contribute to the development of sex differences during the reproductive phase (Fig. 6) it is imperative to consider males and females as separate entities in evolution of obesity.

One frontier of sex research that requires further exploration is that of sex chromosome effects. There has been a longstanding and unresolved observation that male infants experience greater degrees of morbidity and mortality than females in response to nutritional stressors [111]. This suggests that sex differences in metabolism can manifest as a pathological phenotype before sexual maturity, and that differences in parental nutrition history and sex hormones (both estrogens and androgens) likely play a vital role in infant viability in the first few years of life. Others have noted a trend towards an increase in the ratio of female to male births in communities exposed to persistent organic pollutants [112]. Many of these compounds accumulate in adipose tissue and possess estrogenic signalling properties [113] and X-linked genes may regulate lipid metabolism to confer enhanced survival of females in utero. Furthermore, the ability of X-linked genes to escape inactivation through epigenetic modification superimposes an additional layer of complexity to the relationship among sex chromosomes, lipid metabolism, and evolution. In any scenario, parental genetic makeup, nutritional

history, and epigenetics are likely the most important factors in determining infant viability and the evolution of complex traits within a population.

Future outlook

A growing body of evidence suggests that sex differences in the acquisition, trafficking, and metabolism of lipids and other nutrients contributes to the disparities in metabolic disease risk between pre- and post-menopausal women and men. While research into sex differences in metabolism is gaining better attention, many aspects of metabolic research remain biased towards one sex creating an incomplete knowledge base of metabolism, and it will take a multisectorial effort to ensure that sex equity is achieved in our quest towards understanding how metabolic pathways are controlled and coordinated in the whole organism. An understanding of the effects of sex and other non modifiable factors on metabolism opens the possibility for applying novel therapies as tools for optimizing metabolic efficiency tailored to suit a specific sex or life-stage.

Figures

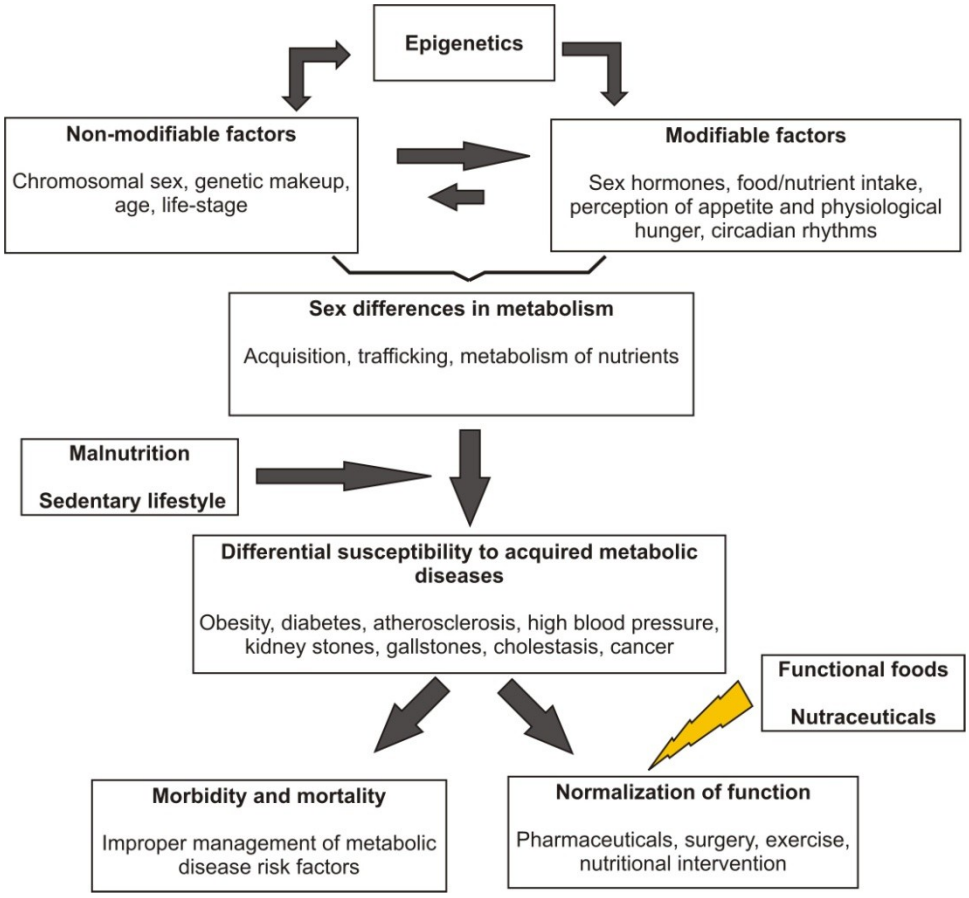

Figure. 1

Framework for incorporating sex differences in metabolism into the current model of metabolic disease. Interactions between the sex chromosomes, sex hormones, and epigenetics lead to sex-specific changes in the acquisition, trafficking, and metabolism of nutrients. These fundamental differences in male/female metabolism contribute to a differential susceptibility to metabolic diseases and metabolic disease risk factors in the context of excess food consumption and sedentary lifestyle. Functional foods and nutraceuticals are emerging as attractive candidates for the delivery of nutrients and xenobiotics involved in disease prevention, potentially reducing the need for dangerous and expensive drugs and surgery. Assessment of sex differences in metabolism will enhance our understanding of metabolism for the management of metabolic disease risk factors.

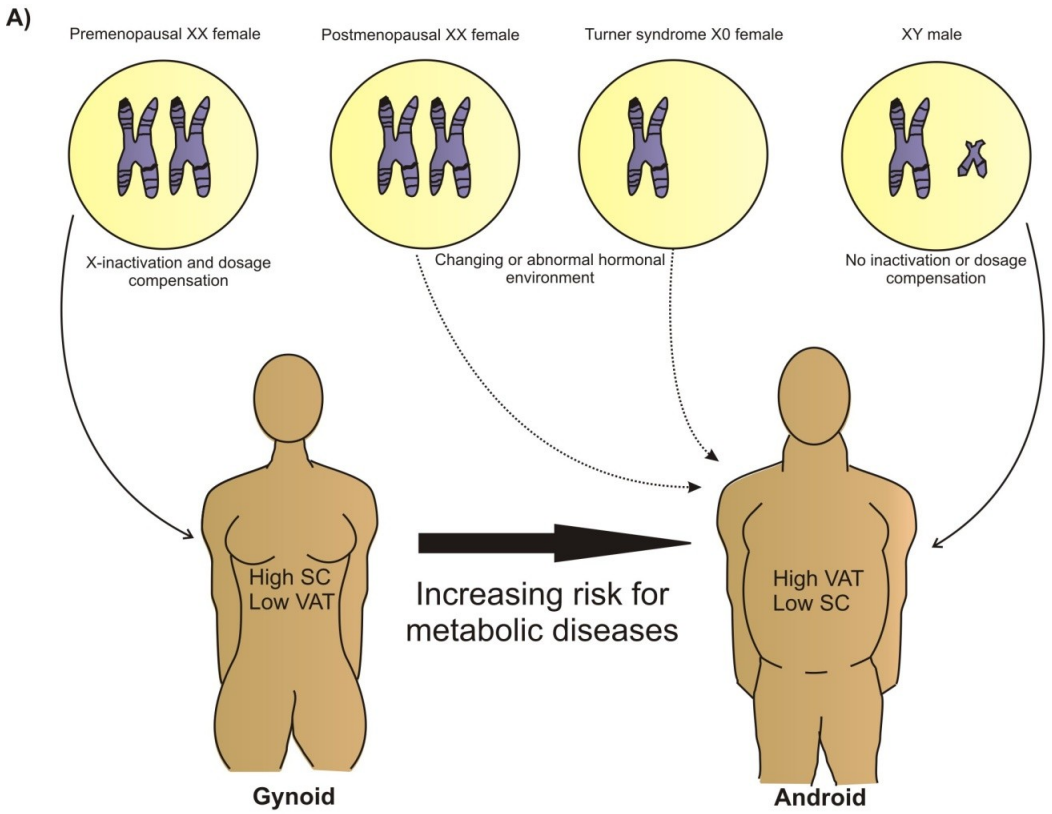

Figure. 2

Sex chromosome complement and gonadal sex are associated with differential risk factors for metabolic disease. In males and premenopausal females, the effects of sex chromosomes, sex hormones, and epigenetics contribute to the development of distinct patterns of body fat distribution (solid lines). The female-associated gynoid body type is characterized by high subcutaneous (SC) adipose tissue, low visceral adipose tissue (VAT), and the preferential storage of fat in the gluteo-femoral region. The male-associated android body type is characterized by higher visceral fat and lower subcutaneous fat in the abdominal region, and is associated with increased morbidity. In postmenopausal and TS women, loss of sex hormone effects (dotted lines) favours a transition from gynoid to android and a concomitant rise in disease risk.

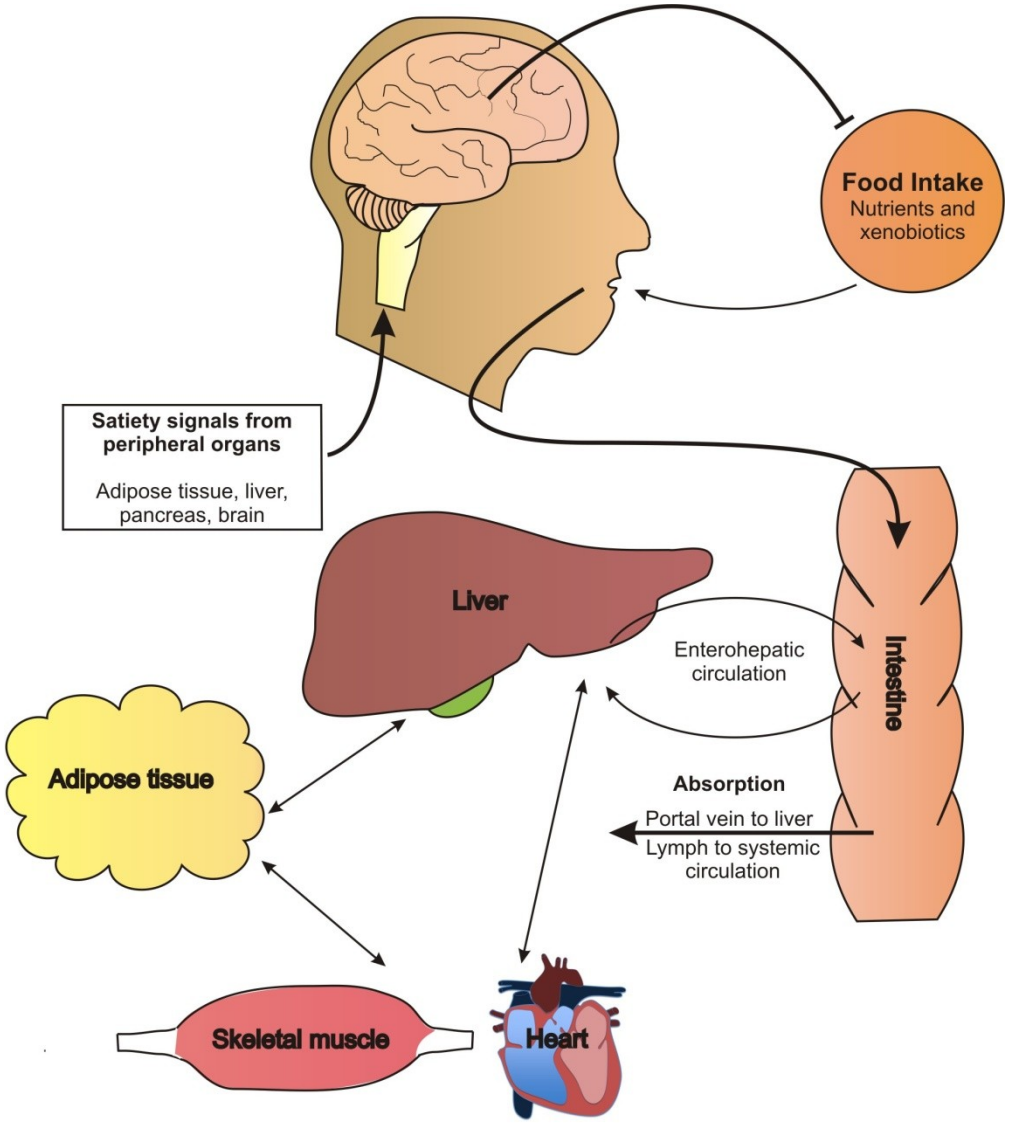

Figure. 3

Major organs involved in lipid metabolism. Food contains a complex mixture of nutrients and xenobiotics that are absorbed along the length of the intestine. Food-derived lipids are incorporated into chylomicrons for delivery to the peripheral tissues. The liver plays an essential role in regulating de novo lipid synthesis as well as bile acid and cholesterol homeostasis. Lipids are stored in the adipose tissue as a long term fuel depot and used in the cardiac and skeletal muscles for oxidation. Satiety hormones derived from the adipose tissue and gut feedback to the satiety centres of the hypothalamus to regulate physiological hunger and food intake.

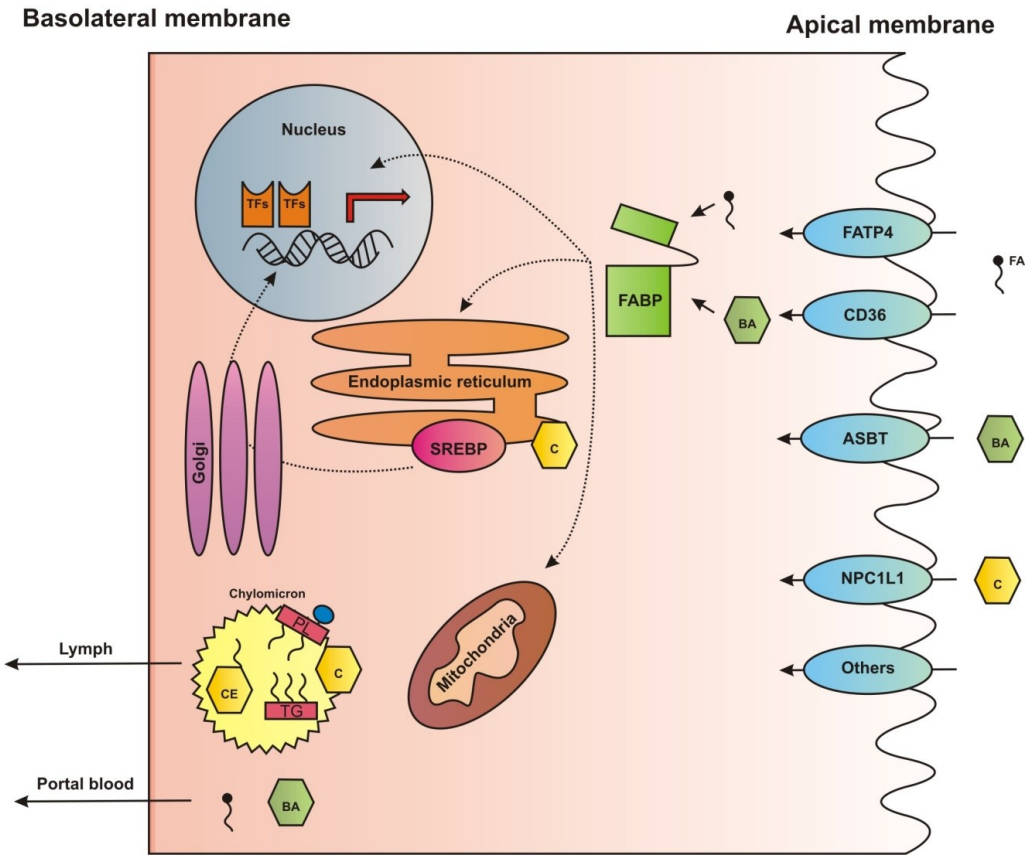

Figure. 4

General scheme for subcellular lipid trafficking and metabolism. Mixed micelles containing lipids and lipid soluble nutrients, are solubilised by bile acids (BA) in the intestinal lumen for absorption. Specialized transporters are involved in the uptake of lipids (e.g., free fatty acids (FA) and cholesterol (C) across the apical membrane. FABPs bind fatty acids and are involved in subcellular trafficking to various organelles where they are used as fuel, substrate for macromolecule synthesis or as ligands for transcription factors to regulate gene expression. Lipids (cholesteryl esters (CE), phospholipids (PL) and triglycerides (TG)) packaged into chylomicrons are released to the systemic circulation via lymph for delivery to peripheral tissues. Fatty acids can also be delivered directly to the liver via the portal circulation.

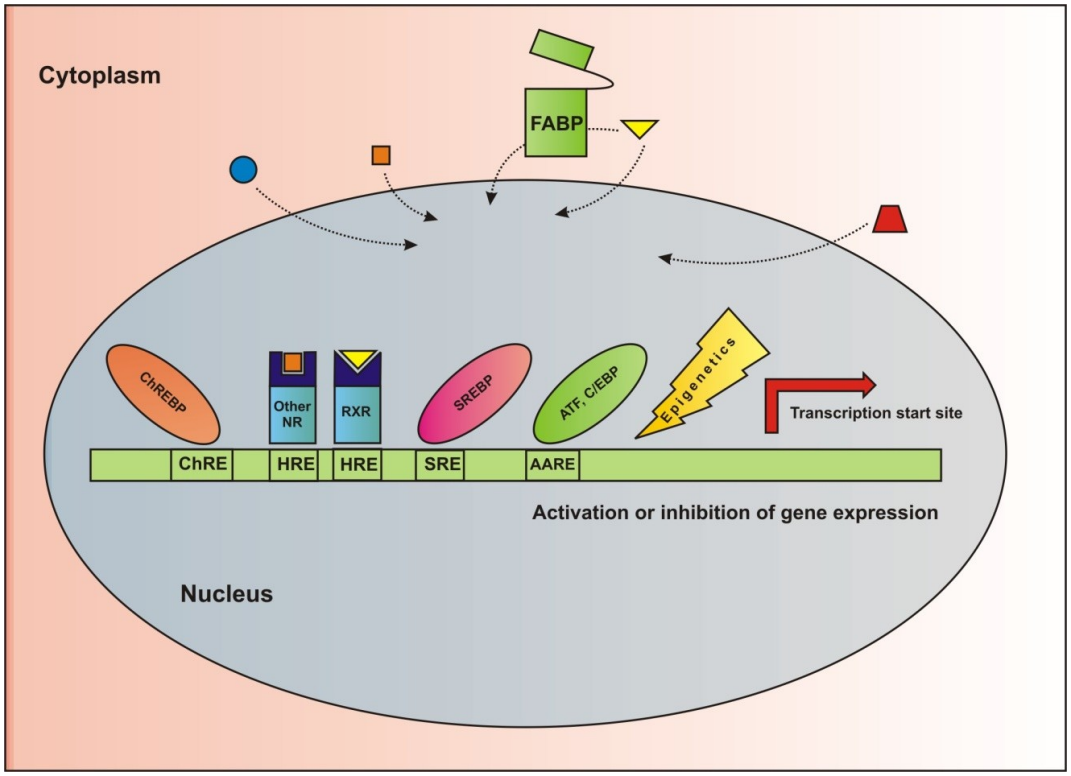

Figure. 5

Nutrient-gene interactions in the control of gene expression. Nutrients and xenobiotics serve as ligands for the nuclear receptor (NR) superfamily of transcription factors, and as regulators of sterol response-element binding proteins (SREBP), carbohydrate response-element binding protein (ChREBP) and transcription factors that bind to the amino acid response elements. These transcription factors bind to specific elements in the promoter region of responsive genes. FABP, fatty acid binding proteins.

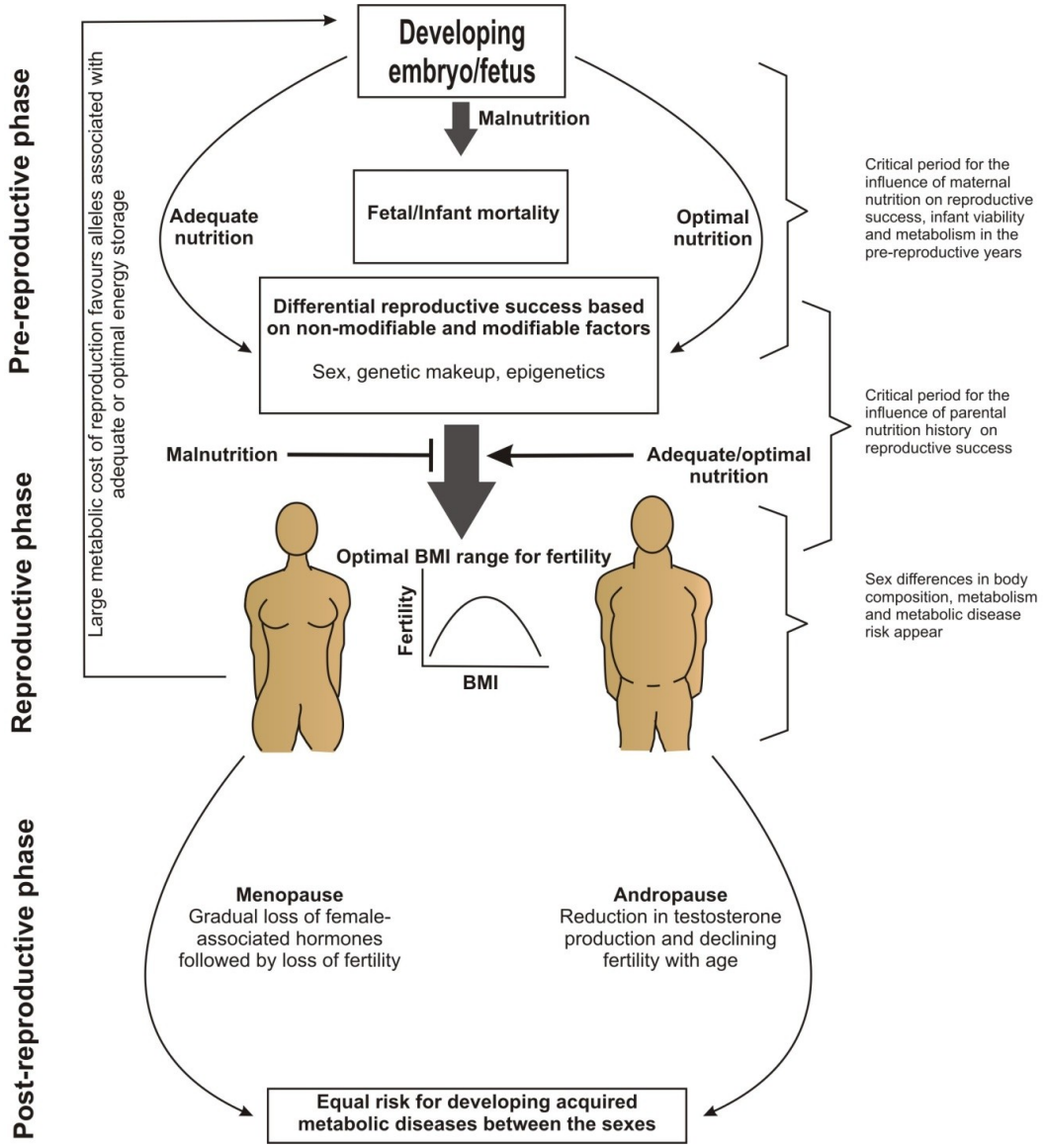

Figure. 6

Factors influencing fertility, fetal and infant mortality and evolution of obesity. Parental metabolism and fat storage capacity are transmitted to the developing fetus through genetic and epigenetic means. These factors directly influence fertility, viability and the development of sex differences in obesity and acquired metabolic diseases in reproductive and post-reproductive phases. BMI, body mass index.

Tables

Table 1.

Examples of known sex differences in lipid metabolism and metabolic disease risk factors

Comparison	Variable	Feature
Female > male	Total proportion of body fat	At comparable BMI
	Subcutaneous fat	At comparable BMI
	Insulin sensitivity	In adipose tissue
	Gallstones	Premenopausal women
	Bile acid pool size	In mice
	Leptin	At comparable BMI
	Lipoprotein lipase activity	In subcutaneous adipose tissue
	Basal FFA appearance in plasma	At comparable BMI
	Basal VLDL clearance	In lean women and men
	Myocellular TG content	Following endurance training regimen
	SREBP-1c mRNA abundance	In rat liver
Male > female	Cardiovascular disease risk	Age-matched women (prior to menopause) and men
	Visceral fat	Compared to premenopausal women
	PPARα mRNA abundance	In liver, not skeletal muscle
	LXR agonist treatment for atherosclerosis	Greater magnitude of protection in males
	FXR agonist treatment for atherosclerosis	In mice susceptible to atherosclerosis
	SREBP-2 protein content	In rat liver

Note: BMI, body mass index; FFA, free fatty acids; VLDL, very low-density lipoprotein; TG, triglycerides; LXR, liver X receptors; and FXR, farnesoid X receptor.

Table 2.

Selection of hormones involved in satiety and energy homeostasis

Hormone	Class	Major source	Function
Leptin	Peptide	Adipose tissue	Hunger suppressant
Apidonectin	Peptide	Adipose tissue	Anti-inflammatory, increases insulin sensitivity
Resistin	Peptide	Adipose tissue	Anatagonizes the effects of insulin
Ghrelin	Peptide	Stomach	Hunger stimulator
Insulin	Peptide	Pancreas	Inhibitor of lipolysis, stimulator of glucose uptake
Neuropeptide Y	Peptide	Hypothalamus	Hunger stimulator
Agouti-related protein	Peptide	Hypothalamus	Hunger stimulator
Brain-derived neurotrophic factor	Peptide	Hypothalamus	Hunger suppressant
Proopiomelanocortin	Peptide	Hypothalamus	Hunger suppressant
Thyrotropin releasing hormone	Peptide	Hypothalamus	Hunger suppressant
α Melanocyte-stimulating hormone	Peptide	Pituitary	Hunger suppressant
Cholecystokinin	Peptide	Intestine	Hunger suppressant
Glucagon-like peptide-1	Peptide	Intestine	Hunger suppressant, delays gastric emptying
Oxyntomodulin	Peptide	Intestine	Hunger suppressant, increases energy expenditure
Oleoylethanolamide	Lipid	Intestine	Produced from dietary fat and acts on cannabanoid receptors in peripheral

			tissues, has anorexigenic effects
Estrogen	Steroid	Ovaries	Varies by tissue, enhances insulin's anabolic effect
Testosterone	Steroid	Testes	Anabolic, possible appetite suppressant
Progesterone	Steroid	Ovaries	Major regulator of lactation
Glucocorticoids	Steroid	Adrenals	Metabolism

Table 3.

Selected nuclear receptors involved in nutrient and xenobiotic metabolism.

Transcription factor	Nutrient regulator
PPARs (NR1C1, NR1C2, NR1C3)	Unsaturated fatty acids Fatty acid metabolites
LXRs (NR1H2, NR1H3)	Oxysterols
FXRs (NR1H4, NR1H5)	Bile acids Guggulsterone (antagonist)
VDR (NR1I1)	Vitamin D Secondary bile acids
RARs (NR1B1, NR1B2, NR1B3), RXR (NR2B1, NR2B2)	Vitamin A, Vitamin A metabolites
PXR (NR1I2)	Xenobiotics Secondary bile acids Guggulsterone
CAR (NR1I3)	Xenobiotics
TRs (NR1A1, NR1A2)	Thyroid hormone (synthesized from tyrosine and iodine)
ERs (NR3A1, NR3A2), AR (NR3C4)	Estrogens and androgens (synthesized from cholesterol)
GR (NR3C1)	Glucocorticoids (synthesized from cholesterol)
SREBPs	Cholesterol
ChREBP	Glucose

Note: PPARs, peroxisome proliferator-activated receptors; LXRs, liver X receptors; FXRs, farnesoid X receptors; VDR, vitamin D receptor; RAR, retinoic acid receptors; RXRs, retinoid X receptors; PXR, pregnane X receptor; CAR, constitutive androstane receptor; TRs, thyroid hormone receptors; ERs, estrogen receptors; AR, androgen receptor; GR, growth hormone receptor; SREBPs, sterol regulatory element-binding protein; ChREBP, carbohydrate response element-binding protein.

Bridge section between manuscript 1 and manuscript 2

In the previous manuscript, evidence for sex differences in lipid metabolism and metabolic disease risk was reviewed for the purpose of defining well known examples of sex differences as well as areas in the collective knowledge base that are lacking on the subject. Past sex biases in research have led to an incomplete understanding of the role of the sex chromosomes and sex hormones on metabolism. Available evidence suggests that these factors sometimes have competing or opposite effects on the metabolism of food-borne nutrients and nutraceuticals, and can modulate the risk for adverse outcomes such as the development of acquired metabolic syndromes.

Previously we examined the effects of genetic sex in mice that are genetically altered to lack Fabp2. Mice lacking Fabp2 display a sex-dimorphic response to high-fat diet feeding, where male mice gain weight and develop fatty liver while female mice lose weight and are protected from fatty liver, suggesting a sex difference in the handling of diet derived lipids in the intestine. In order to gain further insight into the role of Fabp2 in metabolism, we performed a transcriptome analysis of the small intestine in wild-type and *Fabp2*$^{-/-}$ mice fed a low-fat diet using Affymetrix microarrays. Microarrays have emerged as a powerful tool for elucidating pathways that are altered by a particular perturbation such as diet or genetic modification. However since it is a relatively expensive technology most transcriptomics research is performed in only one sex, as studying both sexes amplifies the cost of an experiment. Since the current microarray analysis software packages do not easily accommodate more than one experimental manipulation in the analysis of results, we developed a technique for analyzing the effects of both sex and genotype on the transcriptome. This technique allows for the comparison of multiple experimental manipulations in both sexes, and yields gene lists that are easily sortable. We applied this technique to the study of mice lacking Fabp2, which have been previously shown to exhibit a sex-dimorphic response to diet featuring weight gain and fatty liver in males but not females [3-4, 114]. Using this technique we were able to leverage the power

of microarrays to elucidate metabolic pathways that might be impaired and contribute to the sex-dimorphic phenotype in mice lacking Fabp2.

Manuscript 2: Microarray experiment

Visualization of sex-dimorphic changes in the intestinal transcriptome of *Fabp2* gene-ablated mice

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Abstract

Background/Aims: Sex differences in gene expression program have not been effectively explored at the transcriptome level. We aimed to develop a facile approach for the analysis of transcriptome data to identify sex differences and sex-dimorphic responses to experimental conditions in mice. ***Methods:*** Profiling of the small intestine transcriptome of chow-fed C57BL/6J (wild-type, WT) and *Fabp2*$^{-/-}$ mice was carried out by microarray analysis. Sex-specific and androgynous effects of *Fabp2* gene ablation were examined using FlexArray V1.6 by comparing WT to *Fabp2*$^{-/-}$ mice. The data generated were exported into a single spreadsheet, collated and transformed to identify the differentially expressed genes for pathway analysis. ***Results:*** The method revealed enrichment of 17 sex-dimorphic pathways in the small intestine of WT mice compared to only 4 in *Fabp2*$^{-/-}$ mice. Comparison of the effects of Fabp2 loss in individual sexes revealed a male-specific upregulation of 5 pathways involved in the production of unsaturated fatty acids, and a female-specific downregulation of pathways involved in xenobiotic metabolism. ***Conclusions:*** Our approach detected the common as well as sex-differential pathways that are modified due to the loss of Fabp2. These findings suggest that the pathways involved in nutrient and xenobiotic metabolism in the intestine are regulated by sex-specific mechanisms.

Introduction

The rise in obesity and acquired metabolic syndromes in the sedentary, modernized community has been the impetus for the search for prevention and treatment strategies applicable to the general population. Nutrition research has gained attention due to the ability of nutrients and other food borne compounds to regulate metabolic processes and normalize cellular function. However, the effects of nutritional interventions are typically difficult to quantify because metabolism is influenced by several modifiable (hormones, food intake) and non-modifiable (genetic sex, genomic makeup, age, and life-stage) factors as well as epigenetics and circadian rhythms which are modifiable to a certain extent. Non-modifiable factors can have an overriding influence on the modifiable factors which give rise to the differences in metabolic potential of the organism. Genetic sex is a prime non-modifiable factor; however its effects are often overlooked in metabolic research and this has left a gap in our understanding of metabolism and disease pathogenesis [115].

The field of nutrition and metabolic research has begun to address the inherent limitations associated with single-gene studies by adopting the use of chip-based DNA microarrays that can scan the state of the entire transcriptome at a given genetic and/or nutritional status. Currently, many gene microarray-based studies are usually performed in only one sex (typically males to cover the entire genome) and involve comparison of control and treatment groups to identify global differences in mRNA abundance. Studying sex differences in the context

of a given metabolic condition or treatment requires a more comprehensive analysis and amplifies the financial burden of the experiment.

Sex differences in metabolism not only contribute to differences in disease susceptibility but also the response to therapeutic interventions. This is especially true of the organs responsible for the acquisition, trafficking, and metabolism of nutrients and xenobiotics such as the intestine and liver. Sex differences in the abundance of master regulators of lipid/energy (PPAR signalling pathway) [76, 116-117] and xenobiotic (CYP450 superfamily) [118-121] metabolism are well documented. Consequently, numerous studies have documented sex differences in the response to fibrates, synthetic PPARα agonists designed to normalize blood lipids and reduce metabolic disease risk [80-81, 122-123]. These observations combined with the lack of understanding of sex differences in metabolic pathways have made development of novel treatments for metabolic disease that are equally applicable to both males and females more challenging.

Fabp2 is an abundant cytoplasmic protein that is expressed exclusively in the small intestine [2], and binds a diverse number of lipid species, with a preference for saturated fatty acids [124]. Our previous work on *Fabp2*$^{-/-}$ mice revealed that Fabp2 is not essential for the absorption of dietary fat from the lumen of the small intestine [4]. However the loss of Fabp2 results in a sex dimorphic phenotype [4] [3] [114]. These observations suggest that the loss of *Fabp2* modulates the pathways that control lipid transit through the small intestine in a sex-specific manner. Here we illustrate a method for analyzing microarray data to detect sex-specific and sex-dimorphic responses to specific experimental manipulation such as metabolic challenge or genetic modification, and apply this

method to characterize changes in gene expression pattern in the small intestine of mice due to genetic ablation of the *Fabp2* gene.

Methods

Animals

Wild-type C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, Maine). *Fabp2*$^{-/-}$ mice were produced in house. The *Fabp2*$^{-/-}$ strain had been backcrossed to the C57BL/6J genetic background for 10 generations which included one cross with a male C57BL/6J mouse. The mice used in this study were 10 weeks old and maintained on standard laboratory chow diet (Purina 5001). Use of animals was approved by the institutional animal welfare and policy committee in accordance with the policies of the Canadian Council on Animal Care.

Preparation of RNA and hybridization to DNA microarrays

The small intestines, starting from the base of the stomach to the base of the caecum were taken from the animal, the luminal content of the organ was immediately flushed with cold saline and then the tissue was processed for RNA extraction using the guanidinium isothiocyanate method [125]. Two pools of RNA for each sex of each genotype were created by combining equal amounts of total RNA from two mice. The transcriptome data was generated at the Genomic Analysis and Technology Core Facility, University of Arizona. RNA integrity (RIN > 7) was verified using the Agilent Bioanalyzer 2100. Fluorescently labeled

cDNA probes for each RNA pool was prepared and hybridized to Affymetrix 430A and 430B chips following the procedures specified by the manufacturer. Only one probe was hybridized with each chip. Signals were acquired using the Affymetrix GeneChip Scanner Model 3000. Each chip was assessed for signal pattern fidelity and consistency before use in comparative analyses.

Analysis of microarray data

Analysis of microarray data was carried out using software packages available in the public domain. Our approach calculates the fold change difference of each probeset across four paired comparisons, and combines the powerful statistical algorithms of microarray analysis software with the data processing and global visualization facility of spreadsheet software. Microarray dataset files were imported and visualized using the FlexArray v1.6.1 software package (http://www.gqinnovationcenter.com/services/bioinformatics/flexarray/index.aspx). Imported genechip data was normalized using the optimized Robust Multi-Array Average (JustRMA) [126]. The data from duplicate chips for WT and *Fabp2*$^{-/-}$ mice of each sex were merged for comparison with other groups using the Significance Analysis of Microarrays (SAM) algorithm [127]. Sex-differential gene expression was compared in the WT and *Fabp2*$^{-/-}$ mice separately. The sex-specific effects of *Fabp2* loss were then examined by comparing transcript profiles of male and female *Fabp2*$^{-/-}$ mice to their respective WT counterparts. In the SAM analysis, the order and sequence in which the groups are assigned (i.e., Fabp2-deficient vs. wildtype; male vs. female) remained

consistent for all the comparisons (see Fig. 1). This is a crucial step for building the datasets used in the spreadsheet analysis because the structure of the resulting datasets is what permits the visualization of the behavior of each probeset across multiple experimental conditions. The data for each comparisons for the 430A and 430B chip was exported to a tab delimited text file and merged into a single spreadsheet (LibreOffice v3.3) using the probeset identification as a unique importing and sorting key. Fold change values less than 1 were transformed by taking the reciprocal and multiplying by -1. This enables the representation of the magnitude and direction (positive value for upregulation, negative value for downregulation) of the signal difference compared to the reference. Fold change values greater than 1.5 or less than -1.5 (1.5> FC <-1.5) for each comparison were assigned a value of 1 in a separate column, allowing for significantly up- or downregulated genes to be easily sorted. The data was then organized to display the fold change difference for each of the 4 comparisons in adjacent columns. This approach allows for the rapid visualization of the effects of two or more variables, which is not easily accomplished within the FlexArray program. Probesets with fold changes that were not significantly different (defined as $P > 0.05$) when *Fabp2*$^{-/-}$ mice was compared WT mice for each sex were excluded in the final compilation. Probesets meeting the fold change cutoffs (1.5> FC <-1.5) were segregated into separate lists based on the type of comparison for pathway enrichment analysis. Pathway enrichment was queried using the WebGestalt Gene Set Analysis Toolkit V2 [128] for KEGG pathway analysis [129].

Results

Verification of signal fidelity and the fitness of the method

Data from replicate microarray chips were analyzed by histogram of log expression ratios and linear regression of the expression mean to ensure representative datasets. Within each group, >99% of all probes fell within an expression ratio of log (±0.5) and had R^2 values greater than 0.99. The ability of the method to properly separate known sex-specific markers was verified by examining the segregation of the female-specific X inactive-specific transcript *(Xist*) [130] and the male-specific DEAD box polypeptide 3 Y-linked (*Ddx3y*) [131] transcript. For both genes, male vs. female comparisons revealed the expected sex-differences in expression, and comparison of *Fabp2*$^{-/-}$ males or females to their respective WT counterparts revealed no anomalous segregation of sex-specific transcripts. Signals that were identified as not different in the merged datasets were excluded from the gene lists used for pathway analysis.

Sex-differential gene expression in WT and Fabp2$^{-/-}$ *mice*

To determine the effect of Fabp2 loss on sex differential gene expression patterns, KEGG pathway analysis was performed on WT and *Fabp2*$^{-/-}$ mice (Fig. 1). In WT mice a total of 137 genes displayed differential expression in males and females compared to 115 in the *Fabp2*$^{-/-}$ group. Only 23 genes (23/252) were common between the two genotypes (Fig. 2a). The 137 differentially expressed genes in WT mice corresponded to 17 KEGG pathways (Fig. 2b). Four of the 17 pathways were directly involved in lipid metabolism in the intestine. In contrast,

only 4 KEGG pathways were elucidated from the differentially expressed genes of the *Fabp2*$^{-/-}$ genotype and none were directly related to lipid metabolism (Fig. 2b). Analysis of the common, differentially expressed genes revealed 3 pathways that were maintained in WT and *Fabp2*$^{-/-}$ mice, all of which were related to insulin signaling (Fig. 2b). The considerable variation in sex-differential gene expression between WT and *Fabp2*$^{-/-}$ mice indicates that loss of Fabp2 modulates the expression of differentially expressed genes in a sex-specific manner.

Changes in intestinal gene expression in male and female Fabp2$^{-/-}$ *mice*

Since the effect of genotype on sex-differential gene expression led to distinct differences between WT and *Fabp2*$^{-/-}$ mice with little overlap (Fig. 2a), we examined the effects of Fabp2 loss in individual sexes to determine the sex-specific and androgynous response. The loss of Fabp2 altered the expression of 226 genes in males (Table 1a) and 182 in females (Table 1b). In males approximately 82% (185/226) of the differentially expressed genes contained a Mouse Genome Informatics identifier (MGI ID) annotation while 77% (140/182) of the differentially expressed genes in females was fully annotated. The loss of Fabp2 had differential effects on the number of genes that were up- and downregulated in male and female mice. In males, 132 genes were upregulated while 94 genes were downregulated. In females 41 genes were upregulated while 141 genes were downregulated. A total of 38 significantly enriched biological processes were obtained by querying the male *Fabp2*$^{-/-}$ gene list with associated GO terms compared to only 21 biological processes in the female *Fabp2*$^{-/-}$ mice. Lipid metabolic processes were the most significantly enriched GO term in males

(GO:0006629) and females (GO:0044255) with 22 and 13 unique genes respectively (Table S1 and S2). Both males and females displayed a similar, robust repression of *Fabp2*, which served to confirm that these animals do not produce Fabp2 mRNA. Taken together these results demonstrate that the loss of Fabp2 leads to sex differences in the expression of genes involved in intestinal lipid metabolism.

Upregulated pathways in male and female Fabp2$^{-/-}$ mice

To further elucidate the sex specific effects of Fabp2 loss we created separate gene lists for up- and down- regulated genes and performed a pathway analysis using the KEGG pathway feature of the WebGestalt program. For genes that were upregulated in males, 14 pathways were significantly enriched including PPAR signalling pathway, glycerolipid metabolism, linoleic acid metabolism, arachidonic acid metabolism, and fatty acid metabolism (Fig. 3). A common feature of the upregulated pathways in males is the increased mRNA abundance of members of the cytochrome P450 family. In females only the PPAR signalling pathway was significantly enriched (Fig. 3), although the gene list featured a number of the same genes involved in the male PPAR signalling pathway including stearoyl CoA desaturase-1 (*Scd1*), malic enzyme-1 (*Me1*), and cytochrome P450, family 4, subfamily a, polypeptide 10 (*Cyp4a10*).

Downregulated pathways in male and female Fabp2$^{-/-}$ mice

For genes that were downregulated, KEGG pathway analysis revealed a greater number of significantly enriched pathways in females compared to males,

with 7 and 3 pathways respectively (Fig. 3). In contrast to males, females showed downregulation of the cytochrome P450 pathways for drug and xenobiotic metabolism, pathways which feature *Cyp2b10* and *Cyp2c55*. No identical pathways were downregulated in both sexes, with females displaying reductions in glutathione and nitrogen metabolic pathways, and males displaying reductions in the nicotinate/nicotinamide and adipocytokine signalling metabolic pathways.

Androgynous effects of loss of Fabp2 in male and female mice

By comparing the overlap in the 226 and 182 genes that were differentially expressed in male and female *Fabp2*$^{-/-}$ mice it is possible to determine the androgynous response to Fabp2 ablation. A Venn diagram illustrates the genes that are common and unique-to-male and unique-to-female *Fabp2*$^{-/-}$ mice (Fig. 4a). KEGG pathway analysis of these 74 genes yielded 5 enriched pathways (Fig. 4b). In concordance to the previous analysis of individual sexes, the PPAR pathway was the most significantly enriched with 6 unique genes.

Genes and pathways that are uniquely altered in male or female Fabp2$^{-/-}$ *mice*

We surmise that a comparison of the unique gene lists obtained from *Fabp2*$^{-/-}$ males and females, respectively, would reveal similar pathways to the comparison of WT mice. This would account for the loss of sex-differential gene expression in the transcriptome comparison of male and female *Fabp2*$^{-/-}$ mice. Our analysis of sex-differential gene expression in WT mice (i.e., WT male compared to WT female) revealed the majority of genes (111/137) to be

upregulated in females. However in our analysis of the sex-specific effects of *Fabp2* loss we noted a distinct trend towards upregulation of pathways in males and downregulation of pathways in females (Fig. 3). In fact, 9/14 upregulated pathways in male *Fabp2*$^{-/-}$ mice and 4/7 downregulated pathways in female *Fabp2*$^{-/-}$ mice were obtained in the sex-differential analysis of WT mice (Fig. 2b). The male-specific upregulation of pathways and female-specific downregulation of pathways may account for the androgynization of the *Fabp2*$^{-/-}$ intestine.

In addition to the androgynous response to *Fabp2* ablation there are sex-specific changes in gene expression that contribute to the sex-dimorphic *Fabp2* phenotype. The 152 unique probes that were differentially expressed in males were significantly enriched in 11 KEGG pathways (Fig. 4b). The majority of these pathways are involved in the metabolism of lipids and lipid soluble nutrients. In contrast to the previous analysis of male pathways, the glycerolipid metabolic pathway was the most significantly enriched. There was also significant enrichment in pathways related to the metabolism of the long chain unsaturated fatty acids.

A total of 5 unique pathways were obtained by querying the unique-to-female *Fabp2*$^{-/-}$ gene list in the KEGG pathway database. In concordance with the previous analysis of downregulated pathways, the two most significantly enriched pathways derived from the gene list unique-to-female *Fabp2*$^{-/-}$ mice were the metabolism of drugs and xenobiotics by cytochrome P450 (Fig. 4b). Additionally, the PPAR, nitrogen, and glutathione metabolic pathways were also detected from the unique-to-female *Fabp2*$^{-/-}$ gene list.

Discussion

The aim of this paper was to present a facile approach for detecting sex-specific and sex-differential changes in transcriptome data, and to use this method to analyze the transcriptome of small intestine of WT and *Fabp2*$^{-/-}$ mice. The analysis of WT mice identified sex-differential gene expression enriched in 17 KEGG pathways. The most significantly enriched pathways belonged to the metabolism of drugs and xenobiotics by cytochrome P450, with sex-differential expression of Cyp2 family members (*Cyp2b10, Cyp2c29*, and *Cyp2c55*). Consistent with this finding, studies in rodents and humans have demonstrated sex differences in the abundance of Cyp2 family members in various tissues [118, 120-121], although one study reported no differences in the expression of *Cyp2c18* and *Cyp2c19* in the murine small intestine [119]. A number of pathways involved in the metabolism of long-chain unsaturated fatty acids were significantly enriched and dimorphic in males and females, suggesting that lipid metabolism and transit through the enterocyte are regulated in a sex-specific manner. In support of this idea, postprandial appearance of radiolabelled oleate in plasma is greater in the gastrointestinal circulation of women than men [132]. Sex differences in the intestinal transit of lipids could also affect the bioavailability and efficacy of lipid-soluble micronutrients and drugs that use lipid analogs as vehicles for delivery. We found that 3 genes in the retinal metabolic pathway displayed sex-differential gene expression in WT mice, implying a link between the metabolic processing of lipids and lipid-soluble nutrients in the intestine.

Interestingly, we found that mice lacking Fabp2 have less sex-dimorphic pathways in the small intestine than WT mice, suggesting that loss of *Fabp2* caused the male and female intestine to become more similar (i.e., androgynized). Since the small intestine is responsible for the trafficking of all diet-derived lipids and lipid soluble nutrients, it is possible that the loss of Fabp2 amplifies sex-differences in other organs, such as the liver. Given the role of *Fabp2* in lipid metabolism and the sex-dimorphic response to high fat diet, it is not surprising that loss of *Fabp2* modulates the same pathways that exhibit sex differences in WT mice. Only 3 of the 17 significantly enriched pathways in WT mice were obtained from the sex-dimorphic gene list in *Fabp2*$^{-/-}$ mice, and all 3 involved the insulin signaling pathway. The only sex-dimorphic pathway unique to *Fabp2*$^{-/-}$ mice was the chemokine signaling pathway, with 4 genes significantly upregulated in males compared to females. It is known that pro-inflammatory factors can trigger insulin resistance and lead to aberrant lipid metabolism in the liver, skeletal muscle, and adipose tissue [133-135].

We also observed a similar degree of enrichment and upregulation of the PPAR signaling pathway in both male and female *Fabp2*$^{-/-}$ mice compared to their respective WT counterparts. In particular, the PPAR-controlled lipogenic pathway for the biosynthesis of unsaturated fatty acids was stimulated in male and female *Fabp2*$^{-/-}$ mice. This suggests that loss of Fabp2, the main species of intestinal Fabp involved in binding saturated fatty acids [124], leads to increased expression of the machinery involved in the production of unsaturated fatty acids. This might act as a compensatory mechanism to prevent the accumulation of saturated fatty acids in the cytoplasm, which cause a greater degree of lipotoxicity

and cell death compared to monounsaturated fatty acids [136]. In addition, the enterocytes of *Fabp2*$^{-/-}$ mice might be protected from increased lipogenesis by the liver fatty acid binding protein (Fabp1, encoded by *Fabp1*) which is co-expressed in the small intestine and displays a preference for unsaturated fatty acids and other PPAR ligands [137]. However, our analysis revealed an androgynous stimulation of the PPAR-controlled lipogenic pathway without increasing Fabp1 mRNA abundance on a low-fat diet, suggesting that loss of Fabp2 does not result in a compensatory increase in *Fabp1* transcription. The ability of Fabp1 to compensate for the loss of Fabp2, especially in response to a high dietary fat load, remains to be determined.

When the unique-to-male *Fabp2*$^{-/-}$ gene list (i.e., after removal of androgynous genes) was examined the most significantly enriched pathway was the glycerolipid metabolic pathway, and this was upregulated compared to male WT. Activation of PPARα by a synthetic ligand has been shown to stimulate the glycerolipid metabolic pathway in the intestine, although this was only documented in male mice [138]. Interestingly, the genes that were upregulated in the glycerolipid pathway of the male *Fabp2*$^{-/-}$ mice encoded enzymes with lipase activity, such as pancreatic lipase. Induction of pancreatic lipase gene expression in the small intestine of male rats has been observed previously following acute and chronic fat feeding [139]. This raises the possibility that triacylglycerol (TG) and free fatty acid concentrations in enterocytes might be regulated at the level of TG degradation. [139]. In male *Fabp2*$^{-/-}$ mice, increased lipase expression in the small intestine might decrease the amount of TG available for incorporation into chylomicrons. It has been observed that the ratio of TG to phospholipids in the

intestinal mucosa of *Fabp2*$^{-/-}$ mice is reduced compared to wild-type mice [45], but it is not yet clear if this is a sex-specific effect. The excess fatty acids in the enterocytes are likely shuttled to the liver via the portal circulation, which is likely responsible for the development of fatty liver in male but not female *Fabp2*$^{-/-}$ mice [3]. The intestine of female *Fabp2*$^{-/-}$ mice apparently employs a different strategy for coping with the loss of Fabp2. However, our present transcriptome analysis did not reveal alternate pathways for coping with saturated fatty acids in female *Fabp2*$^{-/-}$ mice; it is possible these pathways are activated on a high-fat diet.

A single nucleotide polymorphism in the *FABP2* gene resulting in the replacement of the alanine-54 codon with a threonine codon (A54T) was originally suggested as being associated with the development of obesity and insulin resistance in Pima Indians [5]. Many of the earlier studies focused on the effect of the variation on fatty acid binding and metabolism. However, the functional studies as well as association studies in different human populations yielded conflicting results. Recently, it became apparent that the *FABP2 T54* allele in Pima Indians is also tightly linked to a promoter variant in this population. When tested in Caco-2 cells, this promoter variant displayed ~3-fold lower basal activity [140]. In this regard, *Fabp2*$^{-/-}$ mice represent a model of extreme attenuation of *FABP2* gene promoter function. Similar polymorphisms have also been found in other human populations [6, 141-143]. The *FABP2* "promoter B" variant [142] which shares the same deletion found in the Pima Indian *FABP2 T54* gene promoter was found to be associated with both the A54 and T54 alleles in a German cohort [6], and individuals from this cohort who are homozygous for both the promoter B and T54 alleles exhibit increased

postprandial lipemia and lower insulin sensitivity [6]. However, the interplay between *FABP2* promoter B and A54 and T54 alleles requires further clarification since the *FABP2* promoter B was found to be associated with reduced risk of type 2 diabetes in male subjects of another German cohort [142].

In summary, we described an approach to visualize sex-dimorphic and sex-specific gene expression patterns in the murine intestinal transcriptome under a given metabolic condition. Using this approach to analyze the small intestine transcriptome of mice lacking *Fabp2,* a gene involved in dietary fat metabolism, we found distinct responses of males and females to the loss of Fabp2 thus indicating that sex is an important modifier of intestinal gene expression. The ability to analyze transcriptome data to identify changes in gene expression program in response to specific metabolic or genetic perturbations simultaneously in both sexes offers a powerful approach for gaining insights into sex differential interactions between nutrients and genes.

Acknowledgments

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Figures

Figure. 1

Schematic of microarray data analysis and pathway enrichment analysis. Data for duplicate chips were merged FlexArray. Fold change data was exported to a spreadsheet for comparison of sex-differential and sex-specific effects of *Fabp2* ablation. Lists of differentially expressed genes from these comparisons were uploaded to the WebGestalt server for pathway enrichment analysis.

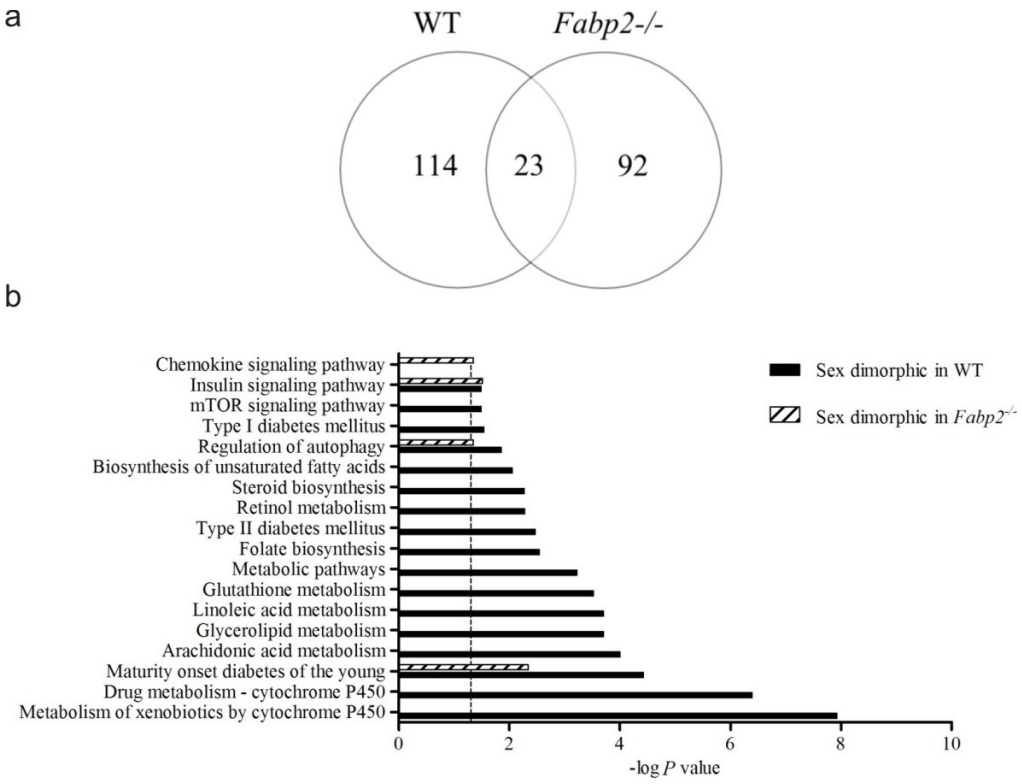

Figure. 2

Sex-dimorphic gene expression and pathway analysis in WT and *Fabp2*$^{-/-}$ mice. Venn diagram showing common and differentially expressed probesets (a). Pathway enrichment analysis from WebGestalt server (b). Black and hatched bars indicate significantly enriched, sex-dimorphic pathways in WT and *Fabp2*$^{-/-}$ mice respectively. The p value of each pathway is represented as a –log p value (>1.3, dashed line) which is equivalent to $P<0.05$.

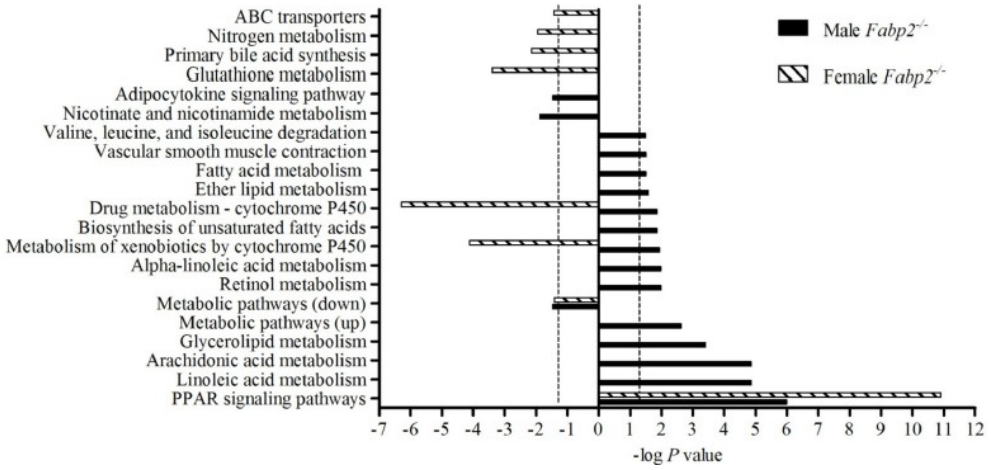

Figure. 3

Up- and downregulated pathways in male and female *Fabp2*$^{-/-}$ mice compared to their respective WT counterparts. Black and hatched bars indicate significantly enriched pathways from sex-specific gene lists in male and female *Fabp2*$^{-/-}$ mice, respectively. The *P* value of each pathway is represented as a –log *P* value (<-1.3 or >1.3, dashed lines) which is equivalent to $P<0.05$. The –log *P* value for downregulated pathways was multiplied by -1 to display downregulated pathways on the left of the y-axis for ease of visualization.

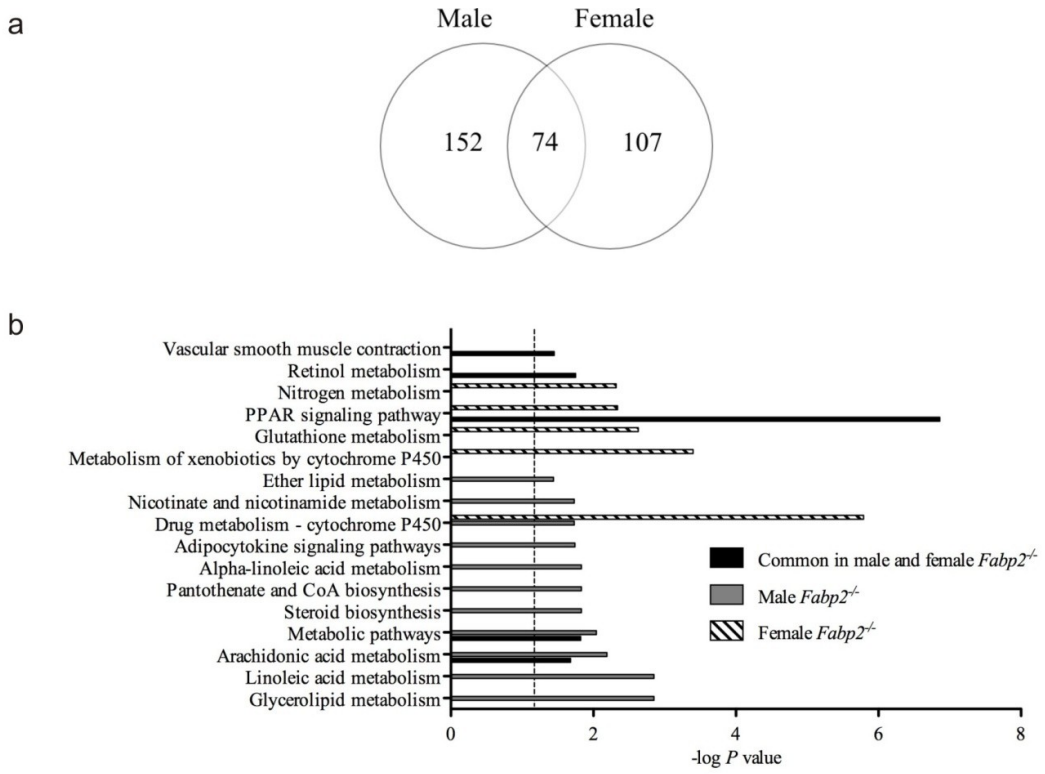

Figure.4

Common and sex-specific effects of *Fabp2* ablation. Venn diagram showing common and differentially regulated probesets that are common to *Fabp2*$^{-/-}$ mice and unique to either sex (a). Pathway enrichment analysis of common and unique probesets from the WebGestalt server (b). Black bars indicate pathway enrichment from probesets differentially expressed in male and female *Fabp2*$^{-/-}$ mice compared to their respective WT counterparts. Grey and hatched bars indicate pathway enrichment from gene lists unique to male and female *Fabp2*$^{-/-}$ mice respectively. The *P* value for each pathway is represented as the – log *P* value (>1.3, dashed line) which is equivalent to $P<0.05$.

Bridge section between Manuscript 2 and Manuscript 3

The previous microarray analysis of the effects of Fabp2 loss in mice revealed several distinct metabolic pathways that are perturbed in a both sexes or in a sex-specific manner. Notably, both male and female *Fabp2*$^{-/-}$ mice displayed an upregulated PPAR signalling pathway, with gene enrichment in the PPARα-lipogenic pathways involved in the production of long chain, unsaturated fatty acids. This was expected, as Fabp2 is suspected of being the primary FABP involved in the intracellular trafficking of saturated fatty acids. In the previous study, mice were fed a standard chow diet to determine the effects of Fabp2 loss without additional experimental perturbations such as high fat and high cholesterol content of the diet. When challenged with a high fat diet, loss of Fabp2 might lead to additional perturbations in the intestinal fatty acid metabolic pathways.

Intestinal lipid metabolism can be broadly categorized by genes involved in either the uptake or intracellular metabolism of fatty acids. Fatty acid uptake at the apical surface of the enterocyte is regulated by a series of transporters, which include the integral membrane proteins CD36 and Fatp4. Once fatty acids are internalized, the intestinal FABPs (Fabp1, 2, and 6) are responsible for shuttling fatty acids and other lipid soluble compounds to various organelles for metabolism and regulation of gene expression. For instance, fatty acids are the natural ligand for the PPAR family of nuclear receptors which regulate the expression of numerous fatty acid metabolic genes including *Srebp1-c* and *Scd-1*. We identified several of the genes involved in fatty acid uptake and intracellular fatty acid metabolism in the previous microarray experiment, suggesting that these processes might be impaired in mice lacking Fabp2. However, it has yet to be determined if Fabp2 loss leads to functional differences in fatty acid uptake capacity and intestinal lipid metabolic gene expression when challenged with a high fat diet. The following experiment was performed to determine if loss of Fabp2 leads to regional differences in the lipid uptake capacity and lipid-metabolic gene expression in the small intestine. Saturated fats were used as the primary source of energy in the high fat diets to stimulate the pathways that

upregulated in male and female *Fabp2*$^{-/-}$ mice under low-fat conditions. Genes identified from the previous microarray experiment as well as other potential candidates for contributing to the *Fabp2*$^{-/-}$ phenotypes were chosen for qPCR analysis. Unlike the microarray experiment which measured gene expression in the whole small intestine, the high fat diet feeding study measured gene expression and lipid uptake capacity in continuous segments of the small intestine in order to construct a high resolution spatial map of regions where lipid uptake and metabolism might be impaired. A secondary objective was to correlate the defect in intestinal lipid metabolism to systemic, whole body changes in metabolism using an intraperitoneal glucose tolerance test. From our analysis, loss of Fabp2 led to distinct differences in lipid uptake capacity, lipid metabolic gene expression, and glucose tolerance, highlighting the importance of lipid metabolism within the enterocyte in the regulation of whole body lipid metabolism.

Manuscript 3: Spatial patterns of lipid uptake and gene expression

Spatial differences in the uptake capacity of long chain fatty acids and expression of lipid metabolic genes in the small intestine of wild-type and *Fabp2* gene-ablated mice in response to low fat and high fat feeding

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A version of this chapter is in preparation for submission.

Key Words: FABPs, small intestine, gene expression, spatial, lipid metabolism, sex differences, lipid uptake, glucose tolerance, fatty liver.

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Abstract

Background: The mammalian small intestine contains three members of the fatty acid binding protein (FABP) family: Fabp1, Fabp2, and Fabp6. Mice lacking Fabp2, the intestinal fatty acid binding protein, display a sex dimorphic response to high fat diet featuring weight gain and fatty liver in males but not females. It has yet to be determined whether spatial differences in intestinal fatty acid uptake capacity and lipid metabolic gene expression contribute to the sex-dimorphic *Fabp2*$^{-/-}$ phenotype.

Methods: *Fabp2*$^{+/+}$ (wild-type) and *Fabp2*$^{-/-}$ mice were conditioned on a low-fat diet or a high saturated fat diet. Capacity of the small intestine to absorb C16:0, C18:0, C18:1, C18:2 fatty acids and cholesterol along the proximal to distal axis was measured ex vivo. In a separate study, mice were fed a diet high in saturated fatty acids with or without 0.2% cholesterol for 3 weeks and the expression of genes involved in the regulation of lipid uptake and intracellular lipid metabolism were assessed by qPCR.

Results: Female *Fabp2*$^{-/-}$ mice conditioned on the high saturated fat diet displayed a greater capacity for the uptake of saturated fatty acids (C16:0 and C18:0) and cholesterol along the proximal and distal intestine than female wild-type mice. Loss of Fabp2 resulted in an increased mRNA abundance of the apical lipid transporter CD36 and intracellular protein Fabp1 in female *Fabp2*$^{-/-}$ mice. High fat-feeding impaired glucose tolerance in male *Fabp2*$^{-/-}$ whereas it was improved in female *Fabp2*$^{-/-}$ mice.

Conclusions: Loss of Fabp2 caused sex-specific changes in lipid uptake capacity and expression of genes involved in intestinal lipid transport and metabolism in distinct regions the small intestine. Female mice might be protected from the adverse effects of Fabp2 loss by a compensatory increase in *Fabp1* expression.

Introduction

The role of fatty acids as an energy source has been clearly defined. However the ability of fatty acids to modulate the gene expression program of an organ system, and subsequently alter the downstream metabolic programs of other organ systems remains a largely unexplored area. Regulation of fatty acid metabolic processes in cells involves a highly coordinated system of proteins involved in the internalization, intracellular transport, and metabolism of fatty acids and lipid soluble nutrients in the cell membrane, cytoplasm, and organelles. Fatty acid binding proteins (FABPs) are a highly conserved and abundant class of 14-15 kDa cytoplasmic proteins involved in the uptake, transport, and metabolism of lipids and lipid soluble nutrients [1]. Since their discovery in 1972, nine members of the multigene FABP family have been identified (FABP1-FABP9) [1]. The members of the FABP family display a diverse range of tissue distribution and are abundant in tissues with a high lipid-metabolizing capacity such as the intestine, liver, adipose tissue, and heart [1]. Although the amino acid sequence of the FABPs share only 20-70% sequence similarity, the tertiary structure is highly conserved [42]. This consists of 10 anti-parallel β-strands with two short α-helices that form a structure known as the β-clam [42, 144]. It is the water-filled interior cavity of the β-clam structure that is responsible for ligand binding, and FABPs are able to bind a diverse range of lipid soluble ligands with varying affinity [145]. Despite their ubiquitous nature, the exact function of FABPs in lipid metabolism is unclear. In vitro studies using recombinant FABPs have suggested a role for FABPs in the transport of fatty acids within the intracellular compartments [146-147]. In this sense, FABPs might act as subcellular gatekeepers, controlling the metabolic fates of their ligands by directing them towards organelles where they are stored as part of the lipid droplet, converted into more complex fatty acids, oxidized for energy, or act as signalling molecules for the regulation of gene expression. Interestingly, studies on FABP gene-ablated mice show that the loss of a single FABP does not result in a lethal phenotype. This is likely attributable to the fact that most tissues express

more than one of the FABP family members, leading to functional overlaps in ligand binding [42].

The mammalian small intestine has been the subject of particular focus in FABP research, as the small intestine is the point of entry for dietary fats and lipid soluble nutrients. The small intestine expresses three members of the FABP family; Fabp1, Fabp2, and Fabp6, which vary in their spatial distribution and ligand preference. Fabp1 is primarily expressed in the proximal half of the small intestine and has a greater affinity for long-chain unsaturated fatty acids [148], while Fabp6 is only expressed in the distal 1/3 of the intestine and is preferentially binds bile acids [2, 149]. Fabp2 is expressed throughout the small intestine but is more abundant in the distal half of the organ and displays a higher affinity for saturated fatty acids [2, 124]. Fabp2 is unique amongst the intestinal FABPs in that it is the only one that is expressed exclusively in the small intestine, which makes *Fabp2* gene-ablated mice useful for probing the functions of FABPs in the intestine. Since Fabp2 expression is confined to the small intestine it was surmised that Fabp2 might be essential for the uptake of dietary fat. However mice lacking Fabp2 were viable and fully fertile, suggesting that Fabp2 is not essential for fat uptake [4]. Interestingly, when these mice were challenged with a high fat in the absence of cholesterol diet they displayed a sex-dimorphic phenotype; male mice gained weight and developed fatty liver while female mice were protected from weight gain, fatty liver, and displayed improved glucose tolerance [3, 114]. We recently performed a transcriptome analysis on the small intestine of low fat-fed wild-type and *Fabp2*$^{-/-}$ mice and found a male-specific upregulation of metabolic pathways involved in the production of long-chain unsaturated fatty acids and a female-specific downregulation of genes involved in xenobiotic-metabolizing pathways (See Manuscript 2). Taken together, these studies suggest that loss of Fabp2 leads to the dysregulation of nutrient and xenobiotic metabolic pathways in the small intestine, which might contribute to the systemic, sex-dimorphic phenotype observed in *Fabp2*$^{-/-}$ mice. Since Fabp1 and Fabp2 display an overlap in their spatial expression pattern and ligand binding affinity, it is possible that Fabp1 can compensate for the loss of Fabp2

function in *Fabp2* gene-ablated mice. A recent study examining the effects of Fabp1 or Fabp2 loss in the small intestine of mice found an increased mRNA abundance of the apical fatty acid and cholesterol transporters CD36 and Npc1l1 in mice lacking either Fabp1 or Fabp2 mice [45]. In a different study, conditioning the small intestine with a high fat diet upregulated the expression of the apical lipid transporters Fatp4 and CD36 while increasing the abundance of Fabp1 and Fabp2 [41]. These studies suggest that Fabp1 and Fabp2 participate in the pathways responsible for lipid uptake.

The current study was undertaken to examine the relationship between lipid uptake capacity and the expression of lipid metabolic genes in continuous segments of the small intestine in male and female wild-type and *Fabp2*$^{-/-}$ mice fed a low fat, high saturated fat, or high saturated fat/ high cholesterol diet. We hypothesized that loss of Fabp2 in the small intestine would lead to a compensatory increase in lipid uptake capacity in regions where Fabp1 is highly abundant. We also predicted that the loss of Fabp2 would lead to changes in the expression of genes involved in the uptake, transport, and metabolism of saturated fatty acids when challenged with a high saturated fat diet. Finally, we predicted that male and female *Fabp2*$^{-/-}$ mice would display sex-dimorphic changes in spatial and total uptake capacity and lipid metabolic gene expression in response to high fat feeding. We observed a female-specific increase in the uptake capacity for saturated fatty acids in the proximal and distal small intestine, as well as an increased abundance of Fabp1 and CD36 mRNA in female *Fabp2*$^{-/-}$ mice, suggesting that females might be protected from the adverse male *Fabp2*$^{-/-}$ phenotype by a transcriptionally controlled, Fabp1-mediated compensatory mechanism.

Methods

Animal Care

Male and female C57BL/6J (wild-type) mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Fabp2*$^{-/-}$ mice were produced in-house and were

backcrossed onto the C57BL/6J background for a minimum of 6 generations. Mice were housed in a controlled environment featuring a 12 hour reversed light/dark cycle and were maintained on a standard low fat chow diet (Purina 5001) for at least 3 weeks before receiving the custom diet. At the onset of the study, wild-type and *Fabp2*$^{-/-}$ mice (n=5/diet/sex) were fed either a low fat chow diet (Purina 5001) or a semi-purified diet (custom prepared by Harlan Teklad, Madison WI) containing high saturated fat (TD10451) or high saturated fat with 0.2% cholesterol (TD10452). Both high fat diets were comprised primarily of palm oil (49%) and provided 42.9% of kcal derived from fat. Mice were housed in standard cages (n=5 per cage) and given free access to food and water with the exception of at the beginning and end of the study when food was withheld over one light cycle in order to measure fasting blood chemistry. After a two week acclimation period to the experimental diets, a subset of wild-type and *Fabp2*$^{-/-}$ mice (n=3/diet/sex) were randomly selected and placed in individual metabolic cages for body weight balance experiments. In these experiments body weight, food intake, food consumption, and fecal output were monitored once daily for a period of three days while on the experimental diets. At the conclusion of the balance experiments all mice were returned to their original cages and maintained on the experimental diet until they were euthanized at the completion of the experiment. The use of animals in this study was approved by the animal care committees at the University of Alberta and McGill University.

Collection of blood and tissue samples

For baseline measurements, food was withheld for 16 hours starting at the beginning of the light cycle and ending four hours into the next dark cycle. At four hours into the dark cycle, blood samples were obtained by saphenous vein puncture and blood was collected in heparinised capillary tubes. Blood was immediately centrifuged to obtain plasma, and plasma was stored at -80°C for later analysis. On the day prior to the end of the study, food was withheld for 16 hours beginning at the start of the light cycle. Mice were anesthetised with 3% isoflurane at a constant flow of 1L/min and were euthanized by cardiac puncture.

Blood was collected in EDTA filled tubes and was centrifuged immediately to obtain plasma. The small intestine was removed distal to the pyloric sphincter and proximal to the ileoceacal valve, rinsed with cold saline and cut into six equal pieces (S1, proximal duodenum; S6, distal ileum). Each of these pieces was placed in RNA*later*® for RNA isolation and stored at -20°C.

Probe and marker compounds for lipid uptake

The [^{14}C]-labelled probes included fatty acids palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) (0.1 mM). The labeled and unlabeled probes were supplied by GE Healthcare Biosciences (Baie d'Urfe, QC) and Sigma Co. (St. Louis, Missouri) respectively. The long chain fatty acids were prepared by solubilizing them in 10 mM taurodeoxycholic acid (St Louis, Missouri) and in Krebs-bicarbonate buffer. The [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume. Probes were shown by the manufacturer to be more than 99% pure by high performance liquid chromatography.

Tissue preparation for lipid uptake assay

Mice (30-40 weeks old) used for the FA uptake assays were from a separate experiment. The animals were euthanized by an intraperitoneal injection of Euthanyl® (sodium pentobarbitol, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with 150 ml cold saline. The intestine was divided into 8 sections of similar length (S1, proximal jejunum; S8, distal ileum). Each section was everted and cut into 7 small rings of approximately 2-4 mm each. Rings from each of the 8 sections were used to examine the uptake of each of the substrates along the entire length of the small intestine [150]

The intestinal rings were immersed in pre-incubation beakers containing oxygenated (O_2-CO_2, 95:5 by volume) Krebs's buffer (pH 7.2) at 37^{o}C, and were allowed to equilibrate for approximately 5 minutes prior to the commencement of the uptake studies. Uptake was initiated by the timed transfer of the tissue rings

from the pre-incubation buffer to a 5 ml plastic vial containing [^{3}H]-inulin and [^{14}C]-labelled substrates in oxygenated Kreb's buffer that had been equilibrated to 37^{o}C in a shaking water bath. Short sections (2cm) of jejunum and ileum were incised and gently scraped with a glass slide to remove the mucosal tissue for determination of the weight of the mucosa and the remainder of the intestinal wall. These tissues were placed on separate glass slides, and were dried overnight in an oven at 55^{o}C. The dry weights of the mucosa and the remainder of the intestinal wall were determined.

Determination of uptake rates

After incubation of the intestinal rings in the lipid substrates for 5 minutes, the uptake of nutrient was terminated by pouring the vial contents onto filters on an Amicon vacuum filtration manifold that was maintained under suction. This was followed by washing the intestinal rings three times with ice-cold saline. The tissue rings were then placed on a glass slide, and were dried overnight in an oven at 55^{o}C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes [151]. The uptake rates were expressed as nmol *100 mg tissue $^{-1}$ min $^{-1}$.

Analysis of lipid uptake data

Mean uptake rates for each individual lipid species assayed was plotted on a line graph for each of the eight intestine segments. Total uptake capacity was calculated by taking the mean area under the curve (AUC) for each group. To determine the uptake capacity for the proximal and distal intestine, the AUC for the four proximal segments and the four distal segments for each group were calculated and plotted on a bar graph. Each uptake assay was performed on 4-6 mice and is represented as mean ± standard error of the mean.

Analysis of plasma

The concentrations of plasma lipids: triglycerides, non-esterified fatty acids, and cholesterol were measured in mice (15-25 weeks old) using commercially available diagnostic kits (Triglyceride Liquicolor® from Stanbio Laboratory Boerne TX, NEFA-C and T-Chol-E assays from Wako Chemicals Richmond VA). Hemolyzed samples were excluded from the analysis.

Analysis of fecal lipids

Fecal samples collected over the three day metabolic cage experiment (15-25 weeks old) were homogenized and extracted using the t-butanol method [152]. Each sample was then assayed for TG, NEFA, and cholesterol using the commercial kits stated above. Total fecal fatty acids were determined by adding the molar content of TG and NEFA in 50mg dried feces.

RNA isolation, quantification, quality control

For gene expression studies, mice (n=5/diet/sex) were fed the diets for 3 duration weeks. Liver and intestine segment total RNA was isolated using a modified version of the Chomczynski technique [125]. Bromochloropropane was substituted for chloroform for separation of aqueous and organic phases [153]. After centrifugation the RNA- containing aqueous phase was transferred to Maxwell® 16 total RNA purification cartridges (Promega Corporation, Madison WI) for RNA isolation using the Maxwell® instrument (Promega Corporation, Madison WI). Total RNA was diluted in 300µL nuclease-free water and an aliquot was taken for immediate quantification and quality control before being stored at -80°C. RNA concentration was measured using the Quant-IT™ standard range RNA assay (Invitrogen, Burlington ON) and the Qubit® fluorometer (Invitrogen, Burlington ON). RNA quality and purity was measured by gel electrophoresis using a boric acid/sodium borate buffer system, stained with ethidium bromide, and imaged under UV light. Samples that did not display the 2:1 ratio of 28S to 18S ribosomal RNA (indicative of RNA integrity) were excluded from the study.

cDNA synthesis

Total RNA (1µg) from small intestine was reverse transcribed using as previously described [154]. Reverse transcription reaction was carried out in a BioRad C1000™ Thermal Cycler (BioRad, Mississauga ON) at the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds.

Quantitative PCR

All qPCR assays were performed using the iQ™ SYBR® Green Supermix on the C1000/CFX96™ real-time detection systems (BioRad, Mississauga ON). All samples were run in duplicate and normalized to Gapdh [155] and Villin mRNA. PCR conditions were as follows: 95°C hot-start for 3 minutes, 95°C denaturation for 10 seconds, 59°C annealing/elongation for 30 seconds, 35 cycles, melting curve analysis from 65-95°C. Gene expression analysis was performed using the CFX96 software with the ΔΔCt method [156] and all genes expressed relative to 0. Runs that did not pass the CFX96 internal quality control test were not included in the final analysis. The mean relative mRNA abundance was represented using line graphs, displaying the mean abundance of each target along the six continuous intestine segments (S1-S6). The mean area under the curve (AUC) for each sex/genotype group was then plotted on a bar graph. Each target was assayed in 3-5 mice and is represented as mean ± standard error of the mean. Primer sequences for qPCR are listed in appendix 1. Primers for qPCR experiment were created in-house or were selected from PrimerBank [157]. Sequences are listed in Table 1.

Intraperitoneal glucose tolerance test (IPGTT)

Male and female WT and *Fabp2*$^{-/-}$ mice (n=4 per group) were maintained on the high saturated fatty acid diet without cholesterol for four weeks. Food was withheld for 12 hours before of the beginning of the test and all mice were given free access to water for the duration of the study. Fasting blood glucose was obtained by saphenous vein puncture and measured with the Contour® USB

Glucometer (Bayer, Toronto, ON). A glucose load of 2.5g/kg body weight was injected into the intraperitoneal cavity using a 25G needle fitted onto a syringe. Blood glucose was measured at 5, 15, 30, 60, and 120 minutes after glucose administration. After the final measurement mice were returned to their original cages. Blood glucose curves were plotted for each of the groups and the AUC was determined.

Statistics

All statistical calculations were performed on GraphPad Prism5 (La Jolla, CA) using two-way ANOVA and t-tests where appropriate. Differences were considered significant when $P<0.05$.

Results

Fatty acid and cholesterol uptake on a chow diet

The uptake capacity of common dietary fatty acid species and cholesterol was examined in eight continuous segments of the small intestine (S1-S8, proximal to distal). In general, lipid uptake capacity tended to be greater in females compared to males regardless of genotype (Fig. 1). Spatial mapping of lipid uptake capacity revealed similar patterns in both male and female wild-type and *Fabp2*$^{-/-}$ mice, with less variability in male mice compared to female mice. The total uptake capacity of C18:2 in female *Fabp2*$^{-/-}$ mice, as determined by taking the area under the curve of the spatial map, was greater compared to male *Fabp2*$^{-/-}$ ($P<0.05$), and can be attributed to an increased capacity for uptake in segments S4-S6 (Fig. 1). Cholesterol uptake capacity tended to increase from proximal to distal in both males and females, although none of these results reached statistical significance (Fig. 1).

Fatty acid and cholesterol uptake on a saturated fatty acid diet

We next examined whether conditioning the mice with a diet high in saturated fatty acids would lead to functional changes in the lipid uptake capacity of male and female wild-type and *Fabp2*$^{-/-}$ mice. In males, uptake capacity of the

saturated fatty acids C16:0 and C18: 0 tended to increase from proximal to distal, and were slightly higher in the *Fabp2*$^{-/-}$ group (Fig. 2). This resulted in a significantly greater total uptake capacity for C16:0 in male *Fabp2*$^{-/-}$ compared to male wild-type (P<0.05) (Fig. 2). In wild-type females, uptake capacity for C16:0 and C18:0 exceeded that of male wild-type (P<0.05). In addition, female *Fabp2*$^{-/-}$ mice displayed a trend towards increase saturated fatty acid uptake capacity compared to female wild-type, reaching statistical significance for the total C16:0 uptake capacity (P<0.05) (Fig. 2). In concordance with the sex difference observed in wild-type mice, female *Fabp2*$^{-/-}$ mice displayed a greater capacity for saturated fatty acid uptake compared to their male counterparts (Fig. 2).

For the unsaturated fatty acids C18:1 and C18:2 a similar pattern was observed. Females tended to have a greater total capacity for unsaturated fatty acid uptake compared to males, although this only reached statistical significance in the wild-type group (P<0.05) (Fig. 2). In *Fabp2*$^{-/-}$ females, uptake capacity of C18:1 and C18:2 was consistently greater in the proximal half of the small intestine (S2, S3) but lower in the distal portion (S7, S8). This resulted in a similar total uptake capacity between female wild-type and *Fabp2*$^{-/-}$ mice.

In concordance with the chow-fed mice, cholesterol uptake capacity increased along the length of the intestine from proximal to distal (Fig. 2). No changes in spatial or total uptake capacity were observed between male wild-type and *Fabp2*$^{-/-}$ mice. However female *Fabp2*$^{-/-}$ mice displayed a significantly greater capacity for cholesterol uptake in segments S3 and S4 as well as a greater total cholesterol uptake capacity (P<0.05) (Fig. 2).

Analysis of uptake capacity by proximal and distal segments

Since the spatial expression pattern of the intestinal FABPs might be related to functional differences in the uptake capacity of the segments where they are expressed, we next analyzed total uptake capacity of saturated fatty acids, unsaturated fatty acids, and cholesterol in the proximal and distal intestine. In agreement with the previous analysis, there was a trend towards chow-fed females having a greater capacity for uptake of saturated and unsaturated fatty acids in

both proximal and distal segments compared to males (Fig. 3a). In both males and female *Fabp2*$^{-/-}$ mice fed the chow diet, the cholesterol uptake capacity was greater in the distal portion of the small intestine compared to the proximal portion (P<0.05) (Fig. 3). No differences in cholesterol uptake capacity between the proximal and distal small intestine were observed in wild-type mice fed the chow diet.

Analysis of the proximal and distal uptake capacity of saturated fat-fed mice revealed similar results to the previous analysis. In general, females displayed an increased capacity for the uptake of saturated fatty acids and unsaturated fatty acids in both the proximal and distal regions of the intestine (Fig. 3). Female *Fabp2*$^{-/-}$ mice had a significantly greater saturated fatty acid uptake capacity in both the proximal and distal regions compared to wild-type females as well as *Fabp2*$^{-/-}$ males (P<0.05) (Fig. 3b). Mice lacking Fabp2 tended to have a greater capacity for unsaturated fatty acid uptake in both proximal and distal regions of the intestine, with the exception of the distal intestine in females. As expected, cholesterol uptake capacity was greater in both the proximal and distal regions of the intestine compared to wild-type females, although this only reaches statistical significance in the proximal half (P<0.05) (Fig. 3).

Validation of intestinal housekeeping genes

Since the aim of these experiments was to compare the spatial expression patterns of lipid uptake capacity with the expression of lipid metabolic genes in male and female wild-type and *Fabp2*$^{-/-}$ mice using qPCR, it was necessary to determine whether the experimental manipulations modulated the expression of putative housekeeping genes in the small intestine. Villin was selected as it is an intestinal epithelial-specific transcript while Gapdh was selected based on its previous use as an internal reference marker. No changes in the ratio of Gapdh to Villin were observed in male and female mice along the length of the small intestine in each of the six segments used for qPCR analysis (Fig. 4). Similarly, genotype and diet had negligible effects on the ratio Gapdh to Villin (Fig. 4), indicating that the various combinations of experimental manipulations did not

alter the constitutive expression patterns of these two markers. Based on these results, Gapdh and Villin are appropriate housekeeping genes for probing the effects of sex, diet, and genotype in the small intestine.

Lipid-metabolic gene expression in mice fed a chow diet

In chow-fed mice, total mRNA abundance of the apical fatty acid transporter CD36 was greater in both male and female *Fabp2*$^{-/-}$ mice compared to wild-type, and greater in female *Fabp2*$^{-/-}$ compared to male *Fabp2*$^{-/-}$ (P<0.05). Spatial mapping of CD36 showed a nearly uniform distribution along the length of the intestine, with a significantly greater abundance in S1-2, and S4-6 in female *Fabp2*$^{-/-}$ and in S2 of male *Fabp2*$^{-/-}$ (P<0.05) (Fig. 5). Spatial mapping of Scd1 mRNA abundance revealed differences in expression patterns. In females, Scd1 mRNA abundance remained constant in segments S1-S4 and then increased dramatically in S5 and S6 (Fig. 5). In contrast Scd1 mRNA abundance in males remained constant throughout the six intestine segments, and displayed a small increase in segments S5 and S6 (Fig. 5). This resulted in the total Scd1 abundance to be higher in females, although this only reached statistical significance in the female *Fabp2*$^{-/-}$ group. No differences in the spatial distribution or total mRNA abundance of Srebp-1c were observed between wild-type and *Fabp2*$^{-/-}$ of either sex (Fig. 5). Both Fabp1 and Fabp6 displayed the expected spatial distribution patterns, with Fabp1 being more abundant in the proximal intestine while Fabp6 was highly abundant in the distal portion of the small intestine (Fig. 5). Of note was a consistent small, but significant decrease in total Fabp6 abundance in *Fabp2*$^{-/-}$ females compared to *Fabp2*$^{-/-}$ males (P<0.05) (Fig. 5).

Lipid-metabolic gene expression in mice fed a diet high in saturated fats without cholesterol

We next examined whether the spatial differences in gene expression found in male and female *Fabp2*$^{-/-}$ mice persisted when the mice were conditioned on a diet high in saturated fat without cholesterol for a period of three weeks. Contrary to the expression pattern of CD36 observed in chow-fed mice, no

differences were found between wild-type and *Fabp2*$^{-/-}$, in either sex. The high fat content of the diet had an overriding effect on the *Fabp2*$^{-/-}$-specific upregulation of CD36 observed in chow-fed mice, leading to normalization of expression levels (Fig. 6). Despite this fact, females had a significantly greater abundance of CD36 in the whole small intestine compared to their male counterparts (P<0.05) (Fig. 6). For Scd1, high-fat diet feeding did not result in spatial changes in mRNA abundance in males, while females displayed a far greater degree of variability, although the total Scd1 abundance did not reach statistical significance (Fig. 6). High fat diet feeding resulted in the increased total abundance of *Srebp-1c* mRNA in male *Fabp2*$^{-/-}$ mice compared to wild-type, due to a significantly greater abundance in segments S2 and S3 (P<0.05) (Fig. 6). Spatial mapping of female Srebp-1c abundance revealed no changes between wild-type and *Fabp2*$^{-/-}$, although female wild-type had a greater abundance compared to male wild-type (P<0.05) (Fig. 6). Fabp1 mRNA abundance followed the expected distribution in all mice, although there was a trend towards increased Fabp1 mRNA in male and female *Fabp2*$^{-/-}$ compared to wild-type. In male *Fabp2*$^{-/-}$ mice, the increase in Fabp1 mRNA was distributed over the entire length of the small intestine while in females the increase occurred in regions of the small intestine where Fabp1 is normally highly abundant. Both male and female *Fabp2*$^{-/-}$ mice had elevated total Fabp1 mRNA compared to their wild-type counterparts although this only reached statistical significance in the female group. High-fat diet feeding also resulted in an increased total Fabp6 mRNA abundance in female *Fabp2*$^{-/-}$ compared to female wild-type and male *Fabp2*$^{-/-}$ mice, which can be attributed to an increases in segments S4-S6.

Lipid-metabolic gene expression in mice fed a diet high in saturated fats with 0.2% cholesterol

The addition of 0.2% cholesterol to the high saturated fat diet had a minimal impact on spatial and total lipid-metabolic gene expression patterns in the small intestine compared to the saturated fat diet without cholesterol. Spatial gene expression mapping on the high saturated fatty acid diet without cholesterol

largely matched the spatial maps obtained from mice fed the high saturated fat diet with cholesterol (Figs. 6, 7). CD36 total mRNA abundance was greater in female *Fabp2*$^{-/-}$ mice compared to male *Fabp2*$^{-/-}$ (P<0.05) (Fig. 7). In addition female *Fabp2*$^{-/-}$ mice displayed an increased abundance of Scd1 mRNA in segment S6 compared to wild-type (P<0.05), although the total Scd1 abundance was not statistically different (Fig. 7). The addition of cholesterol had the effect of minimizing the differences between Srebp-1c abundance in males and females compared to mice fed the saturated fat diet without cholesterol (Fig. 7). In concordance with the increased Fabp1 abundance in female *Fabp2*$^{-/-}$ mice fed the high saturated fat diet, mice fed cholesterol also displayed a similar increase in Fabp1 abundance compared to female wild-type (P<0.05) (Fig. 7). No changes in total Fabp6 abundance were observed in males or females of either genotype (Fig. 7).

Effect of diet on the Scd1:Fabp1 ratio

Our results demonstrate that female *Fabp2*$^{-/-}$ mice have an increased abundance of Fabp1 mRNA in regions of the intestine where they also exhibit an increased capacity for the uptake of saturated fatty acids. Since Scd1 is involved in the conversion of saturated fatty acids to unsaturated fatty acids, the natural ligand for Fabp1, we reasoned that the ratio between Scd1 and Fabp1 in the intestine might indicate regions of the intestine where fatty acid metabolic processes might be compromised. In chow-fed mice, the Scd1:Fabp1 ratio was significantly greater in male *Fabp2*$^{-/-}$ compared to male wild-type and female *Fabp2*$^{-/-}$ (P<0.05) (Fig. 8). The pattern of expression along the length of the small intestine also tended to be more variable in the chow-fed male group compared to female. Mice fed the high saturated fatty acid diet with and without cholesterol displayed a consistent increase in the Scd1:Fabp1 ratio from proximal to distal regardless of sex or genotype (Fig. 8).

Balance and anthropometry

Since our analysis of lipid uptake capacity and lipid-metabolic gene expression in the small intestine revealed differences in male and female *Fabp2*$^{-/-}$ mice, we next determined whether these changes resulted in differences in anthropometry, blood chemistry, and lipid balance. Caloric intake for individual mice on the chow and high saturated fat diets was measured over a three day collection period in a metabolic cage. Female *Fabp2*$^{-/-}$ mice fed the chow diet consumed a greater number of kilocalories in proportion to their body mass compared to male *Fabp2*$^{-/-}$ mice (P<0.05) (Fig. 9a). On the high saturated fat diets, both male and female *Fabp2*$^{-/-}$ mice consumed a greater proportion of kilocalories compared to their respective wild-type counterparts (P<0.05) (Fig. 9a). In addition, female *Fabp2*$^{-/-}$ mice on the high saturated fat diets consumed a greater proportion of kilocalories compared to male *Fabp2*$^{-/-}$ on the same diet (P<0.05) (Fig. 9a). After three weeks on the experimental diets, chow fed male *Fabp2*$^{-/-}$ mice gained more weight than the wild-type male mice fed the same diet. No differences in weight gain were observed between wild-type and *Fabp2*$^{-/-}$ mice of the same sex on either of the high fat diets (Table 2). Male *Fabp2-/-* mice exhibited a significantly greater liver weight as a percentage of body weight when fed the chow diet or the high saturated fat plus 0.2% cholesterol diet (P<0.05) (Fig. 9b). In contrast, the liver weight to body weight ratio in female *Fabp2*$^{-/-}$ mice was significantly lower than wild-type mice on the high saturated fat plus 0.2% cholesterol diet (P<0.05) (Fig. 9b). In chow-fed mice, total fatty acid excretion was greater in female *Fabp2*$^{-/-}$ compared to male *Fabp2*$^{-/-}$ and female wild-type (P<0.05) although male *Fabp2*$^{-/-}$ also displayed greater excretion than male wild-type (P<0.05) (Fig. 9c). No differences in fecal cholesterol output were observed in mice fed the chow diet, although there was a trend towards higher output in mice lacking FABP2 (Fig. 9d).

Plasma lipids

No differences in plasma TG were observed between male and female wild-type and *Fabp2*$^{-/-}$ within each diet group (Table 2). Non-esterfied fatty acid concentration tended to be lower in females fed the high fat diets compared to

males of the same genotype (P<0.05). The one exception to this was the female *Fabp2*$^{-/-}$ group fed the high saturated fat plus 0.2% cholesterol group, which displayed a modest increase in NEFA concentrations compared to wild-type. Regardless of diet, plasma cholesterol was significantly greater in male *Fabp2*$^{-/-}$ mice compared to male wild-type (P<0.05). In addition, the plasma cholesterol in females was significantly lower than males of the same genotype and fed the same diet (P<0.05) (Table 2).

Intraperitoneal glucose tolerance test (IPGTT)

On the basis of our results we surmised that the dietary fat content had a greater impact on the spatial changes in uptake capacity and gene expression than the presence of cholesterol. Since changes in the intestinal uptake and metabolism of dietary lipids might alter fatty acid flux in the liver, leading to impaired glucose tolerance, we performed an IPGTT on a separate group of mice fed the saturated fat diet for 5 weeks. Blood glucose concentrations in male *Fabp2*$^{-/-}$ mice were greater than male wild-type at all time points, reaching statistical significance at baseline (pre-injection) and at 60 and 120 minutes after injection (P<0.05) (Fig. 10). It should be noted that the glucose concentration of male *Fabp2*$^{-/-}$ mice surpassed the upper limit of the assay, and thus the differences between the wild-type group appear minimized. In contrast, glucose concentrations in female *Fabp2*$^{-/-}$ mice tended to be lower than female wild-type throughout the course of the experiment, and reached significance at the 15 and120 minute time points (P<0.05) (Fig. 10). When the area under the curve of the IPGTT compared, male *Fabp2*$^{-/-}$ had a greater blood glucose AUC than wild-type males, while the AUC in female *Fabp2*$^{-/-}$ was significantly lower than male *Fabp2*$^{-/-}$ (P<0.05) (Fig. 10).

Discussion

The aim of this paper was to assess the spatial effects of Fabp2 loss on lipid uptake capacity and lipid-metabolic gene expression in the murine small intestine. Our previous work on Fabp2-deficient mice demonstrated a sex dimorphic response to high-fat diet feeding, resulting in weight gain and fatty

liver in males but not females, as well as enhanced glucose tolerance in females [3, 114]. Transcriptome analysis of the wild-type murine small intestine revealed a number of lipid-metabolic pathways that display sex-differential gene expression patterns that are further modified in mice lacking Fabp2 (See Manuscript 2) [158]. These findings suggest that lipid transit across the enterocyte might be regulated in a sex-specific manner, although this has yet to be assessed at the level of lipid uptake capacity and lipid-metabolic gene expression in the small intestine.

In the present study, analysis of chow-fed mice revealed minimal differences in the spatial and total lipid uptake capacity along the length of the small intestine in wild-type and *Fabp2*$^{-/-}$ of either sex. In addition, there was little variation in the lipid uptake capacity from the proximal to distal regions of the small intestine. Since we measured lipid uptake capacity ex vivo, it remains to be determined whether this corresponds to a physiological property of the small intestine given the unidirectional flow and decreasing concentration of nutrients from proximal to distal [159].

The unique spatial distribution of FABPs in the small intestine [2], and their differential ligand-binding preferences [42], suggests that certain regions of the small intestine might be more efficient at absorbing lipids and lipid soluble nutrients than others. It is possible that the regions where Fabp1, Fabp2, and Fabp6 are expressed are related to local differences in lipid uptake capacity and enterocyte function, thus serving as functional landmarks of lipid metabolic processes. Spatial differences in the expression of nutrient metabolic genes along the length of the intestine have been identified and result in functional differences in discrete intestinal segments [160-162]. For instance, mRNA and protein abundance of the apical fatty acid transporter CD36 is greater in the proximal intestine compared to the distal intestine [163-164]. In another study, loss of CD36 resulted in the dysregulated expression of genes involved in chylomicron synthesis, lipid transport, and cholesterol uptake in the proximal but not mid- or distal intestine [165]. In enterocytes, this led to impaired uptake and metabolism of lipids and lipid soluble nutrients, suggesting that CD36 is required for optimal

lipid metabolism in the proximal intestine [165]. Since Fabp1 and Fabp2 are co-expressed in the proximal portion of the small intestine where CD36 is required for fatty acid uptake, it is possible that loss of either one of these FABPs might result in dysregulated uptake or intracellular metabolism of fatty acids and lipid soluble nutrients. In our study, no changes were observed in the mRNA abundance of Fabp1 in wild-type or *Fabp2*$^{-/-}$ mice fed the low-fat chow diet. This was expected, as the FABPs of the intestine are stimulated under conditions of increased dietary fat [41]. Consistent with previous observations of *Fabp2*$^{-/-}$ mice [45], we found a consistent increase in *CD36* mRNA abundance along the entire length of the small intestine in both male and female *Fabp2*$^{-/-}$ mice. The reasons for this occurrence are not yet clear; however it suggests that Fabp2 is either directly or indirectly involved in regulating the pathways involved in lipid assimilation. Since the increase in CD36 abundance did not correspond to an increased capacity for lipid uptake in *Fabp2*$^{-/-}$ mice, it is possible that CD36 is stimulated due to an overall decrease in lipid absorption efficiency in mice lacking Fabp2. Thus loss of Fabp2 might decrease the efficiency of lipid uptake and lead to a compensatory increase in the expression of genes involved in the assimilation of dietary fat. In support of this notion, overexpression of Fabp1 in mouse fibroblasts enhances fatty acid and cholesterol uptake [166], suggesting that lipid absorption efficiency is proportional to the total abundance of FABPs in cells.

Sex differences in lipid metabolism and metabolic disease risk have been well described [115] (See Manuscript 1), and the ability of fatty acids and cholesterol to modify the metabolic program of an organism is now becoming apparent. Fatty acids, cholesterol, and their metabolic by-products regulate cellular processes by controlling gene expression through transcriptional and post-transcriptional mechanisms. The extent to which sex differences in lipid uptake capacity and lipid-metabolic gene expression in the small intestine contribute to differences in other tissues with high lipid-metabolic activity remains to be determined. Our analysis revealed that the fat content of the diet, and not presence of cholesterol, was the most potent modifier of lipid uptake capacity and intestinal

lipid-metabolic gene expression in wild-type and *Fabp2*$^{-/-}$ mice. In general, female mice had a greater total capacity for the uptake of saturated fatty acids, unsaturated fatty acids, and cholesterol compared to males, and this corresponded to an increased total abundance of CD36 mRNA in the female intestine. Furthermore, the uptake of saturated fatty acids in the proximal and distal intestine of female *Fabp2*$^{-/-}$ mice was significantly greater than their wild-type counterparts. This finding was correlated to an increased abundance of Fabp1 mRNA in the proximal portion of the intestine in female *Fabp2*$^{-/-}$ compared to female wild-type. Since Scd1 is involved in the conversion of saturated fatty acids to unsaturated fatty acids, we predicted that Scd1 abundance would be greater in mice lacking Fabp2, the primary FABP involved in the transport of saturated fatty acids. In contrast to our previous transcriptome analysis of chow-fed mice, no differences in Scd1 abundance were observed between wild-type and *Fabp2*$^{-/-}$ mice of the same sex, although females generally had a greater abundance of Scd1 regardless of diet. Furthermore, no differences in the ratio of Scd1 to Fabp1 were observed in high fat-fed mice, suggesting that differences in Scd1 expression between wild-type and *Fabp2*$^{-/-}$ do not occur at the level of the transcriptome.

Srebp-1c is a cholesterol-regulated transcription factor that functions as a master regulator of triglyceride synthesis [167]. Srebp-1c transcription is positively regulated by oxysterols via the liver X receptors (LXRs) and negatively regulated by PPARs [72], suggesting that Srebp-1c is sensitive to cellular fatty acid and cholesterol status. In our study, loss of Fabp2 also led to an increase in Srebp-1c in male *Fabp2*$^{-/-}$, mainly due to increased an increased abundance in the proximal intestine. It is possible that the stimulation of Srebp-1c that we observed in saturated fat-fed male *Fabp2*$^{-/-}$ mice is another example of an inherent sex difference in PPAR-controlled gene expression. Likewise, the normalization of Srebp-1c abundance in mice fed the diet containing saturated fat with 0.2% cholesterol likely reflects the fact that these mice are sensitive to cellular cholesterol status. Whether the increased Srebp-1c abundance observed in male *Fabp2*$^{-/-}$ mice contributes to the development of obesity and fatty liver requires further study.

The pathophysiology of hepatic insulin resistance and diabetes involves the attenuation of insulin signalling leading to impaired regulation of lipid, carbohydrate, and amino acid metabolism [168]. Fatty liver is commonly associated with insulin resistance [169] and is an indicator of impaired lipid hepatic lipid metabolism. Although the liver is regarded as an important site of energy regulation, the small intestine is responsible for directing the flow of nutrients and other food-borne materials to the liver and other organ systems and determining their metabolic fates. The effects of impaired energy metabolism at the level of the small intestine on the pathophysiology of insulin resistance and diabetes remain to be determined. We previously reported that loss of Fabp2 in mice leads to the development of fatty liver in males and improved glucose tolerance in aged females [3-4, 114]. In addition, mutations in the human FABP2 gene have been linked to the development of insulin resistance and diabetes in various subsets of the population [5-6]. To determine the effects of Fabp2 loss on whole body energy homeostasis we performed an IPGTT on high saturated fat-fed wild-type and Fabp2$^{-/-}$ mice. Our analysis revealed impaired glucose tolerance in male *Fabp2*$^{-/-}$ mice and improved glucose tolerance in female *Fabp2*$^{-/-}$ mice compared to their respective wild-type counterparts. It is possible that the impaired glucose tolerance in male *Fabp2*$^{-/}$ mice is due to an increased delivery of non-esterified fatty acids to the liver via the portal circulation^{-}; however since we did not measure the non-esterified fatty acid content of the portal circulation this mechanism remains to be experimentally confirmed. On the other hand, it is possible that an increased abundance of Fabp1 in female *Fabp2*$^{-/-}$ mice acts to sequester lipids in the enterocyte and prevent an overload of non-esterified fatty acids in the portal circulation. In support of this notion, total fatty acid excretion was higher in female *Fabp2*$^{-/-}$ compared to male *Fabp2*$^{-/-}$. Since our data demonstrates sex differences in lipid flux at the apical surface of the enterocyte and in the expression of the intracellular lipid metabolic machinery, it is likely that the small intestine represents an important site for the regulation of whole body lipid metabolism in males and females.

In Summary, although the ablation of Fabp2 does not impair lipid absorption in mice, it leads to spatial differences in uptake capacity and intestinal lipid metabolic gene expression among males and females. From our analysis it is apparent that the uptake and metabolism of dietary fat in the enterocyte is regulated in a sex-dimorphic manner, and warrants further study to determine the role of intestinal lipid metabolic program in the development of acquired metabolic disorders.

Acknowledgments

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Figures

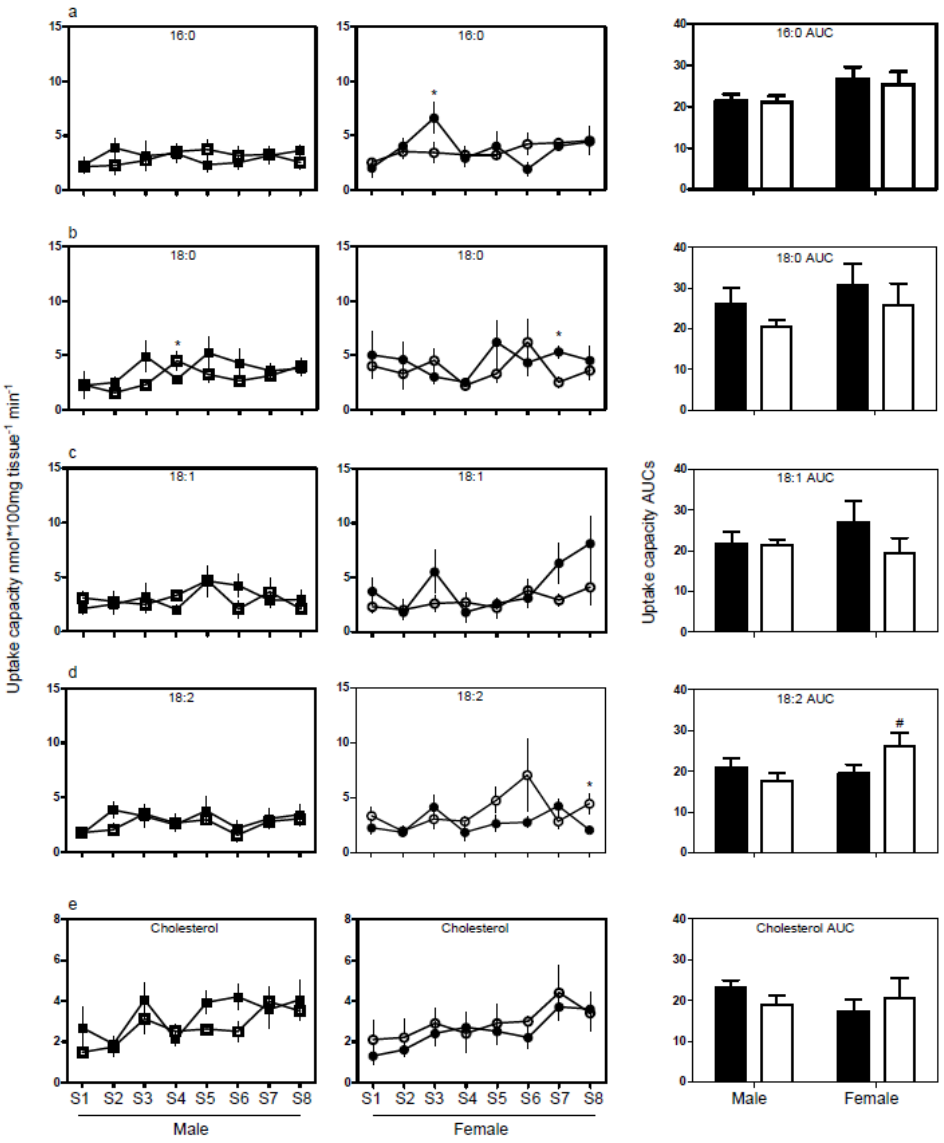

Figure 1.

Spatial and total lipid uptake capacity in intestinal segments of mice fed a chow diet. Intestinal segments are designates S1-S8 from proximal to distal. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

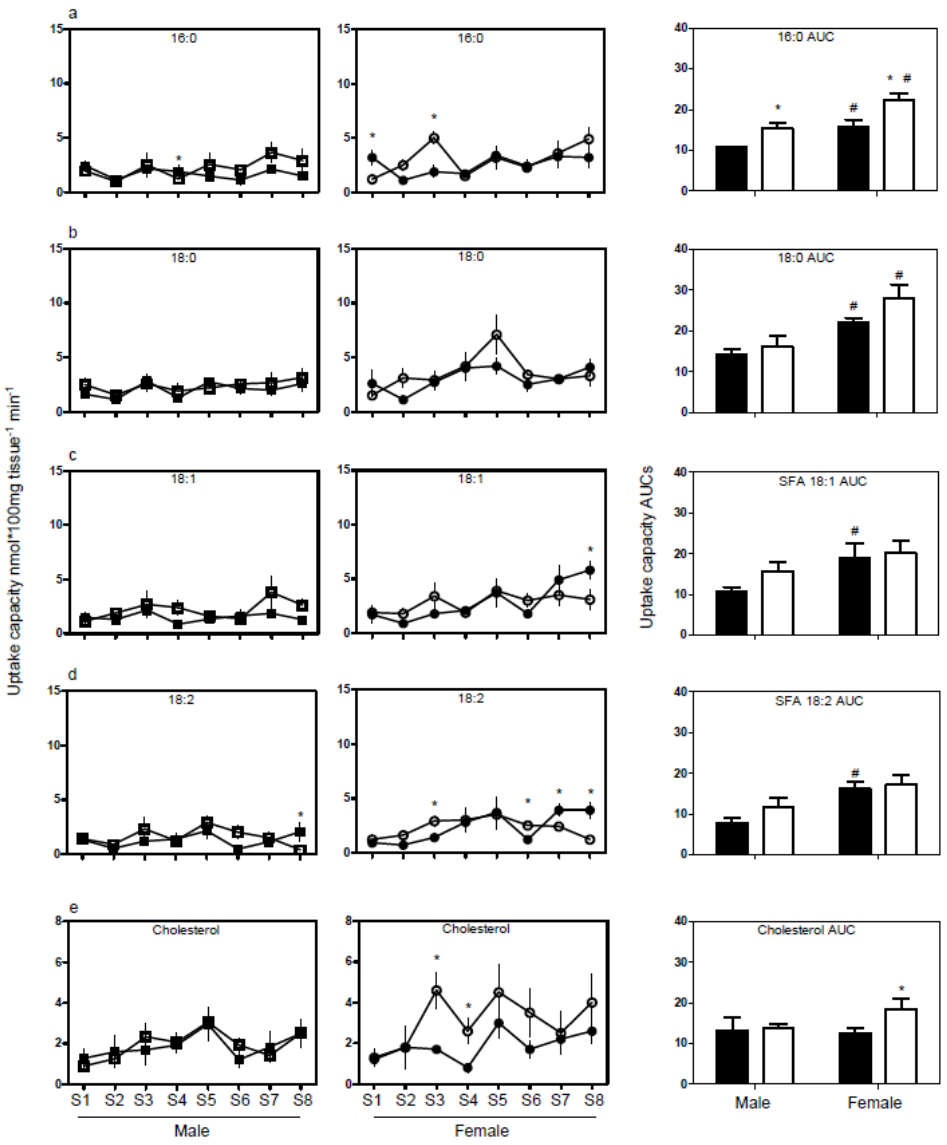

Figure 2.

Spatial and total lipid uptake capacity in intestinal segments of mice fed a saturated fatty acid diet. Intestinal segments are designates S1-S8 from proximal to distal. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). *P<0.05 for the effect of FABP2 loss in individual sexes. # P<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

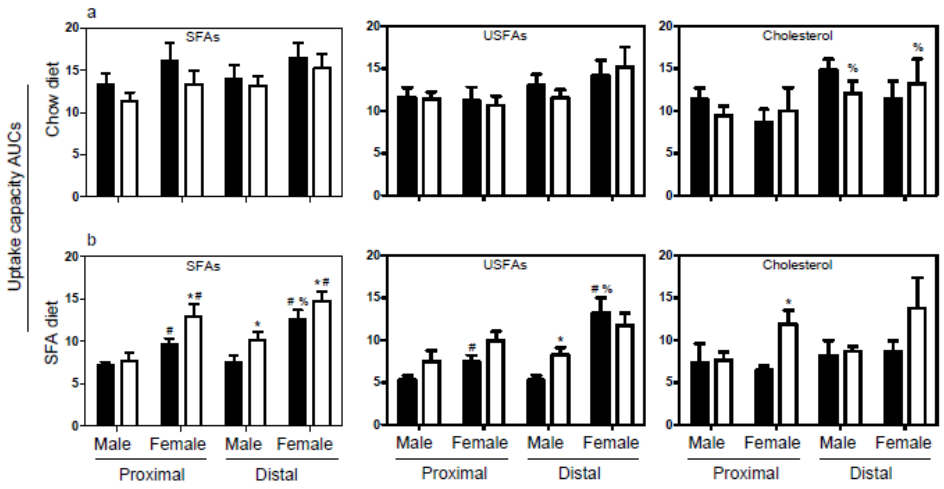

Figure 3.

Lipid uptake capacity in the proximal and distal intestine. Area under the curve for the saturated fatty acids (C16:0, C18:0), unsaturated fatty acids (C18:1, C18:2) and cholesterol. Black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype. % *P*<0.05 between proximal and distal intestine.

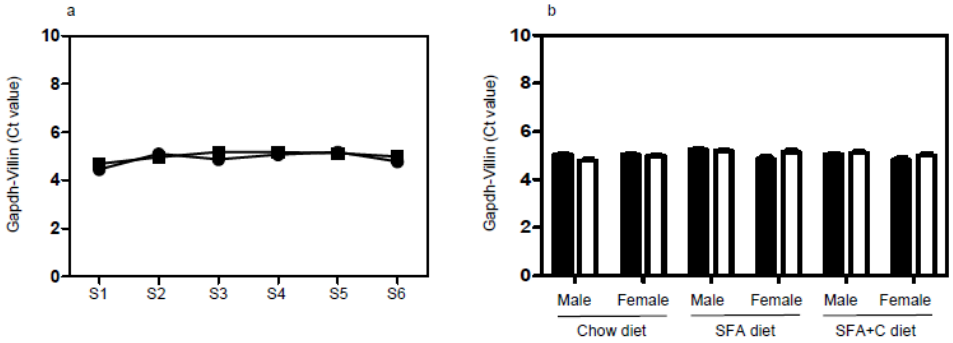

Figure 4.

Validation of intestinal housekeeping genes. (a) Effect of intestinal segment (S1-S6) on the ratio between Villin and Gapdh mRNA abundance. (b) Effect of sex, diet, genotype on the ratio between Villin and Gapdh in the small intestine of male and female wild-type and *Fabp2-/-* mice fed a chow diet, high saturated fat diet, or a high saturated fat plus 0.2% cholesterol diet.

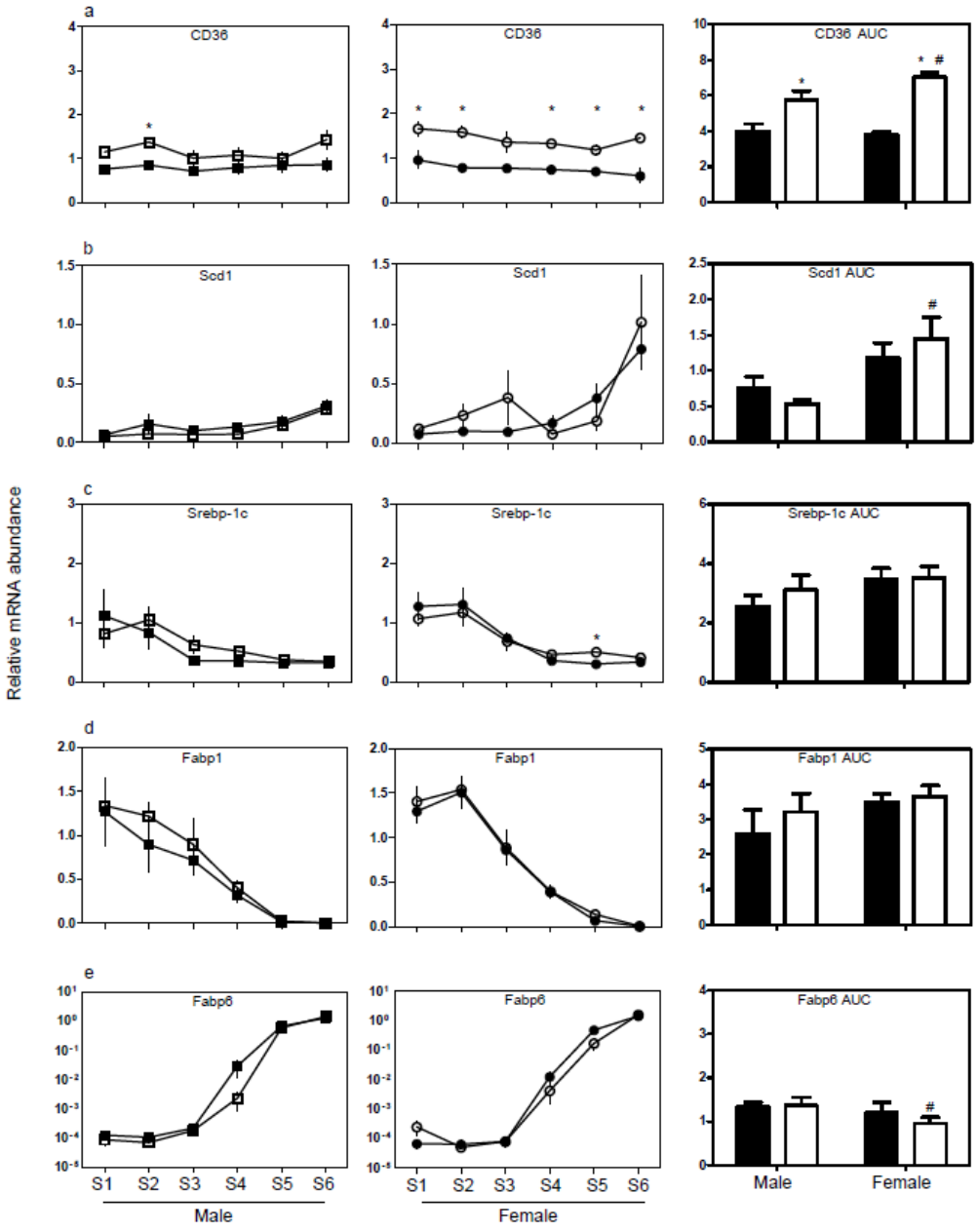

Figure 5.

Spatial and total mRNA abundance of intestinal lipid metabolic genes in chow-fed mice. . Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

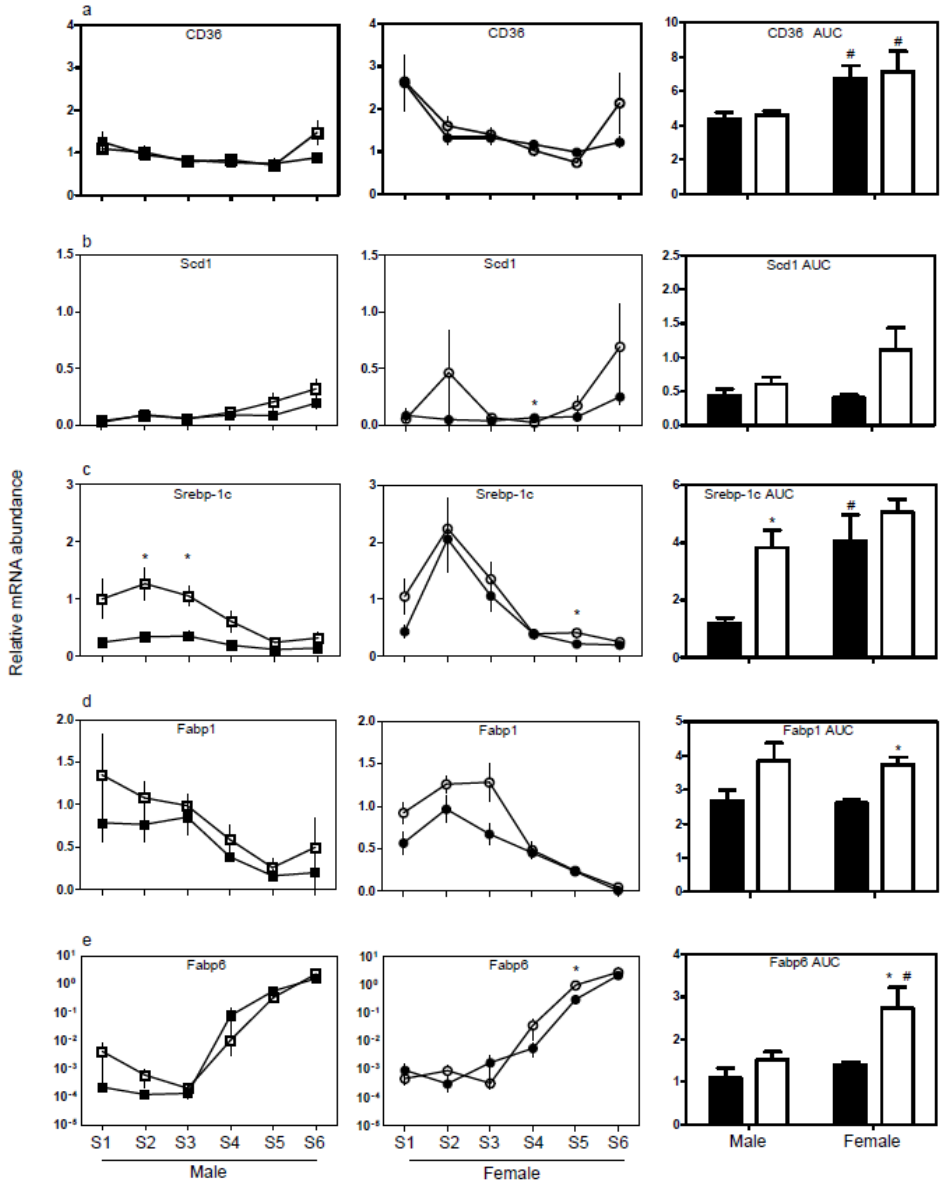

Figure 6.

Spatial and total mRNA abundance of intestinal lipid metabolic genes in saturated fat-fed mice. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). *P<0.05 for the effect of FABP2 loss in individual sexes. # P<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

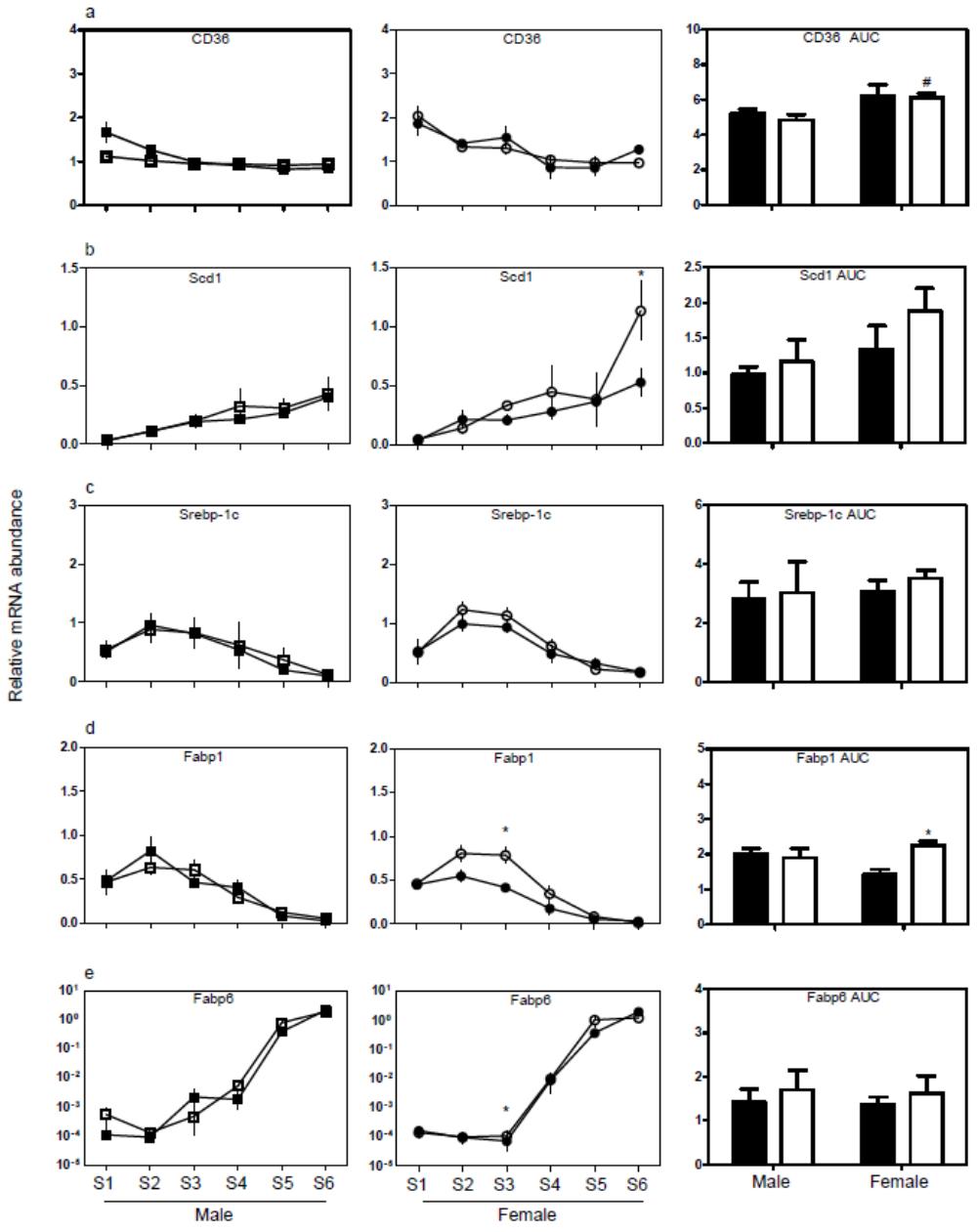

Figure 7.

Spatial and total mRNA abundance of intestinal lipid metabolic genes in saturated fat plus 0.2% cholesterol-fed mice. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). *P<0.05 for the effect of FABP2 loss in individual sexes. # P<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

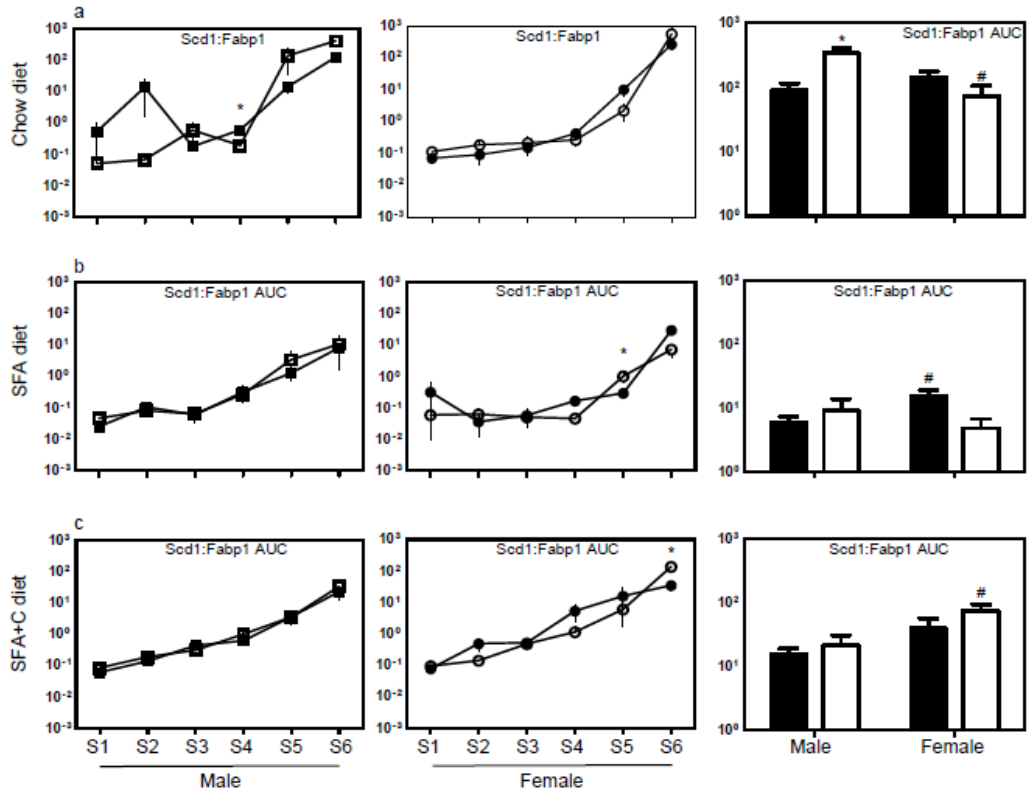

Figure 8.

Ratio of Scd1 to Fabp1 mRNA abundance in the small intestine of male and female wild-type and *Fabp2-/-* mice fed one of three diets. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4-5 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

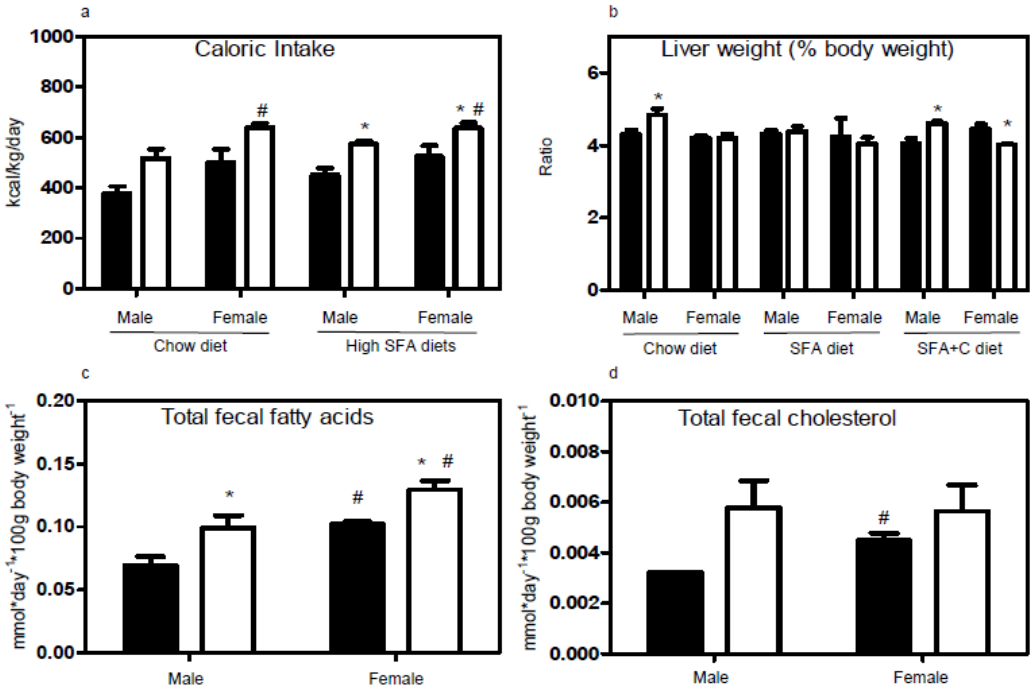

Figure 9.

Anthropometry and lipid balance. (a) Total caloric intake measured over three day metabolic cage collection in chow-fed and saturated fat-fed mice. (b) Liver weight as a percentage of total body weight in mice fed one of the three experimental diets. (c) Total fecal fatty acids (TG+NEFA) in chow-fed mice from the three day metabolic cage collection. (d) Total fecal cholesterol in mice fed the chow diet. For all experiments, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=3 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

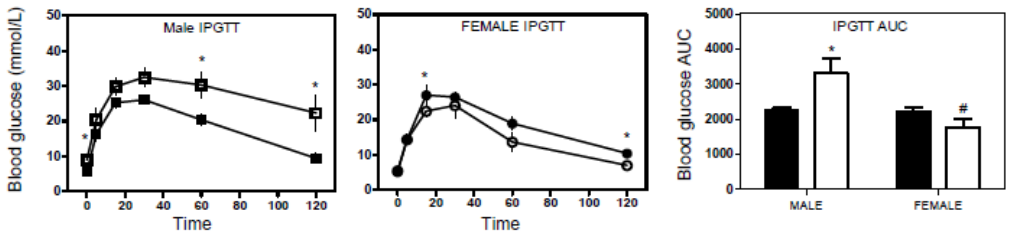

Figure 10.

Intraperitoneal glucose tolerance test in saturated fat-fed mice. Closed and open squares represent wild-type male and *Fabp2*$^{-/-}$ male respectively. Closed and open circles represent wild-type female and *Fabp2*$^{-/-}$ female respectively. For AUCs, black and white bars indicate wild-type and *Fabp2*$^{-/-}$ mice respectively. Data shown is the mean + SEM (n=4 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

Tables

Table 1.

Primer sequences for qPCR experiments.

Name	Sequence
Gapdh	5' GAACGCAAAGCTGAAGTGAGACT 3' 5' TCATTACGCTTGCACTGTTGGT 3'
Villin	5' TCAAAGGCTCTCTCAACATCAC 3' 5' AGCAGTCACCATCGAAGAAGC 3'
Cd36	5' ATGGGCTGTGATCGGAACTG 3' 5' AGCCAGGACTGCACCAATAAC 3'
Srebp-1c	5' GGAGCCATGGATTGCACATT 3' 5' GCTTCCAGAGAGGAGGCCAG 3'
Scd1	5' TCCTCTAGCCCGACTACCAC 3' 5' GCCTCTATTATGGTTGGCTGG 3'
Fabp1	5' TGCAGAGCCAGGAGAACTTTGAGCCA 3' 5' CCCCAGGGTGAACTCATTGCGGAC 3'
Fabp6	5' CCAGCAGGACGGACAGGACTTCACC 3' 5' GCCACCACCTTGCCACCCTCCATC 3'

Table 2.

Anthropometry and blood chemistry in WT and *Fabp2*$^{-/-}$ mice on the indicated low and high fat diets for three weeks. Data shown is the mean + SEM (n=4-5 mice per group). **P*<0.05 for the effect of FABP2 loss in individual sexes. # *P*<0.05 for the effect of sex in the wild-type or *Fabp2*$^{-/-}$ genotype.

	Sex	Weight gain (% initial)	TG mg/dL	NEFA mEq/L	Cholesterol mg/dL
Chow diet					
Fabp2$^{+/+}$	Male	4.5±0.6	23.6±3.3	0.39±0.03	61.8±2.0
Fabp2$^{-/-}$	Male	10.6±2.6*	30.3±7.6	0.39±0.03	99.1±3.5*
Fabp2$^{+/+}$	Female	7.3±1.0	22.9±2.2	0.40±0.06	53.3±1.9#
Fabp2$^{-/-}$	Female	6.5±1.4	30.2±5.5	0.41±0.06	46.1±13#
SFA diet					
Fabp2$^{+/+}$	Male	7.1±2.8	27.4±3.0	0.77±0.08	102.3±1.3
Fabp2$^{-/-}$	Male	9.7±2.1	25.4±11.7	0.74±0.05	130.6±6.2*
Fabp2$^{+/+}$	Female	11.5±0.7	31.3±4.7	0.40±0.06#	78.0±2.3#
Fabp2$^{-/-}$	Female	9.9±3.0	46.9±3.4	0.49±0.03#	93.5±8.8#
SFA+C diet					
Fabp2$^{+/+}$	Male	14.7±1.8	23.6±3.2	0.56±0.06	119.0±4.8
Fabp2$^{-/-}$	Male	11.5±3.1	30.3±7.7	0.53±0.03	147.8±6.8*
Fabp2$^{+/+}$	Female	10.2±1.1#	22.9±2.2	0.38±0.05#	62.4±10.0#
Fabp2$^{-/-}$	Female	15.3±3.9	30.2±5.5	0.61±0.08	81.3±6.3#

Overall Summary and Conclusions

FABPs and nutrient-gene interactions

Recent advances in the fields of molecular biology, genetics, and nutrition have illuminated the importance of nutrients in the regulation of gene expression and the importance of genetic makeup in the control of nutrient metabolism. Nutrient-gene interactions are continuously modified by the nutritional and hormonal milieu, as well as by non-modifiable factors such as genetic sex, and their combined interactions give rise to the metabolic potential of the organism. In the current studies we examined the effect of diet on the expression of genes involved in lipid metabolism in mice lacking Fabp2, a protein involved in intracellular lipid transport in the enterocyte. One of the major findings from our analysis was that female *Fabp2*$^{-/-}$ mice displayed an increased fatty acid uptake capacity and increased abundance of Fabp1 mRNA following high fat-diet feeding. From a teleological perspective, the increased fatty acid uptake capacity and stimulation of Fabp1 gene expression in female mice lacking Fabp2 might act as a compensatory mechanism to ensure that an adequate amount of fat can be stored to meet the metabolic costs of reproduction [106]. It is possible that the female-specific, systemic stimulation of Fabp1 in fat-fed mice represents a sex difference in the PPAR-controlled lipid metabolic pathways. PPARs are a class of nuclear receptors that are involved in regulating cellular lipid metabolic processes, and are activated by unsaturated fatty acids [170]. Fabp1 is transcriptionally regulated by PPARβ/δ in the small intestine [171], and might regulate its own expression by delivering unsaturated fatty acids to the nucleus. In our previous transcriptome analysis of chow-fed mice we observed a similar degree of PPAR-pathway stimulation in male and female *Fabp2*$^{-/-}$ mice with no sex difference in Fabp1 mRNA abundance [158]. It is likely that in the case of the fat-fed female *Fabp2*$^{-/-}$ mice, the high fat content of the diet was responsible for stimulating Fabp1 gene expression through a PPAR-controlled pathway. Sex differences in the abundance [76, 172] and function [77-78] of PPARs in other tissues, such as the liver and heart, have been well documented, and likely contribute to sex differences in the risk of acquired metabolic disorders. Although our study did not

detect sex differences in the abundance of the PPAR target genes Scd1 and Srebp-1c, it is possible that other elements of the PPAR-controlled intestinal metabolic program are regulated in a sex-dimorphic manner.

FABP2 and portal theory

Sex differences in the prevalence of acquired metabolic disorders [115] have provided the impetus for the development of theories which attempt to unify epidemiological data with an underlying physiological mechanism. One such theory posits that excess release of free fatty acids into the portal circulation can lead to increased visceral obesity, fatty liver, and hepatic insulin resistance [173]. This theory, known as 'portal theory', has been tested in various animal models. In rats, intraportal infusion of long chain fatty acids results in impaired glucose tolerance following an intravenous glucose tolerance test [174]. This is similar to the fat-fed male *Fabp2*$^{-/-}$ phenotype that we have reported here and in previous studies [3-4, 114], and supports the concept that excess fatty acids can induce hepatic insulin resistance [175]. Our analysis showed that male *Fabp2*$^{-/-}$ mice conditioned on a saturated fat diet display impaired glucose tolerance and increased accumulation of cholesterol in the plasma compared to male wild-type, while female *Fabp2*$^{-/-}$ mice had a better overall tolerance to the glucose challenge than female wild-type. In agreement with these findings, the liver to body weight ratio of male *Fabp2*$^{-/-}$ mice tended to be higher than male wild-type, while fat-fed female *Fabp2*$^{-/-}$ tended to have a lower liver to body weight ratio than female wild-type. Since we did not measure portal non-esterified fatty acids, it remains to be determined whether loss of Fabp2 leads to an appreciable release of fatty acids into the portal circulation. It is possible that the increased abundance of Fabp1 in the female *Fabp2*$^{-/-}$ mice acts to sequester excess free fatty acids, preventing their release into the portal circulation. If excess free fatty acids are being released into the portal circulation in males, this might indicate an attenuation of chylomicron synthesis, suggesting a role for Fabp2 in chylomicron synthesis in males. On the other hand, if Fabp1 directs fatty acids for storage in the lipid droplet as TG, the higher abundance of Fabp1 in female *Fabp2*$^{-/-}$ mice, combined with the high turnover rate of the intestinal epithelium, would lead to a greater output of lipids

in the feces. In agreement with this idea, we observed an increased output of total fecal fatty acids in the feces of chow-fed female *Fabp2*$^{-/-}$ mice compared to both female wild-type or male *Fabp2*$^{-/-}$.

It should be noted that Fabp1 and Fabp2 employ different mechanisms for the transfer of fatty acids to membranes. Fabp1 transfers fatty acids to the membrane by aqueous diffusion while Fabp2 interacts with the membrane via a collisional mechanism [46]. This is related to functional differences in the ability of these FABPs to target diet derived fatty acids and monoacylglycerols to anabolic or catabolic pathways in the small intestine [45]. Whether these differences contribute to the sex-dimorphic *Fabp2*$^{-/-}$ phenotype on a high-fat diet remains to be determined.

FABP2 mutations in humans

A single nucleotide polymorphism in the second exon of the *FABP2* gene, resulting in the substitution of alanine for threonine at codon 54 (A54T), has been associated with obesity and insulin resistance in the Pima Indian population, known for their high prevalence of diabetes and other acquired metabolic disorders [5]. Subsequent analyses of the *FABP2* A54T mutation in vitro and in different human populations have often led to incongruous results, making it difficult to rationalize the functional significance of the *FABP2* variants on metabolic disease risk. More recently, variations in the *FABP2* promoter (promoter A and promoter B) have been identified and are in strong linkage disequilibrium with the A54T mutation in the Pima Indian population. In vitro analysis of the promoter B variant in Caco-2 cells revealed a threefold lower basal activity [140]. This suggests that our *Fabp2* gene-ablated mice might represent an exaggeration of the *FABP2* promoter B phenotype. Further analysis of the *FABP2* promoter and A54T mutations in human populations have found an increase in postprandial lipemia and a decrease in insulin sensitivity in individuals carrying the promoter B/T54 haplotype [6]. The interplay between the *FABP2* promoter and exon 2 variations require further clarification, as the same group found a decreased risk of type-2 diabetes in individuals carrying the promoter B haplotype after stratification for the A54T polymorphism [142]. Despite these differences,

our data shows that the *Fabp2*$^{-/-}$ mouse model manifests similar clinical signs of metabolic disturbance as carriers of the *FABP2* promoter or A54T variant. Furthermore, the metabolic disturbances associated with FABP2 loss are modified by genetic sex and dietary lipid composition.

Conclusion

In summary, sex differences in lipid metabolism have not been explored at the level of intestinal lipid uptake and intracellular metabolism. We examined the effects of Fabp2 loss and fat content of the diet on the murine small intestine transcriptome to determine if differences in the intestinal lipid metabolic program contribute to the sex-dimorphic *Fabp2*$^{-/-}$ phenotype. Our microarray analysis identified several differentially regulated lipid metabolic pathways in male and female *Fabp2*$^{-/-}$ mice fed a low-fat diet. When challenged with a high saturated fat diet, female *Fabp2*$^{-/-}$ displayed a greater capacity for the uptake of saturated fatty acids than male *Fabp2*$^{-/-}$. This was related to a significant increase in the abundance of Fabp1 mRNA in the proximal half of the intestine of fat-fed female *Fabp2*$^{-/-}$ but not males. In addition *Fabp2*$^{-/-}$ mice displayed a sex-dimorphic response to glucose challenge, suggesting a link between *Fabp2* gene-ablated mice and the risk of diabetes and insulin resistance attributed to *FABP2* mutation in human populations. Based on our analysis, it appears that dietary fat and cholesterol have differential effects on lipid entry into the enterocyte and on the intestinal transcriptome of male and female *Fabp2*$^{-/-}$ mice. Furthermore, the mechanisms surrounding the sex-dimorphic *Fabp2*$^{-/-}$ phenotype are likely attributable to elements of the lipid metabolic program that occur after lipid assimilation.

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