Fatty acid binding protein 2 protects the small intestine from dietary saturated fatty acid-induced endoplasmic reticulum stress

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ABSTRACT

Background: Intake of excess saturated fats, as noted in the western-style diet, is linked with the development of metabolic diseases. Saturated fatty acids, especially in the form of palmitic acids, are known inducers of endoplasmic reticulum (ER) stress causing alteration of cellular functions and manifestation of metabolic diseases such as obesity. The small intestine (SI) contains fatty acid binding proteins (Fabps) that bind diet-derived fatty acids after absorption by enterocytes. In particular, Fabp2 preferentially binds saturated fatty acids. In the Pima Indian population, an FABP2 gene variant with reduced expression is associated with increased incidence of obesity and type 2 diabetes. As well, male mice lacking Fabp2 are prone to adiposity and fatty liver in response a high saturated fat diet. It is hypothesized that Fabp2 is part of a mechanism that protects the SI from the toxic effect of saturated fatty acids. Accordingly, the aim of this project is to determine if loss of Fabp2 increases the sensitivity of the SI to ER stress in response to a high saturated fat diet.

Objectives: The specific objectives of this project are: 1) to compare the efficacy of a high saturated fat diet with and without cholesterol in inducing metabolic disturbance in intact and Fabp2 deficient mice; 2) to determine ER stress status in the SI of mice lacking Fabp2 after challenge with a high saturated fat diet.

Methods: Fabp2+/+ and Fabp2−/− mice matched by age and sex (n=5) were placed on a low-fat diet (2020x), a high saturated fat diet (SF) or a high saturated fat diet with 0.2% (w/w) cholesterol diet (SFC). After four weeks, the mice were assessed for biochemical and metabolic activity by measuring energy expenditure activity and respiratory quotient. Activating transcription factor 4 (ATF4) and spliced x-box protein 1 (XBP1s), mRNA abundance were
measured by quantitative PCR (qPCR) along the longitudinal axis of the SI to determine ER stress status. Fabp1 protein abundance in the SI was evaluated by immunoblotting since both Fabp1 and Fabp2 bind fatty acids and overlap in localization in the SI.

**Results:** Biochemical and whole body metabolism data were consistent with previous studies, with male *Fabp2*/* mice on SF and SFC diets groups being the most at risk of triglyceridemia and hypercholesterolemia, and displaying lower energy expenditure patterns compared to *Fabp2*/*+/* male counterparts. Loss of Fabp2 increased the mRNA abundance of ER stress makers ATF4 and XBP1s, with male *Fabp2*/* mice fed SF and SFC diet reporting presence of ER stress as compared to male *Fabp2*/*+/* mice. Female *Fabp2*/* mice showed low ER stress status, this was accompanied by increased Fabp1 protein abundance in the proximal and medial portions of the SI. Both male and female *Fabp2*/* mice, as well as in female *Fabp2*/*+/* mice had reduced ER stress status on the SFC diet, as compared to the SF diet.

**Conclusion:** This study shows that Fabp2 protects male SI from saturated fatty-acid induced ER stress. The increase in Fabp1 protein abundance in the SI of female *Fabp2*/* mice accounts for the sex-dimorphic phenotype associated with Fabp2 deficiency.
**RESUME**

**Contexte:** La large consommation de graisses saturées, l'une des principaux composants de l'alimentation moderne, est liée au développement des maladies métaboliques. Les acides gras saturés, en particulier sous la forme d'acide palmitique, sont des inducteurs de stress du réticulum endoplasmique (RE), conduisant à l'altération des fonctions cellulaires et la manifestation de maladies métaboliques. Au niveau cellulaire, les acides gras hydrophobes se lient à des protéines de liaison d'acides gras (Fabp). Dans les entérocytes, les acides gras saturés se lient de préférence à Fabp2. Dans la population Indien Pima, une variante du gène *FABP2*, avec une expression réduite, est associée à une incidence accrue d'obésité et de diabète type 2; ce qui est cohérent avec les études animales où les souris mâles dépourvues de Fabp2 sont sujettes à l'adiposité. Sur la base de la capacité des acides gras saturés à se lier à Fabp2; on suppose que Fabp2, fait partie d'un mécanisme qui protège l'intestin grêle (IG) des effets cytotoxiques d'acides gras saturés, en les séquestrant dans le cytoplasme. Le but de ce projet est de déterminer si la suppression de Fabp2 induit le stress du RE dans l’IG en réponse à un régime riche en lipides.

**Objectifs:** Les objectifs spécifiques de cette étude sont: 1) de comparer l'efficacité d'un régime alimentaire riche en graisses saturées avec et sans cholestérol dans l'induction de troubles métaboliques chez des souris *Fabp2*+ et *Fabp2*−/−; 2) afin de déterminer si la perte de Fabp2 augmente la sensibilité de l’intestin pour le stress du RE en réponse à un régime riche en lipides.

**Méthodes:** *Fabp2*+ et *Fabp2*−/− souris appariées pour l'âge et le sexe (n = 5) placées pendant quatre semaines sur soit un régime de référence faible en matières grasses (2020x), soit un régime saturé riche en graisses (SF) ou un régime de gras saturé avec 0.2% (w/w) de cholestérol (SFC), ont été évalués pour activité biochimique et métabolique en mesurant la dépense
d'énergie et le quotient respiratoire. Les marqueurs de stress du RE (facteur d'activation de la transcription 4, jointées x-box protein 1) ont été évalués par PCR quantitative. Fabp1 abondance a été évaluée par immundot parce que Fabp1 et Fabp2 sont tous les deux localisés au même endroit dans l’IG et sont liés aux acides gras saturés.

Résultats: Les résultats à partir des données de métabolisme du corps et biochimiques étaient cohérents avec les études précédentes, avec Fabp2−/− souris mâle sur régime SF et SFC étant les plus risqués aux trouble de triglycéridémie et de hypercholestérolémie, avec une dépenses d'énergie plus faible par rapport à Fabp2+/+ souris mâle. La perte de Fabp2 modifie les marqueurs de stress du RE, avec Fabp2−/− souris mâles nourris de SF et SFC régime rapportant haute intensité de RE stress par rapport aux souris de type sauvage. Fabp2−/− souris femelle ont montré faible activation de stress du RE; ceci a été accompagné par une augmentation de la quantité de Fabp1 dans les parties proximale et médiale de l’intestin. Les deux groupes de souris mâle et femelle Fabp2−/− ainsi que les souris femelle Fabp2+/-, ont un état réduit de RE stress sur le régime SFC, par rapport a l’alimentation SF.

Conclusion: Cette étude montre que chez les souris males, Fabp2 protège les entérocytes de l’induction du stress du RE grâce aux gras saturés. En outre, l'augmentation de l’abondance de Fabp1 dans le SI chez les souris femelles dépouvrues de Fabp2, sur une alimentation riche en lipides, explique la carence en Fabp2 phénotype sexuel dimorphe. Ensemble, les résultats soutiennent l'idée que les Fabps intestinaux font partie d'un mécanisme qui protège le SI de stress du RE causé par une surcharge d'acides gras alimentaires.
CONTRIBUTION OF AUTHORS

Zeina Jamaluddine was the primary author of this Thesis document under the supervision and guidance of Dr. Luis B. Agellon.

Dr. Luis B. Agellon was involved in the concept generation, general design of the experiment, input on data obtained and editing of the manuscript.

Zeina Jamaluddine contributed in the concept research, implementation of the study, laboratory work, data analysis, writing and revision of the manuscript.

A version of this manuscript is being prepared for submission.
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LIST OF ABBREVIATIONS

AMDR: Acceptable macronutrient distribution range

AST: Glutamate-oxaloacetate transaminase

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

AUC: Area under the curve

CD36: Cluster of differentiation 36

CHOP: C/EBP homologous protein

EE: Energy expenditure

eIF2α: Elongation initiation factor 2α

ER: Endoplasmic reticulum

ERAD: ER associated degradation

F: Female

FABP: Fatty acid binding protein

FAS: Fatty acid synthase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GRP78: Glucose-regulated protein 78
IRE1: Inositol-requiring enzyme 1

JNK: Jun N-terminal kinase

LDL: Low-density lipoprotein

LPCAT3: Lysophosphatidylcholine acyltransferase 3.

LXR: Liver X receptors

M: Male

NAFLD: Nonalcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NFκB: Nuclear factor kappa B

PERK: Protein kinase-like ER kinase

PPAR: Peroxisome proliferator-activated receptors

PCR: Real-time polymerase chain reaction

RER: Respiratory exchange ratio

ROS: Reactive oxidative species

SCD1: Stearoyl-coA desaturase 1

SF: Saturated fat

SFC: Saturated fat and cholesterol
SI: Small intestine

SNP: Single nucleotide polymorphism

SREBP: Sterol response element binding proteins

UPR: Unfolded protein response

XBP1: X-box binding protein 1

XBP1s: Spliced variant of XBP1 mRNA
I. Introduction

Questions regarding the detrimental effect of palmitic acid accumulation emerged with increased consumption of palmitic acid in the modern diet. Exposing human Chang liver cells to high amounts of palmitic acid decreases cell viability, elevates cellular generation of reactive oxidative species (ROS) and increases ER dilation [1]. In fact, palmitic acid compromises ER morphology by affecting membrane fluidity causing ER stress [2]. By exceeding cellular amounts of palmitic acid, ER stress triggers the activation of coping responses i.e. unfolded protein response (UPR) pathway, in order to ensure cellular survival [3-7]. UPR corrective strategy activation denoted by amplifying gene expression of pathway components, are indicators of ER stress. Activation of corrective strategies may result in two outcomes, either an adaptive outcome (homeostasis is restored) or a maladaptive outcome (homeostasis not restored). In the case of successful adaptive response ER stress is either averted (normalization of ER function) or ER stress is evaded by persistent modification of the ER function and activation of compensatory mechanism at the expense of the organ leading to metabolic diseases. ER stress responses emerge as a potential mechanism underlining the development of chronic metabolic diseases [6].

The SI is the primary site of nutrient absorption, absorbing around 95% of nutrients from food. It is well documented that the SI adapts its morphology to different nutritional status, due to its high capacity of continuous cellular turnover; replenishing epithelial cells every three days in rodents [8]. Re-feeding rats with a diet rich in fat reverses the decrease in cellular proliferation observed during fasting [9]. Moreover, chronic intake of fat modulates the SI by increasing cellular proliferation, upregulating lipid binding protein, accompanied with improvements in
triglyceride clearance [10]. Thus intestinal cells are able to handle large amounts of fatty acids, some of which are known inducers of ER stress.

The SI plays a major role in palmitic acid entry into the body via absorption and assimilation into chylomicrons. Fatty acid binding protein 2 (Fabp2), in the SI has high affinity for trafficking saturated fatty acid. To date, animal studies indicate that although Fabp2 is not mandatory for dietary fat assimilation, male mice lacking Fabp2 compared to wild-type, independent of the diet (high fat or normal diet) have higher weight gain, higher liver fat content and significantly higher plasma triglyceride levels [11, 12]. Epidemiologic studies associate the coexistence of Fabp2 promoter polymorphism haplotype B and the missense substitution of alanine to threonine (A54T) identified in FABP2 gene of the Pima Indian population, with increased risk of insulin resistance, dyslipidemia and a reduction in promoter activity altering Fabp2 abundance in SI [13-16]. Thus, male Fabp2−/− mice model represents an extreme form of Fabp2 gene promoter attenuation. Sex dimorphism is noted in the literature with regards to Fabp2. In contrast to the observations in male mice, female Fabp2 deficient mice subjected to a high fat diet present a decrease in weight and a protection from the spillover of free fatty acid in the portal circulation [11].

Rationale

Previous work by our laboratory indicates that Fabp2 is not required for assimilation of dietary fatty acids. However, mice lacking Fabp2 exhibit sex dimorphic metabolic defects suggesting that Fabp2 plays an important role in dietary fatty acid metabolism. Under normal physiologic conditions, Fabps in the SI may provide a protective role against the toxic effect of excess free fatty acids by sequestering them in the cytoplasm. In the case of Fabp2, which is
exclusively found in the SI and shows preference for saturated fatty acids, this protein may protect enterocytes from cytotoxic effects of saturated fatty acids. In particular, Fabp2 may be part of a mechanism that protects enterocytes from fatty acid-induced ER stress when consuming diets with high saturated fat content. Figure 1 indicates an illustration of the study hypothesis.

**Hypothesis:**

Fabp2 protects the SI from ER stress in response to a high saturated fat diet.

**Objectives:**

1. Compare the efficacy of a high saturated fat diet with and without cholesterol in inducing metabolic disturbance in intact and Fabp2 deficient mice
2. Determine if loss of Fabp2 increases the sensitivity of the SI to ER stress in response to a high lipid diet.
Figure 1. Study hypothesis: Fabp2 protects the SI from ER stress in response to a high saturated fat diet.
II. Literature review

1. Saturated fatty acids

According to the acceptable macronutrient distribution range (AMDR) requirements, adults daily caloric intake of total dietary lipids including fats (as triglycerides, diglycerides, monoglycerides, and unesterified fatty acids) should represent 20 to 35% of total energy intake [17]. Biochemically, fatty acids are derived from triglycerides, phospholipids and cholesteryl esters. Different classes of fatty acids appear in food: namely saturated, polyunsaturated, monounsaturated and trans- fatty acids. Saturated fatty acids are the most hydrophobic class of fatty acids and are characterized by a fully saturated acyl chain. In food, saturated fatty acids are derived from animal sources such as meat, lard, cream and butter, as well as plant sources such as palm kernel oil, coconut oil and cottonseed oil. Meat and dairy products contribute around 60% of saturated fat in the diet of Canadians and Americans [18]. The typical western-style diet is composed of high amounts of fat especially in the form of saturated fatty acids. Around one-quarter of males and females age 19 years and older, display fat intakes above the AMDR in Canada [19]. The mean intake of saturated fatty acids for Canadian adults is around 10% of total energy [19]. Data obtained from the Center for Disease Control indicate similar pattern in the American population whereby saturated fatty acids consists 11 to 12% of energy intake [17]. Those numbers exceed guidelines given by the American Heart Association’s and Health Canada with regards to recommended intake of saturated fatty acid intake at no more than 7% of total caloric intake [20]. Dietary reference intake recommends that the dietary intake of saturated fatty acids should be as low as possible [17]. More information is required to prescribe the defined level of intake that onsets metabolic disease.
It has been previously noted that high intake of saturated fatty acids is linked with the development of various metabolic diseases including cardiovascular diseases, diabetes, obesity and inflammatory bowel disease [21]. Increased intake of saturated fatty acids is manifested by an increase in total blood cholesterol concentration due to a rise in low-density lipoprotein (LDL) cholesterol concentration [22, 23]. By suppressing expression of LDL receptors, saturated fatty acids cause a rise in blood LDL concentration [24, 25]. Currently, a controversy in the literature has been noted with regards to the detrimental effects of saturated fatty acids. A recent meta-analysis found no evidence linking dietary intake of saturated fat and amplified risk of cardiovascular diseases [26]. Those reports are directing research to distinguish between different types of saturated fatty acids whereby individual fatty acids species within the saturated fatty acid group have different biological activities. Among saturated fatty acids, palmitic acid (hexadecanoic acid; C16:0) is the most common fatty acid found in food (palm oil, coconut oil, meats, cheeses, butter and dairy) and is directly linked with increased risk of cardiovascular disease [17]. Scientific evidence points out that palmitic and myristic (tetradecanoic acid; C14:0) acid have the greatest effect in raising LDL and total cholesterol levels, whereas stearic acid (octadecanoic acid; C18:0) has not been shown to elevate blood cholesterol since it is rapidly converted to oleic acid in vivo [27-29]. More research is yet to be done to fully determine the detrimental effects of specific types of saturated fatty acids.

2. Saturated fatty acids as instigator of ER stress

2.1. ER

The ER, an important component of the cellular reticular network, is crucial for diverse cellular functions. The “rough” ER, studded with ribosomes on the cytoplasmic face, is involved in synthesis, folding, posttranslational modification (N-glycosylation, disulfide bond,
oligomerization) and transport of membrane and secreted proteins [30]. In the lumen of the ER, chaperone proteins, folding enzymes and ER-associated degradation (ERAD) proteins, are responsible for translocation of properly folded protein and for proteosomal degradation of improperly folded proteins, and work together in order to assure quality control of proteins. The “smooth” ER, is accountable for synthesis and assembly of lipids and steroids [30]. The ER also contributes in trafficking of membranes, breakdown of toxins, metabolism of xenobiotic, storage and release of intracellular calcium, regulation of gene expression, signaling between cells and energy metabolism [6, 30].

### 2.2. Loss of ER homeostasis and coping mechanisms

Disturbance in the proper function of the ER jeopardizes essential cellular activities involved in intracellular signaling, transcriptional control pathways, ion fluxes, control of energy metabolism, protein synthesis, synthesis and assembly of lipids [6]. The ability of the ER to sense and respond to stress is a central component of cellular survival. At a cellular level, the ER is able to detect loss of energy and nutrient homeostasis which cause ER stress [6]. Examples of energy and nutrient imbalances triggering a condition of ER stress include: excess or deficiency in nutrients used for ATP production, increase in reactive oxygen species and pH imbalance. With regards to energy imbalance, depletion in ATP levels contributes to ER stress resulting in the inhibition of proper protein folding in the cell due to the decrease in glucose regulatory protein 78 (GRP78) function, an example of a chaperone protein that binds tightly to ATP [31, 32]. In alcoholic disorders, excessive alcohol intake induce ER stress by increasing ROS and altering calcium homeostasis [33].

ER stress results in the activation of corrective strategies aimed at restoring proper ER functions and salvaging the cell. Corrective strategies include: regulation of pH, heat shock
response, repair DNA damage, and UPR (see Section 2.3) which increases chaperone proteins in the cell [6].

The activation of corrective strategies has two outcomes, either an adaptive or a maladaptive response [6]. Figure 2 presents a graphic representation of the different corrective response outcomes. In the case of maladaptive response, homeostasis is not restored and the malfunctioning cells are removed through programmed cell death (apoptosis). In the case of adaptive response, homeostasis is restored and thus the cell is spared from apoptosis. The adaptive response is achieved in two modes: in one mode, ER stress is averted and the cell reverts back to normal function as if nothing happened to the cell; in the other mode, ER malfunction is bypassed by long term modification of cell function via compensatory mechanisms [6]. The main idea underlying the latter case is that the activation of compensatory mechanism at the cellular level provides a survival advantage for the cell but is detrimental to overall metabolic function at the organismal level leading to formation of acquired metabolic disorders. Various examples of metabolic diseases where ER stress is implicated are shown in Table 1.

### 2.3. The UPR, an example of a coping response

#### 2.3.1. Activation of UPR

The objective of coping responses is to alleviate ER stress in order to ensure survival of the cell. Appearance of unfolded and misfolded proteins, following the loss of ER proper folding function, initiates the UPR coping strategy, and is a clear indicator of ER stress. The main consequences of UPR activation are: decrease in protein entry into the ER, increase in proteins involved in folding and increase in ERAD components. Activation of the three arms of the UPR originates from protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6)
or inositol-requiring enzyme 1 (IRE1). In unstressed situations, the UPR is not active, chaperone proteins are bound to the transmembrane proteins IRE1, PERK, ATF6 [34]. During ER stress, accumulation of unfolded protein in the ER lumen leads to the activation of the three key ER transmembrane proteins by the dissociation of GRP78, causing chaperone protein to bind to the misfolded protein in the lumen [35-37]. The dissociation liberates the serine/threonine kinases (PERK and IRE1) and the transcription factor (ATF6). Figure 3 shows the different UPR pathways activation.

2.3.2. PERK-eIF2α-ATF4 pathway

PERK activation leads to the phosphorylation of the cytosolic eukaryotic translation initiation factor 2α (eIF2α), which inhibits protein translation, thus attenuating the entry of new proteins into the ER [38-40]. Another consequence of eIF2α phosphorylation is the selective translation of ATF4, a transcription factor that induces the transcription of genes encoding proteins involved in amino acid biosynthesis and redox balance [41]. In general the PERK pathway stimulates pathways that help the cell offset the increase in unfolded proteins.

2.3.3. IRE1-spliced XBP1

Activation of IRE1 un masks its endoribonuclease activity and results in the removal of a 26-base fragment from the mRNA encoding the transcription of x-box binding protein 1 (XBP1) [42, 43]. The spliced XBP1 (XBP1s) mRNA results in translational frameshift yielding a variant XBP1s transcription factor, which binds to ER stress elements in genes encoding proteins involved in facilitating protein degradation [44, 45].
2.3.4. **ATF6**

Activation of ATF 6 transcription factor leads to translocation of ATF6 to Golgi complex. In the Golgi ATF6 is cleaved by site 1 protease and site 2 protease in order to release the DNA binding domain of the mature ATF6 transcription factor. It is of note that both of the proteases are required for the activation of sterol response element binding proteins (SREBPs), key transcription factors involved in the regulation of sterol and triacylglycerol metabolism. Nuclear ATF6 binds to ER stress elements in the promoters of genes encoding chaperone proteins like ERp 57, GRP94 and protein disulfide isomerase, and proteins involved in ERAD [46].

2.3.5. **Apoptosis**

Stimulation of the PERK and IRE1 branches of UPR can also determine cell survival. For example, initiation of a pro-apoptotic event by Jun N-terminal kinase (JNK) phosphorylation depends on IRE1 phosphorylation [47]. In addition, activation of the PERK pathway reduces the inhibitors of nuclear factor kappa B (NFkB) thus causing activation of NFkB in inflammatory response of cells [48]. Studies have shown that IRE1 interacts with tumour necrosis factor receptor associated factor 2 leading to inflammation and apoptosis initiation via JNK and NFkB. The PERK pathway is also involved in apoptosis via ATF4 regulated pro-apoptotic pathways by the production of the C/EBP homologous protein (CHOP) transcription factor [46]. Indeed, targeted deletion of the gene for CHOP in mice prevents ER stress-mediated apoptosis [49].

2.4. **Palmitic acid and ER stress**

Studies have shown that excess palmitic acid induces ER stress [50-52]. When comparing different types of fatty acids as potential inducers of ER stress, studies indicate that ER stress observed in β-cell of the pancreas is primarily due to lipotoxicity from palmitic acid but not from
Treating preadipocytes with palmitic acid increases the mRNA abundance of CHOP, GRP78, JNK, XBP1s and phosphorylation of eIF2a [4]. Several pathways have been implicated in the mechanism behind palmitic acid induced ER stress. It is noted that by increasing caspase 3 activity palmitic acid induces ER stress causing liver injury in rats [7]. Several suggestions were proposed regarding the link between palmitic acid and ER stress activation. Holzer Ryan et al [53], indicated that ER stress is caused by saturated fatty acid induced expansion of the intracellular membrane microdomains by the alteration of membrane distribution and activation of proto-oncogene tyrosine-protein kinase Src for JNK stimulation. It is also suggested that palmitic acid increases phosphatidylcholine to phosphatidylethanolamine ratio causing perturbation in the calcium transport activity, sarco-ER calcium ATPase 2 pump, resulting in impaired ER calcium retention. Changes in calcium concentration leads to protein misfolding and ER stress by impairing chaperone function [54]. Some reports linked ceramide accumulation to ER stress since palmitate is a precursor of ceramides giving rise to sphingomyelins, the building blocks of raft domains. However, a study indicated that mechanisms independent from ceramide accumulation cause palmitic acid induced ER stress and apoptosis [7]. The main findings indicate that loss of ER fluidity and compromised ER morphology are consequences of the toxic effect of accumulation of excess palmitic fatty acids in ER phospholipid acyl chains causing ER stress [55].

3. Saturated fat, ER stress and nonalcoholic fatty liver disease (NAFLD)

3.1. NAFLD

The link associating nutrients, ER stress and metabolic diseases is proposed. NAFLD is an example of disease implicated in this pathway. NAFLD is a serious and alarmingly prevalent chronic disease in developed countries affecting around 30%-47% of adults and 10% of
children [56]. NAFLD poses major health concerns since it is characterized by fatty liver infiltration, i.e., triglycerides accumulation in the liver, in absence of chronic alcohol consumption and viral or autoimmune liver disease. In later stages, this disease progresses into non-alcoholic steatohepatitis (NASH) characterized by steatosis inflammation, scaring of the liver apoptosis and fibrosis end stage liver disease. The potential sources of macrovascular accumulation of triglycerides in the cytoplasm of hepatocytes are: dietary chylomicron remnants, lipolysis of adipose tissue, de novo lipogenesis from carbohydrates, dysfunction in fatty acid oxidation, reduction in very low density lipoprotein secretion, hepatic uptake of free fatty acid and esterification to triglycerides [57]. The exact pathogenesis of the disease is uncertain; suggestions include insulin resistance causing alteration in glucose and lipid metabolism, while oxidative stress and cytokines result in NASH development [58, 59]. A link between diet and NALFD is established, with increased intake of dietary fructose and fat present in western-style diet being implemented in the development of NAFLD [60]. Moreover, the ER in hepatocytes is well developed since the ER is a major contributor of essential liver functions, contributing to the idea that disruption of ER homeostasis by the diet is involved in the pathogenesis of this disease.

3.2. ER stress and development of NAFLD

The lipotoxic environment created by NAFLD, promoted by an oversupply of lipids, influences ER stress activation [61, 62]. The high intrahepatic levels of fatty acid inducing ER stress can lead to adaptive or maladaptive responses. Activation of UPR by IRE1α expression results in hepatic steatosis as a consequence to chemical induction of ER stress [63]. In rat model with hepatic steatosis, increase in saturated fatty acid loads leads to increase in caspase 3 activity, ER stress and liver injury [7]. Saturated fat especially in form of palmitic acid induces ER stress and apoptosis in human liver cell line (L02 and HepG2) by upregulating CHOP, ATF4
and PERK mRNA abundance [50]. In patients with NAFLD and NASH, ER stress of varying degree is implicated [64].

The mechanism behind the link between ER stress and NAFLD may involve SREBP1c. An interesting link is established between SREBP1c and ER stress. Triglycerides induced ER stress increases the expression of SREBP1c, fatty acid synthase (FAS) and acetyl-coA carboxylase [65]. In this case, induction of ER stress increases the proteolytic processing of SREBP1c [65]. Cleaved SREBP1 causes induction of lipogenic genes and increased triglycerides deposition [65]. In addition SREBP1c has been implicated in the regulation of NAFLD in an animal model of fatty liver [66]. Thus, proposing that the accumulation of SREBP1c under ER stress may cause the development of NAFLD.

In order to re-establish ER homeostasis various arms of the UPR signaling pathway play a role in the regulation of lipid metabolism by promoting lipid homeostasis.

The PERK pathway is associated with stimulation of lipogenesis and hepatic steatosis. In mammary epithelium of mice carrying a deletion of PERK gene, lower free fatty acids levels and reduced expression of lipogenic genes especially FAS, ATP citrate lysate and stearoyl-coA desaturase 1 (SCD1) is observed [67]. Selective elimination of eIF2α decreases expression of peroxisome proliferator-activated receptors (PPARγ) causing a reduction in hepatosteatosis in mice animal fed a high fat diet [68]. In mice, deletion of ATF4 gene protects from diet-induced obesity, hypertriglycemia and hepatic steatosis and is associated with lower PPARγ, SREBP1c, ACC and FAS mRNA abundance [69, 70]. In summary the whole activation of PERK pathway is involved in promotion of lipogenesis during ER stress.

IRE1α-XBP1 pathway activation is also involved in maintaining hepatic lipid homeostasis by regulating hepatic lipogenesis. Under ER stress, IRE1α is involved in maintaining VLDL
secretion; in case of IRE1α deletion VLDL accumulation occurs [71]. In the case of conditional deletion of hepatocytes IRE1α, severe hepatic steatosis is observed and is associated with increased PPARγ mRNA abundance [71] Studies have also implicated XBP1s as a transcription factor that activates the promoters of lipogenic genes SCD1 and diacylglycerol O-acyltransferase 2 [72]. In this case, liver-specific elimination of the gene for XBP1 in mice lowers plasma triglycerides and free fatty acid concentrations [72]. This pathway is mainly involved in maintaining proper lipid biosynthesis.

Interestingly ATF6α can inhibit the transcriptional activity of SREBP2 by direct interaction and formation of a complex [73]. Under ER stress, ATF6 regulates hepatic triglycerides levels by stimulating lipid droplet formation [74]. ATF6-deficient mice on a high fat intake increases SREBP1c and SREBP2 function, and exhibit hepatic steatosis due to the reduction of VLDL secretion via destabilization of apolipoprotein B100 [75]. Thus ATF6 acts to avert ER stress at a cellular level at the expense of the organ.

In summary, all three arms of the UPR regulate lipid stores in the liver. The arms are generally involved in maintaining lipid homoeostasis. The three arms are involved in coping responses allowing the liver cells to survive but at the expense of liver organ and the formation of NAFLD.

4. ER stress and the SI

As pointed out in the previous section, metabolic disorder stems from a loss of energy and nutrient homeostasis. Unresolved ER stress and activation of UPR pathway marked with increase GRP78 expression and XBP1 splicing, in intestinal epithelial cells is documented in both cases of inflammatory bowel disease, Crohn’s disease and ulcerative colitis [76]. With an
inadequate intake of food, causing imbalances in fatty acid nutrient homeostasis at the SI level, ER stress may be initiated in this organ.

4.1. Fabps in the SI

The SI contains three types of Fabps known to bind small hydrophobic ligands such as fatty acids. Fabp family is composed of 9 clamshell-like proteins that can constitute 5-8% of cellular proteins [77]. The exact function of Fabps is not yet clearly defined and little is known about their mechanisms of action. Several suggestions have been proposed in the literature, including trafficking of lipids to different cellular compartments (lipid droplet, mitochondria, peroxisome, enzyme, nucleus), coordination of cellular function in response to lipids, import and storage of fatty acids, and buffering or sequestering functions [77]. The general picture is that Fabps reversibly bind small hydrophobic molecules, such as fatty acids, in order to escort and dictate the fate of these molecules. All Fabps bind fatty acids but individual type of Fabps show differences in ligand selectivity and affinity. All Fabps display similar tertiary structure but a 15 % to 70 % variance in primary structures exists [78]. The general tertiary structure of Fabps consists of a β barrel formed by two orthogonal five stranded β sheets, and a lid formed by two α helices over one end of the β barrel [78]. The ligand-binding site is located inside the β barrel. One important note about Fabps is that increased fatty acid entry into cells leads to an increase in Fabp abundance in most cell types [79].

In the SI, there are three types of Fabps showing different distribution along its length. Each of the Fabps can constitute 5-8% of total enterocyte proteins. Fabp1 is found mainly in the proximal two-thirds region. Fabp2 is detected all along the SI. Fabp6 is most abundantly present in the distal portion and displays a higher binding affinity for bile acid than fatty acids [80]. Fabp1, shows preference for unsaturated and saturated fatty acids and is unique among different
Fabps since it is able to bind two fatty acid molecules simultaneously [81]. Previous studies indicate that Fabp1 and Fabp2 exhibit differences in mechanism of fatty acid loading and off-loading [82]. In in vitro studies, Fabp1 transfers free fatty acids by facilitated diffusion [83, 84]. In contrast, Fabp2 transfers free fatty acids by collisional interaction between the Fabp and membranes [82]. A recent study showed that Fabp6 is involved in the transport of bile acids within enterocytes and plays an important role in maintaining bile acids homeostasis in the enterohepatic circulation [85].

4.2. Fabp2

4.2.1. Epidemiological studies

The human FABP2 gene is located at chromosome 4q28-q31 [86]. A missense mutation is identified in FABP2 gene of the Pima Indian population, resulting in a substitution of an alanine residue with a threonine residue (A54T). This single nucleotide polymorphism (SNP) (from G to A) at codon 54 is associated with insulin resistance and dyslipidemia in this Pima Indian population [13, 14]. However, there is a lack of consensus in the data linking this specific SNP with insulin resistance or obesity in many human populations studied [87]. Recently seven polymorphisms in the promoter of FABP2 were reported and categorized into two haplotypes: A and B. Studies associate allele B with increased risk of type 2 diabetes and hypercholesteremia [15]. In fact, one subject homozygous for haplotype B and the A54T SNP displayed enhanced postprandial levels of insulin and blood triglycerides [16]. It is suggested that the promoter allele B causes a reduction in FABP2 gene promoter activity and thus reduced Fabp2 protein level in enterocytes [16].
4.2.2. Fabp2-deficient mice

Fabp2 binds saturated fatty acids with high affinity and lipophilic drugs with lower affinity [86]. The ability of Fabp2 to bind saturated fatty acids suggests that it plays a role in lipid metabolism. Evidence highlighting the exact function of Fabp2 in vivo is lacking. To date, studies report that Fabp2 is not mandatory for assimilation of dietary fat since Fabp2\(^{−/−}\) mice (lacks Fabp2) are viable and fertile and exhibit no evidence of fat malabsorption from the diet [11, 12]. Studies reveal that, compared to wild type mice, male mice that lack Fabp2, have higher weight gain, higher liver fat content and significantly higher triglycerides independent of the amount of fat in the diet [11]. Interestingly male mice lacking Fabp2 display a phenotype that is similar to humans who are homozygous for the FABP2 gene promoter allele B. Thus, Fabp2\(^{−/−}\) mice model can be considered as an extreme form of FABP2 gene promoter attenuation. In contrast, female Fabp2-deficient mice given the high fat diet show a decrease in weight and unchanged blood triglyceride level [11]. Hyperinsulinemia is observed with males and female Fabp2\(^{−/−}\) mice [11]. The exact reason for the observed sex disparity in Fabp2 deficient mice is not well understood. It is interesting to note that female Fabp2\(^{−/−}\) mice on a high saturated fat diet have increased abundance of mRNAs for Cluster of differentiation 36 (CD36) and Fabp1 [88]. A similar study noted the increase in transporter proteins Niemann-Pick C1-Like 1 and CD36 mRNA with absence of Fabp2 gene; however, in this study no changes in mRNA abundance of genes involved in lipid metabolism (synthesis and oxidation) was observed [89].
<table>
<thead>
<tr>
<th>Nutrient/energy</th>
<th>Levels</th>
<th>Model or Target tissue</th>
<th>Induction of ER stress (Mechanism)</th>
<th>Pathology</th>
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<tr>
<td>Glucose</td>
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<td>Cells, Murine model</td>
<td>UPR activation (PERK, CHOP, IRE1)</td>
<td>Diabetes, Insulin resistance</td>
<td>[90]</td>
</tr>
<tr>
<td>Thiamine (vitamin B1)</td>
<td>Deficiency</td>
<td>Mice model</td>
<td>Upregulation of GRP78 and CHOP, phosphorylation eIF2a, ROS</td>
<td>Neuronal loss in the brain</td>
<td>[91]</td>
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<tr>
<td>Ascorbate (Vitamin C)</td>
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<tr>
<td>Hypoxic stress (O2)</td>
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<td>ROS, altered calcium homeostasis</td>
<td>Ischemia-reperfusion injury, Tumorigenesis</td>
<td>[93]</td>
</tr>
</tbody>
</table>

Table 1. Loss of nutrient homeostasis, triggering ER stress and related metabolic diseases. [94]
Figure 2. ER stress and coping pathways.

This figure was reproduced from Groenendyk J, Agellon LB, Michalak M: Coping with Endoplasmic Reticulum Stress in the Cardiovascular System. Annual review of physiology 2012.
Figure 3. Unfolded protein responses; an example of the activation of coping responses. This figure was reproduced from Groenendyk J, Agellon LB, Michalak M: Coping with Endoplasmic Reticulum Stress in the Cardiovascular System. Annual review of physiology 2012.
**Study Rationale**

Excess saturated fatty acids intakes represented in the modern diet, in the form of palmitic acid, are known inducers of ER stress by increasing the ER stress markers ATF4 and XBP1s. In the SI, primary site of fatty acid absorption, all Fabps bind fatty acids, but Fabp2 prefers to bind saturated fatty acids. Fabp2 is not required for assimilation of dietary fatty acids. However, mice lacking Fabp2 exhibit sex dimorphic metabolic defects suggesting that Fabp2 plays an important role in dietary fatty acid metabolism. Under normal physiologic conditions, Fabps in the SI may provide a protective role against the toxic effect of excess free fatty acids by sequestering them in the cytoplasm. In the case of Fabp2, which is exclusively found in the SI and shows preference for saturated fatty acids, this protein may protect enterocytes from cytotoxic effects of saturated fatty acids. In particular, Fabp2 may be part of a mechanism that protects enterocytes from fatty acid-induced ER stress when consuming diets with high saturated fat content. In this case removing Fabp2 would increase ER stress markers.

**Hypothesis**

Fabp2 protects the SI from ER stress in response to a high saturated fat diet (see Figure 1).

**Objectives:**

This study was organized into two objectives in order to evaluate this hypothesis. The first objective was to compare the efficacy of a high saturated fat diet with and without cholesterol in inducing metabolic disturbance in both sexes of wild-type and Fabp2-deficient mice. The second objective was to determine if markers of ER stress is increased in the SI of Fabp2-deficient mice in response to a high lipid diet.
III. Materials and Methods

Animals

Male and female C57BL/6J mice (15-17 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) while Fabp2\(^{-/}\) mice (C57BL/6J genetic background) were produced in our animal facility. The mice were housed in controlled environment at 22°C, with a reversed 12 h light and 12 h dark cycle starting at 0600 h. McGill University Animal Care Committee approved the use of animals in this study.

High fat feeding of mice

Age and sex matched mice were distributed into 12 groups with n=5/sex/genotype/diet. For duration of four weeks four groups (male Fabp2\(^{+/+}\), male Fabp2\(^{-/-}\), female Fabp2\(^{+/+}\), female Fabp2\(^{-/-}\)) were placed in one of the following diets: a low fat diet (2020x, Teklad Lab Animal Diets, WI, USA), a high saturated fat diet (SF), or a high saturated fat diet supplemented with 0.2% (w/w) cholesterol diet (SFC). Both SF and SFC diets were enriched in palmitic acid, which is a known to inducer of ER stress [51]. The compositions of the diets used in this study are shown in Appendix A.

Measurement of whole body metabolism by indirect calorimetry

Respiratory exchange ratio (RER), energy expenditure rate (EE) and activity rates as sum of beam breaks in the horizontal and vertical plane were measured using the Panlab Oxylet Physiocage System (Panlab-Harvard Apparatus, Barcelona, Spain). Measurements were performed after two weeks on study diets for n=3/sex/genotype/diet mice. Mice were placed in
individual metabolic cages and monitored over the course of 24-hour cycle for 3 days. Days 1-2 were used as adaptation days; data from day 3 was used for analysis.

**Lipid analysis**

After 4 weeks of controlled diets, food was withheld at the start of the light cycle on the last day of the study. Mice were euthanized by cardiac puncture under isoflurane anesthesia. Blood was collected in ethylenediaminetetraacetic acid coated tubes. Total plasma triglyceride and total cholesterol concentrations were measured using a commercial assay kit (Genzyme, MA, USA). Glutamate-oxaloacetate transaminase (AST) activity in plasma was used as indicator of liver health, and was measured using a commercial assay kit (Biotron Diagnostics, CA, USA).

**Analysis of ER stress markers**

After blood collection, the SI was excised, rinsed with cold saline and cut into 6 equal pieces. Segments representing the proximal to distal parts of the SI were place in RAlater® (Life Technologies Inc., ON, Canada) and stored at -20°C. Total mRNA was isolated by single step acid guanidinium thiocyanate-phenol-chloroform extraction [95]. RNA quality was assessed by running samples on 1% agarose gel and comparing the integrity of the 28S and 18S ribosomal RNA bands. Nucleic acid concentration was determined by UV spectrometry. Total RNA was converted into cDNA using M-M-MuLV reverse transcriptase and random hexamers as primer (Enzymatics, MA, USA) and stored at -20°C until analysis. Relative mRNA abundance was determined by quantitative RT-PCR (qPCR). All qPCR assays were performed using qPCR Master Mix on the CFX96™ real-time detection system (Bio-Rad, ON, Canada). For Fabp1 mRNA, samples were run in duplicates and normalized to villin mRNA abundance. For ATF4,
total XBP1 and XBP1s mRNA, samples were run in triplicate and normalized to villin mRNA abundance. Sequences of primer used for qPCR are shown in Appendix B. Relative mRNA abundance was calculated using the ΔΔCt method.

**Analysis of Fabp1 protein abundance**

At necropsy, samples from SI segments 1, 3 and 5 (representing proximal medial and distal portions) were placed on liquid nitrogen then stored in -80°C until analysis. Fabp1 protein abundance was determined by immunoblotting and normalized to the abundance of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. The anti-Fabp1 antiserum was raised in rabbit using recombinant murine Fabp1 as the immunogen. The anti-GAPDH antiserum was purchased (Abcam, MA, USA). Total protein homogenates were extracted and dotted as an array on a nitrocellulose membrane then probed with rabbit antisera against murine Fabp1 or murine GAPDH. After washing, the membranes were reprobed with horseradish peroxidase conjugated anti-rabbit antibodies, washed, and then the immune complexes were visualized using the ECL Western Blotting System (Thermo Scientific, MA, USA). The analysis was done in duplicates. Quantitation was done by densitometry of bands in scanned films using Un-Scan-It® software version 6.1 (Silk Scientific Inc., UT, USA).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5® software (GraphPad® Software Inc., CA, USA) by applying two-way ANOVA or Student’s t-test where suitable. Differences were considered statistically significant when P<0.05 and P<0.001.
IV. Results

Balance and anthropometry

After 4 weeks on the assigned diet no differences in weight gain was observed between $Fabp2^{-/-}$ and $Fabp2^{+/-}$ male mice, in contrast with females where $Fabp2^{-/-}$ mice had lower weight gain than $Fabp2^{+/-}$ across all diets (P<0.05) (Figure 4A). With respect to dietary effects, $Fabp2^{-/-}$ and $Fabp2^{+/-}$ male mice gained more weight with increasing fat composition in the diet. This effect was not evident in female mice (P<0.05) (Figure 4A). The changes in gonadal fat mass paralleled body weight changes across all groups (Figure 4B).

Plasma lipids measurements

Results from biochemical measurements followed similar patterns to previously reported data. Plasma triglycerides of SFC diet fed male mice was greater in $Fabp2^{-/-}$ compared to $Fabp2^{+/-}$ mice (P<0.05) (Table 2). Plasma cholesterol was higher in male $Fabp2^{-/-}$ mice on SF and SFC compared to wild-type male mice of the same diet (P<0.05) (Table 2). No significant differences in plasma triglycerides and cholesterol were noted between the two genotypes of females on all diets. Sex dimorphism in the response of $Fabp2^{-/-}$ mice was evident. Plasma triglycerides levels were higher in SF and SFC diet fed male $Fabp2^{-/-}$ contrasted to females of the same genotype and fed same diet (P<0.05) (Table 2). Female $Fabp2^{-/-}$ mice on the SFC diet had lower cholesterol concentration compared to $Fabp2^{-/-}$ male mice (P<0.05) (Table 2). Plasma AST activity levels are clear indications of liver health. Male $Fabp2^{-/-}$ mice had elevated levels of AST on both dietary groups fed fat with and without cholesterol causing genotype effect (male $Fabp2^{-/-}$ and male $Fabp2^{+/-}$) and significant sex differences (male $Fabp2^{-/-}$ and female $Fabp2^{-/-}$)
Liver injury in male Fabp2<sup>-/-</sup> mice paralleled the results found in plasma triglycerides and cholesterol levels.

**Whole body metabolism**

Ratio between the volume of O<sub>2</sub> inhaled and volume of CO<sub>2</sub> exhaled was measured in order to determine the fuel source metabolized for energy. Accordingly with the diet composition, mice on the 2020x diet regardless of sex and genotype showed an RER of ~1, suggesting the use of carbohydrates as the preferred fuel, while the RER decreased to ~0.75 for mice on the SF and SFC diets (Figure 5A, 6A, 7A).

EE was measured to investigate the reasons for the discrepancies found in data regarding body weight gain of male Fabp2<sup>-/-</sup> mice. Interestingly, regardless of the diet, male Fabp2<sup>-/-</sup> mice in both 2020x and SF diet groups exhibited lower EE especially during the light period, as indicated by significantly lower area under the curve (AUC) compared to male Fabp2<sup>+/+</sup> mice (P<0.05) (Figure 5B, 6B, 7B). The results were accompanied with lower activity of male Fabp2<sup>-/-</sup> mice on 2020x during the dark period (P<0.05), accounting for the decrease in EE observed in this group (Figure 5C). Similar trends were observed for mice on the SF diet, but the differences were not statistically significant. Female Fabp2<sup>+/+</sup> and Fabp2<sup>-/-</sup> mice did not show differences in EE or activity.

**ER stress related gene expression in the SI of mice on 2020x, SF and SFC diet.**

ER stress status was determined by qPCR analysis of ATF4 mRNA abundance as an indicator of PERK activation, XBP1 and XBP1s mRNA abundance as an indicator of IRE1 activation. In general mice on the 2020x diet did not display elevated levels of ER stress, with
the exception of segment 1 of Fabp2−/− male mice which showed increased ATF4 abundance compared to Fabp2+/+ male mice (P<0.05) (Figure 8, 8B). This feature was not evident in female Fabp2−/− mice (Figure 8, 8B). These results suggest that this region of the SI in male Fabp2−/− mice is in a state of ER stress regardless of the diet.

Inducing ER stress via increased palmitic acid intake revealed a systematically elevated amount of ER stress markers among Fabp2-deficient male mice. In fact all along the SI, ATF4, total XBP1 and spliced XBP1 mRNA abundance were significantly higher in male Fabp2−/− opposed to Fabp2+/+ male mice (P<0.05) (Figure 9B, 9C, 9D). Contrasting to the results in males, Fabp2−/− female compared to Fabp2+/+ female mice had lower amounts of total XBP1 corresponding with lower amounts of spliced XBP1 in segment 1 of SI (P<0.05) (Figure 9C, 9D). With regards to the entire SI, differences of AUC of male Fabp2−/− displayed greater total and spliced XBP1 levels in contrast with female Fabp2−/− mice (P<0.01) (Figure 9C, 9D)

Similar patterns were noted with mice fed a SFC diet. In SI segments 1,2,3,4 and 6, ATF4 mRNA abundance was higher in male Fabp2−/− mice compared to male Fabp2+/+ mice (P<0.05) (Figure 10B). In the proximal region of the SI, total XBP1 (in segments 1 and 2) and XBP1s mRNA abundance (in segments 1-4) was higher in Fabp2−/− mice compared to than male Fabp2+/+ mice (P<0.05) (Figure 10C, 10D). Wild-type female mice on SFC diet had higher ATF4 mRNA abundance along the SI compared to female Fabp2−/− mice on the same diet (P<0.05) (Figure 10B). In addition, proximal region of total and spliced XBP1 (segment 1) exhibited higher amounts in Fabp2+/+ contrasted to female Fabp2−/− mice mRNA abundance (Figure 10C, 10D). On the level of sex dimorphism, when analyzing the whole SI AUC, male Fabp2−/− mice displayed elevated amounts of ATF4 (P<0.05), total and spliced XBP1 (P<0.01) compared to female Fabp2 targeted gene elimination mice (Figure 10B, 10C, 10D).
With regards to dietary composition, the SF and SFC diet had similar composition except with an addition of 0.2% (w/w) of cholesterol in the SFC diet. When comparing ER stress markers (ATF4 and XBP1s), taking into account the entire SI, male and female Fabp2−/− mice on SFC diet reported lower amounts of ER stress activation compared to male and female Fabp2+/+ mice on SF diet (P<0.05) (Figure 11A, 11B). Indicating that addition of cholesterol to the diet decreased ER stress in the SI of mice.

**Fabp1 abundance along the SI**

Fabp2 protein is detected all along the SI but is most abundant in the medial portion, while Fabp1 protein is localized mostly in the proximal region, both types of Fabps bind to saturated fatty acids. Since both Fabp1 and Fabp2 can interact with the same fatty acid species and overlap in localization along the SI, determining the fate of Fabp1 mRNA and protein abundance with regards to Fabp2 gene elimination is essential. Fabp1 mRNA abundance data indicated similar patterns observed in previous data, where across all diets both male and female Fabp2−/− mice had elevated total Fabp1 mRNA abundance compared to wild-type counterparts but those results were not significant (Figure 8A, 9A, 10A). Importantly changes in Fabp1 protein abundance were assessed since changes in Fabp1 mRNA levels may not represent Fabp1 protein abundance since ligand binding could significantly alter the protein half-life. As expected measuring Fabp1 protein abundance along the SI indicated minimal amounts of Fabp1 in the distal portion across all groups (Figure 12). In general, data indicated a trend of increased Fabp1 protein expression in both male and female Fabp2−/− mice compared to Fabp2+/+. Female Fabp2−/− mice on 2020x diet had greater amounts of Fabp1 in medial portion of SI compared to female Fabp2+/+ mice on the same diet (P<0.05) (Figure 12A).
In SFC diet group a clear increase in Fabp1 protein was detected in proximal (P<0.01) and medial (P<0.05) portion of the SI comparing Fabp2 gene ablated female mice to wild-type (Figure 12C). Sex dimorphism was noted in medial portion of 2020x diet fed mice and SFC diet fed mice with Fabp2−/− male mice recording lower amounts of Fabp1 proteins compared to Fabp2−/− female mice (P<0.05) (Figure 12A, 12C).
Figure 4. Effect of SF and SFC diets on body weight gain and gonadal adiposity in Fabp2+/+ and Fabp2−/− mice.

Data indicates mean ±SEM (n=5 mice per group), black and white bars indicate Fabp2+/+ and Fabp2−/− mice respectively on 2020x diet, high saturated fat diet (SF) and high saturated fat diet with 0.2% cholesterol (SFC). (A) Body weights after 4 weeks on diet. (B) Gonadal fat mass after 4 weeks on diet. * P<0.05 Fabp2+/+ vs. Fabp2−/− mice. Diet effect comparison was performed using one-way ANOVA with (a) P<0.05.
Figure 5. Effect of 2020x diet on energy metabolism and activity in \(Fabp2^{+/+}\) and \(Fabp2^{-/-}\) mice. Data indicates mean ±SEM (n=3 mice per group). Black and white squares indicate \(Fabp2^{+/+}\) and \(Fabp2^{-/-}\) male mice, respectively. Black and white circles indicate \(Fabp2^{+/+}\) and \(Fabp2^{-/-}\) female mice, respectively. Black and white bars represent \(Fabp2^{+/+}\) and \(Fabp2^{-/-}\) mice respectively. (A) Respiratory exchange ratio (RER), (B) energy expenditure (EE) and area under the curve (AUC) for EE and (C) total activity represents horizontal and vertical (rearing) activities. *P<0.05, \(Fabp2^{+/+}\) vs. \(Fabp2^{-/-}\) mice.
Figure 6. Effect of SF diet on energy metabolism and activity in $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice. Data indicates mean ±SEM (n=3 mice per group). Black and white squares indicate $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Black and white circles indicate $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Black and white bars represent $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. (A) Respiratory exchange ratio (RER), (B) energy expenditure (EE) and area under the curve (AUC) for EE and (C) total activity represents horizontal and vertical (rearing) activities. *P<0.05, $Fabp2^{+/+}$ vs. $Fabp2^{-/-}$ mice.
Figure 7. Effect of SFC diet on energy metabolism and activity in $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice.

Data indicates mean ±SEM (n=3 mice per group). Black and white squares indicate $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Black and white circles indicate $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Black and white bars represent $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. (A) Respiratory exchange ratio (RER), (B) energy expenditure (EE) and area under the curve (AUC) for EE and (C) total activity represents horizontal and vertical (rearing) activities.
Figure 8. Spatial distribution and abundance of intestinal (A) Fabp1, (B) ATF4, (C) total XBP1 and (D) XBP1s mRNA in 2020x diet-fed Fabp2^{+/+} and Fabp2^{-/-} mice.

Black and white squares represent male Fabp2^{+/+} and Fabp2^{-/-} mice, respectively. Black and white circles represent female Fabp2^{+/+} and Fabp2^{-/-} mice, respectively. Small intestine (SI) indicates the segments of the SI. For area under the curve (AUC), black and white bars indicate Fabp2^{+/+} and Fabp2^{-/-} mice respectively. (M) and (F) represents male and female mice area under the curve. Data shown represents the mean ± SEM (n=4-5 mice per group). *P<0.05 Fabp2^{+/+} vs. Fabp2^{-/-} mice. # P<0.05 male vs. female.
Figure 9. Spatial distribution and abundance of intestinal (A) Fabp1, (B) ATF4, (C) total XBP1 and (D) XBP1s mRNA in SF diet-fed Fabp2$^{+/+}$ and Fabp2$^{-/-}$ mice.

Black and white squares represent male Fabp2$^{+/+}$ and Fabp2$^{-/-}$ mice, respectively. Black and white circles represent female Fabp2$^{+/+}$ and Fabp2$^{-/-}$ mice, respectively. Small intestine (SI) indicates the segments of the SI. For area under the curve (AUC), black and white bars indicate Fabp2$^{+/+}$ and Fabp2$^{-/-}$ mice respectively. (M) and (F) represents male and female mice area under the curve. Data shown represents the mean ± SEM (n=4-5 mice per group). *P<0.05 **P<0.01 Fabp2$^{+/+}$ vs. Fabp2$^{-/-}$ mice. # P<0.05 ##P<0.01 male vs. female.
Figure 10. Spatial distribution and abundance of intestinal (A) Fabp1, (B) ATF4, (C) total XBP1 and (D) XBP1s mRNA in SFC diet-fed $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice.

Black and white squares represent male $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Black and white circles represent female $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. Small intestine (SI) indicates the segments of the SI. For area under the curve (AUC), black and white bars indicate $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice respectively. (M) and (F) represents male and female mice area under the curve. Data shown represents the mean ± SEM (n=4-5 mice per group). *P<0.05 **P<0.01 $Fabp2^{+/+}$ vs. $Fabp2^{-/-}$ mice. # P<0.05 ##P<0.01 male vs. female.
Figure 11. Comparison of ER stress markers ATF4 and XBP1s in male and female $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice fed SF and SFC diets.

Black and white bars represent $Fabp2^{+/+}$ and $Fabp2^{-/-}$ mice, respectively. mRNA abundance (A) ATF4 and (B) XBP1s. Data indicates mean ±SEM (n=5 mice per group). *P<0.05, **P<0.01, $Fabp2^{+/+}$ vs. $Fabp2^{-/-}$ mice.
Figure 12. Fabp1 protein abundance along the SI of Fabp2+/- and Fabp2-/- mice.

Black and white bars represent Fabp2+/- and Fabp2-/- mice respectively. (A) 2020x diet fed mice, (B) high saturated fat diet fed mice and (C) high saturated fat with cholesterol diet fed mice. Data indicates mean ± SEM (n=5 mice per group). *P<0.05 and **P<0.01, Fabp2+/- vs. Fabp2-/- mice. # P<0.05, male vs. female mice.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fabp2(^{+/+}) Males</th>
<th>Fabp2(^{-/-}) Males</th>
<th>Fabp2(^{+/+}) Females</th>
<th>Fabp2(^{-/-}) Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2020x diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.381 ±0.015</td>
<td>0.449 ±0.076</td>
<td>0.413 ±0.011</td>
<td>0.420 ±0.019</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.104 ±0.106</td>
<td>1.217 ±0.092</td>
<td>1.481 ±0.346</td>
<td>1.768 ±0.287</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>44.681 ±0.012</td>
<td>46.809 ±0.070</td>
<td>35.106 ±0.009</td>
<td>39.362 ±0.01</td>
</tr>
<tr>
<td><strong>SF diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.533 ±0.062</td>
<td>0.565 ±0.025</td>
<td>0.445 ±0.067</td>
<td>0.413 ±0.011#</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.013 ±0.186</td>
<td>3.153 ±0.143*</td>
<td>2.390 ±0.157</td>
<td>2.696 ±0.219</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>74.46 ±0.017</td>
<td>92.766 ±0.013*</td>
<td>49.574 ±0.017#</td>
<td>48.511 ±0.09#</td>
</tr>
<tr>
<td><strong>SFC diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.617 ±0.038</td>
<td>0.791 ±0.026*</td>
<td>0.536 ±0.062</td>
<td>0.552 ±0.021#</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>3.031 ±0.140</td>
<td>3.671 ±0.095*</td>
<td>2.816 ±0.157</td>
<td>2.870 ±0.254#</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>94.468 ±0.023</td>
<td>117.021 ±0.001*</td>
<td>39.362 ±0.004#</td>
<td>34.681 ±0.07#</td>
</tr>
</tbody>
</table>
Table 2. Plasma parameters concentration

Effect of diet high in saturated fat with or without cholesterol on plasma parameters. Data shown represent the mean ± SEM (n= 4-5 mice per group). *P<0.05 and **P<0.01, Fabp2+/− vs. Fabp2−/− mice. # P<0.05, male vs. female mice.
V. Discussion

Previous studies in our lab indicate that mice lacking Fabp2 display sex dimorphic phenotype, with male Fabp2\(^{-/+}\) mice being more susceptible to metabolic alterations than female Fabp2\(^{-/-}\) mice regardless of the diet [11, 12, 96, 97]. The results of these studies strongly imply that Fabp2 in the enterocytes plays an important role in the metabolism of dietary fatty acids. Fabp2 also shows preference for binding saturated fatty acids, which are known inducers of ER stress [1, 50, 54]. Based on these premises, the present study examined the potential role of Fabp2 in preventing fatty acid-induced ER stress in the intestine resulting from consumption of diets with an excess of saturated fats.

In order to investigate the role of Fabp2 in the SI, first we determined the impact of SF diet without and with cholesterol on whole body metabolism of Fabp2\(^{+/-}\) and Fabp2\(^{-/-}\) mice. However, the body weight changes observed for male Fabp2\(^{-/-}\) mice in the present study did not yield the expected results. One possible explanation may be the aggressive behavior exhibited by male mice in the present study. Another possibility may be related to the new diet formulations used in the present study, which were different from the diets used in the previous studies [11, 12, 96, 97]. Previous diets used Chow diet as a reference compared to 2020x, where 2020x is a purified defined diet clear form phytoestrogen as compared to Chow diet. Nevertheless, the biochemical and whole body metabolism data were consistent with previous studies [11, 96], with male Fabp2\(^{-/-}\) mice on either the saturated fat diet without and with cholesterol supplementation as displaying the highest degree of hypertriglyceridemia and hypercholesterolemia among all the mice analyzed in the present study. Also, whole body metabolism data was consistent with the pattern previously reported for male Fabp2\(^{-/-}\) mice, i.e., male Fabp2\(^{-/-}\) mice had lower EE and activity than male Fabp2\(^{+/-}\) mice [96]. Female Fabp2\(^{-/-}\)
mice reported lower body weight gain compared to female *Fabp2*+/+ counterparts independent of the diet, which is in agreement with previous studies done by our laboratory [11, 12, 96]. In general, these results obtained indicated that the SF and SFC diets caused altered metabolism in *Fabp2*−/− mice in a manner that was similar to that observed in the previous studies [96].

To determine if Fabp2 is part of a mechanism that protects the enterocytes from fatty acid-induced ER stress when consuming diets with high saturated fat content, ER stress status in the SI of male and female *Fabp2*+/+ and *Fabp2*−/− mice was assessed by examining the mRNA abundance of ER stress markers ATF4 mRNA abundance (indicator of PERK pathway activation), total and XBP1s mRNA abundance (indicator of IRE1 pathway status). The results clearly showed an increase in ER disturbance in *Fabp2*−/− male mice fed high saturated fat diet without or with cholesterol. We did not determine mRNA abundance of GRP78 that reflects the activation of ATF6 pathway [98], the third arm of the UPR, since ER stress induction by palmitic acid has been denoted by increase mainly in ATF4 and XBP1s expression [1, 51]. With regards to female *Fabp2*−/− mice, the results showed the opposite effects from male mice whereby the ER of female *Fabp2*−/− mice is protected from saturated fat diet-induced stress. Hodeify et al [99] reported a similar sex dimorphic sensitivity in the case of acute kidney injury, with male mice being more prone to tunicamycin-induced ER stress as indicated by marked increase in CHOP and XBP1s mRNA abundance, while female kidneys remain less susceptible to ER stress activation.

Previously, no change in Fabp1 mRNA abundance was detected in the SI of male and female *Fabp2*−/− mice maintained on the 2020X diet, consistent with the finding of a previous study done by our laboratory [96]. On the SF and SFC diet, Fabp1 mRNA abundance tented to increase along the SI, similar to previous observations [96]. Since both Fabp1 and Fabp2 coexist
in the same portions of the SI, and both can bind saturated fatty acids [80], it is possible that that Fabp1 could contribute to observed sex dimorphism displayed by Fabp2<sup>−/−</sup> mice. As Fabp6 is localized in the distal portion of the SI where Fabp2 protein abundance is at its lowest [80], I opted to analyze Fabp1 protein abundance, instead of Fabp6, since ligand binding could increase the half-life of Fabp1 protein. Moreover Fabp6 primarily binds to bile acids and functions mainly in bile acid transport [85]. The observed increase of Fabp1 protein in female Fabp2<sup>−/−</sup> mice on the SF and SFC diet suggest that Fabp1 can substitute for the Fabp2 and thus limiting ER stress activation. The increase in Fabp1 protein abundance observed in females may be attributed to an increase in half–life of the protein and or an increase in translation efficiency. In support of this concept, estrogen has been reported to increase mRNA stability [100]. Furthermore, Fabp1 is a known target activated by PPARα [101]. Studies on PPARα-deficient mice exhibit sex dimorphic regulation of lipid metabolism [102]. Male PPARα-deficient mice reported increased hepatic and cardiac lipid accumulation [102]. Interestingly this phenotype in males wasn’t observed after 2 weeks on estradiol pretreatment [102]. In light of this information, Fabp1 sex dimorphic response may be modulated by PPARα. Those findings strongly implicate the importance of studying the effects of estrogen in gene expression.

Previous analysis of fatty acid uptake capacity ex vivo showed that female Fabp2<sup>−/−</sup> mice have higher palmitic acid uptake capacity and increased CD36 mRNA abundance compared to male Fabp2<sup>−/−</sup> mice. This is accompanied with increased of fecal excretion of fatty acid [96]. Taken together, the increase in Fabp1 protein abundance in Fabp2<sup>−/−</sup> female mice suggests that Fabp1 is binding excess fatty acids and trapping them in enterocytes until these cells are shed from the body. I anticipated a general increase in ER stress markers when cholesterol was added to the SF diet since it is known that cholesterol is an inducer of ER stress [103]. The results
demonstrate, however, that SF diet-fed mice showed the highest amount of ER stress compared to SFC diet-fed mice. Rong et al demonstrated that oxysterols induces lysophosphatidylcholine acyltransferase 3 (LPCAT3) gene expression via LXR, and this causes an increase in phospholipids with unsaturated fatty acyl chains content and a decrease in ER stress markers [2]. This surprising finding will need further study in order to determine the mechanism how cholesterol decreases ER stress in the SI when present in a diet containing saturated fat.

In summary, the findings support shed the light on the idea that intestinal Fabps are part of a mechanism that protects enterocytes in the SI from ER stress resulting from overload of dietary fatty acids.
VI. Conclusion

In conclusion, the findings of this study support the hypothesis that Fabp2 protects the SI from ER stress resulting from a high saturated fat diet. Additionally, Fabp1 protein abundance was increased in the SI of female $Fabp2^{-/-}$ mice in response to the high fat diet. This female specific response may explain the sex dimorphic phenotype resulting from loss of Fabp2 in mice. Taken together, the findings support the idea that intestinal Fabps are part of a mechanism that protects enterocytes in the SI from ER stress resulting from overload of dietary fatty acids. A surprising finding in this study was the decrease in the extent of high dietary saturated fat-induced ER stress when cholesterol was included in the diet. The mechanism underlying this effect will require further study. Possible explanation may be attributed to the fact that cholesterol in enterocytes induces LXR and XBP1 splicing causing the stimulation of TG synthesis and PL synthesis, respectively [104, 105]. TG and PL are packed into lipoprotein (chylomicrons) and released into the portal circulation causing a reduction in ER stress inducers free fatty acid inside the enterocytes.

This study offers insights regarding two main concepts. The first concept is that perturbation of the ER function is mainly due to disturbance in energy and nutrient homeostasis contributing to the development of metabolic diseases [6]. With regards to this study, a specific type of saturated fatty acids, palmitic acid, have been implicated in this scenario. Palmitic acid in this study has been involved in the development of chronic metabolic diseases via ER stress activation. This is alarming since palmitic acid is a predominant type of fatty acid in western style diet. Thus this study highlights the primary need to focus on limiting the intake of palmitic acid from the diet. The second concept is that protection of cells from ER stress could prevent the development of chronic diseases. This study offers new insight in prevention of metabolic
disease. This study points out that an increase in Fabp2 in the SI may offer protection from ER stress and associated obesity phenotype induced by current dietary trends. The study offers venues for personalized medicine by targeted increase in Fabp2 gene expression in order to prevent ER stress induced by high fatty acids intake.
APPENDIX

Appendix A - Diet composition

<table>
<thead>
<tr>
<th>Diet</th>
<th>2020x</th>
<th>SF</th>
<th>SFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/g)</td>
<td>3.1</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Calorie Carbohydrates (%)</td>
<td>60</td>
<td>35.9</td>
<td>35.8</td>
</tr>
<tr>
<td>Calorie Protein (%)</td>
<td>24</td>
<td>21.2</td>
<td>21.2</td>
</tr>
<tr>
<td>Calorie Fat (%)</td>
<td>16</td>
<td>42.9</td>
<td>42.8</td>
</tr>
</tbody>
</table>

Table A1 - Diet composition based on calories

<table>
<thead>
<tr>
<th>Diet</th>
<th>2020x</th>
<th>SF</th>
<th>SFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates (%)</td>
<td>47</td>
<td>40.1</td>
<td>40</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>19.1</td>
<td>23.7</td>
<td>23.7</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>6.5</td>
<td>21.3</td>
<td>21.3</td>
</tr>
<tr>
<td>C 16:0 Palmitic (%)</td>
<td>0.6</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>C 18:0 Stearic (%)</td>
<td>0.1</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Total saturated fat (%)</td>
<td>0.8</td>
<td>12.3</td>
<td>12.3</td>
</tr>
<tr>
<td>Cholesterol (%)</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table A2 - Diet composition by weight
### Appendix B. Sequences of primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’→3’)</th>
<th>q-PCR run</th>
</tr>
</thead>
</table>
| **Fabp1** | F:TGCAGAGCCAGGAGAACTTTGAGCCA  
R: CCCCAGGGTGAACTCATTGCGGAC | Step 1: 95°C for 3 min.  
Step 2: 95°C for 10 s, 59°C for 30 s.  
Repeated 35 cycles. |
| **Total XBP1** | F: GAGTCCGCGAGCAGGTTGTG   
R: GTGTCAGAGTCCATGGA | Step 1: 95°C for 3 min  
Step 2: 95°C for 20 s, 58°C for 15 s, 72°C for 15 s.  
Repeated 35 cycles. |
| **XBP1s** | F: AAGAACACGCCTTGGGAATGG   
R: ACTCCCCTTGGCCTCCAC | Step 1: 95°C for 3 min  
Step 2: 95°C for 20 s, 58°C for 15 s, 72°C for 15 s.  
Repeated 35 cycles. |
| **ATF4** | F: GGACAGATTGATGTTGGAGAGAAATG   
R: GGAGATGGGCAATTTGGGTTTGCAC | Step 1: 95°C for 3 min  
Step 2: 95°C for 10 s, 58°C for 30 sec.  
Repeated 35 cycles. |
| **Villin** | F: TCAAAAGCTCTCTCAACATCAC   
R: AGCAGTCACCATCGAAGAAGC | Used to normalize in each run |

Table B1-Primer sequences for quantitative PCR.
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