

## Original Research

# Diet-induced differential effects on plasma lipids secretion by the inositol-requiring transmembrane kinase/endoribonuclease 1 $\alpha$

Jahangir Iqbal<sup>1,2,\*</sup>, Ali Al Qarni<sup>1</sup>, Ahmed Bakillah<sup>1,2</sup>

<sup>1</sup>King Abdullah International Medical Research Center (KAIMRC)-Eastern Region, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), King Abdulaziz Hospital (KAH), Ministry of National Guard-Health Affairs (MNG-HA), 31982 Al Ahsa, Saudi Arabia, <sup>2</sup>Department of Cell Biology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA

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## 1. Abstract

Intestinal and hepatic lipid metabolism plays an essential role in regulating plasma lipid levels. These lipids are mobilized on apolipoprotein B (apoB)-containing lipoproteins and their plasma homeostasis is maintained by balancing production and catabolism. Microsomal triglyceride transfer protein (MTP) which is expressed mainly in the intestine and liver plays an essential role in regulat-

ing the assembly and secretion of apoB-lipoproteins. Any imbalance in the production or clearance of lipoproteins leads to hyperlipidemia which is a major risk factor for atherosclerosis, obesity, diabetes, and metabolic syndrome. Here, we identify a new role of inositol-requiring transmembrane kinase/endoribonuclease 1 $\alpha$  (IRE1 $\alpha$ ) in the regulation of plasma lipids. We generated intestine specific IRE1 $\alpha$  knockout mice to study whether intestinal IRE1 $\alpha$

regulates plasma lipids by modulating intestinal lipid absorption. Intestine specific deletion of *Ire1a* gene in mice fed chow diet, significantly reduced plasma cholesterol and triglycerides by 29% and 43% in *Ire1a*<sup>-/-</sup> mice ( $P < 0.01$  &  $P < 0.001$ , respectively). These changes were not associated with any alteration of MTP activity nor its mRNA expression. On the other hand, Western diet increased plasma triglyceride by 37% ( $P < 0.01$ ) without affecting total plasma cholesterol in *Ire1a*<sup>-/-</sup> mice. Interestingly, this effect was associated with a significant increase in the intestinal MTP activity and its mRNA expression (25%,  $P < 0.01$  and 70%,  $P < 0.05$ , respectively). Collectively, our findings reveal key role of intestinal IRE1 $\alpha$  in the regulation of plasma lipids that may provide a therapeutic target for disorders of lipid metabolism.

## 2. Introduction

Hyperlipidemia is a major risk factor associated with atherosclerosis, obesity, diabetes, and metabolic syndrome and ensues due to overproduction or reduced clearance of lipoproteins. There is increasing evidence that alterations in metabolism of triglyceride-rich lipoproteins are of importance in the pathogenesis of atherosclerosis and its clinical consequences [1]. Further, increased hepatic very low density lipoprotein (VLDL) synthesis is the principal defect in subjects with hyperlipidemia and is also an important component of the dyslipidemia of diabetes and obesity [2–4]. Plasma homeostasis of lipids is maintained by balancing production and catabolism of apoB-lipoproteins mainly by the liver and intestine. Molecular mechanisms involved in lipoprotein assembly by the intestine and liver requires MTP, an essential chaperone resident in the endoplasmic reticulum, which transfers several lipids *in vitro* [5–7].

The endoplasmic reticulum (ER) is the major site for lipid synthesis and apoB-lipoproteins assembly. The ER stress, which occurs due to disruption in ER protein folding capacity, leads to activation of an evolutionarily conserved unfolded protein response (UPR) signaling system in order to restore ER homeostasis. The UPR allows cells to manage ER homeostasis through an adaptive mechanism involving IRE1 $\alpha$ , protein kinase R (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [8, 9]. These three proteins act in parallel to transmit information across the ER membrane to decrease protein synthesis and induce transcription factors to enhance the synthesis of chaperones. Increasing evidence suggests that ER stress and UPR activation can regulate cellular processes beyond ER protein folding and can play crucial roles in lipid metabolism [10–12]. Any disturbance in ER homeostasis can stimulate lipogenesis [13] and inhibit hepatic VLDL secretion [14, 15], leading to hepatosteatosis. Deletion of the hepatocyte-specific *Ire1a* gene causes profound hepatosteatosis and hypolipidemia in the mice upon ER stress

[11]. Furthermore, transcription factor X-box binding protein 1 (XBP-1) have been shown to directly regulate hepatic lipogenesis and lipid metabolic pathways [12].

MTP is regulated mainly at the transcriptional level [16, 17]. Because of its essential role in lipoprotein biosynthesis and availability of *in vitro* assays to measure its activity, MTP has been a favorite target to lower plasma lipids. Several antagonists have been identified that inhibit MTP activity and reduce plasma lipids [18, 19]. However, they are associated with substantial side effects. It is hypothesized that toxicities associated with MTP therapy can be avoided by tissue-specific inhibition of the intestinal MTP and sparing hepatic MTP. In fact, several such reagents have been identified [19]. Hence, it is timely to explore intestine specific mechanisms that control lipid absorption.

Besides transcriptional regulation, IRE1 $\beta$ , cleaves MTP mRNA using a novel post-transcriptional regulatory mechanism to down regulate MTP expression [20]. Absence of *Ire1 $\beta$*  expression predisposes mice to hyperlipidemia and atherosclerosis when fed high cholesterol and fat diet [21]. In this study, we asked whether a ubiquitously expressed homolog of IRE1 $\beta$ , IRE1 $\alpha$  known to play a critical role in the UPR or ER stress, is also involved in the regulation of MTP and intestinal lipid absorption. Gene deletion studies have revealed that *Ire1a*<sup>-/-</sup> embryos die between 12.5 and 13 days of gestation [22]. Hence, *Ire1a* floxed mice have been generated to study tissue specific role of IRE1 $\alpha$  [11]. We generated intestine specific IRE1 $\alpha$  knockout mice to study whether intestinal IRE1 $\alpha$  regulates plasma lipids by modulating intestinal lipid absorption.

## 3. Materials and methods

### 3.1 Materials

Infinity Cholesterol (catalog #TR13421), Infinity Triglyceride (catalog #TR22421), and TRIzol<sup>TM</sup> (catalog #15596018) reagents were purchased from Thermo Fisher Scientific (Middletown, VA). Omniscript RT (catalog #205113) kit was purchased from Qiagen (Germantown, MD) and qPCR<sup>TM</sup> core kit for SYBR Green I (catalog #10-SN10-05) was from Eurogentec (San Diego, CA). Western diet (catalog #TD.88137) containing 17.3%, 48.5%, 21.2%, and 0.2% by weight of protein, carbohydrate, fat, and cholesterol, respectively was purchased from Envigo (Indianapolis, IN). All other chemicals and solvents were obtained from Fisher Scientific (Pittsburgh, PA) or VWR International (Bridgeport, NJ).

### 3.2 Animals

*Ire1a*<sup>f/f</sup> mice have been previously described [11] and were crossed with *Vil-CRE* transgenic mice [23] to obtain heterozygous *I-Ire1a*<sup>+/-</sup>. These mice were crossed to generate homozygous *Ire1a*<sup>-/-</sup> mice. *Ire1a*<sup>f/f</sup> mice were used as wild type controls (*I-Ire1a*<sup>+/+</sup>) in the study.

Mice were kept at 21–23 °C on a 12-h dark/light cycle (lights on: 7:00 AM–7:00 PM). To study the effects of Western diet, 12-weeks old male *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice (22–25 g; N = 3 per group) were fed a Western diet for 3 weeks. Mice were fed either chow diet (LabDiet 5001) or Western diet (TD88137, Envigo; Indianapolis, IN) containing 23.9, 48.7, 5, 0.02% (chow diet) 17, 48.5, 21.2, and 0.2% (Western diet) by weight of protein, carbohydrate, fat, and cholesterol, respectively. Animal care and procedures were performed in accordance with the guidelines of and approved by the Institutional Animal Care and Use Committee of State University of New York Downstate Medical Center.

### 3.3 Plasma and tissue lipid measurements

Total cholesterol and triglyceride levels in the plasma and tissues were measured using commercially available kits from Thermo Fisher Scientific (Middletown, VA) as described previously [24]. Plasma lipoproteins were separated by gel filtration (flow rate of 0.2 mL/min) using a Superose 6 10/300 GL column (GE Healthcare Life Sciences, Marlborough, MA), and 200  $\mu$ L fractions were collected to determine the cholesterol and triglycerides in apoB- and non apoB-containing lipoproteins [20].

### 3.4 Determination of MTP activity

Small pieces (0.1 g) of liver or proximal small intestine (~1-cm) were homogenized in low salt buffer (1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM MgCl<sub>2</sub>) and centrifuged, and supernatants were used for protein determination and MTP assay [25].

### 3.5 mRNA quantification

Total RNA from tissues was isolated using TRIzol<sup>TM</sup>. The purity of RNA was assessed by the A<sub>260</sub>/A<sub>280</sub> ratio. RNA preparations with A<sub>260</sub>/A<sub>280</sub> ratios more than 1.7 were used for cDNA synthesis. The first strand cDNA was synthesized using Omniscript RT kit. Each reaction of quantitative PCR was carried out in a volume of 20  $\mu$ L, consisting of 10  $\mu$ L of cDNA sample (1 : 100 dilution of the first strand cDNA sample) and 10  $\mu$ L of PCR master mix solution containing 1X PCR buffer from qPCR<sup>TM</sup> core kit for SYBR Green I. The PCR was carried out by incubating the reaction mixture first for 10 min at 95 °C followed by 40 cycles of 15 sec incubations at 95 °C and 1 min at 60 °C in an ABI 7000 SDS PCR machine (Thermo Fisher Scientific, Middletown, VA). Data were analyzed using  $\Delta\Delta C_T$  method, according to the manufacturer's instructions, and presented as arbitrary units that were normalized to *ArpP0* mRNA.

### 3.6 Xbp-1 mRNA splicing

A two-step RT-PCR method was used to determine *Xbp-1* mRNA splicing. The primers (*Xbp1*-forward 5'-ggccgggtctgctgagt-3' and *Xbp1*-reverse 5'-tccttctgggtagacctctggga-3') were designed to differentiate

the two forms of *Xbp-1*, which differ by 26 nucleotides. The PCR products were separated by 3% agarose gels.

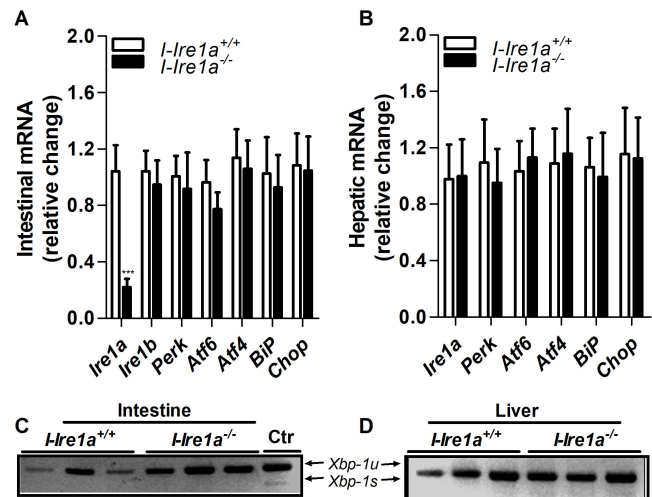
### 3.7 Statistical analysis

Data were analyzed using GraphPad Prism 5 (Windows V5, GraphPad Software). Data are presented as mean  $\pm$  standard deviation (S.D). Comparisons of mean values between two groups were performed by Students' *t* test. Values of *P* < 0.05 were considered significant.

## 4. Results

### 4.1 Deletion of intestinal IRE1 $\alpha$ does not affect ER stress genes in the intestine or liver of chow fed mice

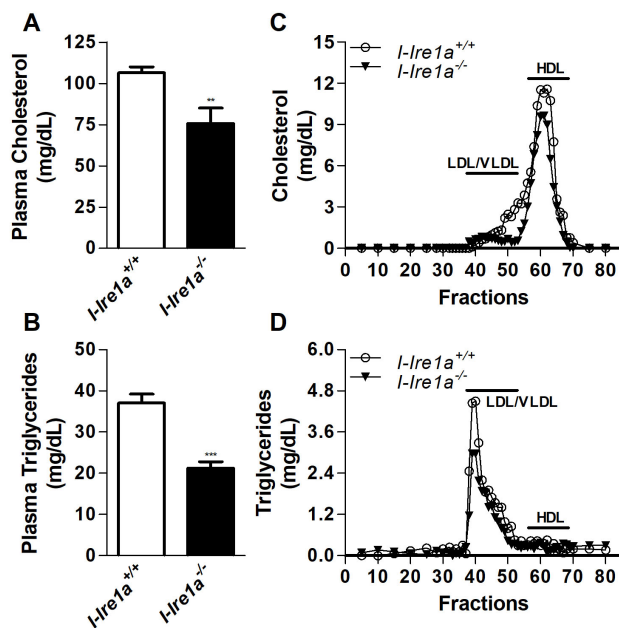
To determine whether deletion of IRE1 $\alpha$  in the intestine affects ER stress, intestinal and liver mRNA from 12 week old *Vil-Cre*<sup>-/-</sup> *Ire1a*<sup>f/f</sup> (*I-Ire1a*<sup>+/+</sup>) and *Vil-Cre*<sup>+/+</sup> *Ire1a*<sup>f/f</sup> (*Ire1a*<sup>-/-</sup>) mice on chow diet was used to quantify the expression of different ER stress genes. Deletion of *Ire1a* gene resulted in a significant knockdown of ~80% in its mRNA levels in the intestine (*P* < 0.001). However, no change was observed in the mRNA levels of *Ire1b* or other ER stress genes (*Perk*, *Atf6*, *Atf4*, *BiP*, and *Chop*) in both the intestine and liver (Fig. 1A,B). Similarly, feeding of chow diet did not result in the splicing of intestinal or hepatic *Xbp-1* mRNA, another marker of ER stress, in both *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice (Fig. 1C,D). These data show that deletion of IRE1 $\alpha$  in the intestine does not result in ER stress in chow fed mice.



**Fig. 1. Expression of ER stress genes in intestine specific ablated *Ire1a* mice fed chow diet.** 12-week old male (*n* = 3) *Vil-Cre*<sup>-/-</sup> *Ire1a*<sup>f/f</sup> (*I-Ire1a*<sup>+/+</sup>) and *Vil-Cre*<sup>+/+</sup> *Ire1a*<sup>f/f</sup> (*Ire1a*<sup>-/-</sup>) mice on chow diet were fasted for 6 h and sacrificed. mRNA from intestine and liver were used to determine expression of various ER stress genes (A and B) and *Xbp-1* splicing (C and D), respectively. Values (mean  $\pm$  SD). \*\*\**P* < 0.001 are significantly different from *I-Ire1a*<sup>+/+</sup> mice. Ctr, positive control for *Xbp-1* splicing.

#### 4.2 Plasma lipids are reduced in *Ire1a*<sup>-/-</sup> mice fed chow diet

To test whether intestinal IRE1 $\alpha$  plays a role in lipid metabolism, 12 week old *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice on chow diet were fasted for 6 h and plasma lipids were measured. Compared with *I-Ire1a*<sup>+/+</sup> control mice, both plasma cholesterol (Fig. 2A) and triglycerides (Fig. 2B) were significantly reduced by ~29–43% in *Ire1a*<sup>-/-</sup> mice ( $P < 0.01$  &  $P < 0.001$ , respectively). The decrease in cholesterol was in both apoB- and non apoB-containing lipoproteins (Fig. 2C) whereas decrease in triglycerides was in mainly in apoB-containing low-density lipoprotein (LDL)/VLDL lipoproteins (Fig. 2D). These results suggest that intestine specific deletion of *Ire1a* decreases plasma lipids.



**Fig. 2. Effect of intestine specific deletion of *Ire1a* on plasma lipids in chow fed mice.** 12-week old male ( $n = 3$ ) *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice on chow diet were fasted for 6 h and blood was collected through retro-orbital bleeding. Plasma was used to measure total cholesterol (A) and triglycerides (B). Pooled plasma from each group was separated by FPLC and lipids were measured in the fractions (C and D). Values (mean  $\pm$  SD) are representative from 2 independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  are significantly different from *I-Ire1a*<sup>+/+</sup> mice.

#### 4.3 Intestinal cholesterol and hepatic triglycerides are decreased in *Ire1a*<sup>-/-</sup> mice fed chow diet with no change in MTP expression

We have shown previously that deletion of IRE1 $\beta$  results in an increase in the expression of intestinal MTP [20]. On the other hand, Wang *et al.* [26] have shown that deletion of IRE1 $\alpha$  in the liver results in decreased MTP activity. To determine whether decrease in plasma lipids in *Ire1a*<sup>-/-</sup> mice on chow diet was due to any changes in the

expression of MTP, we measured MTP activity (Fig. 3A,D) and mRNA levels (Fig. 4A,B) in the intestine and the livers of these mice. We did not observe any significant difference in the intestinal and hepatic MTP activity and mRNA levels between the wild type and intestine specific *Ire1a* gene knockout mice. Next, we looked at the levels of lipids in these tissues that might contribute to any changes in plasma lipids. There was a significant decrease of 35% ( $P < 0.05$ ) in the intestinal cholesterol levels (Fig. 3B) in *Ire1a*<sup>-/-</sup> mice compared to *I-Ire1a*<sup>+/+</sup> mice. This decrease might be due to less uptake of dietary cholesterol from the intestinal lumen since *Ire1a*<sup>-/-</sup> mice exhibited reduced expression of ~67% in *Npc1l1* levels in the intestine (Fig. 4A). Interestingly, we also saw a significant decrease of ~40% ( $P < 0.05$ ) in hepatic triglycerides (Fig. 3F) in these mice which might be secondary to decreased absorption by the intestine. On the other hand, no difference was seen in the intestinal triglycerides (Fig. 3C) or hepatic cholesterol (Fig. 3E) between *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice.

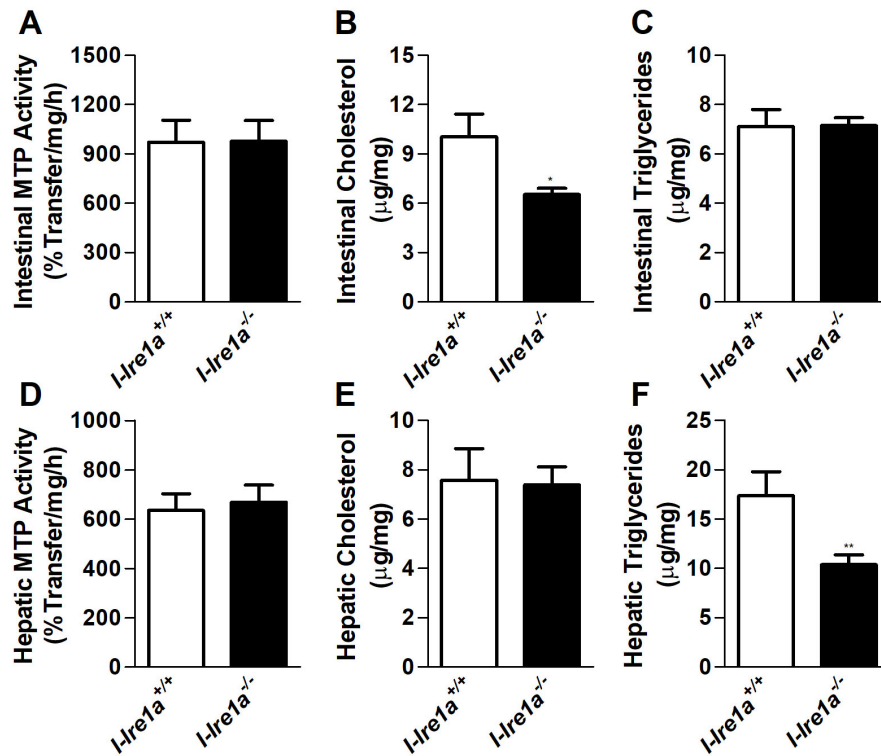
#### 4.4 Expression of lipid metabolism genes in the intestine and liver of chow fed *Ire1a*<sup>-/-</sup> mice

To determine whether deletion of IRE1 $\alpha$  in the intestine affects lipid metabolism genes in the intestine and liver, we isolated mRNA from *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice and performed quantitative PCR of various genes. Besides *Npc1l1*, deletion of intestinal IRE1 $\alpha$  also decreased intestinal *Abca1* levels (Fig. 4A) by 46% ( $P < 0.05$ ) which may be due to less dietary cholesterol being taken up by the enterocytes. We also observed decreased expression of ~49–56% ( $P < 0.05$ ) in *Cpt1 $\alpha$* , *Ppara $\alpha$* , *Acc1 $\alpha$* , *Scd1* and *Mgat2* mRNA levels in the intestine of *Ire1a*<sup>-/-</sup> mice (Fig. 4A) suggesting that both oxidation as well as synthesis of lipids may be reduced in *Ire1a*<sup>-/-</sup> mice. Furthermore, these mice also showed a significant reduction of 55% and 66% in *Ppara $\alpha$*  and *Cpt1 $\alpha$*  mRNA levels ( $P < 0.05$  &  $P < 0.01$ ), respectively in the liver suggesting reduced lipid oxidation (Fig. 4B). On the other hand, levels of *Mgat2*, *Srebp-1c* and *Srebp2* were significantly increased ( $P < 0.01$ ) in the livers of *Ire1a*<sup>-/-</sup> mice compared to *I-Ire1a*<sup>+/+</sup> mice (Fig. 4B) suggesting that liver is working toward increasing the synthesis of lipids to compensate for their reduced uptake from the plasma.

#### 4.5 Western diet affects expression of ER stress genes in intestinal IRE1 $\alpha$ knockout mice

Chow diet resulted in lower plasma and tissue lipids in the intestine specific IRE1 $\alpha$  knockout mice without any significant change in the expression of ER stress genes or MTP (Figs. 1-4). Next, we wanted to check whether Western diet had similar effect on the plasma and tissue lipid levels and tissue gene expression. Feeding of Western diet for 3 weeks significantly reduced the expression of ER stress genes in the intestine of *Ire1a*<sup>-/-</sup> mice compared to *I-Ire1a*<sup>+/+</sup> mice (Fig. 5A). Expression of *Atf4*, *Perk*, *BiP* and *Ire1 $\beta$*  in the intestine was decreased by ~27–





**Fig. 3. Effect of intestine specific deletion of *Ire1a* on MTP activity and tissue lipids in chow fed mice.** 12-week old male ( $n = 3$ ) *I-Ire1a*<sup>+/+</sup> and *I-Ire1a*<sup>-/-</sup> mice on chow diet were fasted for 6 h and sacrificed. Intestine and liver were used to measure MTP activity (A and D), total cholesterol (B and E), and triglycerides (C and F), respectively. Values (mean  $\pm$  SD) are representative from 2 independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  are significantly different from *I-Ire1a*<sup>+/+</sup> mice.

55% ( $P < 0.01$ ), but *Atf6* expression was increased by 58% ( $P < 0.05$ ) in *Ire1a*<sup>-/-</sup> mice compared to *I-Ire1a*<sup>+/+</sup> mice (Fig. 5A). Feeding of Western diet was associated with the splicing of *Xbp-1* mRNA only in the intestine of *I-Ire1a*<sup>+/+</sup> mice but not in *Ire1a*<sup>-/-</sup> mice (Fig. 5C). These data show that deletion of IRE1 $\alpha$  in the intestine may prevent Western diet induced *Xbp-1* splicing and downstream ER stress response. However, increase in the expression of ATF6 levels in the intestine of *Ire1a*<sup>-/-</sup> mice suggest that ATF6 arm of the unfolded protein response may be activated to mitigate the ER stress in the absence of IRE1 $\alpha$ . Next, we measured the levels of ER stress genes in the liver to determine if deletion of intestinal IRE1 $\alpha$  affects their expression in the liver. Expression of ER stress mRNAs (*Ire1a*, *Perk*, *Atf6*, *Atf4*, *BiP*, and *Chop*) were increased by 36–88% ( $P < 0.05$ ) in *Ire1a*<sup>-/-</sup> livers (Fig. 5B). Similarly, there was an increased splicing of *Xbp-1* mRNA (Fig. 5D) in the livers of *Ire1a*<sup>-/-</sup> mice compared to *I-Ire1a*<sup>+/+</sup> mice. These data suggest that Western diet has a differential effect on the expression of ER stress genes in the intestine and liver of *Ire1a*<sup>-/-</sup> mice.

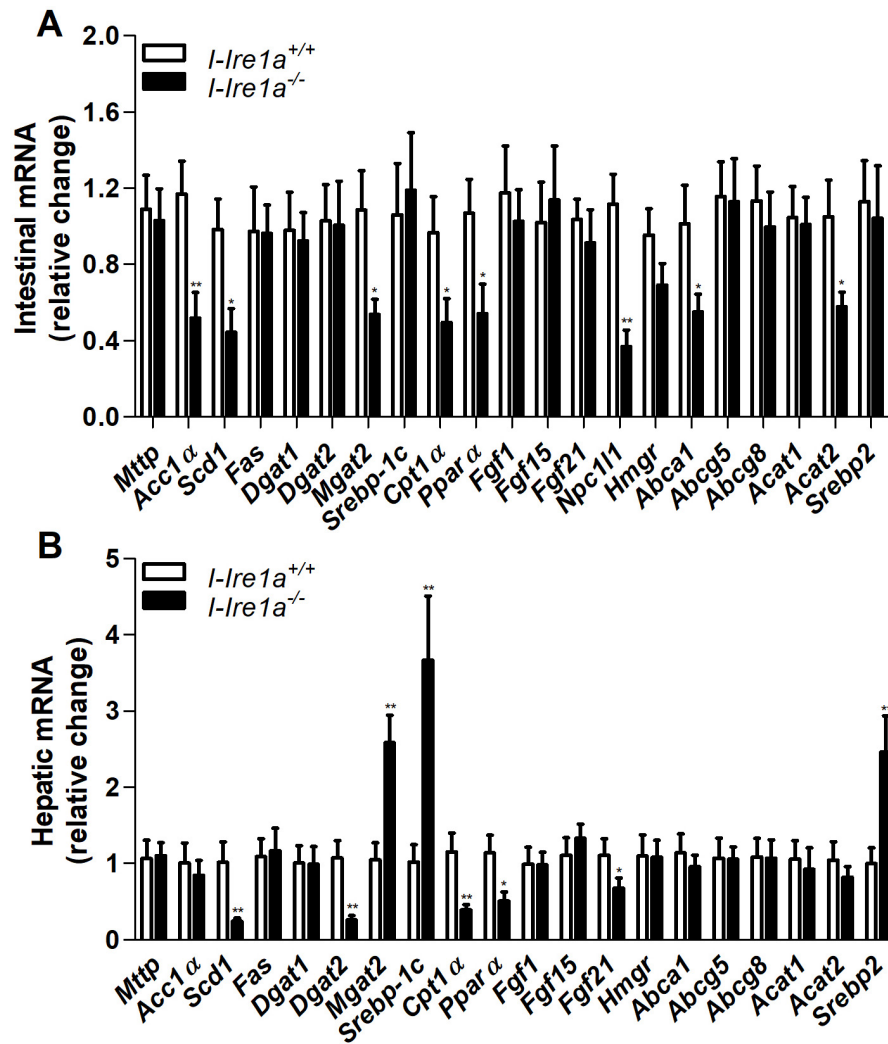
#### 4.6 Plasma triglycerides are increased in *Ire1a*<sup>-/-</sup> mice on Western diet

To determine whether Western diet also affects lipid metabolism in the intestinal IRE1 $\alpha$  knockout mice,

12-week old *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice on Western diet for 3 weeks were fasted for 6 h and plasma lipids were measured. Compared with *I-Ire1a*<sup>+/+</sup> mice, total plasma cholesterol was unaffected (Fig. 6A) but plasma triglycerides (Fig. 6B) were significantly increased by 37% ( $P < 0.01$ ) in *Ire1a*<sup>-/-</sup> mice. Although total plasma cholesterol was not changed, we observed that there was an increase in the levels of cholesterol in apoB-containing lipoproteins with a decrease in non-apoB containing lipoprotein cholesterol (Fig. 6C). Furthermore, increase in the levels of total plasma triglycerides was mainly due to an increase in the triglycerides in apoB-containing lipoproteins (Fig. 6D). These results suggest that Western diet feeding increases the levels of plasma lipids in the apoB-containing lipoproteins in intestine specific *Ire1a* gene deleted mice.

#### 4.7 Western diet affects tissue lipids and MTP expression in *Ire1a*<sup>-/-</sup> mice

We observed earlier that chow diet decreased the plasma and tissue lipids in *Ire1a*<sup>-/-</sup> mice without any change in MTP expression (Figs. 2–4). Contrary to chow diet (Fig. 2C), Western diet increased the levels of plasma triglycerides by 37% in the knockout mice compared to wild type mice (Fig. 6C). To determine whether this increase was due to any change in MTP expression or tissue lipids, we measured the MTP activity and mRNA as well



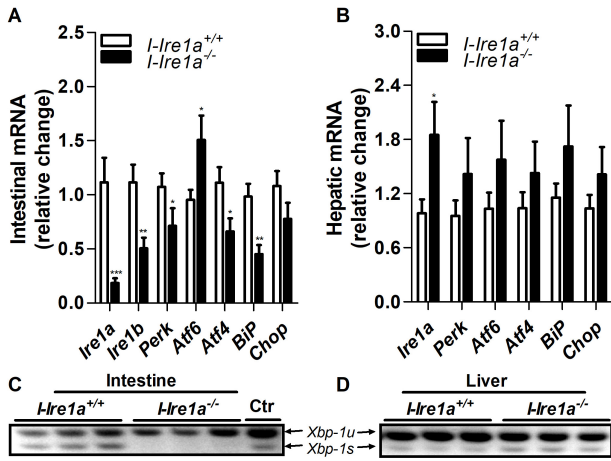
**Fig. 4. Expression of lipid metabolism genes in intestine specific IRE1a knockout mice fed chow diet.** 12-week old male ( $n = 3$ )  $I-Ire1a^{+/+}$  and  $I-Ire1a^{-/-}$  mice on chow diet were fasted for 6 h and sacrificed. mRNA from intestine (A) and liver (B) were used to determine expression of various lipid metabolism genes. Values (mean  $\pm$  SD). \* $P < 0.05$  and \*\* $P < 0.01$  are significantly different from  $I-Ire1a^{+/+}$  mice.

as cholesterol and triglycerides in the intestine and liver of  $I-Ire1a^{+/+}$  and  $I-Ire1a^{-/-}$  mice. Feeding of Western diet resulted in a significant increase of  $\sim 25\%$  ( $P < 0.01$ ) in the intestinal MTP activity (Fig. 7A) and 70% ( $P < 0.05$ ) in the intestinal MTP mRNA levels (Fig. 8A) in  $I-Ire1a^{-/-}$  mice compared to  $I-Ire1a^{+/+}$  mice. On the other hand, there was an insignificant decrease of 22% and a significant decrease of 56% ( $P < 0.001$ ) in the levels of intestinal cholesterol and triglycerides, respectively in  $I-Ire1a^{-/-}$  mice (Fig. 7B,C). These data suggests that increase in plasma lipids may be due to increased secretion of apoB-containing lipoproteins by the intestine. Next, we looked at the effect of Western diet on the hepatic MTP expression and lipids in  $I-Ire1a^{+/+}$  and  $I-Ire1a^{-/-}$  mice. Contrary to increased intestinal MTP expression, there was a reduction of around 45% ( $P < 0.001$ ) in the levels of MTP activity (Fig. 7D) and mRNA (Fig. 8B) in the livers of knockout mice. This reduction in MTP expression was accompanied by  $\sim 8$ -fold accumula-

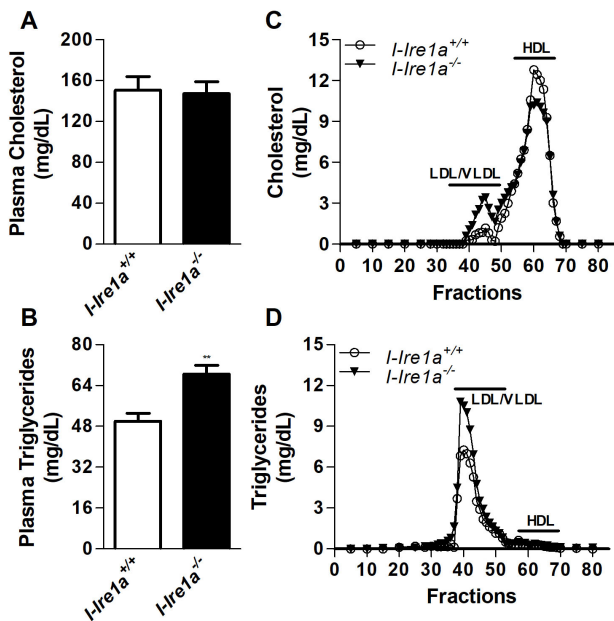
tion in the levels of hepatic triglycerides in  $I-Ire1a^{-/-}$  mice compared to  $I-Ire1a^{+/+}$  mice (Fig. 7F). Again, no significant difference was seen in the hepatic cholesterol (Fig. 7E) between  $I-Ire1a^{+/+}$  and  $I-Ire1a^{-/-}$  mice fed a Western diet for 3 weeks.

#### 4.8 Expression of lipid metabolism genes in the intestine and liver of $I-Ire1a^{-/-}$ mice fed Western diet

Overall feeding of Western diet did not change the expression of triglyceride synthesis genes in the intestine of  $I-Ire1a^{+/+}$  and  $I-Ire1a^{-/-}$  mice (Fig. 8A). However, there was a reduction of 56% and 44% in the expression of  $Cpt1\alpha$  and  $Ppar\alpha$  ( $P < 0.05$ ), respectively in the knockout mice intestine suggesting a lower fatty acid oxidation compared to wild type mice. Similar to chow fed mice, we observed a decrease of 55% (0.05) in the expression of  $Npc111$  levels in the intestine of  $I-Ire1a^{-/-}$  mice fed a Western diet (Fig. 8A) which may suggest that these mice take up less cholesterol



**Fig. 5. Expression of ER stress genes in intestine specific ablated *Ire1a* mice fed Western diet.** 12-week old male ( $n = 3$ ) *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice on Western diet for 3 weeks were fasted for 6 h and sacrificed. mRNA from intestine and liver were used to determine expression of various ER stress genes (A and B) and *Xbp-1* splicing (C and D), respectively. Values (mean  $\pm$  SD). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  are significantly different from *I-Ire1a*<sup>+/+</sup> mice. Ctr, positive control for *Xbp-1* splicing.



**Fig. 6. Effect of intestine specific deletion of *Ire1a* on plasma lipids in Western diet fed mice.** 12-week old male ( $n = 3$ ) *I-Ire1a*<sup>+/+</sup> and *Ire1a*<sup>-/-</sup> mice on Western diet for 3 weeks were fasted for 6 h and blood was collected through retro-orbital bleeding. Plasma was used to measure total cholesterol (A) and triglycerides (B). Pooled plasma from each group was separated by FPLC and lipids were measured in the fractions (C and D). Values (mean  $\pm$  SD). \*\* $P < 0.01$  are significantly different from *I-Ire1a*<sup>+/+</sup> mice.

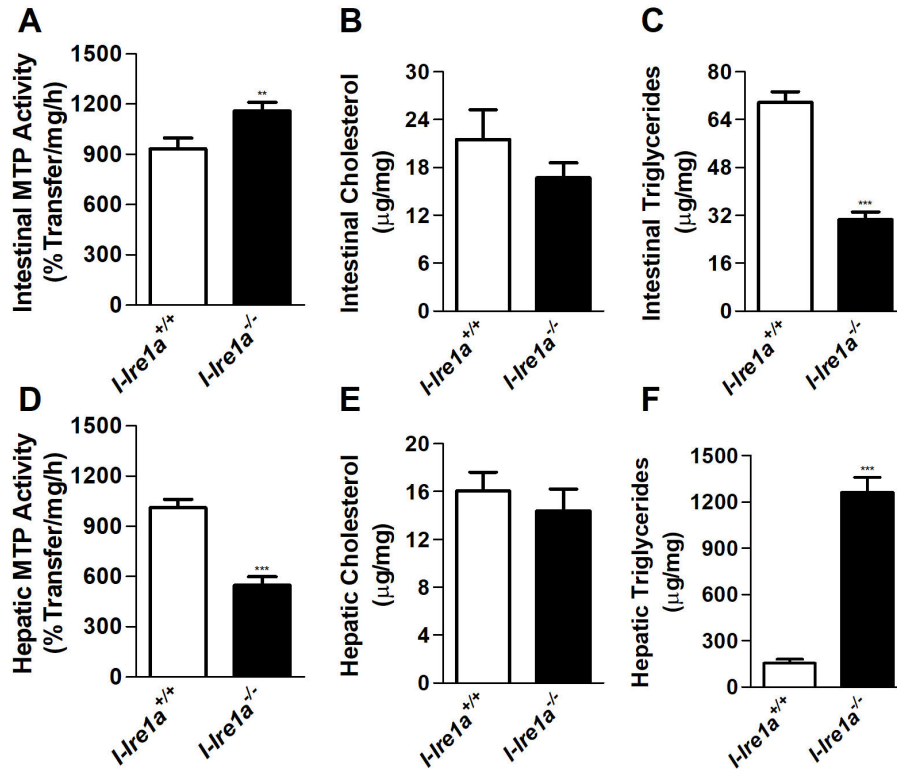
by the enterocytes. Next, we looked at the expression of lipid metabolism genes in the liver. Interestingly, expression of lipid metabolism genes such as *Fas*, *Dgat1*, *Dgat2*,

*Mgat2*, *Srebp-1c*, *Cpt1 $\alpha$* , *Ppar $\alpha$* , *Abcg8*, etc. was significantly increased by Western diet in the livers of *Ire1a*<sup>-/-</sup> mice (Fig. 8B). Besides, lipid metabolism genes, there was also an increase of  $\sim 3.7$ -fold in the expression of *Fgf21* in the livers of *Ire1a*<sup>-/-</sup> mice (Fig. 8B). These results along with decreased activity and expression of MTP may explain the increased levels of triglycerides in the liver of *Ire1a*<sup>-/-</sup> mice fed a Western diet.

## 5. Discussion

Intestinal and hepatic MTP expression plays an essential role in regulating plasma lipid and lipoprotein levels. MTP expression is controlled at transcriptional and post-transcriptional levels and we have shown that intestinal *IRE1 $\beta$*  down regulates MTP expression to decrease plasma lipid levels [20]. In this study, we asked whether ubiquitously expressed homolog of *IRE1 $\beta$* , *IRE1 $\alpha$*  is also involved in the regulation of plasma lipid metabolism. The molecular and physiological implications of ER stress sensor, *IRE1 $\alpha$*  as well as its mechanistic action in metabolic disorders have been well described [27, 28]. Gene deletion studies have revealed that *Ire1a*<sup>-/-</sup> embryos die between 12.5 and 13 days of gestation [22]. Therefore, we generated intestine specific *IRE1 $\alpha$*  knockout mice to study its role in plasma lipid levels.

*IRE1 $\alpha$*  knockout mice (*Ire1a*<sup>-/-</sup> and *Ire1a*<sup>-/-</sup>) fed with either chow diet or Western diet for 3 weeks did not result in any significant changes in body weight (data not shown). Analysis of gene expression in the intestine and liver revealed that deletion of intestine specific *IRE1 $\alpha$*  does not affect the expression of ER stress gene markers nor *Xbp-1* splicing on chow diet (Fig. 1). In contrast, Zhang *et al.* [29] reported increased expression of *Chop* and other ER stress markers in colon epithelial cells of intestine specific *Ire1a*<sup>-/-</sup> mice. This discrepancy may be due to the differences in either the age (16–18 weeks) or the sex (females) of mice used in the study. In the current study, we used 12 weeks old male mice to study the role of *IRE1 $\alpha$*  in the regulation of plasma lipids. It was reported that females were more susceptible to develop intestinal dysfunction such as colitis in the absence of intestinal *IRE1 $\alpha$*  [29]. These authors also reported increased mortality only after the age of around 16–18 weeks suggesting that intestinal dysfunction and, therefore, ER stress may be exacerbated in the later stages of life in these mice. We noticed decreased *Xbp-1* splicing in the intestine of *Ire1a*<sup>-/-</sup> mice fed a Western diet for 3 weeks (Fig. 5C). This is consistent with the reduced expression of *IRE1 $\alpha$*  in the intestine. On the other hand, Western diet increased *Xbp-1* splicing in the intestine of wild type mice. We have shown previously that *IRE1 $\beta$*  does not contribute to the increase in *Xbp-1* splicing in the intestine of Western diet fed mice [20]. During chronic stress, Zhang *et al.* [29] also reported a decreased expression of spliced variant of *Xbp-1* in the intestinal ep-



**Fig. 7. Effect of intestine specific deletion of *Ire1a* on MTP activity and tissue lipids in Western diet fed mice.** 12-week old male ( $n = 3$ ) *I-re1a*<sup>+/+</sup> and *I-re1a*<sup>-/-</sup> mice on Western diet for 3 weeks were fasted for 6 h and sacrificed. Intestine and liver were used to measure MTP activity (A and D), total cholesterol (B and E), and triglycerides (C and F), respectively. Values (mean  $\pm$  SD). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  are significantly different from *I-re1a*<sup>+/+</sup> mice.

ithelial cells of 16-18 weeks old female *Ire1a*<sup>-/-</sup> mice. The combined data suggests that chronic ER stress either due to Western diet feeding or older age of mice augments *Xbp-1* mRNA splicing and IRE1 $\beta$  cannot compensate for the absence of IRE1 $\alpha$  in the splicing of *Xbp-1* mRNA.

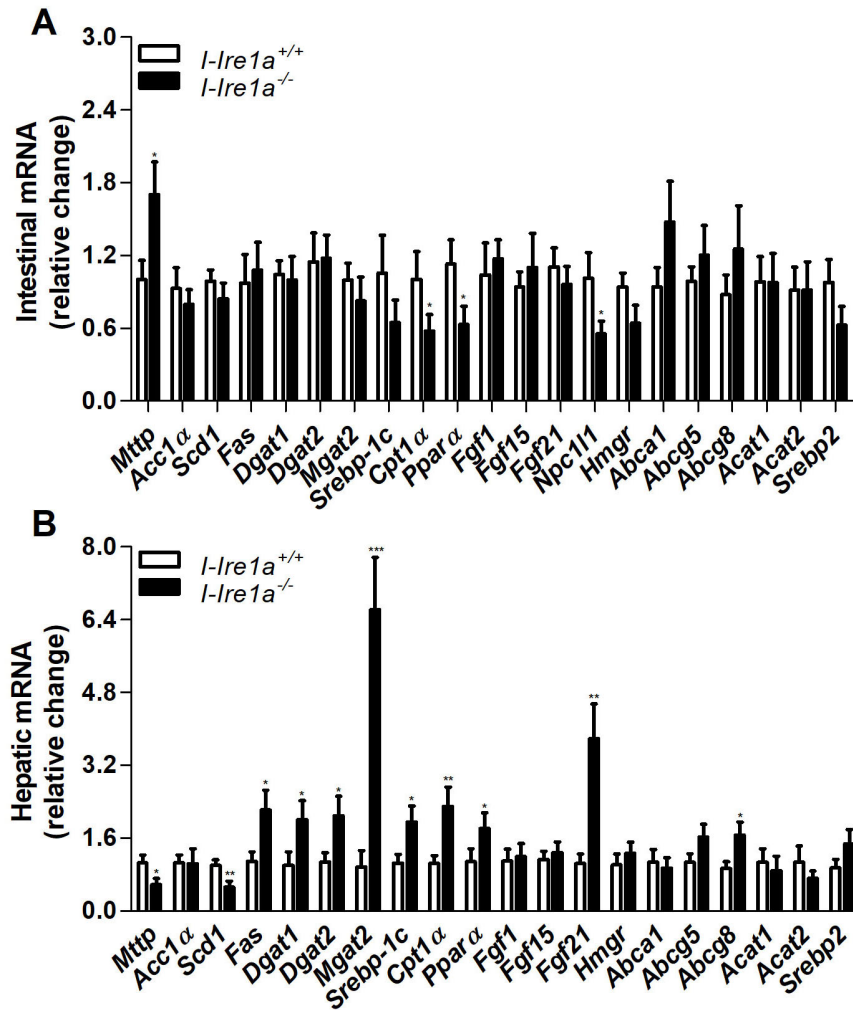
In addition, we have shown previously that absence of IRE1 $\beta$  in the intestine of chow diet fed mice does not change plasma lipids significantly [20]. In the present study, our data indicate that deletion of intestine-specific IRE1 $\alpha$  decrease plasma cholesterol and triglycerides in chow diet fed mice and this decrease was independent of changes in intestinal MTP activity or mRNA levels (Figs. 2-4). These mice also showed a reduction in intestinal cholesterol levels. Determination of mRNA levels in the chow fed *Ire1a*<sup>-/-</sup> suggest a decrease in the intestinal *Npc1l1* and *Abca1* levels suggesting that both uptake as well as efflux of cholesterol by the enterocytes may be reduced in the knockout mice resulting in reduced plasma cholesterol levels. Interestingly, our data revealed a decrease in hepatic triglyceride levels. This reduction in hepatic triglycerides may be due to reduced absorption by the intestine. Quantification of mRNA levels suggested changes in the expression of several lipid metabolism genes in the liver which may be secondary to the changes in the lipid absorption by the intestine. This increase may be due to compensatory changes

in the liver to boost up the synthesis because of less lipids coming from the intestine. These results are consistent with the intestine-specific MTP knockout mice which show a decrease in hepatic lipid levels with a compensatory increase in lipogenic genes due to less lipid absorption from the intestine [30, 31].

Using liver specific IRE1 $\alpha$  knockout mice, Wang *et al.* [26] showed that IRE1 $\alpha$  in the liver reduces plasma lipids primarily due to a defective triglyceride-rich VLDL secretion. However, they did not find any defects in lipogenesis or apoB synthesis and secretion in the *L-Ire1a*<sup>-/-</sup> hepatocytes. Furthermore, similar to our data, these authors reported that expression of MTP was not affected by the deletion of IRE1 $\alpha$ . However, they noticed a decrease of MTP activity in the liver due to reduction in the expression of protein disulfide isomerase (PDI), a subunit of MTP that is necessary for its normal activity [32]. It is possible that IRE1 $\alpha$  has different roles in regulating MTP activity in the intestine and liver to affect lipid metabolism.

Contrary to chow diet, Western diet caused an increase in plasma and a decrease in the intestinal triglycerides level in *Ire1a*<sup>-/-</sup> mice (Figs. 6,7). This may be due to increased MTP activity and mRNA levels in the intestine of these mice. Interestingly, we observed increased triglycerides accumulation in the livers of *Ire1a*<sup>-/-</sup> mice.





**Fig. 8. Expression of lipid metabolism genes in intestine specific IRE1 $\alpha$  knockout mice fed Western diet.** 12-week old male ( $n = 3$ ) *I-Ire1a*<sup>+/+</sup> and *I-Ire1a*<sup>-/-</sup> mice on Western diet for 3 weeks were fasted for 6 h and sacrificed. mRNA from intestine (A) and liver (B) were used to determine expression of various lipid metabolism genes. Values (mean  $\pm$  SD). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  are significantly different from *I-Ire1a*<sup>+/+</sup> mice.

This increase was accompanied by a decreased MTP activity and mRNA expression in the livers of *Ire1a*<sup>-/-</sup> mice fed a Western diet (Figs. 7,8). Additionally, several lipogenic genes, in conjunction with decreased MTP expression, were up-regulated in the livers of *Ire1a*<sup>-/-</sup> mice fed a Western diet which may explain the increased accumulation of triglycerides in these mice. Our results also show that Western diet increased the expression of ER stress genes and *Xbp-1* mRNA splicing in the livers of *Ire1a*<sup>-/-</sup> mice (Fig. 5B,D). Hepatic steatosis is known to cause increased expression of spliced variant of *Xbp-1* mRNA [33]. However, deletion of XBP-1 or IRE1 $\alpha$  in the liver is also known to cause hepatic steatosis [11, 34]. It is possible that active XBP-1 (spliced variant) is essential to regulate hepatic lipids and its absence leads to hepatic steatosis. On the other hand, increased expression of spliced variant of *Xbp-1* mRNA may be a protective mechanism to counteract the effects of higher lipids in the liver due to steatosis. How intestine specific IRE1 $\alpha$  deletion causes the dramatic

changes in the expression of ER stress or lipogenic genes in the liver needs further investigation.

In summary, we identified an intestine specific role of IRE1 $\alpha$  in regulating plasma lipid levels. The regulatory mechanism is independent of MTP activity or expression in chow diet mice. However, similar to IRE1 $\beta$ , intestinal IRE1 $\alpha$  may be required to reduce lipid absorption and MTP expression on a Western diet. In future studies, it will be important to determine whether IRE1 $\alpha$  directly regulates MTP expression similar to IRE1 $\beta$  by an internal cleavage of its mRNA [20]. In addition, comparing data from *L-Ire1a*<sup>-/-</sup> and *Ire1a*<sup>-/-</sup> mice suggest that IRE1 $\alpha$  may act differentially on the MTP expression in the livers and intestines to exert its effects on plasma lipids. Taken together, our findings provide novel insights into regulation of lipid homeostasis by modulating intestinal IRE1 $\alpha$ . Thus, manipulation of the intestine specific IRE1 $\alpha$  mediated signaling may provide a unique approach to treat hyperlipidemia.

## 6. Author contributions

Jl contributed to the conception and design of the study, conducted experiments, performed data analysis and interpretation and wrote the manuscript. AB and AAQ performed data analysis and interpretation and wrote the manuscript.

## 7. Ethics approval and consent to participate

Animal care and procedures were performed in accordance with the guidelines of and approved by the Institutional Animal Care and Use Committee of State University of New York Downstate Medical Center.

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## 10. Conflict of interest

The author declares no conflict of interest.

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**Abbreviations:** ApoB, apolipoprotein B; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; HDL, high density lipoproteins; IRE1 $\alpha$ , inositol-requiring transmembrane kinase/endoribonuclease 1 $\alpha$ ; IRE1 $\beta$ , inositol-requiring transmembrane kinase/endoribonuclease 1  $\beta$ ; LDL, low density lipoproteins; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; PERK, protein kinase R (PKR)-like ER kinase; UPR, unfolded protein response; VLDL, very low density lipoproteins; XBP-1, X-box binding protein 1.

**Keywords:** Lipoproteins; Lipids; Atherosclerosis; Obesity; Diabetes; Endoplasmic reticulum; Unfolded protein response; Microsomal triglyceride transfer protein; Inositol-requiring transmembrane kinase/endoribonuclease 1 $\alpha$ ; Inositol-requiring transmembrane kinase/endoribonuclease 1  $\beta$

**Send correspondence to:** Jahangir Iqbal, King Abdullah International Medical Research Center (KAIMRC)-Eastern Region, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), King Abdulaziz Hospital (KAH), Ministry of National Guard-Health Affairs (MNG-HA), 31982 Al Ahsa, Saudi Arabia, Department of Cell Biology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA, E-mail: [iqbalja@ngha.med.sa](mailto:iqbalja@ngha.med.sa)