DOWNREGULATION OF INTESTINAL FARNESOID X RECEPTOR IN ERYTHROPOIETIC PROTOPORPHYRIA

SCHOOL OF PHARMACY
UNIVERSITY OF PITTSBURGH

This thesis was presented

by

Komal Dilip Pradhan

It was defended in
2017
and approved by
Da Yang, MD, PhD, Assistant Professor, School of Pharmacy, University of Pittsburgh
Vinayak Sant, PhD, Assistant Professor, School of Pharmacy, University of Pittsburgh
Xiaochao Ma, PhD, Associate Professor, School of Pharmacy, University of Pittsburgh

[Thesis Director/Dissertation Advisor]: Xiaochao Ma, PhD, Associate Professor, School of Pharmacy, University of Pittsburgh

DOWNREGULATION OF INTESTINAL FARNESOID X RECEPTOR IN ERYTHROPOIETIC PROTOPORPHYRIA

Komal Pradhan, B.Pharm
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ABSTRACT

Erythropoietic protoporphyria (EPP) is characterized by accumulation of protoporphyrin IX (PPIX) in the body. The liver is the major organ responsible for PPIX excretion through the biliary system. Because PPIX is highly hydrophobic, an excess amount of PPIX will precipitate in bile ducts, which can physically block bile flow and result in cholestatic liver injury. Bile acids are natural ligands of Farnesoid X Receptor (FXR) and FXR is a ligand dependent transcription factor. In EPP-associated liver injury, bile ducts are blocked by PPIX which can decrease intestinal exposure to bile acids. Therefore, we hypothesize that PPIX-mediated bile duct blockage will decrease intestinal exposure of bile acids and suppress FXR signaling pathway. By using a genetically engineered EPP mouse model, we confirmed our hypothesis by revealing that the FXR target genes including FGF15, Bsep and Shp, were significantly suppressed in the intestine. In summary, this project demonstrated that the intestinal FXR function is suppressed in EPP.

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1.0 INTRODUCTION

Erythropoietic protoporphyria (EPP) is a genetic disorder characterized by decreased activity of the mitochondrial enzyme, Ferrochelatase (FECH), required for the last step of heme biosynthesis. The inheritance of this mutation is found to be complex, and is demonstrated in both autosomal and recessive patterns of inheritance [1-3]. EPP is a heterozygous disorder and until now, 24 different mutations have been established in 27 different unrelated patients [4]. Molecular analysis has detected several different mutations in EPP including, missense, nonsense, and splice-site mutations, along with insertions and deletions [5]. Due to the mutation in the FECH gene, there is a malfunction in the process of heme formation from Protoporphyrin IX (PPIX), inhibiting the iron insertion in the PPIX molecule. This is responsible for progressive deposition of PPIX in hepatocytes, bile canaliculi, blood and erythrocytes.

The PPIX molecule, due to its insoluble and hydrophobic nature, is excreted in bile than in urine. The gradual deposition of PPIX observed in EPP is responsible for its accumulation in hepatocytes and cholangiocytes forming the foundation for bile duct blockage and hence liver damage [6, 7]. The therapeutic approach in EPP, to address this complication, involves basically using liver transplantation or cholecystectomy methods to remove the damaged liver and gall bladder respectively. However, these therapies involved in EPP are limited to the treatment of symptoms and not the mechanistic cause behind the symptom. The other disadvantage of these therapies is involvement of using invasive methods to resolve the complication. Hepatotoxicity is amongst the most fatal complication in EPP. Hence it is of utmost importance to investigate the mechanisms involved in the hepatotoxicity associated with EPP. Hence this project will provide

a novel insight into the mechanism of hepatotoxicity involved in EPP, which further can promote the use of non-invasive methods to aid hepatotoxicity observed in EPP patients.

1.1 PPIX SYNTHESIS AND DISPOSITION

Heme biosynthesis is an eight step process, where PPIX formation is the second last step. The heme biosynthesis partly takes place in cytoplasm and partly in the mitochondria. The reaction is initiated by formation of δ- Aminolevulinic acid (ALA) from glycine and succinyl CoA in the presence of ALA synthase (ALAS) [8]. This reaction takes place in the mitochondria and then ALA is transported into the cytoplasm. ALAS is the rate limiting step in the PPIX formation [9]. Heme itself acts as a co-repressor in inhibiting the gene expression of ALAS. Additionally, control of heme biosynthesis in erythrocytes originates in several other sites, where incorporation of iron in PPIX molecule, catalyzed by FECH, is one of them. FECH is an iron-sulfur cluster protein and its expression increases during erythroid differentiation. **Figure 1** briefly explains the pathway involved in heme synthesis. Although heme is synthesized in all the cells, 80% of PPIX production takes place in the bone marrow cells. In EPP, due to the decreased activity of FECH, PPIX accumulates in the maturing red blood cells during hematopoiesis. When the red blood cells enter the circulation, free protoporphyrin diffuses across the red cell membrane and binds to the plasma protein. PPIX is disposed from the hepatocytes rather than in urine. The liver extracts the PPIX from the plasma and most of which is excreted unchanged into the biliary system. The remainder is metabolized by the liver FECH to heme, while a part of it is reabsorbed into the enterohepatic circulation.

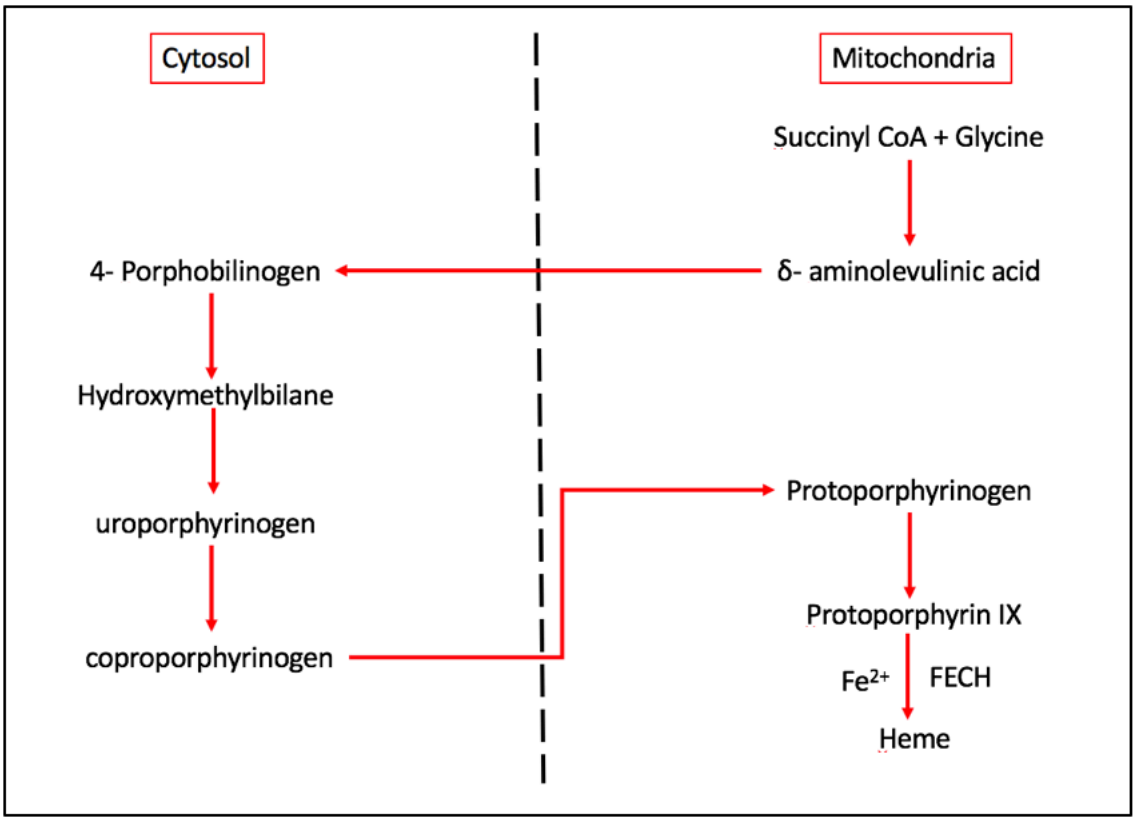

Figure 1: Heme biosynthesis pathway. The pathway depicts the 8 step reaction to form heme from succinyl CoA and glycine. Ferrochelatase (FECH) is required in the last step of heme synthesis to insert iron in the PPIX molecule. Deficiency of this enzyme in EPP leads to accumulation of PPIX in the liver.

1.2 MECHANISM OF PPIX INDUCED LIVER INJURY

Hepatotoxicity in EPP is the most clinically serious manifestation and occurs in 5% of the EPP population [10]. It is characterized by accumulation of the PPIX in the hepatocytes and bile ducts. Cholecystectomy is needed to reduced the PPIX accumulation and hence prevent liver injury in the EPP patients [11]. In some patients, due to the progressive liver disease, they require the transplantation of the liver for increasing the survival rate [12]. Bone marrow contributes to a major source for PPIX accumulation, followed by the hepatic de novo synthesis of PPIX.

PPIX accumulation can damage not only the hepatocytes but also the cholangiocytes and increased accumulation of PPIX will impede its own excretion, since the hepatocytes are the sole excretory organs for PPIX. This also affects the bile acid composition and the bile flow. Increased accumulation of PPIX can further lead to inflammation of the liver and hence liver injury. Since bile acids are themselves toxic in nature, biliary blockage, along with bile acid accumulation can cause oxidative stress and inflammation [13]. This further contributes to the PPIX mediated hepatotoxicity.

1.3 FXR AND FXR SIGNALING IN LIVER GUT AXIS

Enterohepatic circulation aids in circulation and uptake of bile acids and other steroidal moieties produced in the liver, into the intestine and then resending them back to the liver. FXR is one of the major nuclear receptors which assist in this process. It is a bile acids responsive ligand gated transcription factor and has a crucial role in bile homeostasis. FXR is highly expressed in the liver, small intestine, kidney and adrenal gland; and helps in maintaining the metabolism of bile acids, cholesterol, lipids, glucose as well as inflammatory responses.

Cyp7a1 is the rate limiting enzyme in the bile acids synthesis pathway. FXR exhibits its repression on this enzyme by three pathways: Shp dependent, Fibroblast growth factor 15(mouse) and 19 (humans) FGF15/19; and JNK pathway. Small heterodimer partner (Shp) is an atypical member of the Nuclear receptor (NR) family, lacking the DNA binding domain [14]. Shp interacts with several other transcription factors negatively like, LXR, LRH-1, HNF4α [15]. It has been reported that the Shp represses the nuclear receptor transcriptional activity via two

mechanisms: competition with the nuclear receptors co-activators and via direct transcriptional repression activity [16]. LRH-1, and HNF4α are the key positive regulators of the Cyp7a1 gene transcription. HNF4α is highly expressed in the liver, and is responsible for the liver specific transcription induction of various genes involved in the homeostasis of glucose, lipids and cholesterol. LRH-1 not only is the key motif for Cyp7a1 gene expression but also is a key factor required for the acidic pathway rate limiting enzyme Cyp8b1. FXR downregulates the Cyp7a1 expression via bile acids activated Shp induction. This Shp further, interacts with the LRH-1 and HNFα, which are transcriptional factors required for the Cyp7a1 gene expression. This phenomenon was confirmed in the Shp-/- mice, where they also studied the Shp mediated the repression of the Cyp8b1 expression [17]. FXR mediated Shp induction, also represses SREBP1c which is required for lipid metabolism, along with the bile acid transporters like ASBT, OATPs, NTCP and OSTα/β, involved in bile acid trafficking along the enterohepatic circulation. The second pathway of FXR mediated cyp7a1 repression involves the gene induction of FGF15. Following the induction, FGF15 has two chief effects; primarily FGF15 circulates into the enterohepatic circulation and enters the liver. In liver, FGF15 binds to the complex formed by surface receptor with tyrosine kinase activity FGFR4 and the βKlotho, a single transmembrane protein. This investigation was confirmed in the FGFR4 KO, FXR KO and βKlotho KO mice, which helped in the FGF15-mediated Cyp7a1 repression [18, 19]. Secondly, the FGF15 is also found to be involved in the gall bladder filling. Recent studies have established the FGF15 mediated relaxation of gall bladder smooth muscles, which assist in the gall bladder filling even when its full and in fasted state [20].

Bile acids once, synthesized in the liver are transported to the small intestine through portal system, where it is conjugated to taurine or glycine to form secondary bile acids by bile acids-CoA synthetase (BACS) and bile acids- CoA N-aminoacetyltransferase (BATS) respectively [21]. Bile acids are also conjugated by UGT2B4 and SULT2A1. FXR is responsible for direct transcriptional upregulation of these conjugation catalyzing enzymes [22].

FXR is also directly responsible for the transcriptional regulation of several bile acid transporters required for the efflux of the bile acid: Bile salt export protein (Bsep), multidrug resistance protein 2 (MRP2, ABCC2) and the multidrug resistance p-glycoprotein3 (MDR3, ABCB4) [23-25]. These efflux transporters secrete bile acids from the hepatocytes into the bile canaliculi. Apart from the transporters and the conjugating enzymes, FXR also regulates the levels of the I-BABP (ileum bile acid binding protein), which is associated in the enterohepatic circulation [26].

Thus, FXR has a crucial role in the bile acid synthesis, metabolism, as well as secretion and conjugation, hence, is required in maintaining the bile acid homeostasis. FXR also plays an important role in inflammation and maintenance of intestinal barrier [27]. Apart from FXR's effect in liver health maintenance, it is also required for antibacterial defense and liver regeneration [28, 29]. Since EPP involves blockage of bile ducts, and hence liver disease, we wanted to know if the FXR was involved in the EPP. Due to various assenting effects of FXR in liver physiology management the aim of this project is to determine the intestinal FXR function in EPP. In this project, we hypothesized that, in genetically engineered mice model of EPP, the FXR target genes including FGF15, Bsep, and Shp were suppressed, suggesting the downregulation of intestinal FXR function in EPP.

2.0 MATERIALS AND METHODS

2.1 ANIMAL MODEL:

Ferrochelatase mutant mice were generated by Dr. J. C. Deybach's team using chemical mutagen ethylnitrososurea. FECH mutant mice is associated with inherited FECH mutation represented as Fech-mut or $Fech^{m1pas}$/ $Fech^{m1pas}$. Fech-mut mice show a 90% depletion of FECH activity, depicting the severe form of hepatic disease in EPP [30]. Wildtype (WT) mice were purchased from Taconic Biosciences, Inc. (Hudson, NY). Both the mice sets were kept in a 12 h light and dark cycle with water, food ad libitum. The handling of mice was performed according to the Institutional Animal Care and Use Committee. Blood, liver, intestine samples were collected, snap frozen and stored at -80^{o} C till use.

2.2 BIOCHEMICAL ASSAYS:

Alanine amino transferase (ALT), and alkaline phosphatase (ALP) were used as biomarkers for checking the presence of liver injury. Serum was obtained by centrifuging blood samples collected in heparinized tubes at 10,000 g for 10 mins at room temperature. 10 μl of sample was incubated with 200 μl of reagent mixture using standard kit procedure (Pointe Scientific, INC). Absorbance was measured using spectrophotometer (BioRad) at 37^{o} C. The wavelength used for measuring the absorbance of ALT and AST was 340 nm and 450 nm for ALP.

2.3 HISTOLOGICAL ANALYSIS:

Liver tissue was fused in 4% formaldehyde phosphate buffer. Fixed tissue was subjected for dehydration in serial concentrations of alcohol and xylene. This was finally embedded in paraffin. Four μm sections were cut using microtome and stained using Hematoxylin and Eosin (H&E) dye.

2.4 PPIX AND BILE ACIDS QUANTIFICATION:

PPIX was measured using UPLC-QTOFMS (Waters Corp, Milford, MA, USA). Thirty μl of serum, along with 70 μl of methanol was vortexed for 30 s. This mixture was centrifuged at 15,000 g for 10 mins. Supernatent was transferred in an autosampler vial. One μl was injected into the UPLC-QTOFMS. Masslynx version 4.1 software was used to quantify the MS data with Quanlynx program (Waters Corp, Milford, MA, USA). Serum bile acids were measured using a Total Bile Acid Assay Kit (BQ Kits, San Diego, CA). UPLC-MS (Waters, Milford, MA) was used as mentioned previously.

2.5 GUT PERMEABILITY ASSAY:

Fluoroscein isothiocyanate conjugated dextran was used to perform the intestinal permeability assay. The mice were fasted overnight and were given 100 mg/kg of FITC Dextran next morning by oral gavage. Mice were sacrificed after 4 h of fasting and the serum samples were collected. Absorbance was measured at 490 nm and 530 nm to detect the amounts of FITC Dextran in the serum. The concentration of FITC-Dextran was calculated by comparing the fluorescence measured with standard curve.

2.6 RNA ANALYSIS:

RNA analysis from the intestinal and liver tissue were performed using TRIzol reagent (Ambion life technologies). Quantitative PCR (qPCR) was performed using cDNA generated from 0.5 μg of total RNA using Invitrogen kit. qPCR carried out using sybr green reagent in Quant studio 7600 (Applied Biosystems). qPCR was quantified using the comparative cycle threshold (Ct) method and samples were normalized to Cyclophilin D.

2.7 STATISTICAL ANALYSIS:

Data was expressed as mean ± SEM. Statistical significance between two groups was determined by the two tailed Students t test and ANOVA. P value of less than 0.05 was considered as statistically significant.

3.0 RESULTS/ DISCUSSION:

3.1 Genotyping results:

EPP in most patients is associated with 10 to 30% of normal functional FECH activity, while several patients exhibit one mutant allele and one low expression normal allele, that may possibly result in the low expression of FECH [31]. Hence to mimic this condition we used Fech-mut mouse model. As mentioned previously Fech-mut mice exhibit a 90 % of reduced FECH activity. Genotyping results in **Figure 2** show the presence of FECH mutation in Fech-mut mice, which is absent in the WT mice.

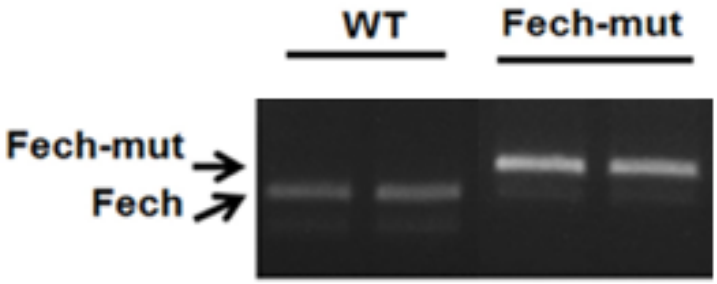

Figure 2. Genotyping result of Fech-mut mice and WT mice.

3.2 CHARACTERIZATION OF THE PPIX MEDIATED LIVER INJURY

EPP is a disorder where there is deposition of the PPIX crystals in the liver [32]. The presence of PPIX crystals were observed in the liver samples of Fech-mut mice and WT mice. The Fech-mut

mice showed extensive deposition of the PPIX in the liver, proving the presence of EPP (**Figure 3A**). Serum ALT and ALP are the most common biomarkers used for the detection of liver injury. Liver injury can be expressed as ALT levels to be more than three times the upper limit of normal (ULN) level or ALP levels to be more than twice the ULN. ALT is a biomarker for hepatocellular injury while serum ALP levels suggest cholestatic injury [33]. The EPP mouse model (Fech-mut mice) used showed a marked increase of almost 6-fold increase in the serum ALT level (**Figure 3B**) suggesting hepatocellular injury, along with almost 3-fold increase in the serum ALP levels (**Figure 3C**). The EPP mouse model hence showed a well characterized cholestatic liver injury. Histology studies performed also suggested the presence of bile plugs in the portal vein section of the liver of Fech-mut mice, proving the presence of PPIX mediated blockage and hence cholestasis in the EPP model (**Figure 3D**). In cholestasis, due to the bile blockage, excessive bile is observed in the serum samples. To reduce the pressure developed due to the blockage, the bile tends to enter the circulation and hence high levels of bile acids are observed in the serum. Hence, to further characterize the cholestatic injury in Fech-mut mice, the serum samples of the Fech-mut mice and the WT mice were subjected to bile acid analysis [34]. Bile acids analyzed here were taurocholic acid (TCA), tauro-alpha-muricholic acid (T-α-MCA), tauro-beta-muricholic acid (T-β-MCA), taurodeoxycholic acid (TDCA), tauromurideoxycholic acid (TMDCA), tauroursodeoxycholic acid (TUDCA). The serum bile acids showed a well distinguished increase in the bile acids levels in the Fech-mut mice in comparison to the WT mice (**Figure 3E**).

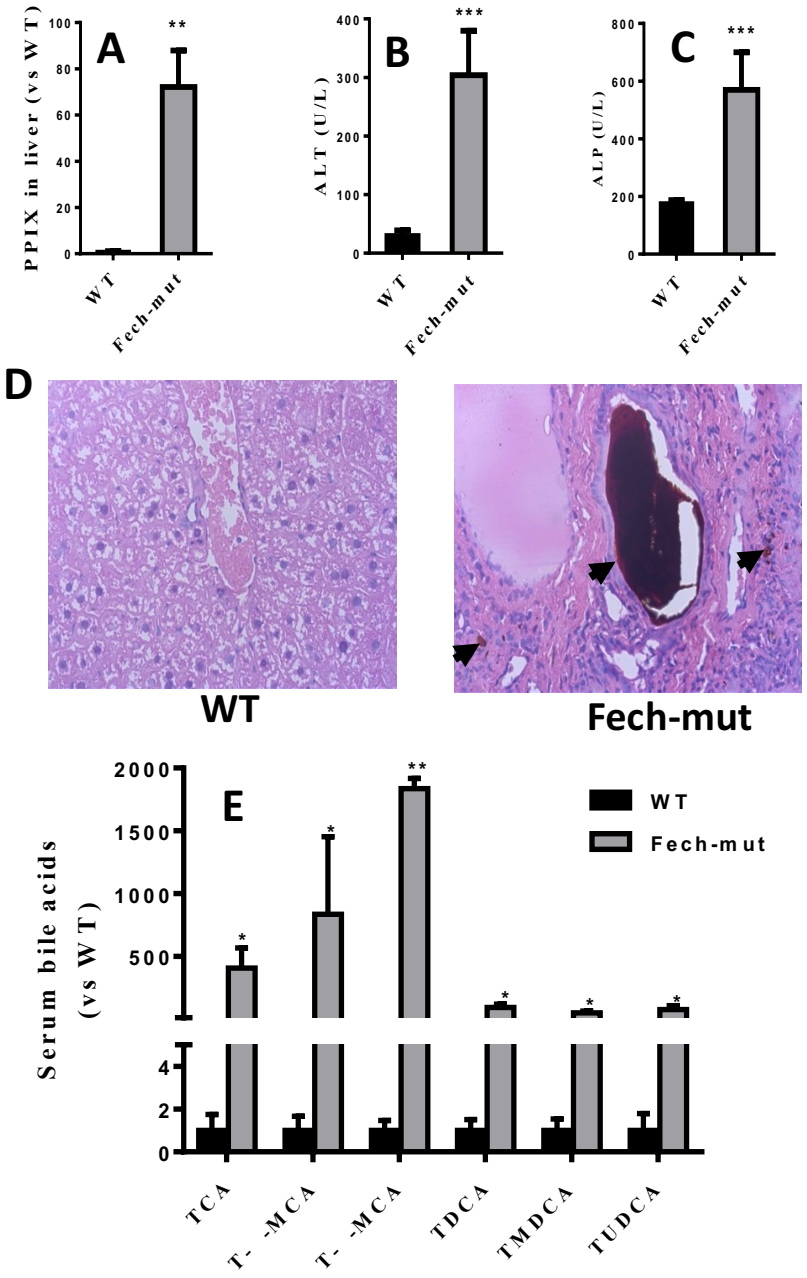

Figure 3. PPIX mediated liver injury in Fech-mut mice. A. Liver PPIX level in WT and Fech-mut mice measured using UPLC-QTOFMS in positive mode. B,C. Serum ALT and ALP levels in WT and Fech-mut mice. D. Histological analysis of liver section of WT and Fech-mut mice analyzed by Hand E staining. The arrows point to bile pigments. E. Serum bile acids level in WT and Fech-mut mice measured using UPLC-QTOFMS in negative mode. All data are expressed as mean ± SEM (n=4). **P*<0.05,***P*<0.01, *** *P*<0.005. The data in WT were set as 1. WT: wild type; PPIX: protoporphyrin IX; TCA: Taurocholic acid; T-α-MCA: Tauro-alpha-muricholic acid; T-β-MCA:tauro-beta-muricholic acid; TDCA: Taurodeoxycholic acid; TMDCA:Tauromurideoxycholic acid ; TUDCA: Tauroursodeoxycholic acid.

3.3 INTESTINAL FXR SIGNALING IN FECH MUT MICE:

FXR is extensively expressed in the small intestine, especially in the ileum and is primarily responsible for the target gene expression of Bsep, FGF15 and Shp in the small intestine [35]. We looked into the expression levels of FXR and its targets genes. In our studies, we could observe that the FXR expression in the ileum was intact (**Figure 4A**) but its function was downregulated. Hence to understand more about the FXR functioning we also looked into the mRNA expression of target genes of FXR using qPCR. The primary FXR target genes required in the bile acid homeostasis include FGF15, Bsep and Shp. Shp and FGF15 are required for the negative feedback mechanism for the bile acid synthesis. The Shp and FGF15 expression levels were observed to be low in Fech-mut mice (**Figure 4B** and **4C** respectively). Bsep, which is an exporter of bile acids in intestine was also found to be suppressed in the Fech-mut mice (**Figure 4D).** This provides evidence that the function of FXR is downregulated in diseased mice. Since Bsep, Shp and FGF15 are involved in the bile acid homeostasis, downregulation of FXR function in EPP might potentiate the cholestasis observed in the disease. Further studies will be carried out to know the exact role of FXR in the mechanism of the cholestatic injury observed in the EPP mouse model.

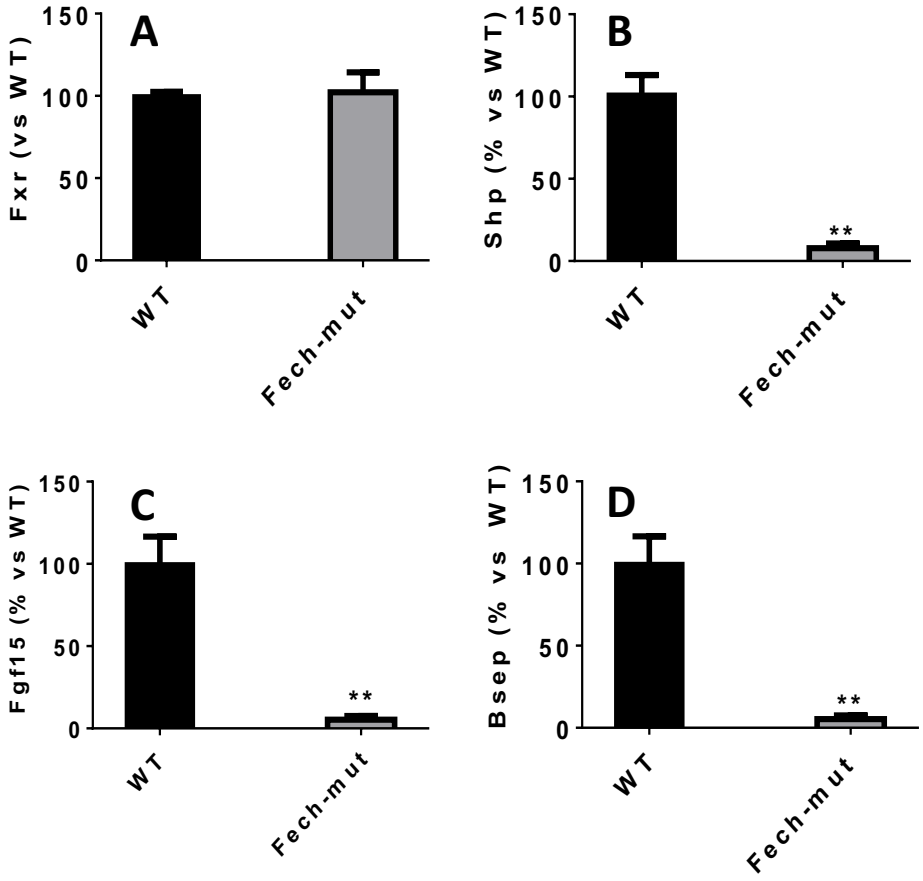

Figure 4. Intestinal FXR signaling in Fech-mut mice. A. Fxr, B. small heterodimer partner (Shp), C. fibroblast growth factor (FGF) 15 and D. bile salt export pump (Bsep) mRNA expression analyzed by qPCR. All data are expressed as mean ± SEM (n=4). ***P*<0.01, as compared with WT. The data in WT were set as 100. WT: Wild type.

3.4 INTESTINAL PERMEABILITY AND LPS SIGNALING IN THE LIVER

Bacterial lipopolysaccharide is the most common surface endotoxin in all gram negative bacteria and are potent innate immunity stimulator [36]. Lipopolysaccharide can be recognized by the pattern recognition receptors like Toll like receptors (TLRs). Most enzymes and genes required for the lipopolysaccharide production are found in the *E. coli* [37]. The role of

lipopoysaccharide in liver diseases has been extensively studied, since LPS commonly accentuates the existing liver disease and can complicate the health of the liver [38, 39].

The toxicity of this lipopolysaccharide is prevented by activation of pattern recognition receptor, TLR4. TLR, surface receptors, comprise of 13 different family members, where each TLR detects different pathogen associated molecular pattern (PAMPS) from different microbes [40]. These TLRs basically function as sensors of microbial infection and can lead to inflammatory and immune response [41, 42]. Since the bacterial component recognized by the TLRs are not unique, they are cannot sense the difference between commensal and pathogenic bacteria and hence initiate responses against both the bacteria [43, 44]. TLRs, apart from the immune function, are also involved in the maintenance of the epithelial homeostasis, along with its protective function from the direct injury to the epithelial tissue. They induce the expression of several heat shock proteins like TNFα, IL-6, cytoprotection by cyclooxygenase 2 (COX2), keratinocyte growth factor 1 (KGF-1), keratinocyte growth factor 2 (KGF2), vascular endothelial growth factor (VEGF) and Angiogenin 4 [44-48]. TLR activate the same signaling components as that of Interleukin 1 (IL-1) receptor, the nuclear factor kappa B (NFκB) pathway [49]. The NFκB controls the expression of proinflammatory cytokines and chemokines, along with the upregulation of the co stimulatory molecules.

LBP, Lipopolysaccharide binding protein is the first molecule which comes in contact with the LPS endotoxin. LPS is produced in the liver and has high affinity binding for the lipid A of the LPS, the bond is required for the binding of the LPS to the LPS receptor, TLR4. This complex homodimerizes with MD-2 and CD14 which act as accessory proteins for the activation of the

receptor [50]. MD-2 is a secretory glycoprotein which is present as an extracellular adaptor, essential for the activation of TLR4 [51]. Knockout studies using MD-2 mice have shown that MD-2 is essential for the TLR4 recognition of LPS [52]. The LPS mediated NFκB signaling requires a couple of adaptor proteins, myeloid differentiation factor D 88 (MyD88), MyD88 adaptor like protein (TIRAP), TIR containing adaptor mole (TRIF/ TICAM-1), TRIF related adaptor molecule (TRAM, TICAM-2) [53, 54]. TLR-4 is the only subtype from the family which activates NFκB via MyD88 dependent as well as independent pathway. In the MyD88 pathway, the LBP, LPS, CD-14, MD-2 and TLR4 complex heterodimerizes with the TIRAP and MyD88 receptor complex, which further associates with the IRAF family, (IL- 1R associated kinases) [55]. These all complexes further lead to activation of several downstream events of the MyD88 pathway, thereby activating the NFκB and MAPK pathways. In the MyD88 independent pathway, TRIF and TRAF-6 complex formation is essential for the IFN- β pathway activation [56]. The downstream events for the MyD88 independent pathway is similar to that of the MyD88 dependent pathway, where ultimately TNFα, IFN-β (Interfon 1β) and iNOS (inducible NO synthase) are activated [57].

Hence we looked into the levels of interleukin 1b, iNOS, COX1, COX2, TLR4, CD14, LBP and MD-2 in the ileum using qPCR (**Fig 5A, B, C, D, F, G, H,** and **I**). We could comprehend from the results that the mRNA expression of the above genes was upregulated in the diseased mice model. This suggests that the LPS molecule from the small intestine is interacting with the PPMPs in the liver. Hence to further validate the leakiness of the gut and leakage of the LPS into the blood circulation, we performed the FITC dextran assay. **Figure 5E** indicates the leakiness of

the gut. Further studies will be carried out to test the lipopolysaccharide mediated cholestatic inflammation of the liver.

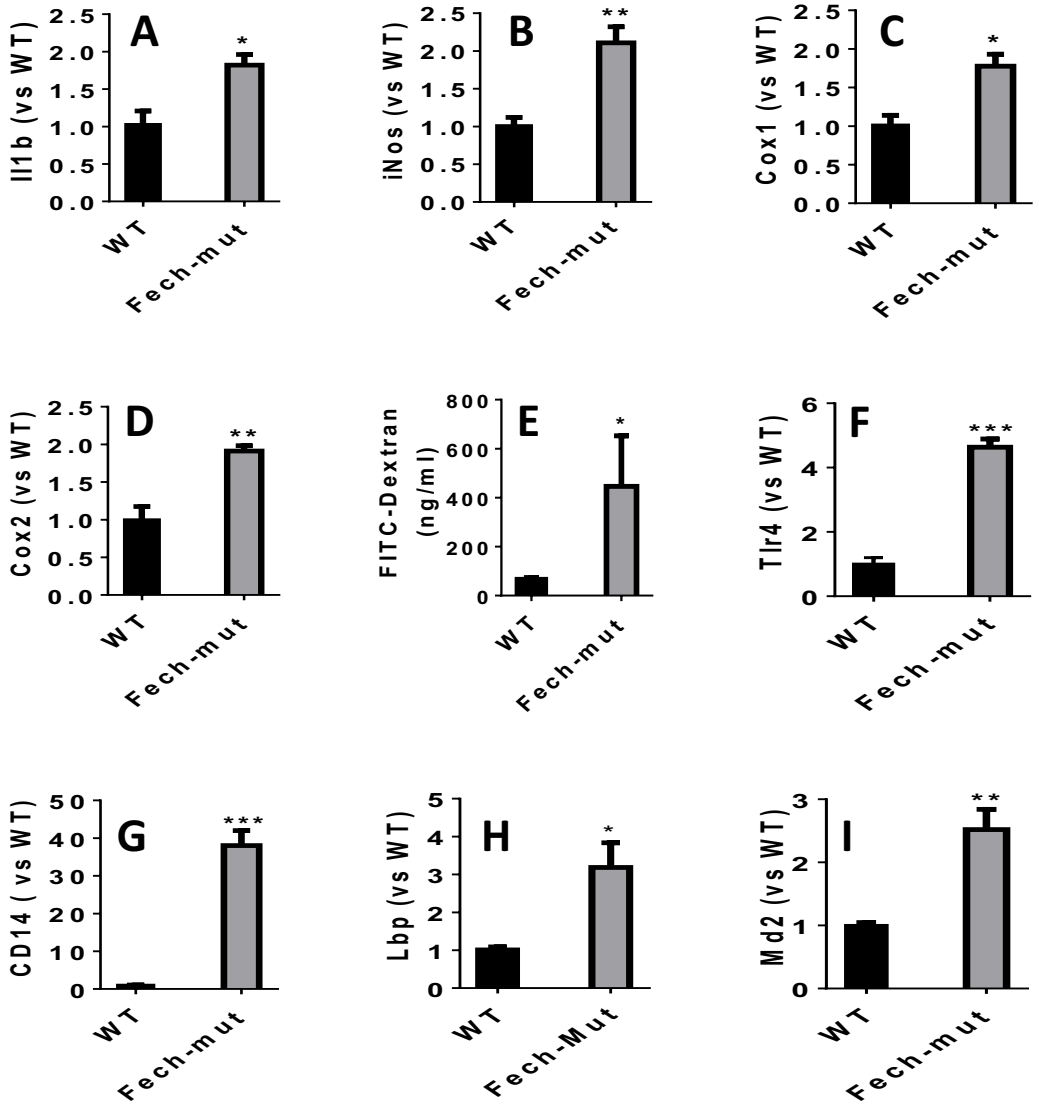

Figure 5. Effect of EPP-associated liver injury on inflammation and permeability in intestine and LPS signaling in liver. A. Inducible nitric oxide synthase (iNOS), B. interleukin-1 beta (Il1β), C. cyclooxygenase (COX) 1 and D. Cox2 mRNA expression in ileum of WT and Fech-mut mice analyzed by qPCR. E. FITC labeled dextran concentration in the serum of WT and Fech-mut mice. F. Toll-like receptor (TLR) 4, G. cluster of differentiation (CD14), H. lipopolysaccharide-binding protein (LBP) and I. MD-2 mRNA expression in liver of WT and Fech-mut mice analyzed by qPCR. All data are expressed as mean ± SEM (n=4). **P*<0.05 ***P*<0.01, *** *P*<0.005 as compared with WT. The data in WT were set as 1. WT: Wild type.

3.5 INFLAMMATION IN THE LIVER:

NFκB activation, activates several inflammatory pathways. The inflammation in the liver was confirmed by checking the mRNA levels of these NFκB markers of inflammation. As discussed previously, the ILβ and TNFα pathways were checked in the liver samples of the diseased mice. The Fech-mut mice showed a clear induction of the downstream signaling molecules of inflammation. It was found that all these markers including the cluster of differentiation (CD68), monocyte chemoattractant protein-1 (Mcp1), F4/80, interleukin-1 beta (Ilβ) and tumor necrosis factor alpha (TNFα) were upregulated suggesting the presence of inflamed liver (**Figure 6A, B, C, D,** and **E).**

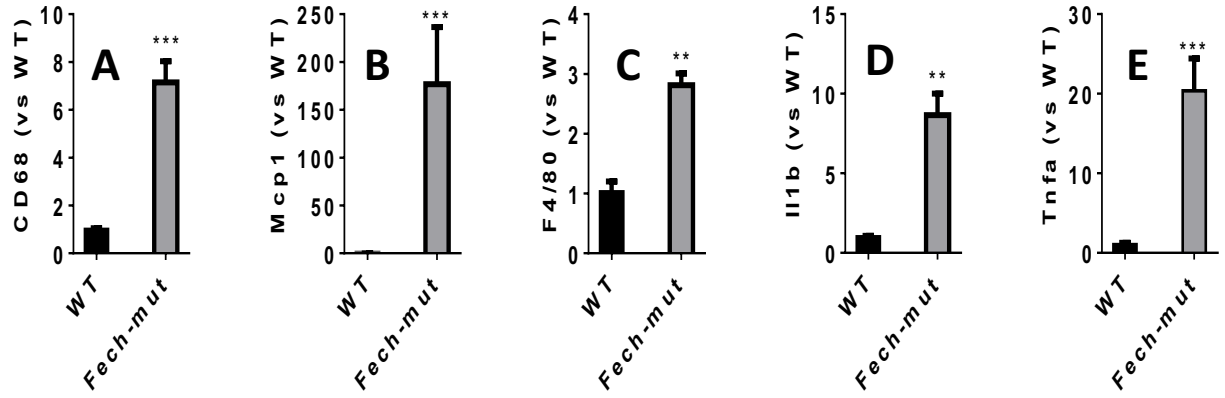

Figure 6. Effect of EPP-associated liver injury on inflammation in Fech-mut mice. A. Cluster of differentiation (CD) 68, B. F4/80, C. monocyte chemoattractant protein-1(Mcp1), D. interleukin-1 beta (Il1β) and E tumor necrosis factor alpha (TNFα). mRNA expression in liver of of WT and Fech-mut mice analyzed by qPCR. All data are expressed as mean ± SEM (n=4). **P*<0.05, ***P*<0.01, *** *P*<0.005. The data in WT were set as 1. WT: Wild type.

4.0 CONCLUSION AND FUTURE PLAN

FXR has been extensively studied in the field of cholestasis and is thought to be a potential target for the treatment of cholestasis [58, 59]. Based on these studies, we have reported the involvement of this receptor in the PPIX mediated cholestasis in EPP mouse model. We could successfully demonstrate the downregulation of the FXR target genes in the small intestine. We could also find that the LPS signaling was activated due to the downregulation of FXR in small intestine.

The **Figure 7** depicts the accumulation of PPIX, leading to the blockage of the bile ducts and canaliculus, and hence the bile duct blockage and injury. The blockage may be further responsible for the oxidative stress and inflammation in the liver, which if progresses can lead to liver injury. The blockage, also reduces the bile flow into the intestine. Since bile acids are the natural ligands of the FXR, present in intestine, inefficiency of the bile acids reaching the intestine can be a reason of reduced functioning of intestinal FXR. The downregulation of intestinal FXR is directly and indirectly responsible for the decrease in FGF15 along with the increase in LPS signaling respectively, and hence further investigations are required to know if these pathways are accountable for the liver injury observed in the EPP.

Hence, this study can be used as a guide to use the FXR agonists for the treatment of cholestasis in EPP patients. Further investigations must be carried out to have a deeper insight of the

molecular mechanisms involved in FXR mediated liver injury and this shall be used to reduce the liver failure observed in EPP patients.

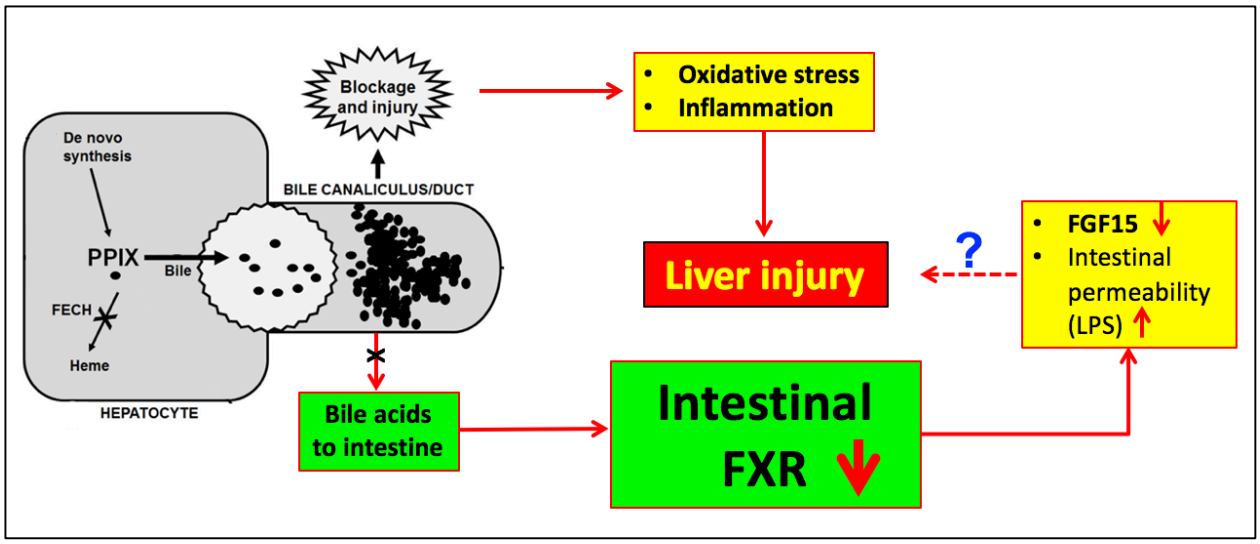

Figure 7. Project summary and future plan. This model of EPP demonstrates the accumulation of PPIX leading to the blockage of the bile ducts and canaliculus, followed by the bile duct blockage and injury. The blockage being responsible for the oxidative stress and inflammation in the liver, can further progress in to liver injury. The blockage, also reduces the bile flow into the intestine. Since bile acids are the natural ligands of the FXR, present in intestine, inefficiency of the bile acids reaching the intestine can be a reason of reduced functioning of intestinal FXR. The downregulation of intestinal FXR is directly and indirectly responsible for the decrease in FGF15 along with the increase in LPS signaling respectively, and hence further investigations are required to know if these pathways are accountable for the liver injury observed in the EPP.

5.0 ACKNOWLEDGEMENTS

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