Published in final edited form as:

Biochem Pharmacol. 2018 August ; 154: 474–481. doi:10.1016/j.bcp.2018.06.011.

Liver Metabolomics in a Mouse Model of Erythropoietic Protoporphyrria

Pengcheng Wang¹, Madhav Sachar¹, Grace L. Guo², Amina I. Shehu¹, Jie Lu¹, Xiao-bo Zhong³, and Xiaochao Ma¹

¹Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA

²Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ 08854 USA

³Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, CT 06269, USA

Abstract

Erythropoietic protoporphyria (EPP) is a genetic disease that results from the defective mutation in the gene encoding ferrochelatase (FECH), the enzyme that converts protoporphyrin IX (PPIX) to heme. Liver injury and even liver failure can occur in EPP patients because of PPIX accumulation in the liver. The current study profiled the liver metabolome in an EPP mouse model caused by a Fech mutation (Fech-mut). As expected, we observed the accumulation of PPIX in the liver of Fech-mut mice. In addition, our metabolomic analysis revealed the accumulation of bile acids and ceramide (Cer) in the liver of Fech-mut mice. High levels of bile acids and Cer are toxic to the liver. Furthermore, we found that the major phosphatidylcholines (PC) in the liver and the ratio of total PC to PPIX in the bile were decreased in Fech-mut mice compared to wild type mice. A decrease of the ratio of PC to PPIX in the bile can potentiate the accumulation of PPIX in the liver because PC increases PPIX solubility and excretion. These metabolomic findings suggest that the accumulation of PPIX, together with the disruption of the homeostasis of bile acids, Cer, and PC, contributes to EPP-associated liver injury.

Graphical Abstract

Correspondence: Xiaochao Ma, Ph.D., Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261. Tel. (412) 648-9448; mxiaocha@pitt.edu. Author contributions

PW, MS, and JL performed the experiments. PW, GLG, AIS, XZ, and XM conducted data analysis. PW and XM contributed to the new reagents, analytic tools, and animal models. PW and XM conceived the project and wrote the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

Erythropoietic protoporphyria; liver; metabolomics; protoporphyrin IX; bile acids

1. Introduction

Erythropoietic protoporphyria (EPP) is the third most common type of porphyria, and usually first diagnosed in early childhood [1]. EPP results from the defective mutation of the gene encoding ferrochelatase (FECH), the enzyme that is responsible for the final step of heme synthesis [2–4]. FECH catalyzes the insertion of a ferrous iron into protoporphyrin IX (PPIX) to form heme, which mainly occurs in the bone marrow and liver [2, 5, 6]. The defective mutation of the FECH gene reduces FECH activity and leads to PPIX accumulation in the bone marrow, erythrocytes, plasma, and liver. Liver injury frequently occurs in EPP patients because of PPIX accumulation in the liver. It has been estimated that up to 20% of the EPP patients have liver injury and approximately 2–5% develop serious liver damage or even liver failure [7–10].

PPIX-mediated bile duct blockage is the major cause of the EPP-associated liver disease [2, 11]. The liver removes PPIX from the body and secretes it into the biliary system [6, 12, 13]. Because PPIX is highly hydrophobic, excessive PPIX can precipitate in the bile ducts and cause bile duct blockage [9]. Consequently, PPIX-mediated bile duct blockage results in cholestatic liver injury, which can further lead to fibrosis, cirrhosis and liver failure [7–10, 14]. Various treatments have been used to manage EPP-associated liver injury in clinical practice, such as suppressing PPIX production by iron therapy [15] or intravenous administration of hemin [16], reducing PPIX pool by plasmapheresis or hemodialysis [17], interrupting the enterohepatic circulation of PPIX by charcoal [18], increasing bile flow by chenodeoxycholic acid or ursodeoxycholic acid [19, 20], and inhibiting oxidative stress by vitamin E [21]. However, the efficiency of these approaches has not been proved in large numbers of patients and none of them is consistently effective in all cases [5, 9, 10]. Liver transplantation can restore normal liver function, but it does not correct the metabolic disorder in the bone marrow and it can lead to the recurrence of injury in the transplanted liver [5].

Understanding the biochemical changes in the liver of EPP will be helpful for developing novel strategies for the treatment of EPP-associated liver injury. Metabolomics uses advanced analytical techniques and computational methods to analyze the global

biochemical alterations in a biological system, thus it can provide unique and fundamental insights into disease progression [22, 23]. The current study used a metabolomic approach to profile the metabolite alterations in the liver of a mouse model of EPP [24, 25]. The results from our study can be used to explore the mechanisms of EPP-associated liver disease and guide the development of novel therapies for EPP.

2. Materials and methods

2.1. Chemicals and reagents

PPIX was purchased from Sigma-Aldrich (St. Louis, MO). Taurocholic acid (TCA), tauro- α -muricholic acid (T- α -MCA), tauro- β -muricholic acid (T- β -MCA), and β -muricholic acid (β -MCA) were obtained from Steraloids, Inc. (Newport, RI). 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PC 34:2) was purchased from Avanti Polar Lipids (Avanti Polar Lipids Inc., Alabaster, AL). N-palmitoyl-D-erythro-sphingosine (C16:0 Cer), N-stearoyl-D-erythro-sphingosine (C18:0 Cer), and N-nervonoyl-D-erythro-sphingosine (C24:1 Cer) were purchased from Cayman Chemical Company (Ann Arbor, MI). All solvents for ultra-performance liquid chromatography and time-of-flight mass spectrometry (UPLC-TOFMS) analysis were of the highest grade commercially available.

2.2. Mice

Wild-type (WT) and Fech-mutant (Fech-mut) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and maintained under a standard 12 h dark/light cycle with water and chow provided ad libitum. Blood, liver, and gall bladder samples were collected from 2–4 month-old (male) mice. A section of liver tissue was excised and immediately fixed in a 4% formaldehyde phosphate buffer for histological analysis. The remaining liver tissues were flash-frozen in liquid nitrogen and stored at -80°C until further analysis. All animal studies were conducted in accordance with study protocols approved by the University of Pittsburgh Medical Center Institutional Animal Care and Use Committee.

2.3. Serum biochemical and liver pathological analysis

Serum biochemical and liver histological analysis were conducted to evaluate liver injury. Serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities were analyzed according to the standard assay kit procedures. Fixed liver tissues were subjected to dehydration in serial concentrations of alcohol and xylene followed by paraffin embedding. Four-micrometer serial sections were cut and stained with hematoxylin and eosin (H&E).

2.4. Sample preparation for metabolite analysis

Liver samples were weighed and homogenized in water (100 mg of tissues in 500 μL of water). Two separate extraction methods were used to analyze liver metabolites [26]. For aqueous extracts, 200 μL of acetonitrile: methanol (1:1, v/v) was added to 100 μL of each homogenate, and followed by vortexing and centrifugation at 15,000 g for 10 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (15,000 g for 10 min). For organic extracts, 2 mL of dichloromethane: methanol (2:1, v/v) and 400 μL water were added to 100 μL of liver homogenate. The mixture was then vortexed for 1 min

and centrifuged at 4,500 g for 5 min. The organic phase was transferred to a glass tube and dried under a gentle stream of nitrogen. The residue was reconstituted with 100 μ L of acetonitrile: isopropyl alcohol: H₂O (1:1:1, v/v/v) and centrifuged at 15,000 g for 10 min. Bile samples were prepared by adding 200 μ L of acetonitrile: isopropyl alcohol: H₂O (1:1:1, v/v/v) to 2 μ L of bile, and then vortexed for 30s and centrifuged at 15,000 g for 10 min. Each supernatant from above was transferred to an autosampler vial and analyzed by UPLC-TOFMS (Waters Corporation, Milford, MA). The total phosphatidylcholine in the bile was measured with a commercial colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI).

2.5. UPLC-TOFMS analysis

Samples were analyzed by an Acquity BEH C18 column (2.1 $\times$ 100 mm, 1.7 μ m, Waters, Milford, MA). For the analysis of aqueous extracts, the flow rate of the mobile phase was 0.5 mL/min using a gradient ranging from 2% to 98% acetonitrile/water containing 0.1% formic acid in 12 min, then held for 8 min and equilibrated with the initial ratio for 4 min. For the analysis of the organic extracts, solvent A was 40% water, 60% acetonitrile containing 0.1% formic acid, and solvent B was 90% isopropanol, 10% acetonitrile containing 0.1% formic acid. The initial conditions were 65% of A and 35% of B, which was increased to 95% of B in 12 min, held at 95% of B until 18 min before returning to the initial conditions for column equilibration for 2 min. The flow rate of the mobile phase was 0.4 mL/min and the column temperature was maintained at 50 $^{\circ}$ C. TOFMS was operated in both positive and negative mode with electrospray ionization, respectively. The source and desolvation temperatures were set at 150 and 500 $^{\circ}$ C. Nitrogen was applied as cone and desolvation gas, and the gas flow rates were set as 50 and 800 L/h, respectively. Argon was applied as collision gas. The capillary and cone voltages were set at 0.8 kV and 40 V. The data were acquired from 50 to 1000 Da in centroid format. TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine encephalin (m/z 556.2771 for positive mode and m/z 554.2615 for negative mode) in real time. Tandem mass fragmentation scans with collision energy ramping from 15 to 45 eV were used for structural elucidations of metabolites.

2.6. Metabolomic analysis

Progenesis QI software (Nonlinear Dynamics, Newcastle, UK) was used for data analysis. Each UPLC-MS run was imported as an ion intensity map, including retention time and m/z . A data matrix was generated through alignment of all the runs, peak picking, isotope and adduct deconvolution. The data matrix generated by Progenesis QI was further exported into SIMCA-P+ software (Version 13, Umetrics, Kinnelon, NJ). The data were transformed by mean-centering and Pareto-scaling to increase the importance of low abundant ions without significant amplification of noise. Principal component analysis (PCA) was performed to analyze the metabolome difference between two groups and orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to maximize the class discrimination. S-plot was generated and the variables that significantly contributed to the discrimination between groups were considered as potential markers. Metabolites were identified by MS/MS fragmental analysis and by searching the metabolomics database, including METLIN, Human Metabolome Database (HMDB) and Lipid MAPS [27–29], with Δ ppm < 10. The

identity of major metabolites was further confirmed by comparing the retention time and MS/MS spectrum with the authentic standards.

2.7. Quantitative polymerase chain reaction (qPCR) analysis

Total mRNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using complementary DNA (cDNA) generated from 1 µg of total RNA with a SuperScript II Reverse Transcriptase kit and random oligonucleotides (Invitrogen). qPCR, containing 25 ng cDNA, 150 nM of each primer, and 5 µL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 µL, was carried out on an ABI-Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Values were quantified using the comparative CT method, and mRNA abundance was normalized to cyclophilin.

2.8. Statistical analysis

All quantified data are expressed as mean $\pm$ SD. Statistical analysis was performed with two-tailed Student's *t* tests using GraphPad Prism 6 (GraphPad Software, San Diego, CA). A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. PPIX accumulation and liver injury in Fech-mut mice

As expected, PPIX level was dramatically increased in the liver of Fech-mut mice (Fig. 1A). Compared to WT mice, PPIX level increased to 72-fold in the liver of Fech-mut mice. The biliary system is responsible for PPIX excretion. However, PPIX is highly hydrophobic and excessive amount of PPIX will precipitate in the bile and block bile flow. We observed plugs in the bile ducts of Fech-mut mice, but not in WT mice (Fig. 1B and 1C). In addition, the serum ALT, AST, and ALP activities were significantly increased in Fech-mut mice, suggesting that liver damage occurred (Fig. 1D-1F).

3.2. Liver metabolome in Fech-mut mice

PCA score plot demonstrated a clear separation between the liver metabolome of WT and Fech-mut mice (Fig. 2A). The corresponding loading S-plots generated from OPLS-DA showed ions that contributed to the group differentiation (Fig. 2B and 2C). PPIX, bile acids, phosphatidylcholine (PC), and ceramide (Cer) were the major altered metabolites in the liver of Fech-mut mice compared to WT mice (Fig. 2B, 2C and Table 1).

3.3. Bile acid accumulation in the liver of Fech-mut mice

Bile acids were identified as the top increased ions that contributed to the group discrimination (Fig. 2B and Table 1). Compared to WT mice, T- β -MCA, TCA, T- α -MCA, β -MCA, and TCDCA increased to 9.7, 4.1, 6.7, 6.7, and 67.5-fold in the liver of Fech-mut mice, respectively (Fig. 3A). We next examined the hepatic expression of the genes encoding enzymes and transporters that are associated with bile acid homeostasis. Cholesterol 7 α -hydroxylase (CYP7A1), hydroxycholesterol 7- α -hydroxylase (CYP7B1), 8 β -hydroxylase (CYP8B1) and sterol 27-hydroxylase (CYP27A1) are the

enzymes involved in bile acid biosynthesis [30]. We found that the expression of Cyp7a1 was not changed, but the expressions of Cyp8b1, Cyp27a1, and Cyp7b1 were significantly decreased in the liver of Fech-mut mice compared to WT mice (Fig. 3B). We also examined the expression of genes that regulate bile acid biosynthesis, including farnesoid X receptor (FXR) and its target gene small heterodimer partner (SHP) [30]. No significant difference was found in the expression of FXR and SHP in the liver of WT and Fech-mut mice (Fig. 3C). These data suggest that the accumulation of bile acids in the liver of Fech-mut mice is not caused by the increase of bile acid biosynthesis.

For the bile acid uptake transporters, we observed a dramatic downregulation of organic anion transporter 1 (Oatp1), and a moderate decrease in Na⁺-taurocholate cotransporting polypeptide (Ntcp) in the liver of Fech-mut mice (Fig. 3D). In addition, we observed a significant upregulation of transporters that are involved in basolateral excretion of bile acids, including multidrug resistance-associated protein 1, 4, and 5 (Mrp1, 4, and 5) in the liver of Fech-mut mice (Fig. 3E). Furthermore, the expression of transporters that are involved in biliary excretion of bile acids, including Mrp2 and bile salt export pump (Bsep), did not show a significant change between WT and Fech-mut mice (Fig. 3F). Upregulation of basolateral excretion and downregulation of uptake transporters of bile acids are considered as an adaptive response to cholestatic liver injury [31]. These data suggest that the accumulation of bile acids in the liver of Fech-mut mice is caused by PPIX-mediated bile duct blockage.

3.4. PC in the liver and bile of Fech-mut mice

PC 34:2, PC 38:6, PC 36:2, and PC 40:6 are the major phospholipids in the liver [32]. We found that the levels of these PCs were decreased in the liver of Fech-mut mice compared to WT mice (Fig. 4A). However, the expression of the genes encoding the enzymes involved in hepatic PC biosynthesis, including choline kinase α and β (CHK α and CHK β), phosphate cytidyltransferase 1 α (PCYT1 α), and phosphatidylethanolamine N-methyltransferase (PMET) [33], did not show significant difference between WT and Fech-mut mice (Fig. 4B). These results suggest that the alteration of hepatic PC in Fech-mut mice is not due to PC biosynthesis. In addition, PCs are the dominant phospholipids in the bile, which can facilitate the dissolution of PPIX in the bile [11]. We found that the level of total PC in the bile did not show significant differences between WT and Fech-mut mice (Fig. 4C). However, due to the increase of PPIX in the bile (Fig. 4D), the ratio of total PC to PPIX was dramatically decreased in the bile of Fech-mut mice when compared to WT mice (Fig. 4E).

3.5. Accumulation of Cer in the liver of Fech-mut mice

The levels of C16:0 Cer, C18:0 Cer, and C24:1 Cer were dramatically increased in the liver of Fech-mut mice (Fig. 5A). Compared to WT mice, the levels of C16:0 Cer, C18:0 Cer, and C24:1 Cer increased to 5.3, 6.9, and 5.9-fold, respectively, in the liver of Fech-mut mice. Cer is synthesized through *de novo* synthesis, salvage pathway, and sphingomyelinase (SMase) pathway [34, 35]. We found that the expressions of the genes that are involved in the *de novo* synthesis of Cer, including serine palmitoyltransferase, long chain base subunit 1 and 2 (Sptlc 1 and 2), were significantly increased in the liver of Fech-mut mice (Fig. 5B). In addition, the expressions of the hepatic genes that are involved in the salvage pathway of Cer

synthesis, including ceramide synthases 2, 4, 5, and 6 (Cers 2, 4, 5, and 6), were also increased in Fech-mut mice (Fig. 5C). Furthermore, the expression of sphingomyelin phosphodiesterase 3 (Smpd 3) that contributes to the neutral-SMase pathway of Cer formation increased to 42-fold in the liver of Fech-mut mice compared to WT mice (Fig. 5D). These results suggest that the accumulation Cer in the liver of Fech-mut mice is due to the upregulation of Cer biosynthesis.

4. Discussion

The current study profiled the liver metabolome in Fech-mut mice in comparison with WT mice. We found that: (1) PPIX level was increased in the liver and bile; (2) bile acids were increased in the liver; (3) major PCs were decreased in the liver and the total PC to PPIX ratio was decreased in the bile; and (4) ceramides were increased in the liver (Fig. 6). We believe that all these metabolic changes may contribute to the EPP-associated liver disease.

PPIX accumulation in the liver can cause liver injury by activating c-Jun N-terminal protein kinase and inducing mitochondrial permeability transition [36, 37]. In addition, excessive PPIX will precipitate in the bile ducts, cause bile duct blockage, and affect the excretion of bile acids, which is supported by our observation of plugs in bile ducts and accumulation of bile acids in the liver of Fech-mut mice (Figs. 1C, 2B, and 3A). Bile acids are activators of FXR that upregulate the expression of SHP and inhibit the transcription of CYP7A1 [30, 38]. However, the expressions of SHP and CYP7A1 were not significantly altered in the liver of Fech-mut mice (Fig. 3B and 3C), suggesting that other factors, such as inflammation (Fig. 1C), might be responsible for the dysregulation of bile acid biosynthesis in the liver of Fech-mut mice. In addition, we ruled out the possibility that the accumulation of bile acids is caused by the increase of synthesis, because the synthesis enzymes including Cyp7b1, 8b1, and 27a1 were downregulated in the liver of Fech-mut mice (Fig. 3C).

The accumulation of bile acids, especially hydrophobic bile acids, in the liver can cause inflammation, as well as hepatocyte and biliary injury [39, 40]. Therefore, bile acid accumulation in the liver may potentiate the progression of EPP-associated liver injury. In this regard, attenuation of hepatic bile acid accumulation can be used as a potential therapeutic approach for EPP-associated liver injury. Indeed, hydrophilic bile acids, such as chenodeoxycholic acid and ursodeoxycholic acid, have been used in the clinic to treat EPP-associated liver injury, because these two bile acids can increase bile flow and increase overall bile acid excretion [19, 20]. However, further studies are needed to evaluate the efficacy of hydrophilic bile acids for treatment in EPP patients [5, 9, 10].

Our metabolomic analysis also found that the major PCs were significantly decreased in the liver of Fech-mut mice (Fig. 4A). PC is the major phospholipid in biological membranes and plays critical roles in the maintenance of membrane integrity [33, 41]. Depletion of hepatic PC can lead to liver failure [42] and dietary PC supplementation can protect against liver diseases [43, 44]. In addition, PC has both hydrophobic tail and hydrophilic head, which can facilitate biliary PPIX dissolution by forming micelles [11]. Although we did not observe the change of total PC in the bile of Fech-mut mice, the ratio of total PC to PPIX was

significantly decreased (Fig. 4E), which can reduce PPIX solubility in the bile, exaggerate PPIX-mediated bile duct blockage, and therefore potentiate liver injury.

Furthermore, our metabolomic analysis discovered Cer accumulation in the liver of Fech-mut mice. Cer is a component in the lipid bilayer of cell membranes [45] and plays important roles in cellular proliferation, differentiation, oxidative stress, and inflammation [35]. Liver is the key site for Cer biosynthesis [35]. We illustrated that the accumulation of Cer in the liver of Fech-mut mice was through the upregulation of Cer biosynthesis pathways (Fig. 5). However, we cannot rule out the potential effects of intestinal FXR on Cer homeostasis in the liver, because FXR can upregulate Cer synthesis in the intestine [46, 47]. Cer has been found to inhibit hepatic mitochondrial complex III and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), and to increase reactive oxygen species (ROS) [35, 48]. Since oxidative stress and inflammation can be caused by PPIX and bile acids in EPP [11, 24, 49], Cer accumulation may further worsen these cellular processes.

In summary, the present study provided a metabolomic perspective of EPP-associated liver injury. We found the accumulation of PPIX, bile acids, and Cer in the liver of Fech-mut mice. We also found a decrease of the major PC in the liver and total PC to PPIX ratio in the bile of Fech-mut mice. All these metabolic changes may contribute to EPP-associated liver injury (Fig. 6). Our data can be used to guide the development of novel therapies for EPP-associated liver injury by modulating the homeostasis of PPIX, bile acids, PC, and Cer.

Acknowledgments

This work was supported in part by the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK090305), the National Institute of Allergy And Infectious Diseases (R01AI131983), and the National Institute of General Medical Sciences (R01GM118367).

Abbreviations

Cer	ceramide
EPP	erythropoietic protoporphyria
FECH	ferrochelataase
MS/MS	tandem mass spectrometry
OPLS-DA	orthogonal projection to latent structures-discriminant analysis
PC	phosphatidylcholine
PCA	principal component analysis
PPIX	protoporphyrin IX
TOFMS	time-of-flight mass spectrometry
UPLC	ultraperformance liquid chromatography

References

- [1]. APF, <http://www.porphyrifoundation.com/about-porphyrria/types-of-porphyrria/EPP>, PLoS one (2014).
- [2]. Puy H, Gouya L, Deybach J-C, Porphyrrias, *The Lancet* 375(9718) (2010) 924–937.
- [3]. Becker DM, Viljoen JD, Katz J, Kramer S, Reduced ferrochelatase activity: a defect common to porphyria variegata and protoporphyria, *British journal of haematology* 36(2) (1977) 171–9. [PubMed: 871431]
- [4]. Kramer S, Viljoen JD, Erythropoietic protoporphyria: evidence that it is due to a variant ferrochelatase, *The International journal of biochemistry* 12(5–6) (1980) 925–30. [PubMed: 7450150]
- [5]. Anstey AV, Hift RJ, Liver disease in erythropoietic protoporphyria: insights and implications for management, *Gut* 56(7) (2007) 1009–18. [PubMed: 17360790]
- [6]. Balwani M, Naik H, Anderson KE, Bissell DM, Bloomer J, Bonkovsky HL, Phillips JD, Overbey JR, Wang B, Singal AK, Liu LU, Desnick RJ, Clinical, Biochemical, and Genetic Characterization of North American Patients With Erythropoietic Protoporphyria and X-linked Protoporphyria, *JAMA dermatology* 153(8) (2017) 789–796. [PubMed: 28614581]
- [7]. Park PJ, Hwang S, Choi YI, Yu YD, Park GC, Jung SW, Yoon SY, Song GW, Ha TY, Lee SG, Liver transplantation for acute-on-chronic liver failure from erythropoietic protoporphyria, *Clinical and molecular hepatology* 18(4) (2012) 411–5. [PubMed: 23323258]
- [8]. Bloomer JR, The liver in protoporphyria, *Hepatology* 8(2) (1988) 402–7. [PubMed: 3281889]
- [9]. Anstey AV, Hift RJ, Liver disease in erythropoietic protoporphyria: insights and implications for management, *Postgrad Med J* 83(986) (2007) 739–48. [PubMed: 18057171]
- [10]. Casanova-Gonzalez MJ, Trapero-Marugan M, Jones EA, Moreno-Otero R, Liver disease and erythropoietic protoporphyria: a concise review, *World journal of gastroenterology : WJG* 16(36) (2010) 4526–31. [PubMed: 20857522]
- [11]. Lyoumi S, Abitbol M, Rainteau D, Karim Z, Bernex F, Oustric V, Millot S, Letteron P, Heming N, Guillmot L, Montagutelli X, Berdeaux G, Gouya L, Poupon R, Deybach JC, Beaumont C, Puy H, Protoporphyrin retention in hepatocytes and Kupffer cells prevents sclerosing cholangitis in erythropoietic protoporphyria mouse model, *Gastroenterology* 141(4) (2011) 1509–19, 1519 e1–3. [PubMed: 21762662]
- [12]. Bloomer JR, Hill HD, Kools AM, Straka JG, Heme synthesis in protoporphyria, *Current problems in dermatology* 20 (1991) 135–47. [PubMed: 1935205]
- [13]. Samuel D, Boboc B, Bernuau J, Bismuth H, Benhamou JP, Liver transplantation for protoporphyria. Evidence for the predominant role of the erythropoietic tissue in protoporphyrin overproduction, *Gastroenterology* 95(3) (1988) 816–9. [PubMed: 3294082]
- [14]. Singal AK, Parker C, Bowden C, Thapar M, Liu L, McGuire BM, Liver transplantation in the management of porphyria, *Hepatology* 60(3) (2014) 1082–9. [PubMed: 24700519]
- [15]. Gordeuk VR, Brittenham GM, Hawkins CW, Mukhtar H, Bickers DR, Iron therapy for hepatic dysfunction in erythropoietic protoporphyria, *Ann. Intern. Med* 105(1) (1986) 27–31. [PubMed: 3717807]
- [16]. Bloomer JR, Pierach CA, Effect of hematin administration to patients with protoporphyria and liver disease, *Hepatology* 2(6) (1982) 817–21. [PubMed: 7141394]
- [17]. Spiva DA, Lewis CE, Erythropoietic protoporphyria: therapeutic response to combined erythrocyte exchange and plasmapheresis, *Photo-dermatology* 1(5) (1984) 211–20. [PubMed: 6531299]
- [18]. Gorchein A, Foster GR, Liver failure in protoporphyria: long-term treatment with oral charcoal, *Hepatology* 29(3) (1999) 995–6. [PubMed: 10189233]
- [19]. Van Hattum J, Baart H de la Faille, Van den Berg JW, Edixhoven-Bosdijk A, Wilson JH, Chenodeoxycholic acid therapy in erythrohepatic protoporphyria, *Journal of hepatology* 3(3) (1986) 407–12. [PubMed: 3559148]
- [20]. Gross U, Frank M, Doss MO, Hepatic complications of erythropoietic protoporphyria, *Photodermatology, photoimmunology & photomedicine* 14(2) (1998) 52–7.

- [21]. Komatsu H, Ishii K, Imamura K, Maruyama K, Yonei Y, Masuda H, Tsuchihashi T, Sajima Y, A case of erythropoietic protoporphyria with liver cirrhosis suggesting a therapeutic value of supplementation with alpha-tocopherol, *Hepatology research : the official journal of the Japan Society of Hepatology* 18(3) (2000) 298–309. [PubMed: 11058833]
- [22]. Johnson CH, Ivanisevic J, Siuzdak G, Metabolomics: beyond biomarkers and towards mechanisms, *Nat. Rev. Mol. Cell Biol* 17(7) (2016) 451–9. [PubMed: 26979502]
- [23]. Wishart DS, Emerging applications of metabolomics in drug discovery and precision medicine, *Nat. Rev. Drug Discov* (2016).
- [24]. Tutois S, Montagutelli X, Da Silva V, Jouault H, Rouyer-Fessard P, Leroy-Viard K, Guenet JL, Nordmann Y, Beuzard Y, Deybach JC, Erythropoietic protoporphyria in the house mouse. A recessive inherited ferrochelatase deficiency with anemia, photosensitivity, and liver disease, *J. Clin. Invest* 88(5) (1991) 1730–6. [PubMed: 1939658]
- [25]. Boulechfar S, Lamoril J, Montagutelli X, Guenet J-L, Deybach J-C, Nordmann Y, Dailey H, Grandchamp B, de Verneuil H, Ferrochelatase structural mutant (Fechm1Pas) in the house mouse, *Genomics* 16(3) (1993) 645–648. [PubMed: 8325637]
- [26]. Want EJ, Masson P, Michopoulos F, Wilson ID, Theodoridis G, Plumb RS, Shockcor J, Loftus N, Holmes E, Nicholson JK, Global metabolic profiling of animal and human tissues via UPLC-MS, *Nature protocols* 8(1) (2013) 17–32. [PubMed: 23222455]
- [27]. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G, METLIN: a metabolite mass spectral database, *Ther. Drug Monit* 27(6) (2005) 747–751. [PubMed: 16404815]
- [28]. Sud M, Fahy E, Cotter D, Brown A, Dennis EA, Glass CK, Merrill AH, Jr, Murphy RC, Raetz CR, Russell DW, Lmsd: Lipid maps structure database, *Nucleic Acids Res.* 35(suppl_1) (2006) D527–D532. [PubMed: 17098933]
- [29]. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, HMDB 4.0: the human metabolome database for 2018, *Nucleic Acids Res.* 46(D1) (2017) D608–D617.
- [30]. Chiang JYL, Ferrell JM, Bile acid metabolism in liver pathobiology, *Gene Expr.* (2018).
- [31]. Alrefai WA, Gill RK, Bile acid transporters: structure, function, regulation and pathophysiological implications, *Pharm. Res* 24(10) (2007) 1803–23. [PubMed: 17404808]
- [32]. Nakanishi H, Iida Y, Shimizu T, Taguchi R, Separation and quantification of sn-1 and sn-2 fatty acid positional isomers in phosphatidylcholine by RPLC-ESIMS/MS, *J. Biochem* 147(2) (2010) 245–56. [PubMed: 19880374]
- [33]. Li Z, Vance DE, Phosphatidylcholine and choline homeostasis, *J. Lipid Res* 49(6) (2008) 1187–94. [PubMed: 18204095]
- [34]. Kitatani K, Idkowiak-Baldys J, Hannun YA, The sphingolipid salvage pathway in ceramide metabolism and signaling, *Cell. Signal* 20(6) (2008) 1010–8. [PubMed: 18191382]
- [35]. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP, Role of ceramides in nonalcoholic fatty liver disease, *Trends Endocrinol. Metab* 23(8) (2012) 365–71. [PubMed: 22609053]
- [36]. Xu H, Sun Y, Zhang Y, Wang W, Dan J, Yao J, Chen H, Tian F, Sun X, Guo S, Protoporphyrin IX induces a necrotic cell death in human THP-1 macrophages through activation of reactive oxygen species/c-Jun N-terminal protein kinase pathway and opening of mitochondrial permeability transition pore, *Cell. Physiol. Biochem* 34(6) (2014) 1835–1848. [PubMed: 25502879]
- [37]. Pastorino JG, Simbula G, Gilfor E, Hoek JB, Farber JL, Protoporphyrin IX, an endogenous ligand of the peripheral benzodiazepine receptor, potentiates induction of the mitochondrial permeability transition and the killing of cultured hepatocytes by rotenone, *J. Biol. Chem* 269(49) (1994) 31041–31046. [PubMed: 7983042]
- [38]. de Aguiar Vallim TQ, Tarling EJ, Edwards PA, Pleiotropic roles of bile acids in metabolism, *Cell Metab.* 17(5) (2013) 657–69. [PubMed: 23602448]
- [39]. Li T, Apte U, Bile Acid Metabolism and Signaling in Cholestasis, Inflammation, and Cancer, *Adv. Pharmacol* 74 (2015) 263–302. [PubMed: 26233910]
- [40]. Chiang JYL, Bile acid metabolism and signaling in liver disease and therapy, *Liver research* 1(1) (2017) 3–9. [PubMed: 29104811]

- [41]. Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A, Vance DE, The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis, *Cell Metab.* 3(5) (2006) 321–331. [PubMed: 16679290]
- [42]. Li Z, Agellon LB, Vance DE, Phosphatidylcholine homeostasis and liver failure, *J. Biol. Chem* 280(45) (2005) 37798–802. [PubMed: 16144842]
- [43]. Buang Y, Wang Y-M, Cha J-Y, Nagao K, Yanagita T, Dietary phosphatidylcholine alleviates fatty liver induced by orotic acid, *Nutrition* 21(7) (2005) 867–873. [PubMed: 15975496]
- [44]. Lieber CS, Robins SJ, Li J, DeCarli LM, Mak KM, Fasulo JM, Leo MA, Phosphatidylcholine protects against fibrosis and cirrhosis in the baboon, *Gastroenterology* 106(1) (1994) 152–9. [PubMed: 8276177]
- [45]. Hannun YA, Obeid LM, Principles of bioactive lipid signalling: lessons from sphingolipids, *Nat. Rev. Mol. Cell Biol* 9(2) (2008) 139–50. [PubMed: 18216770]
- [46]. Jiang C, Xie C, Li F, Zhang L, Nichols RG, Krausz KW, Cai J, Qi Y, Fang ZZ, Takahashi S, Tanaka N, Desai D, Amin SG, Albert I, Patterson AD, Gonzalez FJ, Intestinal farnesoid X receptor signaling promotes nonalcoholic fatty liver disease, *J. Clin. Invest* 125(1) (2015) 386–402. [PubMed: 25500885]
- [47]. Xie C, Jiang C, Shi J, Gao X, Sun D, Sun L, Wang T, Takahashi S, Anitha M, Krausz KW, Patterson AD, Gonzalez FJ, An Intestinal Farnesoid X Receptor-Ceramide Signaling Axis Modulates Hepatic Gluconeogenesis in Mice, *Diabetes* 66(3) (2017) 613–626. [PubMed: 28223344]
- [48]. Holland WL, Summers SA, Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism, *Endocr. Rev* 29(4) (2008) 381–402. [PubMed: 18451260]
- [49]. Davies R, Schuurman A, Barker CR, Clothier B, Chernova T, Higginson FM, Judah DJ, Dinsdale D, Edwards RE, Greaves P, Gant TW, Smith AG, Hepatic gene expression in protoporphyric Feh mice is associated with cholestatic injury but not a marked depletion of the heme regulatory pool, *Am. J. Pathol* 166(4) (2005) 1041–53. [PubMed: 15793285]

Fig. 1. Liver injury in Fech-mut mice.

(A) Abundance of PPIX in the liver. (B and C) Histological analysis of liver by H&E staining (400X). Scale bar, 10 μ m. PV, portal vein. The arrows point to bile plugs. (D-F) Serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities. All quantified data are expressed as mean $\pm$ S.D. (n = 4). ** P < 0.01 vs WT mice.

Fig. 2. Metabolomic analysis of liver samples from WT (n = 4) and Fech-mut mice (n = 4). (A) Separation of liver samples from WT and Fech-mut mice in a PCA score plot. (B and C) Loading S-plots generated by OPLS-DA analysis of the aqueous (B) and organic (C) extracts. The x-axis is a measure of the relative abundance of ions, and the y-axis is a measure of the correlation of each ion to the model. TCA, taurocholic acid; T- α -MCA, tauro- α -muricholic acid; T- β -MCA, tauro- β -muricholic acid; β -MCA, β -muricholic acid; TCDCA, taurochenodeoxycholic acid; PPIX, protoporphyrin IX; PC, phosphatidylcholine; Cer, ceramide.

Fig. 3. Accumulation of bile acids in the liver of Fech-mut mice.

Bile acids were quantified by UPLC-TOFMS. (A) Abundance of major bile acids in the liver. (B-F) Relative expression of the genes responsible for bile acid synthesis (B), regulation (C), uptake (D), basolateral (E), and biliary (F) excretion. All data are expressed as mean $\pm$ S.D. (n = 4). The data in WT mice were set as 1. * P < 0.05; ** P < 0.01; *** P < 0.001 vs WT. FXR, farnesoid X receptor; SHP small heterodimer partner; Ntcp, Na⁺-taurocholate cotransporting polypeptide; Oatp, organic anion transporter; Mrp, multidrug resistance-associated protein; Bsep, bile salt export pump.

Fig. 4. Alterations of phosphatidylcholines (PCs) in the liver and bile of Fech-mut mice. (A) Relative abundance of major PCs in the liver. (B) Expression of the genes responsible for PC biosynthesis in the liver. (C and D) Total PC and PPIX in the bile of WT and Fech-mut mice. (E) The ratio of total PC to PPIX in the bile of WT and Fech-mut mice. All data are expressed as mean $\pm$ S.D. (n = 4). The gene expression data in WT group were set as 1. ** $P < 0.01$ vs WT.

Fig. 5. Accumulation of Cer in the liver of Fech-mut mice.

(A) Relative abundance of Cer in the liver. (B-D) Expression levels of genes responsible for *de novo*, sphingomyelinase, and salvage pathways of Cer biosynthesis. All data are expressed as mean $\pm$ S.D. (n = 4). The data in WT group were set as 1. *** P < 0.001, ** P < 0.01 vs WT. Cer, ceramide; Sptlc, serine palmitoyltransferase, long chain base subunit; Cers, ceramide synthases; Smpd, sphingomyelin phosphodiesterase.

Fig. 6. The summary of the metabolic changes in the liver of an EPP mouse model and their associations with EPP-associated liver injury.

EPP is caused by the dysfunction of ferrochelatase, which can decrease the conversion of protoporphyrin IX (PPIX) to heme and lead to PPIX accumulation. (1) PPIX accumulation can directly cause liver injury and indirectly cause liver injury through PPIX-mediated bile duct blockage. (2) Bile acids accumulation that ensued from PPIX-mediated bile duct blockage can cause liver injury. (3) Decrease of biliary total phosphatidylcholine (PC) to PPIX ratio reduces PPIX dissolution in the bile, which can potentiate PPIX-mediated bile duct blockage and liver injury. (4) Ceramide accumulation in EPP condition can cause oxidative stress and liver injury.

Table 1.

Summary of metabolites that are altered in the liver of Fech-mut mice vs WT mice.

Metabolites	RT (min)	Observed m/z	Calculated m/z	Mass Error (ppm)	Predicted Molecular Formula	Identification
Increased						
1	4.98	514.2837	514.2838	-0.2	C ₂₆ H ₄₄ NO ₇ S	T-β-MCA
2	5.67	514.2835	514.2838	-0.6	C ₂₆ H ₄₄ NO ₇ S	TCA
3	4.90	514.2839	514.2838	0.2	C ₂₆ H ₄₄ NO ₇ S	T-α-MCA
4	6.33	407.2796	407.2797	-0.2	C ₂₄ H ₃₉ O ₅	β-MCA
5	6.30	498.2885	498.2889	-0.8	C ₂₆ H ₄₄ NO ₆ S	TCDCa
6	2.18	563.2655	563.2658	-0.5	C ₃₄ H ₃₅ N ₄ O ₄	PPIX
7	8.17	630.6188	630.6189	-0.2	C ₄₂ H ₈₀ NO ₂	C24:1 Cer
8	7.02	520.5093	520.5094	-0.2	C ₃₄ H ₆₆ NO ₂	C16:0 Cer
9	7.40	548.5401	548.5407	-1.1	C ₃₆ H ₇₀ NO ₂	C18:0 Cer
Decreased						
10	6.48	758.5712	758.5700	1.6	C ₄₂ H ₈₁ NO ₈ P	PC 34:2
11	6.18	806.5690	806.5700	-1.2	C ₄₆ H ₈₁ NO ₈ P	PC 38:6
12	7.01	786.6011	786.6013	-0.3	C ₄₄ H ₈₅ NO ₈ P	PC 36:2
13	6.75	834.6003	834.6013	-1.2	C ₄₈ H ₈₅ NO ₈ P	PC 40:6

RT, retention time; ppm, part per million. TCA, taurocholic acid; T-α-MCA, tauro-α-muricholic acid; T-β-MCA, tauro-β-muricholic acid; β-MCA, β-muricholic acid; TCDCa, taurochenodeoxycholic acid; PPIX, protoporphyrin IX; PC, phosphatidylcholine; Cer, ceramide.