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MICE WITH HUMAN-LIKE BILE ACID COMPOSITION DEVELOP PFIC-2- LIKE CHOLESTASIS

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ABSTRACT

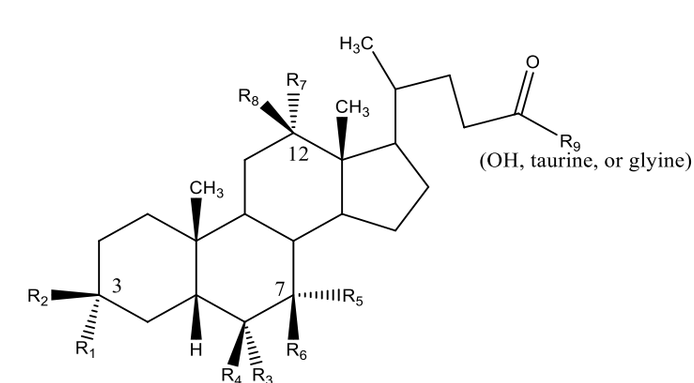
Bile acids (BAs) are essential for lipid absorption, bile formation, and regulation of intermediary metabolism. Primary BAs are synthesized in the liver and conjugated with glycine or taurine (T) before biliary excretion. Humans produce two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA). BA synthesis is regulated by the farnesoid X receptor (FXR), which represses Cyp7a1, the rate-limiting enzyme of BA synthesis. Although mice are commonly used models, mice efficiently hydroxylate CDCA by Cyp2c70 at the 6β-position; thus, their dominant BAs are the hydrophilic muricholic acids (MCAs), in contrast to the hydrophobic human BA-pool. These species differences complicate the translational research of BAs. For example, because of the hydrophilic BA composition, the PFIC-2 model, bile salt export pump (Bsep)-null mouse shows moderate changes in phenotype compared to human PFIC-2 patients. To better understand the role of human-like BA metabolism, we engineered Cyp2c70-null mice.

BA concentrations in liver, bile, and plasma, and hepatic gene expression, were quantified in male and female wild-type and Cyp2c70-null mice. Hepatic and plasma concentrations of total (Σ)BAs increased in male mice (liver: +73%, plasma: +5-fold), and were markedly higher in female null mice (liver: +1.79-fold, plasma: +14-fold) compared to WT mice. MCA formation was practically abolished, and CDCA and its metabolites increased markedly, creating a human-like, hydrophobic BA composition in Cyp2c70-null mice. Contrary to significant increase in concentrations of CDCA and LCA in livers of Cyp2c70-null mice, hepatic FXR-SHP and intestinal FXR-Fgf15 pathways were minimally activated. Biliary excretion of ΣBAs decreases in male (-18%) and more in female (-25%) Cyp2c70-null mice, contrary to increased biliary excretion of CDCA (~16-fold) and LCA (~32-fold). Decreased ΣBA excretion was due to reduced biliary excretion of TCA in male mice (-19%), more markedly in female mice (-46%). The ratio of TCDCA/TCA in WT liver is 0.12, whereas it is 1.6 in male and 2.9 in female Cyp2c70-null mice. Murine Bsep has a higher affinity for TCDCA than TCA; therefore, TCDCA can inhibit biliary transport of TCA, especially in female (-46%) Cyp2c70-null mice. Since (T)CA is the natural ligand for murine FXR, due to lower excretion of TCA, the intestinal FXR-Fgf15 pathway (responsible for Cyp7a1 suppression) is not activated. In contrast, the hepatic FXR-pathway is activated, causing12-α-hydroxylase Cyp8b1 suppression, especially in female Cyp2c70-null mice. In summary, Cyp2c70-null mice have a human-like hydrophobic BA-pool and exhibit PFIC-like cholestatic features, which will help clarify the pathomechanisms of various pediatric liver diseases.

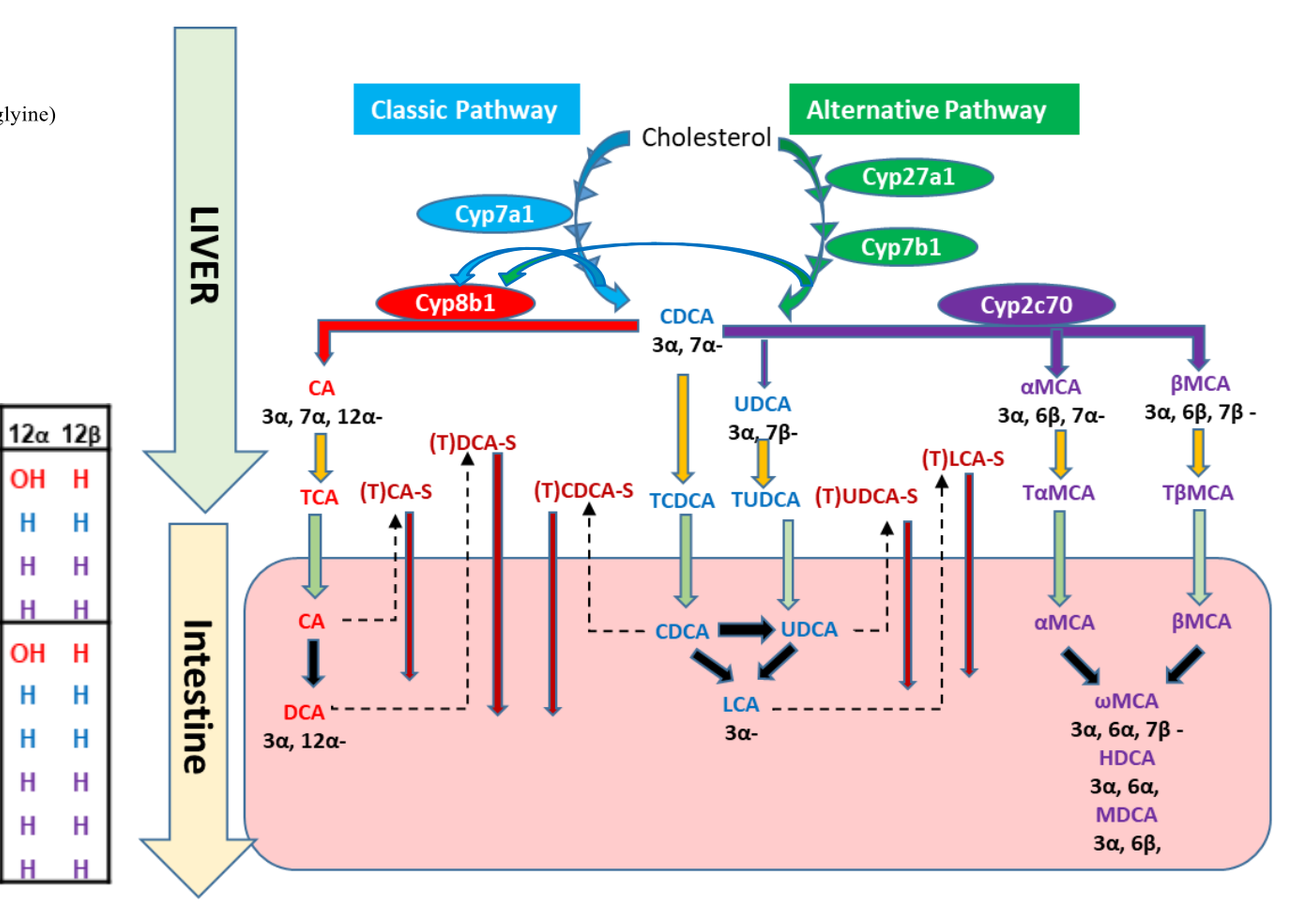
INTRODUCTION

- **Bile acids (BAs) are detergent-like molecules synthesized by the liver (Fig. 1) with essential roles in the digestion and absorption of lipids and fat-soluble vitamins**
- **BAs also have critical roles in energy balance and intermediate metabolism (Hofmann and Hagey, 2014; Li and Chiang, 2014).**
- **BAs are hepatotoxic and are implicated in various pediatric and adult human diseases (cholestatic liver disease, PBC, PSC, NAFLD, cancers, etc.)**
- **Mice are commonly used for modeling pediatric and adult liver diseases. However, there are several differences between human and mice BA homeostasis, which complicates translational research.**
- **The most important difference is that humans produce cholic acid (CA) and chendeoxycholic acid (CDCA) as primary BAs, whereas in mice in addition to CA and CDCA produce 6β-hydroxylated muricholic acids (MCAs)**
- **Recently, the rodent-specific Cyp2c70 was identified as being responsible for 6β-hydroxylation**
- **Due to the formation of MCAs, mice have more hydrophilic BA pools than humans**
- **In addition, the effect of these primary BAs are different because CDCA and CA act like agonists, whereas MCA acts as an antagonist on the Farnesoid X Receptor (FXR), the major regulator of BA homeostasis**
- **To better model human diseases, creating Cyp2c70-null would allow us to study the role of human-like BA composition in mouse models**
- **Therefore, our laboratory engineered Cyp2c70-null mice**

Fig 1. Bile Acid Metabolism in Mice



Bile acids	Abbrev.		3α	3β	6α	6β	7α	7β	12α	12β
cholic acid	CA	Primary	OH	H	H	H	OH	H	OH	H
chenodeoxycholic acid	CDCA	Primary	OH	H	H	H	OH	H	H	H
α-muricholic acid	α-MCA	Primary	OH	H	H	OH	OH	H	H	H
β-muricholic acid	β-MCA	Primary	OH	H	H	OH	H	OH	H	H
deoxycholic acid	DCA	Secondary	OH	H	H	H	H	H	OH	H
ursodeoxycholic acid	UDCA	Secondary	OH	H	H	H	H	OH	H	H
lithocholic acid	LCA	Secondary	OH	H	H	H	H	H	H	H
ω-muricholic acid	ωMCA	Secondary	OH	H	OH	H	H	OH	H	H
murideoxycholic acid	MDCA	Secondary	OH	H	H	OH	H	H	H	H
hyodeoxycholic acid	HDCA	Secondary	OH	H	OH	H	H	H	H	H



***Left panel*: Structures of major types of bile acids, and their hydroxylation position.**
***Right panel*: A simplified diagram of BA synthesis.**
CA and its metabolites (red), CDCA and its metabolites (blue), 6-OH BAs (purple), sulfated-BAs (brown) are indicated.

- **In the liver, cholesterol is converted to CDCA and CA. The rate-limiting enzyme of BA synthesis is cholesterol 7α-hydroxylase (CYP7A1), which converts cholesterol to 7α-hydroxycholesterol in the classical pathway.**
- **The sterol 12α-hydroxylase enzyme (Cyp8b1) is solely responsible for the biosynthesis CA. Therefore the activity of CYP8B1 controls the ratio of CDCA to CA**
- **In rodents Cyp2c70 forms 6β-hydroxylated hydrophilic BAs (Takahashi et al., 2016; de Boer et al., 2020).**
- **BAs are conjugated with either glycine or taurine before they are transported into bile, stored in the gall bladder, and enter the duodenum upon food ingestion.**
- **The majority of BAs secreted into the intestine are reabsorbed in the terminal ileum and recirculated back to the liver via the portal vein.**
- **~10-12% of the BAs escape reabsorption and reach the large intestine where bacterial enzymes are capable of metabolizing BAs. All BAs formed due to bacterial modifications of primary BAs are termed secondary BAs.**
- **The dehydroxylation of BAs results in more toxic BAs, for example, the conversion of CDCA to LCA, which is considered the most toxic BA.**
- **After reabsorption, BAs can also be sulfated at the 3 and 7 positions, which decreases their toxicity (Alnouti, 2009;** Zhang and Klaassen, 2010; Huang et al., 2011).
- **Sulfation is considered the most important detoxification of LCA, the most toxic BA (Hofmann, 2004).**

PURPOSE

To obtain a new mouse model with human-like hydrophobic BA composition to model various pediatric liver diseases.

MATERIALS AND METHODS

ANIMALS:
- **Wildtype (WT) C57BL/6J mice were purchase from Jackson labs (Bar Harbor, Maine).**
- **Exon one of the Cyp2c70 gene was targeted using CRISPR/Cas-mediated genome editing on fertilized eggs generating 9 pups.**
- **Two mouse lines were kept for development and backcrossed against WT mice**
- **After 3 generations of backcrossing, homozygote Cyp2c70-null mice were generated for initial characterization**

TISSUE COLLECTION:
- **Male and female WT and both genotypes of Cyp2c70-null mice (n=5-6) were collected at 3 months of age**
- **Anesthesia: Urethane (1.2 g/kg)**
- **Blood was collected from suborbital veins; livers were collected and snap-frozen in liquid nitrogen.**

BILE COLLECTION (from separate sets of mice):
- **Male and female WT and Cyp2c70-null mice were collected at 3 months of age**
- **Anesthesia: Urethane (1.2 g/kg)**
- **Common bile duct was cannulated and bile samples were collected for 40-min.**
- **Volume of bile was determined gravimetrically.**

RNA EXTRACTION:
- **Stranded mRNA purified by TRIzol® Reagent with Phase Lock GelTM and extracted by**
- **QC results (RIN 7.6-8.7) were obtained using Agilent TapeStation 4000**

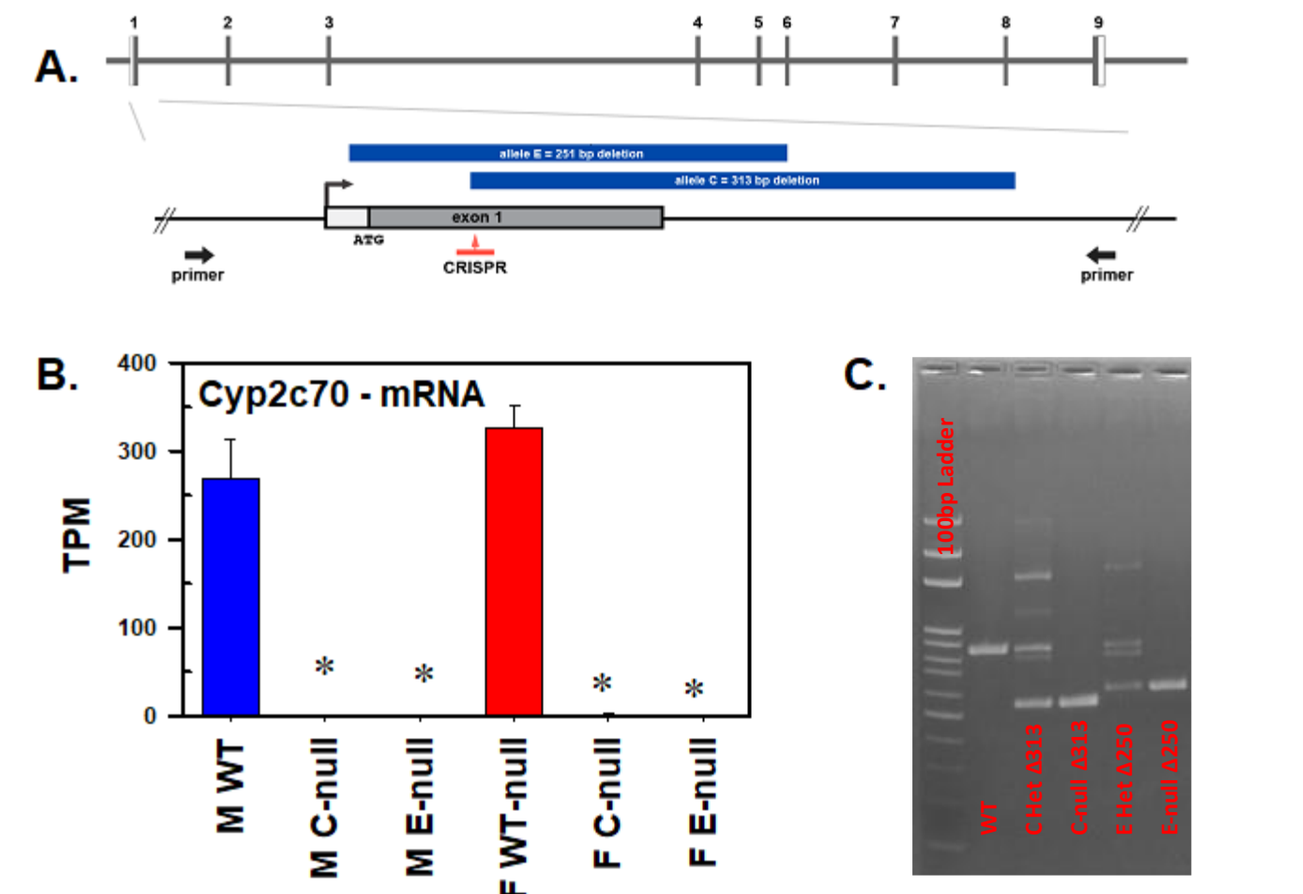
MESSENGER RNA QUANTIFICATION:
- **The mRNA expression of genes in liver were quantified by next-generation sequencing using NovaSeq 6000 with a NuGEN Universal Plus RNA Assay and NuGEN Universal Plus RNA UDI.**
- **Gene were annotation using GENCODE (GRCm38-version M24, Ensembl 99)**

BILE ACID ANALYSIS:
- **BAs were quantified by reverse-phase ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) [3;4].**

STATISTICS:
- **BA concentration values were log-normalized**
- **Statistical significance was determined by ANOVA and Duncan's post hoc for WT vs KO and by Student's *t*-test for sex differences. All tests used a *p*-value of 0.05. Asterisks (*) indicate differences between WT and KO mice; # indicates difference between males and females of the same genotype**

RESULTS

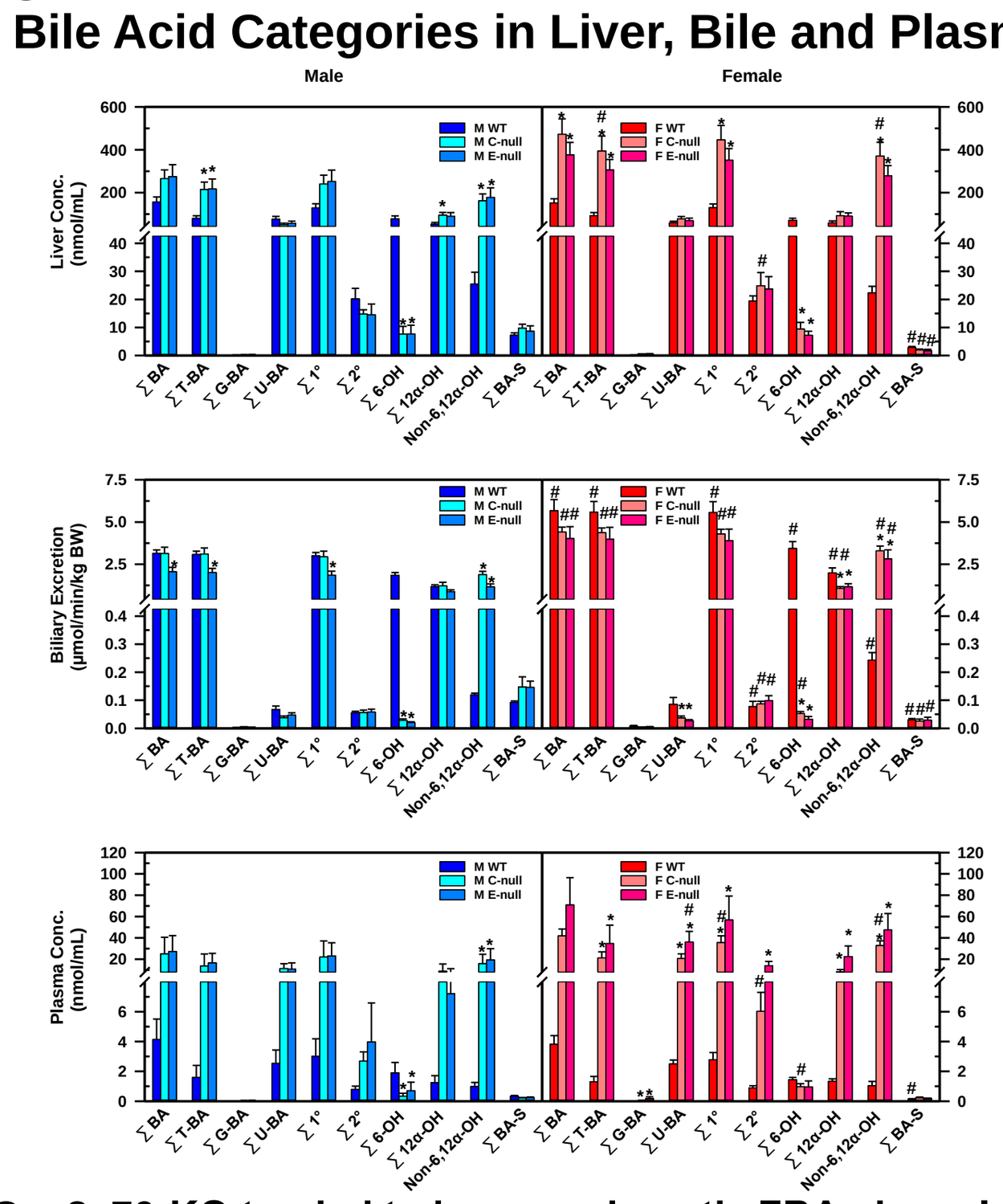
Fig 2. Generation of Cyp2c70-null Mice



A. CRISPR target site in the Cyp2c70 gene and deletions in the "C-null" and "E-null" lines.
- **C-null is a 313 bp deletion, in which the first 19 amino acids are present**
- **E-null is a 251 bp deletion. The entire coding sequence of exon one is deleted**

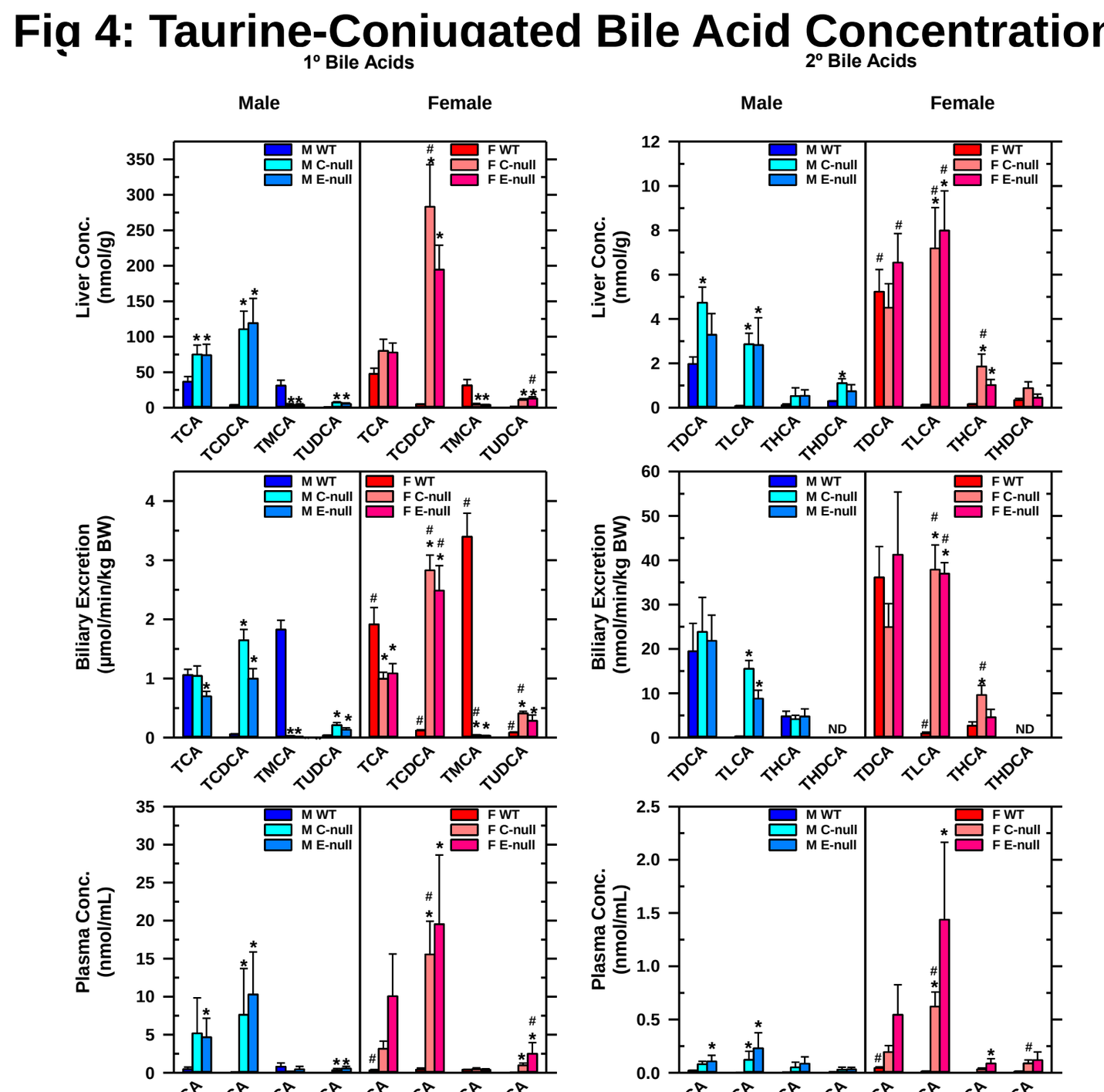
- **B. mRNA expression of Cyp2c70 in WT, C-null and E-null mice. Cyp2c70 expression tends to be higher in female WT**
- **C. Genotyping of experimental mouse lines. WT band=862 bp**

Fig 3. Concentration & Excretion Values for Major Bile Acid Categories in Liver, Bile and Plasma



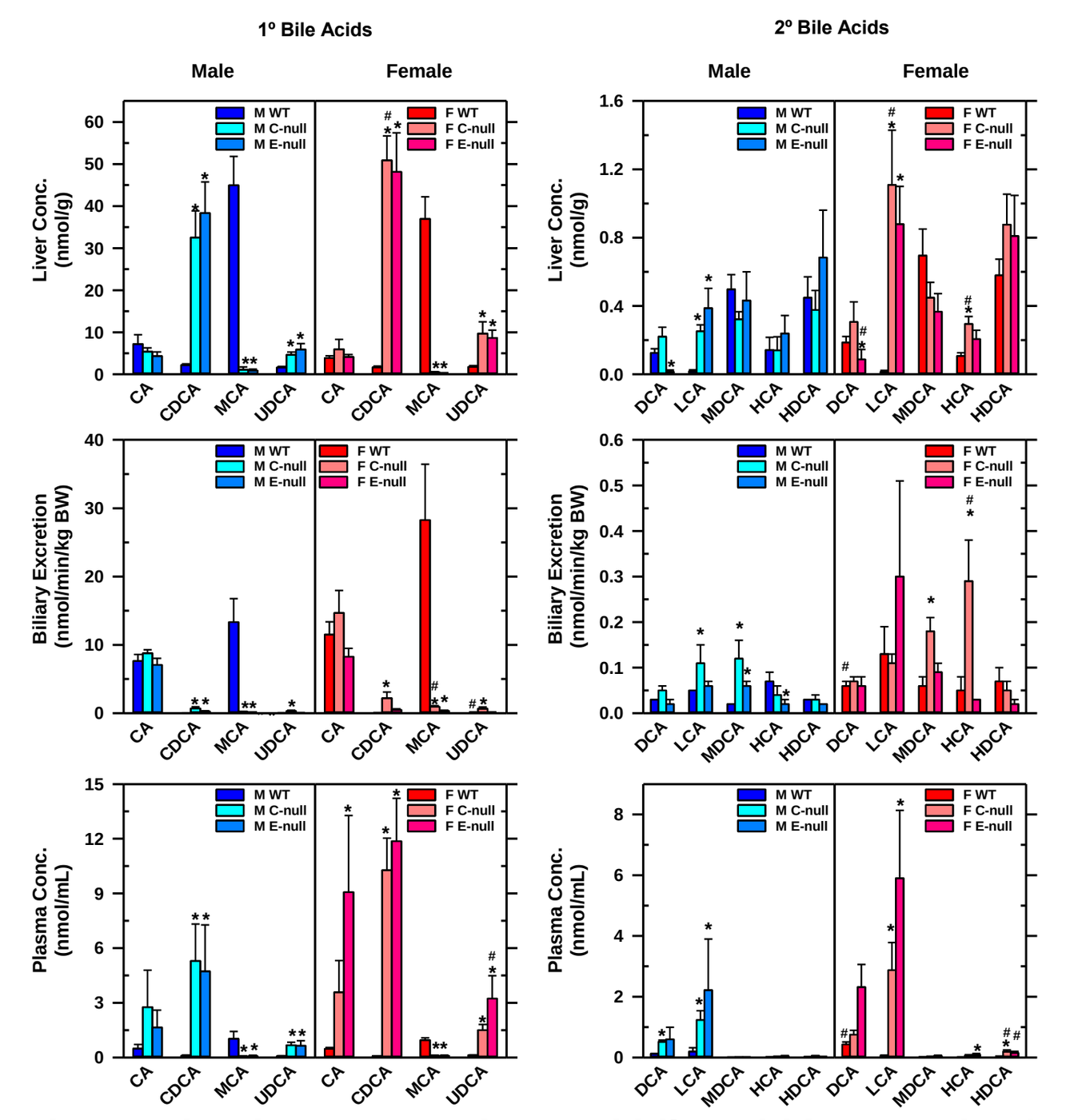
- **Cyp2c70-KO tended to increase hepatic ΣBAs in males (+70%) and significantly increased ΣBAs in females (+150%)**
- **Cyp2c70-KO resulted in sharp decreases in bile acids with hydroxy groups at the 6C position for both males (-90%) and females (-87%)**
- **Non-6,12αOH bile acids were significantly increased in the livers of both males (5-fold) and females (12-fold)**
- **Despite increased hepatic ΣBAs, females experienced decreased biliary excretion (25%) following cyp2c70-KO, particularly among unconjugated (-57%) and 12α-hydroxylated bile acids (-42%)**
- **In both liver and bile, sulfation of bile acids is significantly higher in males, regardless of KO (+60% liver, +67% bile)**
- **Cyp2c70-KO caused elevated bile acid concentrations in plasma, especially in females**

Fig 4: Taurine-Conjugated Bile Acid Concentrations



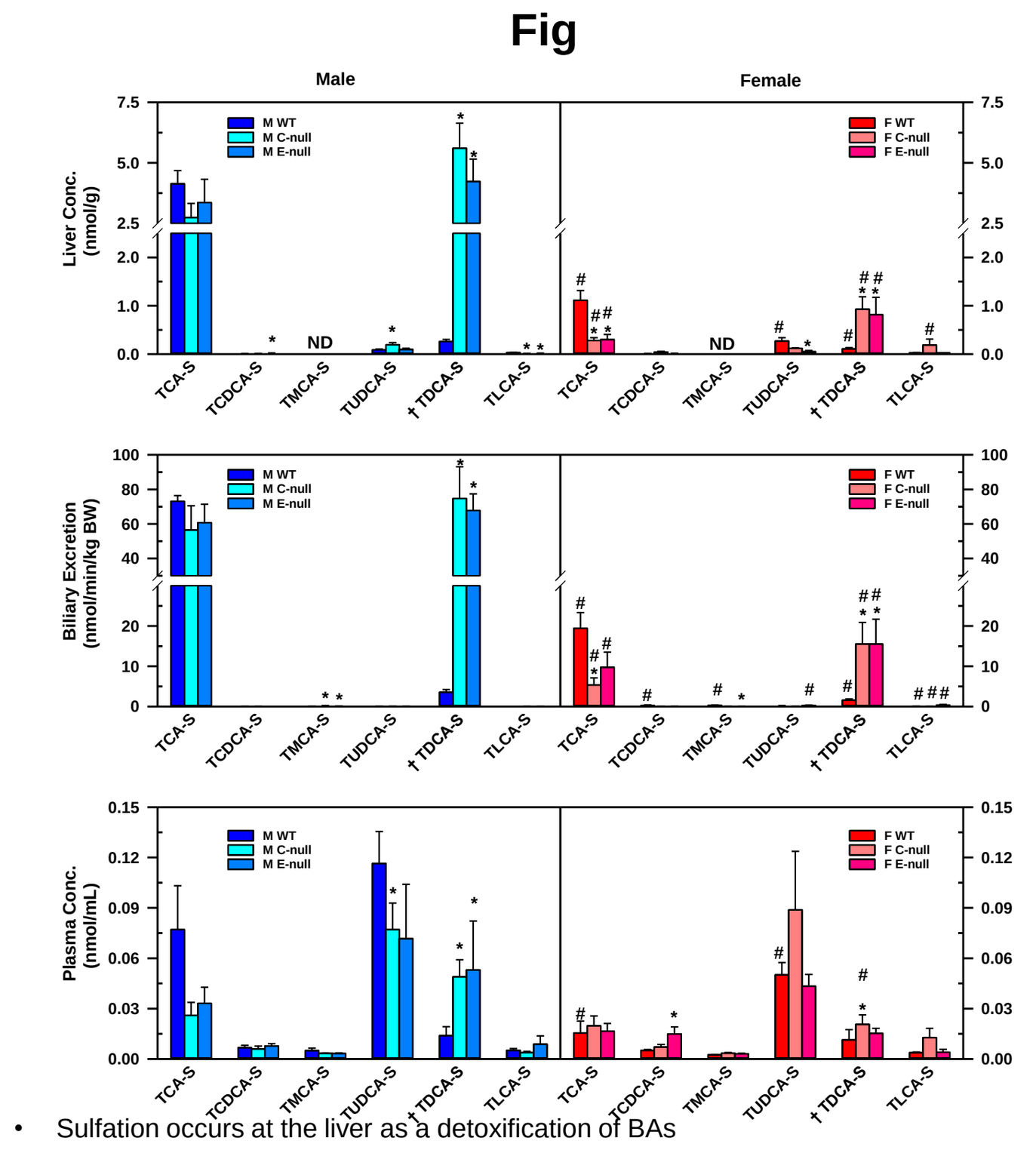
- **Cyp2c70-KO eliminated TMCA, but otherwise caused increases in all other primary bile acids, especially in females**
- **TCDCA, the precursor of TMCA, increased most significantly (+28-fold M; +34-fold F)**
- **TCDCA and TCA compete for excretion via the bile salt export protein (BSEP), but TCDCA has a much higher affinity (5.7 µmol/L vs. 30.3 µmol/L, Noe et al.)**
- **TLCA, a secondary form of CDCA & the most hydrophobic and toxic bile acid, was significantly increased in all tissues, with greater increases in female mice compared to male mice**
- **Female mice experienced significantly decreased excretion of TCA (-44%), the predominant 12α-hydroxylated bile acid, and natural agonist of FXR in mice**
- **Plasma BA concentrations increased for KO mice, especially in females.**
- **Cyp2c70-null mice experience increased hepatic and plasma BA concentrations, with decreased excretion. This is characteristic of**

Fig. 5: Unconjugated Bile Acid Concentrations



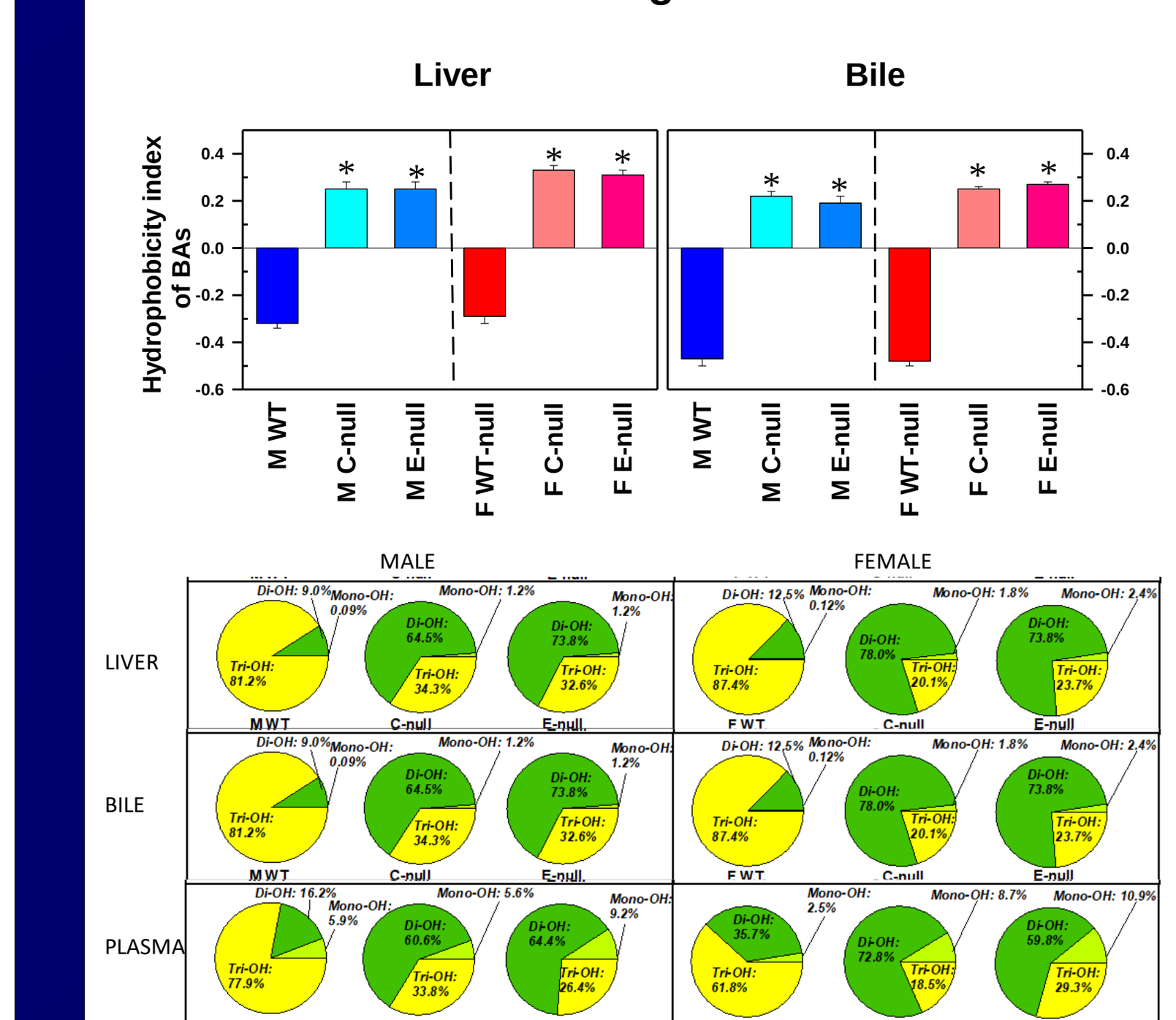
- **The predominant unconjugated bile acid in WT mice is MCA, which is practically abolished with cyp2c70-KO (<-90% in all tissues)**
- **Following cyp2c70-KO CDCA concentrations increase significantly in the liver of both male (13-fold) and female (28-fold) mice**
- **Unconjugated bile acids have greatly reduced affinities for BSEP**

Fig



- Sulfation occurs at the liver as a detoxification of BAs
- Sult2a8 accomplishes 7-OH sulfation (TCA-S, TCDCA-S, TMCA-S, TUDCA-S, TDCA-S), and is male dominant
- Sult2a1 accomplishes 3-OH sulfation (TLCA-S) and is female dominant
- In WTs, TCA-S is the major sulfated BA in liver and bile, but TUDCA-S is the major sulfated BA in plasma
- In Cyp2c70-KOs † TDCA-S increases 15-fold in males and 6-fold in females, becoming the major BA sulfate in liver and bile. † TDCA-S formation remains male predominant.
- In the liver, TCA-S and † TDCA-S are the major sulfated BAs, and have significantly higher concentrations in males, regardless of cyp2c70-KO
- Cyp2c70-KO caused significantly increased sulfation of † TDCA-S in livers for both males and females
- TLCA-S, which is sulfated at the 3-OH position by Sult2a1, tends to be higher in females, and increases after cyp2c70-KO.

Fig



RESULTS

Fig 7.

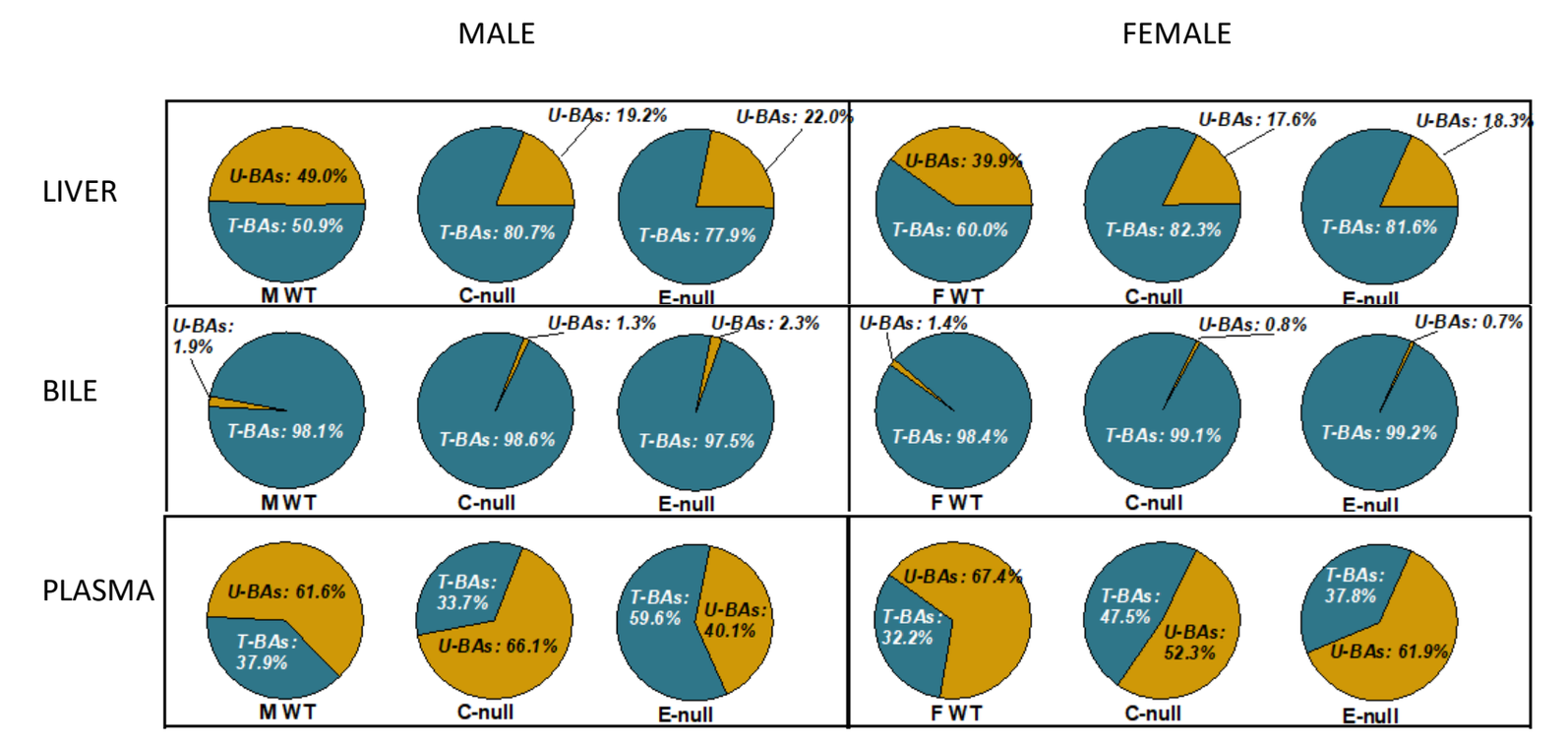
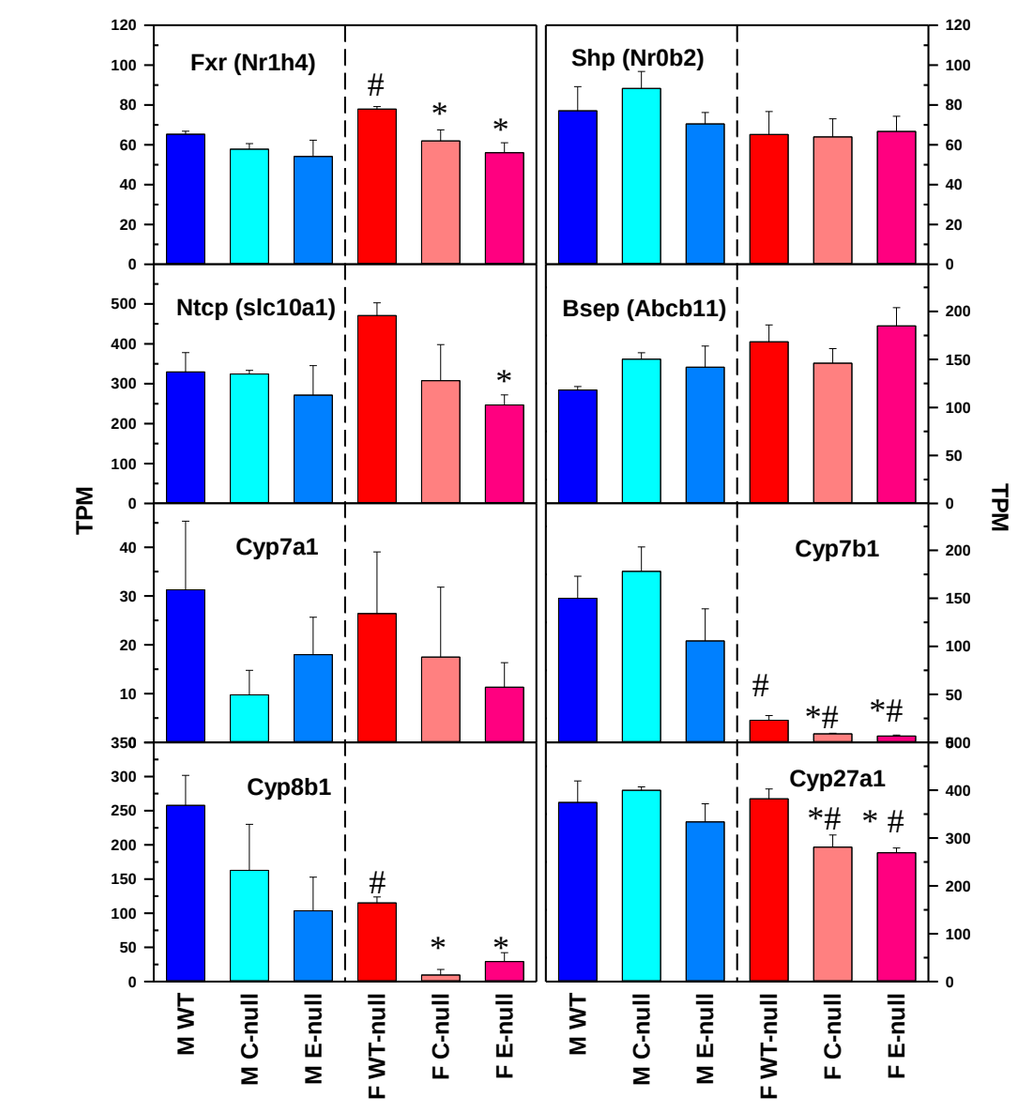
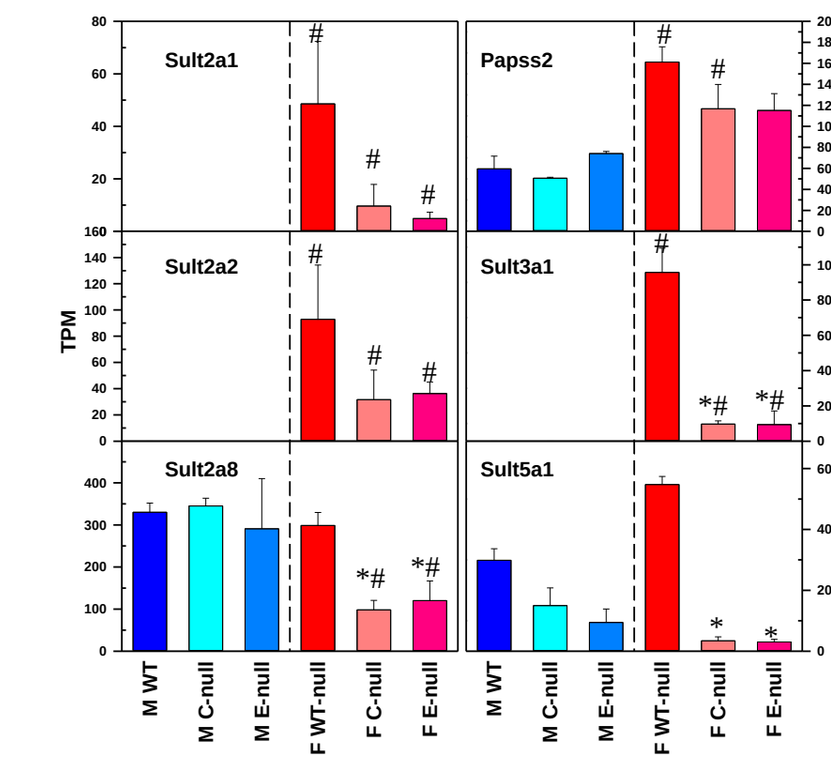


Fig 7.



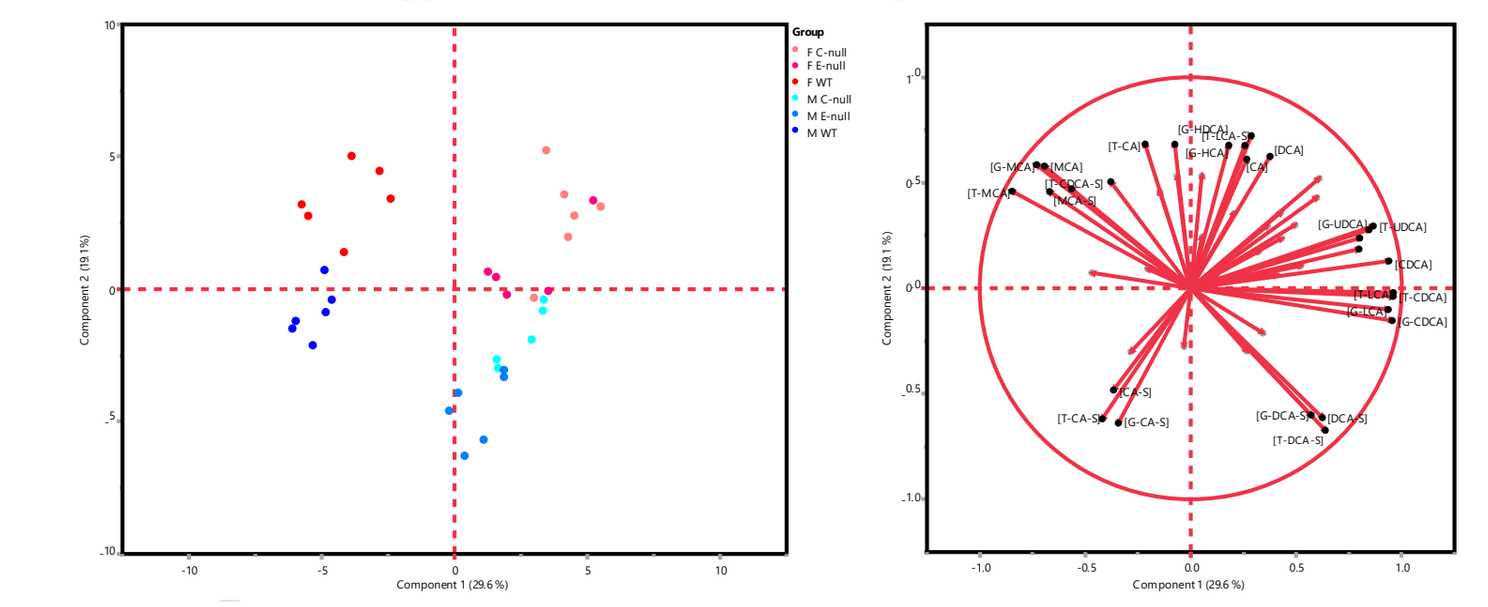
- **Cyp2c70-KO significantly decreases hepatic FXR expression only in females**
- **Hepatic Shp expression is not altered by cyp2c70-KO**
- **Uptake transporter (Ntcp) expression tends to decrease following Cyp2c70-KO, especially in females**
- **Bile acid synthesis enzymes tend to decrease after Cyp2c70-KO**
  - **Cyp7a1, the rate limiting enzyme for BA synthesis, tends to decrease in both males and females**
  - **Cyp7b1, already significantly lower in females, decreases significantly with cyp2c70-KO**
  - **Cyp8b1, responsible for CA formation, is significantly decreased in females**

Fig 9.



- **Sult2a1, Sult2a2, and Sult3a1 are all essentially absent in male mice, and decrease in females following cyp2c70-KO (-80%, -60%, and -90%, respectively)**
- **Sult2a8 carries out 7α-OH sulfation. Cyp2c70-KO does not alter expression of Sult2a8 in males, but significantly decreases expression compared to female WT (-60%) and compared to male KOs (-60%)**
- **Papss2 generates the active sulfate for Sult enzymes. It is female dominant**
  - **Papss2 tends to decrease in females only (-28%) after cyp2c70-KO**

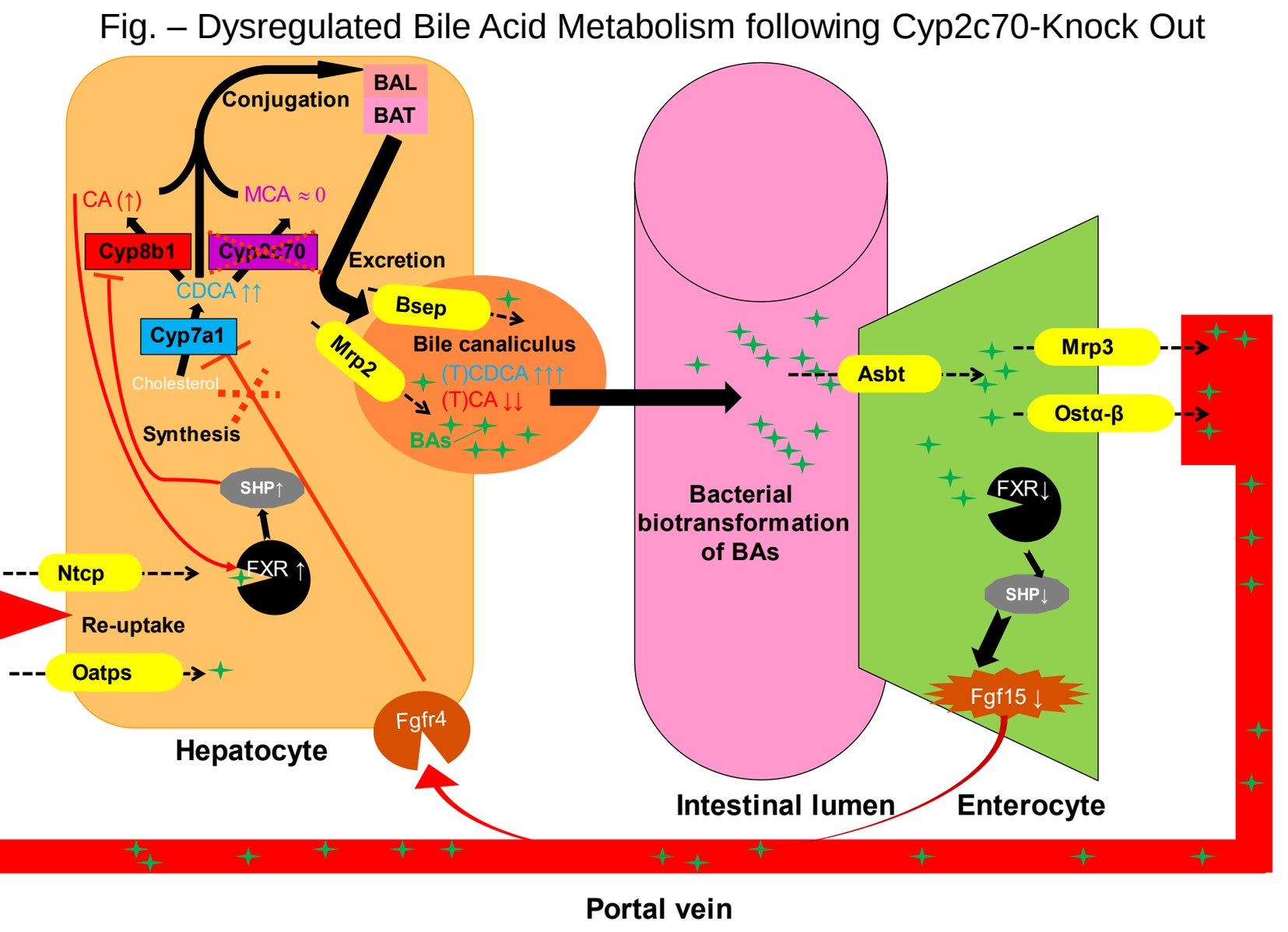
Fig 7. PCA of Biliary Excretion



- **The first two principal components account for 50% of the variation within the samples**
- **Component 1 separates WTs and KOs. MCA and metabolites are strongly negatively correlated with component 1 while CDCA and metabolites are strongly positively correlated**
- **Component 2 separates males from females and C-null from E-null. Six of the top 10 most determinant bile acids for component 2 are sulfated BAs.**
- **Increased concentrations of forms CA-S and † DCA-S, the dominant sulfated BAs, cluster samples towards the negative y-axis.**
- **Increased LCA-S and conjugates are female dominant and cluster samples towards the positive y-axis**
- **PCA shows that excretion CDCA and metabolites causes WT and KO groups to cluster separately**
- **Similarly, sulfation causes separation between female and male groups, with Cyp E-null experiencing increased sulfation of**
- **UDCA-S excretion helps differentiate C-null and E-null strains. UDCA-S excretion is increased in C-null mice, but decreased in E-null mice (data not shown).**

SUMMARY

Fig. – Dysregulated Bile Acid Metabolism following Cyp2c70-Knock Out



- **Cyp2c70-KO creates a hydrophobic bile acid pool consisting of mainly CDCA and conjugates**
- **TCDCA and TCA compete for excretion via BSEP**
- **TCDCA has a much higher affinity for BSEP**
- **Therefore (T)CA cannot be excreted, and accumulates in the liver**
- **TCA is the natural ligand for FXR in mice**
- **Hepatic FXR regulates bile acid composition via Cyp8b1 through SHP**
- **Intestinal FXR regulates overall bile synthesis via Cyp7a1 through Fgf15**
- **Only hepatic FXR is activated due to the lack of excreted TCA**
- **Therefore only CA synthesis, but not overall bile acid synthesis is downregulated**
- **Cyp2c70-KO alters bile acid metabolism to resemble human values in hydrophobicity and composition**
- **This model can simulate PFIC-2 conditions, previously not possible in mouse**
- **Sulfation becomes critical for Cyp2c70-null mice, as in human bile acid metabolism**

REFERENCES

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