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# Bile Salts and Nuclear Receptors in Biliary Epithelial Cell Pathophysiology

Nicolas Chignard

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**Bile Salts and Nuclear Receptors  
in Biliary Epithelial Cell Pathophysiology**

by Dr. Nicolas Chignard

Friday, the 11th of May 2012

**Post-Doctoral Thesis Committee :**

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## Acknowledgments

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I would like to express my deepest respect to Pr. Raoul Poupon. He has set a standard that few can meet but many should intend to follow. His incessant curiosity is nourished by a tremendous and ever growing knowledge, but most extraordinary is his ability to connect and translate information into scientific concepts. His acceptance to chair my post-doctoral committee is a great honor.

I deeply appreciate Pr. Chantal Housset's strong support throughout all these years. She shaped the way I perform my work today. Among many other examples, she showed me how to simply and scientifically present one's work, a skill that I use on a daily basis whether as a teacher or a researcher.

It was a delight to interact with Dr. Annick Paul who supervised my first steps in research while undertaking my Ph.D. I still miss our day-to-day interactions.

Thank you also to Dr. Laura Beretta who gave me an innovative and ambitious project while I was a post-doctoral fellow in her laboratory at the University of Michigan. This opportunity to complete a research program allowed me to pursue my career as an associate professor at Université Pierre et Marie Curie.

The work presented in this post-doctoral thesis was primarily performed by students that I had the pleasure to supervise. I'm grateful to all of them. I especially would like to acknowledge the following: Marie-Jeanne Biyeyeme Bi Mve, my first student. I think she taught me as much as I taught her. Emilie d'Aldebert, who then came along and helped me prepare my first senior author paper. Delphine Firrincieli, the first Ph.D. student I supervised. She is the main reason why I submitted my post-doctoral thesis. I have the utmost respect for her will and capability for success. I am fortunate to be able to work with her daily.

Finally, my heartfelt gratitude goes to my family for their support and affection. I am indebted to my father for his constant support and precious advice. Last but not least, I want to express all my affection and admiration to Laetitia. I have become a better person being beside her. My most caring thoughts go to Thomas, Arthur and Bérénice, who have the tremendous power to make life bigger and better.

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# 1. Curriculum Vitae

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Date of birth: 20 August 1975

Place of birth: Schiltigheim, France

Nationality: French

## 1.1. Education

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2010	Animal Scientific Procedures Personal Licence, UPMC
2008	«From the doctoral project management to a PhD career development» Doctoral Studies Institute, UPMC
1999-2003	Ph.D. in Physiology and Pathophysiology from UPMC “Regulation of human gallbladder epithelial secretory functions by bile acids” Inserm U402, Hospital Saint-Antoine, Paris
1998-1999	MSc in Cellular and Molecular Physiology, UPMC

## 1.2. Professional Experience

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Since 2006	Associate Professor in Saint Antoine Research Center; UMR_S938 of UPMC
2004-2006	Assistant Professor in UMR_S680 “Pathology of the adipocyte and hepatic cells” of UPMC
2003-2004	Post-Doctoral researcher in the Dpt. of Microbiology and Immunology. University of Michigan, Ann Arbor, Michigan, USA. “Transcriptomic and proteomic analysis of hepatocellular carcinoma” (Dr. Laura Beretta)
Jan-Feb 2002	Trainee in the Liver and Hepatobiliary Unit, Liver Research Laboratories, Clinical Research Block, Queen Elizabeth Hospital, Birmingham, UK (Dr. Ruth Joplin)

## 1.3. Professional Activities

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### i) Teaching activities

- Head of the teaching units of “Hepatic Pathophysiology” and “Science and Society” from the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC
- Member of the committee evaluating the laboratory training of first year students from the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC

- Member of the committee attributing the final diploma to students from the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC
- Tutor of students of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” and of the “Physiology and Pathophysiology” Doctoral School of UPMC

## ii) Supervision Activities

2011	Timothé Denaës, 1st year training of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC  Project: Involvement of VDR in hepatic inflammation
2011	Thomas Pudlarz, 3rd year training of the “MD/PhD” program of the Medical School of UPMC  Project: Involvement of VDR in macrophages: Relevance to NASH
2010	Marko Jevtic, 1st year training of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC  Project: Involvement of VDR in the control of hepatic E-Cadherin expression
2009-2010	Silvia Zuniga, Ph.D. thesis from the Doctoral School of « Physiology and Pathophysiology » of UPMC and the Pontifica Universidad de Chili  Project: Involvement of the vitamin D nuclear receptor in fatty liver disease
Since 2008	Delphine Firrincieli, Ph.D. thesis from the Doctoral School of « Physiology and Pathophysiology » of UPMC  Project: Involvement of the vitamin D nuclear receptor in biliary pathophysiology
2008	Delphine Firrincieli, 2nd year training of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC  Project: Epithelial to mesenchymal transition of biliary epithelial cells: Relevance to primary biliary cirrhosis
2007	Emilie d’Aldebert, 2nd year training of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC  Project: Bile acids control cathelicidin expression in human biliary epithelial cells: Implication of the vitamin D nuclear receptor
2005-2006	Marie-Jeanne Biyeyeme Bi Mve, 1st and 2nd year training of the “Physiology and Pathophysiology” program of the Master of “Sciences and Technologies”, Specialization “Integrative Biology and Physiology” of UPMC  Project: Regulation of the vitamin D nuclear receptor by bile acids in human biliary epithelial cells

**iii) Administrative Activities**

- Member of the recruiting committee of the Department of Physiology of UPMC
- Member of the recruiting committee of the Department of Physiology of UPEC

**iv) Scientific Evaluation Activities**

- Expert for peer-reviewed journals: Journal of Hepatology, Hepatology, Gastroenterology, Cellular Physiology and Biochemistry, Clinical Gastroenterology and Hepatology
- Scientific expert for the “Conseil Régional Nord-Pas de Calais, Direction de l’Enseignement Supérieur, Recherche et Nouvelles Technologies”
- Scientific expert for the “Dutch Digestive Foundation”

**v) Honors and Distinctions**

- Fellow of the French Department of Higher Education and Research (Oct 1999-Oct 2002)
- First prize awardee in 2003 of the French National Association of Cell Biology Teachers
- Grantee of the French Association for the Study of the Liver (AFEF) - 2009
- Awardee of UPMC for research investment - 2010

**vi) Professional Society**

- French Association for the Study of the Liver (AFEF)

**1.4. Scientific Communications**

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**vii) Publications**

Note: Supervised students appear underlined

1. 2001 : **Chignard N**, Mergey M, Veissière D, Parc R, Poupon R, Capeau J, Paul A, Housset C. Bile acid transport and regulating functions in the human biliary epithelium. *Hepatology* 33:496-503. (IF = 9.5)
2. 2002 : Corpechot C, Barbu V, Wendum D, **Chignard N**, Housset C, Poupon R, Rosmorduc O. Hepatocyte growth factor and c-Met inhibition by hepatic cell hypoxia : a potential mechanism for liver regeneration failure in experimental cirrhosis. *Am. J. Pathol.* 160:613-620. (IF = 6.9)
3. 2003 : **Chignard N**, Mergey M, Veissière D, Poupon R, Capeau J, Paul A, Housset C. Bile



salts potentiate adenylate cyclase activity and  $\beta$ -adrenergic induced secretion in the human gallbladder epithelium. *Am. J. Physiol.- Gastrointest. Liver Physiol.* 284:G205-G212. (IF = 3.4)

4. 2004 : **Chignard N** and Beretta L. Proteomics for hepatocellular carcinoma marker discovery. *Gastroenterology* 127:S120-S125. (IF = 13)

5. 2004 : Poupon R, **Chignard N**, Rosmorduc O, Barbu V, Housset C. La fonction biliaire et sa régulation. *Med. Sci. (Paris)* 20:1096-1099. (IF = 0.1)

6. 2005 : **Chignard N**, Mergey M, Barbu V, Finzi L, Tiret E, Paul A, Housset C. VPAC1 expression is regulated by FXR agonists in the human gallbladder epithelium. *Hepatology* 42:549-557. (IF = 9.8)

7. 2006: Demeilliers C, Jacquemin E, Barbu V, Mergey M, Paye F, Fouassier L, **Chignard N**, Housset C, Lomri N. Altered hepatobiliary gene expressions linked to FIC1 defect: Evidence for CFTR down-regulation. *Hepatology* 43:1125-1134. (IF = 10.4)

8. 2006 : **Chignard N**, Shang S, Wang H, Marrero J, Bréchet C, Hanash S, Beretta L. Cleavage of endoplasmic reticulum proteins in hepatocellular carcinoma: detection of generated fragments in patient sera. *Gastroenterology* 130:2010-2022. (IF = 12.4)

9. Fouassier L, Rosenberg P, Mergey M, Saubaméa B, Clapéron A, Kinnman N, **Chignard N**, Jacobsson-Ekman G, Strandvik B, Rey C, Barbu V, Hultcrantz R, Housset C. Ezrin-radixin-moesin-binding phosphoprotein (EBP50), an estrogen-inducible scaffold protein, contributes to biliary epithelial cell proliferation. *Am J Pathol.* 2009 Mar;174(3):869-80. (IF = 5.5)

10. D'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M, Wendum D, Firincieli D, Coilly A, Fouassier L, Corpechot C, Poupon R, Housset C, **Chignard N**. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the human biliary epithelium. *Gastroenterology.* 2009 Apr;136(4):1435-43. (IF = 12.4)

11. **Chignard N**, Poupon R. Targeting farnesoid X receptor in hepatic and biliary inflammatory diseases. *Gastroenterology.* 2009 Aug;137(2):734-5. (IF = 12.4)

12. Firincieli D, Boissan M, **Chignard N**. Epithelial-mesenchymal transition in the liver. *Gastroenterol Clin Biol.* 2010 Oct;34(10):523-8. (IF = 0.9)

13. Boissan M, De Wever O, Lizarraga F, Wendum D, Poincloux R, **Chignard N**, Desbois-Mouthon C, Dufour S, Nawrocki-Raby B, Birembaut P, Bracke M, Chavrier P, Gespach C, Lacombe ML. Implication of metastasis suppressor NM23-H1 in maintaining adherens junctions and limiting the invasive potential of human cancer cells. *Cancer Res.* 2010 Oct 1;70(19):7710-22. (IF = 7.5)

14. Zuniga S, Firincieli D, Housset C, **Chignard N**. Vitamin D and the vitamin D receptor in liver pathophysiology. *Clin Res Hepatol Gastroenterol.* 2011 Apr; 35(4):295-302. (IF = 0.9)

15. Firincieli D, Zuniga S, Poupon R, Housset C, **Chignard N**. Role of nuclear receptors in the biliary epithelium. *Dig Dis.* 2011; 29(1):52-7. (IF = 1.5)

16. Debray D, Rainteau D, Barbu V, Rouahi M, El Mourabit H, Lerondel S, Rey C, Humbert L, Wendum D, Cottart CH, Dawson P, **Chignard N**, Housset C. Defects in Gallbladder Emptying and Bile Acid Homeostasis in Mice with Cystic Fibrosis Transmembrane Conductance Regulator Deficiencies. *Gastroenterology.* 2012 Feb 24.

**viii) Abstracts relating to supervised students work**

1. 2008 : d'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M, Wendum D, Coilly A, Fouassier L, Corpechot C, Poupon R, Housset C, **Chignard N**. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the biliary epithelium. Falk Symposium 165 "Bile acids biology and therapeutic actions", Amsterdam, June 2008.
2. 2008 : d'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M, Wendum D, Coilly A, Fouassier L, Corpechot C, Poupon R, Housset C, **Chignard N**. Les acides biliaries contrôlent l'expression de la cathélicidine *via* des récepteurs nucléaires dans les cellules épithéliales biliaries humaines. Journées de l'Association Française pour l'Etude du Foie, Paris, Sept 2008.
3. 2009 : Firincieli D, Boissan M, Housset C, **Chignard N**. La transition épithélio-mésenchymateuse des cellules épithéliales biliaries : Implication de l'immunité innée. 65ème Journées de l'AFEF. Centre de Congrès de Disney's Hotel New York, Paris, Sept 2009.
4. 2009 : Firincieli D, Rey C, Wendum D, Lasnier E, Cadoret A, Boissan M, Housset C, **Chignard N**. Role of the vitamin D nuclear receptor on liver injury in bile-duct ligated mice. The Henry M. and Lillian Stratton Basic Research Single Topic Conference Pattern Recognition Receptors and Innate Immune Signaling Pathways in Liver Diseases (AASLD). Atlanta, USA. Sept 2009.
5. 2009 : Firincieli D, Rey C, Wendum D, Lasnier E, Cadoret A, Boissan M, Housset C, **Chignard N**. Role of the vitamin D nuclear receptor on liver injury in bile-duct ligated mice. The 60th annual meeting of the american association for the study of liver diseases (AASLD). Hynes Convention Center, Boston, USA. Oct 2009.
6. 2010 : Firincieli D, Zúñiga S, Rey C, Wendum D, Lasnier E, Rainteau D, Cadoret A, Boissan M, Housset C, **Chignard N**. L'invalidation du récepteur nucléaire de la vitamine D favorise les lésions hépatiques d'origine biliaire. 66ème Journées de l'AFEF. Palais du Pharo, Marseille, Sept 2009.
7. 2010 : Zúñiga S, Firincieli D, Lasnier E, Miquel JF, Housset C, **Chignard N**. L'invalidation du récepteur nucléaire de la vitamine D favorise la stéatose hépatique. 66ème Journées de l'AFEF. Palais du Pharo, Marseille, Sept 2009.
8. 2010 : Firincieli D, Zúñiga S, Rey C, Wendum D, Lasnier E, Rainteau D, Cadoret A, Boissan M, Housset C, **Chignard N**. Invalidation of the vitamin D nuclear receptor promotes biliary-type liver injury. The 61th annual meeting of the american association for the study of liver diseases (AASLD). Hynes Convention Center, Boston, USA. Oct 2010.
9. 2010 : Zúñiga S, Firincieli D, Lasnier E, Miquel JF, Housset C, **Chignard N**. Invalidation of the Vitamin D Nuclear Receptor promotes liver steatosis. The 61th annual meeting of the american association for the study of liver diseases (AASLD). Hynes Convention Center, Boston, USA. Oct 2010.
10. 2010 : Firincieli D, Zúñiga S, Rey C, Wendum D, Lasnier E, Rainteau D, Cadoret A, Boissan M, Housset C, **Chignard N**. Invalidation of the vitamin D nuclear receptor promotes biliary-type liver injury. Falk Symposium 175 "Bile acids as metabolic integrators and therapeutics", Freiburg, Oct 2010.

11. 2010 : Zúñiga S, Firincieli D, Lasnier E, Miquel JF, Housset C, **Chignard N**. Invalidation of the Vitamin D Nuclear Receptor promotes liver steatosis. Falk Symposium 175 “Bile acids as metabolic integrators and therapeutics”, Freiburg, Oct 2010.
12. 2011 : Firincieli D, Zuñiga S, Rey C, Wendum D, Lasnier E, Rainteau D, Cadoret A, Boissan M, Housset C, **Chignard N**. Invalidation of the vitamin D nuclear receptor promotes biliary-type liver injury. The International Liver Congress (EASL), March 2011, International Congress Centrum (ICC), Berlin, Germany.
13. 2011 : Zúñiga S, Firincieli D, Lasnier E, Miquel JF, Housset C, **Chignard N**. Invalidation of the Vitamin D Nuclear Receptor promotes liver steatosis. The International Liver Congress (EASL), March 2011, International Congress Centrum (ICC), Berlin, Germany.

#### ix) Book Chapters

1. 2001 : **Chignard N**, Mergey M, Veissière D, Parc R, Poupon R, Capeau J, Paul A, Housset C. Regulation of biliary epithelial cell secretion by bile acids. In : van Berge-Henegouwen G P, Keppler D, Leuschner U, Paumgartner G, Stiehl A, editors. Biology of bile acids in health and disease. Dordrecht: Kluwer Academic Publishers. p 152-157.
2. 2004 : **Chignard N**, Fouassier L, Paul A and Housset C. Regulation of secretion in human gallbladder epithelial cells. In : Alpini G, Alvaro D, Marzoni M, LeSage N, LaRusso N, editors. The pathophysiology of biliary epithelia. Landes Bioscience. p 149-158.
3. 2006 : **Chignard N**, Chazouillères O, Housset C. La sécrétion biliaire. EMC (Elsevier SAS, Paris), Hépatologie, 7-006-B-10
4. 2009 : **Chignard N**, Housset C. Physiologie des voies biliaires et applications à la clinique. In: Chazouillères O, Regimbeau J, eds. Maladies des voies biliaires. Rueil-Malmaison: Doin editions.
5. 2009 : d'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M, Wendum D, Coilly A, Fouassier L, Corpechot C, Poupon R, Housset C, **Chignard N**. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the biliary epithelium. In : van Berge-Henegouwen G P, Keppler D, Leuschner U, Paumgartner G, Stiehl A, editors. Biology of bile acids in health and disease. Dordrecht: Kluwer Academic Publishers. p 152-157.

#### x) Invited speaker

1. 2006 : Pathophysiology of biliary secretion : An update. Department of Hepatology, Saint Antoine Hospital, Paris, France.
2. 2009 : Involvement of the vitamin D nuclear receptor in hepatic pathophysiology. Unit of Innate defenses and Inflammation, Pasteur Institute, Paris, France.
3. 2010 : Bile salts and nuclear receptors in hepato-gastroenterology. Department of Hepato-Gastroenterology, Saint Antoine Hospital, Paris, France.

4. 2010 : Poster highlights. 66ème Journées de l'AFEF. Palais du Pharo, Marseille, France.
5. 2010 : Role of nuclear receptors in biliary epithelium. XXI International Bile Acid Meeting on "Bile Acids as Metabolic Integrators and Therapeutics", Freiburg, Germany.
6. 2011 : Involvement of vitamin D in liver pathophysiology. Saint Antoine Research Center, Paris, France.

### **1.5. Patents**

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Vitamin D compounds for the treatment of biliary diseases – European Patent EP08305061.7

## 2. Research activities

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The liver is a multi-cellular organ in which non-parenchymal cells represent forty percent of the total cell number. Even though less abundant than hepatocytes, alterations of non-parenchymal liver cell function are central to the development of liver diseases. The latter observation highlights the involvement of these cells in hepatic physiology. Among non-parenchymal liver cells, the biliary epithelial cells (BECs) display two major biological functions: protection and secretion.

### 2.1. Introduction

---

Bile secretion facilitates intestinal lipid absorption, controls cholesterol homeostasis and enables the excretion of endogenous and exogenous molecules. Bile originates from hepatocytes, where it is produced and secreted in the bile canaliculi. Hepatic bile secretion is controlled by the activity of transporters expressed at the apical pole of hepatocytes. The crucial role of these transporters is highlighted by the severe phenotypes that arise when the genes encoding these transporters are mutated. The expression of these transporters is also altered in acquired cholestatic diseases. Medical treatment of these pathological entities may both slow disease progression and normalize clinical, biochemical and histological parameters. Currently, the only drug available for the treatment of these diseases is ursodeoxycholic acid or UDCA (1, 2). The therapeutic efficacy of UDCA has been well documented in the prototypical biliary disease, primary biliary cirrhosis (PBC) (3-5). However treatment is not efficient in normalizing laboratory parameters and improving liver histology in subgroups of patients (6). Furthermore, in other biliary diseases, the therapeutic benefit of UDCA remains controversial. In the context of ineffective UDCA treatment, the only remaining therapeutic option relies on liver transplantation. Thus medical therapies of biliary-type liver diseases able to potentiate or supersede UDCA therapy are urgently needed. In order to achieve this goal the molecular mechanisms supporting UDCA therapeutic benefit and/or the regulation of biliary secretion should be better defined.

BECs, the primary targets of biliary-type liver diseases, form a mono-cellular epithelial sheet that seals bile ducts. Bile ducts, beside pure conveying functions, actively modify the volume and composition of canicular bile, mainly through vectorial transport of water, electrolytes and macromolecules across BECs. Both hydroelectrolytic transport and mucin secretion occur in intra- and extrahepatic portions of the biliary tract (7-10). Biliary mucins ensure cytoprotection of the epithelium, while transepithelial fluid secretion prevents the stagnation and subsequent toxic effects of bile along the entire biliary tract. Thus, the secretory activity of BECs allows both the efficient delivery of bile to the intestine and the protection of the biliary epithelium.

The driving force for fluid secretion across the biliary epithelium is the extrusion of chloride ions through apical chloride channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) (11, 12). In humans, this secretory activity is mainly stimulated by the “Vasoactive Intestinal Peptide” (VIP), which acts through basolateral membrane receptors coupled to the cAMP signaling pathway (9, 13).

## 2.2. Bile salt transport and function in human biliary epithelial cells

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Biliary epithelial cell secretion is mainly controlled by molecules circulating in blood or secreted by intrinsic neurons, but regulating signals also arise from the biliary pole of the cells. The direct involvement of luminal factors, such as bile salts, in the regulation of bile ductal secretion is highlighted by the expression of the apical sodium-dependent bile acid transporter (ASBT) in rat intrahepatic bile duct epithelial cells (14, 15). Furthermore, bile salt feeding in rat causes an increase in the amount of secretin receptors in BECs, and may thereby amplify ductal secretion in response to the hormone (16). It has also been established that bile salts stimulate biliary mucin secretion by a non-detergent mechanism in human (10) and dog (17) gallbladder-derived BECs. Bile salts exert their effects by a calcium-dependent pathway in humans (10), while in dog it is not mediated by classical signal transduction pathways (17, 18).

Because the effect of bile salts on BECs secretion activities is elicited by non-detergent mechanisms, the hypothesis arose that UDCA, which is a hydrophilic bile salt, could directly affect BECs function. This assumption is further supported by the fact that UDCA has a documented efficacy in the treatment of diseases with altered BEC function, such as as PBC (19), cystic fibrosis liver disease (20) or cholelithiasis (21). The therapeutic efficacy of UDCA has long been attributed to an effect on hepatocytes, including the stimulation of canalicular bile acid secretion in cholestatic patients (22), anti-inflammatory properties (23) and anti-apoptotic effects (24, 25). Yet, the observation that UDCA reduces the nucleation-promoting activity in bile (26), modulates BECs apoptosis (27) and stimulates BEC secretion at high concentration (28), suggests that it may also control BEC biology.

Thus, our study aimed at determining if human gallbladder-derived BEC exhibit bile acid transport activity that affect their secretory functions. Human gallbladder-derived BEC were used as easy access prototypical BECs. BECs lining intrahepatic bile ducts or the gallbladder display functional differences, however, they share many common features that distinguish them from other liver cells, thus they can be considered as a unique cell type. Accordingly, we compared the effects of tauroursodeoxycholate (TUDC) and of the endogenous bile salt, taurochenodeoxycholate (TCDC), on human gallbladder-derived BECs secretory responses.

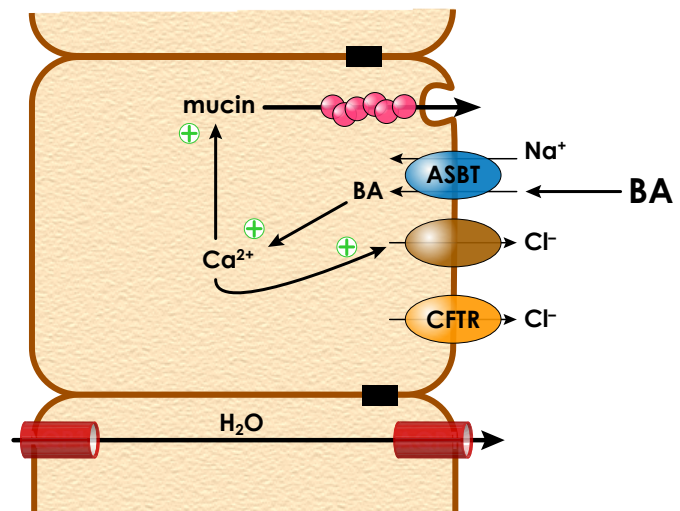
In our study we could show that ASBT is expressed and functional in primary cultures of human BEC. ASBT has been initially identified as the ileal sodium bile acid cotransporter, and cDNA was first isolated in hamster by means of an expression cloning strategy (29). Subsequently, the human, rat and mouse ASBT cDNAs have been cloned (30-32). Besides ileal expression in all these species, ASBT has been identified in hamster, rat, and human tubular epithelial cells (29, 31, 33) and in rat cholangiocytes (14, 15). We further showed that human gallbladder-derived BEC express ASBT both at the transcript and protein levels, and that the protein is located in the plasma membrane, as ascertained by a surface glycoprotein biotinylation assay. These observations highlight the fact that intrahepatic and extrahepatic (*i.e.* gallbladder) BECs share common features, such as bile salt transport.

We also evidenced that taurocholate is transported in a sodium-dependent manner in primary cultures of BECs. This sodium-dependent transport activity was characterized by an apparent Km of 66  $\mu\text{mol/L}$ , within the range of values previously reported in rat cholangiocytes (14, 15) and in transfected cells expressing ASBT (29, 32, 33). Human BECs also displayed a sodium-independent TC uptake activity that could have



been mediated by the organic anion transporting polypeptide OATP-A (34). Indeed, semiquantitative RT-PCR suggested that even though the level of OATP-A expression is low in BEC, it is similar to hepatocytes (35). Based on these observations and on previous findings in rat cholangiocytes (15, 36), we postulated that bile salt sodium-dependent uptake occurs across the apical membrane of human BEC whereas sodium-independent transport probably occurs across both the apical and basolateral domains. These observations suggest that bile salt transporters in BEC may provide a cholehepatic shunt pathway for conjugated bile salts. The cholehepatic shunt could subserve a defense mechanism by preventing prolonged exposure of the biliary tract lining epithelium to extramucellar bile salts. The latter assumption is supported by the observation that fluorescent bile salts are visualized within the liver following their injection in a mouse gallbladder isolated from the common bile duct by cystic duct ligation (personal data).

Our study also documents direct stimulation of chloride and mucin secretion by conjugated bile salts in human BEC. Although it was previously shown that UDCA at high concentration activates chloride conductance in the Mz-ChA-1 cell line (28), this report established that conjugated bile salts, below their critical micellar concentration (37), induce chloride secretion in BEC. Bile salt-induced chloride and mucin secretion was to a large extent sodium-dependent, consistent with the necessity for conjugated bile acids to be transported within BEC *via* ASBT to elicit a biologic response. The findings of our study suggest that once transported within BEC, bile salts will induce hydroelectrolytic and mucin secretion. This observation sheds light on the ability of bile salts to promote epithelial protection against their own toxicity.



*Effects of bile salts on BEC secretions (Chignard et al, Hepatology 2001)*

TUDC has been shown to be efficiently transported by ASBT (33), an observation supported by the results of TC uptake inhibition in BEC of our study. Our results further indicate that TUDC uptake is accompanied by the stimulation of secretion in human BEC. However, as compared with TCDC, TUDC induced significantly lower mucin secretion, a differential effect that may be related to the fact that TUDC and TCDC trigger different intracellular signaling pathways within BEC. Indeed, by using inhibitors of the major intracellular transduction pathways, we could show that TCDC stimulates mucin secretion mainly *via* the activation of  $Ca^{2+}$ /calmodulin-dependent protein kinase II, whereas the effect of TUDC is mediated by a PKC-dependent pathway. In line with these findings, it was previously shown that in hepatocytes, TUDC is more potent in inducing PKC translocation than more hydrophobic bile acids such as TCDC (38). Chloride

secretion also tended to be lower in response to TUDC. However, although mucin secretion in response to TUDC was on average fourfold lower than in response to TCDC, chloride secretion was on average 1.8-fold lower, and the latter difference was not statistically significant. Therefore and in comparison with more hydrophobic endogenous bile acids, TUDC induced a shift in the balance between chloride and mucin secretion towards lower mucin over chloride secretion.

Under UDCA treatment, the general toxicity bared by the bile acid pool, and delivered to hepatocytes is decreased. Our findings imply that enrichment of the bile acid pool in TUDC may moreover cause a decrease in mucin secretion whereas hydroelectrolytic secretion is maintained. This could be of benefit, not only in cholelithiasis in which mucins accelerate cholesterol nucleation (39), but also in cholestatic liver diseases to avoid excessive toxic accumulation in bile ducts by promoting a flushing process.

### **2.3. Bile salts control hydroelectrolytic secretion through cAMP**

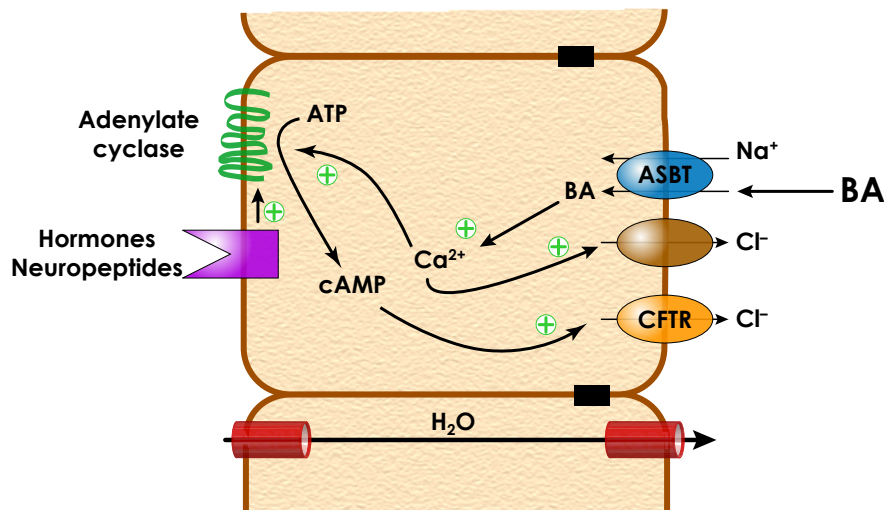
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In BECS, the secretion process is mainly elicited by the vasoactive intestinal polypeptide (VIP) (40) or by secretin (9) that transduce signal through the intracellular second messenger cAMP (12). The production of cAMP results from the activation of G protein-coupled receptors specific for  $\beta$ -adrenergic agonists, VIP or secretin (41, 42). Following activation of these receptors by their ligands, the G protein  $\alpha$ s subunit is released and stimulates an adenylate cyclase enzyme, which will convert ATP into cAMP. Adenylate cyclase enzymes form a superfamily of nine isoforms termed AC1 to AC9. While stimulation through the  $G_s$   $\alpha$  subunit is the major mechanism by which all adenylate cyclases are activated, individual isoforms have different regulatory properties that allow complex signal integration. AC1, 3, 8 may be stimulated or inhibited by intracellular free calcium and by calmodulin, while AC2, 5, 7 are stimulated by protein kinase C (PKC). AC4 is stimulated by the  $\beta\gamma$  subunit of G proteins, while low calcium concentrations inhibit AC6. Lastly, AC9 is insensitive to either calcium, PKC or  $\beta\gamma$  subunit (43).

In the biliary epithelium, the extrusion of chloride ions through the apical cAMP-dependent chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR) is a major driving force for fluid secretion (12, 44). Because we and others had shown that BECs secretion is elicited by bile salts (16, 28, 45), we explored the action of TCDC and of TUDC on the cAMP-dependent secretory pathways in human gallbladder-derived BECs.

In this study, we were able to show that bile salts potentiate cAMP-regulated secretion in BECs. Indeed, both TCDC and TUDC increased forskolin-induced cAMP accumulation, without affecting basal levels, consistent with a regulation of adenylate cyclase activity. The latter observation has been challenged by the observation that cAMP concentration is increased in gallbladder epithelial cells in response to taurocholate (TC) and taurolithocholate (TLC), while these cells express the bile salt membrane receptor, TGR5 (46). This discrepancy could arise either from the difference in bile salts used or from the used method for cAMP detection. In depth analysis of adenylate cyclase expression allowed us to show that human gallbladder-derived BECs express six isoforms of adenylate cyclase, namely AC3, AC4, AC5, AC6, AC7, and AC9, while AC1, AC2 and AC8 were not detected by RT-PCR. Thus, we showed that among the adenylate cyclase isoforms expressed in human BECs some are known to be positively regulated by PKC





Effects of bile salts on BEC cAMP-regulated secretions (Chignard et al, Am J Physiol 2003)

(AC5 and AC7) (47, 48) or by the  $\beta\gamma$  subunit of *pertussis* toxin-sensitive  $G_i$  proteins (AC4 and AC7) (49, 50). However, the potentiating effect of TCDC on forskolin-induced cAMP production was insensitive to *pertussis* toxin (51), making unlikely the possibility that bile salts modulate cAMP synthesis through the release of  $\beta\gamma$  subunit from  $G_i$  protein in BECs. By contrast and in support to a PKC-mediated regulation, the potentiating effects of bile salts on cAMP production were mimicked by PMA and were abrogated by GF109-203X, an inhibitor of PKC $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  isoforms (52). Furthermore, the potentiation of cAMP production by bile salts was suppressed by PKC down-regulation (10, 53). In line with these results, we showed by immunoanalysis that PKC $\alpha$  and PKC $\delta$  were translocated to the membranes of BECs in response to bile salts. These observations therefore suggest that bile salts potentiate cAMP production by stimulating adenylate cyclase activity through PKC  $\alpha$  and  $\delta$ .

We also showed in this report that cAMP production triggered by  $\beta$ -adrenergic stimulation (*i.e.* isoproterenol) was also potentiated by bile salts. The effect of bile salts (TCDC or TUDC) on cAMP production translated into a potentiation of chloride secretion induced by isoproterenol. Together with the previous demonstration that TCDC induces a rapid rise of  $[Ca^{2+}]_i$  in gallbladder-derived BECs (10), inhibition of calcium-dependent chloride channel inhibition by DIDS and chelation of intracellular calcium by BAPTA/AM indicated that the secretory response to bile salts alone was mediated by calcium-dependent chloride channels (54). By contrast, in the presence of concomitant  $\beta$ -adrenergic stimulation, bile salts increased chloride secretion mainly *via* a cAMP-dependent pathway. Indeed, chloride secretion in this setting was inhibited by DPC, a broad chloride channel inhibitor, but not by DIDS, consistent with an effect on the cAMP-dependent chloride channel, CFTR (44, 55-58). Because cAMP-dependent chloride secretion promotes bicarbonate and fluid secretion in the gallbladder-derived biliary epithelium (12, 44), the results of our study suggest that bile salts potentiate hormonal and neurogenic stimulation of fluid secretion that will both facilitate the progression of bile in bile ducts and assist gallbladder emptying after feeding.

Furthermore and given that TUDC induces lower mucin secretion than TCDC (45), the findings of this study, that show similar effects of TCDC and TUDC on cAMP-dependent anion secretion, reinforce the concept that the protection of BECs triggered by UDCA may arise from its ability to flush toxics out of the biliary tract.

#### **2.4. Bile salts control VPAC1 expression through nuclear receptors**

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The regulation of bile secretion occurs at different levels of the biliary tree. Bile is formed primarily in hepatocyte canaculi by an osmotic process resulting from active bile salt secretion (59). In bile ducts and in the gallbladder, bile is then modified by the absorption or secretion of water and ions (9, 60). Thus, bile delivered to the intestine results from vectorial transport occurring in the different epithelial cell types lining the biliary tree, *i.e.* hepatocytes, intrahepatic bile duct and extrahepatic epithelial cells. Among regulatory peptides, VIP induces bile secretion by stimulating transport activities both in hepatocytes and in BECs. In hepatocytes, VIP induces an increase in bile salt-dependent bile secretion (61). In BECs, VIP stimulates a bicarbonate-rich secretion with higher potency than all other secretagogues (61-63). In most systems, VIP effects are mediated by cAMP production following the activation of high or low affinity receptors, namely the vasoactive intestinal peptide receptor-1 (VPAC1) and the vasoactive intestinal peptide receptor-2 (VPAC2) (64, 65). Among these two types of VIP receptors, only VPAC1 is expressed in the liver (64).

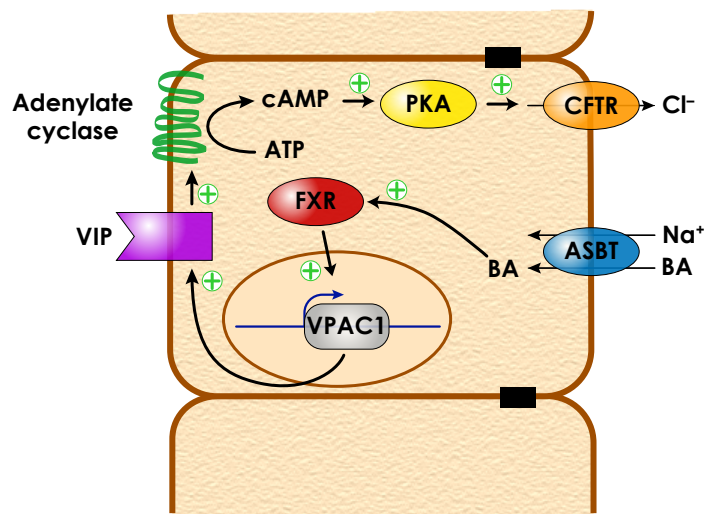
In cancer cell lines, VPAC1 gene transcription is regulated by members of the nuclear receptor superfamily (66-68). The nuclear receptor superfamily comprises the farnesoid X receptor (FXR) for which bile salts serve as natural ligands (69). Ligand-bound FXR regulates gene transcription upon heterodimerization with the retinoid X receptor alpha (RXR $\alpha$ ), a permissive nuclear receptor activated by 9-cis retinoic acid (9-cis Ra) (70). The FXR/RXR $\alpha$  heterodimer controls different aspects of bile salt synthesis and transport in hepatocytes and in enterocytes (71-73), in part through transcriptional activation of the short heterodimer partner (SHP) (72, 74, 75). In animal models, the synthetic FXR agonist, GW4064 (76), has been reported to protect against cholestatic liver injury through the regulation of genes ensuring bile formation in hepatocytes (77). Although no FXR expression had been reported in BECs at the time of our study, bile salts had been shown to induce ductal secretion in rats through transcriptional control (16).

Thus, we first undertook a study to determine the level of VPAC1 expression in the different cell types lining the human biliary tree, *i.e.* hepatocytes, intrahepatic bile duct and gallbladder epithelial cells. Our results indicate that VPAC1 is expressed in all major cell types participating in bile formation. VPAC1 displays a gradient of expression along the human biliary tree, the gallbladder showing the highest level of expression. In primary cultures of gallbladder-derived BECs, VIP elicited both cAMP production and chloride secretion. In line with previous studies (61-63, 78, 79), these observations indicate that VIP through VPAC1 activation is a major regulator of cAMP-dependent hydroelectrolytic secretion in the biliary epithelium.

We then ascertained whether bile salts control VPAC1 expression in BECs. Analysis of the human VPAC1 gene revealed the presence of potential FXR response elements in the 5' end and promoter region of the gene. Furthermore, we found that gallbladder-derived BECs, as well as bile duct-derived BECs, express

both FXR and RXR $\alpha$ . Interestingly, pharmacological activation of FXR by GW4064 induced a significant increase in VPAC1 transcripts in BECs. In physiological settings, FXR activation mainly arises from bile salts present in bile. Bile salt concentrations in human bile ducts (80) and in the gallbladder (81) are higher than the half-maximal effective concentration required for bile salt activation of FXR, *i.e.* 50  $\mu\text{mol/L}$  (71). Thus, our results indicate that bile salts have the potential to induce VPAC1 transcriptional regulations through FXR along the biliary tree.

Human bile is mostly composed of chenodeoxycholate, cholate and deoxycholate (38%, 36% and 16% of the total bile salt pool, respectively), while other bile salts, such as ursodeoxycholate, lithocholate and sulfolithocholate, are present in trace amount (82). Even though all major bile salts have the ability to activate FXR *in vitro* (69, 71), CDC has been identified as the principal FXR activator present in bile by sequential purification (83). In human BECs, the ability of CDC to activate endogenous FXR was demonstrated by gel-shift experiments and by an increase in SHP protein expression. We could also show that in these cells CDC regulates VPAC1 expression both at the transcript and protein level. Consistent with previous results showing that GW4064 was more potent than CDC in inducing gene expression in hepatocytes (84), we show that CDC induces VPAC1 gene expression to a lesser extent than GW4064. Taken together our results indicate that bile salts effectively induce VPAC1 expression in BECs through the activation of FXR.



*Transcriptional control of BEC secretion by bile salts (Chignard et al, Hepatology, 2005)*

In bile duct-ligated rats, GW4064 was shown to protect against liver injury through FXR activation (77). Moreover, administration of GW4064 to mice fed a lithogenic diet prevents the development of cholesterol gallstone disease (85). These protective effects have been attributed to the transcriptional regulation of genes involved in the metabolic and transport functions of hepatocytes. Our findings suggest that the therapeutic effects of FXR activation may also result from regulations occurring in BECs. We postulate on the basis of the present data that in animal models of cholestasis, GW4064 treatment may modulate ductal secretion through the control of gene expression. As demonstrated in this study, GW4064 may increase VPAC1 expression through FXR activation in BECs and thus favor choleresis by enhancing ductal secretion. Furthermore, we anticipate that in cholesterol gallstone disease FXR-induced VPAC1 expression would enhance the stimulation of fluid secretion in gallbladder. The resulting increase in bile dilution is expected to reduce cholesterol supersaturation and crystallization.

In conclusion, we have shown that VPAC1 is a major receptor regulating secretory functions in BECs and that VPAC1 expression is increased by FXR activation in these cells. These results suggest that bile

salts through FXR-regulated VPAC1 expression could favor VIP-induced bile delivery to the intestine after feeding. Moreover and as already highlighted, our results indicate that bile salts through post-translational or transcriptional control may exert protective activities towards the biliary epithelium by avoiding the stagnation of deleterious compounds.

## 2.5. Proteomic analysis of hepatocellular carcinoma

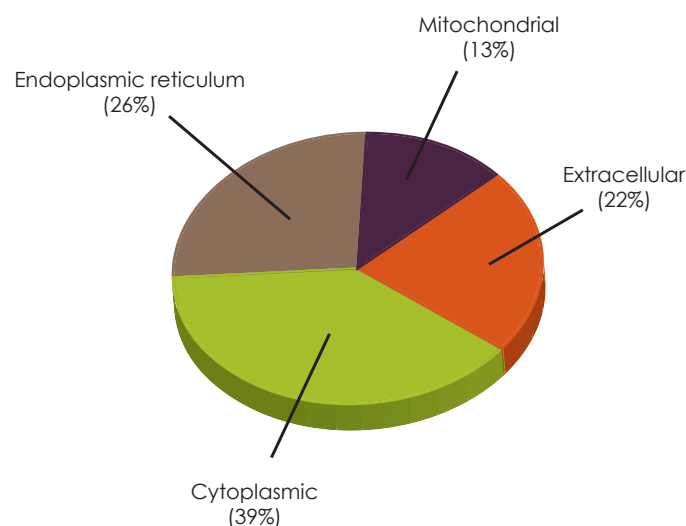
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Patients with cirrhosis linked to biliary-type liver diseases have a high risk of developing hepatocellular carcinoma (HCC) (86, 87). HCC, the most common form of primary liver cancer, usually develops on cirrhotic liver (88). The worldwide etiology of cirrhosis is mainly associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) chronic infection (89).

The global incidence of HCC has been rising in the last decades thus leading to an increase in HCC-related mortality (90, 91). The similarity between incidence and mortality rates of HCC highlights the rapid death after diagnosis, with a 5-year survival rate of less than 5% (91). The poor survival of patients with HCC is largely related to the lack of reliable tools for early diagnosis. At-risk patients are screened for HCC with blood (*i.e.*  $\alpha$ -fetoprotein) or ultrasonographic tests (92), however the usefulness of these tests has been overshadowed by their inability to diagnose efficiently early-stage tumors (93, 94). The lack of efficiency of  $\alpha$ -fetoprotein (AFP) for HCC surveillance or diagnosis has led to assessment of other serologic markers such as des- $\gamma$ -carboxyprothrombin (95, 96), glycipan-3 (97) and others (98). Because these potential markers still lack efficiency, the development of new molecular targets for HCC diagnostics and therapeutics is eagerly awaited.

Proteomics bear the promises for the discovery of biomarkers for early HCC detection and diagnosis (99). Proteomics-based profiling uniquely allows delineation of global changes in expression patterns resulting from transcriptional and post-transcriptional control, post-translational modifications, and shifts in proteins between cellular compartments. Therefore, we performed a study applying proteomic tools to the comparative analysis of protein profiles between HCC and adjacent non-tumor liver tissues to identify potential molecular markers.

Our study performed using 2-D PAGE followed by mass spectrometry allowed us to identify a large number of proteins modified in HCC compared with adjacent non-tumor tissues (99). In agreement with previous studies performed with the same technique, we observed a decrease in carbamoyl-phosphate synthase 1, cytochrome b5, liver carboxylesterase, liver-type arginase, protein disulfide isomerase (PDI), superoxide dismutase [Cu-Zn], and enoyl-CoA hydratase expression in tumor tissues compared with non-tumor counterparts (100, 101). We also observed an increase in Hsp60, GRP78, triosephosphate isomerase, and ferritin light chain expression in HCC, as previously reported (100-103). Most proteins we identified as differentially regulated in HCC are cytoplasmic (39%), while other important groups included mitochondrial proteins (13%), extracellular proteins (22%), and components of the endoplasmic reticulum (ER) (26%). In the human proteome, the proportion of proteins assigned to the mitochondria and ER compartments is 4% and 3%, respectively (104). Our findings therefore suggest that mitochondria- and ER-associated cellular functions may be specifically dysregulated in HCC.



*Proportion of differentially identified proteins in HCC according to subcellular localization (Chignard et al, Gastroenterology, 2006)*

Protein separation by 2-D PAGE may discriminate between post-translationally modified or processed protein isoforms. Most studies reported before our work have not presented any information regarding specific protein isoforms, with the exception of the identification of aldehyde dehydrogenase protein alterations (105) and aldose reductase-like protein (106) in HCC. In our analysis, 27% of the proteins we identified as modified in HCC corresponded to proteolytic cleavages as demonstrated by a smaller apparent molecular weight and a clustering of the peptides identified by mass spectrometry within a specific region of the protein sequence. Interestingly, most of them corresponded to proteins expressed in the ER, including calreticulin, PDIA3, PDI, GRP78, and liver carboxylesterase.

We further demonstrated in this study that, following proteolytic cleavage, fragments of calreticulin, GRP78, PDIA3 and PDI are released in the extracellular compartment. These fragments of calreticulin and PDIA3 can be detected at significantly higher levels in serum of patients with HCC compared with serum from healthy individuals, from patients with chronic hepatitis or from patients with cirrhosis, suggesting that they could represent novel HCC biomarkers. Furthermore, these proteins are ER chaperones and folding enzymes that not only assist the proper folding and assembly of newly synthesized proteins, but that also retain immature proteins in the ER (107). As an example, calreticulin, together with calnexin and PDIA3, are responsible for glycoprotein quality control (108, 109). GRP78 binds to many secretion incompetent proteins to sequester them in the ER. Because the abundance of these proteins influences ER release, a decrease in their hepatic ER levels leads to an increase in the amount of hepatic serum protein secretion (110-113). Taken together, these observations suggest that the cleavage of ER chaperone proteins in HCC can directly represent biomarkers of the disease or that loss of ER chaperoning function has the potential to give rise to biomarkers that remain to be discovered.

In conclusion, we identified novel potential HCC biomarkers that are attractive candidates to perform full validation studies. Finally and in line with other studies (114, 115), our observations suggest that specific protein isoforms and protein cleavage products could represent specific markers for HCC.



## 2.6. Bile salts control innate immunity through nuclear receptors

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HCC has been shown to spontaneously arise in mice lacking FXR (116). The control of hepatic tumor development by FXR has been linked to its ability to antagonize hepatic inflammation through the inhibition of NF- $\kappa$ B activity (117). This observation may account for the fact that the liver while being constantly exposed to endotoxins and xenobiotics is virtually devoid of inflammation.

The biliary tract is also a sterile milieu, despite being exposed to bacteria and bacterial products derived from the intestine. This pathogen-free environment is maintained by multiple defense factors such as bile flow, mucous secretion, IgA and bile salts (118). In disease states such as PBC, sterility may however be disrupted as evidenced by the presence of endotoxins in BECs (119). Interestingly in these patients, administration of UDCA, causes a decrease in intracellular endotoxin accumulation and in circulating anti-endotoxin antibodies (120, 121). These observations suggest that bile salts may be central in the antibacterial defense mechanisms of the biliary tract.

Bile salts are indeed amphipatic molecules bearing direct bacteriolytic properties (122). Bile salts may also have the potential to reduce epithelial bacterial colonization by increasing mucus thickening and bile flow (45). As we have previously shown, bile salt-induced bile flow is driven by the stimulation of chloride secretion in BECs through calcium and cyclic AMP-dependent signalling pathways (123), but may also result from nuclear receptor activation (124). This property may be relevant to epithelial innate defenses because nuclear receptors prone to bile salt activation, such as the farnesoid X receptor (FXR) or the vitamin D nuclear receptor (VDR) (69, 71, 125), have been reported to control antibacterial activity in epithelial cells (126-128).

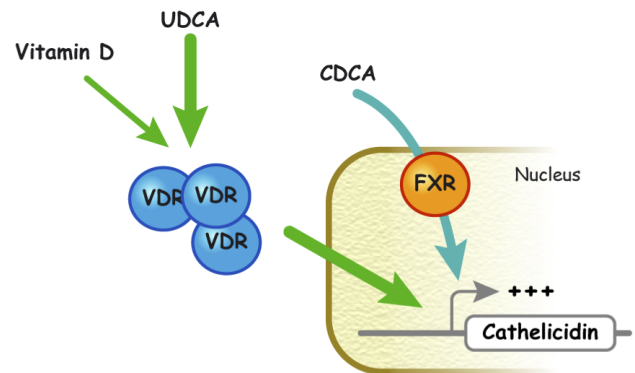
As an example, activation of VDR results in the induction of cathelicidin expression (126, 128), an antimicrobial peptide known to be protective *in vivo* against bacterial infection (129-131). Of particular interest, cathelicidin exerts microbicidal activity against *Escherichia Coli* (132), a bacterium suspected to either cause or exacerbate PBC (119). From these data, we hypothesized that bile salts may control innate immunity in BECs through VDR-induced cathelicidin expression.

We could demonstrate in our study that cathelicidin is expressed in the human biliary epithelium and that it is secreted into the lumen of the biliary tract in response to overt bacterial infection, suggesting a role of this peptide in the innate immune defense of the liver. While the cathelicidin gene was previously identified as a direct target of the vitamin D nuclear receptor (VDR) (126, 128), the liver harbors VDR in BECs, but not in hepatocytes, as shown by our study and in a previous report (133). Therefore, we addressed the possibility that cathelicidin expression may be regulated by VDR in BECs. Our data that shows that VD3 induces cathelicidin expression through VDR in cultured BECs confirmed this possibility. We could also demonstrate that bile salts have also the ability to increase cathelicidin expression in these cells, suggesting that these amphipatic molecules may contribute to biliary tract sterility by eliciting antimicrobial defenses.

Both CDCA and UDCA induced cathelicidin expression, although only UDCA was effective through VDR. UDCA and CDCA were both able to increase the expression of VDR protein and the activity of a VDRE driven promoter, however UDCA had the unique faculty to induce the nuclear translocation of VDR and its binding to specific response elements. Furthermore, the regulatory effect of UDCA on cathelicidin

expression was prevented by VDR siRNA knock-down, whereas the effect of CDCA on cathelicidin expression was minimally affected by VDR knock-down. This discrepancy may be related to the ability of FXR to bind various consensus sequences (134), a possibility that was further supported by the increase in VDRE driven promoter activity under FXR pharmacological activation by GW4064. Dominant negative experiments provided additional evidence that the effect of CDCA was mediated by FXR. Therefore, while both CDCA and UDCA are able to control VDR expression in biliary epithelial cells, UDCA specifically triggers VDR nuclear activity and cathelicidin expression through this pathway. Conversely, the effect of CDCA on cathelicidin expression is independent of VDR and is mediated by FXR.

An important finding of our study is that the combination of CDCA or UDCA with VD3, induces greater cathelicidin expression than either bile salt or VD3 alone. The effects of VD3 and UDCA were additive, indicating that an increased amount of VDR protein induced by UDCA was available for concomitant activation by VD3. The combination of CDCA with VD3 was more than additive with respect to cathelicidin expression, which may be attributed to the activation of two distinct nuclear receptors, *i.e.* FXR and VDR. It was previously suggested that targeting FXR may



Representation of vitamin D and bile salt action on cathelicidin expression in BECs (d'Aldebert et al, *Gastroenterology*, 2009)

be useful in the treatment of patients with cholestatic liver diseases (135). Accordingly, pharmacological activation of FXR caused a significant protection against liver injury in cholestatic rats (77). Against the latter assumption, mice invalidated for FXR are protected from biliary obstruction when compared to wild type littermates (136). Despite these discrepancies in preclinical models, a FXR agonist is currently in phase II clinical trial for the treatment of PBC ([www.interceptpharma.com](http://www.interceptpharma.com)). Evidence is also increasing to indicate that VDR should be considered as a therapeutic target in inflammatory biliary diseases. As an example, a significant association between susceptibility or complications of PBC and VDR polymorphisms has been previously identified (137-142). Here, the data suggest that the therapeutic benefit provided by UDCA could result, in part, from the ability of this bile salt to increase cathelicidin expression. Consistent with this assumption and with a VDR-mediated effect, VDR and cathelicidin expressions are both increased by UDCA in the liver of patients with PBC. Abnormal accumulation of endotoxins in biliary epithelial cells is a feature of PBC (121). Thus, cathelicidin, which is known to neutralize the deleterious effects of bacterial products (143), could account for the clearance of endotoxins that was previously observed in PBC patients under UDCA treatment (120, 121). Furthermore, because the stimulation of cathelicidin expression in BECs was higher when UDCA was combined with vitamin D, our results suggest that in inflammatory biliary diseases involving bacterial factors, a significant benefit would arise from a strategy systematically combining UDCA with vitamin D.

### 3. Research program

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Vitamin D through VDR activation covers a wide spectrum of biological actions elicited in various tissues. Despite the fact that the liver is central to vitamin D homeostasis, the involvement of the hormone on liver biology is poorly known. Interestingly, vitamin D serum levels are invariably decreased in gastrointestinal and liver diseases (144-146), suggesting that alterations in the vitamin D-VDR axis may either be a consequence or may precede or worsen these disorders.

The possibility that the vitamin D-VDR axis is actively involved in liver disease is supported by the observation that VDR polymorphisms have been identified in PBC and autoimmune hepatitis (AIH) (138-142). VDR polymorphisms have also been associated with decreased bone mineral density in PBC (137), indicating that VDR genetics might not only influence the susceptibility of inflammatory liver diseases but also associated complications.

The impact of vitamin D or VDR in hepatic pathophysiology has however been poorly evaluated, mostly because VDR expression is low or absent in the liver (147). Indeed, VDR expression is virtually absent from hepatocytes, while clearly expressed in all non-parenchymal cells (133). As observed for PBC, the vitamin D-VDR axis could thus be involved in liver pathophysiological settings with altered non-parenchymal cells functioning.

Steatosis, which can be modulated by Kupffer cell biology (148), could represent a pathological entity modulated by the vitamin D-VDR axis. In line with this assumption, phototherapy or vitamin D ameliorate diet-induced steatohepatitis in the rat (149). Moreover, lower vitamin D levels are associated with increased severity of steatosis, necroinflammation and fibrosis in patients with non-alcoholic liver disease (NAFLD) (150, 151).

The potential of the vitamin D-VDR axis to modulate fibrosis has also been discussed in the context of chronic hepatitis C (152), following the observation that serum vitamin D levels are inversely correlated with fibrosis in HCV patients (153). Consistently, low vitamin D serum levels have been correlated with the severity of inflammation and fibrosis in chronic hepatitis C (153). However, no genetic association between VDR polymorphism and HCV infection has yet been evidenced (154). In HBV, VDR polymorphisms have been associated with infection susceptibility and clinical course (155-158). These observations are of particular interest because hepatic stellate cells do not express nuclear receptors apart from VDR (159).

Taken together, these observations suggest that the vitamin D-VDR axis may be of particular relevance in liver diseases involving non-parenchymal liver cells. We thus developed a research program that will address three specific points pertaining to liver pathophysiology. First, we will study the role of VDR in biliary-type liver disease. Second, we will assay the involvement of VDR in hepatic steatosis. Third, we will analyze the consequence of VDR invalidation on hepatic inflammation.

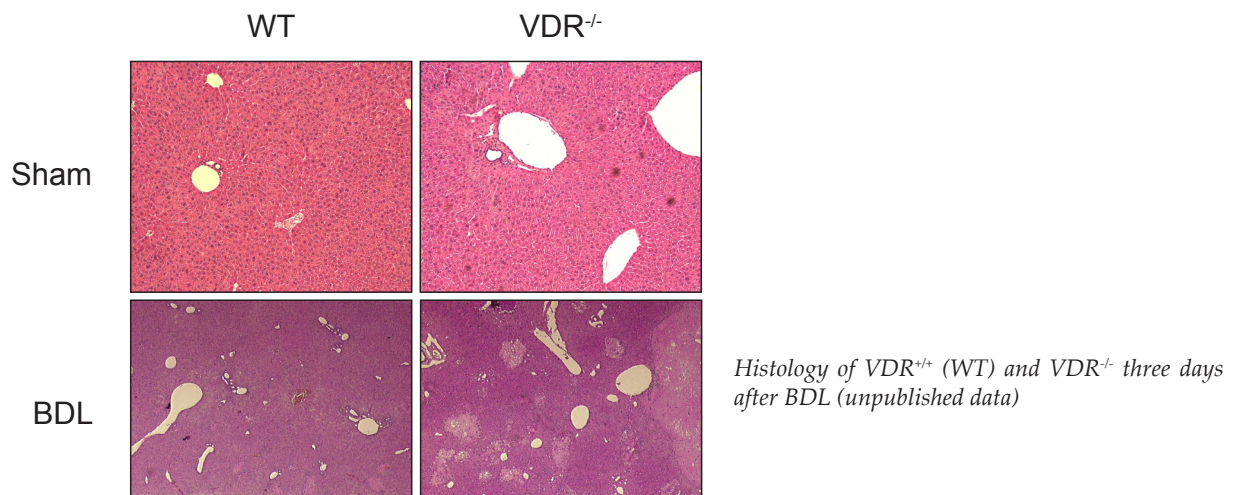


### 3.1. VDR in biliary-type liver diseases

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The first aim of the project will be conducted in VDR ablated mice (VDR<sup>-/-</sup>) and in wild type littermate mice (WT) with biliary-type liver disease.

Biliary-type liver disease will be induced by bile duct ligation. Our preliminary data indicate that hepatic histology was more altered in VDR<sup>-/-</sup> mice than in wild type littermates three days after BDL. The latter observation was confirmed by a higher increase in serum alanine and aspartate aminotransferase. Because VDR is mainly expressed in BECs and because VDR was shown to control cell-cell interaction in the colon (160), we will specifically analyze E-Cadherin and ZO-1 expression in bile ducts. E-cadherin staining indicates that VDR<sup>-/-</sup> BDL mice have 51% of disrupted intrahepatic bile ducts when compared to 29% in wild type BDL littermates. This observation suggests that parenchymal alteration may result from bile spill over due to bile duct alterations.



In order to confront this hypothesis to experimental evidences, we will ascertain the following elements in mice:

- Analysis of ZO-1 staining in bile ducts
- Expression of detoxifying enzymes, such as Cyp3a11 by RT-QPCR
- Molecular analysis of tissue repair markers by RT-QPCR, immunoblotting and immunohistochemistry in liver tissue

We will also develop at least one of the following strategies in the biliary epithelial cell line, Mz-ChA-1 (161):

- VDR knockdown by siRNA
- VDR overexpression by plasmid transfection

In these cells, we will then analyze:

- Cellular morphological aspect
- Expression of E-Cadherin and ZO-1 by RT-QPCR, immunoblotting and immunocytostaining

These strategies should allow us to ascertain the impact of VDR on bile duct integrity, on detoxification and on scar tissue production. We should also be able to describe the molecular link between the absence of VDR and pathophysiological consequences.

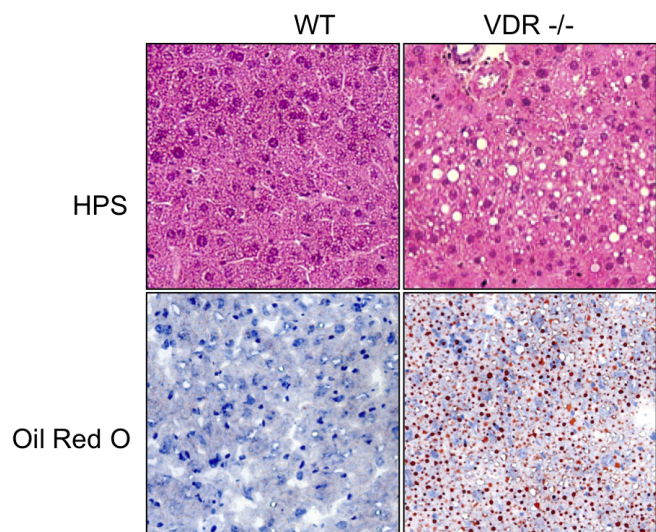
### 3.2. VDR in fatty liver diseases

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The second aim of the project will be to compare VDR<sup>-/-</sup> mice with WT mice for liver steatosis. As already previously mentioned, the rationale for this study relies on the observations that vitamin D circulating levels are inversely correlated with the extent of fatty liver disease in humans (150, 151). Therefore, we hypothesized that the vitamin D-VDR axis may be altered in fatty liver diseases, the goal of our project being to decipher the existing link between VDR and hepatic steatosis.

Our preliminary results indicate that 57% of male VDR<sup>-/-</sup> mice develop steatosis while no steatosis was evidenced in WT littermates under basal conditions as observed following hematoxylin phloxine saffron staining (HPS) and Oil Red O staining. To better delineate the molecular mechanisms involved in steatosis development, we will explore:

- Expression of genes involved in the metabolism of hepatic lipids by RT-QPCR.
- Liver lipid profile by lipidomic analysis
- Metabolic parameters and hepatic enzymes by serum chemistry



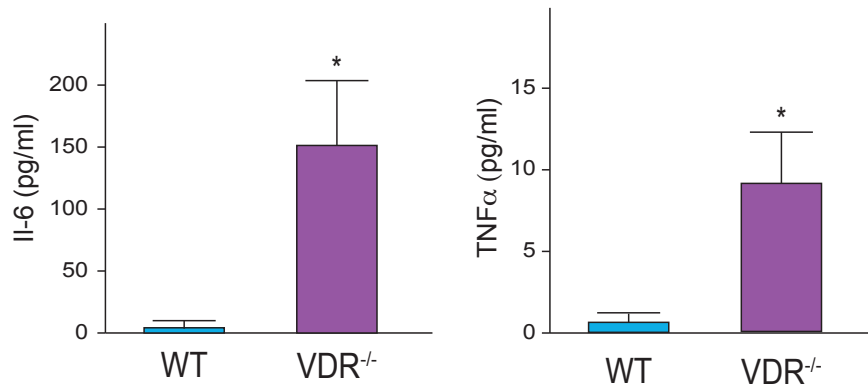
*Illustration of the steatosis observed in VDR<sup>-/-</sup> mice (unpublished data)*

This analysis should allow us to decipher the involvement of VDR in hepatic steatosis. The identification of perturbations arising from extra-hepatic tissues will be under scrutiny.

### 3.3. VDR in hepatic inflammation

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The last aim of our project refers to hepatic inflammation. This study will be undertaken because Kupffer cells, the resident macrophage of the liver, express VDR (133) and because nuclear receptors have been linked to hepatic inflammation (117). To assess the involvement of VDR in hepatic inflammation, we will focus our work in 12 month aged VDR<sup>-/-</sup> and WT mice.



*Serum cytokines levels in WT and VDR<sup>-/-</sup> mice (unpublished data)*

Our preliminary results indicate that VDR<sup>-/-</sup> mice have increased circulating levels of inflammatory cytokine. To directly assay the level of liver inflammation we will analyze the:

- Expression of cytokines in the liver by RT-QPCR, cytokine array and ELISA
- Number and localization of Kupffer cells by immunohistochemistry
- Expression and phosphorylation of liver NFκB and IκB

We will also induce VDR overexpression by plasmid transfection in murine macrophages (RAW cells) to then ascertain cell response to inflammatory stimuli (*i.e.* LPS).

This experimental design should also us to shed light on the direct role of VDR in hepatic inflammation.

## 4. Qualifications

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The proposed research program will require the association of knowledge and proficiency in cell biology, molecular biology, animal surgery and histological analysis, all of which can be found in the vicinity of the Saint Antoine Research Center.

The technical resources of the laboratory have full potential to enable the project to thrive. Indeed, the laboratory is fully equipped for cell culture, molecular biology and animal surgery. The laboratory is also equipped with devices needed for cellular and molecular biology. Among others, we are for example equipped with a Nanodrop apparatus (Thermo scientific) and LightCycler 480 (Roche Diagnostics).

For in vivo studies, two facilities are available for animal housing. In these facilities, whether conventional or high barrier SPF, animals are housed in ventilated racks. The laboratory is authorized to execute surgical protocols on animals and possesses state of the art equipment for anesthesia and animal surgery. Animal protocols have been submitted to and approved by an in house ethical committee on animal experimentation.

We have a longstanding collaboration with the Clinical Biochemistry Department and Pathology Department of Saint Antoine Hospital that allows us to perform expert biochemical and histological analysis, respectively. We have also set up a collaborative effort with the mass spectrometry facility of Saint Antoine Hospital for lipidomic analyses.

My central position in the project organization will allow me to oversee and coordinate the work force around the specified tasks. My goal is to make sure that resources are optimally used in order to successfully achieve the project. The project is thus undertaken by individuals (*i.e.* technicians, students) encouraged to optimally use their skills in order to play an effective part in accomplishing set tasks. Each participant is usually given a specific task or subtask and is asked to comply with task in ascribed timeframe. Milestone meetings take place every month to monitor the progress of the project.

I have developed a strong expertise in liver pathophysiology over the last decade. My Ph.D. project, supervised by Pr Housset, was dedicated to study the effects of bile salts on the secretory functions of human biliary epithelial cells. I defended successfully my Ph.D. in March 2003. The technical expertise I acquired during the course of this project, that includes cell isolation, cell culture and cell signaling, allows me to conduct effectively these aspects of our research. My Ph.D. work was rewarded by the publication of three original articles in top-level international peer-reviewed journals and by a first prize from the French national association of cell biology teachers (ANEBC).

After my Ph.D., I worked from May 2003 to June 2004 as a post-doctoral fellow in the Microbiology Department of the University of Michigan (MI, USA). In the laboratory of Laura Beretta, my project was dedicated to unravel new HCC biomarkers by a proteomic approach. During this time period, I wrote a review and an original paper pertaining to my project, that were published in the best journal of the field. Furthermore, I started to supervise the work of others as the Beretta Lab essentially functioned with graduate or undergraduate students.

In 2004, I was hired as an assistant professor in the department of physiology of UPMC. I was awarded a tenure track and an associate professor position in 2006. I am since in charge of the academic course entitled “Hepatic pathophysiology” and “Science and Society” delivered to students in their last year of the Master of Integrative Biology and Physiology. My working time is partitioned equally between my teaching and research activities. My research activity is held at Saint Antoine Research Center in the team directed by Pr. Housset.

My ability to manage research is stressed by the successful defense of three master’s degrees and the funding of a doctoral trainee obtained on the basis of the work performed under my supervision. Furthermore, the research work performed by students under my management was evaluated positively by external reviewers, as outlined by accepted meeting presentations and publications (see *curriculum vitae* for details).

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