博士論文（要約）

Studies on pathophysiological roles of bile acids in canine gallbladder mucocele

（犬の胆嚢粘液嚢腫における胆汁酸の病態生理学的役割に関する研究）

Toshiaki Kakimoto

柿元 俊彰

Contents

General Introduction

Canine gallbladder mucocele (GBM) is defined as an excess accumulation of abnormally gelatinous bile within the gallbladder (Mesich et al. 2009; Norwich 2011; Kesimer et al., 2015), and it has been reported that the frequency of diagnosis has increased during the last two decades (Besso et al., 2000; Pike et al., 2004; Aguirre et al., 2007). As the histopathologic findings of GBM, cystic mucinous hyperplasia of the gallbladder mucosa and excess secretion of gel-forming mucins, such as Muc5ac and Muc5b, have been reported (Kovatch et al., 1965; Pike et al., 2004; Worley et al., 2004; Kesimer et al., 2015). Moreover, it was revealed recently that the gallbladder epithelium acquires a well-differentiated, mucin-secretory phenotype during GBM formation (Kesimer et al., 2015). Dogs with GBM show clinical signs, including vomiting, anorexia, abdominal pain, and icterus, when the over-accumulation of mucus bile causes gallbladder rupture or common bile duct obstruction (Besso et al. 2000; Pike et al. 2004). Although cholecystectomy results in a good prognosis for survival, perioperative mortality for affected dogs ranges from 21.7% to 40% (Smalle et al., 2015). Since there is little evidence for the effectiveness of drug therapy, elucidation of the mechanism of GBM formation is necessary for the development of therapy. However, the pathogenesis of GBM has remained unclear.

On the contrary, several studies have identified the predisposing factors for GBM. It was reported that older small- to medium-breed dogs, such as Shetland Sheepdogs, Miniature Schnauzers, American Cocker Spaniels, Pomeranians, and Chihuahuas, are predisposed to the disease (Aguirre et al., 2007; Norwich 2011; Kutsunai et al., 2014). Certain endocrine diseases, including hypothyroidism and hyperadrenocorticism but not diabetes mellitus, abnormal lipid metabolism, and the use of imidacloprid-containing drugs

are also reported as risk factors for GBM (Mesich et al., 2009; Kutsunai et al., 2014; Gookin et al., 2015). Moreover, it was revealed that postprandial gallbladder hypomotility is observed in dogs with GBM (Tsukagoshi et al., 2012). It is assumed that these predisposing factors are useful for investigating the pathogenesis of GBM.

Bile acids are physiological detergents that facilitate intestinal absorption and transport of dietary lipids and vitamins. Basically, bile acids are known to belong to 2 major bile acids groups, hydrophobic and hydrophilic, and it was also revealed that the hydrophobic bile acids have more cytotoxic effects than hydrophilic ones (Thomas et al., 2008; Behar et al., 2013). Although species differences in bile acid composition were reported, the main canine hydrophobic bile acids are taurodeoxycholic acid and taurochenodeoxycholic acid (Washizu et al., 1991). Gallbladder mucus hypersecretion is described as one of the characteristic histopathological findings in GBM (Kesimer et al., 2015). In a previous study, it was demonstrated that these two hydrophobic bile acids accelerate mucin secretion by dog gallbladder epithelial cells (Klinkspoor et al., 1995). Recently, it was also revealed that the hydrophobic bile acids have an inhibitory effect on gallbladder smooth muscle contraction by inducing formation of H_2O_2 (Xiao et al., 2002) or acting on G protein-coupled bile acid receptor (TGR5) (Lavoie et al., 2010). Since the aforementioned gallbladder hypomotility was observed in dogs with GBM, the hydrophobic bile acids might be associated with GBM formation. In canine models of hyperadrenocorticism, a shift in bile acid composition towards an increased concentration of hydrophobic, unconjugated bile acids was reported (Kook et al., 2011). Moreover, apart from the role of bile acids in dietary lipid absorption, it has been revealed that the hydrophobic bile acids play an important role in triglyceride and cholesterol homeostasis as

endogenous ligands for the farnesoid X receptor and TGR5 (Thomas et al., 2008). These descriptions also support the assumption that the hydrophobic bile acids are involved in the pathogenesis of GBM, because both hyperadrenocorticism and abnormal lipid metabolism were known as risk factors for GBM. Recently, it was found that the hydrophobic bile acids upregulate the function of the cystic fibrosis transmembrane conductance regulator (CFTR) by activating TGR5 (Keitel et al., 2009). Since abnormally jellied bile, which is one of the characteristic features of GBM, is also observed in cystic fibrosis patients (Kobelska-Dubie et al., 2014) and its animal model (Meyerholz et al., 2010; Sun et al., 2014), bile acid-induced malfunction of CFTR might also have an effect on the pathological processes. Therefore, in the present thesis, I investigated the involvement of bile acids in the pathogenesis of GBM.

This thesis consists of three chapters. In Chapter 1, I have described the effects of dietary lipid overload on gallbladder bile acid composition and gallbladder motility in healthy dogs to estimate the changes of bile acid metabolism and gallbladder motility in dogs with abnormal lipid metabolism. In Chapter 2, I have detailed the actual bile acid composition of gallbladder contents in dogs with GBM. In Chapter 3-1, I presented the examination of mRNA and protein expression levels of TGR5 and CFTR based on the results described in Chapter 2. Moreover, in Chapter 3-2, I have detailed a preliminary study conducted on the development of a functional CFTR assay by using canine hepatobiliary organoids.

Chapter 1

Effect of a high-fat/high-cholesterol diet on gallbladder bile acid composition and gallbladder motility in dogs

本章の以降の内容は学術論文として出版する計画があり、出版社との契約条件によって公表できない。本章の内容は American Journal of Veterinary Research にて出版予定である。

Chapter 2

Bile acid composition of gallbladder contents in dogs with gallbladder mucocele and biliary sludge

本章の以降の内容は、学術論文として出版予定であり、出版社との契約条件によって公表できない。本章の内容が掲載されている学術論文（American Journal of Veterinary Research）は、2017 年に出版予定である。

Chapter 3

Association of the membrane-bound bile acid receptor TGR5 and the cystic fibrosis transmembrane regulator with canine gallbladder mucocele

Chapter 3-1

Expression levels of the membrane-bound bile acid receptor TGR5 and the cystic fibrosis transmembrane regulator in gallbladder tissues of dogs with gallbladder mucocele

本章の以降の内容は学術論文として出版する計画があるため公表できない。

2 年以内に公表予定。

Chapter 3-2

Investigation of a functional cystic fibrosis transmembrane conductance regulator assay using canine hepatobiliary organoids: a preliminary study

本章の以降の内容は学術論文として出版する計画があるため公表できない。

5 年以内に公表予定。

General discussion and conclusion

Canine gallbladder mucocele (GBM) is currently one of the most common canine hepatobiliary diseases; however, the precise pathogenic mechanism of GBM has not been elucidated. Gallbladder is a unique organ for the storage of bile and constantly contains bile acids, which have various effects on the gallbladder. To date, it has been demonstrated that the bile acids, in particular hydrophobic ones, have effects on gallbladder, causing mucin hypersecretion, smooth muscle dysfunction, and water secretion (Klinkspoor et al., 1995; Xiao et al., 2002; Keitel et al., 2009). Interestingly, these effects of hydrophobic bile acids are also the characteristic features of GBM (Tsukagoshi et al., 2012; Kobelska-Dubie et al., 2014; Kesimer et al., 2015). However, the association of bile acids with the pathogenesis of GBM was poorly understood. Therefore, in the present thesis, I addressed the pathogenic mechanism of GBM from the viewpoint of the involvement of bile acids.

Abnormal lipid metabolism was previously identified as one of the risk factors for GBM, although its role in the pathogenesis of GBM was unclear (Kutsunai et al., 2014). Since bile acids are widely known to play a critical role in lipid metabolism, I hypothesized that the abnormal lipid metabolism causes GBM by changing the gallbladder bile acid compositions. Thus, in Chapter 1, I described the effect of dietary lipid overload on bile acid compositions in six healthy dogs by feeding them a high-fat/high-cholesterol diet (HFCD) for 2 weeks. I found that the 2 weeks of HFCD feeding significantly increased both the concentration and compositional ratio of taurochenodeoxycholic acid and taurolithocholic acid, which are types of hydrophobic bile acids, in bile. On the contrary, HFCD feeding caused a significant decrease in both the concentration and compositional ratio of taurocholic acid and tauroursodeoxycholic acid, which are types of hydrophilic bile acids. Moreover, I found that the HFCD feeding causes gallbladder hypomotility through a decrease in gallbladder

cholecystokinin sensitivity. However, these results have to be clarified by further investigations because the sample size was small and these HFCD-fed dogs did not develop GBM. In Chapter 2, I have detailed the bile acid compositions of gallbladder contents in 18 actual GBM cases. On the basis of the results presented in Chapter 1, I hypothesized that the fraction of the hydrophobic bile acids in GBM dogs is higher than those of control dogs. Interestingly, the results showed that the concentrations of most bile acids in the GBM group were significantly lower than those in the control group. Moreover, the compositional ratios of taurodeoxycholic acid and taurolithocholic acid, which are hydrophobic bile acids, were significantly lower in the GBM cases, regardless of plasma triglyceride and total cholesterol levels. These results presented in Chapter 2 were quite different from my hypothesis and were not consistent with the results presented in Chapter 1.

One of the reasons for the conflicting results between Chapter 1 and 2 might be the difference of the time of sampling. I thought that the results presented in Chapter 1 were reasonable and it was acceptable to speculate that an increase in the hydrophobic bile acids is involved in the initial pathogenesis of GBM. Mucinous metaplasia of the gallbladder epithelium, which indicates excessive mucin secretion, was reported as one of the characteristic histopathologic findings of GBM (Kesimer et al., 2015). This histopathologic finding supports the above-mentioned speculation because the hydrophobic bile acids are known to have an effect on gallbladder, causing mucin hypersecretion (Englert et al., 1977; Klinkspoor et al., 1995). Moreover, I found that the 2 weeks of HFCD feeding caused gallbladder hypomotility through a decrease in gallbladder cholecystokinin sensitivity. This result was also consistent with that observed in a previous report, which showed that gallbladder motility decreased in dogs with GBM (Tsukagoshi et al., 2012). It was known that

the hydrophobic bile acids inhibit smooth muscle contraction by generating oxidative stress (H_2O_2) and causing lipid peroxidation, and by their aggressive effects on tissues because of their high detergency (Xiao et al., 2001; Lavoie et al., 2010; Behar et al., 2013). This result suggested that the increases in hydrophobic bile acids are involved in the gallbladder hypomotility. In a previous study on dogs, it was reported that the iatrogenic hypercortisolemic state causes a reversible shift in bile acid composition toward an increased concentration of cytotoxic, hydrophobic, unconjugated bile acids (Kook et al., 2011). Since both hyperlipidemia and hyperadrenocorticism were previously identified as risk factors for GBM, and the increases in the hydrophobic bile acids were consistently observed in both exogenous steroid-administrated dogs and dietary lipid-overloaded dogs, it was assumed that the increases in hydrophobic bile acids play a role in initial GBM formation. On the contrary, taurodeoxycholic acid and taurolithocholic acid, which decrease in GBM cases, are secondary bile acids. The secondary bile acids are produced by intestinal microbiota and return to the liver through the enterohepatic circulation. In mice cholestasis models, it was shown that gallbladder dysmotility causes a decrease in the fractions of the secondary bile acids owing to a disorder of bile acid enterohepatic circulation (Debray et al., 2012). As mentioned in Chapter 1, I found that the HFCD feeding causes gallbladder hypomotility. This result suggested that the increases in hydrophobic bile acids trigger the gallbladder dysmotility. Moreover, it was also revealed that more severe gallbladder hypomotility is observed in dogs with GBM. Therefore, I assumed that the change of bile acid compositions in GBM cases is the secondary event of gallbladder hypomotility accompanying GBM progression. Taken together, from the results presented in Chapters 1 and 2, I speculated that there are two patterns of bile acid compositions depending on the stage of dyslipidemia-associated GBM

progression. That is, hyperlipidemia causes the increase in hydrophobic bile acids and this increase induces gallbladder hypomotility and mucin hypersecretion as an initial pathogenic mechanism of GBM. Subsequently, when the bile acid enterohepatic circulation begins to be affected by the gallbladder hypomotility accompanying GBM progression, the secondary bile acids might be decreased.

Additionally, I thought that the low levels of the secondary bile acids, in particular taurodeoxycholic acid, also contribute to the progression of GBM. Taurodeoxycholic acid is known as one of the potent endogenous agonists of TGR5. In the epithelium of the gallbladder of humans, it was reported that activated TGR5 induces activation of CFTR that contributes to fluid and chloride secretion into the gallbladder lumen (Keitel et al., 2009). More interestingly, it was demonstrated that accumulation of abnormally mucinous bile within the gallbladder and cystic hyperplasia of gallbladder epithelium, which are characteristic histopathologic findings in GBM, are also observed in cystic fibrosis patients and its animal model (Meyerholz et al., 2010; Kobelska-Dubie et al., 2014; Sun et al., 2014). Therefore, I speculated that fluid secretion insufficiency caused by low taurodeoxycholic acid fractions is involved in canine GBM formation. Additional studies are necessary to clarify the effects of the compositional changes of gallbladder bile acids on GBM in dogs.

I showed that the fraction of taurodeoxycholic acid decreases in the gallbladder bile of dogs with GBM as presented in Chapter 2. Taurodeoxycholic acid is known to upregulate TGR5 protein expression (Zhou et al., 2015) Moreover, similar to dogs with GBM, abnormally jellied bile has also been observed in human patients with cystic fibrosis (Kobelska-Dubie et al., 2014) and animals experimentally treated to suppress the expression of functional CFTR protein (Meyerholz et al., 2010; Sun et al., 2014). As mentioned in

Chapter 3-1, I evaluated the difference in mRNA and protein expression levels of TGR5 and CFTR between the gallbladder tissues of normal dogs and those of dogs with GBM. I hypothesized that low expression levels of TGR5 and CFTR are observed in GBM. The results of these analyses revealed that levels of TGR5 mRNA significantly decreased in dogs with GBM. However, there was no significant difference in TGR5 protein expression levels between control and GBM samples. In contrast, no difference in CFTR expression at either the mRNA or the protein level could be detected between control and GBM samples. As hypothesized, I observed a decrease in TGR5 mRNA expression levels in dogs with GBM. Although the difference in protein levels of TGR5 was not detected, the semi-quantitative nature of western blot analysis might have contributed to the results. However, since no differences in the expression levels of CFTR mRNA and protein were observed between the control and GBM, I thought that I should focus on the functional abnormality rather than the expression levels of CFTR to elucidate its pathophysiological role in GBM. Further studies that investigate the association between TGR5 expression level and CFTR function might provide more insight into the pathogenesis of GBM.

As mentioned in Chapter 3-2, I attempted to develop a method for quantification of TGR5–CFTR signaling pathway function using canine hepatobiliary cells. Recently, Dekkers et al. (2013) demonstrated an organoid swelling assay, which is a novel and rapid quantitative assay for CFTR function using three-dimensional human intestinal stem cell cultures, so-called intestinal organoids. Fortunately, it was reported more recently that hepatic organoids have been established from canine hepatic progenitor cells (Nantasanti et al., 2015). I thought that if canine gallbladder organoids can be developed, the effect of TGR5 expression levels on CFTR function might be determined using the organoid

swelling assay. Therefore, the first aim of this study was to generate canine gallbladder organoids and to examine the expression levels of TGR5 mRNA in these organoids. The second aim was to investigate the utility of canine gallbladder organoids in the organoid swelling assay. I have successfully developed canine gallbladder organoids from a canine gallbladder tissue, and the canine gallbladder organoids showed significantly higher expression levels of TGR5 mRNA than those of hepatic and cholangiocyte-like organoids. Moreover, canine gallbladder organoids tended to expand more than hepatic organoids upon forskolin stimulation. The swelling of both of these organoids decreased with CFTR inhibition. These results suggest that canine gallbladder organoids are applicable in an assay of TGR5–CFTR signaling pathway function. However, as preliminary supplementary data, I found that neither canine hepatic nor gallbladder organoids swell in response to TGR5 stimulation. I speculate that the localization of TGR5 is responsible for this unfortunate consequence, because TGR5 is reported to be localized in the apical membranes of gallbladder epithelial cells, but not in the basolateral membranes (Keitel et al., 2009). This means that there is a possibility that the TGR5 activator does not reach the luminal surface of organoids. Further studies, which attempt to reverse the polarity (inside-out, with the basal surface facing the central lumen) of organoids, might ensure canine gallbladder organoids are applicable to a functional assay of TGR5–CFTR signaling.

From the results obtained a series of studies, I illustrated the novel hypothetical concepts of the pathogenic mechanism of canine hyperlipidemia-associated GBM, which are as follows. First, hyperlipidemia induces an increase in hydrophobic bile acids, such as taurochenodeoxycholic acid and taurolithocholic acid, in gallbladder bile. Subsequently, an increase in the hydrophobic bile acids causes gallbladder hypomotility and excess mucin

secretion. Additionally, as gallbladder motility decreases, the fraction of the secondary bile acid, taurodeoxycholic acid, also decreases owing to the disorder of bile acid enterohepatic circulation. As the next step, TGR5 expression is downregulated by the low levels of the taurodeoxycholic acid fraction. Finally, when the malfunction of CFTR is caused by the low expression of TGR5, abnormal highly viscous bile formation and accumulation within the gallbladder lead to the development of mucocele. However, the causal relationship between the changes of bile acid composition and GBM is not fully understood in these studies. Since there is no useful animal model for GBM, it seems difficult to investigate causality. The use of canine gallbladder organoids has the potential to address this issue, because it is known that organoids mimic some of the structure and function of real organs (Willyard, 2015). Future studies that investigate the effects of bile acids on canine gallbladder organoids will provide knowledge for further understanding of the pathogenic mechanism of canine GBM.

Acknowledgments

I would like to express my heartfelt gratitude to Dr. Koichi Ohno for his guidance and unwavering support, throughout the course of my work. I express my gratitude to Prof. Hajime Tsujimoto and Drs. Hideyuki Kanemoto, Kenjiro Fukushima, Yuko Koshino, Hirotaka Tomiyasu, and all the members of the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, for their invaluable support and guidance in completing my work.

I would like to greatly thank Drs. Louis C. Penning, and Bart Spee, and Ms. Loes A. Oosterhoff (Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University) for their valuable suggestions and excellent technical support in culturing organoids.

Moreover, I would like to thank all of the patients, their owners, and experimental beagles included my work for their tremendous help.

Finally, I am most grateful to my family. Words can not express how grateful I am to my mother and father for all of the sacrifices that you've made on my behalf.

References

Aguirre, A.L., Center, S.A., Randolph, J.F., Yeager, A.E., Keegan, A.M., Harvey, H.J., Erb, H.N., 2007. Gallbladder disease in Shetland Sheepdogs: 38 cases (1995-2005). Journal of the American Veterinary Medical Association 231, 79-88.

Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245-5250.

Beaver, M.H., Wostmann, B.S., Madsen, D.C., 1978. Bile acids in bile of germfree and conventional dogs. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine 157, 386-389.

Becker, S., Reinehr, R., Grether-Beck, S., Eberle, A., Häussinger, D., 2007. Hydrophobic bile salts trigger ceramide formation through endosomal acidification. Biological Chemistry 388, 185–196.

Behar, J., Mawe, G., Carey, M., 2013. Roles of cholesterol and bile salts in the pathogenesis of gallbladder hypomotility and inflammation: cholecystitis is not caused by cystic duct obstruction. Neurogastroenterology and Motility 25, 283-290.

Besso, J.G., Wrigley, R.H., Gliatto, J.M., Webster, C.R., 2000. Ultrasonographic appearance and clinical findings in 14 dogs with gallbladder mucocele. Veterinary Radiology & Ultrasound 41, 261-271.

Brinkhof, B., Spee, B., Rothuizen, J., Penning, L.C., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. Analytical biochemistry 356, 36-43.

Carpino, G., Cardinale, V., Gentile, R., Onori, P., Semeraro, R., Franchitto, A., Wang, Y., Bosco, D., Iossa, A., Napoletano, C., Cantafora, A., D'Argenio, G., Nuti, M., Caporaso, N., Berloco, P., Venere, R., Oikawa, T., Reid, L., Alvaro, D., Gaudio, E., 2014. Evidence for multipotent endodermal stem/progenitor cell populations in human gallbladder. Journal of hepatology 60, 1194–1202.

Chen, Y., Kong, J., Wu, S., 2015. Cholesterol gallstone disease: focusing on the role of gallbladder. Laboratory Investigation 95, 124-131.

Choi, M., Moschetta, A., Bookout, A.L., Peng, L., Umetani, M., Holmstrom, S.R., Suino-Powell, K., Xu, H.E., Richardson, J.A., Gerard, R.D., et al., 2006. Identification of a hormonal basis for gallbladder filling. Nature Medicine 12, 1253-1255.

Debray, D., Rainteau, D., Barbu, V., Rouahi, M., El, M, H., Lerondel, S., Rey, C., Humbert, L., Wendum, D., Cottart, C, H., Dawson, P., Chignard, N., Housset, C., 2012. Defects in gallbladder emptying and bile Acid homeostasis in mice with cystic

fibrosis transmembrane conductance regulator deficiencies. Gastroenterology. 142, 1581-91.

Dekkers, J.F., Wiegerinck, C.L., de Jonge, H.R., Bronsveld, I., Janssens, H.M., de Winter-de Groot, K.M., Brandsma, A.M., de Jong, N.W.M., Bijvelds, M.J.C. Scholte, B.J., Nieuwenhuis, E.E.S., van den Brink, S., Clevers, H., van der Ent, C.K., Middendorp, S., Beekman, J.M., 2013. A functional CFTR assay using primary cystic fibrosis intestinal organoids. Nature medicine 19, 939–945

Dray -Charier, N., Paul, A., Combettes, L., Bouin, M., Mergey, M., Balladur, P., Capeau, J., Housset, C., 1997. Regulation of mucin secretion in human gallbladder epithelial cells: Predominant role of calcium and protein kinase C. Gastroenterology 112, 978-990.

Duboc, H., Taché, Y., Hofmann, A.F., 2014. The bile acid TGR5 membrane receptor: From basic research to clinical application. Digestive and Liver Disease 46, 302-312.

Englert, E., Harman, C.G., Freston, J.W., Straight, R.C., Wales, E.E., 1977. Studies on the pathogenesis of diet-induced dog gallstones. The American Journal of Digestive Diseases 22, 305-314.

Ghoneim, R.H., Ngo Sock, E.T., Lavoie, J.M., Piquette-Miller, M., 2015. Effect of a high-fat diet on the hepatic expression of nuclear receptors and their target genes: relevance to drug disposition. The British Journal of Nutrition 113, 507-516.

Gookin, J.L., Correa, M.T., Peters, A., Malueg, A., Mathews, K.G., Cullen, J., Seiler, G., 2015. Association of gallbladder mucocele histologic diagnosis with selected drug use in dogs: A matched case-control study. Journal of Veterinary Internal Medicine 29, 1464-1472.

Han, S.I., Studer, E., Gupta, S., Fang, Y., Qiao, L., Li, W., Grant, S., Hylemon, P.B., Dent, P., 2004. Bile acids enhance the activity of the insulin receptor and glycogen synthase in primary rodent hepatocytes. Hepatology 39, 456-463.

Ji, S., Jung, S., Kim, B., Jung, J., Yoon, J., Choi, M., 2014. Feasibility of percutaneous contrast ultrasound-guided cholecystography in dogs. Veterinary Radiology & Ultrasound 56, 296-300.

Jonderko, K., Ferré, J.P., Buéno, L., 1992. Real-time ultrasonography as a noninvasive tool for the examination of canine gallbladder emptying: a validation study. Journal of Pharmacological and Toxicological Methods 27, 107-111.

Jonkers, I.J.A.M., Smelt, A.H.M., Ledeboer, M., Hollum, M.E., Biemond, I., Kuipers, F., Stellaard, F., Boverhof, R., Meinders, A.E., Lamers, C.H.B.W., et al., 2003. Gall bladder dysmotility: a risk factor for gall stone formation in hypertriglyceridaemia and reversal on triglyceride lowering therapy by bezafibrate and fish oil. Gut 52, 109-115.

Kakimoto, T., Kanemoto, H., Fukushima, K., Ohno, K., Tsujimoto, H., 2016. Preliminary study on the association between serum bile acid composition and gallbladder disease in dogs. Journal of Pet Animal Nutrition 19, 67-73.

Kälin, N., Claass, A., Sommer, M., Puchelle, E., Tümmler, B., 1999. DeltaF508 CFTR protein expression in tissues from patients with cystic fibrosis. The Journal of clinical investigation 103, 1379-1389.

Keitel, V., Cupisti, K., Ullmer, C., Knoefel, W. T., Kubitz, R., Häussinger, D., 2009. The membrane-bound bile acid receptor TGR5 is localized in the epithelium of human gallbladders. Hepatology 50, 861-870.

Kesimer, M., Cullen, J., Cao, R., Radicioni, G., Mathews, K.G., Seiler, G., Gookin, J.L., 2015. Excess secretion of gel-forming mucins and associated innate defense proteins with defective mucin un-packaging underpin gallbladder mucocele formation in dogs. PloS One 10, e0138988.

Klinkspoor, J.H., Kuver, R., Savard, C.E., Oda, D., Azzouz, H., Tytgat, G.N., Groen, A.K., Lee, S.P., 1995. Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. Gastroenterology 109, 264-274..

Klinkspoor, J.H., Yoshida, T., Lee, S.P., 1998. Bile salts stimulate mucin secretion by cultured dog gallbladder epithelial cells independent of their detergent effect. The Biochemical Journal 332, 257-262.

Kobelska-Dubiel, N., Klincewicz, B., Cichy, W., 2014. Liver disease in cystic fibrosis. Przegląd Gastroenterologiczny 9, 136-141.

Kook, P.H., Schellenberg, S., Rentsch, K.M., Reusch, C.E., Glaus, T.M., 2011. Effect of twice-daily oral administration of hydrocortisone on the bile acids composition of gallbladder bile in dogs. American Journal of Veterinary Research 72, 1607-1612.

Kovatch R.M., Hilderbrandt, P.K., Marcus, L.C., 1965. Cystic mucinous hypertrophy of the mucosa of the gall bladder in the dog. Pathol Vet 2, 574-584.

Kutsunai, M., Kanemoto, H., Fukushima, K., Fujino, Y., Ohno, K., Tsujimoto, H., 2014. The association between gall bladder mucoceles and hyperlipidaemia in dogs: A retrospective case control study. The Veterinary Journal 199, 76-79.

LaMont, J.T., Carey, M.C., 1992. Cholesterol gallstone formation. 2. Pathobiology and pathomechanics. Progress in Liver Diseases 10, 165-191.

Lavoie, B., Balemba, O.B., Godfrey, C., Watson, C.A., Vassileva, G., Corvera, C.U., Nelson, M.T., Mawe, G.M., 2010. Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels. The Journal of Physiology 588, 3295-3305.

Li, F., Zhang, X.W., Li, K.W., Ding, J., Wang, G.Y., 2014. Effects of cholecystectomy on the changes of motility of Beagle dogs' sphincter of Oddi. Acta Cirúrgica Brasileira 29, 237-244.

Malek, S., Sinclair, E., Hosgood, G., Moens, N.M.M., Baily, T., Boston, S.E., 2013, Clinical findings and prognostic factors for dogs undergoing cholecystectomy for gall bladder mucocele. Veterinary Surgery 42, 418-426.

Mesich, M.L., Mayhew, P.D., Paek, M., Holt, D.E., Brown, D.C., 2009. Gall bladder mucoceles and their association with endocrinopathies in dogs: A retrospective case-control study. The Journal of Small Animal Practice 50, 630-635.

Meyerholz, D.K., Stoltz, D.A., Pezzulo, A.A., Welsh, M.J., 2010. Pathology of gastrointestinal organs in a porcine model of cystic fibrosis. The American journal of pathology 176, 1377-1389.

Nantasanti, S., Spee, B., Kruitwagen, H.S., Chen, C., Geijsen, N., Oosterhoff, L. A., Van Wolferen, M.E., Pelaez, N., Fieten, H., Wubbolts, R.W., Grinwis, G.C., Chan, J., Huch, M., Vries, R.R.G., Clevers, H., De Bruin, A., Rothuizen, J., Penning, L.C., Schotanus, B.A., 2015. Disease modeling and gene therapy of copper storage disease in canine hepatic organoids. Stem Cell Reports 5, 895–907.

Norwich, A., 2011. Gallbladder mucocele in a 12-year-old cocker spaniel. Canadian Veterinary Journal 52, 319–321.

Okuyama, S., Kokubun, N., Higashidate, S., 1979. A new analytical method of individual bile acids using high performance liquid chromatography and immobilized 3α-hydroxysteroid dehydrogenase in column form. Chemistry Letters 12, 1443-1446.

Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E., Mangelsdorf, D.J., 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell 93, 693-704.

Peters, I.R., Peeters, D., Helps, C.R., Day, M.J., 2007. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. Veterinary Immunology and Immunopathology 117, 55-66.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnology Letters 26, 509-515.

Pike, F.S., Berg, J., King, N.W., Penninck, D.G., Webster, C.R., 2004. Gallbladder mucocele in dogs: 30 cases (2000-2002). Journal of the American Veterinary Medical Association 224, 1615-1622.

Portincasa, P., Di Ciaula, A., Wang, H.H., Palasciano, G., van Erpecum, K.J., Moschetta, A., Wang, D.Q., 2008. Coordinate regulation of gallbladder motor function in the gut-liver axis. Hepatology 47, 2112-2216.

Reinehr, R., Sommerfeld, A., Keitel, V., Grether-Beck, S., 2008. Häussinger, D., Amplification of CD95 activation by caspase 8-induced endosomal acidification in rat hepatocytes. The Journal of Biological Chemistry 283, 2211–2222.

Silver, N., Best, S., Jiang, J., Thein, S.L., 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Molecular Biology 7, 33.

Smalle, T.M., Cahalane, A.K., Köster, L.S., 2915. Gallbladder mucocoele: A review. Journal of the South African Veterinary Association 86, 1318

Stenman, L.K., Holma, R., Korpela, R., 2012. High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids. World Journal of Gastroenterology 18, 923-929.

Strah, K.M., Pappas, T.N., Melendez, R.L., Debas, H.T., 1986. Contrasting cholinergic dependence of pancreatic and gallbladder responses to cholecystokinin. The American Journal of Physiology 250, G665-G669.

Sun, X., Olivier, A.K., Yi, Y., Pope, C.E., Hayden, H.S., Liang, B., Sui, H., Zhou, W., Hager, K.R., Zhang, Y., Liu, X., Yan, Z., Fisher, J.T., Keiser, N.W., Song, Y., Tyler, S.R., Goeken, J.A., Kinyon, J.M., Radey, M.C., Fligg, D., Wang, X., Xie, W., Lynch, T.J., Kaminsky, P.M., Brittnacher, M.J., Miller, S.I., Parekh, K., Meyerholz, D.K., Hoffman, L.R., Frana, T., Stewart, Z.A., Engelhardt, J.F., 2014. Gastrointestinal pathology in juvenile and adult CFTR-knockout ferrets. The American Journal of Pathology 184, 1309-1322.

Takayama, K., Mitani, S., Nagamoto, Y., Sakurai, F., Tachibana, M., Taniguchi, Y., Sekiguchi, K., Mizuguchi, H., 2016. Laminin 411 and 511 promote the cholangiocyte differentiation of human induced pluripotent stem cells. Biochemical and Biophysical Research Communications 474, 91–96.

Tanimizu, N., Miyajima, A., Mostov, K.E., 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Molecular biology of the cell 18, 1472–1479.

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., Schoonjans, K., 2008. Targeting bile-acid signalling for metabolic diseases. Nature Reviews Drug Discovery 7, 678-693.

Tsukagoshi, T., Ohno, K., Tsukamoto, A., Fukushima, K., Takahashi, M., Nakashima, K., Fujino, Y., Tsujimoto, H., 2012. Decreased gallbladder emptying in dogs with biliary sludge or gallbladder mucocele. Veterinary Radiology & Ultrasound 53, 84-91.

Uno, T., Okamoto, K., Onaka, T., Fujita, K., Yamamura, H., Sakai, T., 2007. Hourly changes of total bile acids and conjugated bile acid fractions in the serum before and after meals, and compositional ratio of conjugated bile acid fractions from the serum and the gallbladder in healthy dogs. Journal of Animal Clinical Medicine 16, 125-128.

van der Mark, V.A., de Jonge, H.R., Chang, J.C., Ho-Mok, K.S., Duijst, S., Vidović, D., Carlon, M.S., Oude Elferink, R.P., Paulusma, C.C., 2016. The phospholipid flippase ATP8B1 mediates apical localization of the cystic fibrosis transmembrane regulator. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1863, 2280–2288.

Vandesompele, J., De, Preter, K., Pattyn, F., Poppe, B., Van, Roy, N., De, Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, research0034.1–0034.11

Vang, S., Longley, K., Steer, C.J., Low, W.C., 2014. The unexpected uses of urso- and tauroursodeoxycholic acid in the treatment of non-liver diseases. Global Advances in Health and Medicine 3, 58-69.

Vassileva, G., Golovko, A., Markowitz, L., Abbondanzo, S.J., Zeng, M., Yang, S., Hoos, L., Tetzloff, G., Levitan, D., Murgolo, N.J., Keane, K., Davis, H.R., Hedrick, J., Gustafson, E.L., 2006. Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. Biochemical Journal 398, 423–430.

Wang, A.Z., Ojakian, G.K., Nelson, W.J., 1990. Steps in the morphogenesis of a polarized epithelium. I. Uncoupling the roles of cell-cell and cell-substratum contact in establishing plasma membrane polarity in multicellular epithelial (MDCK) cysts. Journal of cell science 95, 137–151.

Wang, H.H., Portincasa, P., Wang, D.Q., 2016. The cholecystokinin-1 receptor antagonist devazepide increases cholesterol cholelithogenesis in mice. European Journal of Clinical Investigation 46, 158-169.

Watanabe, M., Houten, S.M., Mataki, C., Christoffolete, M.A., Kim, B.W., Sato, H., Messaddeq, N., Harney, J.W., Ezaki, O., Kodama, T., et al., 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 439, 484-489.

Watanabe, M., Houten, S.M., Wang, L., Moschetta, A., Mangelsdorf, D.J., Heyman, R.A., Moore, D.D., Auwerx, J., 2004. Bile acids lower triglyceride levels via a pathway

involving FXR, SHP, and SREBP-1c. The Journal of Clinical Investigation 113, 1408-1418.

Washizu, T., Ishida, T., Washizu, M., Tomoda, I., Kaneko, J.J., 1994. Changes in bile acid composition of serum and gallbladder bile in bile duct ligated dogs. The Journal of Veterinary Medical Science 56, 299-303.

Washizu, T., Tomoda, I., Kaneko, J.J., 1991. Serum bile acid composition of the dog, cow, horse and human. The Journal of Veterinary Medical Science 53, 81-86.

Willyard, C., 2015. The boom in mini stomachs, brains, breasts, kidneys and more. Nature 523, 520-522.

Worley, D.R., Hottinger, H.A., Lawrence, H.J., 2004. Surgical management of gallbladder mucoceles in dogs: 22 cases (1999–2003). J Am Vet Med Assoc 225,1418–1422.

Zhang, Y., Edwards, P.A., 2008. FXR signaling in metabolic disease. FEBS letters 582, 10-18.

Zhao, C., Dahlman-Wright, K., 2010. Liver X receptor in cholesterol metabolism. The Journal of Endocrinology 204, 233-240.

Zhou, H., Zhou, S., Gao, J., Zhang, G., Lu, Y., Owyang, C., 2015. Upregulation of bile acid receptor TGR5 and nNOS in gastric myenteric plexus is responsible for delayed gastric emptying after chronic high-fat feeding in rats. American journal of physiology. Gastrointestinal and liver physiology 308, G863-G873.

Xiao, Z.L., Chen, Q., Biancani, P., Behar, J., 2001. Abnormalities of gallbladder muscle associated with acute inflammation in guinea pigs. American Journal of Physiology. Gastrointestinal and Liver Physiology 281, G490-G497.

Xiao, Z.L., Rho, A.K., Biancani, P., Behar, J., 2002. Effects of bile acids on the muscle functions of guinea pig gallbladder. American Journal of Physiology. Gastrointestinal and Liver Physiology 283, G87-G94.