Histol Histopathol (2019) 34: 69-79

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Distribution of bile acid receptor TGR5 in the gastrointestinal tract of dogs

Paula R. Giaretta[1], Jan S. Suchodolski[2], Anna K. Blick[1], Joerg M. Steiner[2], Jonathan A. Lidbury[2] and Raquel R. Rech[1]

[1]Department of Veterinary Pathobiology and [2]Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA

Summary. Takeda-G-protein-receptor-5 (TGR5) is a receptor for bile acids and its expression has been described in a variety of tissues and species. Characterization of TGR5 distribution and function has been investigated in drug discovery for the treatment of metabolic diseases in humans. Because dogs are one of the species used in biomedical research and share some similarities with human gastrointestinal diseases, the objective of this study was to characterize the distribution of TGR5 receptor in the canine species. This study characterizes the distribution of TGR5 receptor in the gastrointestinal tract, liver, gallbladder, and pancreas of 8 dogs. The distribution of TGR5 antigen and mRNA expression was investigated using immunohistochemistry and RNA in situ hybridization, respectively. TGR5 immunolabeling was located in the cell membrane or in the cell membrane and cytoplasm. TGR5 immunolabeling was broadly distributed in macrophages, endothelial cells, ganglion cells, and leiomyocytes throughout all the examined tissues. Epithelial cells from tongue, stomach to rectum, as well as from gallbladder, biliary and pancreatic ducts demonstrated TGR5 immunolabeling. In endocrine cells, TGR5 immunolabeling was observed in intestinal enteroendocrine cells and islets of Langerhans in the pancreas. The hepatocytes had a unique pattern of immunolabeling located on the canalicular surface of the cell membrane. TGR5 mRNA expression was located mainly in the nucleus and the only negative cells throughout all examined tissues were striated muscle from tongue and esophagus, muscularis mucosae, esophageal glands, and hepatic sinusoids. These findings indicate that the bile acid receptor TGR5 is ubiquitously distributed in the canine gastrointestinal tract.

Key words: GPBAR1, Bile salts, Protein, Nucleic acids, Dog

Offprint requests to: Paula R. Giaretta, Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, 4467 TAMU, 77843, College Station, TX, USA. e-mail: pgiaretta@cvm.tamu.edu

DOI: 10.14670/HH-18-025

Introduction

Primary bile acids are synthesized from cholesterol in hepatocytes as cholic and chenodeoxycholic acids, then conjugated with glycine or taurine and secreted with the bile into the intestinal lumen (Costanzo, 2013). In the ileum and large intestine, the microbiota containing 7α-dehydroxylase can modify primary bile acids to the secondary bile acids deoxycholic and lithocholic acids (Vlahcevic et al., 1996). Bile acids (BA) undergo enterohepatic circulation being actively reabsorbed through the apical sodium dependent bile acid transporter (ASBT) or through passive diffusion by the small and large intestinal mucosa (Aldini et al., 1996).

Digestive functions of BA include hepatic secretion of cholesterol, induction of bile flow, and facilitation of the absorption of cholesterol, triglycerides, and lipid-soluble vitamins (Chiang, 2009; Russel, 2009). The most well-understood examples of BA receptors are the nuclear farnesoid X receptor (FXR) and the

transmembrane G protein-coupled bile acid receptor GPBAR-1, also known as TGR5 (Schaap et al., 2014). FXR is an intestinal and hepatic nuclear receptor and its activation leads to increased excretion of BA by hepatocytes and reduced hepatic uptake and de novo synthesis of BA (Grober et al., 1999). The membranous receptor TGR5 has a broader distribution in tissues, including widespread expression in the human gastrointestinal tract, nervous tissue, and macrophages, functioning as a regulator of metabolism (Duboc et al., 2014). TGR5 transmits its signal by increasing intracellular concentrations of cyclic AMP (cAMP) (Kawamata et al., 2003). The secondary BA lithocholic acid is the strongest endogenous agonist for the receptor, followed by deoxycholic acid, chenodeoxycholic acid, and cholic acid (Kawamata et al., 2003).

BA and their receptors, including TGR5, represent novel targets for the treatment of metabolic and inflammatory disorders (Guo et al., 2016). The function and expression of TGR5 have been partially investigated in humans, mice, rats, and rabbits, with variation in the distribution of the receptor and in bile acid metabolism among species (van Nierop et al., 2017). Dogs are the non-rodent species most commonly used in preclinical drug development (Gad, 2010) and spontaneously develop diseases that share characteristics with humans, which make the dog being increasingly recognized in the field of clinical translational research as an animal model (Paoloni and Khanna, 2008). Bile acid dysmetabolism is commonly associated with gastrointestinal diseases in dogs, similar to humans (Melgarejo et al., 2000; Guard et al., 2017). The objective of this study was to characterize the immunohistochemical and mRNA distribution of TGR5 receptor in the canine gastrointestinal tract.

Materials and methods

Samples and tissue preparation

Eight adult dogs (five male and three female of different breeds; mean age 5 years, range 1-13 years) were included in this study. The dogs presented to the Texas A&M Small Animal Emergency Service and were euthanized due to traumatic injuries, in consensus with their owners. None of the dogs had clinical signs of gastrointestinal disease. No animal was euthanized for the purpose of this study and this was not an animal experiment as samples were collected postmortem. The study was carried out in accordance with the Animal Welfare act (AWA) and was exempted by the Texas A&M University Institutional Animal Care and Use Committee.

Samples from the gastrointestinal tract were collected 10-20 minutes after euthanasia. Tissue samples were collected from tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas. Samples were fixed in 10% neutral buffered formalin for 24 hours, followed by routine histological processing. For histological examination, 4 μm thick sections routinely stained with hematoxylin and eosin were examined by light microscopy and were scored following the World Small Animal Veterinary Association (WSAVA) gastrointestinal guidelines (Day et al., 2008). No significant histologic changes were found in the gastrointestinal tract of the eight dogs.

Immunohistochemistry

Immunohistochemistry was performed in samples of eight dogs. Four μm thick sections were deparaffinized and hydrated in a series of decreasing ethanol concentration. Antigen retrieval was performed by heating the slides to 121°C for 10 min in 10 mM sodium citrate buffer, pH 6.0. Blocking included incubation with 3% hydrogen peroxide diluted in distilled water for 10 min followed by a protein free blocking buffer (#X0909; Dako, Carpinteria, CA, USA) diluted 1:10 in distilled water for 7 min, both at room temperature (approximately 22°C). Tissues were then incubated with an anti-human TGR5 polyclonal antibody (#PA5-27076; Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 for 1 h at room temperature. For negative controls, the primary antibody was substituted with rabbit IgG in consecutive sections from each tissue. Slides were washed 3 times for 5 min each in 1x Tris-buffered saline with Tween 20 (TBST) and detection was performed using a horseradish peroxidase polymer (#TL-060-HL; Thermo Fisher Scientific, Fremont, CA, USA), followed by incubation for 1 min with 1% 3,3'-diaminobenzidine chromogen (#34002; Thermo Fisher Scientific, Rockford, IL, USA). Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with a xylene-based non-aqueous mounting media. Slides of tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas were examined by conventional light microscopy (Olympus BX43). The intensity for TGR5 immunolabeling was recorded for each cell type (i.e., hepatocytes, simple columnar epithelium, ganglion cells, etc.) in the different tissues as follows: (-) no positive cells, (+) only membranous labeling, (++) membranous and weak cytoplasmic labeling, and (+++) membranous and strong cytoplasmic labeling. Slides were photographed with a DP73 camera using the Olympus cellSens Standard platform (Olympus, Tokyo, Japan) and images were exported into Adobe Photoshop CC (Adobe, San Jose, CA, USA) for adjustment of contrast and final preparation of figures.

RNA in situ hybridization

In situ hybridization (ISH) using the RNAscope 2.5 assay (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) was performed with 18 specific probes targeting the 46-1,309 region of predicted canine G protein-coupled bile acid receptor 1/TGR5 (reference sequence:

XM_005640641.2) for detection of mRNA in individual cells. The assay was performed in samples of tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas of two control dogs. Four μm thick formalin-fixed, paraffin-embedded sections of the tissues were mounted on charged slides and incubated in an oven at 60°C for 1 h, then deparaffinized in xylene, followed by dehydration with 100% ethanol and distilled water. Tissue sections were air-dried and incubated with hydrogen peroxide for 10 min at room temperature and rinsed in distilled water. Then, samples were boiled for 30 min at 98-102°C in a pretreatment solution and washed in distilled water followed by 100% ethanol. Slides were air-dried and incubated with RNAscope protease reagent for 30 min at 40°C in a hybridization oven. After being rinsed with distilled water, tissues were hybridized with GPBAR1 or control probes at 40°C for 2h in a hybridization oven. Hybridization with AMP1-AMP6 reagents was performed as follows: AMP1 (40°C for 30 min), AMP2 (40°C for 15 min), AMP3 (40°C for 30 min), AMP4 (40°C for 15 min), AMP5 (room temperature for 1 h), and AMP6 (room temperature for 15 min). Between each hybridization step, slides were washed with wash buffer for three times of 5 min each. The signal amplification was performed with a horseradish peroxidase-based system followed by chromogenic detection with 3,3'-diaminobenzidine. Slides were counterstained with Gill's hematoxylin and mounted with a xylene-based non-aqueous mounting media. Consecutive sections were incubated with a positive control probe targeting canine RNA polymerase II subunit A (POLR2A) to verify RNA quality and a negative control probe targeting non-specific bacterial RNA (dapB gene). All slides were examined by conventional light microscopy (Olympus BX43) and photographed with a digital camera (Olympus DP73) using Olympus cellSens Standard platform (Olympus, Tokyo, Japan). For semi-quantitative evaluation of ISH slides, five 40x fields with groups of cell types were photographed for each slide. The number of cells and dots were manually counted and recorded. Each dot represents one copy of mRNA (Wang et al., 2012). The mean number of dots per cell was calculated. The labeling was categorized into four scores, according with the manufacture's guidelines: (-) negative, no staining or less than 1 dot to every 10 cells; (+) mild, 1-3 dots/cell; (++) moderate, 4-10 dots/cell with very few dot clusters, (+++) marked, >10 dots/cell with dot clusters (Wang et al., 2012). Images were exported into Adobe Photoshop CC (Adobe, San Jose, CA, USA) for adjustment of contrast and final preparation of figure panels.

Colocalization of TGR5 mRNA and IBA-1 antigen in mononuclear cells

Because TGR5-positive mononuclear cells throughout the gastrointestinal tract had moderate amount of cytoplasm, we hypothesized that these cells were macrophages. For characterization of TGR5-positive mononuclear cells, double labeling for TGR5 using RNA ISH and immunohistochemistry for ionized calcium-binding adapter molecule 1 (IBA-1)[18] was performed. IBA-1 is considered a pan-macrophage marker because it is expressed in monocytes and subpopulations of macrophages (Köhler, 2007). RNA ISH was performed using a brown chromogen in a sample of ileum. Slides were rinsed and incubated with anti-IBA1 antibody (#019-19741, Wako Pure Chemical Industries, Richmond, USA) diluted to 1:400 for 1 h at room temperature. The primary antibody incubation was followed by rinsing with 1x TBST and incubation with a polymer (#TL-060-HL; Thermo Fisher Scientific, Fremont, CA, USA). Detection was performed with a red 3,3'-diaminobenzidine chromogen diluted at 1% and incubated for 1 min (#SK-5105; Vector Laboratories, Burlingame, CA, USA), followed by counterstaining with Mayer's hematoxylin. Slides were dehydrated and mounted with a xylene-based non-aqueous mounting media.

Results

Immunohistochemistry localization and scoring

TGR5-immunoreactive cells were broadly distributed in gastrointestinal tissues with membranous or membranous and cytoplasmic immunolabeling (Table 1). In the tongue, the membrane of epithelial cells in the stratum basale and stratum spinosum was positive (Fig. 1A). In the connective tissue, the cytoplasm and membrane of ganglion cells, mononuclear cells, and endothelial cells were labeled. The glossal striated muscle was negative.

In the esophagus, throughout the lamina propria of the mucosa to the adventitia, ganglion cells, endothelial cells, and mononuclear cells were consistently positive. Although the submucosal glands were negative, surrounding myoepithelial cells showed strong cytoplasmic and membranous immunolabeling (Fig. 1B). In contrast, the stratified squamous epithelium, the muscularis mucosae, and the muscularis externa were negative.

In the stomach, the simple columnar epithelium in the fundus and pylorus was positive, mainly in the region of the mucous neck cells of the gastric pits (Fig. 1C). The cytoplasm and membrane of parietal cells showed a strong signal (Fig. 1D). On the other hand, chief cells were positive only on the cell membrane (Fig. 1D). Both the inner and outer layers of the muscularis externa had immunolabeling in the membrane and cytoplasm of leiomyocytes. Mononuclear cells, endothelial cells, and ganglion cells in the submucosal and myenteric plexi were consistently positive. The muscularis mucosae was negative.

In the small and large intestine, the distribution was similar throughout all the segments. The membrane of enterocytes and goblet cells was positive (Fig. 2A). Enteroendocrine cells in the crypts and mononuclear cells distributed in all layers, including Peyer's patches, showed a strong cytoplasmic and membranous signal (Fig. 2B). Ganglion cells (Fig. 2C) and endothelial cells were consistently positive. In the muscularis externa, both the inner and outer layers had membranous and cytoplasmic immunolabeling (Fig. 2D). In contrast, the muscularis mucosae was negative.

In the liver, hepatocytes had membranous labeling on the canalicular surface (Fig. 3A). Kupffer cells, epithelial cells of bile ducts (Fig. 3B), and endothelial cells of portal and centrilobular veins (Fig. 3C) as well as portal arterioles were positive both in the cytoplasm and the cell membrane. In contrast, hepatic sinusoids were negative. In the gallbladder, the simple columnar epithelium (Fig. 3D) and smooth muscle showed labeling in the membrane and cytoplasm. In the lamina propria of the gallbladder, mononuclear cells, ganglion cells, and endothelial cells were also consistently positive.

In the pancreas, approximately 40% of the cells in the islets of Langerhans exhibited membranous and cytoplasmic labeling (Fig. 3E). Although the epithelial cells of the pancreatic ducts were labeled (Fig. 3F), the exocrine acinar cells were consistently negative. Mononuclear cells, ganglion cells, as well as endothelial cells were positive.

Semi-quantitative scoring of RNA in situ hybridization

Throughout the gastrointestinal tract, TGR5 mRNA expression was located mainly in the nucleus of the majority of the cells, but occasionally also in the cytoplasm (Table 1).

In the tongue, esophagus, stomach, and small and large intestines, mild TGR5 mRNA expression was detected in epithelial cells (insets of Figs. 1A,D, 2A), mononuclear cells, endothelial cells, and ganglion cells (inset of Fig. 2C). Mucous neck cells of the gastric pits showed moderate expression of TGR5 mRNA (inset of Fig. 1C). Myoepithelial cells around the esophageal glands (inset of Fig. 1B), enteroendocrine cells (inset of Fig. 2B), and the muscularis externa of stomach and intestines (inset of Fig. 2D) also had mild TGR5 mRNA expression. The esophageal glands, the muscularis mucosae from esophagus to rectum and the striated muscle from tongue and esophagus were negative.

In the liver, mild TGR5 mRNA expression was detected in hepatocytes (inset of Fig. 3A), epithelium of bile ducts (inset of Fig. 3B), and endothelium of centrilobular (inset of Fig. 3C) and portal veins and portal arterioles. Additionally, Kupffer cells had mild mRNA expression. In contrast, the hepatic sinusoids were negative. The smooth muscle in the gallbladder showed moderate TGR5 mRNA expression, while the epithelium (inset of Fig. 3D), endothelial cells,

Table 1. Distribution and scoring of TGR5 protein (immunohistochemistry) and mRNA (in situ hybridization) expression along the canine gastrointestinal tract.

Tissues/Cells	IHC[a]	ISH[b]
Tongue		
Keratinized stratified squamous epithelium	+	+
Macrophages	+++	+
Endothelial cells	++	+
Ganglion cells	++	+
Striated muscle	-	-
Esophagus		
Non-keratinized stratified squamous epithelium	-	+
Macrophages	+++	+
Esophageal glands	-	-
Myoepithelial cells	+++	+
Endothelial cells	++	+
Ganglion cells	++	+
Muscularis mucosae	-	-
Striated muscle	-	-
Stomach		
Simple columnar epithelium	+	+
Mucous neck cells	++	++
Parietal cells	+++	+
Chief cells	+	+
Macrophages	+++	+
Endothelial cells	++	+
Ganglion cells (submucosal and myenteric plexi)	++	+
Muscularis mucosae	-	-
Muscularis externa	++	+
Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum		
Enterocytes/goblet cells	+	+
Enteroendocrine cells	+++	+
Macrophages	+++	+
Endothelial cells	++	+
Ganglion cells (submucosal and myenteric plexi)	++	+
Muscularis mucosae	-	-
Muscularis externa	++	+
Liver		
Hepatocytes	+	+
Biliary epithelium	++	+
Kupffer cells	+++	+
Sinusoids	-	-
Endothelial cells of veins and arteries	++	+
Gallbladder		
Simple columnar epithelium	++	+
Macrophages	+++	+
Endothelial cells	++	+
Ganglion cells	++	+
Smooth muscle	++	++
Pancreas		
Islet cells	++	+
Acinar cells	-	+
Pancreatic ductular epithelium	+	+
Macrophages	+++	+
Endothelial cells	++	+
Ganglion cells	++	+

[a]: (-) no positive cells, (+) only membranous labeling, (++) membranous and weak cytoplasmic labeling, and (+++) membranous and strong cytoplasmic labeling. [b]: (-) negative, no staining or less than 1 dot to every 10 cells; (+) mild, 1-3 dots/cell; (++) moderate, 4-10 dots/cell with very few dot clusters.

mononuclear cells, and ganglion cells had mild expression. In the pancreas, the expression of TGR5 mRNA in exocrine acinar cells, islet cells (inset of Fig. 3E), ductular epithelium (inset of Fig. 3F), ganglion cells, and mononuclear cells was mild.

Colocalization of TGR5 mRNA and IBA-1 antigen in mononuclear cells

Mononuclear cells in the lamina propria of the ileum demonstrated both TGR5 mRNA expression in the nucleus and IBA-1 immunolabeling in the cytoplasm. This indicates that cells of monocytic/macrophagic lineage in dogs express TGR5.

Discussion

Here, we characterized the distribution of TGR5 in cells of the canine gastrointestinal tract using two in situ methods: immunohistochemistry and RNA in situ hybridization (RNA ISH). We elected to investigate TGR5 expression in the gastrointestinal tract to target the tissues where BA acids are produced, excreted, and found with the highest concentration within the body. We demonstrated that all the cells that expressed TGR5 antigen also expressed TGR5 mRNA. Most of the previous studies in other species have used real-time reverse transcription PCR (RT-qPCR) to describe TGR5 expression (Kawamata et al., 2003; Vassileva et al.,

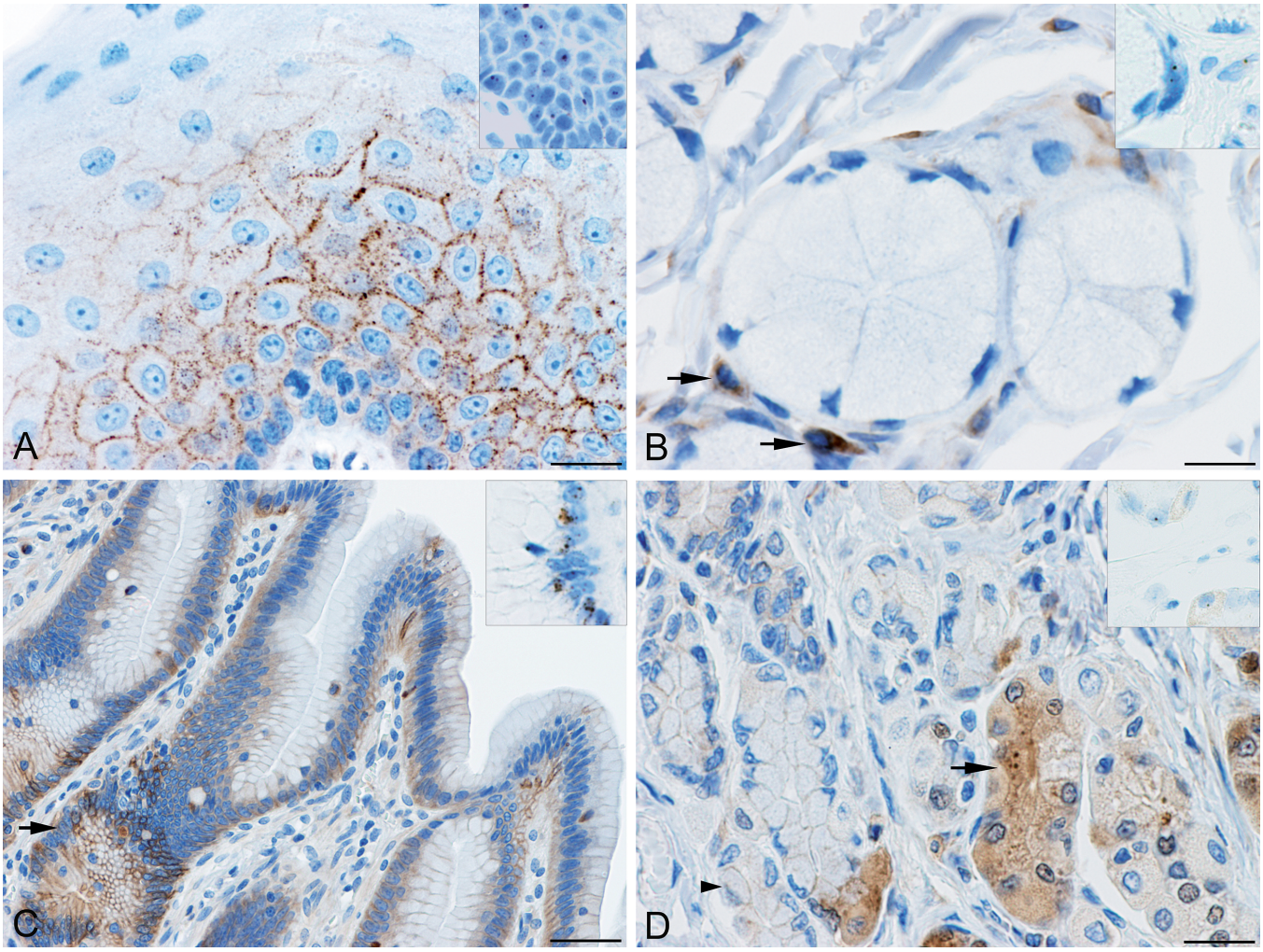

Fig. 1. Immunohistochemistry and RNA in situ hybridization (insets) for TGR5 in the canine upper gastrointestinal tract. **A.** Tongue. Epithelial cells of the stratum basale and spinosum with membranous immunolabeling for TGR5. **B.** Esophagus. The cytoplasm of myoepithelial cells (arrows) surrounding esophageal glands is positive. **C.** Stomach. Superficial simple columnar epithelium with membranous immunolabeling while the mucous neck cells in the gastric pits are labeled in the membrane and cytoplasm (arrow). **D.** Stomach. Parietal cells are markedly positive (arrow) while chief cells (arrowhead) show only membranous labeling. Insets: The same cells that were positive on immunohistochemistry demonstrated mRNA nuclear expression on in situ hybridization. Scale bars: A, D, 20 μm; B, 15 μm; C, 50 μm.

2006; Watanabe et al., 2006). While RT-qPCR is considered the gold-standard method to evaluate gene expression in tissues (Wong and Medrano, 2005) this technique does not preserve the tissue morphology and does not allow the localization of RNA within individual cells (Wang et al., 2012). On the other hand, RNAscope, a novel RNA ISH, is a highly sensitive and specific technology to demonstrate and quantify mRNA within cells (Wang et al., 2012) and therefore was used to cross-validate the immunohistochemistry results in this study.

TGR5 receptors were ubiquitously distributed in the upper and lower gastrointestinal tract as well as in the liver, gallbladder, and pancreas of dogs. Overall, the broad distribution of TGR5 in dogs is similar to what has been described in other species (Stepanov et al., 2013). In mice, TGR5 mRNA has a high expression in the gallbladder (Vassileva et al., 2006), liver, intestine, and brown adipose tissue (Watanabe et al., 2006). In our study, the gallbladder and stomach were the organs containing cells with the highest expression of TGR5 mRNA. Beyond the gastrointestinal tract, expression of TGR5 has been reported in many human and animal tissues, including heart, blood vessels, spleen, kidney, nervous system, and placenta (Kawamata et al., 2003).

Although TGR5 is a membranous receptor, internalization of the receptor and cytoplasmic distribution have been previously reported (Kawamata et al., 2003; Cao et al., 2013). This distribution was confirmed by our study, where the antigen was observed on the membrane only, or on the membrane and

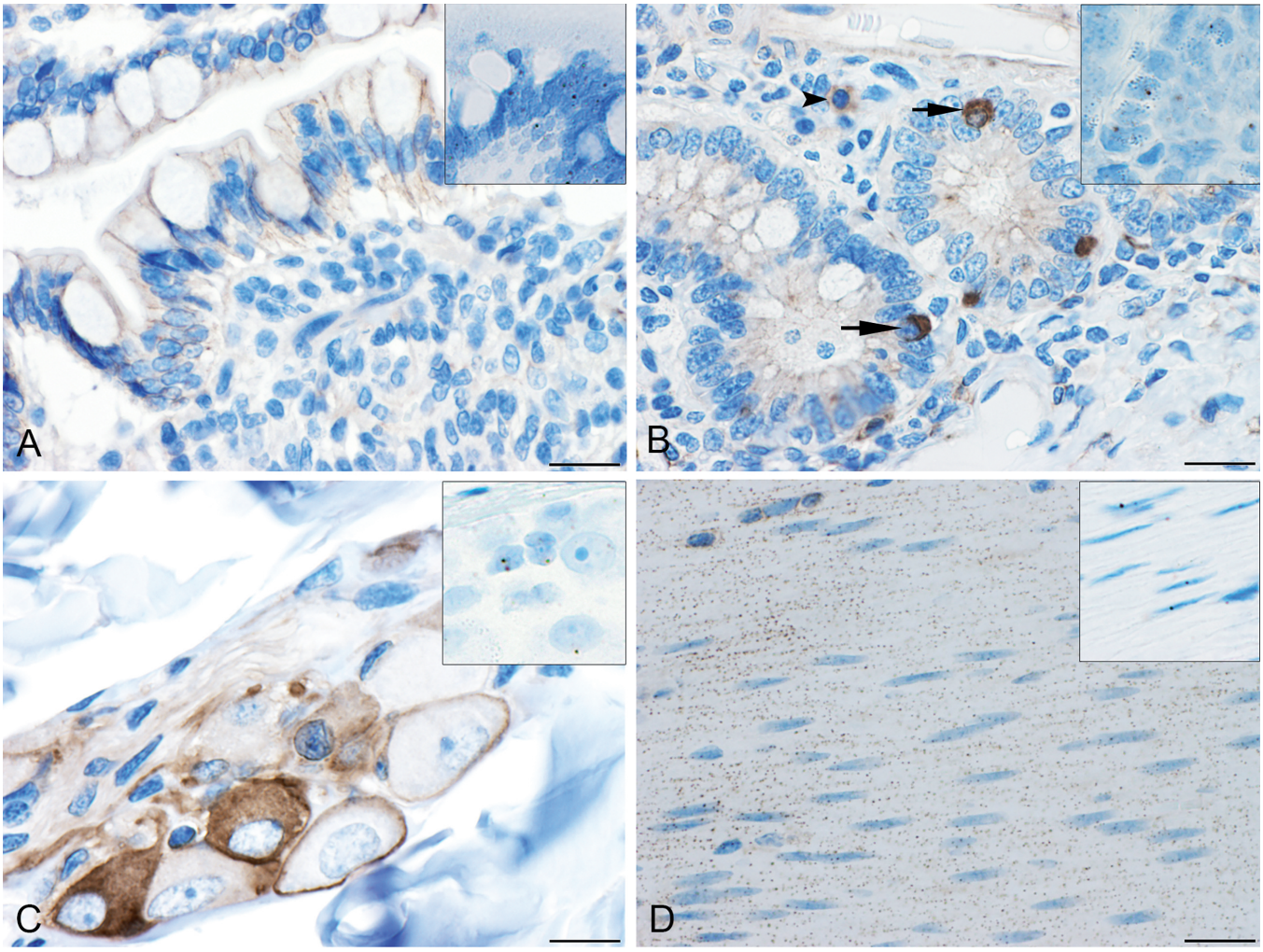

Fig. 2. Immunohistochemistry and RNA in situ hybridization (insets) for TGR5 in the canine lower gastrointestinal tract. **A.** Enterocytes and goblet cells with membranous immunolabeling in the ileum. **B.** Enteroendocrine cells in the crypts (arrows) and macrophage (arrowhead) in the lamina propria of the duodenum with strong cytoplasmic and membranous immunolabeling. **C.** Ganglion cells in a submucosal plexus of the duodenum show labeling in the cytoplasm and membrane. **D.** Punctate immunolabeling in the membrane and cytoplasm of leiomyocytes in the muscularis externa of the jejunum. Insets: The same cells with TGR5 antigen demonstrated mRNA nuclear expression. Scale bars: A, B, D, 20 μm; C, 15 μm.

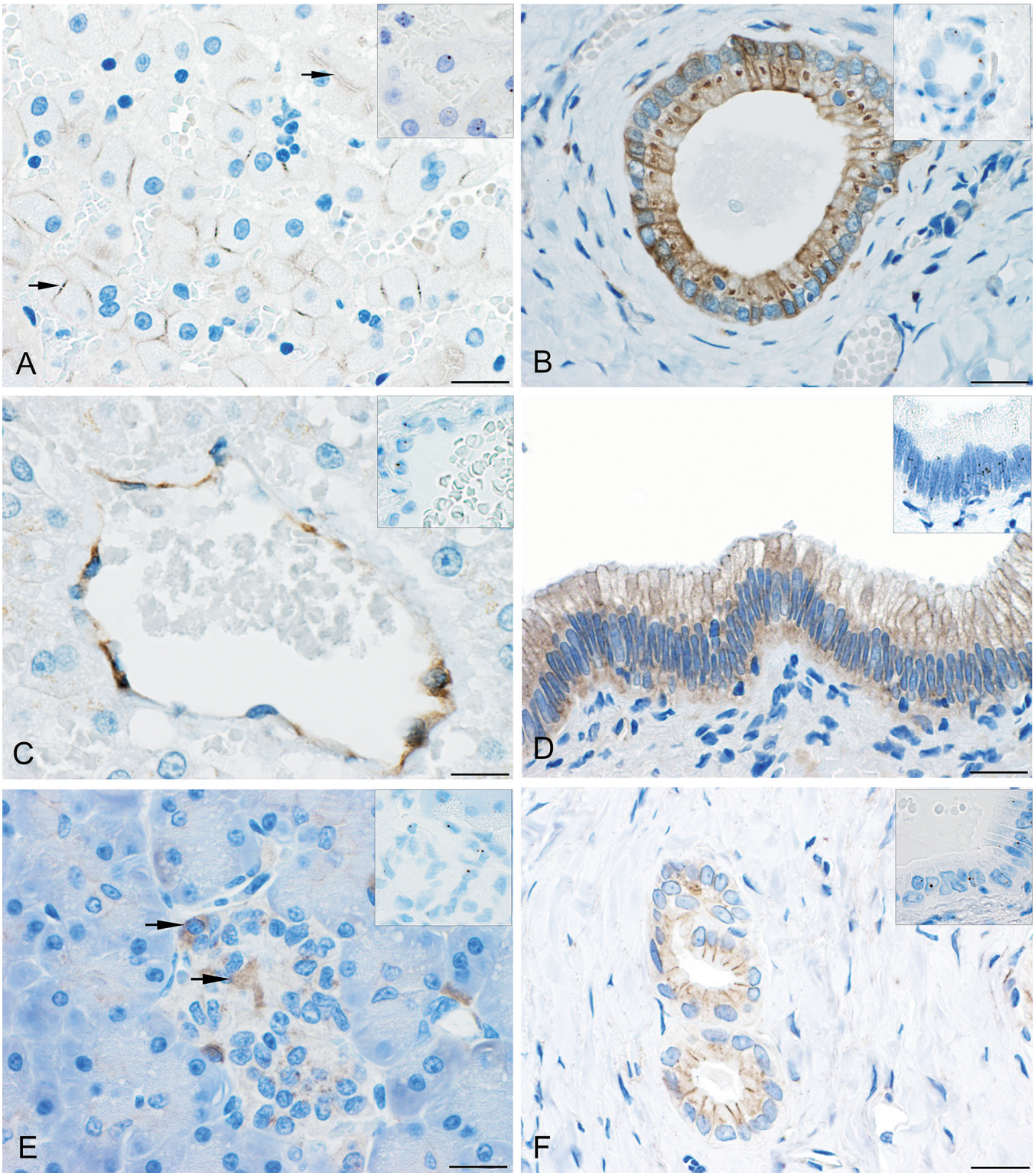

Fig. 3. Immunohistochemistry and RNA in situ hybridization (insets) for TGR5 in the canine liver, gallbladder, and pancreas. **A.** Liver. The canalicular surface of the hepatocellular membrane (arrows) is positive and the sinusoidal surface is negative. **B.** Liver. Epithelial cells of bile ducts are immunolabeled in the cytoplasm and membrane. **C.** Liver. Endothelial cells of a centrilobular vein with granular antigen expression in the cytoplasm and membrane. **D.** Gallbladder. Epithelial cells with cytoplasmic and membranous immunolabeling. **E.** Pancreas. Cells in the islets of Langerhans (arrows) were positive for TGR5 antigen in the cytoplasm and membrane. **F.** Pancreas. Epithelial cells of the interlobular ducts are labeled on the membrane. Insets: The same cells with TGR5 antigen demonstrated mRNA nuclear expression. Scale bars: A, B, D, F, 20 μm; C, E, 15 μm.

cytoplasm. Hepatocytes showed a unique pattern of immunolabeling, expressing TGR5 on the canalicular surface of the cell membrane, but not on the sinusoidal surface. Expression of TGR5 RNA in hepatocytes has previously been demonstrated in a human cell line derived from hepatocellular carcinoma (Yang et al., 2007). Our findings, however, differ from rats, where TGR5 is not expressed by hepatocytes but it is found in sinusoidal endothelial cells (Keitel et al., 2007). Despite the fact that the esophageal mucosa and the acinar cells of the pancreas were negative on immunohistochemistry, RNA ISH showed that these cells express TGR5 mRNA. This divergence could be due to the fact that gene expression techniques, such as ISH, are more sensitive than immunohistochemistry or because the levels of expressed genes are not high enough for translated protein expression (Pascal et al., 2008). Alternatively, TGR5 may be transcribed, but not translated, but this would seem unlikely.

Overall, macrophages and endothelial cells distributed across all examined gastrointestinal segments were consistently positive for TGR5, reinforcing the concept that bile acids present not only in the gastrointestinal lumen but also in the systemic circulation are relevant for activation of this receptor (Schaap et al., 2014). Many tissue macrophages, including Kupffer cells, alveolar macrophages, microglia, as well as monocytes of humans, mice, rats, and rabbits have been shown to express TGR5 (Kawamata et al., 2003; Keitel et al., 2007, 2008; Yanguas-Casas et al., 2017). TGR5 activation in macrophages and Kupffer cells has an anti-inflammatory effect due to inhibition of the nuclear factor NF-$\varkappa$B pathway, decreasing the expression of inflammatory interleukins and tumor necrosis factor-α (TNFα) (Keitel et al., 2008; Pols et al., 2011; Wang et al., 2011). TGR5 expression is increased in the colon of patients with Crohn's disease due to infiltration of macrophages (Cipriani et al., 2011; Yoneno et al., 2013). Interestingly, in a murine model of colitis, TGR5 agonists have been shown to shift classically activated macrophages to an alternatively activated phenotype (Biagioli et al., 2017). TGR5 agonists increase the production of nitric oxide and suppress the expression of adhesion molecules in the endothelial cells of bovine and human vessels (Kida et al., 2013). A study with infusion of low doses of a TGR5 agonist in dogs provoked an undesirable and marked reduction in peripheral vascular tone and blood pressure (Fryer et al., 2014). This might be explained by the broad expression of TGR5 in endothelial cells of dogs, with receptor activation possibly leading to vasodilation and hypotension.

Regarding cells that promote gastrointestinal motility, TGR5 was widely distributed in ganglion cells of the submucosal and myenteric plexi and in leiomyocytes of the muscularis externa of the stomach, intestines, and gallbladder. Similar expression has been observed in the muscularis of the gallbladder and muscularis externa of the small intestine of mice (Poole et al., 2010; Li et al., 2011) and in the gastrointestinal ganglion cells of mice (Poole et al., 2010) and rats (Duboc et al., 2016). TGR5 activation in myenteric ganglion cells (Poole et al., 2010) or leiomyocytes of the stomach (Rajagopal et al., 2013) and gallbladder (Lavoie et al., 2010) causes smooth muscle relaxation. In mice, TGR5 agonists produce increased gallbladder filling and can potentially increase the risk of gallstone formation (Lavoie et al., 2010; Li et al., 2011). In the colon of rats, TGR5 agonists inhibit ganglion cells of the submucosal plexi, leading to decreased colonic secretions (Duboc et al., 2016). The muscularis mucosae of the examined tissues as well as the striated muscle of tongue and esophagus did not express TGR5 in dogs. To date, there are no reports describing TGR5 expression in the muscularis mucosae of other species. Although the muscularis mucosae is composed of smooth muscle, its distinct autonomic innervation and function (Uchida and Kamikawa, 2007), with independent peristaltic movement from the muscularis externa (Ross and Pawlina, 2015), could account for the lack of expression of TGR5. Despite the expression of TGR5 in the skeletal muscle of humans (Kawamata et al., 2003; Watanabe et al., 2006), the predominant type of striated fibers found in the esophagus of dogs greatly differ from the canine skeletal striated muscle, which could explain the differences in expression (Mascarello et al., 1984).

In the enteric endocrine system, enteroendocrine cells distributed in the crypts throughout the small and large intestine expressed TGR5. In humans and mice, TGR5 is known to be expressed in L cells, where ligands induce secretion of glucagon-like peptide-1 (GLP-1), which promotes insulin release from pancreatic β cells and increases insulin sensitivity (Katsuma et al., 2005). Although the type of enteroendocrine cells was not investigated in this study, L cells in dogs can be present in all portions of small and large intestines (Kubes et al., 1974; Peranzi and Lehy, 1984). Interestingly, in our study, cells of the islets of Langerhans were positive for TGR5. In mice, both α and β islet cells can express TGR5 (Whalley et al., 2011). The function of TGR5 activation in β cells is to increase insulin secretion (Kumar et al., 2012).

As far as gastrointestinal epithelial cells, TGR5 expression has been described in the esophageal (Hong et al., 2010), gastric (Cao et al., 2013), and gallbladder epithelium (Keitel et al., 2009) of humans as well as in biliary (Keitel et al., 2010) and pancreatic ducts (Kowal et al., 2015). TGR5 is expressed in the small intestine (Poole et al., 2010), gallbladder (Vassileva et al., 2006), and in bile duct epithelia (Keitel et al., 2010) of mice as well as in the large intestine (Ward et al., 2013), bile ducts (Masyuk et al., 2013), and pancreatic acini of rats (Kowal et al., 2015), similarly to what we observed in the dogs. TGR5 activation in colonocytes of rats has been shown to play an anti-secretory role (Ward et al., 2013; Duboc et al., 2016). Its activation in cholangiocytes has a protective effect, inducing secretion of chloride and bicarbonate as well as regulating cell

proliferation (Li et al., 2011; Masyuk et al., 2013). In the pancreatic ductular epithelium, TGR5 can stimulate a sodium/calcium exchange (Kowal et al., 2015). In disease, the receptor has been demonstrated to be overexpressed in esophageal dysplasia, esophageal adenocarcinoma (Marketkar et al., 2017), and in the intestinal-type of gastric adenocarcinoma in people (Cao et al., 2013). TGR5 agonists can induce proliferation in cells of esophageal adenocarcinoma (Hong et al., 2010). Noteworthy, the reflux of bile into the stomach and esophagus is a risk factor for the development of gastric and esophageal cancer in humans (Lundegårdh et al., 1994).

Preclinical studies have suggested that targeting TGR5 might be promising for the treatment of several metabolic conditions in humans, such as type 2 diabetes, obesity, and non-alcoholic steatohepatitis (van Nierop et al., 2017). In addition, the anti-inflammatory effect of TGR5 in macrophages has drawn attention to a possible modulatory role of bile acids in patients with inflammatory conditions, such as colitis and atherosclerosis (Pols et al., 2011; Biagioli et al., 2017). Dogs can serve as a model to study type 2 diabetes (Ionut et al., 2010) as well as develop chronic enteropathy that shares similarities with inflammatory bowel disease in humans (Cerquetella et al., 2010). In addition, clinical cancer research has become more common in companion animals and the gained information may ultimately benefit both pets and humans (Burton and Khanna, 2014). Because dogs are one of the species used in preclinical studies, it is imperative to better understand their bile acids metabolism and distribution of their receptors. The broad distribution of the TGR5 receptor in multiple tissues as observed in this study remains the greatest challenge for drug development and targeted therapy due to systemic effects.

Acknowledgements. PRG has a fellowship from the Brazilian National Council for Scientific and Technological Development (CNPq).

References

Aldini R., Montagnani M., Roda A., Hrelia S., Biagi P.L. and Roda E. (1996). Intestinal absorption of bile acids in the rabbit: Different transport rates in jejunum and ileum. Gastroenterology 110, 459-468.

Biagioli M., Carino A., Cipriani S., Francisci D., Marchiano S., Scarpelli P., Sorcini D., Zampella A. and Fiorucci S. (2017). The bile acid receptor GPBAR1 regulates the M1/M2 phenotype of intestinal macrophages and activation of GPBAR1 rescues mice from murine colitis. J. Immunol. 199, 718-733.

Burton J. and Khanna C. (2014). The role of clinical trials in veterinary oncology. Vet. Clin. North Am. Small Anim. Pract. 44, 977-987.

Cao W., Tian W., Hong J., Li D., Tavares R., Noble L., Moss S.F. and Resnick M.B. (2013). Expression of bile acid receptor TGR5 in gastric adenocarcinoma. Am. J. Physiol. Gastrointest. Liver Physiol. 304, 322-327.

Cerquetella M., Spaterna A., Laus F., Tesei B., Rossi G., Antonelli E., Villanacci V. and Bassotti G. (2010). Inflammatory bowel disease in the dog: differences and similarities with humans. World J. Gastroenterol. 16, 1050-1056.

Chiang J.Y. (2009). Bile acids: regulation of synthesis. J. Lipid Res. 50, 1955-1966.

Cipriani S., Mencarelli A., Chini M.G., Distrutti E., Renga B., Bifulco G., Baldelli F., Donini A. and Fiorucci S. (2011). The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. PLoS One 8, e10.1371.

Costanzo L.S. (2013). Gastrointestinal physiology. In: Physiology. 5th ed. Costanzo LS (ed). Elsevier. Philadelphia. pp 339-393.

Day M.J., Bilzer T., Mansell J., Wilcock B., Hall E.J., Jergens A., Minami T., Willard M. and Washabau R. (2008). Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J. Comp. Pathol. 138, S1-43.

Duboc H., Tache Y. and Hofmann A.F. (2014). The bile acid TGR5 membrane receptor: from basic research to clinical application. Dig. Liver Dis. 46, 302-312.

Duboc H., Tolstanova G., Yuan P.Q., Wu V., Kaji I., Biraud M., Akiba Y., Kaunitz J., Million M., Tache Y. and Larauche M. (2016). Reduction of epithelial secretion in male rat distal colonic mucosa by bile acid receptor TGR5 agonist, INT-777: role of submucosal neurons. Neurogastroenterol. Motil. 28, 1663-1676.

Fryer R.M., Ng K.J., Nodop Mazurek S.G., Patnaude L., Skow D.J., Muthukumarana A., Gilpin K.E., Dinallo R.M., Kuzmich D., Lord J., Sanyal S., Yu H., Harcken C., Cerny M.A., Hickey E.R. and Modis L.K. (2014). G protein–coupled bile acid receptor 1 stimulation mediates arterial vasodilation through a K(Ca)1.1 (BK(Ca))-dependent mechanism. J. Pharmacol. Exp. Ther. 348, 421-431.

Gad S.C. (2010). Nonrodent animal studies. Drug safety evaluation. In: Pharmaceutical sciences encyclopedia. 2nd ed. Gad S.C. (ed). John Wiley & Sons. New York. pp 400-440.

Grober J., Zaghini I., Fujii H., Jones S.A., Kliewer S.A., Willson T.M., Ono T. and Besnard P. (1999). Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. J. Biol. Chem. 274, 29749-29754.

Guard B.C., Honneffer J.B., Jonika M.M., Lidbury J.A., Steiner J.M., Jergens A. and Suchodolski J.S. (2017). Tu1864 - Longitudinal characterization of dysbiosis and unconjugated bile acid profiles in the feces of dogs with inflammatory bowel disease. Gastroenterology 152, S992.

Guo C., Chen W.D. and Wang Y.D. (2016). TGR5, not only a metabolic regulator. Front. Physiol. 7, e646.

Hong J., Behar J., Wands J., Resnick M., Wang L.J., DeLellis R.A., Lambeth D., Souza R.F., Spechler S.J. and Cao W. (2010). Role of a novel bile acid receptor TGR5 in the development of oesophageal adenocarcinoma. Gut 59, 170-180.

Ionut V., Liu H., Mooradian V., Castro A.V.B., Kabir M., Stefanovski D., Zheng D., Kirkman E.L. and Bergman R.N. (2010). Novel canine models of obese prediabetes and mild type 2 diabetes. Am. J. Physiol. Endocrinol. Metab. 298, e38-48.

Katsuma S., Hirasawa A. and Tsujimoto G. (2005). Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem. Biophys. Res. Commun. 329, 386-390.

Kawamata Y., Fujii R., Hosoya M., Harada M., Yoshida H., Miwa M., Fukusumi S., Habata Y., Itoh T., Shintani Y., Hinuma S., Fujisawa Y. and Fujino M. (2003). A G protein-coupled receptor responsive to bile acids. J. Biol. Chem. 278, 9435-9440.

Keitel V., Reinehr R., Gatsios P., Rupprecht C., Gorg B., Selbach O., Haussinger D. and Kubitz R. (2007). The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology 45, 695-704.

Keitel V., Donner M., Winandy S., Kubitz R. and Haussinger D. (2008). Expression and function of the bile acid receptor TGR5 in Kupffer cells. Biochem. Biophys. Res. Commun. 372, 78-84.

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

Keitel V., Gorg B., Bidmon H.J., Zemtsova I., Spomer L., Zilles K. and Haussinger D. (2010). The bile acid receptor TGR5 (Gpbar-1) acts as a neurosteroid receptor in brain. Glia 58, 1794-1805.

Kida T., Tsubosaka Y., Hori M., Ozaki H. and Murata T. (2013). Bile acid receptor TGR5 agonism induces NO production and reduces monocyte adhesion in vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 33, 1663-1669.

Köhler C. (2007). Allograft inflammatory factor-1/Ionized calcium-binding adapter molecule 1 is specifically expressed by most subpopulations of macrophages and spermatids in testis. Cell Tissue Res. 330, 291-302.

Kowal J.M., Haanes K.A., Christensen N.M. and Novak I. (2015). Bile acid effects are mediated by ATP release and purinergic signalling in exocrine pancreatic cells. Cell Commun. Signal. 13, 28.

Kubes L., Jirasek K. and Lomsky R. (1974). Endocrine cells of the dog gastrointestinal mucosa. Cytologia 39, 179-194.

Kumar D.P., Rajagopal S., Mahavadi S., Mirshahi F., Grider J.R., Murthy K.S. and Sanyal A.J. (2012). Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic beta cells. Biochem. Biophys. Res. Commun. 427, 600-605.

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

Li T., Holmstrom S.R., Kir S., Umetani M., Schmidt D.R., Kliewer S.A. and Mangelsdorf D.J. (2011). The G protein-coupled bile acid receptor, TGR5, stimulates gallbladder filling. Mol. Endocrinol. 25, 1066-1071.

Lundegårdh G., Adami H.O., Helmick C. and Zack M. (1994). Risk of cancer following partial gastrectomy for benign ulcer disease. Br. J. Surg. 81, 1164-1167.

Marketkar S., Li D., Yang D. and Cao W. (2017). TGR5 expression in benign, preneoplastic and neoplastic lesions of Barrett's esophagus: Case series and findings. World J. Gastroenterol. 23, 1338-1344.

Mascarello F., Rowlerson A. and Scapolo P.A. (1984). The fibre type composition of the striated muscle of the oesophagus in ruminants and carnivores. Histochemistry 80, 277-288.

Masyuk A.I., Huang B.Q., Radtke B.N., Gajdos G.B., Splinter P.L., Masyuk T.V., Gradilone S.A. and LaRusso N.F. (2013). Ciliary subcellular localization of TGR5 determines the cholangiocyte functional response to bile acid signaling. Am. J. Physiol. Gastrointest. Liver Physiol. 304, 1013-1024.

Melgarejo T., Williams D.A., O'Connell N.C. and Setchell K.D. (2000). Serum unconjugated bile acids as a test for intestinal bacterial overgrowth in dogs. Dig. Dis. Sci. 45, 407-414.

Paoloni M. and Khanna C. (2008). Translation of new cancer treatments from pet dogs to humans. Nat. Rev. Cancer 8, 147-156.

Pascal L.E., True L.D., Campbell D.S., Deutsch E.W., Risk M., Coleman I.M., Eichner L.J., Nelson P.S. and Liu A.Y. (2008). Correlation of mRNA and protein levels: Cell type-specific gene expression of cluster designation antigens in the prostate. BMC Genomics 9, 246.

Peranzi G. and Lehy T. (1984). Endocrine cell populations in the colon and rectum of cat, dog, and monkey: fine structure, immunocytochemistry, and distribution. Anat. Rec. 210, 87-100.

Pols T.W., Nomura M., Harach T., Lo Sasso G., Oosterveer M.H., Thomas C., Rizzo G., Gioiello A., Adorini L., Pellicciari R., Auwerx J. and Schoonjans K. (2011). TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. Cell Metab. 14, 747-757.

Poole D.P., Godfrey C., Cattaruzza F., Cottrell G.S., Kirkland J.G., Pelayo J.C., Bunnett N.W. and Corvera C.U. (2010). Expression and function of the bile acid receptor GpBAR1 (TGR5) in the murine enteric nervous system. Neurogastroenterol. Motil. 22, 814-825.

Rajagopal S., Kumar D.P., Mahavadi S., Bhattacharya S., Zhou R., Corvera C.U., Bunnett N.W., Grider J.R. and Murthy K.S. (2013). Activation of G protein-coupled bile acid receptor, TGR5, induces smooth muscle relaxation via both Epac- and PKA-mediated inhibition of RhoA/Rho kinase pathway. Am. J. Physiol. Gastrointest. Liver Physiol. 304, 527-535.

Ross M.H. and Pawlina W. (2015). Histology: a text and atlas: with correlated cell and molecular biology. 6th ed. Wolters Kluwer Health. Baltimore. pp 929.

Russell D.W. (2009). Fifty years of advances in bile acid synthesis and metabolism. J. Lipid Res. 50, S120-125.

Schaap F.G., Trauner M. and Jansen P.L.M. (2014). Bile acid receptors as targets for drug development. Nat. Rev. Gastroenterol. Hepatol. 11, 55-67.

Stepanov V., Stankov K. and Mikov M. (2013). The bile acid membrane receptor TGR5: a novel pharmacological target in metabolic, inflammatory and neoplastic disorders. J. Recept. Signal. Transduct. Res. 33, 213-223.

Uchida K., and Kamikawa Y. (2007). Muscularis mucosae - the forgotten sibling. J. Smooth Muscle Res. 43, 157-177.

van Nierop F.S., Scheltema M.J., Eggink H.M., Pols T.W., Sonne D.P., Knop F.K. and Soeters M.R. (2017). Clinical relevance of the bile acid receptor TGR5 in metabolism. Lancet Diabetes Endocrinol. 5, 224-233.

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. Jr, Hedrick J. and Gustafson E.L. (2006). Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. Biochem. J. 398, 423-430.

Vlahcevic Z., Heuman D., and Hylemon P. (1996). Physiology and pathophysiology of enterohepatic circulation of bile acids. In: Hepatology: a textbook of liver disease. 3rd ed. Zachim D. and Boyer T.D. (eds.). Saunders. Philadelphia. pp 376-417.

Wang Y.D., Chen W.D., Yu D., Forman B.M. and Huang W. (2011). The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-kappaB) in mice. Hepatology 54, 1421-1432.

Wang F., Flanagan J., Su N., Wang L.C., Bui S., Nielson A., Wu X., Vo

H.T., Ma X.J. and Luo Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 14, 22-29.

Ward J.B., Mroz M.S. and Keely S.J. (2013). The bile acid receptor, TGR5, regulates basal and cholinergic-induced secretory responses in rat colon. Neurogastroenterol. Motil. 25, 708-711.

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

Whalley N.M., Pritchard L.E., Smith DM, and White A. (2011). Processing of proglucagon to GLP-1 in pancreatic alpha-cells: is this a paracrine mechanism enabling GLP-1 to act on beta-cells? J. Endocrinol. 211, 99-106.

Wong M.L. and Medrano J.F. (2005). Real-time PCR for mRNA quantitation. BioTechniques. 39, 75-85.

Yang J.I., Yoon J.H., Myung S.J., Gwak G.Y., Kim W., Chung G.E., Lee S.H., Lee S.M., Kim C.Y. and Lee H.S. (2007). Bile acid-induced TGR5-dependent c-Jun-N terminal kinase activation leads to enhanced caspase 8 activation in hepatocytes. Biochem. Biophys. Res. Commun. 361, 156-161.

Yanguas-Casas N., Barreda-Manso M.A., Nieto-Sampedro M. and Romero-Ramirez L. (2017). TUDCA: an agonist of the bile acid receptor GPBAR1/TGR5 with anti-inflammatory effects in microglial cells. J. Cell Physiol. 232, 2231-2245.

Yoneno K., Hisamatsu T., Shimamura K., Kamada N., Ichikawa R., Kitazume M.T., Mori M., Uo M., Namikawa Y., Matsuoka K., Sato T., Koganei K., Sugita A., Kanai T. and Hibi T. (2013). TGR5 signalling inhibits the production of pro-inflammatory cytokines by in vitro differentiated inflammatory and intestinal macrophages in Crohn's disease. Immunology 139, 19-29.

Accepted July 12, 2018