Title: Hypercalcemia of malignancy in a dog diagnosed with cholangiocellular carcinoma

L. Martínez-Sogues[a,b], A. Vila[a], X. Roura[a], J. Pastor[b], R. Novellas[a,b], A.J. Marco[c], M. Cuvertoret-Sanz[c], J. Martínez[c] and L. Solano-Gallego[b]

[a]*Hospital Clínic Veterinari (HCV),* [b]*Departament de Medicina i Cirurgia Animals, Facultat de Veterinària and* [c]*Departament de Sanitat i Anatomia Animals, Servei de Diagnòstic de Patologia Veterinària, Universitat Autònoma de Barcelona, Edifici V, Campus UAB, Bellaterra, Spain.*

Correspondance to: L. Solano-Gallego[1]

[1]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària. Universitat Autònoma de Barcelona, Edifici V, Campus UAB, Bellaterra, Spain. Telephone number (+34) 935868533. Email: laia.solano@uab.cat.

Abstract

A four-year-old, neutered male Golden Retriever was presented with a one-week history of weight loss, polyuria and polydipsia. The diagnostic workup showed an increased ionized calcium concentration, mild increase in serum creatinine and urea concentration, and severe hyperlipasemia. A complete abdominal ultrasound revealed multiple hepatic nodules. A cytological diagnosis of malignant epithelial neoplasia, highly suggestive of bile duct adenocarcinoma was made. In order to confirm the presumptive diagnosis of hypercalcemia of malignancy due to the presence of a hepatic neoplasia, serum parathormone-related peptide concentration (PTH-rP) was measured, and the result revealed an increased concentration. The dog was hospitalized and received supportive treatments consisting of intravenous furosemide and fluid therapy. After ruling out lymphoma and hypoadrenocorticism, oral prednisone was initiated, and ionized calcium concentration decreased gradually down to normal concentration after seven days of hospitalization. Chemotherapy with intravenous epirubicin was initiated based on the cytological diagnosis. One month after diagnosis, and due to the worsening of its clinical condition, the dog was humanely euthanized. Post-mortem examination confirmed a cholangiocellular carcinoma. To our knowledge, this is the first report of malignant hypercalcemia associated with cholangiocellular carcinoma in a dog.

Keywords: canine; neoplasia; liver; parathyroid hormone-related protein; ionized calcium

Introduction

Hepatobiliary neoplasia is uncommon in dogs and cats and accounts for less than 1.5% of all canine and 1% to 2.9% of all feline tumors. [1]

Bile duct carcinoma or cholangiocellular carcinoma is the second most common hepatic tumor in dogs, followed by hepatocellular carcinoma, accounting for 22% to 41% of all malignant liver tumors in this species. [1] Bile duct carcinoma has an aggressive biologic behavior. Metastasis is common, particularly to regional lymph nodes and the lungs [1] with median survival times usually being less than six months. [2]

The most frequent underlying cause of hypercalcemia in dogs is neoplasia. [3] A variety of tumors have been associated with hypercalcemia of malignancy in dogs, the two most common of which are lymphoma and apocrine gland adenocarcinoma of the anal sac. [3] However, hypercalcemia of malignancy has been documented with other tumors such as multiple myeloma, thyroid carcinoma, thymoma, squamous cells carcinoma, mammary gland carcinoma/adenocarcinoma, melanoma, primary lung tumors, chronic lymphocytic leukemia, renal angiomyxoma, and parathyroid gland tumors. [3,4]

There is lack of information in the veterinary literature regarding malignancy–related hypercalcemia associated with hepatic tumors in dogs. The case presented here describes a case of hypercalcemia of malignancy associated with cholangiocellular carcinoma in a dog.

Case presentation

A four-year-old, neutered male Golden Retriever was presented for a one-week history of anorexia, polyuria, polydipsia, and weight loss.

On presentation, the dog was depressed and dehydrated, and a IV/VI grade heart murmur was ausculted. The remainder of the physical exam including rectal examination, was otherwise unremarkable.

Minimal data base evaluation at presentation revealed a markedly increased ionized calcium concentration (2.73 mmol/L; reference interval 1.25 – 1.5). Initially, the dog received fluid therapy (sodium chloride 0.9%; B. Braun Medical, VetCare SA, Rubí, Barcelona) and treatment with intravenous furosemide (2 mg/kg BID; Seguril 20mg/2mL Sanofi-Aventis SA, Barcelona).

Clinical laboratory tests performed prior to referral included a complete blood cell count, serum biochemistry panel and urinalysis. Significant findings included an inflammatory leukogram, consisting of mild neutrophilia (15,392 neutrophils/µL; reference interval 2,940 – 12,670), with a left shift (187 bands/µL; reference interval 0 – 170), marked total hypercalcemia (16.0 mg/dL; reference interval 8.2 – 11.9), severe hyperlipasemia (15,557 IU/L; reference interval 75 – 784, spectrophotometric method at 37ºC), mild azotemia (serum creatinine 1.6 mg/dL; reference interval 0.5 – 1.5, and serum urea of 70 mg/dL; reference interval 21.4 – 59.9), mild hyperbilirubinemia (0.34 mg/dL; reference interval 0.01 – 0.31), and a mild increase in AST activity (113 IU/L; reference interval 16 – 89). Phosphorus concentration was in the lower reference limit (2.7 mg/dL; reference interval 2.7 – 6.7). Urinalysis performed on a urine sample obtained by cystocentesis revealed isosthenuria (urine specific gravity 1.008). Analysis using urine dipstick and urine sediment examination were unremarkable.

Because lymphoma and hypoadrenocorticism are potential causes of hypercalcemia in dogs, thoracic radiographs, abdominal ultrasound and ACTH stimulation test were

performed. The results of the ACTH stimulation test were unremarkable, ruling out hypoadrenocorticism in this dog.

Thoracic radiographs showed, on the ventrodorsal projection, an increase of cardiac silhouette cranial to the right atrium; whereas the laterolateral projection revealed an increased opacity at the dorsocranial level of the cardiac silhouette without tracheal displacement. The main differential diagnosis related to these findings were cardiac valvular disease or, less likely, neoplasia; a cardiac ultrasound was recommended but declined by the owners.

An abdominal ultrasound revealed a mild amount of abdominal fluid and multiple hepatic nodules of heterogeneous echogenicity, with moderately defined margins and variable size, ranging from 0.8 to 3.5 cm (Fig 1). Mild mesenteric lymphadenomegaly was also identified. Neither renal calcification nor other remarkable findings were observed. Ultrasound-guided fine needle aspirates were obtained from the hepatic nodules for cytological evaluation and stained with the Quick Panoptic® stain (Fig 2) (Química Clínica Aplicada, Amposta, Spain). A cytological diagnosis of malignant epithelial neoplasia, highly suggestive of bile duct adenocarcinoma, with mild neutrophilic inflammation and hepatic cholestasis was made. A metastatic adenocarcinoma from a primary extrahepatic tumor or hepatic carcinoma were also considered as differentials. Hepatic biopsy with histopathological examination was recommended but the owners declined further investigations.

Hypercalcemia of malignancy was suspected due to the presence of a hepatic neoplasm. In order to confirm the presumptive diagnosis, serum parathormone-related protein (PTH-rP) concentration was measured and was found to be increased (5 pmol/L, reference value < 2 pmol/L). Serum pre- and post-bile acids concentration were also

measured to evaluate liver function, in order to modify the chemotherapy dosage or protocol in case the liver function was impaired. The results of bile acid stimulation test revealed normal liver function.

Initially, supportive treatment consisted of intravenous furosemide (2 mg/kg BID; Seguril 20mg/2mL Sanofi-Aventis SA, Barcelona) and fluid therapy (sodium chloride 0.9%; B. Braun Medical, VetCare SA, Rubí, Barcelona). This did not decrease the ionized calcium concentration. The initial azotemia also worsened over the first 24h in hospital despite fluid therapy (serum creatinine 2.06 mg/dL; reference interval 0.5 – 1.5, and serum urea 95.7 mg/dL; reference interval 21.4 – 59.9).

After ruling out lymphoma and hypoadrenocorticism, oral prednisone treatment (1mg/kg SID; Dacortin 30 mg, Merck S.L, Madrid) was initiated. Oral prednisone and intravenous furosemide were administered simultaneously during the first 3 days of hospitalization and ionized calcium concentrations decreased steadily from 2.73 mmol/L to 2.02 mmol/L (reference interval 1.25 – 1.5). Due to this improvement, oral prednisone was continued but furosemide treatment was ceased. After 7 days of hospitalization, ionized calcium concentration returned to normal (1.47 mmol/L; reference interval 1.25 – 1.5), and the dog's general condition improved. Further aggressive treatment, such as administration of bisphosphonates, sodium bicarbonate, calcium channel blockers or dialysis, were not deemed necessary given the improvement.

Based on the cytological diagnosis, chemotherapy with intravenous epirubicin was started at day seven of hospitalization (30 mg/m^2; Accord, 2mg/mL Injectable Solution, epirubicin hydrochloride, Accord Healthcare SA, Barcelona) and prescribed every three weeks for four sessions. Additional chemotherapy, using a metronomic protocol and

including piroxicam (0.3 mg/kg SID) and cyclophosphamide (10mg/m^2 SID) was planned at week three after epirubicin treatment.

The dog was discharged from the hospital with oral prednisone (1mg/kg SID, Dacortin 30 mg, Merck S.L, Madrid). As the dog developed gastrointestinal signs during hospitalization (e.g. vomiting and diarrhea), treatment with metronidazole (7.5mg/kg BID, Flagyl 250 mg tablets, Sanofi-Aventis SA, Barcelona), maropitant (1mg/kg SID, Cerenia 60mg tablets, Zoetis Belgium, Louvain-la-Neuve, Belgium) and omeprazole (1mg/kg SID, Omeprazol Normon 40mg, S.A, Tres Cantos, Madrid) were also prescribed. The dog was also started on a prescription hepatic diet (Royal Canin® Hepatic).

One week after being discharged, the ionized calcium was mildly elevated (1.58 mmol/L; reference interval 1.25 – 1.50) and serum creatinine and urea were within normal limits; although in the upper reference limit (1.5 mg/dL; reference interval 0.5 – 1.5; and 58.2 mg/dL; reference interval 21.4 – 59.9, respectively). Ten days after the last examination, the dog returned with a 4 day history of vomiting, diarrhea and generalized weakness. At that time, ionized calcium was markedly increased (2.4 mmol/L; reference interval 1.25 – 1.50). Due to the poor prognosis and clinical progression, the owners elected for euthanasia.

On post-mortem examination, 5mL of serosanguinous peritoneal fluid was collected. The liver was enlarged with multiple, pale color and firm hepatic nodules of variable size, ranging from 0.5 to 5cm, with typical umbilicate appearance. The heart showed mild mitral valve thickening.

Tissue samples from the lungs, spleen, kidney, normal liver parenchyma, hepatic nodules, myocardium, adrenal glands, stomach, intestine, pancreas, bone marrow, brain

and cerebellum were obtained and fixed in 10% neutral buffered formalin, paraffin embedded, and routinely processed for histologic examination. Sections (4μm) were stained with hematoxylin and eosin (HE). Histological sections of the hepatic nodules revealed multiple sites of local invasion of hepatic parenchyma by neoplastic epithelial cells (Fig 3 and Fig 4). Further assessment was performed to confirm the suspicious of cholangiocellular carcinoma. Periodic acid Schiff (PAS) staining of the liver's histological section was positive (Fig 5). The kidneys showed marked mineralization, principally affecting the epithelium of cortical tubules, producing degeneration and loss of normal epithelium architecture. The stomach glandular epithelium showed a small amount of mineralization. Examination of the hypophysis of the pituitary revealed a small sized cystic lesion (2mm) containing basophilic acellular material. There was no evidence of metastasis to the regional lymph nodes or distant organs.

Based on the histological findings and PAS stain, the final diagnosis was an intrahepatic cholangiocellular carcinoma, with renal tubular mineralization and a pituitary cyst.

To determine if neoplastic cells from cholangiocellular carcinoma secreted PTH-rP, immunohistochemistry (IHC) for PTH-rP was performed at the Ohio State University College of Veterinary Medicine. Histologic sections (4μm) were deparaffinized, hydrated and pretreated in target retrieval solution (Dako, Santa Clara, CA, USA). Sections were incubated with primary anti-PTH-rP antibody for 30 minutes (Dako, Santa Clara, CA, USA, diluted 1:100), rinsed and incubated with biotinylated rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA, USA, diluted 1:200) in protein block for 30 minutes. After rinsed, sections were incubated with Vector RTU ABC Elite complex (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. The chromogen used for color development was 3,3' diaminobenzidine tetrahydrochloride (DAB) (Dako, Santa Clara, CA, USA). Finally, sections were counterstained with

hematoxylin. For the negative control, sections were incubated with goat IgG for 30 minutes (Vector Laboratories, Burlingame, CA, USA, diluted 1:100). Thyroid follicular carcinoma served as positive control. The cholangiocellular carcinoma exhibited minimal, nonspecific background staining leading to a negative result.

Discussion

This is to our best knowledge, the first case report of malignant hypercalcemia that could be associated with cholangiocellular carcinoma in a dog. Several case reports in human medicine have described hypercalcemia of malignancy associated with cholangiocellular carcinoma. [5,6,7,8,9] In people, cholangiocellular carcinoma has a poor prognosis but the tumor is rarely associated with a paraneoplastic syndrome. The prognosis for patients with cholangiocellular carcinoma that produces PTH-rP is even worse, where the interval between the diagnosis of hypercalcemia and the patient's death is usually less than one year. [7]

The increased PTH-rP concentration was considered the most likely cause of hypercalcemia in the dog described in this report; however, PTH-rP concentration was only mildly increased in this case as compared to other reports of canine hypercalcemia of malignancy induced by PTH-rP [10, 11]. In these previous reports, PTH-rP concentration was markedly increased, being 15.3 pmol/L (reference value < 2pmol/L) [10] and 21 pmol/L (reference value < 2pmol/L) [11]. This could suggest that PTH-rP might have be the main factor responsible for the induction of hypercalcemia in this dog. Several mechanisms of hypercalcemia of malignancy have been described in human and veterinary medicine include humoral hypercalcemia of malignancy due to circulating tumor-produced hormones or cytokines, and hypercalcemia resulting from primary bone tumors or metastases with local osteolytic bone resorption. [3, 12] The

negative result of the IHC for PTH-rP could suggest additional factors secreted by neoplastic cells that may have a synergistic or additive effect with PTH-rP. [3, 12] Additional factors could include osteoclast stimulatory factors, such as interleukin 1 and 6, tumor necrosis factor-α, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, transforming growth factor-β, calcitriol, and macrophage inflammatory protein-1α. [12] Unfortunately, IHC or plasma tests for additional cytokines or calcitriol were not performed in this case.

Although the neoplastic cells in the present case did not exhibit IHC positivity for PTH-rP, they could contain PTH-rP messenger RNA, as occurs in humoral hypercalcemia of malignancy in dogs with anal sac adenocarcinoma where neoplastic cells stain weakly positive for PTH-rP but have abundant PTH-rP mRNA. [13] Unfortunately, PTH-rP mRNA expression was not analyzed in this dog. Furthermore, the body was frozen for 36-48hr before post-mortem examination was performed, thus leading to tissue autolysis. Although post-mortem interval for at least 24h does maintain protein stability and IHC staining intensity for certain classes of antigens; careful interpretation of autolyzed material would be necessary since some proteins may degrade and lose detectable staining. [14] For that reason, the length of time before post-mortem examination in our case could have affected the IHC for PTH-rP. Furthermore, neoplastic cells may up-regulate and down-regulate gene expression, resulting in the absence of expression of expected antigens or the expression of new antigens. [15]

A definitive diagnosis of intrahepatic cholangiocellular carcinoma was made by histopathology. Since histological morphology of cholangiocellular carcinoma and carcinoids might be similar, hepatic carcinoid could not be completely ruled out as a diagnosis. The presence of acini, tubules, intraluminal secretion and mucin are common

features of cholangiocellular carcinoma. Mucin was demonstrated with PAS stain and is common in cholangiocellular carcinomas but is not a feature of carcinoids. [16]

Renal failure can result in hypercalcemia and may have been a contributing factor in this case. The urine specific gravity, azotemia and histologic evidence of renal pathology strongly indicated renal disease in this case. However, hypercalcemia was not considered to be secondary to renal disease. Dogs with chronic renal failure have usually normal to low ionized calcium concentration. [15] The increased serum PTH-rP concentration was consistent with hypercalcemia of malignancy, and the evidence of renal tubular mineralization strongly indicates that hypercalcemia was the primary cause of the renal pathology.

The biochemistry profile revealed a marked increase in serum pancreatic lipase activity with minimal simultaneous increase in serum amylase activity. This finding has been described in dogs with pancreatic and hepatic tumors. [17] The study published by Quigley et al., (2001) concluded that marked and unexplained hyperlipasemia may be used as a biochemical marker for pancreatic and hepatic epithelial malignant neoplasia in dogs. [17] Another consideration for the increase in serum pancreatic lipase activity in this dog was pancreatitis, since the serum biochemistry results and clinical findings (e.g. hyperbilirubinemia, hypercalcemia, weakness, gastrointestinal signs, polyuria and polydipsia) could be related to acute pancreatitis. Although hypocalcemia is most commonly associated with acute pancreatitis in dogs and cats [18], previous studies published in humans [19] and cats [20] have suggested that hypercalcemia induces pancreatic injury by increasing the permeability of the pancreatic duct to molecules such as pancreatic enzymes, followed by the accumulation and activation of proteases that results into pancreatitis. It is well known that serum lipase and amylase activities are not useful and should not be used for detection of pancreatic disease due to their low

sensitivity and specificity [21]. Another condition associated with increased lipase and/or amylase activities is renal disease, although renal disease was not considered in this case, since hyperlipasemia was severe. Other situations associated with hyperlipasemia are hepatic, intestinal, neoplastic diseases and corticosteroid administration [21]. The post-mortem examination did not reveal pancreatic disease. Histopathology is considered the gold standard for the diagnosis of pancreatitis [21]. However, exclusion of pancreatitis based on histopathology can be difficult since the inflammatory lesions are often focal and can be easily missed. In the present case, multiple sections of the pancreas were evaluated, increasing the likelihood of detection of microscopic lesions, and precluding the diagnosis of pancreatitis or pancreatic neoplasia in this dog.

The liver function parameters, including bile acids, were within normal limits, suggesting that liver function was preserved; however, the dog exhibited mild hyperbilirubinemia most likely associated with hepatic and post-hepatic causes, secondary to cholangiocellular carcinoma and hepatic cholestasis.

Post-mortem examination did not reveal any neoplasia other than the cholangiocellular carcinoma. Although the IHC could not confirm the expression of PTH-rP on neoplastic cells, the absence of other neoplasia or diseases suggest that the increase in serum PTH-rP is most likely associated with cholangiocellular carcinoma.

Conclusion

This is, to our knowledge, the first case report of malignant hypercalcemia associated with cholangiocellular carcinoma in a dog. A differential diagnosis of cholangiocellular carcinoma should be included in dogs affected by hypercalcemia of malignancy of unknown origin.

Authors Contributions/Acknowledgments

The authors would like to thank all the veterinarians and technicians at the HCV who were involved in the care of this dog. The authors are grateful to CPMPSR Histology/IHC Core Lab at the Ohio State University for performing the PTH-rP immunohistochemistry.

Conflict of Interest Statement

The authors declare no conflicts of interest with respect to publication of this manuscript.

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Figure Legends

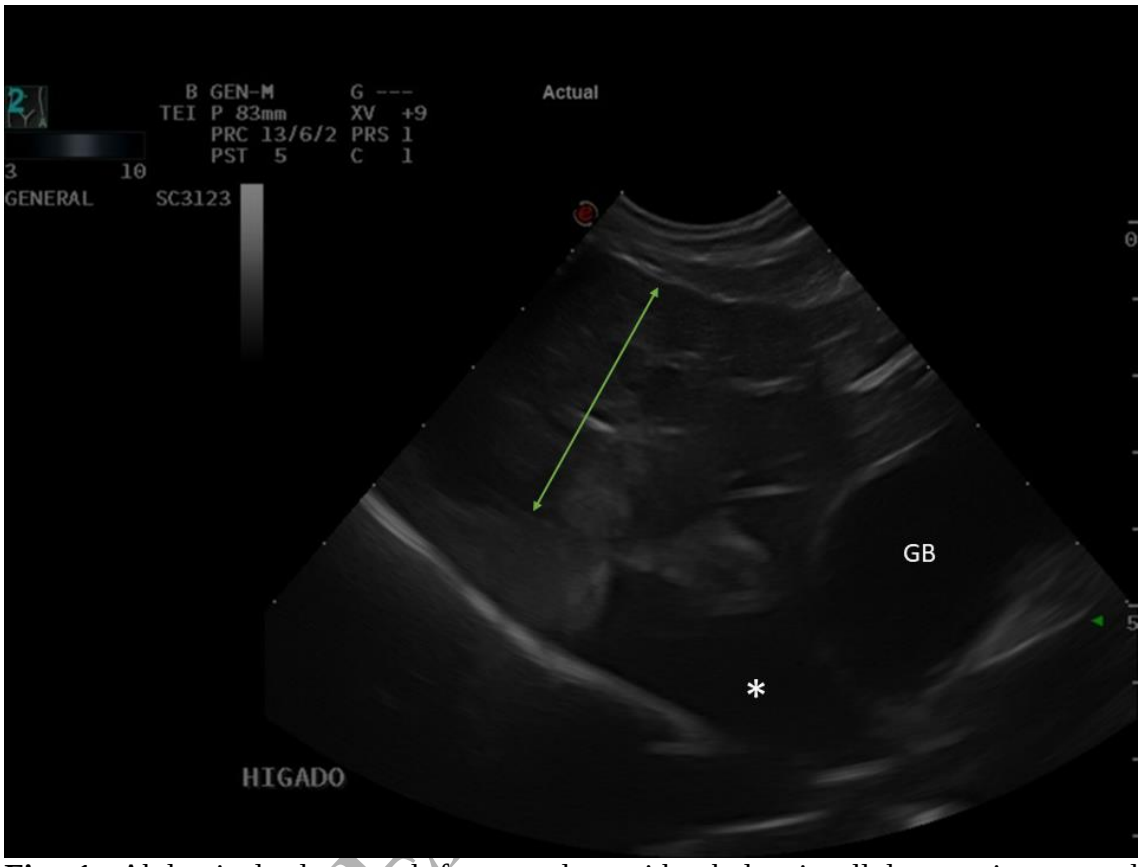

Fig. 1. Abdominal ultrasound from a dog with cholangiocellular carcinoma and hypercalcemia of malignancy showing a hepatic nodule of heterogenous echogenicity (arrow). A small amount of abdominal effusion is noted (*). GB (gall bladder).

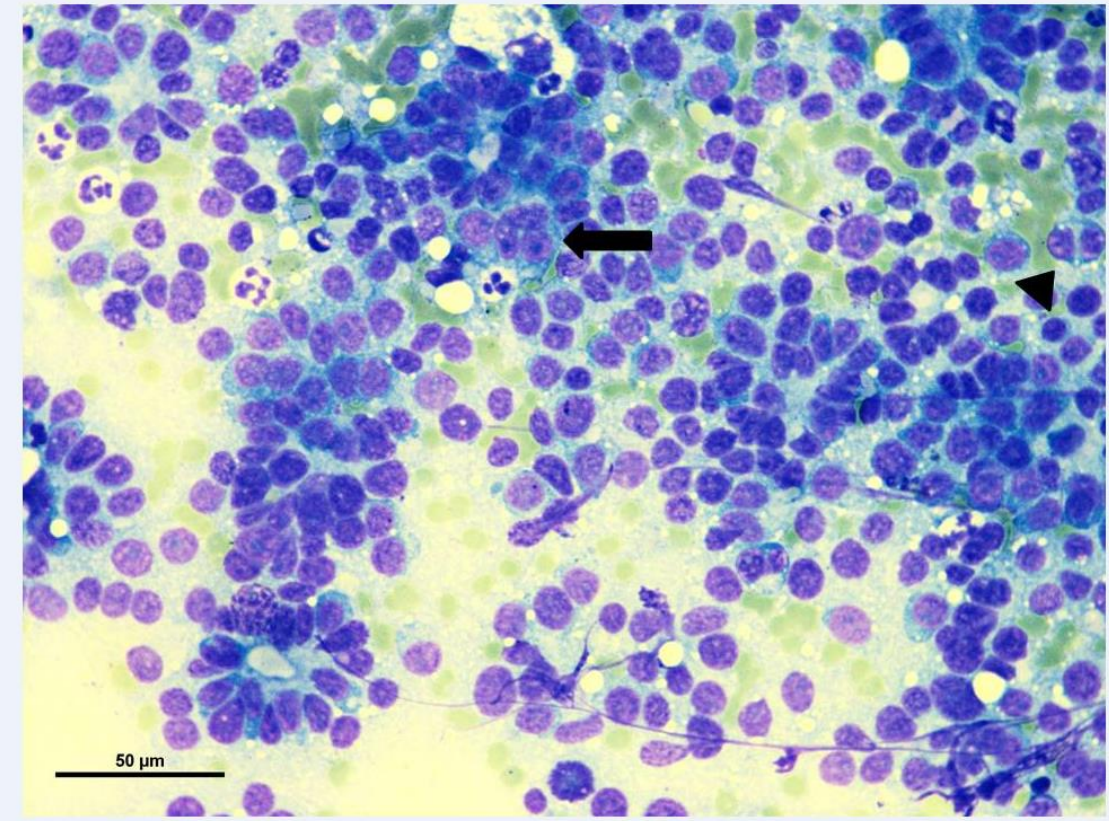

Fig. 2. Fine needle aspirate from the hepatic nodule of a dog with cholangiocellular carcinoma and hypercalcemia of malignancy. Small epithelial cells (10 to 12 µm) with round to cuboidal morphology, mostly individually organized or in small clusters with acinar arrangement, occasionally containing grey amorphous material are noted. The epithelial cells display an elevated nucleus: cytoplasm ratio, with mild anisocytosis and anisokaryosis. Binucleated (arrowhead) and multinucleated cells (arrow) are observed. Quick Panoptic Stain®.

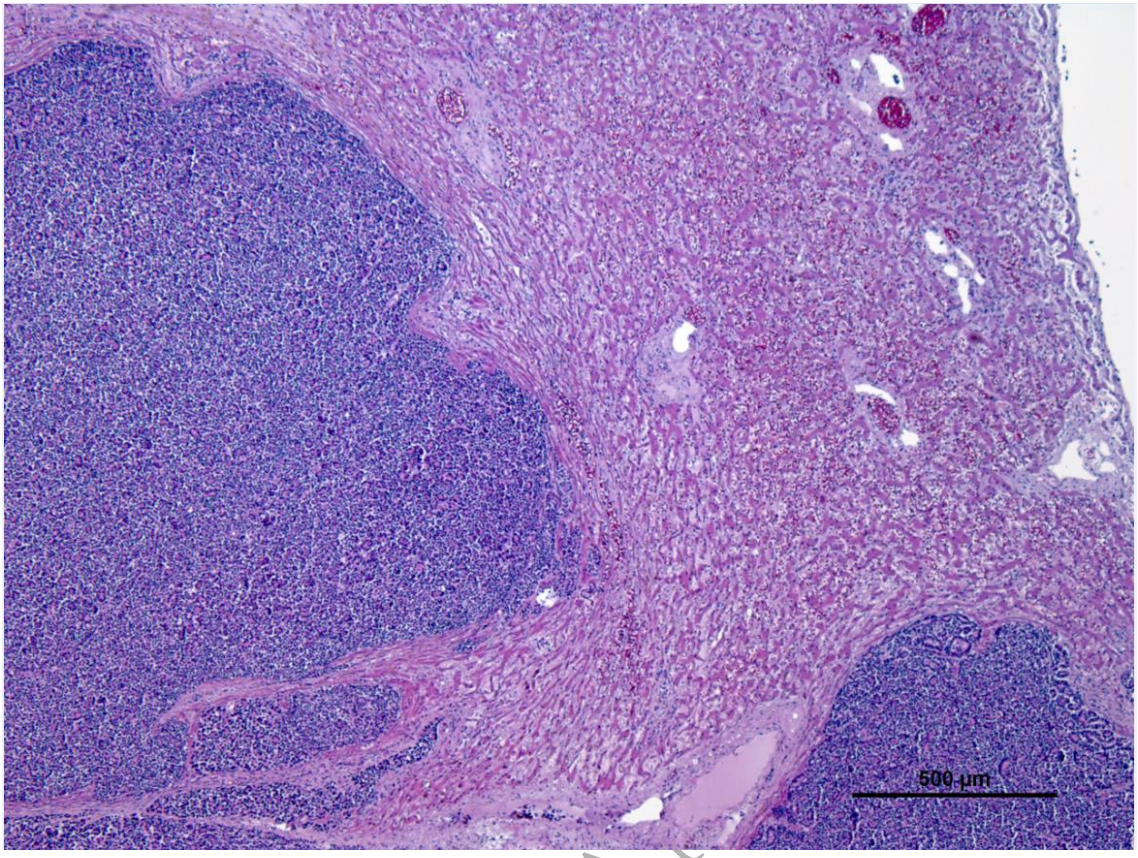

Fig. 3. Histological section of the cholangiocellular carcinoma from the patient in this report. Multiple sites of local invasion of surrounding hepatic parenchyma by neoplastic cells are noted. Tumor cells proliferated in a multifocal to coalescent, multilobulated, and encapsulated nodules. Hematoxylin and Eosin stain.

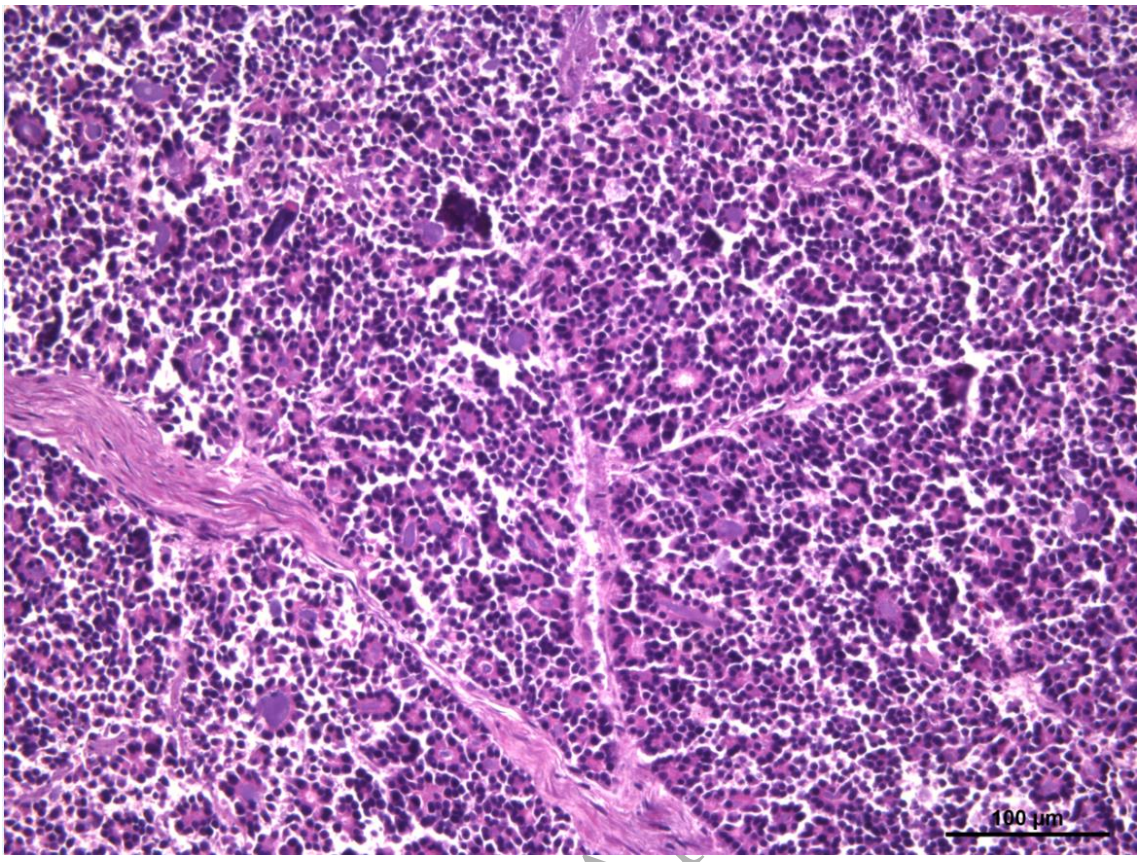

Fig. 4. Histological section of the cholangiocellular carcinoma described in this report. Medium sized neoplastic epithelial cells with cuboidal to columnar morphology, with small amount of eosinophilic cytoplasm and discrete margins are observed. The neoplastic epithelial cells form well-differentiated ductal and acini structures, occasionally with eosinophilic to weakly basophilic secretory material within the lumen, suggestive of mucin, and separated by fibrous connective-tissue stroma. Hematoxylin and Eosin stain.

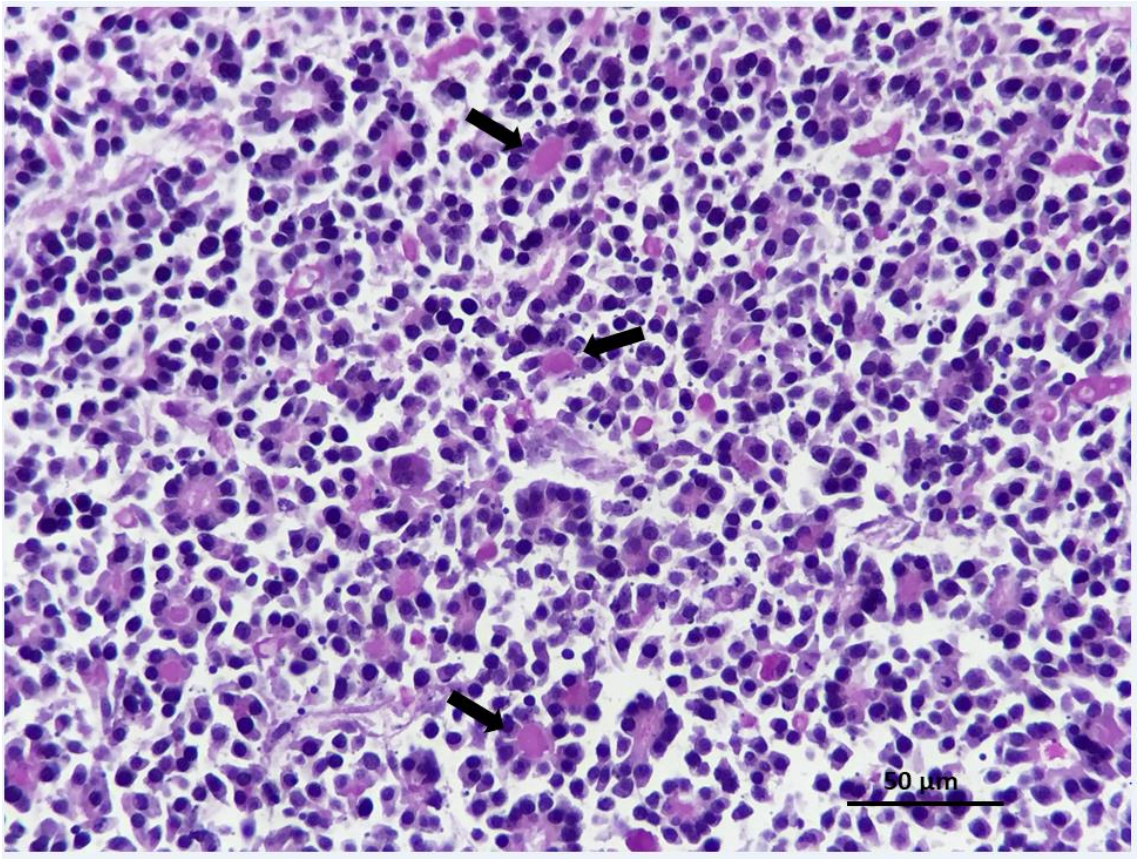

Fig. 5. Histological section of the cholangiocellular carcinoma described in this report. Positive Periodic acid Schiff (PAS) stain confirms the mucin nature of the secretory material (arrow), a common feature of cholangiocellular carcinoma. Hematoxylin and Eosin and PAS stain.