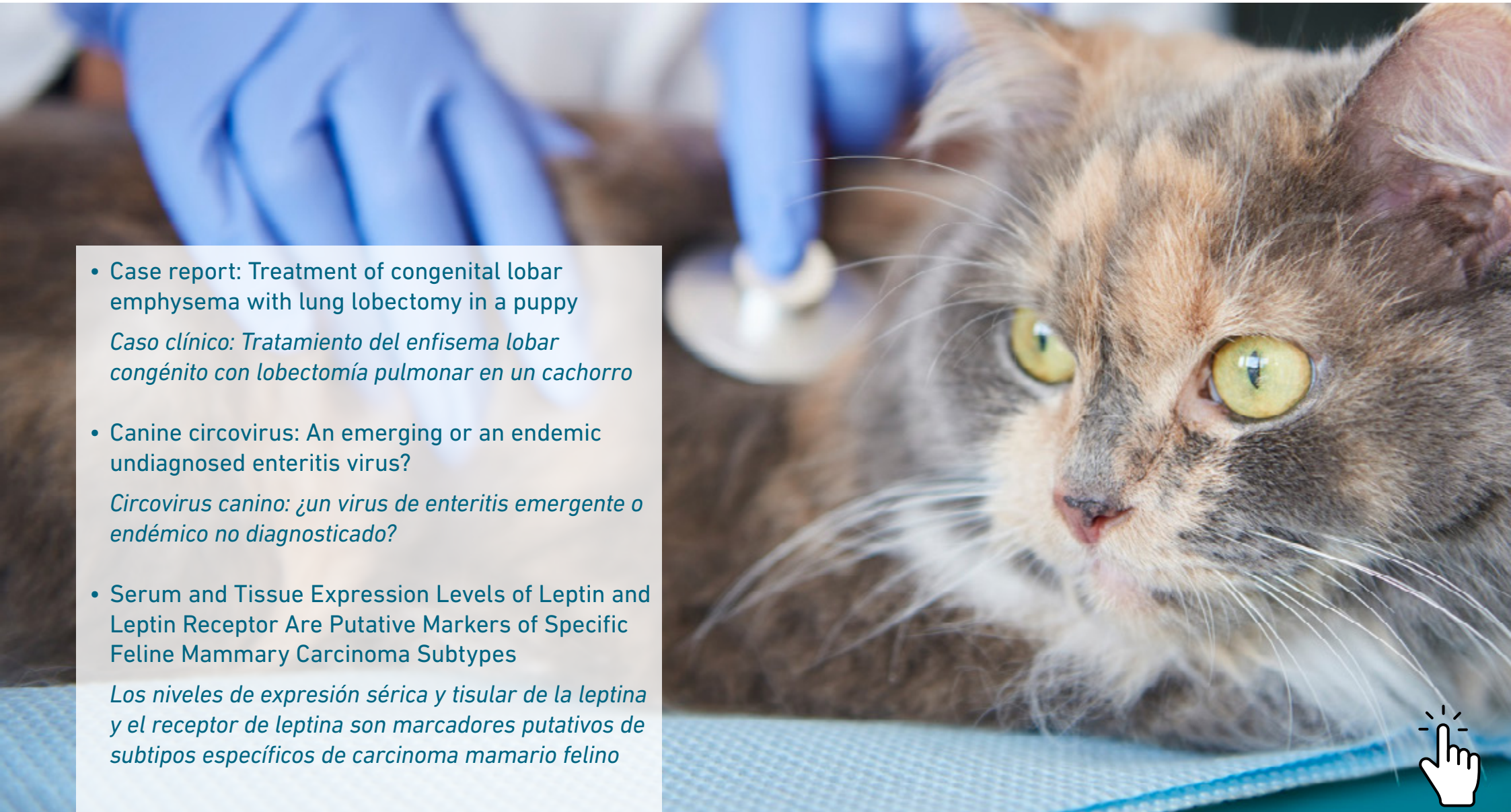
(R) evolution

PEQUEÑOS ANIMALES

Nº 5



- Case report: Treatment of congenital lobar emphysema with lung lobectomy in a puppy

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SUMARIO

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Case report: Treatment of congenital lobar emphysema with lung lobectomy in a puppy

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Palabras clave:
canino, lobectomía pulmonar, enfisema lobar, congénito, tomografía computarizada

Keywords:
canine, lung lobectomy, lobar emphysema, congenital, computed tomography

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Una perra Catahoula Leopard hembra sexualmente intacta de 11 semanas de edad fue evaluada por una historia de varias semanas de intolerancia al ejercicio y períodos intermitentes de dificultad respiratoria. Las radiografías torácicas revelaron un campo pulmonar derecho marcadamente hiperinflado, con compresión de los lóbulos pulmonares circundantes. La tomografía computarizada torácica localizó aún más la hiperinflación en el lóbulo pulmonar medio derecho, con sospecha de enfisema lobar congénito. Se realizó con éxito una toracotomía intercostal derecha con lobectomía pulmonar media derecha. Los resultados histopatológicos confirmaron hipoplasia del cartílago bronquial con enfisema marcado y fibrosis pleural. El cachorro se recuperó de la cirugía sin incidentes y fue dado de alta del hospital sin complicaciones postoperatorias. A los 18 meses después de la operación, el perro estaba clínicamente normal sin retorno de dificultad respiratoria. Este informe de caso describe el tratamiento quirúrgico exitoso de un cachorro de raza grande con la condición poco común de enfisema lobar congénito.

An 11-week-old, sexually intact female Catahoula Leopard dog was evaluated for a multiple-week history of exercise intolerance and intermittent periods of respiratory distress. Thoracic radiographs revealed a markedly hyperinflated right lung field, with compression of the surrounding lung lobes. Thoracic computed tomography further localized the hyperinflation to the right middle lung lobe, with suspicion of congenital lobar emphysema. A right intercostal thoracotomy with right middle lung lobectomy was performed successfully. Histopathology results confirmed bronchial cartilage hypoplasia with marked emphysema and pleural fibrosis. The puppy recovered from surgery uneventfully and was discharged from the hospital without any postoperative complications. At 18 months postoperatively, the dog was clinically normal with no return of respiratory distress. This case report describes successful surgical treatment of a large breed puppy with the uncommonly reported condition of congenital lobar emphysema.

Introduction

Congenital lobar emphysema (CLE) is a rare lower respiratory tract disease that most commonly presents in young dogs and cats, with age ranges in the literature from 6 weeks to 24 months of age (1–8). The literature predominately reports CLE in small or toy breed dogs, with rare reports in large breed dogs (9). Clinical signs reported in the literature include respiratory signs such as exercise intolerance, coughing, tachypnea or dyspnea, and cyanosis (1, 3–6, 8–13). Additionally, subcutaneous emphysema, pneumothorax, and pneumomediastinum have been noted on imaging and physical examination (3, 4, 7, 8, 13, 14). Congenital lobar emphysema is characterized by alveolar air accumulation resulting in hyperinflation of the affected lung lobes, most commonly due to bronchial cartilage hypoplasia, dysplasia, or aplasia leading to bronchial collapse (1–6, 8, 10, 11, 13–18). In the human literature, there are 3 recognized etiologies of lobar emphysema: bronchial cartilage dysplasia, which may range from hypoplastic and flaccid cartilage to a complete absence of tissue; external bronchial compression; and idiopathic (19, 20). Idiopathic etiologies have also been reported in veterinary patients, in which no bronchial abnormalities were identified on histo-

Abbreviations: *CLE, congenital lobar emphysema; CT, computed tomography.*

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pathology (7, 9, 10, 12). While many cases reported in the veterinary literature have bronchial abnormalities, ~80% of people diagnosed with CLE have an undetermined etiology, and only 20% have associated bronchial abnormalities (19). Surgical intervention with lung lobectomy of the affected lobes has been reported to result in successful treatment and recovery; however, older literature predominantly reported death or euthanasia in dogs affected with CLE (10–14, 16, 21). The case report described here details diagnosis and treatment of a large-breed puppy with CLE.

Case presentation

Informed consent was obtained from the owner for publication of this case report. An 11-week-old, sexually intact, female Catahoula Leopard dog with a weight of 6 kg was evaluated for a multiple-week history of exercise intolerance and intermittent periods of respiratory distress. At 8 weeks of age, the primary veterinarian performed thoracic radiographs to investigate for causes of exercise intolerance and respiratory distress (**Figures 1A**, **B**). The thoracic radiographs revealed a markedly hyperinflated and hyperlucent right lung lobe with compression of the surrounding lobes, displacement of the cardiac silhouette, and a leftward mediastinal shift. The dog was subsequently referred to a specialty hospital for further diagnostics and care with a differential diagnosis including congenital lobar emphysema based on the above radiographic changes.

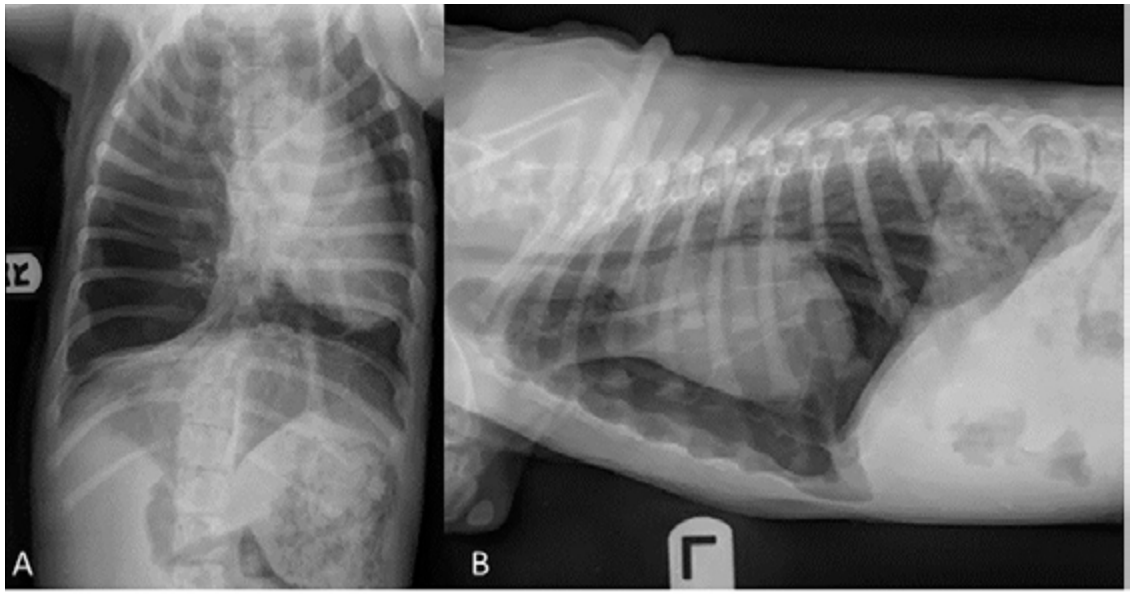

Figure 1. *Ventrodorsal (**A**) and lateral (**B**) radiographic projections of an 11-week old, sexually intact, female Catahoula Leopard dog with congenital lobar emphysema. Noted in image (**A**), is a left mediastinal shift, and images (**A, B**) depict a hyperlucent right lung lobe and displacement of the cardiac silhouette.*

On presentation to the soft tissue surgery service at the referral hospital, physical examination revealed absent right lung sounds and increased, harsh left lung sounds on thoracic auscultation. The dog's respiratory effort was moderately increased on presentation. Her temperature (100.4° F, 38°C), pulse (150 beats per min), and the remainder of her physical examination were within normal limits. Bloodwork abnormalities were consistent with the young age of the patient, including a mild anemia (hematocrit 35.1%; reference range 40.5–59.9%), lymphocytosis (lymphocytes 5.18 x 10^3/µL; reference range 1.10–3.96 x 10^3/µL), mild panhypoproteinemia (albumin 2.9 g/dL; reference range 3.2–4.3 g/dL and globulins 1.5 g/dL; reference range 1.9–3.1 g/dL), hyperphosphatemia (7.8 mg/dL; reference range, 2.5–5.9), hyperkalemia (4.9 mmol/L; reference range, 2.-4.7 mmol/L) and a mild elevation in ALP (295 U/L; reference range, 13–240 U/L).

To further characterize the pulmonary pathology, a thoracic computed tomography (CT) scan was performed under general anesthesia with the intention to proceed directly to surgery pending the results of the CT scan. The patient was sedated with an intramuscular injection of butorphanol (0.4 mg/kg) and alfaxalone (2 mg/kg), after which anesthesia was induced with an intravenous injection of ketamine (5 mg/kg) and midazolam (0.25 mg/kg). General anesthesia was maintained with vaporized sevoflurane and continuous rate infusions of ketamine (10–20 mg/kg/h) and fentanyl (5–10 µg/kg/h).

A transverse multislice submillimeter helical dataset was obtained from the thoracic inlet to the cranial abdomen with a 40-slice helical CT scanner (Philips Brilliance-40, Philips International B.V., Amsterdam, Netherlands). To decrease risk of pulmonary rupture and pneumothorax, a breath hold was not utilized when acquiring the CT images. A pre- and post-contrast study was completed using iodinated contrast material (Optiray, 0.45 mL/kg of 350 mg I/mL IV).

Images revealed a severely distended right middle lung lobe with hypoattenuating parenchyma relative to the remaining lung lobes. Furthermore, the pulmonary vessels throughout the right middle lung lobe were decreased in size and the primary bronchus was mildly dilated, with abrupt narrowing in the distal bronchus.

Distension of the right middle lung lobe resulted in a marked leftward displacement of the heart and other mediastinal structures as well as the right cranial, right caudal and accessory lung lobes (**Figure 2**). Consequential compression of the left caudal and left cranial lung lobes also occurred. Multifocal regions of increased pulmonary attenuation and ground glass patterns throughout the displaced and compressed lung lobes were noted and attributed to atelectasis. A diagnosis of CLE was suspected based on hyperinflation of the right middle lung lobe and abrupt narrowing of the bronchus within the right middle lung most likely related to bronchial hypoplasia and less likely bronchial compression. The remaining changes of the thoracic cavity were deemed likely to be secondary to compression and displacement from the right middle lung lobe.

With careful consideration for the age of the patient and prognosis without treatment, it was elected to continue with surgical intervention. The dog proceeded to surgery immediately following the CT scan under the same anesthetic event. A fentanyl loading dose of 5 µg/kg was administered intravenously, and Normosol-R intravenous fluids were administered at a rate of 3 ml/kg/h. In addition to the vaporized sevoflurane, continuous rate infusions of ketamine (10–20 mg/kg/h) and fentanyl (5–10 µg/kg/h) were utilized to maintain a surgical plane of anesthesia.

Following aseptic preparation of the right lateral thoracic wall, the patient was positioned for surgery in left lateral recumbency. Mechanical ventilation was started prior to incision of the thoracic wall. A right lateral intercostal thoracotomy was performed with a 12 cm incision made in a dorsoventral direction along the 5th intercostal space. A standard intercostal thoracotomy approach with sharp and blunt dissection of the subcutaneous tissues and thoracic wall musculature was performed, and finochietto retractors were used for retraction of the ribs to aid in visualization.

The markedly inflated right middle lung lobe was manipulated to access the hilus, resulting in a majority of the lobe being externalized from the thoracic cavity (**Figure 3**). A DST Series™ TA™ Stapler (Medtronic, Minneapolis, MN) with a 30 mm V3 Vascular (Medtronic, Minneapolis, MN) cartridge was used to seal the bronchus, artery and vein at the hilus of the right middle lung lobe. A scalpel was then used to excise the lung lobe distal to the staples. An exploratory exam of the right hemithorax was performed and was within normal limits except for notation of an atelectatic right caudal lung lobe.

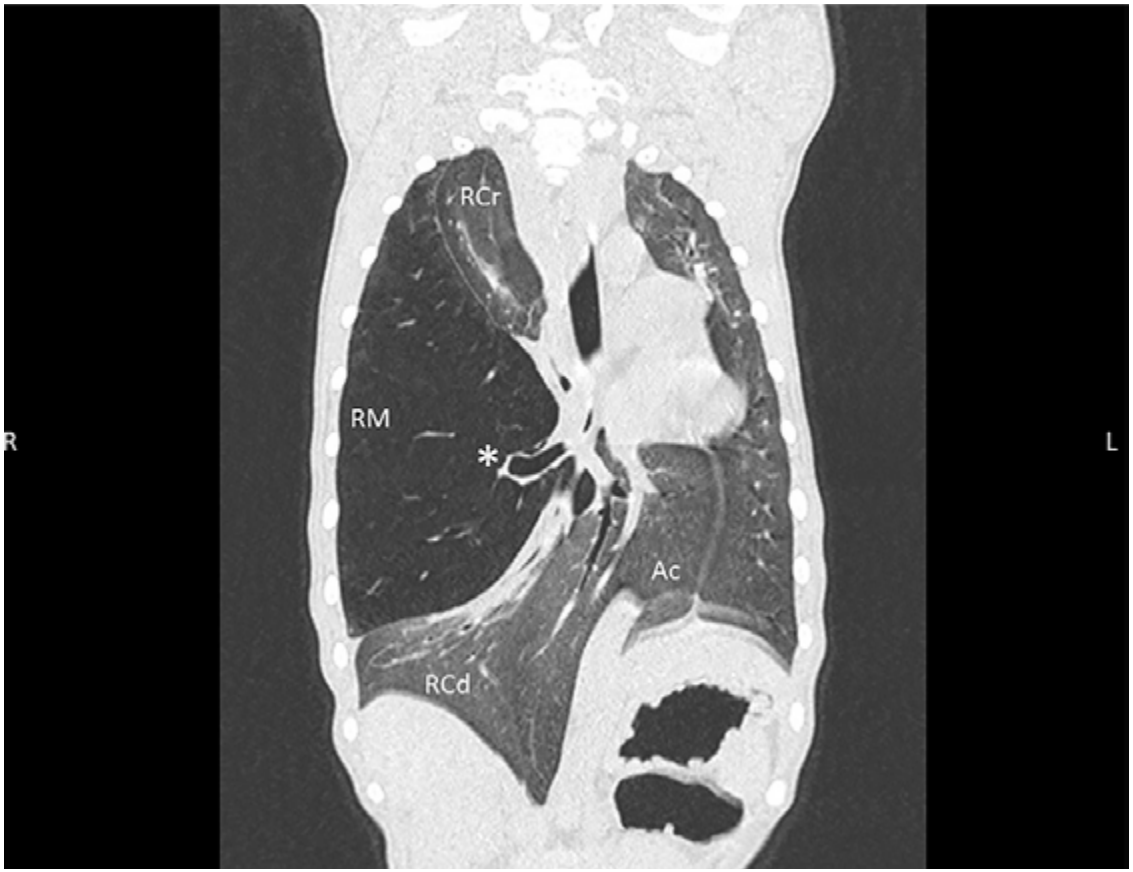

Figure 2. *Thoracic CT dorsal reconstruction images from an 11-week old, sexually intact, female Catahoula Leopard dog with congenital lobar emphysema. Note the hyperinflated right middle lung lobe (RM) with an abrupt narrowing of the main stem bronchus (asterisk); leftward deviation of the heart, mediastinal structures, and accessory lung lobe (Ac); and diffuse increased attenuation/ground glass pattern within remaining right cranial (RCr) and right caudal (RCd) lung lobes as well as left lung.*

A local block of bupivacaine (6 mg; 1 mg/kg) and lidocaine (6 mg; 1 mg/kg) was placed into the intercostal muscles cranial to, at the level of, and caudal to the incision. A red rubber catheter connected to a 3-way stopcock and 20 mL syringe was passed through the incision prior to closure for evacuation of air from the thorax. The ribs were apposed using simple interrupted sutures of 3-0 PDS, and the musculature of the thoracic wall was apposed with 3-0 Monocryl in a simple continuous pattern. Prior to closure of the skin, the anesthetist was consulted as to whether substantial resistance was noted during hand-ventilation. Air was suctioned from the thoracic cavity until anesthesia noted minimal resistance to ventilation. Negative pressure was not re-established within the thoracic cavity due to concern of re-expansion injury to the remaining lung lobes. The thoracic catheter used for air evacuation was removed prior to closure of the skin, and the skin was apposed in an intradermal pattern using 4-0 Monocryl.

Histopathology was performed including hematoxylin and eosin (H&E) stain, Masson's trichrome stain to identify collagen, and immunohistochemistry antibodies for AE1/AE3 cytokeratin to identify epithelial cells and α-smooth muscle actin (α-SMA) to evaluate bronchial smooth muscle of the resected right middle lung lobe (**Figures 4**–**7**).

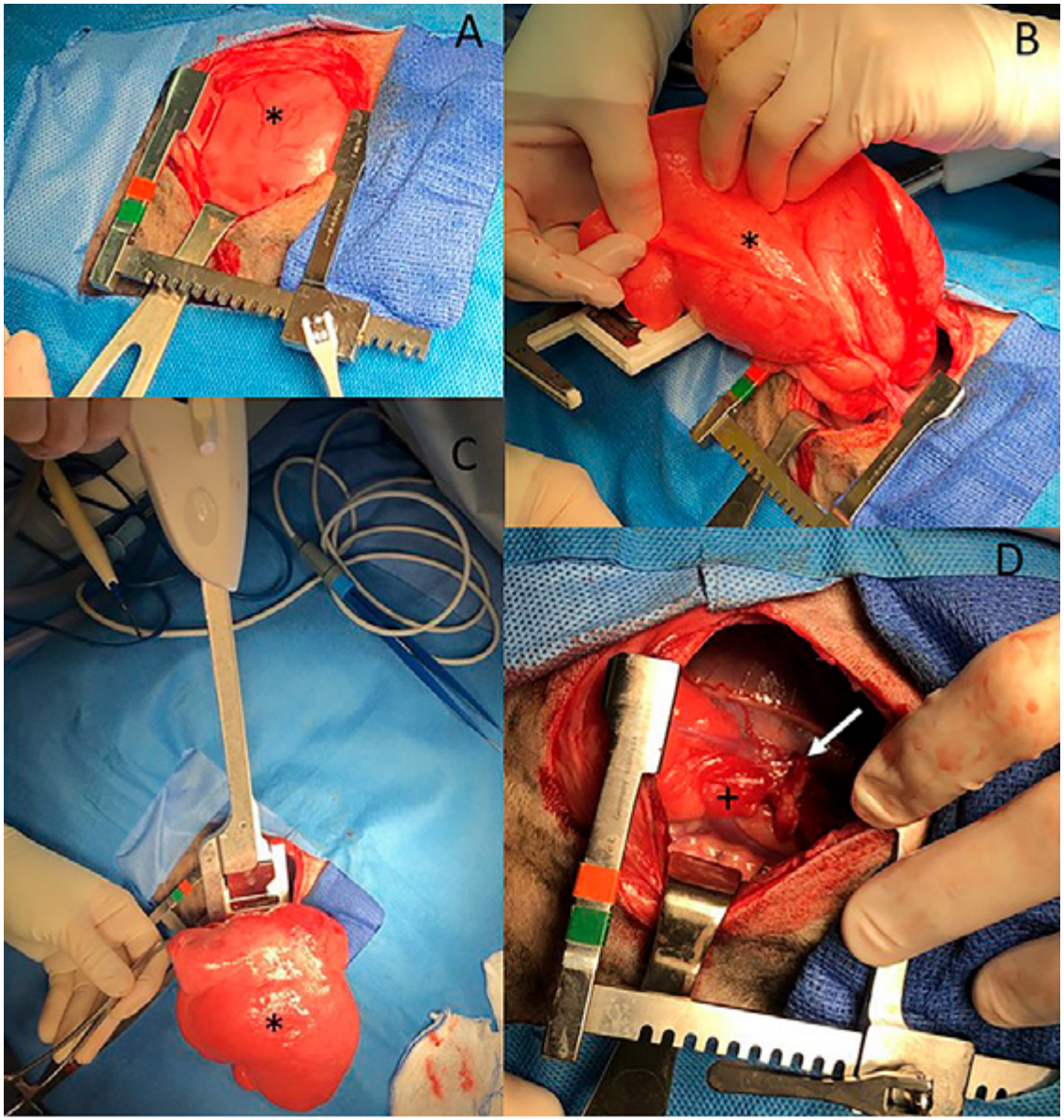

Figure 3. *(**A–D**) Intraoperative images from an 11-week old, sexually intact, female Catahoula Leopard dog with CLE. The head is to the right and dorsal is on the bottom of all images. The right middle lung lobe (asterisk in all images) is occupying a majority of the right thoracic cavity (**A**) and is hyperinflated (**A, B**). A lung lobectomy was performed with a DST Series TA Stapler using a 30 mm V3 vascular stapler (**C**). The right middle lung hilus can be seen with the staple line (white arrow) following lobectomy, and the right caudal lung lobe has an area of atelectasis (+) (**D**).*

Results revealed bronchial cartilage hypoplasia characterized by variable quality cartilage to the absence of cartilage, dilated and coalescing alveoli consistent with marked emphysema, abnormal bronchial smooth muscle architecture, and expanded connective tissues and collagen with pleural fibrosis (**Figures 4–7**). The microscopic findings were consistent with the suspected clinical diagnosis of congenital lobar emphysema.

The dog recovered uneventfully from anesthesia and was maintained on a fentanyl constant rate infusion (2–5 µg/kg/h IV, titrated to effect), gabapentin (10 mg/kg PO every 8 h), and carprofen (2 mg/kg PO every 12 h). Oxygen supplementation at an FiO2 of 30–40% was provided for 8 h via an oxygen cage, and the dog had a normal respiratory rate and effort the following day out of oxygen supplementation. The dog was discharged 24 h postoperatively with no apparent complications. The owners reported the dog continued to recover well from surgery. As of 18 months postoperatively, the dog was doing clinically well, with no dyspnea or exercise intolerance per owner communication.

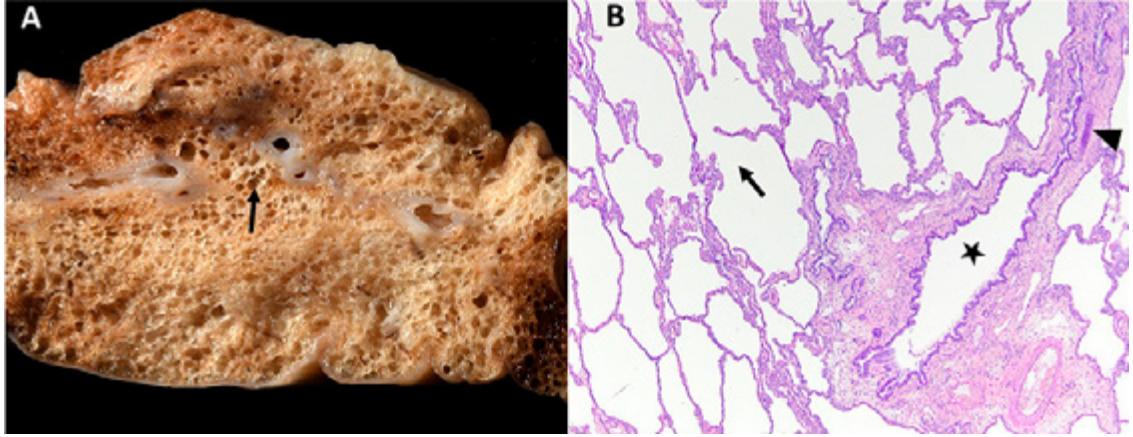

Figure 4. *Gross (**A**) and histopathologic (**B**) images of the right middle lung lobe affected by CLE are depicted. Ruptured alveoli are present in the lung lobe: grossly this is visible as air pockets within the lung tissue (surrounding the arrow, **A**) and microscopically as discontinuity of the alveolus wall (arrow within alveolus, **B**). In image (**B**), bronchial cartilage is present as a small discontinuous basophilic band (arrow head), which should fully surround a normal bronchus. This represents bronchial cartilage hypoplasia and is the cause of the bronchus in image (**B**) (star) being partially collapsed instead of rounded.*

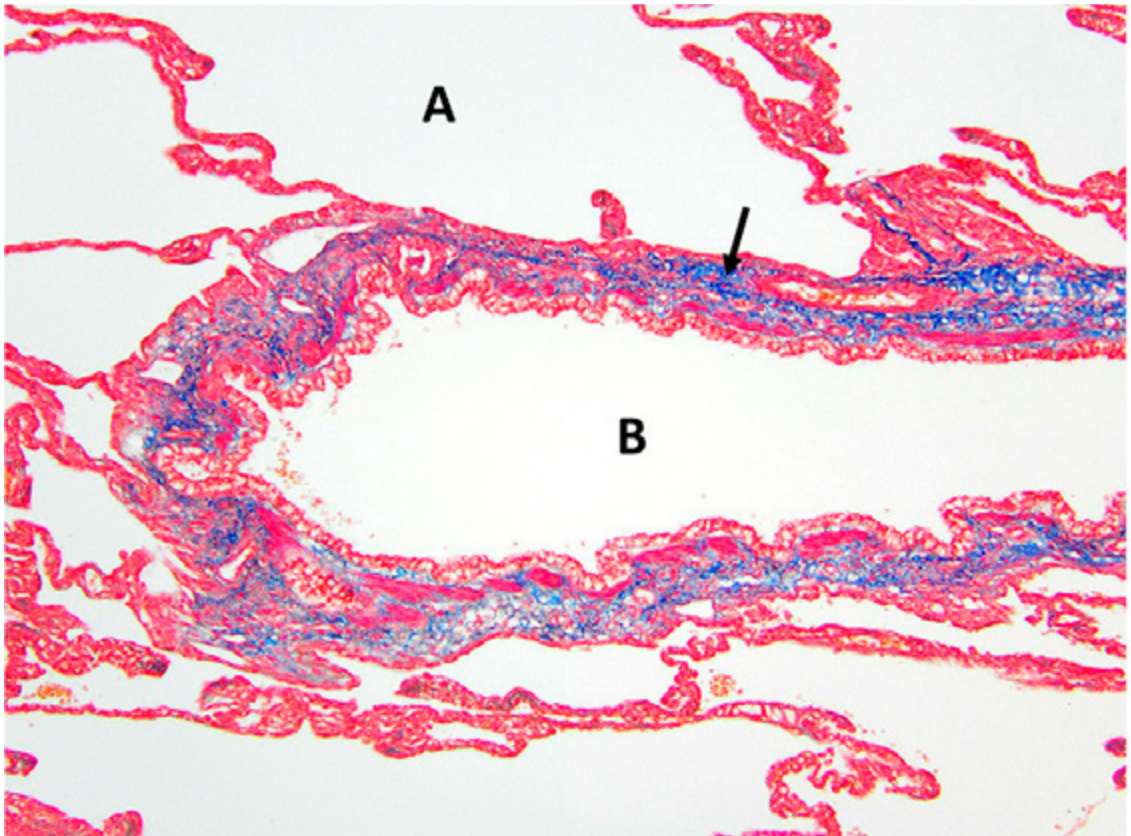

Figure 5. *Masson's trichrome staining of the right middle lung lobe highlights the abundant collagen/connective tissues (arrow) surrounding the bronchus B with no cartilage present. A, alveolus.*

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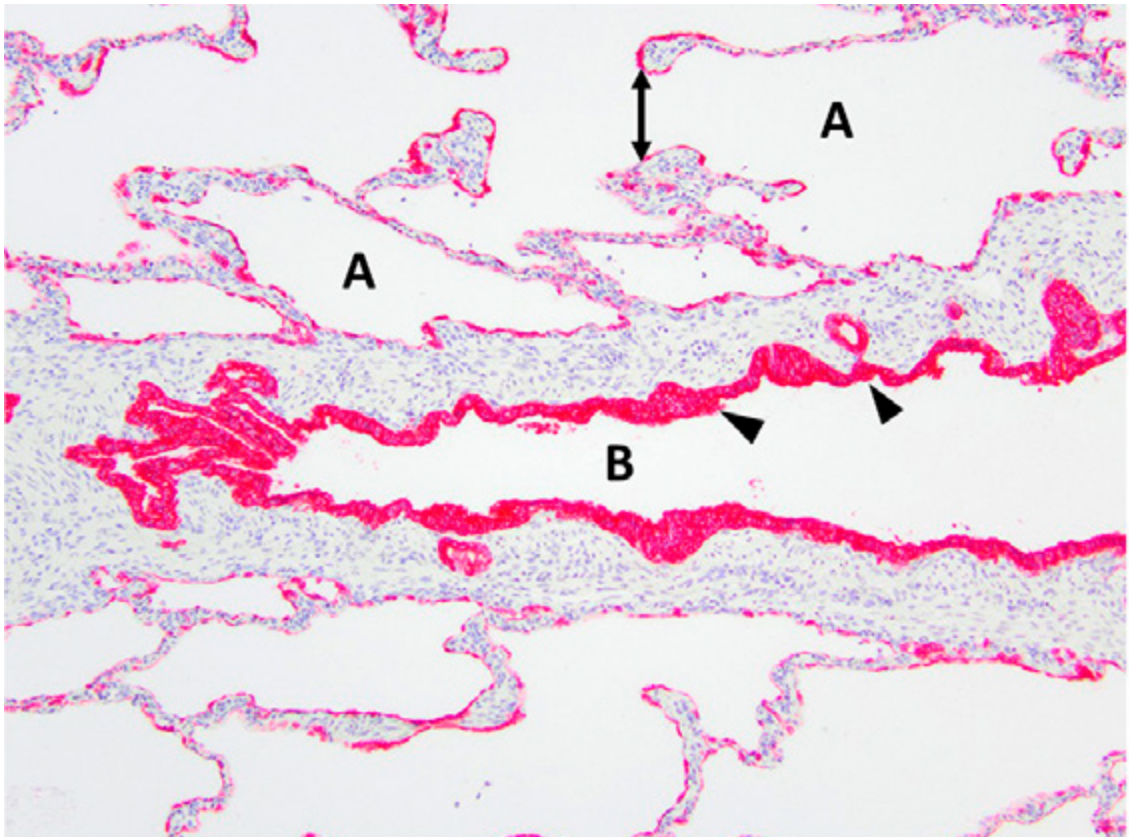

Figure 6. *Immunohistochemistry AE1/AE3 cytokeratin of the right middle lung lobe highlights the bronchial epithelium with multilayered columnar to cuboidal epithelium and invaginations (arrowheads). The bronchus is identified as* ***B****. The alveoli are identified as* ***A*** *and contain ruptured septa with clubbed ends (double arrow).*

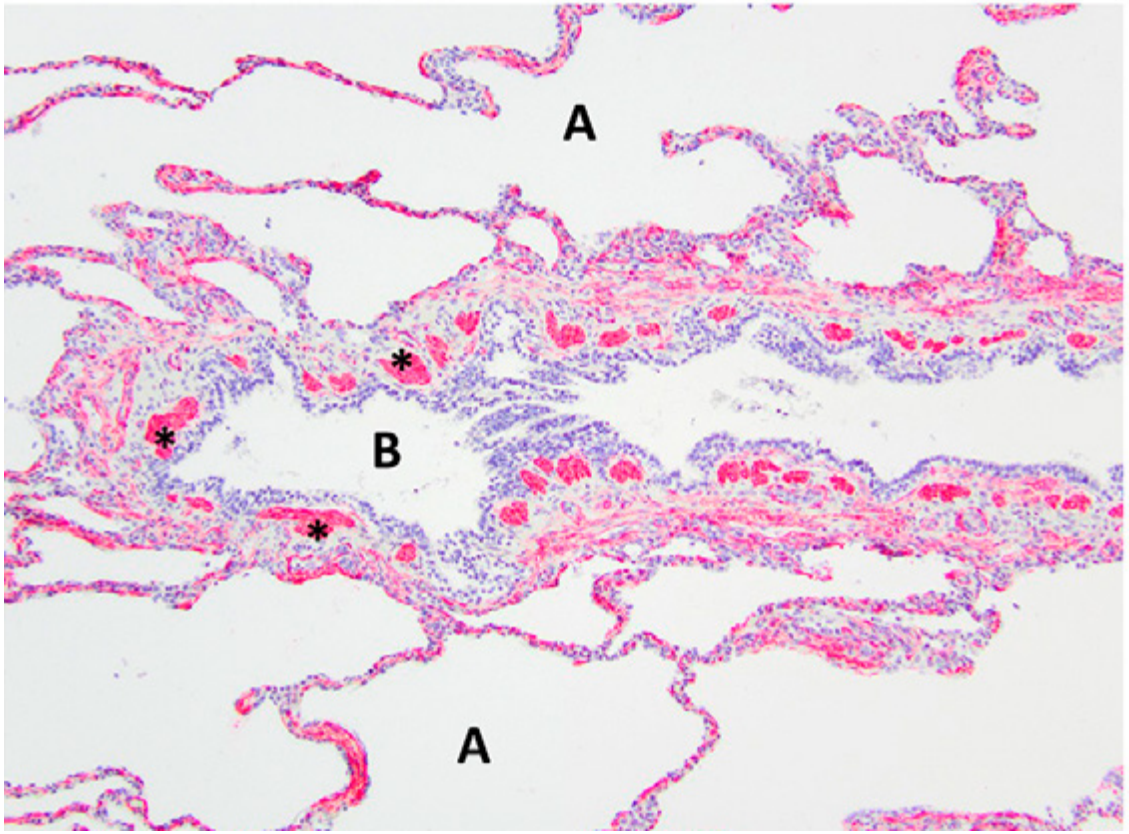

Figure 7. *Immunohistochemistry α-SMA for smooth muscle of the right middle lung lobe highlights the abnormal bronchial smooth muscle architecture as the discontinuous smooth muscle bundles (asterisks) surrounding the bronchus B. A, alveoli.*

Discussion

This case report details the clinical treatment of a young dog diagnosed with CLE and undergoing successful surgical management of this condition. The diagnostic imaging, surgical, and histopathologic findings were all considered characteristic of CLE. Congenital lobar emphysema is a rare lower respiratory tract disease that is characterized by alveolar air accumulation with resulting hyperinflation of the affected lung lobes. Air accumulation is believed to be due to dynamic airway collapse, in which entry of air on inspiration occurs normally, however, abnormal bronchial collapse during expiration does not allow air to escape (1, 2, 9, 13, 19). It is this overdistension due to hyperinflation that causes the lung to be generally non-functional. Abnormal bronchial collapse and obstruction on expiration has been reported to be associated with bronchial cartilage hypoplasia, dysplasia, and aplasia; external bronchial compression; and idiopathic etiologies (1–7, 10–12, 14, 17, 18).

Histopathology of the lung lobe removed from the dog reported here revealed bronchial cartilage hypoplasia with marked emphysema and pleural fibrosis. The microscopic findings of this lung lobe including the abundant collagen with no cartilage around the bronchus on the Masson's trichrome

stain, the abnormal bronchial smooth muscle architecture on the α-SMA immunostaining, the cuboidal and invaginated epithelium lining the bronchus on the AE1/AE3 immunostaining, and the many areas of discontinuous ruptured alveolar septa were consistent with a diagnosis of CLE and previously reported (7, 8, 14). The pathologist commented that the deficient bronchial cartilage and bronchial atresia resulting in defects in the bronchial walls caused greater volumes of air to enter the affected lobe on inspiration than exited on expiration, resulting in air trapping and the clinical presentation of a hyperinflated lung lobe. The bronchial hypoplasia diagnosed here has been previously documented as associated with CLE in 10 reported cases in the literature (4–6, 14–17). Pleural fibrosis has been noted in other dogs with histopathologic analysis of the affected lung lobes, however the clinical significance of pleural fibrosis, as it pertains to CLE in dogs, is not described (7, 8, 14). Although some reported cases in dogs have determined CLE to be idiopathic in nature, it appears the dog in this report developed CLE due to the aforementioned bronchial abnormalities (7, 9, 10, 12).

The dog in this report was 11 weeks old at the time of surgery with clinical signs starting as early as 8 weeks old. Although CLE is often diagnosed in young animals, the dog in this report is one of the youngest dogs to have been clinically affected by CLE and successfully treated with surgical excision of the affected lung lobe. The 10 previous CLE patients with descriptions of successful surgical treatment consisted of nine dogs, ranging from 6 weeks to 10 years of age, and one 5-month-old kitten (1–9, 18). On evaluation of the previously reported cases, the median age at the time of successful treatment of CLE in the nine dogs was 20 weeks and was also 20 weeks in the only reported cat (1–9, 18). Importantly, this case report highlights a successful anesthetic and invasive surgical event for a pediatric puppy (< 12 weeks old), which comes with additional risks due to the immature age including reduced ability to respond to cardiovascular changes; immature renal, hepatic, and thermoregulatory functions; and reduced pulmonary reserve with high oxygen consumption (22, 23).

Of the 10 case reports with successful surgical treatment described previously, lobectomy has been performed on the following affected lung lobes in dogs and cats with CLE: 7/10 for the right middle lung lobe, 2/10 for the left caudal subsegment of the left cranial lung lobe, and 1/10 for the left caudal subsegment of the left cranial lung lobe and accessory lobe simultaneously (1–9, 18). A recent retrospective study of 14 dogs and 3 cats with lobar emphysema suspected a congenital etiology in 14/17 animals (17). In the same report, 8/17 animals underwent lung lobectomy with confirmed CLE diagnosis of the right middle lung lobe in all 8 cases, though one dog and one cat also had the right cranial and right caudal lobes affected, respectively (17). The dog in the case reported here, similar to other cases, was affected with CLE in the right middle lung lobe.

Although a variety of large-breed dogs with CLE have been reported (9, 11, 12, 17), descriptions of successful surgical intervention in the literature for large breed dogs are limited to a single case report of an Old English Sheepdog (9). Therefore, the description of successful surgical intervention for the dog in this report supplements the literature for surgical intervention in large-breed dogs. Although it is possible that other large-breed dogs were treated surgically, breed descriptions for dogs undergoing surgery were not available for one report (17). The majority of reports with successful surgical treatment are in small breed dogs (1, 3–8, 18).

Hyperinflation of the affected lung lobe on the thoracic radiographs resulted in pursuit of a CT scan to further define pulmonary pathology in the dog described here. Evidence of hyperinflation and hyperlucency of the affected lung lobes, mediastinal shift, pulmo-

nary atelectasis, and elevation of the cardiac silhouette from the sternum were common findings in 15 previous cases that utilized radiography in their diagnosis of CLE (1–13, 16, 18). Of the 15 cases diagnosed by thoracic radiography and supporting clinical signs, only five went on to utilize CT for surgical planning (2, 3, 5, 6, 8). The CT scan in this dog revealed severely expanded pulmonary parenchyma with hypoattenuation compared to the surrounding lung lobes consistent with hyperinflation, which was also seen in all previously reported cases undergoing CT (2, 3, 5, 6, 8). The value of CT lies in the ability to diagnose other concurrent conditions in the pulmonary parenchyma and thoracic cavity such as pneumothorax, pneumomediastinum, bullae or blebs, bronchial abnormalities, and concurrent abnormalities in other lung lobes, which are all variably reported in the literature (2, 3, 5, 6, 8). It is important to note that multiple cases have been reported involving more than one lung lobe based on CT diagnosis (5, 17). Therefore, if a CT scan is not performed prior to surgical intervention, consideration should be made to approach the thoracic cavity via a median sternotomy instead of an intercostal thoracotomy if there is any concern that additional lung lobes on opposite sides of the thoracic cavity may be affected. This will allow for effective exploration of both hemithoraces. The additional findings in the dog of this case report due to use of the CT scan included abrupt narrowing of the bronchus in the affected lung lobe, compression with atelectasis of local lung lobes, and displaced thoracic structures.

It should be noted that not all patients are immature at the time of CLE diagnosis, and some patients may be asymptomatic for CLE (4, 8, 16–18). However, most patients do exhibit clinical signs related to CLE and at an immature age, similar to the dog of this report. Out of the total 18 cases with surgical intervention for CLE reported in the literature (1–9, 18), only one case of perioperative death has been reported (1–9, 17, 18). In addition, 7/18 reported cases also detail long-term follow-up revealing clinically normal dogs (1–6, 8). Given the reported good short- and long-term outcomes, the literature supports surgical removal of affected lung lobes as treatment of choice for CLE, particularly for clinically affected dogs. Prognosis for the dog of this case report is excellent given the lack of dyspnea or exercise intolerance at 18 months postoperatively, as well as confirmation on preoperative imaging that the remaining lung lobes were within normal limits.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because this is a case report of a clinical case and underwent no changes to clinical care as a result of this case report. Written informed consent was obtained from the owners for the participation of their animals in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

CL, SH, and ME were involved in the clinical management of this case. CL, SH, and LE discussed the case. LE was responsible for drafting the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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(R) evolution

PEQUEÑOS ANIMALES

Tabla de contenido:

Inhibidores de la enzima de conversión de angiotensia, aún seguimos ahí

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Introducción

Los inhibidores de la enzima de conversión de angiotensina (IECAs) son profármacos que inhiben la síntesis a nivel renal de angiotensina II que es un potente vasoconstrictor.

Actúan, por tanto, como vasodilatadores y se recomiendan en el tratamiento de la enfermedad renal crónica (ERC), hipertensión arterial, proteinuria y del fallo cardiaco congestivo (FCC).

El benazepril, enalapril, imidapril y ramipril están aprobados para el tratamiento de perros con FCC y su eficacia está demostrada ya que mejoran la función hemodinámica y los signos clínicos, aumentando la supervivencia. Son prodrogas que se administran por vía oral y se activan dentro del organismo.

Al inhibir el paso de angiotensina I a angiotensina II presentan un efecto hipotensivo leve a moderado.

Existe también evidencia de que los IECAs están recomendados en perros y gatos con ERC ya que reducen la presión capilar glomerular, tienen efectos antiproteinúricos, tienden a retrasar la progresión de la enfermedad y limitan la extensión de las lesiones renales.

Una de las mayores ventajas es su tolerabilidad.

En perros presentan eliminación renal (45%) y hepática (55%) y en gatos se excretan principalmente por el hígado (casi 85%).

Sistema renina-angiotensina-aldosterona (RASS)

Sistema de emergencia que conecta el corazón con el riñón para regular la presión arterial.

Está siempre preparado, el hígado libera continuamente angiotensinógeno.

Cuando hay un problema cardiaco, el corazón no bombea la sangre con eficiencia y el resultado es la reducción del volumen sanguíneo circulante. Lo detecta el riñón y provoca en el aparato yuxtaglomerular la liberación de renina, que desencadena la activación del eje. El angiotensinógeno se transforma en angiotensina I, casi sin actividad, que en los capilares pulmonares se encuentra a la ECA y se transforma en angiotensina II.

La angiotensina II es un potente vasoconstrictor que actúa a nivel de la corteza adrenal para liberar aldosterona. La angiotensiona II provoca vasoconstricción y la aldosterona reabsorción de sodio y agua aumentando la volemia. Ambos provocan el aumento de la presión arterial.

La aldosterona se une a los receptores mineralocorticoides del corazón provocando alteraciones ventriculares, arritmias, fibrosis cardiovascular y edema pulmonar. Por tanto, la activación del RAAS puede provocar la sobrecarga del corazón.

La acción completa sobre el eje requiere el control sobre la angiotensina II y la aldosterona. Los IECAs controlan la transformación de angiotensina I a II, pero no el escape de la aldosterona.

El escape de la aldosterona (EA) es una condición en la cual la IECA y/o el receptor bloqueador de angiotensina fallan en suprimir la actividad del sistema angiotensina-aldosterona. El bloqueo incompleto del SRAA es común en perros con EVM que están recibiendo un IECA. Mientras que el mecanismo del EA es probablemente multifactorial y aún no se conoce bien, la existencia demostrada de EA en perros ofrece la posibilidad de mejorar el pronóstico de la EVM con la adición de un bloqueante del receptor mineralocorticoide a las terapias actuales. En un estudio se comprobó que, aproximadamente, el 30% de los perros en tratamiento por cardiopatía y FCC presentaban EA. La identificación de subpoblaciones de pacientes que experimentan EA puede ayudar a guiar el diseño de futuros estudios y la toma de decisiones clínicas.

La deshidratación, hipotensión, FCC grave, la administración de dosis elevadas de diuréticos o una alteración renal previa, son algunas de las situaciones que incrementan el riesgo de complicación renal durante la terapia con IECAs.

La funcionalidad renal debe evaluarse antes de iniciar una terapia y los valores de urea y creatinina deberán ser controlados periódicamente durante el tratamiento

Fallo cardiaco congestivo

Una de las principales causas de morbilidad y mortalidad en perros. Al igual que en humanos, la activación del SRAA juega un papel central en la patofisiología del FCC, su activación crónica contribuye a la progresión de la enfermedad. Uno de los principales tratamientos es el uso de IECAs que mitigan el SRAA.

Los IECAs en perros disminuyen la presión arterial sistémica (PA), la fracción de regurgitación mitral y la presión de la aurícula izquierda.

Dosis más altas de 0,5 mg/kg/12h bajan los niveles de AngII y aumentan los niveles de Ang 1-7 comparando con niveles más bajos, lo que es más favorable ya que se mantiene la ruta de protección con efecto antifibrótico, antihipertrófico, vasodilatador y antioxidante pero en un estudio no se vieron diferencias relevantes entre los grupos de dosis más bajas y más altas en

cuanto a la presión arterial o las variables ecocardiográficas. El conocimiento de las alteraciones dependientes de la dosis en los biomarcadores de las vías clásicas y alternativas del SRAA podría ayudar a optimizar el tratamiento.

La edad, aumento progresivo de tamaño cardiaco (A y VI), incremento de la velocidad de flujo transmitral (E), aumento del NT-proBNP y el aumento en reposo de la frecuencia cardiaca son, al menos, moderadamente predictivos de la progresión de la EVM y pueden ayudar a identificar perros con riesgo de FCC.

Las repeticiones en la aparición del edema pulmonar indica un mal ajuste de la medicación y deben revisarse la terapia diurética y neurohormonal (IECAs).

El objetivo fundamental del tratamiento de la insuficiencia cardiaca consiste en reducir la severidad de los signos clínicos, mejorar la calidad de vida, prolongar la vida y reducir el riesgo de muerte súbita.

- Reducir edema y derrame
  - Diuréticos: furosemida, espironolactona, hidroclorotiazida.
  - Vasodilatadores venosos: IECAs, pimobendan
- Mejorar el gasto cardiaco:
  - Inotropos positivos: pimobendan
  - Vasodilatadores arteriales: IECAs, amlodipino, pimobendan
- Manejo de las arritmias:
  - Diltiazem, digoxina, sotalol, amiodarona, β-bloqueantes
- Reducir ejercicio físico
- Control nutricional
- Terapia múltiple recomendada por el ACVIM:
- IECA: evitar el paso de angiotensina I a angiotensina II
- Espironolactona: evitar escape aldosterona
- Furosemida: evitar el edema pulmonar
- Pimobendan: Inodilatador

Lo racional para el uso de IECAs es que la supresión del SRAA tiene el potencial de conducir a una situación más favorable hemodinámicamente ya que producen vasodilatación, contrarrestan la retención de líquidos y la progresión de la remodelación auricular y ventricular que ocurre en respuesta a la regurgitación mitral.

Sin embargo, dos estudios, con perros asintomáticos, no mostraron diferencias en el retraso en el desarrollo de FCC entre perros que se les administró enalapril y otros con placebo. Lo que indica que pueden no ser eficaces en el tratamiento de perros asintomáticos.

Por tanto, se recomienda empezar tratamiento con IECAs en estadio C, episodio de FCC que no es refractario al tratamiento estándar. En fase C el tratamiento recomendado por el ACVIM:

- Diurético: furosemida o torasemida. Se recomienda uso de furosemida hasta que falle y entonces torasemida. No cortar el tratamiento si ya ha habido FCC.
  - Furosemida: 2-4 mg/kg/8-12 horas
  - Torasemida 0,2 mg/kg/8-12h
- Inodilatador:
  - Pimobendan: Estudio QUEST. 0,2-0,3 mg/kg/12h
- Espironolactona + IECAs: benazeprilo el más utilizado: Estudio BESST. Mejora la esperanza y calidad de vida. Reduce el riesgo de edema pulmonar.
  - Espironolactona: 2 mg/kg/24h
  - IECAs: 0,25 mg/kg/12-24h

En cuanto a la nutrición se está estudiando el efecto de añadir ciertos nutrientes al tratamiento del FCC. Lo importante es que el animal coma adecuadamente y cubra sus necesidades calóricas y proteica (60 Kcal/kg/dia).

- Ácidos grasos omega 3: reducen las arritmias y tratamiento caquexia cardiaca
- Proteínas: no restringir proteínas, acelera la caquexia cardiaca
- Estudio de supervivencia reducida en perros que consumían dieta sin grano
- L-carnitina y taurina
- Modesta restricción sodio

También es importante la restricción del ejercicio físico dando solo paseos con correa.

Se deben medir la creatinina sérica y electrolitos 14 días después de empezar con el tratamiento. Posible desarrollo de ERA si las concentraciones de creatinina sérica aumentan en ≥30% de la concentración inicial.

Los IECAs son inhibidores enzimáticos competitivos. Se precisa inhibir >95% del ECA para inhibir significativamente la formación de Angiotensina II. Dosis altas demostraron mayores beneficios. A mayor grado de insuficiencia cardiaca, se deben administrar dosis mayores. No son útiles para reducir la presión en AI ya que se ha visto que la presión se reduce muy poco a pesar de la reducción de la postcarga.

La administración crónica de furosemida debe acompañarse de la administración de IECAs para contrarrestar la activación de mecanismos vasoconstrictores y desequilibrios electrolíticos que puedan surgir. Los IECAs y la reducción en la ingesta de sodio ayudan a reducir la dosis diaria de diuréticos.

La aldosterona provoca retención de sodio y líquidos, estimula la inflamación de arterias vasos, desajusta baroreceptores, puede provocar hipertensión y arritmias.

La secreción persistente de aldosterona, a pesar de la presunta reducción de la actividad plasmática de la ECA y de la producción de angiotensina II, se ha denominado "escape de aldosterona". Tanto los IECAs como los diuréticos del asa como el amlodipino, provocan escape de aldosterona. Hay diferentes rutas de producción de aldosterona y escapa a su ruta habitual, además, los IECAs bloquean el SRAA plasmático no el tisular. No se sabe con certeza por qué se produce este escape.

La espironolactona (2.0 mg/kg PO q12 - 24 h) se recomienda como terapia adyuvante para el tratamiento crónico en fase C. El beneficio se basa en ser antagonista de la aldosterona.

En el estudio DeLay se falló en demostrar que la administración combinada de espironolactona y benazepril retrasara la aparición de FCC en perros en fase preclínica. Sin embargo, el tratamiento proporciona efectos beneficiosos en la remodelación cardiaca y estos resultados sí pueden tener relevancia clínica. El bloqueo de los receptores de mineralocorticoides reduce la remodelación cardiaca y con el tratamiento se vio que mejoraron valores como el índice vertebral cardiaco o VHS (vertebral heart score), ratio aurícula izquierda/aorta, diámetro interno del ventrículo izquierdo en diástole normalizado (LVEDDn) y el flujo transmitral que son factores predictivos negativos. NT-proBNP tampoco aumento en el grupo tratado y si en el placebo.

La espironolactona bloquea receptores de aldosterona, es un diurético muy débil, previene la reabsorción distal de sodio y ahorra potasio, antagoniza efectos cardiotóxicos de la aldosterona, previene la fibrosis miocárdica y contrarresta el escape de la aldosterona a los IECAs. Las dosis sugeridas son de 0,5-2,0mg/kg VO una o dos veces al día.

Hay pocos estudios sobre el manejo terapéutico del FCC en gatos. Normalmente la terapia crónica consiste en:

- Furosemida PO 1-3 mg/kg q12 -24h o menor
- IECA PO, enalapril o benazepril 0.25 a 0.5 mg/kg q12-24h
- Diltiazem PO 1.5-2.5 mg/kg q8h (no en gatos con FCC)

A la mayoría de los gatos se les trata con IECAs y furosemida. Beta bloqueantes cuando no hay FCC o para tratar arritmias u obstrucción severa del tracto de salida del ventrículo izquierdo. El diltiazem es más eficaz, pero se usa menos por su administración más frecuente.

En un estudio con benazepril utilizado en gatos con CMH en fase subclínica se vió que se mejoraba la función diastólica y la hipertrofia ventricular aunque se consideró que los hallazgos podían ser inicidentales.

No se ha demostrado efecto sobre el tiempo de aparición de FCC, ni que se retrase el paso de estadio C a D, aunque se sigue usando por algunos cardiólogos.

Ne necesitan más estudios en pacientes felinos para evaluar su eficacia.

Enfermedad renal crónica

Enfermedad irreversible y progresiva que avanza a glomeruloesclerosis y fibrosis intersticial y, en ocasiones, a estadios finales de fallo renal.

Varios sistemas endocrinos y citoquinas están involucradas en el desarrollo de hipertensión renal y progresión de la enfermedad. La hiperactivación del SRAA juega un papel primordial.

La AngII contrae las arteriolas eferentes aumentando la presión capilar glomerular y la filtración. A su vez, contrae los vasos sanguíneos periféricos y, por tanto, eleva la presión arterial sistémica. Estimula la corteza adrenal para secretar aldosterona que reduce la pérdida de sodio y fluidos en túbulos distales, contribuyendo también al incremento de la presión arterial.

La hipótesis de la hiperfiltración nos dice que el aumento de la filtración glomerular provoca daños físicos en los glomérulos, lo que lleva a una lesión renal progresiva. El exceso de AngII desempeña su papel patógeno principal al aumentar la presión de perfusión glomerular provocando lesiones tubulointersticiales, daño en las nefronas remanentes y agravando el deterioro y la disfunción estructural renal.

La proteinuria es un fuerte factor de progresión y su reducción tiene efectos críticos.

Todos los perros con proteinuria renal o azotemia deben ser evaluados para detectar hipertensión. Los gatos mayores, en particular aquellos con enfermedad renal crónica (ERC), deben someterse a exámenes de presión arterial desde el inicio de la enfermedad y durante el curso de la misma ya que aproximadamente el 10 % tendrá hipertensión.

La indicación general es reducir la presión arterial <150 mm Hg

Se cree que el mecanismo de la hipertensión renal implica la retención de líquidos corporales asociada con la insuficiencia renal, el aumento del gasto cardíaco y la resistencia de los vasos periféricos, la activación del sistema renina-angiotensina-aldosterona (RAA) y la supresión del sistema calicreína-cinina-prostaglandina.

Las inhibir la ECA y por tanto la producción de AngII obtendremos un efecto renoprotector con reducción en la presión arterial sistémica, la presión capilar glomerular y el volumen de filtrado glomerular.

En los gatos, la pérdida de masa renal provoca la activación del SRAA y la hipertensión renal asociada que juegan un papel fundamental en la progresión de la insuficiencia renal.

La respuesta a los IECAs es menor que en perros, es posible que exista heterogeneidad en el grado de afectación del SRAA en gatos con enfermedad renal natural y, por tanto, en la respuesta a la inhibición de la ECA. Su administración en estos pacientes provoca una reducción moderada de la hipertensión sistémica. Aun así, pueden ser un tratamiento eficaz para frenar la tasa de progresión de la insuficiencia renal en gatos ya que disminuyen la hipertensión glomerular presumiblemente por su efecto en el SRAA intrarrenal.

En algunos gatos se pueden desarrollar disminuciones transitorias y reversibles de la TFG en respuesta a la vasodilatación arteriolar eferente durante el tratamiento con inhibidores de la ECA.

En un estudio con benazepril, corrigió la hipertensión sistémica y redujo significativamente la angiotensina II y la aldosterona. Se redujeron los valores de creatinina sérica y la proteinuria. Además, se excreta la mayoria en bilis (85%), de esta forma no se acumula en animales con disfunción renal lo que indica su potencial terapéutico seguro en ERC.

En perros, la ERC suele tener un origen glomerular y por eso presentan más proteinuria que los gatos. En IRIS I no suelen presentar signos clínicos a menos que esté muy baja la albúmina o en glomerulonefritis.

La hipertensión sistémica también es común en perros con ERC y puede conducir a una progresión más rápida de la insuficiencia renal. La activación del SRAA es una de las principales causas y el tratamiento con IECA se considera una de las estrategias terapéuticas más eficaces.

Los IECAs producen disminución en la hipertensión capilar glomerular y la gravedad de la proteinuria y un retraso en la progresión de las lesiones renales. Presentan un efecto positivo sobre la TFG y se reduce la liberación de matriz extracelular y colágeno de las células mesangiales y tubulares que disminuye la fibrosis glomerular e intersticial.

Estudios adicionales deberían investigar si los IECA pueden minimizar la hipercoagulabilidad renal.

La administración de benazepril en perros parece ser segura y bien tolerada, con efectos adversos mínimos.

Proteinuria

La hipertensión glomerular causa proteinuria debido al aumento de la tasa de filtración glomerular (TFG) de una sola nefrona que aumenta el radio de los poros dentro de la barrera de filtración y, por lo tanto, se produce la ultrafiltración de proteínas.

La pérdida renal de proteínas plasmáticas puede contribuir a la hipoalbuminemia, alteraciones en los factores de coagulación, disminución de la inmunidad y desarrollo de hiperlipidemia en algunos casos.

Se ha demostrado que la proteinuria crónica se asocia con fibrosis intersticial, así como con degeneración y atrofia tubular.

La proteinuria es un importante marcador para diagnóstico precoz y pronóstico. Está altamente relacionada con la reducción de la supervivencia en perros y gatos azotémicos y no azotémicos. En perros azotémicos, un ratio proteína/creatinina en orina (UPC por sus siglas en inglés) igual o mayor a 1 está asociado con un mayor riesgo de crisis urémica y muerte.

Las fuerzas hemodinámicas influyen en el movimiento transglomerular de

las proteínas, por lo que alterar la hemodinámica renal sería eficaz para reducir la proteinuria.

Se debe medir la proteinuria en varios controles y descartar la pre y la postrenal.

Los perros normales y la mayoría de los gatos normales deben tener una relación proteína: creatinina en la orina inferior a 0,4 e inferior a 0,2, respectivamente.

Los perros que tienen proteinuria renal y un UPC de 2,0 o mayor suelen tener enfermedad glomerular, mientras que los perros con un UPC inferior a 2,0 pueden tener enfermedad glomerular o enfermedad tubulointersticial. Las enfermedades glomerulares ocurren con mucha menos frecuencia en los gatos, pero deben sospecharse cuando el UPC es 2 o mayor. La hipoalbuminuria concurrente es evidencia adicional de que hay enfermedad glomerular

El sistema renina-angiotensina-aldosterona (SRAA) ha sido el principal sistema objetivo de este enfoque para reducir la proteinuria. La administración de IECAs y/o bloqueadores de los receptores de angiotensina (ARB) se consideran un tratamiento estándar en perros y gatos con proteinuria renal.

Los mecanismos propuestos para estos efectos incluyen la disminución de la resistencia arteriolar glomerular eferente que conduce a una disminución o normalización de la presión hidráulica transcapilar glomerular, reducción de la pérdida de sulfato de heparina glomerular, disminución del tamaño de los poros endoteliales de los capilares glomerulares, mejora del metabolismo de las lipoproteínas, crecimiento y proliferación mesangial glomerular más lentos. En perros y gatos, el objetivo terapéutico ideal es una reducción de la UPC, una reducción de la UPC del 50 % o más sin un empeoramiento inapropiado de la función renal.

La hiperpotasemia parece ser un efecto secundario común de la inhibición de SRAA en perros con enfermedad renal, pero probablemente sea poco común en gatos.

El tratamiento de la proteinuria en perros reduce la progresión de la ERC y, en un estudio, el grupo tratado con enalapril mostró una reducción significativa del UPC tras 30 días de tratamiento. Además, demostró minimizar los signos clínico de uremia y mantener una óptima condición corporal.

Hipertensión

La hipertensión no tratada pone en riesgo de daño a otros órganos diana (cerebro, corazón, ojo, riñón, grandes vasos), lo que aumenta la morbilidad y la mortalidad.

Se estima una incidencia en gatos del 19.5%.

La presión arterial sistólica (PAS) media debe ser de 120 mmHg (rango 110–132 mmHg) pero aumenta con la edad y en gatos con ERC.

Se distinguen varios tipos de hipertensión:

- Situacional
- Primaria o idiopática
- Secundaria

Si un gato presenta hipertensión sostenida >160 mmHg en las siguientes revisiones, debe tratarse.

Ideal obtener presiones desde los 3 años, cada 12 meses (ACVIM)

En el tratamiento es fundamental valorar cada caso individualmente, tratar la causa y que la reducción sea gradual.

En gatos hipertensos se recomienda una reducción de 20-30 mmHg y los tratamientos de elección son el amlodipino (antagonista canales del Ca) y el telmisartán (inhibidor de angiotensina II). Los IECAs se utilizan más en caso de proteinuria.

En perros, los IECAs constituyen el tratamiento de primera elección, seguidos del amlodipino, inhibidores de la angiotensina II y los beta-bloqueantes.

Objetivo de la terapia: disminuir al máximo el riesgo de daño en órganos diana (PAS < 150 mm Hg y PAD < 95 mm Hg). La terapia debe ajustarse si la PA es > 150/95 mm Hg o <120 mm Hg.

Los IECAs en hipertensión presentan una serie de ventajas e inconvenientes:

- Ventajas:
  - Dilatan las arteriolas eferentes
  - Bajan la presión intraglomerular
  - Frecuentemente bajan la magnitud de la proteinuria
  - Los efectos adversos cardiacos y renales de la angiotensina II y aldosterona pueden ser atenuados por esta clase de agentes.
- Inconvenientes:
  - Una consecuencia secundaria de la dilatación arteriolar eferente es una tendencia teórica a la disminución de la tasa de filtración glomerular (TFG)., poco importante en perros y gatos
  - Un modesto incremento en los valores de creatinina sérica
  - No deberían usarse en pacientes deshidratados en los cuales la TFG puede descender precipitadamente
  - En gatos, no se recomiendan como monoterapia ya que el descenso de la presión arterial es muy leve

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Canine circovirus: An emerging or an endemic undiagnosed enteritis virus?

Circovirus canino: ¿un virus de enteritis emergente o endémico no diagnosticado?

Palabras clave: CaninoCV, caninos, enteritis, diarrea, coinfección

Keywords: *CanineCV, canines, enteritis, diarrhea, coinfection*

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Tabla de contenido:

El Circovirus Canino (CanineCV) pertenece a la familia Circoviridae. Es un virus emergente descrito por primera vez en 2011; Desde entonces, se ha detectado en diferentes países y se puede definir como virus de distribución mundial. CanineCV infecta cánidos domésticos y silvestres y se relaciona principalmente con enteritis hemorrágica en caninos. Sin embargo, se ha identificado en muestras fecales de animales aparentemente sanos, donde en la mayoría de los casos se encuentra en coinfección con otros agentes virales como el parvovirus canino tipo 2 (CPV).

La prevalencia/frecuencia estimada de CanineCV ha sido variable en las poblaciones y países donde se ha evaluado, llegando del 1 al 30%, y todavía hay muchos conceptos para definir las características epidemiológicas del virus. La caracterización molecular y los análisis filo-evolutivos que permiten postular el origen silvestre y la distribución intercontinental del virus. Esta revisión se centra en la importancia de continuar la investigación y establecer sistemas de vigilancia para este virus emergente.

Canine Circovirus (CanineCV) belongs to the family *Circoviridae*. It is an emerging virus described for the first time in 2011; since then, it has been detected in different countries and can be defined as worldwide distribution virus. CanineCV infects domestic and wild canids and is mainly related to hemorrhagic enteritis in canines. However, it has been identified in fecal samples from apparently healthy animals, where in most cases it is found in coinfection with other viral agents such as the canine parvovirus type-2 (CPV). The estimated prevalence/frequency of CanineCV has been variable in the populations and countries where it has been evaluated, reaching from 1 to 30%, and there are still many concepts to define the epidemiological characteristics of the virus. The molecular characterization and phylo-evolutive analyses that allow to postulate the wild origin and intercontinental distribution of the virus. This review focuses on the importance on continuing research and establish surveillance systems for this emerging virus.

Introduction

The *Circoviridae* family comprises non-enveloped viruses with a diameter of 15–25 nm and a single-stranded circular DNA genome of less than 2,500 nt in length. According to the international committee on virus taxonomy (ICTV[1]), two genera have been recognized within this family: *Circovirus* and *Cyclovirus* (1–3). Within the genus *Circovirus*, almost 50 species have been identified that infect birds and mammals (1), including the clinically relevant beak and feather disease (BFDV), goose circovirus, pigeon circovirus, canary circovirus, porcine circovirus and the most recently recognized canine circovirus (CanineCV) (4–6).

CanineCV has a covalently closed single-stranded DNA genome of 2062–2063 nt (7, 8). It has an ambisense genome with two main ORFs arranged inversely; ORF1 encodes the viral replicase gene (Rep) (nt 1–912) necessary for the initiation of the replication cycle, and ORF2 encodes the capsid protein (Cap) (nt 1,116–1928), which participates in the immune response of the host (1, 7). The Cap and Rep genes of CanineCV share, respectively, between 25 and 50% identity with the other known animal circoviruses (2). A third ORF (ORF3) has been proposed in samples from Thailand; this ORF is located between nt 470 and 889 in the antisense region in ORF1 (5). Studies in porcine circovirus type 2 (PCV2) relate ORF3 to a protein associated with viral pathogenesis and the induction of apoptosis; however, in the case of CanineCV, its function is not yet being clarified (5, 9).

(R) evolution

PEQUEÑOS ANIMALES

Tabla de contenido:

Virus replication

As with other circoviruses, CanineCV has 2 noncoding intergenic regions. The first corresponds to the 5'-intergenic region of 135 nt located between the start codons of the 2 ORFs and comprises the origin of replication (Ori). The latter is a thermodynamically stable conserved stem–loop structure with a characteristic sequence of 9 nt for the initiation of replication (TAGTATTAC), a palindromic sequence of 12 nt pairs and an open loop of 10 nt (CATAGTATTA) (2). The second intergenic region is the 3' region of 203 nt, located between the termination codons of the two main ORFs (**Figure 1**). These highly conserved sequences in circoviruses are associated with rolling circle replication (2, 10).

The rolling circle mechanism constitutes the process of replication of circular DNA genomes. It has also been described in plasmids, plant viruses and other families of animal viruses, such as *Geminiviridae* and *Nanoviridae*. For circoviruses, the study model has been mainly that of porcine circovirus (PCV). Due to the reduced size of the viral genome that limits its coding capacity, this type of replication depends largely on host cell factors and requires cellular enzymes expressed during the S phase of the cell cycle (11, 12). It is estimated that the virus uses glycosaminoglycans as binding receptors to target cells and penetrates the cell by clathrin-mediated endocytosis (9). The ssDNA genome is transported to the nucleus, where it is converted to a supercoiled intermediate double strand that will serve as a template to generate viral DNA. In *Geminivirus* and *Nanovirus* species, the primer for the negative strand is composed of DNA or RNA with multiple 5' ribonucleotides or RNA synthesized by the host after infection; however, the primer for the negative strand in circoviruses has not yet been determined (12).

Once the double strand of the genome is formed, an initiation complex composed of Rep and Rep' proteins that have endonuclease activity is attached to the stem–loop structure to cleave the loop, generating a free 3'OH end to which cellular DNA polymerase binds to initiate replication. When a unit of the genome has replicated, the Rep complex closes the loop covalently, leading to the release of a positive circular single-stranded parent molecule and a circular double-stranded DNA molecule, the latter composed of a hybrid of a negative parental strand and a positive strand newly synthesized (11, 12). The single-stranded DNA molecule can be encapsulated or involved in a second replication cycle. In *in vitro* studies in PCV, two different pathways have been suggested for the release of the virus from the cell at the end of the replication cycle: the first is the budding of groups of virions, and the second is cell lysis by apoptosis; however, the *in vivo* mechanism is still poorly understood [**Figure 2**; (11, 13)].

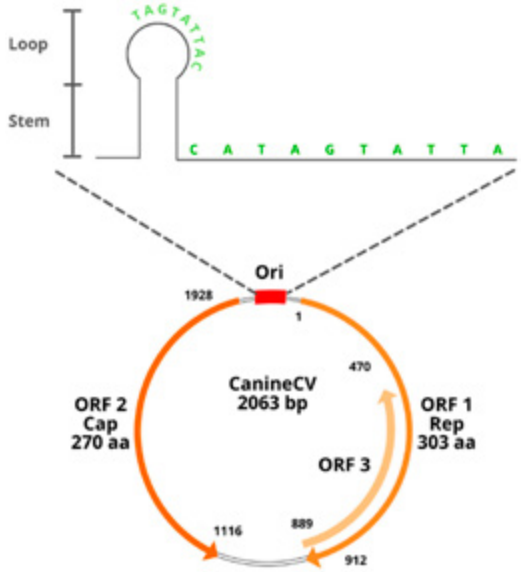

Figure 1. *Structural diagram of the CanineCV genome. See text for references.*

The "melting-pot" model has been proposed to explain the formation of the double-stranded intermediary. In this model, Rep proteins bind to palindromic sequences, generating a sphere of instability known as a melting pot. In this environment, the four inverted repeat strands present a "fused" state since there is an absence of hydrogen bonds between the positive and negative genomes. In this structure, not any double helix formation can be maintained,

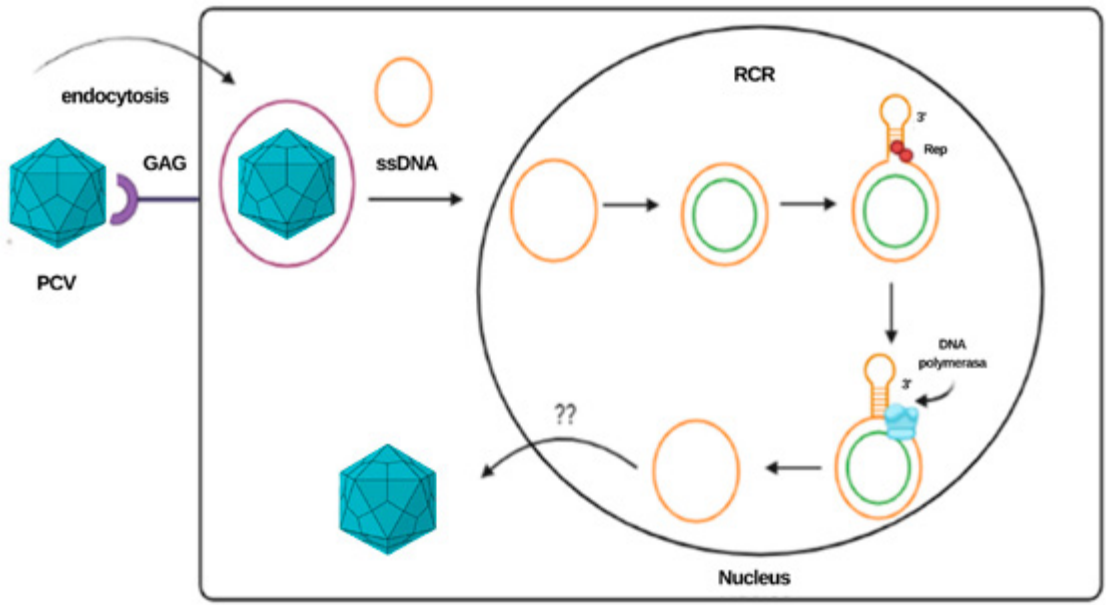

Figure 2. *Representation of the PCV replication cycle and the rolling circle mechanism. This same mechanism is proposed for the CanineCV.*

but the four strands remain very close to each other, juxtaposed in a tertiary structure that allows a possible exchange between the template strands during the synthesis of the genome, which provides two templates for the synthesis of the palindromic sequences during the initiation and termination of replication (11, 13). The availability of two template chains during the synthesis of the main chain contributes to the flexibility and the increase in the frequency of mutations in the Ori; however, the correction mechanism inherent to the melting pot allows mutations in the palindromic sequences to be eliminated, corrected, or regenerated through the mechanism of template change, allowing the integration of the nt sequence in the Ori (11).

Molecular epidemiology

CanineCV was identified for the first time in 2011 by Kapoor et al. (2) in serum samples from six of 205 clinically healthy dogs, confirming the first non-porcine circovirus to infect mammals (2, 4, 6). From this first report and to date, different studies of detection, genetic sequencing and phylogenetic analysis have been carried out in different countries worldwide, both in domestic dogs and in wild canids. The overall objective of such investigations has been mainly to establish the circulation of CanineCV at the demographic level in the region or area (2, 4). Virus detection studies have been based on molecular techniques including conventional and quantitative PCR (qPCR) (14, 15). Virus isolation has been unsuccessful. In MDCK cells infected with the CanineCV D1056 strain from Brazil only DNA was detected by qPCR in low amounts during the inoculation phase perhaps due to the amount of viral DNA found by qPCR for CanineCV was low (16). The same negative result has been reported by using Italian samples in MDCK and D17 cells (8). However, it is well known that most of the circoviruses such as PCV, are generally considered non-cytopathogenic and the low growth efficiency of PCVs in cultured cells has become a major obstacle to the establishment of diagnostic methods (17). Nevertheless, few reports have showed the cytopathic effect of PCV2 in specific cell lines (18, 19).

CanineCV is defined as a worldwide distribution agent since its circulation has been reported in 4 of 5 continents. The phylogenetic analyses of the strains reported to date have been prepared based on the complete genome and with the nucleotide sequences or concatenated amino acids of the Rep and Cap proteins (3, 4, 7). Multiple effort has been made to stablish a classification

system that helps to understand virus origin and evolution. Sun et al. in China in 2018 based the phylogenetic analysis of the detected CanineCV strains on ORF2 sequences and proposed a classification into two groups: CanineCV-1 and CanineCV-2 (10). Subsequently, it was reported that the existing strains of CanineCV at the global level could be divided into four different groups: CanineCV-1, which includes strains from Italy, four from the United States, two from Germany, one from Argentina, and one from China. CanineCV-2 group strains from China, of which three were detected in 2014, 13 in 2015 and 24 in 2017. CanineCV-3 includes strains from China identified between 2014 and 2015, and CanineCV-4 contains a strain from the United States. three strains from the province of Guangxi and included the five strains detected in their research. The authors indicate that to differentiate the 4 groups, it is possible to use polymorphisms of individual nucleotide sequences (20). On the other hand, in the analysis conducted by Piewbang et al. in 2018 (5), it was concluded that the CanineCV sequences detected in their study were different from the majority of the sequences reported and were mainly related to the UCD3-478 strain from the United States. Additionally, the authors highlight that the phylogenetic trees based on the Rep and Cap genes of the CanineCV genomes circulating in Thailand are divergent and reveal uneven patterns when comparing both genes, which is associated with recombination events (5).

In South America, CanineCV has been detected in Argentina, Brazil and Colombia. Kotsias F et al. (21) detected the UBA-Baires strain of CanineCV in an outbreak of enteric disease in puppies between 2014 and 2015 in Argentina. Phylogenetic evaluation of whole genome sequences showed that this strain is grouped with viruses from Europe, mainly from Italy, and differed considerably from strains reported in the United States and China (21). In contrast, strain D1056 reported in Brazil by Cruz et al. (16) showed high similarity (<80%) with the NY214 strain reported in the United States. Giraldo-Ramírez et al. Colombia confirmed the separation of CanineCV strains into four different genotypes, designating clades according to geographical distribution and with the new sequences reported as follows: Genotype China includes sequences found in China, Genotype Asia-1 and Asia-2 include sequences from Thailand and China, and Cosmopolitan Genotype contains sequences from Italy, Germany, China, the United States, Argentina and the strain found in Colombia, also defining a clade of CanineCV sequences identified in foxes [**Figure 3**; (22)].

Phylogenetic analysis of CanineCV sequences identified in arctic and red foxes in Norway (23) showed a distinguishable clustering in five groups in the tree constructed with sequences of complete genomes and in the tree of concatenated amino acids of the Rep and Cap proteins. Group 1 included sequences identified in dogs, wolves and a badger found in Europe, Asia and the United States. Groups 2, 3 and 4 included sequences identified in dogs in Asia, and the sequence 09-10F from 2011 of a red fox in Italy, Group 5, was made up of sequences identified in foxes. The UCD3-478 strain did not fit into any of the previous groups (23). The presence of the sequence 09-10F/2011 in Group 4 indicates that the segregation of the groups does not occur based on the host where they were identified. The authors also define that the UCD3-478 strain may represent an intermediary between the groups 4 and 5 or the only sequence of a possible sixth group (23).

Recent analysis from Middle east including Turkish and Iranian samples belonging from diarrheic dogs through analysis of the partial Rep and Cap nt sequences, had found that the Iranian CanineCV strains were more closely related to strains detected in Turkey, allowing to hypothesize a possible introduction of the virus from this neighbor country (24–26). However, the most recent complete genome nucleotide

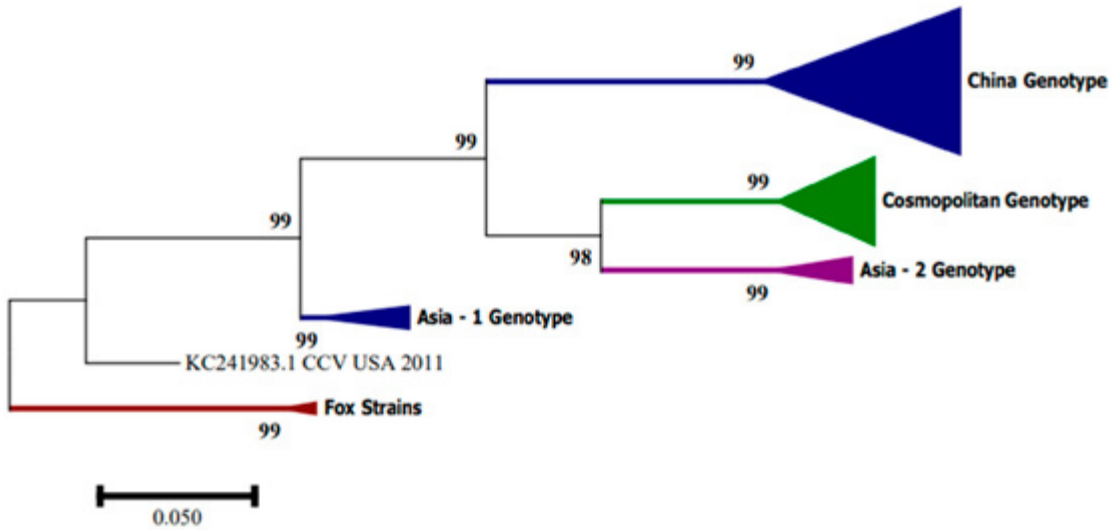

Figure 3. *Phylogenetic tree based on complete CanineCV genome sequences. The phylogenetic analysis was performed using MEGA 7.0 for Windows by maximum likelihood with 1,000 bootstrap replicates and the 3-parameter Tamura nucleotide substitution model.*

sequences analysis of CanineCV strains obtained from Iranian dogs belongs to a separate independent clade (24).

Additionally, in 2020, the first systematic report on the use of codons and adaptation to the hosts of CanineCV was carried out in which all available sequences were used. The results suggest that CanineCV descends from the bat and that the sequences have been dominated mainly by natural selection, making it possible to propose a transmission hypothesis between species in which CanineCV may have evolved from bats (analysis of origin) and, subsequently, have adapted to domestic and wild canids (27).

Disease in dogs

In 2013, Li et al. characterized the complete genome of CanineCV from the liver of a dog with severe hemorrhagic gastroenteritis, granulomatous lymphadenitis, and vasculitis. Since then, CanineCV infection has been associated with clinical profiles of acute gastroenteritis, hemorrhagic diarrhea, signs of vasculitis, lymphadenitis, thrombocytopenia, neutropenia, and lymphopenia, which is related to immunosuppressive activity due to lymphoid tissue damage; the latter has also been described in infection by PCVs in pigs and in birds suffering Beak and Feather Disease Virus (BFDV) (3, 6, 28). Other authors have also associated CanineCV with respiratory signs such as dyspnea, nasal discharge, cough, or rales in the lungs (5). However, the virus has also been detected in samples of asymptomatic canines, so its pathogenesis and epidemiology cannot yet be clearly defined.

Different forms of infection could be suggested, such as subclinical, clinical or coinfection with other infectious agents, and the fecal route as the main route for transmission (3, 6, 28). With the objective of understanding and establishing the behavior of CanineCV infection, in addition to its prevalence and genetic diversity, multiple investigations have been carried out worldwide in dogs with symptoms compatible with those described and in apparently healthy dogs.

The prevalence of CanineCV in different countries, both in samples of patients with compatible signs and asymptomatic animals, is variable. Li et al. in 2013 in the United States (3) established a prevalence in stool samples of 11.3% in dogs with diarrhea, 6.9% in healthy dogs, and 3.3% in serum samples in dogs as signs of thrombocytopenia, neutropenia or history of ticks. The prevalence between positive samples from animals with diarrhea and samples from healthy dogs was not significant (3). In Asia, several studies have been conducted in different countries. In Taiwan, Hsu et al. (4) found a high-

ly significant correlation between the presence of diarrhea and CanineCV, with a prevalence of 28.02% in dogs with diarrhea and 11.9% in healthy dogs; the authors suggested that the difference in the results with respect to the USA may be related to geographic distribution, molecular technique or primer design (4). In Northeast China, a prevalence of CanineCV of 15.6% in dogs with diarrhea and 6.7% in healthy dogs was found; also, authors found a significant difference between both prevalences (20).

Sun W et al. in 2019, in the southern region of Guangxi established a molecular prevalence of CanineCV in serum samples of dogs with diarrhea of 8.75% and in healthy dogs of 9.25% (10). In Germany, it was established a prevalence of CanineCV of 20.1% in dogs with diarrhea and 7.3% in healthy dogs, indicating that the virus can be found significantly more frequently in dogs with diarrhea (29). Nevertheless, no significant difference between the detection of CanineCV in healthy dogs and dogs with hemorrhagic diarrhea, with prevalence rates of 4.5 and 3.6%, respectively has been found also in Germany (28). In Vietnam, the evaluation for CanineCV was performed on 81 CPV-2c positive fecal samples, establishing that 19.8% of the samples were positive for CanineCV in diarrheic puppies less than 7 months of age (30).

In middle east region, the presence of CanineCv has been studied in Turkey and Iran. In Turkey, from a total of 150 rectal swab samples collected from animals with manifestations of gastrointestinal disease, nine positive samples were obtained (6%) with coinfection with the other two viruses between 2 to 5% (26). To investigate the prevalence and genomic characteristics of CanineCV in Iranian dogs, a total of 203 fecal samples from dogs were collected between February and November 2018 from five different geographical regions and examined by real-time PCR (qPCR). Thirteen dogs (6.4%) tested positive for CanineCV DNA, and all were detected in coinfection with CPV-2. Later in 2022 a second study was carried out in the same way using real-time PCR in 156 rectal swabs from clinically healthy canines. 14 positive samples for CanineCV were obtained (8.9% prevalence). The CanineCV circulation in non-diarrheal dogs in Iran highlighting the need for further epidemiological investigations (24, 25).

The only study reported to date on seroprevalence of CanineCV was conducted with samples taken between 2016 and 2017 in northeast China (31). Authors found a seroprevalence of 39.82%, with differences between the different cities ranging from 23 to 43%. Similarly, the study showed a significant difference in prevalence between dogs with diarrhea and healthy dogs (31). A compilation of the studies carried out to date in different countries and obtained from databases such as PubMed and Science Direct, with the purpose for the detection of CanineCV in domestic dogs is shown in **Table 1**.

The prevalence of CanineCV varies over a wide range. The differences between the studies may be due to multiple factors as mentioned before. In addition, it is not yet possible to define whether the virus is found more frequently in dogs with diarrhea than in healthy animals, as shown by the variations in the results of the reports published to date. Therefore, it is important to conduct molecular and research that clearly defines the epidemiological characteristics of the virus and its role as an infectious agent in gastrointestinal clinical disease in domestic dogs.

Histopathological findings

Necropsy and immunohistochemistry of retrospective cases of dogs with signs compatible with CanineCV has shown critical lesions, such as necrotizing vasculitis in the intestine and spleen, granulomatous lymphadenitis and histiocytic drainage in Peyer's patches and lymph nodes, and microscopic lesions in the kidney (3). Clinico-Histopathological findings in a case report in Connecticut, United States,

Country	Year	Type of sample	Clinical signs	Sampled animals	Reference
United States	2013	Stool, blood	Diarrhea, thrombocytopenia, neutropenia	35 sick 14 healthy	(3)
Italy	2014	Tissues	Hemorrhagic diarrhea	1 sick	(7)
United States	2016	Feces, tissues	Hemorrhagic gastroenteritis	3 sick	(6)
China	2016	Rectal swabs, feces	Diarrhea	58 sick, 19 healthy	(4)
Italy	2017	Rectal swabs, feces	Acute gastroenteritis	71 sick, 19 healthy	(32)
Germany	2017	Feces	Diarrhea	37 sick 6 healthy	(29)
Germany	2017	Feces	Hemorrhagic diarrhea	55 sick, 66 healthy, 54 CPV-2 positives	(28)
Thailand	2018	Oral or nasal swab, tissues	No	9 sick	(5)
China	2019	Serum	Diarrhea	81 sick 79 healthy	(10)
China	2019	Feces	Diarrhea	15 sick 3 healthy	(20)
United States	2019	Tissues	Hemorrhagic gastroenteritis, multisystemic vasculitis	1 sick	(33)
Argentina	2019	Tissues	Hemorrhagic diarrhea	3 sick	(21)
Turkey	2019	Feces	Diarrhea	150 sick	(26)
Colombia	2020	Feces	Hemorrhagic diarrhea	5 sick	(22)
Brazil	2020	Feces	Hemorrhagic gastroenteritis	1 sick	(16)
China	2020	Serum	Diarrhea	417 total	(27)
Vietnam	2021	Feces	Diarrhea	81 total	(30)
Iran	2022	Rectal swabs	No	156 total	(24)

Table 1. *Studies conducted for the detection of CanineCV in domestic canines.*

showed macroscopically pale mucous membranes, blue–black small intestine, black stools in the colon, splenomegaly, and multiple enlarged and hemorrhagic lymph nodes. At the microscopic level, circumferential transmural vasculitis and necrosis of crypt cells and lymphocyte necrosis in the lymph nodes and spleen were observed in the intestine, and vasculitis was also found in the liver, kidneys, lung, meninges, and brain (33). Similarly, lesions such as friability of the small intestine, focal congestion of Peyer's patches, aqueous and hemorrhagic content in the small intestine, large intestine with multifocal petechiae and melena inside has been reported (6).

Histologically, loss of microvilli, necrosis in crypt cells, lymphoid necrosis in Peyer's patches in the ileum, mesenteric lymph nodes and spleen, granulomatous inflammation, and increased histiocytes in lymph nodes were found (6). Compiling the above, it is possible to establish that the histopathological findings associated with CanineCV have a close similarity, both in the affected organs and in the lesions found, the most common being blue–black coloration in the intestine, lymphocyte necrosis and granulomatous inflammation. A similar histopathological profile has also been described in PCV2 infection in pigs; however, causality studies that demonstrate by *in situ* hybridization or immunohistochemistry the presence of this viral agent in compatible lesions are still lacking (33).

Infection in wild carnivores

Different studies have confirmed the presence of CanineCV in wild carnivores. A retrospective analysis from samples taken between 2013 and 2014, detected and molecularly characterized CanineCV in samples of organs from domestic dogs, wolves, foxes and badgers collected in regions of central and southern Italy; CanineCV was found in nine out of 34 wolves, eight out of 209 dogs, and one out of 10 badgers, confirming that the circulation of the virus is not restricted to domestic dogs (8).

CanineCV has been also confirmed in foxes in the United Kingdom, from samples taken from foxes suffering meningoencephalitis between 2009 and 2013. High frequencies of infection in histological samples and in serum of foxes measured bye by real-time PCR. Also, phylogenetic analysis revealed a close relationship with CanineCV identified in

2011 in the USA with an identity of 92 and 89% in the amino acid sequence in the Rep protein and in the nt sequence, respectively; however, the sequences of CanineCV from dogs and CanineCV from foxes grouped in separated clades (34). Notably, circovirus infection in wild foxes is associated with neurological signs, in contrast to domestic dog disease which was associated with gastrointestinal, respiratory and vasculitis. The fact that circoviruses that infect foxes and those that infect domestic dogs are very similar viruses but with different symptoms raises several questions for future research regarding the transmission and pathogenesis of both viruses between species of the same family (34). Also, seeking to evaluate the presence of CanineCV in red foxes (*Vulpes vulpes*) from Italy by using molecular and phylogenetic approaches, CanineCV DNA was detected in one of 32 processed stool samples; confirming that dogs and foxes could share phylogenetic related circoviruses (35).

In another retrospective analysis on samples of 51 arctic foxes (*Vulpes lagopus*) from the Svalbard archipelago, Norway and 59 red foxes (*Vulpes vulpes*) from Northern Norway (mainland) sampled from 1996 to 2018, CanineCV DNA was detected in 10 red foxes and 11 arctic foxes. Positive DNA were found even in samples from Artic foxes sampled in 1996, fifteen years before the first report in domestic dogs in USA, suggesting that wild carnivores could have harbored an ancestor of CanineCV that could later be transmitted to domestic canids, thus highlighting the role of wild carnivores in the transmission of pathogens to the domestic dogs populations (23).

Coinfection and comparative analysis to PCV

In the studies on the detection of CanineCV carried out to date, coinfection with other infectious agents has also been analyzed. Among these, the presence of bacteria such as *Salmonella* sp., *Campylobacter* sp. and enterotoxins of *Clostridium* sp.; parasites such as *Giardia* sp. and *Cryptosporidium* sp.; and viruses such as canine parvovirus type 2 (CPV-2), canine coronavirus and canine distemper has been found (3, 6, 7). In similar way, coinfection between several pathogens and circovirus has already been described in pigs, where it is relatively common (36). PCVs have been widely studied for several decades, and four different subtypes have been identified: PCV1, PCV2, PCV3 and PCV4 (36–38). PCV1 corresponds to a nonpathogenic type, described in 1974 as a contaminant in PK-15 pig kidney cell lines (39, 40). PCV2 is highly pathogenic, and it has been associated with a series of syndromes and pathologies classified into four forms of presentation that affect pig production worldwide, generating countless economic losses in the industry (41). PCV3 is associated with two clinical presentations called the systemic form and the reproductive form (42). Finally, PCV4 is the most recent discovery and has been reported only in China (43).

PCV2 was initially associated with multisystem wasting syndrome (PMWS); however, after multiple investigations, the association of PCV2 with other clinical conditions, such as hepatitis, reproductive failure, respiratory disease and nephropathy (PDNS), was established. It has also been associated with porcine respiratory complex disease (PRDC) (36, 38). Currently, it is recognized as the causative agent of some conditions known as porcine circovirus associated diseases (PCVAD), which mainly affect fattening pigs from 7 to 16 weeks of age, where there are clinical manifestations such as weight loss, hypertrophy of lymph nodes, jaundice, growth retardation, dyspnea, and abortions. Histopathological findings include lymphoid depletion, replacement by histiocytes in lymphoid tissue, bronchiolitis and interstitial pneumonia with mononuclear infiltrate in the lungs (41). There are several reports and studies on the role of coinfection with other infectious agents since it is unusual for PCV2 to generate clinical pictures as a single

agent (36). It is generally found together with other microorganisms, such as *Salmonella* sp., *Mycoplasma hyopneumoniae*, swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV). Coinfection has even been described between different PCV2 genotypes. Also, it has been suggested that coinfections are associated with a greater severity of infection (41, 44).

The adverse effect of PCV2 coinfections with the aforementioned agents could be explained by the repercussions on the immune system of pigs since the virus targets lymphoid tissues. The replication of PCV2 destroys the normal architecture of the lymphoid follicles, causing lymphoid depletion that is then replaced by histiocytes. Replication of the virus has been evidenced in organs such as the spleen, tonsils, lungs, kidney, liver and thymus; in the latter, PCV2 alters the selection of T lymphocytes, particularly in younger animals. It also infects dendritic cells, limiting their function; such effects generate severe lymphoid depletion and severe immunosuppression in pigs, increasing the probability of contracting other infections (41, 44).

There are also reports of the detection of PCV2 and PCV3 in dogs. To date, PCV2 has been discovered in other reservoirs, such as calves, goats, mice, and in carnivores, such as mink and foxes, indicating that transmission to nonporcine hosts is possible. Close contact between animals is probably the main route of transmission, particularly in multispecies farms and sites accessible to wildlife (45, 46). The reported studies of PCV2 in nonporcine hosts have associated the presence of the virus with gastrointestinal signs in dogs and with sterility in raccoons. However, the authors conclude on the need to continue with studies that allow defining the direct association between PCV2 and the clinical profile described. Similarly, the PCV3 genome has been detected in populations of domestic dogs, indicating its transmission to non-porcine hosts, and CPV/PCV3 coinfection has even been found in dogs (47, 48).

Similar to PCV2 infection, the pathogenesis of CanineCV and its participation as a coinfecting agent is not yet clearly defined. Current research suggests is that its role as a pathogen may be to exacerbate clinical conditions, like PCV2 infection, weakening the immune system and favoring the entry and dissemination of other viruses and bacteria. This is because, in the majority of the CanineCV detections performed, the virus has been found to be accompanied by other infectious agents, such as CPV with coinfection rates of up to 100% (22). To date, few reports confirmed CanineCV as a single agent in dog suffering acute gastroenteritis or hemorrhagic diarrhea (3, 21).

Based on the findings of the genetic material of CanineCV in lymphoid tissues, it has been proposed different hypotheses regarding the clinical manifestation of the CanineCV infection (6, 28). First, the replication of CanineCV in lymphoid tissues could be favored by previous infection with CPV, without apparent clinical manifestation or generation of disease. Second, the presence of a synergistic effect where CanineCV infection causes immunosuppression and allows the development of the clinical disease by CPV, even in vaccinated individuals, a situation that has been described in pigs as mentioned above.

It has been also evaluated the possible role of CanineCV in acute hemorrhagic diarrhea syndrome. According to the results, it has been reported a low probability that CanineCV acts as a putative agent of the syndrome, but CanineCV may be a predisposing element for bacterial or viral infections. The prevalence of CanineCV in the study was 4.6%, indicating that it could be only an incidental finding where the virus would be part of the normal intestinal microbiota of canines (28); this has also been described in pigs where PCV1 is present in asymptomatic animals as a nonpathogenic agent (49). The most significant finding of the study was the finding of a higher mortality in dogs that presented CPV2/CanineCV coinfection. There are previous reports where

a greater susceptibility or increased severity of infection was found when CPV2 manifests in coinfection with other pathogens, such as enteric coronavirus. The authors suggest that the same can happen with CanineCV, as has been observed and reported in pigs in PCV2 infection.

Final remarks

The discovery of emerging viruses such as CanineCV raises an important research topic in domestic and wild animals. The evidence of multiple gaps in the knowledge of this virus in aspects such as pathogenic and epidemiological characteristics raises the need to create and continue with studies that allow expanding and defining the knowledge about CanineCV infection. Scientific evidence supports that this virus poses a risk to the health and well-being of the canine population, so work must be done to define many concepts and gaps in the understanding of this emerging pathogen. Therefore, its necessary to strength the knowledge of veterinarians and clinicians about the possible manifestations as a disease not only as a single agent but also in coinfection with other pathogens. Additionally, the role of wild canids should be highlighted, since, according to recent research, it is evident that in this population, CanineCV seems to be more pathogenic, and its participation in the transmission of the virus to domestic canines seems to be relevant, especially because human invasion of wild habitats favors contact between both populations, which contributes to the transmission of interspecies pathogens.

The role of CanineCV as a primary agent or coinfecting in enteric diseases in canines remains uncertain; existing research suggests the importance of continuing with other study models that provide new information to define the concepts that remain doubtful until now. As can be observed in the development of this article, CanineCV could have different pathways of disease development in canines, either as a primary agent that leads to generating the clinical signs described or to cause subclinical infection where there are no signs but where the animal could spread the virus or contribute to the severity of the disease in coinfection with other agents. In addition, CanineCV, like the other circoviruses, could play an important role in the immune response of the animal, favoring the entry of other infectious agents or preventing its recovery (**Figure 4**).

Conclusion

The pathogenic and epidemiological characteristics of CanineCV are currently poorly understood, so CanineCV represents a multidisciplinary exploration challenge both in the field of research and in the clinic. Understanding the particularities of this new virus either in healthy animals or coinfected with other agents could provide valuable information regarding prophylactic or therapeutic practices in enteric diseases in canines, contributing to the health and well-being of canine populations. Thus, it is necessary to continue carrying out research in different canine populations that allow the evaluation of the epidemiology of CanineCV in geographical sites not yet explored and its behavior in healthy or coinfected animals, in addition, to monitoring the clinical evolution of the infected animals to help to understand the pathogenesis of the CanineCV.

Tabla de contenido:

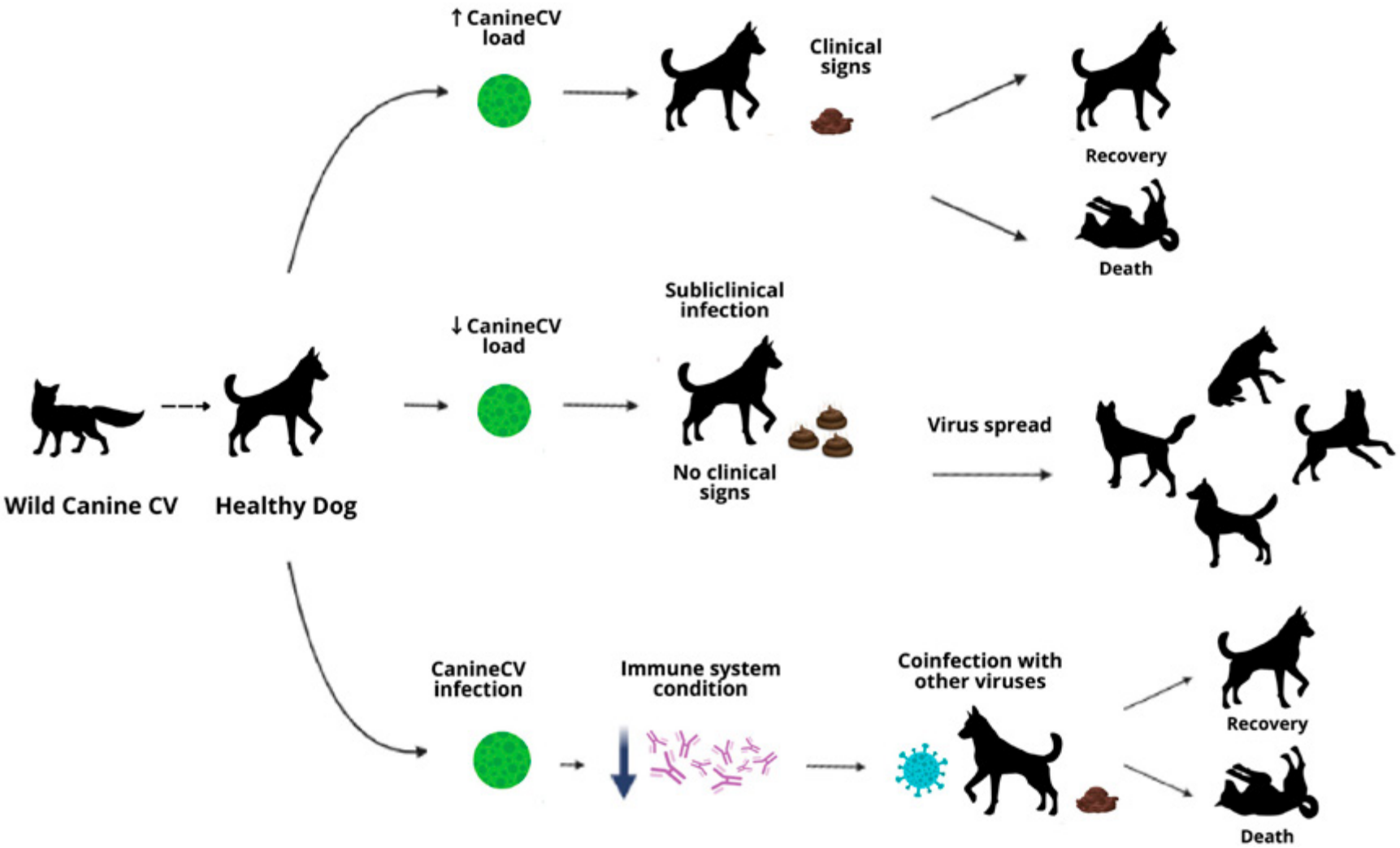

Figure 4. *Diagram of possible disease routes in CanineCV infection in domestic dogs.*

Author contributions

JR-S and JJ: conceptualization, validation, resources, and data curation. DG-B and DV-B: methodology, formal analysis, and investigation. DG-B: writing—original draft preparation. DG-B, DV-B, SG-R, JJ, and JR-S: writing—review and editing. SG-R: software and visualization. JR-S: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Footnotes

1. http://www.ictvonline.org/virustaxonomy.asp

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Serum and Tissue Expression Levels of Leptin and Leptin Receptor Are Putative Markers of Specific Feline Mammary Carcinoma Subtypes

Los niveles de expresión sérica y tisular de la leptina y el receptor de leptina son marcadores putativos de subtipos específicos de carcinoma mamario felino

Palabras clave:
carcinoma mamario felino, leptina, receptor de leptina, índice de leptina libre, biomarcadores

Keywords:
feline mammary carcinoma, leptin, leptin receptor, free leptin index, biomarkers

https://www.frontiersin.org/articles/10.3389/fvets.2021.625147/full

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Tabla de contenido:

La obesidad es un factor de riesgo establecido para el cáncer de mama en mujeres posmenopáusicas, que se asocia con niveles séricos elevados de leptina. Aunque el sobrepeso es una condición común en el gato, el papel de la leptina y su receptor en el carcinoma mamario felino sigue sin resolverse. En este estudio, se investigaron los niveles séricos de leptina y receptores de leptina (ObR) en 58 gatos con carcinoma mamario y se compararon con los de animales sanos, al igual que los niveles de expresión de leptina y ObR en tejidos tumorales. Los resultados mostraron que el Índice de Leptina Libre está significativamente disminuido en gatos con carcinoma mamario (p = 0,0006), particularmente en aquellos con tumores luminales B y HER2 positivos, y que estos animales también presentan niveles de leptina sérica significativamente más bajos (p < 0,0001 y p < 0,005, respectivamente).

Obesity is an established risk factor for breast cancer in post-menopausal women, being associated with elevated serum levels of leptin. Although overweight is a common condition in cat, the role of leptin and its receptor in feline mammary carcinoma remains unsettled. In this study, serum leptin and leptin receptor (ObR) levels were investigated in 58 cats with mammary carcinoma and compared with those of healthy animals, as were the expression levels of leptin and ObR in tumor tissues. The results showed that the Free Leptin Index is significantly decreased in cats with mammary carcinoma ($p = 0.0006$), particularly in those with luminal B and HER2-positive tumors, and that these animals also present significantly lower serum leptin levels ($p < 0.0001$ and $p < 0.005$, respectively). Interestingly, ulcerating tumors ($p = 0.0005$) and shorter disease-free survival ($p = 0.0217$) were associated to serum leptin levels above 4.17 pg/mL. In contrast, elevated serum ObR levels were found in all cats with mammary carcinoma ($p < 0.0001$), with levels above 16.89 ng/mL being associated with smaller tumors ($p = 0.0118$), estrogen receptor negative status ($p = 0.0291$) and increased serum levels of CTLA-4 ($p = 0.0056$), TNF-α ($p = 0.0025$), PD-1 ($p = 0.0023$), and PD-L1 ($p = 0.0002$). In tumor samples, leptin is overexpressed in luminal B and triple-negative carcinomas ($p = 0.0046$), whereas ObR is found to be overexpressed in luminal B tumors ($p = 0.0425$). Altogether, our results support the hypothesis that serum levels of leptin and ObR can be used as biomarkers of specific feline mammary carcinoma subtypes, and suggests the use of leptin antagonists as a therapeutic tool, reinforcing the utility of the cat as a cancer model.

Introduction

Feline mammary carcinoma (FMC) is a high prevalence disease (12–40% of all tumors in cat) that shows similar clinicopathological and genetic features (1), comparing to human breast cancer (2), supporting its use in comparative oncology studies (3, 4), and allowing to improve therapeutic protocols for women and cats (5). Despite the cat is considered a suitable cancer model, especially for the most aggressive mammary carcinomas subtypes, HER2-positve (2, 6) and triple-negative (6–8), further efforts are needed to track disease progression (9). Some common biomarkers are already identified, as for example, the androgen receptor (1), the PD-1 (10) and the CTLA-4 (11), which represent potential molecular therapeutic targets. Likewise, obesity is a common nutritional disorder in the

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cat, with higher prevalence in indoor and sterilized animals above 3 years of age (12). In humans, obesity induces a chronic inflammatory status, being a risk factor for breast cancer (13–15).

Leptin is a 16 kDa adipocytokine, encoded by the *obese* gene and involved in the central regulation of food intake, energy homeostasis, modulation of reproductive function and peripheral metabolic processes, such as breast/mammary gland development, cellular proliferation and angiogenesis (16–18). In tissues and serum, leptin expression is modulated by fat mass, with healthy cats showing lower serum leptin levels than obese animals (12), as reported in humans (13, 14). Interestingly, although this protein is mainly secreted by adipocytes, it can also be expressed by pathologically altered cells, such as cancer cells (19, 20). Thus, malignant cells can regulate their metabolic activities (21), promoting uncontrolled cell growth via Wnt/β-catenin (22), migration, invasion and angiogenesis (15, 23), and downregulating apoptosis through a Bcl-2-dependent mechanism (21, 24). Accordingly, leptin overexpression is detected in breast cancer cells and neighboring adipocytes, contrasting with normal breast glandular epithelial cells (15, 25), promoting the expression of several tissue factors (26), which suggest an oncogenic role for this adipocytokine (14). Furthermore, studies in human breast cancer patients showed that leptin overexpression has paracrine effects, not always reflected in serum levels, but associated with more aggressive tumors and therapy resistance (25). Additionally, in overweight human patients a positive correlation was found between leptin overexpression in the tumor microenvironment and estrogen receptor (ER) positive breast cancer, and with a human epidermal growth factor receptor 2 (HER 2)-positive status frequently related to a more invasive tumor phenotype (27).

In parallel, the leptin receptor (ObR, 150–190 kDa) was found to be involved in innate and adaptive immunity (28), being expressed in several organs, including breast and peripheral tissues, as well as in adipocytes (29, 30) and immune cells. ObR has an extracellular N-terminus domain, a transmembrane domain and a cytoplasmic C-terminus domain. Upon leptin ligation, ObR homodimerizes and the associated JAK monomer is auto phosphorylated to activate the downstream signaling pathways (19); in the case of ObR forms with lack of auto phosphorylation capabilities, auxiliary kinases are important (29). The soluble ObR form is a 146 kDa protein (31) that can be generated by cellular apoptosis or by the proteolytic cleavage of the extracellular anchored protein domain, with this shedding being more frequent in shorter intracellular isoforms. In serum, ObR modulates the leptin bioavailability, being decreased in obese humans (19). In breast cancer patients, ObR is overexpressed independently of the ER status (14), being correlated with low overall survival (OS) (20). Furthermore, the ratio between leptin/ObR serum levels (Free Leptin Index—FLI) is considered a useful predictor of leptin activity, reflecting the individual metabolic status (32) and when increased it is an important risk factor for breast cancer development (33). In parallel, studies in breast cancer patients found an association between leptin and ObR overexpression with a chronic inflammatory status, conditioning T-cell immune responses (increase Th1- and decrease Th2-responses) (34) and the activation of immune checkpoint inhibitors (29). Indeed, some studies in humans have shown a positive correlation between overexpression of leptin and ObR with several immunomodulatory molecules (e.g., Cytotoxic T-Lymphocyte Associated Protein 4–CTLA-4; Tumor Necrosis Factor α-TNF-α; Programmed Cell Death-1–PD-1 and Programmed Cell Death-ligand 1–PD-L1) (35, 36). While CTLA-4 is a protein related to the inflammatory response that is increased in breast cancer patients, contributing to immune downregulation (37), TNF-α is a pro-inflammatory cytokine that induces apoptosis promoted by

the absence of leptin (38). Moreover, the overexpression of PD-1 in T-cells is associated with ObR overexpression in humans with distinct tumor types (39), induced through the AKT pathway activation by oestrogens (40) and is responsible for the PD-1 mediated T-cell dysfunction (41).

As mentioned above, obesity is associated with increased leptin levels, which induces resistance to chemotherapy (42, 43). Therefore, the leptin/ObR axis has been widely studied (44) as a target for an adjuvant therapy, not only in ER-positive tumor status (42), but also in triple-negative tumors (45), in which the lack of hormonal receptors reduces the therapeutic options. Nowadays, different therapeutic strategies targeting the leptin/ObR axis are being used, namely leptin antagonists, that downregulate the leptin downstream pathways (e.g., Wnt and STAT3) (42, 44–46), leptin and ObR specific monoclonal antibodies or nanoparticles, that prevent leptin/ObR binding and, finally, soluble ObR molecules that enclose plasmatic leptin, regulating its availability (46).

To the best of our knowledge, this study is the first to evaluate the serum leptin and ObR levels, as well as tumor tissue expression of leptin and ObR in cats with mammary carcinoma. Thus, the main goals of this study were to: (1) compare the serum leptin and ObR levels of cats with mammary carcinoma stratified by molecular subtype with those of healthy animals; (2) investigate the leptin and ObR expression in tumor tissues and compare it with normal mammary tissues; (3) search for statistical associations between serum leptin/ObR levels and leptin/ObR IHC scores in tumor mammary tissues; and (4) test for statistical associations between serum leptin/ObR levels and clinicopathological features, in order to evaluate the utility of leptin and ObR as diagnostic and/or prognosis biomarkers or promising drug targets in cats with mammary carcinoma.

Materials and Methods

Animal Population

Paired tumor and serum samples were collected from 58 female cats, with fully documented history of FMC, exhibiting a mean age at diagnosis of 11.5 years (range 6.5–18 years), with the majority showing an undifferentiated breed and presenting an average body condition score (1–9) of 3.73 (ranging between 1 and 7). Also, 24 serum samples from healthy cats presented for elective ovariohysterectomy showing a mean age of 1.37 years (range 0.5–5.5 years) and an average body condition score (1–9) of 5.0 (ranging between 4 and 6), were collected at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon. All the procedures involving manipulation of animals were consented by the owners. For each animal enrolled in the study, the clinicopathological data were recorded, including: age; breed; body weight; reproductive; and contraceptive administration status; treatment status (none, mastectomy or mastectomy plus chemotherapy); number, location, size, and histopathological classification; ER status, PR status, HER2 status (47), and Ki-67 index (48) of tumor lesions; malignancy grade, scored using the Elston and Ellis system (49); presence of tumor necrosis, lymphatic invasion, lymphocytic infiltration, and/or cutaneous ulceration; regional lymph node involvement; and clinical stage (TNM system) (**Table 1**). Regarding the molecular subtyping of feline mammary carcinomas (2, 50), animals were stratified in luminal A (n = 10), luminal B (n = 17), HER2-positive (n = 15) and triple-negative (n = 16) groups. The animals were anesthetized before surgical procedures and blood samples were collected without interfering with the animals' well-being. Briefly, all tissue samples were embedded in paraffin after fixation in 10% buffered neutralized formalin (pH 7.2), during 24–48 h, while serum samples were separated from clotted blood by centrifugation (1,500 g, 10 min, 4°C) and stored at −80°C until further use. All samples that showed haemolysis were discarded, as recommended (2, 51).

TABLE 1 | Clinicopathological features of the female cats with mammary carcinomas enrolled in this study.

Clinicopathological feature	Number of animals (%)	Clinicopathological feature	Number of animals (%)
Breed		**Size**	
Undifferentiated	44 (75.9%)	<2 cm	22 (37.9%)
Siamese	7 (12.1%)	≥2 cm	36 (62.1%)
Persian	5 (8.6%)	**Animal weight**; 23 unknown	
Norwegian Forest	2 (3.4%)	<3 kg	6 (10.3%)
Age		3–5 kg	24 (41.4%)
<8 years old	4 (6.9%)	>5 kg	5 (8.6%)
≥8 years old	54 (93.1%)	**Treatment**; 3 unknown	
Reproductive status; 1 unknown		Mastectomy	49 (84.5%)
Spayed	20 (34.5%)	Mastectomy + Chemo	4 (6.9%)
Pill	21 (36.2%)	None	2 (3.4%)
Both	9 (15.5%)	**Multiple tumors**	
Any	7 (12.1%)	Yes	35 (60.3%)
Lymph node status; 4 unknown		No	23 (39.7%)
Positive	19 (32.8%)	**Malignancy grade**; 1 unknown	
Negative	35 (60.3%)	I	3 (5.2%)
Stage (TNM)		II	8 (13.8%)
I	15 (25.9%)	III	46 (79.3%)
II	6 (10.3%)	**Necrosis**	
III	31 (53.4%)	Yes	42 (72.4%)
IV	6 (10.3%)	No	16 (27.6%)
Lymphatic invasion		**Lymphocytic infiltration**; 2 unknown	
Yes	7 (12.1%)	Yes	37 (63.8%)
No	51 (87.9%)	No	19 (32.8%)
HER2 status		**Tumor ulceration**	
Positive	14 (24.1%)	Yes	8 (13.8%)
Negative	44 (75.9%)	No	50 (86.2%)
ER status		**Ki67 index**; 1 unknown	
Positive	31 (53.4%)	Low (< 14%)	18 (31%)
Negative	27 (46.6%)	High (≥ 14%)	39 (67.2%)
PR status			
Positive	36 (62.1%)		
Negative	22 (37.9%)		

n = 58; TNM, Tumor, Node, Metastasis; ER, Estrogen Receptor; PR, Progesterone Receptor.

Table 1. *Clinicopathological features of the female cats with mammary carcinomas enrolled in this study.*

Measurement of Serum Leptin and ObR Levels

The serum levels of leptin and ObR, CTLA-4, TNF-α (11), PD-1 and PD-L1 (10) were quantified by using commercial ELISA-based kits (R&D Systems, Minneapolis, USA; DY398-05, DY389, DY476, DY2586, DY1086, and DY156, respectively). For each assay, a standard curve was plotted using 10-fold serial dilutions of the recombinant proteins provided by the manufacturer, and the r^2 values were calculated using a quadratic regression [r^2 = 0.9976 for leptin, r^2 = 0.9632 for ObR, r^2 = 0.99 for PD-1, and r^2 = 0.96 for PD-L1 (10)], whereas serum CTLA-4 and TNF-α concentrations were determined by using a curve-fitting equation (r^2 > 0.99), as previously reported (11). Briefly, a 96-well plate was prepared by adding the capture antibody to each well and incubate overnight. Plates were then treated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h, to prevent non-specific binding. Standards and diluted serum samples were added to sample wells and incubated for 2 h at room temperature (RT), followed by incubation with the detection antibody for 2 h at RT. Afterwards, the streptavidin-conjugated to horseradish peroxidase (HRP) was added to each well and incubated at RT for 20 min previous to the addition of the substrate solution in 1:1 H_2O_2 and tetramethyl-benzidine to each well (20 min at RT in the dark). The reaction was interrupted by adding a stop solution ($2NH_2SO_4$) and the absorbance was measured by a spectrophotometer (FLUOStar OPTIMA, Microplate Reader, BMG, Ortenberg, Germany), using 450 nm as the primary wavelength and 570 nm as a reference wavelength. After serum leptin and ObR measurement, the FLI was calculated based on the ratio between leptin/ObR serum levels (32).

Assessment of the Leptin and ObR Status by Immunohistochemistry (IHC)

Initially, the feline mammary carcinoma formalin fixed paraffin-embedded (FFPE) samples were stained with haematoxylin-eosin to select a representative tumor area (n = 58) and a normal tissue area to be used as control (n = 20). FFPE samples were sectioned in slices with 3 µm thickness (Microtome Leica RM135, Newcastle, UK) and mounted on a glass slide (SuperFrost Plus, Thermo Fisher Scientific, Massachusetts, USA). On PT-Link module (DAKO, Agilent, Santa Clara, USA), samples were deparaffinized, hydrated and antigen retrieval was performed for 20 min at 96°C using Tris-EDTA buffer pH 9.0 (EnVision™ Flex Target Retrieval Solution High pH, DAKO). Then, slides were cooled for 30 min at RT and immersed twice for 5 min in distilled water. IHC technique

was performed with commercial solutions from the Novolink™ Max Polymer Detection System Kit (Leica Biosystems, Newcastle, UK). Before antibody incubation, tissue samples were treated with Peroxidase Block Novocastra Solution (Leica Biosystems) for 15 min and the unspecific antigenic recognition was inhibited by incubation with Protein Block Novocastra Solution (Leica Biosystems) for 10 min. Finally, tissue samples were incubated at RT for 1 h, in a humidified chamber, with the following primary antibodies: anti-leptin antibody (ab3583, Abcam, Cambridge, UK) and anti-ObR antibody (ab104403, Abcam), both diluted at 1:200. The slides were washed twice, for 5 min, between all the incubation steps, using a PBS solution at pH 7.4. Then, the detection polymer was incubated for 30 min at RT, and detection was performed using diaminobenzidine (DAB substrate buffer and DAB Chromogen, Leica Biosystems) for 5 min. Later, samples were counterstained with Gills haematoxylin (Merck, New Jersey, USA) for 5 min, dehydrated in an ethanol gradient and xylene, and mounted using Entellan mounting medium (Merck). Antibodies were predicted to react with the feline proteins, occurring in cell membrane and cytoplasm (52, 53). Human breast tissue and feline liver were used as positive controls for the leptin staining, showing a positive staining in glandular cells (25, 52), hepatocytes (54, 55), and a faint staining in cholangiocytes (56), respectively. For the ObR, human and feline kidney were used as positive controls, showing a immunostaining in tubular and some glomerular cells, as previously reported (55, 57). Sections of the feline mammary tissues analyzed were used as a negative controls.

Leptin and ObR were evaluated in the glandular epithelium of the tumor, stromal tissue and tumor infiltrating inflammatory cells. To access proteins immunoreactivity we used a previously reported scoring system (14, 25, 52) and the H-Score published by the American Society of Clinical Oncology (ASCO). The final IHC score was obtained by multiplying the positive cells (0 = absence of staining; 1 = all cells stained), by the highest staining intensity (**Table 2**), varying from 0 to 3, with tissue samples scored as 0 considered negative, and samples scored as 3 as highly reactive. All slides were subjected to blind scoring, by two independent and experienced pathologists.

Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism software, version 5.04 (California, USA), with two-tailed $p < 0.05$ considered statistically significant for a 95% confidence level ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) and the average values were represented with the standard deviation.

The non-parametric Kruskal–Wallis test was performed to compare leptin and ObR results between healthy cats and cats with mammary carcinomas stratified by tumor subtype. Receiver-operating characteristic (ROC) curves were performed to choose the optimal cut-off value for serum leptin and ObR levels, and to determine the specificity and sensitivity of the technique to diagnose the disease. The non-parametric Mann-Whitney test was used to compare the serum levels of both proteins with several clinicopathological features. Survival analysis was performed using the Kaplan–Meier test to evaluate the disease-free survival (DFS) in cats with mammary carcinomas. Correlations between serum ObR levels and the previously reported serum CTLA-4, TNF-α, PD-1, and PD-L1 concentrations (10, 11) were investigated using the Spearman's rank correlation coefficient.

	Staining intensity	
	Score	**Interpretation**
Stained tumor cells (0–1)	0	No staining
	1	Weak
	2	Moderate
	3	Strong†

Final IHC score = stained tumor cells x staining intensity score (0–3).
Three microscopic fields were analyzed at 400× magnification (†High protein reactivity).

Table 2. *Scoring criteria of immunostaining assay for leptin and ObR.*

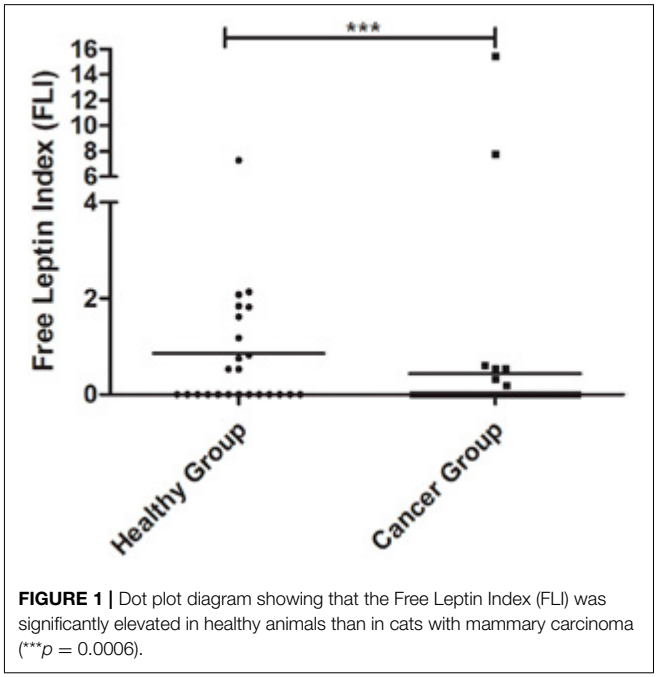

FIGURE 1 | Dot plot diagram showing that the Free Leptin Index (FLI) was significantly elevated in healthy animals than in cats with mammary carcinoma (***$p = 0.0006$).

Figure 1. *Dot plot diagram showing that the Free Leptin Index (FLI) was significantly elevated in healthy animals than in cats with mammary carcinoma (***p = 0.0006).*

Results

Cats With Mammary Carcinoma Showed Lower Free Leptin Index

The Free Leptin Index (FLI) was determined in the serum samples of cats with mammary carcinomas and compared with healthy animals. Results obtained showed that cats with disease had a significantly lower FLI than the control group (0.44 vs. 0.86, $p = 0.0006$, **Figure 1**). Moreover, the same results were obtained if the outliers were removed from the analysis ($p = 0.0005$).

In addition, results revealed that body weight did not influence serum leptin and ObR levels, both in the control group ($p = 0.0760$ and $p = 0.8432$, respectively, **Figures 2A,B**) and in the cancer group ($p = 0.3294$ and $p = 0.9722$, respectively, **Figures 2C,D**).

Cats With Luminal B or HER2-Positive Mammary Carcinomas Showed Decreased Serum Leptin Levels

Regarding the serum leptin levels, results obtained showed that cats with luminal B or HER2-positive mammary carcinomas had lower serum leptin levels than healthy animals (0.00 vs. 13.89 pg/ml, $p < 0.01$; 0.83 vs. 13.89 pg/mL, $p < 0.05$, respectively, **Figure 3A**), and considering the analysis with no outliers, the same results could be reported ($p = 0.0021$). The optimal cut-off value to predict mammary carcinoma was 4.17 pg/ml with an area under the ROC curve (AUC) of 0.7045 ± 0.0757 (95% CI: 0.5561–0.8528, $p = 0.0103$; sensitivity = 96.9%; specificity = 43.5%; **Figure 3B**). Considering this analysis with no outliers, an AUC = 0.6732 ± 0.0731 (95% CI: 0.5299–0.8164, $p = 0.0158$; sensitivity = 92.9%; specificity = 43.4%) was obtained, leading to the same results. Further statistical analysis, revealed that serum leptin levels above the cut-off value were associated with tumor

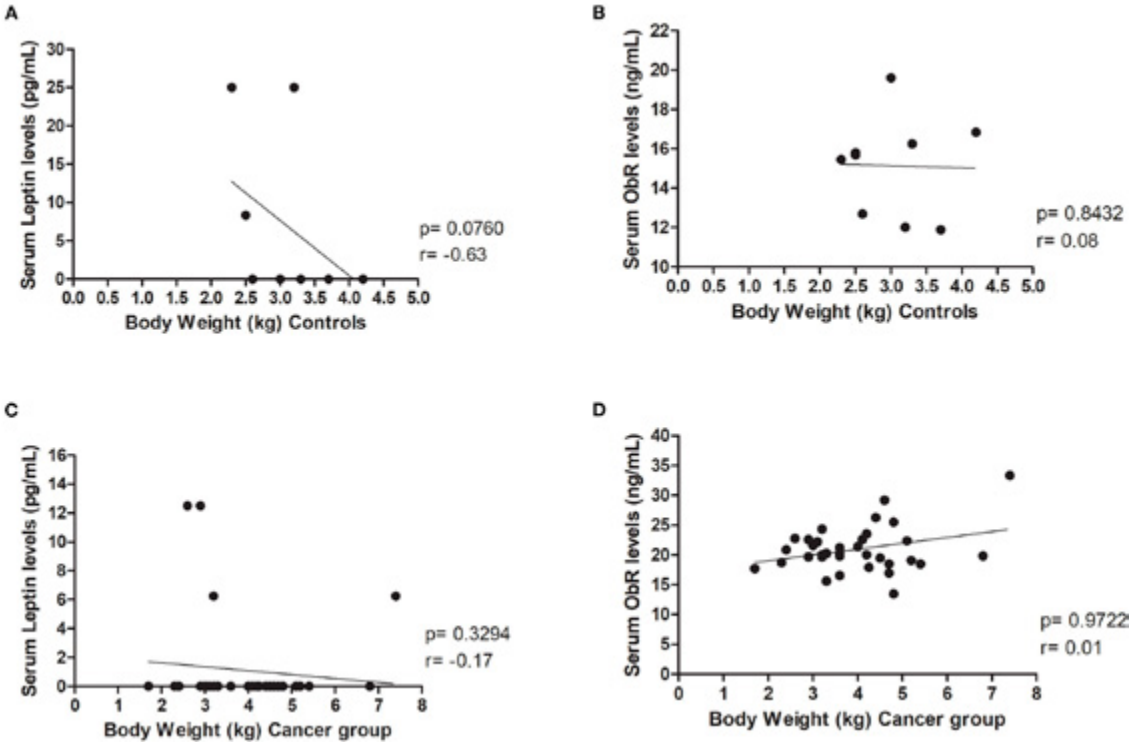

Figure 2. *Body weight did not influence leptin, neither ObR serum levels in healthy and diseased animals. Correlations were not found between (A) serum leptin (p = 0.0760) or (B) ObR (p = 0.8432) levels and body weight in the control group. Furthermore, evaluating the cancer group, no correlations were detected between (C) serum leptin (p = 0.3294) or (D) ObR (p = 0.9722) levels and feline body weight.*

ulceration (p = 0.0005, **Figure 3C**; or p = 0.0009, if no outliers were considered) and shorter DFS (117 vs. 314 days, p = 0.0217, **Figure 3D**; or p = 0.0245, if the outliers were removed).

Cats With Mammary Carcinoma Showed Elevated Serum Levels of ObR and of Inflammation Mediators

Considering the above results, the serum ObR levels were also evaluated. When the animals were grouped according to the tumor subtype, a significant difference was found between the mean ranks of at least one pair of groups (p < 0.0001, with or without outliers). Results revealed that serum ObR levels were significantly higher in animals with mammary carcinoma than in controls, independently of molecular subtype (control group 15.67 ng/ml; luminal A 23.04 ng/ml, p < 0.0001; luminal B 20.18 ng/ml, p < 0.001; HER2-positive 28.99 ng/ml, p < 0.0001; triple-negative 21.70 ng/ml, p < 0.0001; **Figure 4A**). Furthermore, the optimal cut-off value calculated for cats with mammary carcinoma was 16.89 ng/ml, with an AUC of 0.9408 ± 0.0288 (95% CI: 0.8842–0.9973, p < 0.0001; sensitivity = 94.8%; specificity = 87.0%; **Figure 4B**). If the outliers were removed from the analysis, the same results were obtained, with an AUC = 0.9397 ± 0.0293 (95% CI: 0.8823–0.9972, p < 0.0001; sensitivity = 94.7%; specificity = 87.0%).

In addition, elevated serum ObR levels were associated with smaller tumors (p = 0.0118, **Figure 4C**; p = 0.0248, if no outliers were considered) and with cats had an ER-negative status (p = 0.0291, **Figure 4D**; p = 0.0452, if no outliers were considered). Finally, a positive correlation was found between serum ObR levels and serum levels of CTLA-4 (r = 0.38, p = 0.0056, **Figure 5A**), TNF-α (r = 0.40, p = 0.0025, **Figure 5B**), PD-1 (r = 0.42, p = 0.0023, **Figure 5C**), and PD-L1 (r = 0.50, p = 0.0002, **Figure 5D**). Removing the outliers from our data, the same results could be reported (CTLA-

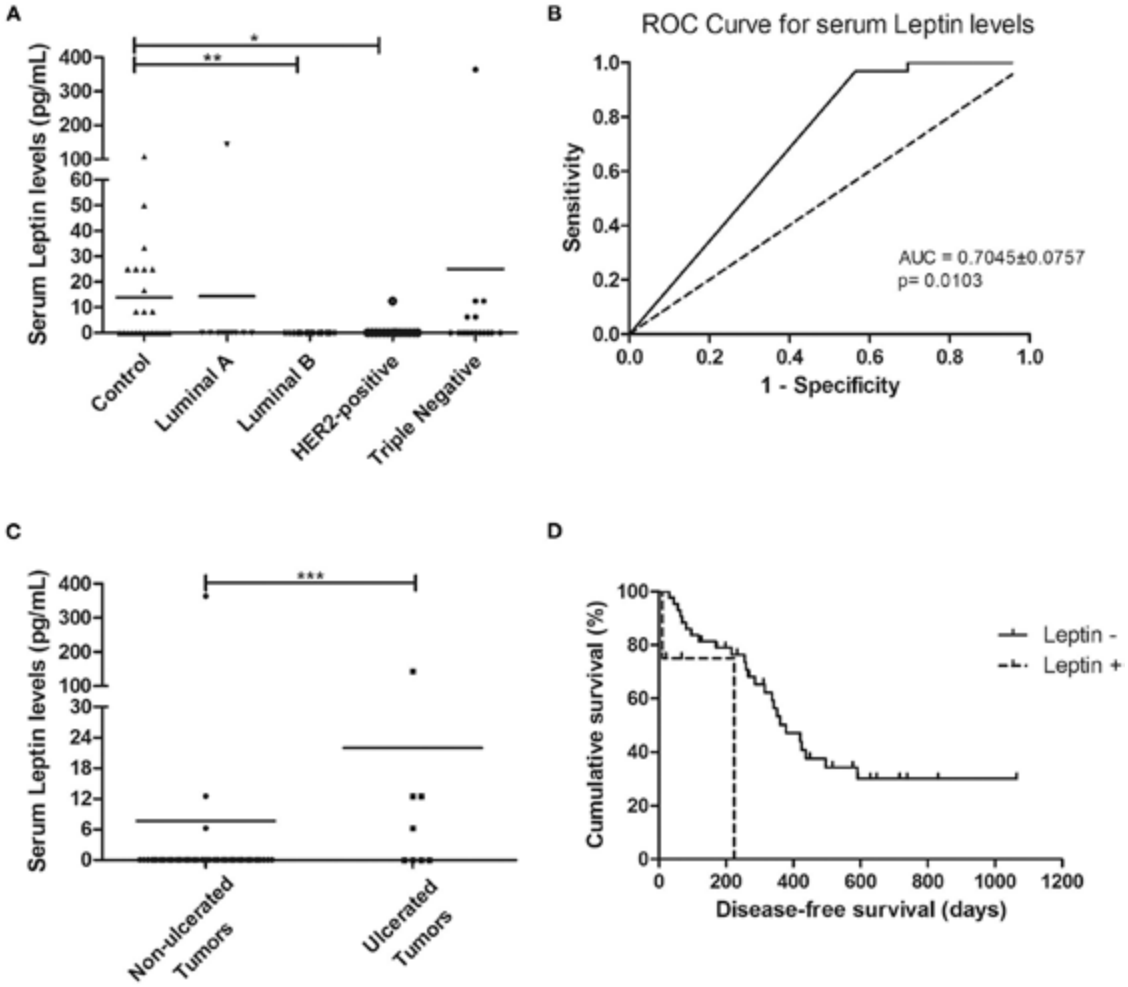

Figure 3. *Cats with luminal B and HER2-positive mammary carcinomas showed decreased serum leptin levels, although cats with ulcerated tumors exhibited serum leptin levels above the cut-off value of 4.17 pg/mL, being associated with shorter disease-free survival. (A) Dot plot diagram showing the distribution of serum leptin levels (pg/mL) among healthy animals (control) and cats stratified by the mammary carcinoma subtype. Significant decreased serum levels of leptin were found in cats presenting luminal B or HER2-positive subtypes in comparison to healthy animals (p = 0.0025). (B) The optimal cut-off of serum leptin levels to predict mammary carcinoma was determined to maximize the sum of the sensitivity and specificity (4.17 pg/mL; AUC = 0.7045 ± 0.0757, 95% CI: 0.5561–0.8528, p = 0.0103; sensitivity = 96.9%; specificity = 43.5%). (C) Dot plot diagram showing that serum leptin levels were significantly higher in cats with ulcerated tumors (p = 0.0005). (D) Cats with mammary carcinoma and serum leptin levels higher than 4.17 pg/mL had a lower DFS (p = 0.0217). *p < 0.05; **p < 0.01; ***p < 0.001.*

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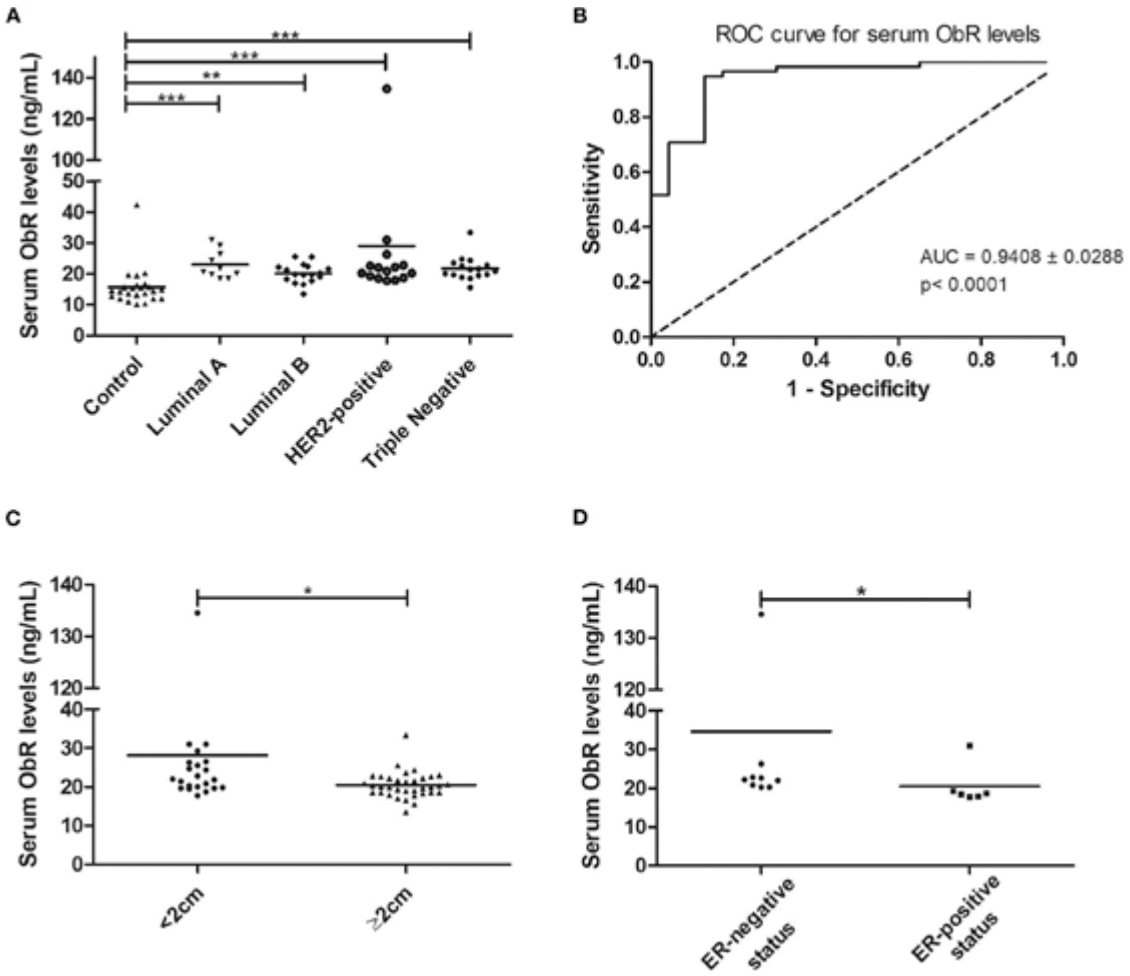

Figure 4. *Cats with mammary carcinoma showed elevated serum ObR levels, with serum concentrations above 16.89 ng/mL being associated with smaller tumors and an ER-negative status. (A) Dot plot diagram showing the distribution of serum ObR levels (ng/mL) in heathy animals (control) and in cats with mammary carcinoma stratified by the molecular subtype. Significant higher serum levels of ObR were found in all tumor subtypes in comparison to healthy animals ($p < 0.0001$). (B) The optimal cut-off value of serum ObR levels to predict cats with mammary carcinoma was 16.89 ng/mL with an AUC of 0.9408 ± 0.0288 (95% CI: 0.8842–0.9973, $p < 0.0001$; sensitivity = 94.8%; specificity = 87.0%). (C) Dot plot diagram showing that serum ObR concentrations were significantly low in tumors larger than 2 cm ($p = 0.0118$). (D) Dot plot diagram displaying a positive association between higher serum ObR levels and ER-negative status ($p = 0.0291$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

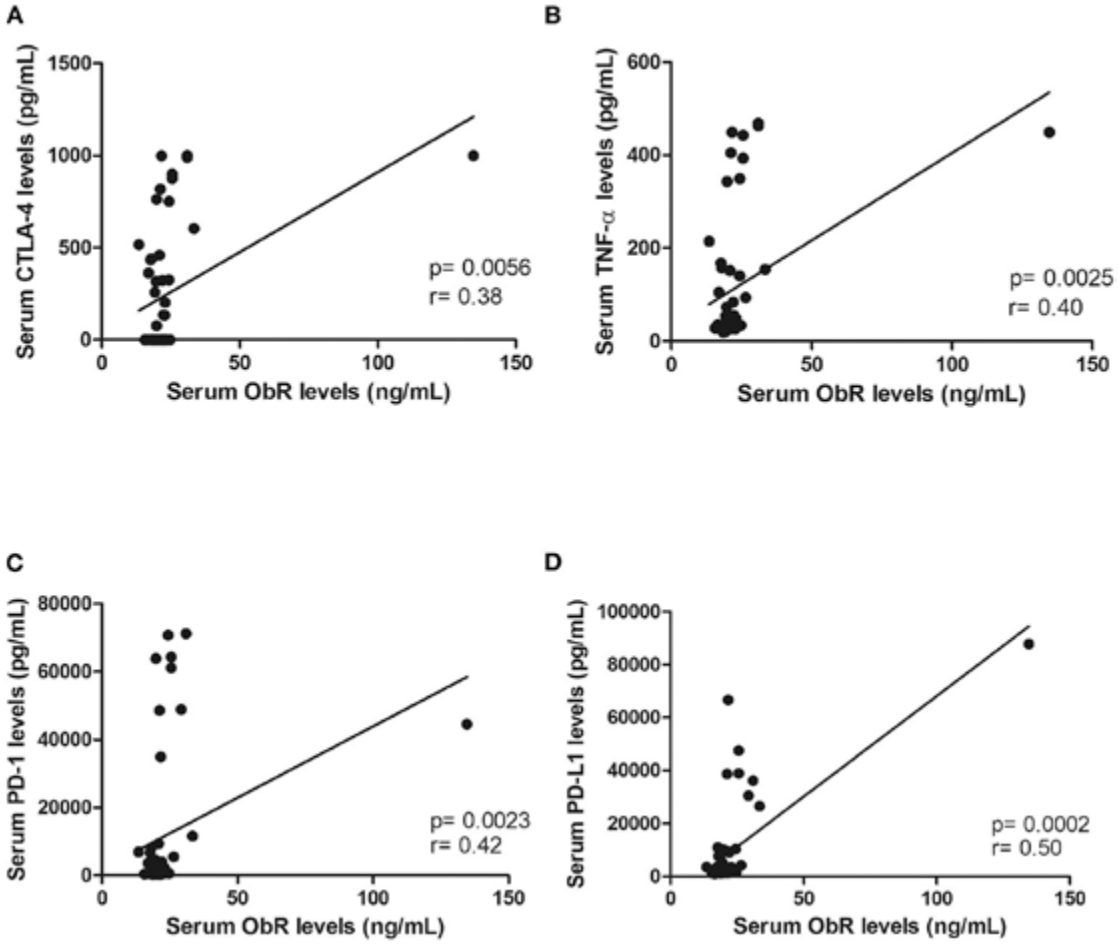

Figure 5. *Serum ObR levels showed a positive correlation with inflammatory mediators, namely (A) serum CTLA-4 levels (p = 0.0056), (B) serum TNF-α levels (p = 0.0025), (C) serum PD-1 levels (p = 0.0023), and (D) serum PD-L1 levels (p = 0.0002).*

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4: $r = 0.34$, $p = 0.0153$; TNF-α; $r = 0.37$, $p = 0.0064$; PD-1: $r = 0.39$, $p = 0.0002$; and PD-L1: $r = 0.47$, $p = 0.0007$).

Leptin and ObR Are Overexpressed in Luminal B and Triple-Negative Mammary Carcinomas

The obtained results revealed that cats with luminal B or triple-negative mammary carcinoma showed a higher leptin IHC score in the tumor glandular cells, comparing to the healthy control samples (1.93 vs. 1.34, $p < 0.05$; 2.00 vs. 1.34, $p < 0.05$, respectively; **Figures 6A**, **7A,B**). Regarding the leptin receptor, the IHC score was also significantly higher in animals with a luminal B tumor subtype than in healthy animals (2.50 vs. 1.75; $p = 0.0425$; **Figures 6B**, **7C,D**).

Furthermore, the immunostaining reveals to be positive in the stroma cells, in 72.2 and 18.2% of the tumors, for leptin (IHC score of 0.79) and ObR (IHC score of 1.1), respectively. Moreover, was observed that, independently of the tumor subtype, a mean of 81 ± 2.5% of the tumor inflammatory mononuclear cells presented to be positive for the leptin staining (mean IHC score of 2.0 for the macrophages and mean IHC score of 1.6 for the lymphoid cells, **Figure 8A**). The same analysis, considering the ObR revealed a positive staining for a mean of 87.6 ± 2.5% of the tumor inflammatory mononuclear cells (mean IHC score of 2.62 for the macrophages and mean IHC score of 1.33 for the lymphoid cells, **Figure 8B**).

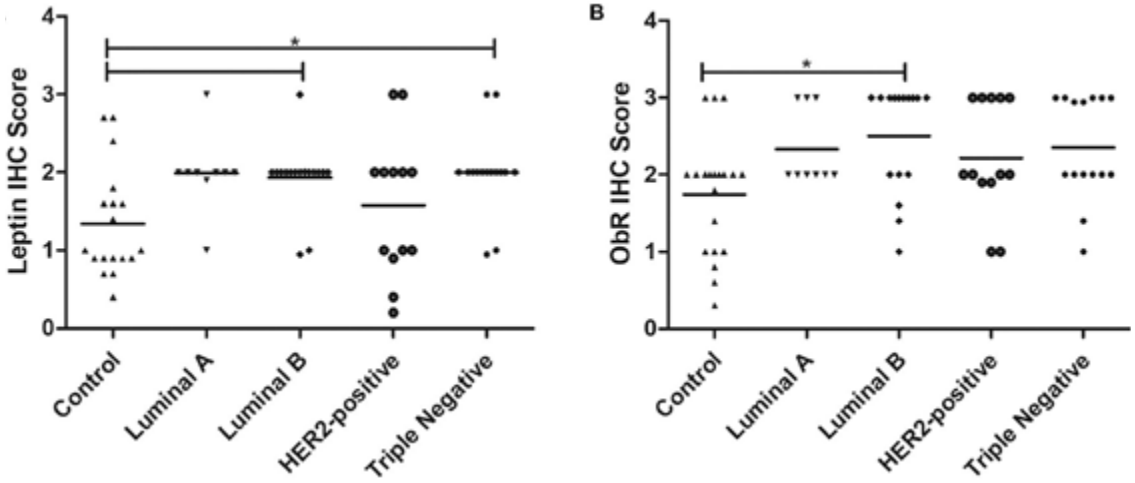

Figure 6. *Final IHC scores for leptin (**A**) and ObR (**B**) in cats with mammary carcinoma stratified by the tumor subtype and compared with controls. (**A**) Leptin expression was significantly higher in luminal **B** and triple-negative subtypes ($p = 0.0046$). (**B**) Expression of ObR was statistically higher in luminal **B** tumor subtype ($p = 0.0425$).*

In addition, our findings revealed that serum ObR levels are negatively correlated with the ObR IHC score, with cats presenting higher serum ObR levels showing mammary tumors with lower ObR IHC scores ($p = 0.0103$, **Figure 9**; $p = 0.0244$ if the outliers were removed from the analysis).

Discussion

Although spontaneous FMC has been proposed as a suitable model for human breast cancer studies, the role of the leptin/ObR axis has never been evaluated in cats. In humans, previous studies showed that leptin and ObR overexpression are associated with pro-inflammatory and pro-tumorigenic effects, particularly in overweight women (13, 15). Moreover, some studies reported increased serum leptin levels with aging (58, 59). In this study, the healthy group presented a mean age lower than the tumor group, and despite what is reported in human and rats (58, 59), the results obtained demonstrated that cats with mammary carcinoma have a reduced Free Leptin Index (FLI) in comparison to the healthy group ($p = 0.0006$), not only due to the increase in serum ObR levels (60), but

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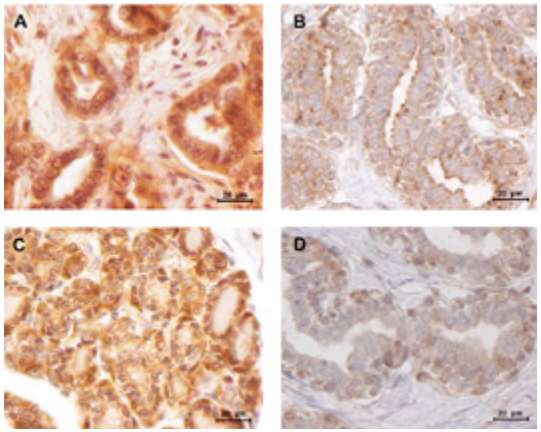

Figure 7. *Leptin and ObR were overexpressed in luminal **B** mammary carcinomas. (**A**) Leptin overexpression in a luminal **B** mammary carcinoma (IHC score of 1.93) contrasting with (**B**) a low staining intensity detected in normal mammary tissues (IHC score of 1.34). (**C**) Luminal **B** mammary tumors showed a higher staining intensity for ObR (IHC score of 2.50), (**D**) than normal mammary tissues (IHC score of 1.75) (400× magnification).*

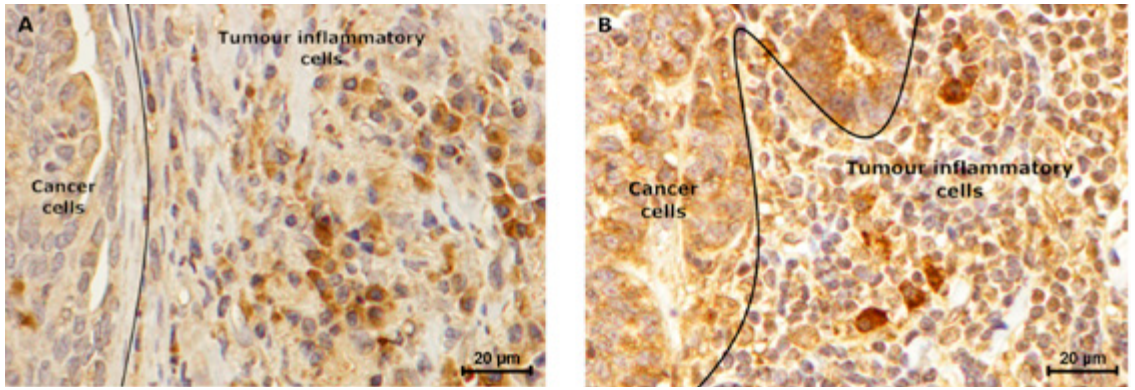

Figure 8. *Tumor inflammatory cells express leptin and ObR. Luminal B carcinoma subtype showed a (A) positive leptin staining (IHC score of 1.5), which is lower when compared to the (B) ObR immunostaining (IHC score of 2.5) of tumor inflammatory cells. Furthermore, in both samples higher staining intensity was observed in macrophages, when compared to lymphoid cells (IHC score of 2.0 vs. 1.2, respectively for leptin, and IHC score of 3.0 vs. 2.0, respectively for ObR) (400× magnification).*

also suggesting that diseased animals may have decreased soluble leptin levels, as reported in pre-menopausal women with breast cancer (61) and colon cancer patients (62). These results indicate that serum leptin may be recruited by mammary cancer cells to promote tumor growth and cell migration (23). Indeed, cats with luminal B or HER2-positive mammary carcinoma showed significantly lower serum leptin levels when compared with controls ($p < 0.001$ and $p < 0.05$, respectively), revealing that serum leptin levels are downregulated in tumors with PR-positive status (14) and/or HER2-positive status (27). In contrast, cats with luminal A showed elevated serum leptin levels, indicating that ER overexpression in the tumor may promotes leptin expression (14). Regarding the elevated serum leptin levels found in cats with triple-negative mammary carcinomas, studies demonstrated that leptin induces cell proliferative capacity (e.g., via Wnt/β-catenin pathway) (22, 43) and promotes cell survival by interacting with Bcl-2 proteins, being associated with more aggressive tumors (63). Indeed, our results revealed that elevated serum leptin levels occur in an advanced stage of the disease, being significantly associated with tumor ulceration ($p = 0.0005$) and shorter DFS ($p = 0.0217$), as reported for women with breast cancer (14, 15).

In parallel, as documented in breast cancer patients (14, 64), all cats with mammary carcinoma showed higher serum ObR levels than healthy controls ($p < 0.0001$). Also higher serum ObR levels were correlated with smaller tumor size ($p = 0.0118$), suggesting that ObR shedding occurs in small tumors, modulating the serum levels of free leptin (28). Moreover, our results further support the hypothesis that malignant cells in larger tumors maintain the ObR expression on its surface to increase their survival and growth (19). Interestingly, the higher serum ObR levels were found in cats with mammary carcinomas presenting a HER2-positive/ER-negative status ($p = 0.0291$), as reported for human breast cancer patients (14), confirming the crosstalk between the leptin/ObR axis

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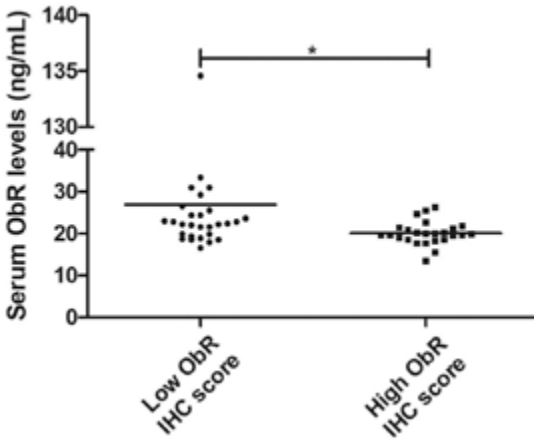

Figure 9. *Dot plot diagram showing a negative correlation between serum ObR levels and tumor ObR IHC score (*p = 0.0103).*

and the EGFR downstream signaling pathway (65).

In addition, this study discloses the utility of leptin and ObR as promising diagnostic biomarkers to differentiate animals with FMC from healthy cats (cut-off value of 4.17 pg/mL for leptin and 16.89 ng/mL for ObR).

We also found that serum ObR levels were positively correlated with serum CTLA-4 ($p = 0.0056$), TNF-α ($p = 0.0025$), PD-1 ($p = 0.0023$) levels as reported in breast cancer patients (39), and with serum PD-L1 levels ($p = 0.0002$). Indeed, previous studies showed that activation of the leptin/ObR axis can result in a chronic inflammatory status (35, 66), a well-known risk factor for breast cancer, with leptin being involved in CD4+ T-regulatory cells differentiation due to ObR overexpression on lymphocyte plasm membrane (67). These activated CD4+ T-regulatory cells express CTLA-4(35) and PD-1, two immune-inhibitory checkpoint molecules that downregulate T-cell immune responses (37), leading to tumor development (68), and contributing to cell growth (69). On the other hand, in an attempt to control the tumorigenesis process, CD4+ T-regulatory cells secrete TNF-α (38), a molecule that shows a dual role in immunomodulation, being also expressed by cancer cells (70), acting as an autocrine growth factor (71). Altogether, these findings provide support for the crosstalk between the leptin/ObR axis and tumor immunoediting mechanisms, contributing to an immunosuppressive status in cats with mammary carcinoma (10, 11).

The immunostaining analysis of the tumor and normal tissue samples revealed that luminal B and triple-negative mammary carcinoma subtypes ($p < 0.05$) showed leptin overexpression. Although a strong ObR expression was only detected in luminal B mammary carcinomas ($p = 0.0425$), as described in human breast cancer (25). Furthermore, several studies suggest that leptin and ObR are overexpressed in tumor tissues, due to hypoxia and/or as a response to insulin, IgF-1 and/or to estradiol (64, 72). In addition, the higher IHC scores for leptin found in luminal B carcinomas also support the previously reported association between the expression of this adipocytokine and aromatase expression, an enzyme that catalyzes the conversion of androgen into estrogen to promote tumor development via an ER-dependent mechanism (14). The overexpression of leptin detected in triple-negative mammary carcinomas is also in concordance with previous results in triple-negative breast cancer, where leptin signaling is crucial for tumor growth (29, 63), being associated with ERK and Akt pathways, both involved in breast cancer cells proliferation (23). Furthermore, the tumor inflammatory mononuclear cells revealed to be positive for leptin and ObR immunostaining, with a higher proteins expression in macrophages. In fact, leptin/ObR axis are reported as activating the inflammatory response (66, 73). Finally, our results demonstrated that cats with low ObR-expressing mammary tumors had higher serum ObR levels, indicating a negative feedback between tumor microenvironment and serum, probably due to a shedding mechanism that leads to a reduction of serum leptin levels (23, 60). Furthermore, the data obtained emphasizes the possibility of blocking the leptin/leptin receptor axis, as an adjuvant therapy in cats with luminal B and triple-negative mammary carcinoma subtypes, as reported for breast cancer patients (42, 44–46).

In conclusion, our data provide a rationale use for leptin/ObR as diagnostic and prognostic biomarkers. Indeed, cats with mammary carcinoma showed a decreased FLI, coupled with decreased serum leptin levels in animals with luminal B or triple-negative mammary carcinoma subtypes. A significant increase in serum ObR levels, was detected in all samples, independently of the tumor subtype, being associated to an immunosuppressive status. Altogether, our data indicate that cats presenting luminal B and triple-negative tumors could benefit from adjuvant therapies targeting leptin, and support the utility of spontaneous FMC as a model for comparative oncology.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by CIISA - Faculdade de Medicina Veterinária. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author Contributions

AG and FF: designed the research. AG, CN, ACU, JC, and FF: performed the research. AG, CN, and FF: analyzed the data. AG and FF: wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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This article is part of the Research Topic

Comparative Oncology - Advances in Veterinary Molecular Oncology

Modificaciones dietéticas para el manejo del perro con enteropatía perdedora de proteínas

Irma Villanueva, DVM, PhD,
Scientific Communication Manager

La enteropatía perdedora de proteínas (EPP) en perros es un síndrome que se asocia con diferentes enfermedades entre las que se encuentran principalmente la enfermedad inflamatoria intestinal (EII) y la linfangiectasia intestinal (LI), aunque también puede ser debida a linfoma gastro-intestinal o a algunas enfermedades infecciosas y parasitarias.

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Independientemente de la causa, los mecanismos por los que se produce esta pérdida de proteínas incluyen (1):

- Obstrucción linfática (ya sea física o funcional) que da lugar a pérdida de linfa.
- Liberación de mediadores celulares que afectan la permeabilidad vascular y provocan salida de fluidos hacia los tejidos.
- Inflamación de la mucosa digestiva, ya sea con o sin ulceración.

El resultado es la acumulación de un fluido rico en proteínas, sobre todo albúmina, en el espacio intersticial que pasa a la luz del tracto digestivo a través de su mucosa. La presentación clínica de los perros es muy similar, independientemente de la causa que la produjo (2).

La LI se piensa que puede ser de origen congénito o ser una enfermedad primaria, pero también puede ser secundaria a un proceso inflamatorio (2). En un estudio retrospectivo reciente, se observó que en las biopsias intestinales de un 53% de los perros con EII, también había algún grado de dilatación de los capilares linfáticos, y de los perros que presentaban hipoalbuminemia, el 76% también tenían dilatación de estos. (3). Asimismo, en un estudio previo se había visto que en todos los perros con enteritis linfoplasmocitaria también había un cierto grado de dilatación de los capilares linfáticos de las vellosidades intestinales y esta dilatación se relacionaba con los valores de albúmina sérica (4). Además, hay que tener en cuenta que la linfangitis puede tener una distribución segmentaria y cabe la posibilidad de no ser siempre detectada en las muestras recogidas para biopsia.

“El pronóstico de las EPP es reservado independientemente de que la causa subyacente sea EII, LI o ambas”

En 2019 Craven y Wasabau (1) revisaron 23 artículos que incluían un total de 469 perros con EPP y concluyeron que, en la mayoría de los perros, se asociaba más a enteritis linfoplasmocitaria que a linfangiectasia primaria intestinal. Además, observaron que las razas más prevalentes eran el Yorkshire Terrier, el Border Collie, el Pastor Alemán y el Rottweiler. La mayoría se presentaban con distensión abdominal, vómitos, diarrea, poliuria/polidipsia, anorexia, pérdida de peso y letargia. Los exámenes clínicos revelaban ascitis, caquexia, pérdida de masa muscular, debilidad, disnea/taquipnea y dolor abdominal.

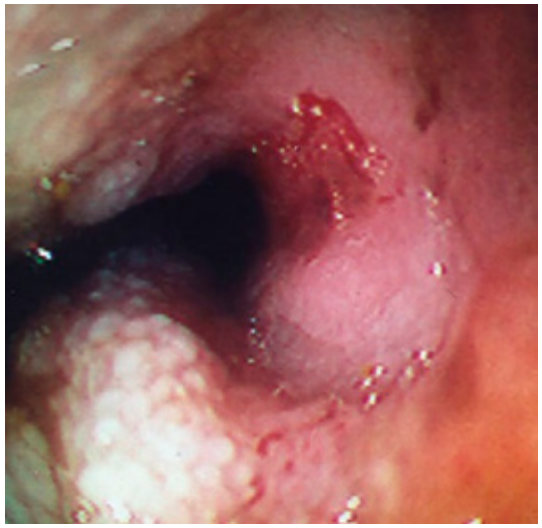

Imagen endoscópica del duodeno de un perro con diagnóstico confirmado de linfangiectasia. © Rance Sellon

El pronóstico de las EPP es reservado independientemente de que la causa subyacente sea EII, LI o ambas y la mortalidad como consecuencia de la enfermedad se produce en el 54,2% de los casos. La malnutrición es muy prevalente en estos pacientes y llevar a cabo una valoración nutricional en la consulta es muy importante porque está relacionada con el pronóstico (5).

En razas predispuestas a padecer enfermedades linfáticas como **Yorkshire Terrier, Shar-Pei, Malteses, Lundehund noruego o Rottweiler**, incluso con inflamación predominante en biopsias intestinales, la LI intestinal siempre debe ser considerada como una posibilidad en cualquier perro con EPP y el abordaje más seguro de estos pacientes es presuponer que ambas patologías pueden estar presentes, particularmente en los casos más severos, donde lo recomendable es una terapia individualizada (6).

La importancia del tratamiento dietético está fuera de toda duda en pacientes con EPP. Una restricción estricta de grasa es clásicamente lo más recomendado para perros con LI para disminuir la formación de quilomicrones y evitar así la dilatación linfática. Sin embargo, la recomendación más común para perros con EII son las dietas a base de proteína novel o proteína hidrolizada ya que la causa posible es una reacción adversa al alimento. Así pues, puede resultar difícil elegir la dieta más adecuada cuando sospechamos que podemos estar ante ambos problemas.

“La importancia del tratamiento dietético está fuera de toda duda en pacientes con EPP”

En muchos de estos perros, al comienzo de la terapia puede no conocerse cuál sería el manejo nutricional más adecuado y con frecuencia es necesario seguir un enfoque de ensayo y error con la dieta que se elija hasta dar con la mejor para cada caso individual.

En teoría, la dieta ideal debe ser altamente digestible, a base de proteína hidrolizada en cantidad adecuada y restringida en grasas, es decir con menos de 20g de grasa/Mcal. Esta opción ya está comercialmente disponible y puede ser una primera opción para comenzar el ensayo dietético. En perros con EPP causada por LI se suele observar una notable mejoría con una dieta baja en grasas, pero en algunos casos el paciente puede requerir una restricción de grasas todavía mayor que la que pueden proporcionar las dietas comerciales, y en perros con EII junto con una LI significativa, puede ser necesaria una dieta casera formu-

lada por un veterinario especialista en nutrición para poder abordar ambos problemas (7).

La mayoría de los perros que responden a la dieta lo hacen en las dos semanas siguientes al comienzo del ensayo, aunque se han reportado casos en los que han sido necesarias hasta ocho semanas. Sin embargo, la respuesta individual de cada paciente va a depender de la composición de la dieta seleccionada y múltiples ensayos dietéticos pueden ser necesarios antes de considerar que nos enfrentamos a una enfermedad que no responde a la dieta.

Además, a veces también nos enfrentamos al reto de un paciente inapetente que no acepta la dieta que se va a probar. Una lenta transición al nuevo alimento y la modificación del entorno incluyendo presentar pequeñas cantidades de alimento de forma frecuente, ofrecer la comida en diferentes lugares e incluso diferentes tipos de comederos puede ser de ayuda.

También se debe considerar la posibilidad de alimentación por sonda en pacientes con enfermedad grave y en estado catabólico que no comen voluntariamente.

El manejo nutricional de los pacientes con EPP puede ser frustrante en muchas ocasiones para el cuidador del animal. Es muy importante que, desde el principio, el clínico advierta que pueden ser necesarios varios ensayos dietéticos y que el seguimiento del animal va a ser imprescindible.

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