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Pathological findings in rescued hedgehogs

Achados patológicos em ouriços resgatados

Gabriela Fernandes da Silva

Orientador: Professora Doutora Irina Amorim

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RESUMO

Os ouriços são pequenos mamíferos e a sua proximidade com o ser humano tem vindo a intensificar-se. No entanto, o conhecimento de potenciais doenças transmissíveis por estes animais, bem como possíveis doenças predisponentes destas espécies, é limitado.

A maioria das patologias que afetam os ouriços são diagnosticadas *post mortem*, pelo que é essencial a partilha de conhecimento entre clínicos e patologistas, a fim de reconhecer doenças predisponentes e estabelecer planos de diagnóstico e terapêuticos adequados. Para além disso, uma vez que as ferramentas existentes para o correto diagnóstico de patologias em ouriços não estão direcionadas para esta espécie, torna-se imprescindível a otimização e validação de métodos de diagnóstico complementares comumente utilizados em laboratórios de patologia.

Este estudo teve como objetivo a descrição dos achados macroscópicos e microscópicos mais relevantes identificados durante o exame de necropsia de um grupo de doze ouriços resgatados, realizado durante um período de quatro meses. Os resultados proporcionaram novos dados respeitantes às várias patologias que podem ser encontradas nestes animais. Com a análise de apenas doze casos, foi possível obter um conjunto significativo e diversificado de patologias de valor considerável do ponto de vista da patologia comparada. Esta panóplia de lesões reflete a importância deste pequeno mamífero no contexto eco-epidemiológico das doenças, dado o seu potencial para transmitir doenças zoonóticas e o seu importante papel no conceito de 'One Health', atuando como 'sentinelas' do ambiente em que se encontram.

PALAVRAS-CHAVE: Ouriços; *Mycobacterium* spp.; Carcinoma da tiroide; Colangiocarcinoma

ABSTRACT

Hedgehogs are small mammals, whose proximity to humans has been intensifying. However, knowledge of potential transmissible diseases by these animals, as well as possible predisposing diseases of these species, is limited.

Most of the pathologies that affect hedgehogs are diagnosed *post mortem*, so it is essential to share knowledge between clinicians and pathologists in order to recognize predisposing diseases and establish adequate diagnostic and therapeutic plans. Furthermore, since the existing tools for the correct diagnosis of pathologies in hedgehogs are not directed to this species, it is essential to optimize and validate complementary diagnostic methods commonly used in pathology laboratories.

This study aimed at describing the most relevant macro- and microscopic findings identified during the necropsy examination of a group of twelve rescued hedgehogs, carried out over a period of four months. The results provided new information regarding pathological diseases that can be found in *post mortem* examinations of these animals. With the analysis of only twelve cases, it was possible to obtain a significant and diverse set of pathologies with considerable value from the point of view of comparative pathology. This panoply of injuries reflects the importance of this small mammal in the eco-epidemiological context of diseases, given its potential to transmit zoonotic diseases and its important role in the concept of ‘One Health’, acting as 'sentinels' of the environment in which they are found.

KEY-WORDS: Hedgehogs; *Mycobacterium* spp.; Thyroid carcinoma; Cholangiocarcinoma

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ABREVIATURAS

CC	Cholangiocarcinoma
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EnLP	Endogenous lipoid pneumonia
HCC	Hepatocellular carcinoma
HE	Hematoxylin and eosin
HPF	High-power field
ICBAS	Institute of Biomedical Sciences Abel Salazar
IHC	Immunohistochemistry
IPATIMUP	Institute of Molecular Pathology and Immunology of the University of Porto
IUCN	International Union for Conservation of Nature
MCT	Mast cell tumor
MUC-1	Mucin 1
NSE	Neuron-specific enolase
PAS	Periodic acid–Schiff
PCR	Polymerase chain reaction
SCC	Squamous cell carcinoma
TPO	Thyroperoxidase
TSH	Thyroid stimulating hormone
WHS	Wobbly hedgehog syndrome

1. Introduction

1.1 Biology and behavior

Hedgehogs are small, nocturnal and insectivorous mammal animals, that belong to the order Eulipotyphla, family Erinaceidae (Amori, 2016; Riley & Chomel, 2005). They have a life expectancy of 3–4 years in the wild and 5–8 years in captivity, and they are classified according to the shape and pattern of their spines, ear length and skull morphology (Del Aguila et al., 2019; Heatley, 2009). Worldwide, 16 species of hedgehogs are recorded, placed into five genera: *Hemiechinus* (two species), *Atelerix* (four species), *Erinaceus* (four species), *Paraechinus* (four species), and *Mesechinus* (two species), however, the most common are the African pygmy hedgehog (*Atelerix albiventris*) and the European hedgehog (*Erinaceus europaeus*) (Ruszkowski et al., 2021). African pygmy hedgehog is actually commonly kept as a pet. However, European hedgehogs are free-living animals and therefore, according to the Berne Convention for the Conservation of European Wildlife and Natural Habitats, keeping them as pets without special permissions is illegal (Keeble & Koterwas, 2020; Ruszkowski et al., 2021).

Hedgehogs are nocturnal in captivity but during the day will emerge from their nest. In the wild, these creatures live in different habitats where they dig their burrows, rest by day, and at night move around to forage (Heatley, 2009). The body weight of African pygmy hedgehogs ranges from 400-600 g in adult males and 250-400 g in adult females (Pollock & Parmentier, 2010). European hedgehogs are considerably larger in size with body weight ranging from 800-1200 g in adult males and 400-800 g in adult females (Pollock & Kanis, 2015). The integumentary system consists of short spines that are modified hairs with a spongy matrix inside the outer fibrous cortex, which cover the dorsum of the body (Heatley, 2009; Riley & Chomel, 2005). The normal stance is plantigrade and although most hedgehogs have five toes on all feet, the African pygmy hedgehog only has four toes, hence it is known as the “Four-toed hedgehog” (Heatley, 2009).

When they are scared, they have the ability to roll into a tight ball to get a defensive posture and protect themselves from predators (Riley & Chomel, 2005). This happens due to the action of a complex muscle system that erects and positions the spines and curls the hedgehog into a spiny ball that generally vibrates and hisses. If necessary, the hedgehog can stay in this position for hours. All species of hedgehogs have a behavior called "anting" or "anoiting": when they find a substance, they lick it until

they begin to hypersalivate and spread excess saliva on the spines and on the skin. The purpose of this behavior is unknown, but some theories refer objectives such as reducing skin parasites, avoiding predators, attracting partners and communicating with other hedgehogs through the odor of each animal (Heatley, 2009). Smell plays an important role in the hedgehogs' ability to survive in nature, so they have a very sensitive olfactory system and well-developed olfactory lobes. They also have a well-developed hearing system (ultrasound range), but their vision is poor and essentially monochromatic (Ivey & Carpenter, 2012).

In cold conditions, all hedgehogs can enter into a torpid state to reduce energy needs and survive when food is scarce (Ivey & Carpenter, 2012). Hibernation is characterized by a decrease in body temperature, oxygen consumption and heart rate, resulting in a drastic reduction in the overall metabolic rate (Johnson, 2011). The European hedgehog hibernates from November to March and, in the wild, the African pygmy hedgehog can hibernate when the weather is cold and dry. During hibernation, hedgehogs may become immunosuppressed and predisposed to infections, so there is no need for captive hedgehogs to hibernate, and they do not when kept warm and well fed (Garcês et al., 2020; Ivey & Carpenter, 2012; Johnson, 2011).

The sex of hedgehogs can be determined by their external anatomy. Males have a prepuce located midway along the ventral abdomen, the penis is spineless and there is no scrotal sac, but the testicles are palpable in the subcutaneous para-anal recesses. As in other rodent species, the female's vulva is located close to the anus, resulting in a decreased anogenital distance (Heatley, 2009).

1.2 Geographic distribution and habitat

The African pygmy hedgehog is originally from Central Africa and can be found in a variety of habitats, such as grassland, scrub, savannah and suburban gardens. Nowadays, its demand as a pet and breeding in captivity have increased (Heatley, 2009). The European hedgehog is commonly found in urban areas, gardens and parks near buildings (Garcês et al., 2020). This species is endemic in Europe and according to The International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species, in Portugal this species is classified as “Least Concern” (Amori, 2016).

1.3 Zoonoses

All pets have a flora of parasites and microbes, some of which have zoonotic potential. Although exotic animals are increasingly becoming pets, knowledge about the

potential of many of these animals to transmit zoonotic agents is limited, which carries a risk of developing diseases with zoonotic potential. On the other hand, the rescue and recovery of sick hedgehogs perpetuates and increases exposure to zoonotic agents (Riley & Chomel, 2005). Bacterial, viral and fungal pathogens with zoonotic potential in domestic and wild hedgehogs are reviewed in Table 1.

Table 1. Zoonotic agents isolated from wild and pet hedgehogs (Ruszkowski et al., 2021)

	Bacterial	Fungal	Viral
Wild hedgehogs	*Anaplasma phagocytophilum* *Borrelia burgdorferi sensu lato* *Borrelia miyamotoi* *Coxiella burnetii* *Rickettsia helvetica* *Leptospira interrogans* *Leptospira ballum* *Leptospira borgpetersenii* *Mycobacterium avium* ssp. *paratuberculosis* *Mycobacterium bovis* *Staphylococcus aureus* *Staphylococcus aureus (MRSA)* *Salmonella* Enteritidis* *Salmonella* Typhimurium* *Streptococcus pyogenes*	*Candida albicans* *Trichophyton erinacei**	TBEV SFTSV
Pet hedgehogs	*Corynebacterium* sp. *Mycobacterium marinum* *Salmonella* Stanley* *Salmonella tilene** *Streptococcus dysgalactiae*	*Candida albicans* *Microsporum* spp.* *Trichophyton erinacei**	

*Most common zoonoses

Tick-Borne Encephalitis Virus (TBEV); Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)

Although rare in hedgehogs, some mycobacterial organisms are reported in these species. *Mycobacterium* spp. are rod-shaped, aerobic, acid-fast, non-spore-forming bacteria and their ecology varies depending on the species (Blume et al., 2021). As preys and scavengers, hedgehogs can become infected with *Mycobacterium* spp. in a variety of ways like contamination of skin wounds, oral and aerosol transmission, which makes them an important element in the epidemiology of the infections caused by these bacteria (Blume et al., 2021; Ruszkowski et al., 2021). In aerosol or oral infection, mycobacteriosis in hedgehogs is usually systemic and the most affected organs are the cervical, lung, subcutaneous, spleen, liver and heart lymph nodes. Typical histological findings consist

of necrotizing granulomatous lesions composed by foamy and epithelioid macrophages, multinucleated giant cells and numerous intracytoplasmic acid-fast bacilli (Blume et al., 2021).

1.4 Common diseases

Among mammals, hedgehogs are one of the most admitted species in wildlife rehabilitation centers, sanctuaries and veterinary hospitals and the main reasons include skin, respiratory and gastrointestinal diseases, malnutrition, hypothermia and traumatic injuries. However, they are resilient animals that are relatively easy to keep in captivity, recover well, and when released back in the wild, adapt and survive (Garcês et al., 2020).

Acariasis has been reported in hedgehogs as a major health problem worldwide. Hedgehogs are commonly infested by mites belonging to the genera *Sarcoptes*, *Notoedres*, *Otodectes*, *Chorioptes* and *Caparinia*, sometimes with concurrent diseases such as dermatophyte infection (Keeble & Koterwas, 2020).

Alimentary common diseases include dental problems, enteritis by salmonella, candidiasis (*Candida albicans*) and cryptosporidiosis (Carpenter & Lindemann, 2015).

Lungworms are a cause of pneumonia and *Pasteurella* spp. and *Bordetella bronchiseptica* can cause respiratory infections in European hedgehogs, possibly also important in African pygmy hedgehog. Other common diseases include hepatic lipidosis and dilated cardiomyopathy, a common *post mortem* finding with an incidence of 38% in captive African hedgehogs, and renal disease with 50% prevalence in a necropsy survey (Carpenter & Lindemann, 2015).

Wobbly hedgehog syndrome (WHS) is an insidious, progressive and incurable neurologic process that is a leading cause of neurologic disease in African pygmy hedgehogs affecting 10% of these in North America (Graesser et al., 2006). A similar disease has been reportedin European hedgehogs. The etiology of WHS is unknown, but an inherited condition is suspected (Díaz-Delgado et al., 2018; Graesser et al., 2006). At an early stage, hedgehogs with WHS have mild ataxia, showing lack of coordination, becoming off balance, stumbling, tripping or wobbling. Paralysis is commonly ascending from hindlimbs to forelimbs and over the months the signs tend to become progressively more severe, including falling consistently to one side, tremors, exophthalmos, scoliosis, seizures, muscle atrophy, self-mutilation, and difficulty in regulating body temperature. Dysphagia and the inability to prehend food can cause weight loss in terminal stages of the disease (Graesser et al., 2006). According to Graesser et al. (2006), disease progression is variable, with 60% of cases immobilized within 9 months after onset of ataxia, and 90% after 15 months.

Other reported causes of progressive paralysis in hedgehogs include brain tumors, intervertebral disc disease and hepatic encephalopathy, but definitive diagnosis of WHS can only be made by *post mortem* histopathological examination of the CNS (Central nervous system) (Graesser et al, 2006). WHS is described as a "spongy myelinopathy", which microscopic hallmark is vacuolization of the white matter of the CNS, typically bilateral and symmetrical, with degeneration and loss of myelin, affecting the cerebellum, medulla oblongata and cervical and thoracic spinal cord. Spongiosis, axonal edema, and microgliosis are other common histological findings (Díaz-Delgado et al., 2018).

Neoplasia is an extremely common pathology, representing the most widely reported disease in hedgehogs, most notably in the African pygmy hedgehog (Johnson, 2020). In a recent study from 2012 to 2017, 100 African pygmy hedgehogs were examined, and neoplastic lesions were detected in 60%, with 74.6% ofthese tumors being classified as malignant, which is consistent with previous studies (Okada et al., 2018).

Oral squamous cell carcinoma (SCC) is highly reported in hedgehogs. Hedgehogs with this tumor usually have an oral that invades the oral soft tissues and bones and may become visible externally as a mandibular and maxillary swelling (Juan-Sallés& Garner, 2007). Histologically, the lesions consist of moderate-to-marked epithelial hyperplasia. Neoplastic squamous cells are arranged in nests or islands, often polyhedral to elongated with abundant cytoplasm and perinuclear clear vacuoles. Anisocytosis, anisokaryosis, acantholysis and nuclear pleomorphism are usually moderate or high and mitosis can be frequent (Del Aguila et al., 2019; Juan-Sallés & Garner, 2007). In hedgehogs, oral SCC is slow to metastasize, usually metastasizing to the lungs. Macroscopically and radiographically, it may resemble bacterial mandibular osteomyelitis (Juan-Sallés & Garner, 2007). As this species is prone to develop oral lesions, it is important to differentiate this neoplasm from common non-neoplastic lesions such as periodontal abscesses and gingival hyperplasia (Del Aguila et al., 2019).

Table A1 summarizes some common diseases in hedgehogs.

2. Aims and Objectives

Some retrospective studies describing the most common diseases of these species are available. However, the description of diseases and occurrence might depend on specific geographical considerations where these studies took place.

Hedgehogs are increasingly becoming pets and playing an important role in society. They are considered companion animals, contributing to the physical, social and emotional development of their owners. However, knowledge of potential transmissible diseases through these animals by pet owners and non-veterinary health professionals is limited (Riley & Chomel, 2005).

Likewise, wild hedgehogs like European hedgehogs have become regular in cities and people end up taking them home or to wildlife rehabilitation centers to rescue them (Ruszkowski et al., 2021). These behaviors end up increasing humans' exposure to potential zoonotic agents. The relationship between humans and hedgehogs does not only entail risks for humans, since due to their preference for inhabiting urban areas, wild hedgehogs are subject to a greater risk of anthropogenic source, such as traps, roadkill and poisons (Garcês et al., 2020).

As most of the pathologies that affect hedgehogs are diagnosed *post mortem*, it is essential to share knowledge between clinicians and pathologists, in order to recognize predisposing diseases and establish appropriate diagnostic and therapeutic plans. Additionally, necropsy acts as a surveillance tool to monitor for emerging or foreign animal diseases with potential public health impact.

Therefore, the aim of this study was to describe the most relevant macroscopic and microscopic findings identified during the necropsy examination of a group of rescued hedgehogs, performed during a period of 4 months.

3. Materials and Methods

3.1 Study design

Twelve rescued hedgehogs, namely five African pygmy hedgehogs (*Atelerix albiventris*) and seven European hedgehogs (*Erinaceus europaeus*), composed of eight female and four male animals, were submitted to necropsy examination at the Veterinary Pathology Laboratory of ICBAS-UP, between February and June 2021. These animals were rescued by the association *Amigos Picudos* and died of natural causes or were euthanized. It is important to note that no animal was euthanized for the purpose of this study. Further details about the study group are compiled in Table 3.

3.2 Histopathology and Histochemistry

The necropsy examination was performed and representative samples of tissues, with or without relevant macroscopic alterations were collected and fixed in 10% neutral

buffered formalin for microscopical examination. Samples taken from most animals include respiratory, gastrointestinal and urogenital tract, liver, spleen, and heart. Given the presentation of neurological alterations, in cases 1, 10 and 12, the CNS was also collected. Tissue samples were routinely processed and paraffin-embedded and 2µm thick serial sections were cut and stained with hematoxylin and eosin (HE). Ancillary histochemical stains were performed whenever necessary (Table 4).

3.3 Immunohistochemistry

According to the case and purpose, immunohistochemistry (IHC) was performed applying a panel of specific antibodies for different antigens (Table 2). Novolink Max-Polymer detection system (Novocastra) was used according to the manufacturer's instructions.

Table 2. Antibodies used for immunohistochemical study.

Marker	Clone	Supplier	Dilution	Antigen UM	IT	Positive control	Case applied
Thyroglobulin	Polyclonal	Dako, Denmark	1:2000	RS/WB	ON	Hedgehog normal thyroid	3
TSH	4C1	Bio-Rad, USA	1:450	RS/WB	ON	Hedgehog normal thyroid	3
TPO	MoAb47	Abcam, UK	1:20	EDTA pH9/WB	ON	Hedgehog normal thyroid	3
NSE	VI-H14	Imgenex, USA	1:500	RS/WB	ON	Canine pancreas	3
AE1/AE3	Polyclonal	Thermo, USA	1:300	RS/WB	ON	Canine uterus and internal control	3, 11
c-Kit	Polyclonal	Dako, Denmark	1:450	RS/WB	ON	Canine mast cell tumor	11
MUC-1	MRQ-17	Cell Marque, USA	ready to use	RS/WB	ON	Canine mammary gland	11
Synaptophysin	SP11	Thermo, USA	1:150	RS/WB	ON	Canine pancreas	3

TSH: thyroid stimulating hormone; NSE: neuron-specific enolase; MUC-1: mucin 1, TPO: thyroperoxidase
Antigen UM: antigen unmasking; RS: Retrieval solution; WB: water bath; IT: incubation time; ON: overnight.

3.4 Additional diagnostic tests

Extraction, Polymerase chain reaction (PCR) amplification and sequencing of DNA

In cases 1, 5 and 6, DNA was extracted from 5 consecutive slices of 20 µM paraffin-embedded lung tissues, using a DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. For cases 5 and 6, partial fragments of mitochondrial 12S rRNA (330 bp) and nuclear 18S rRNA (1700 bp) genes were amplified using two sets of primers (12SF: 5'-CGGGAGTAAAGTTTTGTTTAAACCG-3' and 12SR: 5'-ATTGACGGATGGTTTGTACCAC-3'; NC18SF1: 5'-AAAGATTAAGCCATGCA-3' and NC5BR: 5'-GCAGGTTCACCTACAGAT-3', respectively). The PCR amplification was performed using KAPA Taq DNA polymerase (Kapabiosystems, Massachusetts, USA). Genomic fragments were amplified using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 60 s; 55 °C for 60 s, 72 °C for 60 s; and a final extension at 72 °C for 7 min, as previously described (Barradas et al., 2020). The amplicons were purified (GRS PCR & Gel band purification kit, Grisp, Portugal) and bidirectionally sequenced, using the same primers as for PCR, employing the BigDye ® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems] in an automated sequencer (3130XL Genetic Analyzer, Applied Biosystems), available at IPATIMUP.

In case 1, real-time PCR was performed in Institute for Research and Innovation in Health (i3S), which allowed the exclusion of *Mycobacterium tuberculosis* and *Mycobacterium africanum*.

4. Results

Based on the macroscopic and histological findings of the examined samples, results were grouped according to the different systems in which most significant changes were verified, being subclassified as inflammatory, degenerative, neoplastic and traumatic alterations.

Details about the study group, such as species, sex, age, body weight and main pathological findings of each animal, are compiled in the Table 3.

Table 3. Detailed information about the animals studied.

Case	Species	Sex	Age (years)	Body weight (g)	Main pathological findings
1	African pygmy hedgehog (*Atelerix albiventris*)	F	2	245	Disseminated granulomatous inflammation (*Mycobacterium* spp.); Wobbly hedgehog syndrome
2	European hedgehog (*Erinaceus europaeus*)	M	<1	566	Mycotic pneumonia; Mycotic hepatitis and peritonitis
3	African pygmy hedgehog (*Atelerix albiventris*)	F	>3	336	Oral squamous cell carcinoma; Thyroid carcinoma with splenic metastasis; pyogranulomatous hepatitis;
4	European hedgehog (*Erinaceus europaeus*)	F	>3	398	Fracture of the right zygomatic arch with entrapment and fusion of the right arch of the mandible
5	European hedgehog (*Erinaceus europaeus*)	M	>3	500	Parasitic bronchopneumonia; Hepatic steatosis
6	European hedgehog (*Erinaceus europaeus*)	M	<1	204	Parasitic bronchopneumonia; Subacute interstitial hepatitis; Chronic interstitial nephritis
7	European hedgehog (*Erinaceus europaeus*)	F	<1	489	Chronic interstitial pneumonia; Subacute hepatitis with cholestasis and intrahepatic hemosiderosis
8	African pygmy hedgehog (*Atelerix albiventris*)	F	_	_	Chronic gingivostomatitis
9	European hedgehog (*Erinaceus europaeus*)	F	_	500	Subacute interstitial pneumonia; Chronic periportal hepatitis;
10	European hedgehog (*Erinaceus europaeus*)	F	_	680	Chronic pneumonia and pulmonary edema; Subacute interstitial hepatitis
11	African pygmy hedgehog (*Atelerix albiventris*)	F	5	625	Lipoid pneumonia; Hepatic steatosis; Cholangiocarcinoma; Subcutaneous mast cell tumor; Ovarian fibroma
12	African pygmy hedgehog (*Atelerix albiventris*)	M	_	240	Metastatic renal calcification; glomerulosclerosis and glomerulonephritis; Subacute interstitial hepatitis with cholestasis and intrahepatic hemosiderosis; Central nervous system demyelination

M: male; F: female

Table 4. Additional histochemical stains performed.

Case	Histochemical stain	Structure identification	Tissue	Result
1	Ziehl-Neelsen	*Mycobacterium* spp. (acid-fast bacilli)	Lung; Spleen; Liver; Kidney	+
	Luxol fast blue	Demyelination	CNS	+
2	Periodic acid–Schiff	Fungal hyphae	Lung; Liver	+
	Grocott methenamine silver	Fungal microorganisms	Lung; Liver	+
	Gram stain	Bacteria	Lung; Liver	-
	Fite Faraco staining	Acid-fast bacilli	Lung; Liver	-
	Ziehl-Neelsen	*Mycobacterium* spp. (acid-fast bacilli)	Lung; Liver	-
3	Ziehl-Neelsen	*Mycobacterium* spp. (acid-fast bacilli)	Liver	-
	Congo red	Amyloids	Cervical mass	-
7	Perls Prussian Blue	Hemosiderin	Liver	+
	Hall's Bilirubin Stain	Bilirubin	Liver	+
	Periodic acid–Schiff	Fungal hyphae	Lung	-
	Gram stain	Bacteria	Lung	-
9	Gram stain	Bacteria	Lung	-
	Periodic acid–Schiff	Fungal hyphae	Lung	-
	Ziehl-Neelsen	*Mycobacterium* spp. (acid-fast bacilli)	Lung; Spleen	-
	Gomori trichrome	Connective tissue (muscle)	Lung	+
10	Gram stain	Bacteria	CNS	-
	Grocott methenamine silver	Fungal microorganisms	CNS	-
11	Grocott methenamine silver	Fungal microorganisms	Lung	-
	Periodic acid–Schiff	Fungal hyphae	Lung	-
		Glycogen	Liver	+
	Gram stain	Bacteria	Lung	-
	Giemsa stain	Mast cells	Cervical nodule	+
	Masson's trichrome stain	Connective tissue (fibrosis)	Ovary	+
		Connective tissue (fibrosis)	Liver	+
12	Luxol fast blue	Demyelination	CNS	+
	Perls Prussian Blue	Hemosiderin	Liver	+
	Hall's Bilirubin Stain	Bilirubin	Liver	+

4.1 Skin and subcutaneous tissue

In seven of the 12 necropsied animals it was possible to observe ectoparasites, such as ticks, fleas and maggots.

Neoplasia: In case 11, microscopic analysis of a subcutaneous cervical nodule measuring 2.0 cm in diameter showed a nodular neoplastic lesion with well-defined borders, consisting of pleomorphic cells with numerous basophilic and Giemsa positive intracytoplasmic granules, was diagnosed as a subcutaneous mast cell tumor (MCT) (Figure 1). Positive immunoreactivity for c-Kit allowed the confirmation of this diagnosis (Figure1b).

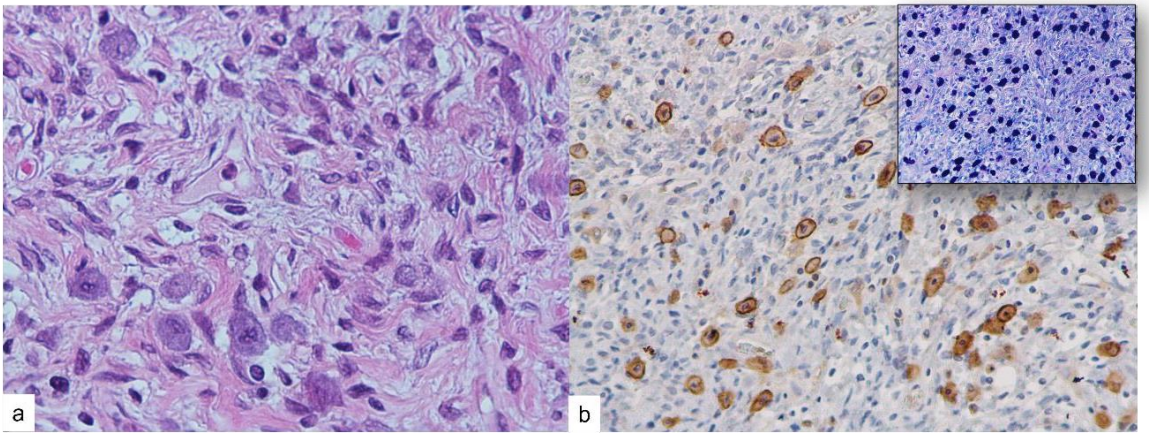


Figure 1. Hedgehog, subcutaneous tissue. Case 11, Mast cell tumor. **a**. Presence of isolated round cells of variable size, supported by abundant collagenous stroma. Cells show high pleomorphism and variable amount of intracytoplasmic basophilic granules. (HE, 100x) **b.** C-kit immunostaining revealed strong and both membranous and cytoplasmic immunopositivity of neoplastic mast cells. Inset: neoplastic mast cells with metachromatic and Giemsa-positive cytoplasmic granules (Giemsa, 200x).

4.2 Respiratory tract

Inflammation: In the present study seven animals were diagnosed with pneumonia. Histological examination allowed the detection of granulomatous and pyogranulomatous pneumonia (cases 1 and 2, respectively), parasitic bronchopneumonia (cases 5 and 6), subacute interstitial pneumonia (case 9), chronic interstitial pneumonia (case 10) and lipoid pneumonia (case 11).

In case 1, macroscopic examination of the lungs revealed the presence of about 12 yellowish-white, rounded and protruding masses, measuring approximately 1-5 mm in diameter disseminated through the organ. Histological examination revealed a chronic granulomatous pneumonia affecting 98% of the pulmonary parenchyma with the presence of multiple to coalescent centers of caseous necrosis surrounded by macrophages, multinucleated giant cells (Langhans cells) and lymphocytes. These were subjected to Ziehl-Neelsen staining which allowed the identification of several intracytoplasmic acid-fast bacilli, located mainly inside macrophages, potentially belonging to the genus *Mycobacterium* spp. Similar lesions were also detected in the kidney, liver and spleen.

In another hedgehog with pyogranulomatous pneumonia (case 2), fungal structures (PAS and Grocott positive) were identified in the lung parenchyma and within vascular structures. These were associated with abundant mixed inflammatory infiltrate, peribronchiolar fibrosis and bronchial epithelial hyperplasia.

Parasitic bronchopneumonia was identified in two cases (cases 5 and 6), which histological examination revealed the presence of transverse and tangential sections of worms in the lumen of the bronchi, bronchioles, and occasionally inside alveolae, surrounded by mixed inflammatory cell infiltrate (Figure 2a-b). In both cases, marked epithelial hyperplasia and peribronchiolar fibrosis were noticed. The spiculated morphology of these worms suggests a nematode parasite. An attempt was made to identify these parasites through conventional PCR. Although a compatible band with appropriate molecular weight was visualized through electrophoresis, after sequencing it was not possible to reach a consistent result.

Cases 9 and 10 presented subacute and chronic interstitial pneumonias, respectively, both associated with peribronchiolar fibrosis (Figure 2c), pulmonary edema and hemorrhage.

Macroscopically, case 11 showed marked pulmonary alterations with lungs displaying compact, red and white marbled color and areas of compensatory emphysema at the periphery of the lobes. Microscopically, the organ was almost completely filled by numerous lipid droplets forming a collection of foamy macrophages that were responsible for pushing the nuclei to the cell periphery, associated with an intense mixed inflammatory infiltrate (Figure 2d).

4.3 Gastrointestinal tract

Oral cavity

Inflammation: Active chronic gingivostomatitis was detected after analysis of an oral mass measuring 0.7 cm in diameter (case 8).

Neoplasia: In case 3, an irregular mass composed of whitish tissues of hard consistency and measuring 2.0 cm was detected in the right maxilla and projecting into the palate (Figure 3a). Microscopically, an infiltrative neoplastic lesion consisting in proliferation of epithelial cells arranged in nests and exhibiting central squamous differentiation was observed (Figure 3b). The neoplastic cells presented high cellular pleomorphism and low mitotic index (two mitotic figures per 10 high-power field (HPF)).

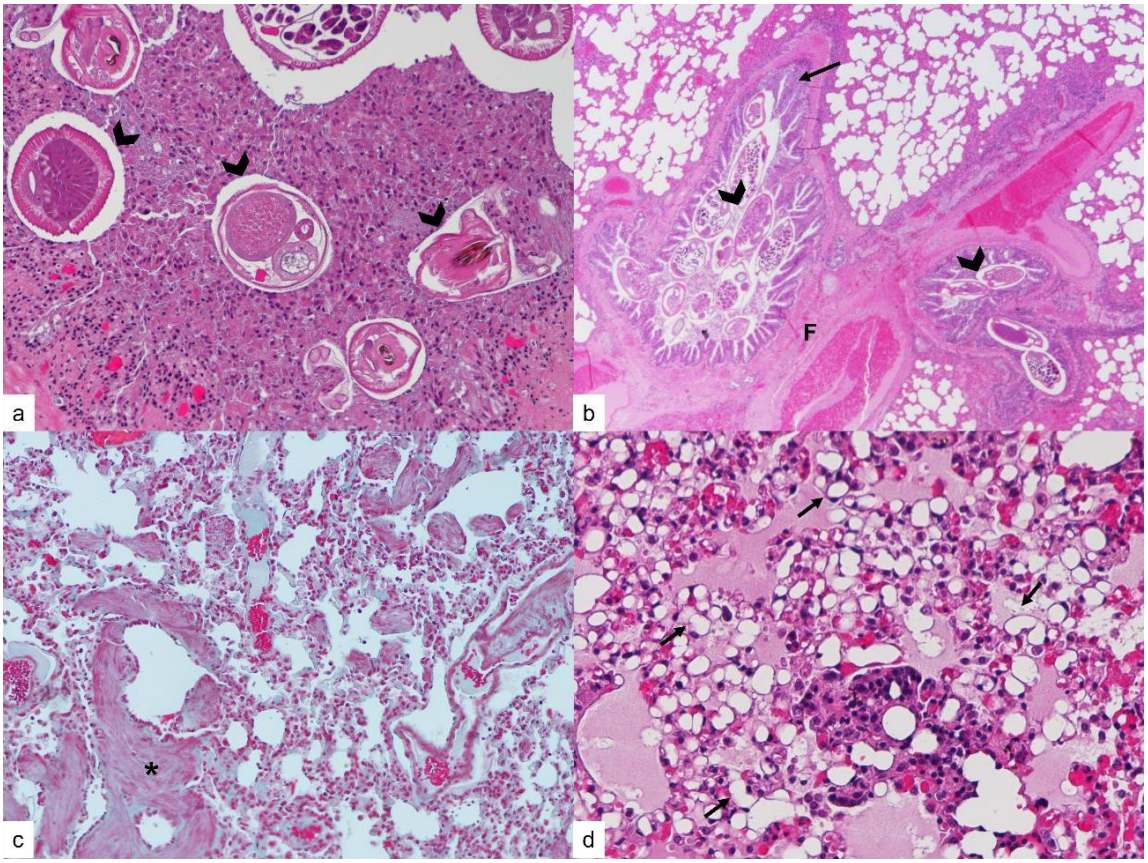


Figure 2. Hedgehog, lung. **a**. Case 5, Parasitic pneumonia. Presence of worms (arrowhead) within the lung parenchyma, associated with mixed inflammatory infiltrate (HE, 100x). **b**. Case 6, Parasitic pneumonia. Presence of several worms (arrowhead) inside the lumen of bronchioles, associated with hyperplasia of the bronchiolar epithelium (arrow) and peribronchiolar fibrosis (F) (HE, 20x). **c.** Case 9, smooth muscle hyperplasia (*) (pink stained) adjacent to the bronchiolar epithelium (Gomori trichome, 100x). **d.** Case 11, Lipoid pneumonia. Intra-alveolar and interstitial accumulation of lipid-laden macrophages (arrows) (HE, 200x).

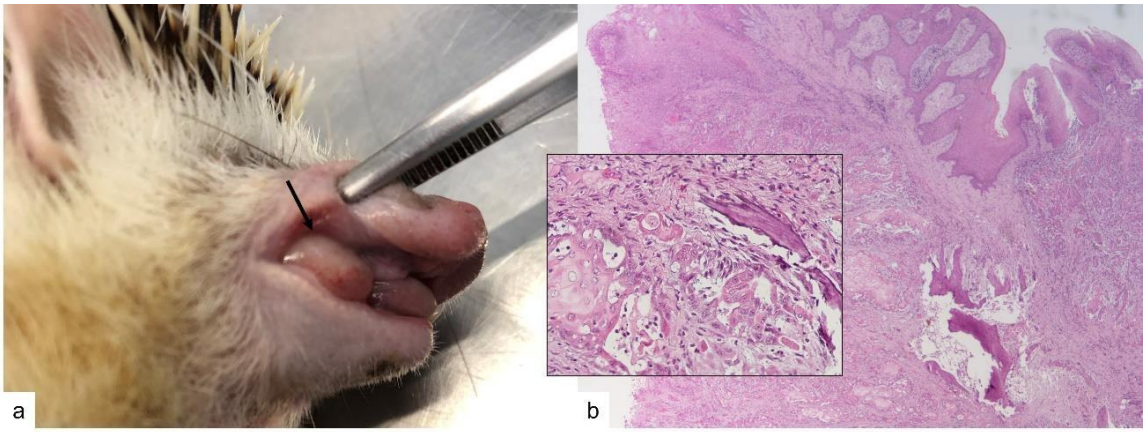


Figure 3. Hedgehog, oral cavity. Case 3, Squamous cell carcinoma. **a.** Gross appearance of the oral infiltrating and protruding mass in the right maxilla. **b.** The mass consisted in neoplastic proliferation of epithelial cells organized in nests. The neoplastic lesion originates from the superficial epithelium and infiltrates the underlying layers, including bone (HE, 20x). Inset: note the atypia of the neoplastic cells and the squamous cell differentiation foci (HE, 200x).

Liver

Inflammation: From the inflammatory lesions, we highlight cases 1 and 3 with granulomatous and pyogranulomatous hepatitis, respectively, case 2 with fibrinopurulent hepatitis, cases 6, 7, 10 and 12 with subacute hepatitis and case 9 with chronic hepatitis.

In case 1, although macroscopically the liver did not show relevant alterations, the histological examination revealed granulomatous lesions identical to those described in the lung. Again, Ziehl-Neelsen was performed allowing the clear detection of acid-fast bacilli compatible with *Mycobacterium* spp.

In case 3, microscopic examination of the liver allowed the visualization of multiple foci of pyogranulomatous reaction with neutrophils, eosinophils, lymphocytes, plasma cells and some multinucleated giant cells. Ziehl-Neelsen staining was performed to try to identify possible infectious agents, which proved to be negative.

Mycotic fibrinopurulent hepatitis was observed in case 2 after histological examination of a liver with an intra-abdominal abscess involving its right lobe. Fungal structures (PAS and Grocott positive) were visualized within foci of an inflammatory reaction, containing degenerated neutrophils supported by a dense fibrin network, associated with severe hyperplasia of the bile ducts (Figure 4a-c).

Cases 6, 7, 10 and 12 presented subacute interstitial hepatitis with hepatocyte swelling and perivascular fibrosis. Moderate bile duct hyperplasia (case 6), intrahepatic cholestasis and hemosiderosis (cases 7 and 12) were also observed.

Degenerative: Degenerative liver lesions were observed in cases 5 and 11, both with hepatic steatosis. Hepatomegaly and nutmeg pattern were relevant gross findings in the liver of case 11 (Figure 4d). In both cases, macrovacuolar hepatocellular degeneration and cellular swelling were observed, however, it is more evident in case 11, where the lesions completely distort the liver's architecture.

Neoplasia: A tumor of the biliary system was diagnosed in this last case (case 11) as hepatic cholangiocarcinoma (CC). Histologically, tubular or acinar arrangements of epithelial neoplastic cells, often centered in periportal areas and associated with intense fibrosis and hepatic hemorrhage, were observed. Neoplastic cells consisted of cubic and cylindrical cells with homogenous and sometimes granular eosinophilic cytoplasm, resembling bile duct epitheliums and PAS-positive amorphous material was presence in the lumen of neoplastic acinar and tubular structures (Figure 4e-g).

Megakaryocytes suggestive of extramedullary hematopoiesis were a relatively common microscopic finding in the liver of some animals (cases 7, 11 and 12).

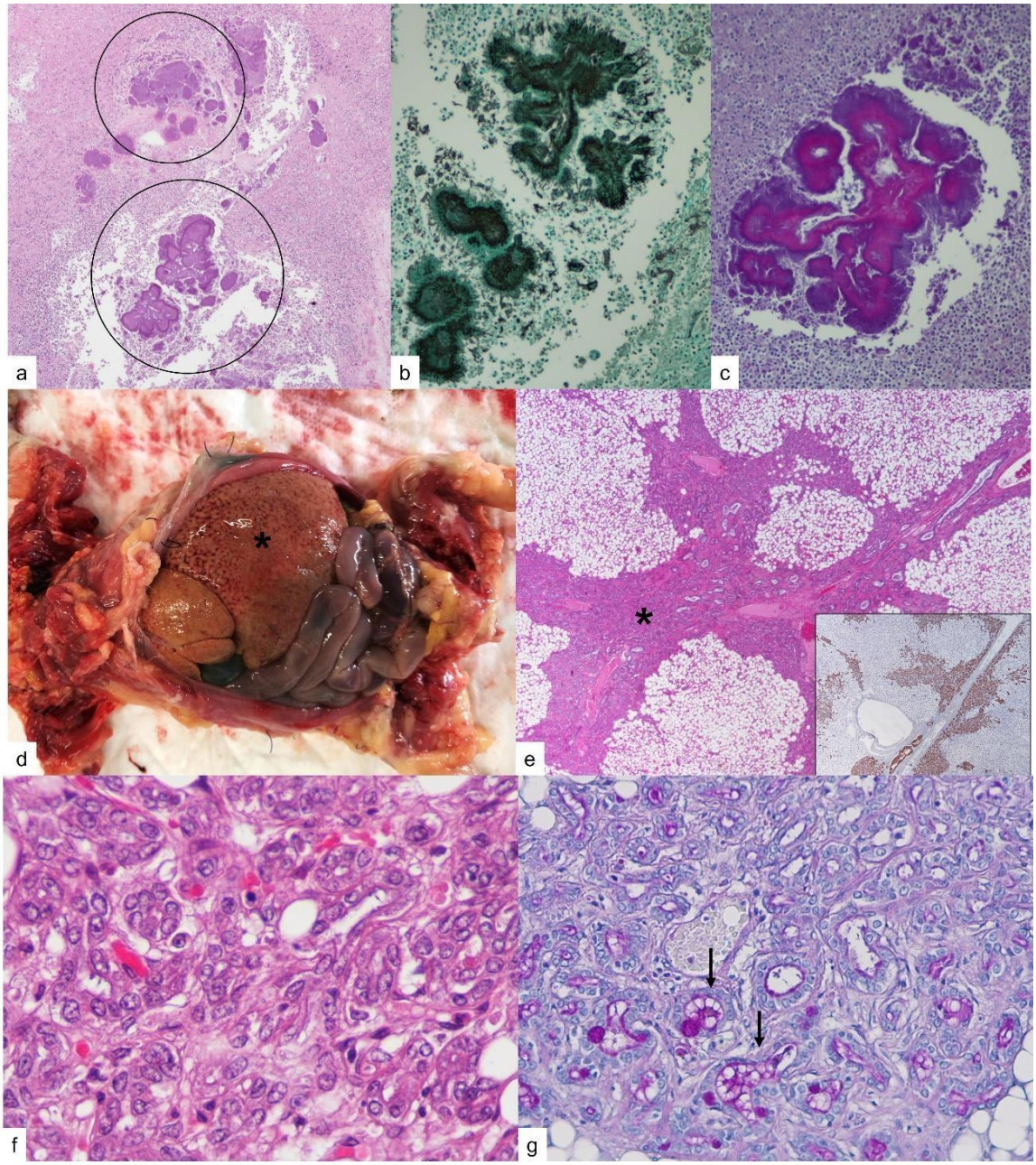


Figure 4. Hedgehog, liver. **a.** Case 2, Mycotic hepatitis. Severe purulent exudate surrounding numerous fungal structures (circle) (HE, 40x). **b.** and **c.** These hyphae were Grocott-positive and PAS-positive, respectively (100x). **d.** Case 11, Cholangiocarcinoma. Macroscopic image of the abdominal cavity demonstrating hepatomegaly (*) and the nutmeg pattern of the liver. **e.** Case 11, Cholangiocarcinoma Microscopic appearance of the organ, showing diffuse steatosis and irregular and multifocal proliferation of neoplastic epithelial cells (*) centered in periportal spaces (cholangiocarcinoma) (HE, 20x). Inset: Neoplastic cells showed AE1/AE3 immunoreactivity, confirming the epithelial histogenesis of the lesion (20x). **f.** Case 11, Cholangiocarcinoma Note the tubular arrangement and the polyhedric shape of neoplastic cells, showing loss of polarity and a large, clear and pleomorphic nuclei (HE, 400x). **g.** Case 11, Cholangiocarcinoma. Some of the tubular structures present PAS-positive secretion lining the apical membrane of neoplastic cells (arrows) (PAS, 200x).

4.4 Urinary system

Inflammation: Kidney lesions were found in three animals. In case 1, the presence of about 9 multifocal round masses measuring <1 mm in diameter was macroscopically observed. Histologically, the same granulomatous lesions that were seen in the lung and liver of this animal were detected in the kidney (Figure 5).

A chronic interstitial nephritis was presented by one animal (case 6) with the presence of a mononuclear inflammatory infiltrate and case 9 presented multifocal metastatic calcification, discrete glomerulosclerosis and glomerulonephritis, and hyaline cysts.

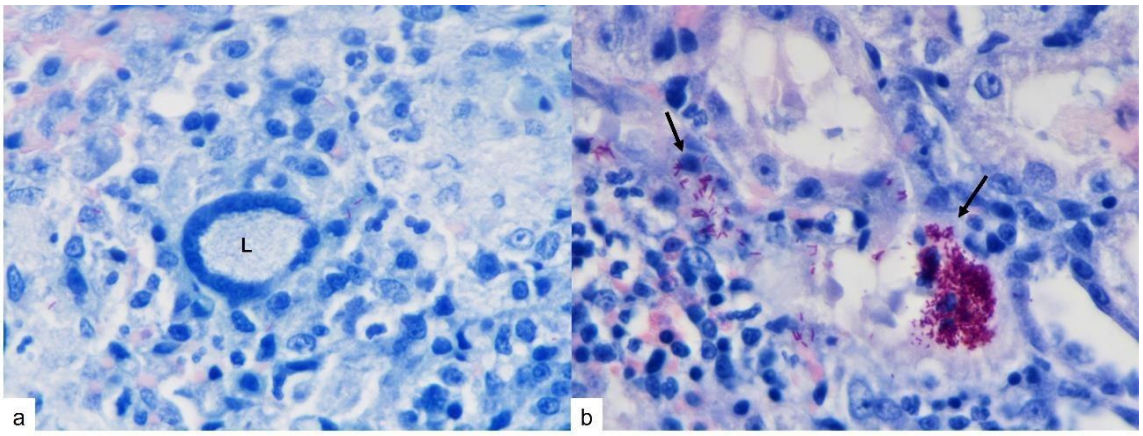


Figure 5. Hedgehog, kidney. Case 1, *Mycobacterium* spp. (Ziehl-Neelsen, 600x). **a.** Detail of the granuloma lesion, showing a multinucleated giant cell composed of several nuclei at the cell periphery (Langhans giant cell) (L). **b.** Presence of numerous acid-fast bacilli in the cytoplasm of epithelioid cells (arrows).

4.5 Endocrine organs

Neoplasia: The macroscopic examination of case 3 allowed the visualization of a bilobed cervical mass measuring 1.5 cm, with white tissues and well-defined limits, located adjacent to the thyroid (Figure 6a). Subsequent histological analysis detected a multinodular neoplastic lesion replacing almost 90% of the organ and involving both thyroid lobes, compatible with solid carcinoma with probable origin in the thyroid. The lesion is constituted by a population of epithelial cells, forming solid groups and supported with scarce extracellular matrix (Figure 6b-d). Neoplastic cells presented large and clear nucleus, showing moderate to high pleomorphism and a finely vacuolated acidophilic cytoplasm. Mitoses reach 4 mitosis figures per 10 HPF. Histochemical Congo red staining was evaluated through polarized light but no signs of amyloid were detected. Immunohistochemical results are summarized in Table 5.

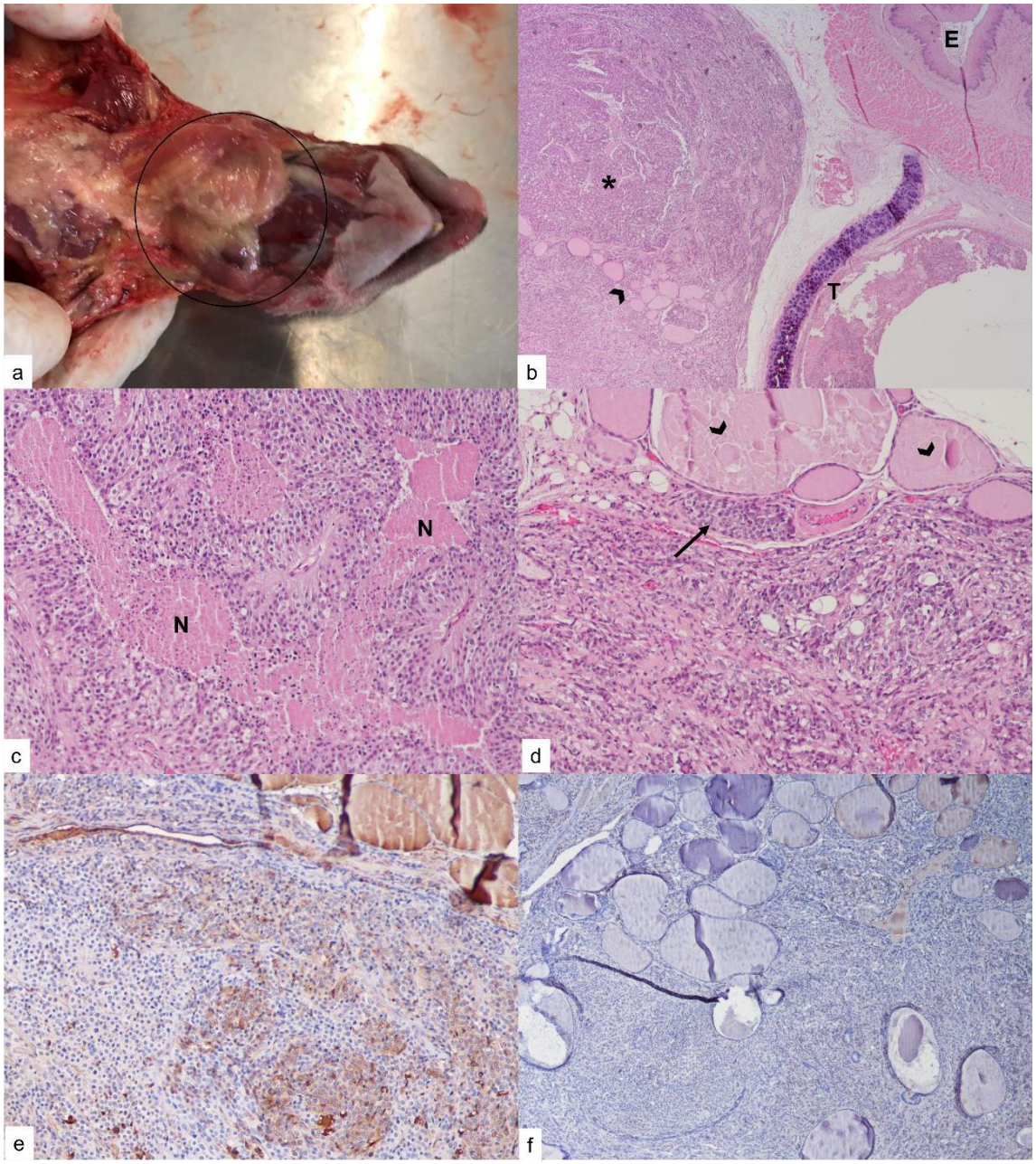


Figure 6. Hedgehog, thyroid. Case 3, Carcinoma. **a.** Gross appearance of the bilateral and multilobulated cervical mass completely involving the organ (circle). **b.** The lesion is composed of neoplastic epithelial cells (*), forming a compact and solid arrangement with undefined and irregular borders that compress the adjacent structures: esophagus (**E**) and trachea (**T**) (HE, 20x). **c.** Multifocal areas of intratumoral necrosis (**N**) (HE, 100x). **d.** Note the foci of extracapsular tumor growth (arrow) and few remaining colloid follicles retained within the lesion (arrowhead) (HE, 100x) **e.** Considerable percentage of neoplastic cells present weak to moderate thyroglobulin-immunopositivity (100x). **f.** Low percentage of neoplastic cells present weak immunoreactivity to synaptophysin (100x).

Table 5. Immunohistochemical panel performed in thyroid neoplastic lesion.

Immunomarker	Results
Thyroglobulin	Mild to moderate and heterogenous cytoplasm immunostaining affecting 70% of the neoplastic population (Figure 6e).
TPO	Weak and generalized immunopositivity affecting 50-75% neoplastic cells.
Synaptophysin	Weak immunostaining in 30% of neoplastic cells, more evident in cells adjacent to the outer capsule (Figure 6f).
NSE	Intense and generalized cytoplasm immunostaining of all the neoplastic cells.

4.6 Lymphoreticular system

Spleen

Inflammation: The granulomatous lesions present in case 1 and compatible with bacteria belonging to the genus *Mycobacterium* spp. were also seen in the spleen.

Neoplasia: In case 3, five whitish nodular masses with well-defined limits measuring between 1.0-5.0 mm in diameter were macroscopically identified in the spleen (Figure 7a). Histologically, multifocal nodular neoplastic lesions were observed, with the cells appearing to be of epithelial origin (Figure 7b). IHC was performed for AE1/AE3 (Figure 7c), thyroglobulin (Figure 7d) and TSH and the result was positive.

Megakaryocytes suggestive of extramedullary hematopoiesis were a relatively common microscopic finding in the spleen of the studied animals.

4.7 Central nervous system

Degenerative: Case 1 was diagnosed with WHS, which histological examination of the brain and spinal cord presented neurodegenerative processes. Multifocal areas of myelin degeneration of the cerebellum and medulla oblongata, characterized by myelin loss, neuronal degeneration, microgliosis and spongiosis were observed (Figure 8a). Luxol fast blue staining allowed the visualization of myelin loss in the segments of the medulla oblongata (Figure 8b).

Case 12 also showed signs of CNS neuronal degeneration and Luxol fast blue staining showed slight myelin loss, however, without sufficient criteria to be diagnosed with WHS.

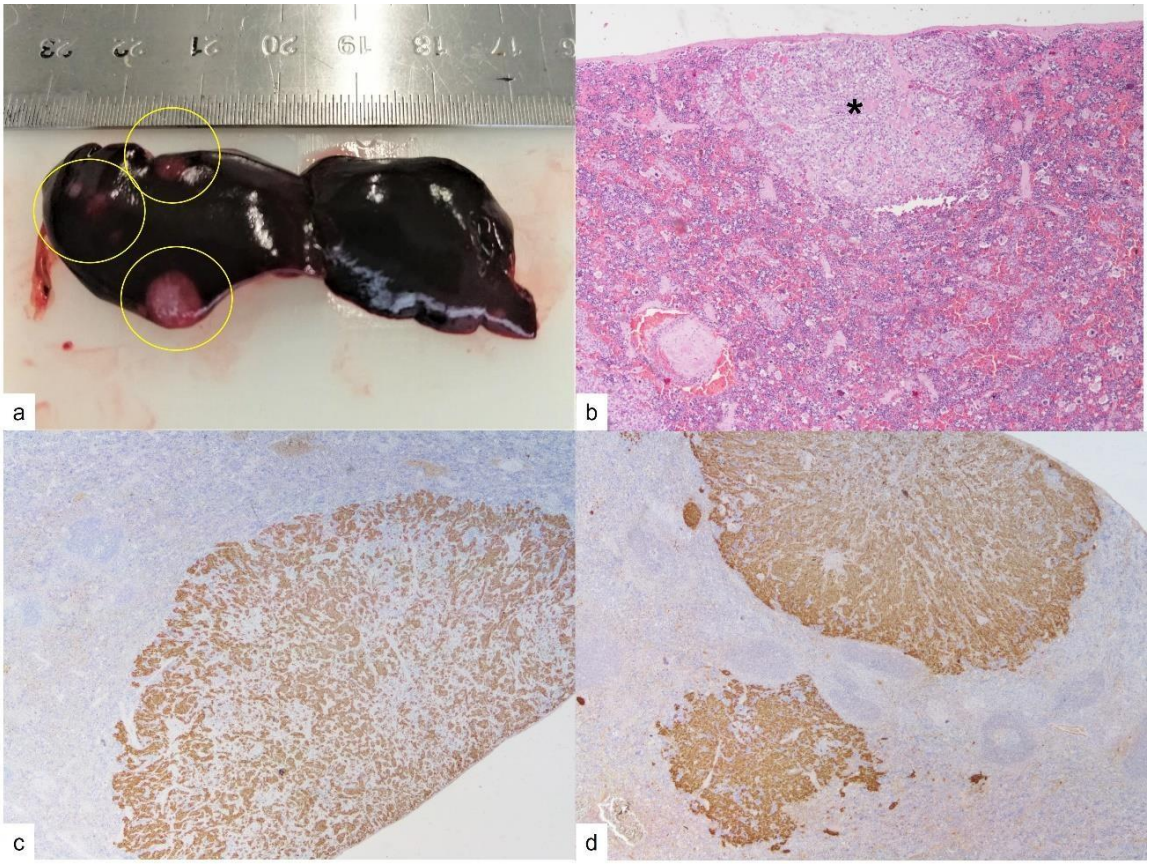


Figure 7. Hedgehog, spleen. Case 3, Metastasis from thyroid carcinoma. **a**. Presence of several, round, whitish and multifocal nodular masses, measuring 1.0-5.0 mm diameter (circle). **b.** Well-demarcated nodular and marginal nodular lesion (*****), composed of epithelial cells in a solid pattern (HE, 20x). **c.** and **d.** The great majority of neoplastic cells presented strong immunoreactivity for AE1/AE3 and thyroglobulin, respectively (20x).

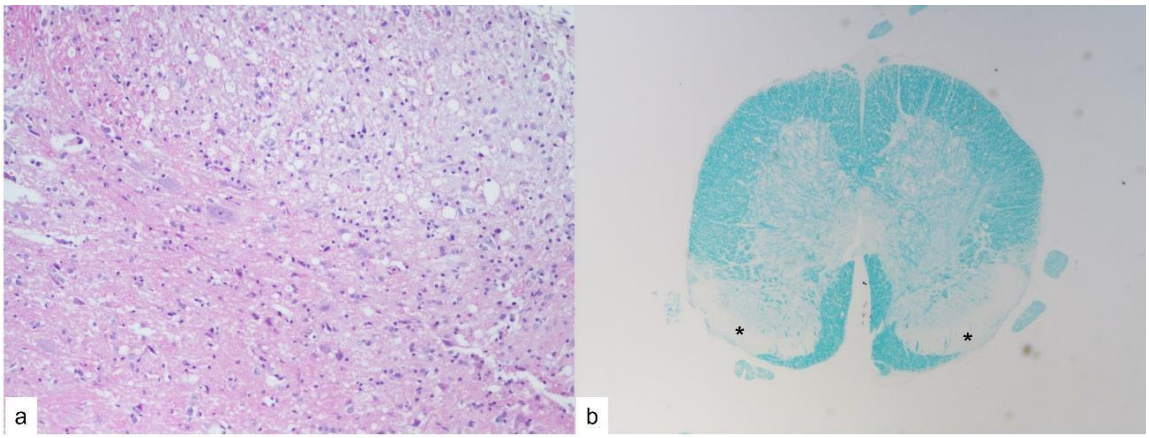


Figure 8. Hedgehog, CNS (Medulla). Case 1, Wobbly hedgehog syndrome. **a.** Note the area of white matter spongiosis (HE, 100x). **b.** Bilateral pale staining areas representing loss of myelin clearly evident in the lower tracts (*) (Luxol fast blue, 20x).

4.8 Skeletal and joints

Trauma: Temporomandibular joint immobility was detected in case 4. *Post mortem* radiographic examination showed a fracture of the right zygomatic arch (Figure 9), with consequent imprisonment of the right branch of the mandible.

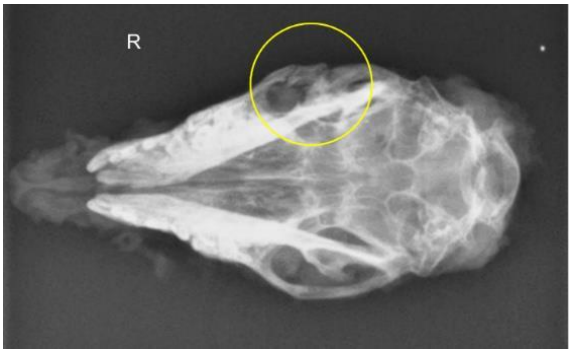


Figure 9. Hedgehog, skull. Case 4, fracture. Dorsoventral radiograph of the head evidencing a fracture of the right zygomatic arch.

4.9 Reproductive tract

Neoplasia: Only one animal (case 11) in the study was diagnosed with reproductive tract neoplasia. Macroscopic examination of the ovary allowed the observation of a multinodular mass measuring 4.0x4.0x1.5 cm, which on histological examination was diagnosed as ovarian fibroma. The multinodular neoplastic lesion has well-defined limits, moderate cell pleomorphism and a low mitotic index. Masson's Trichrome stain confirmed the fibrous nature of connective stroma.

5. Discussion

The present study aimed at analyzing the macro- and microscopic findings of a collection of 12 hedgehogs. In animals rescued from nature, there is a lack of information regarding their clinical history and their condition in nature. Furthermore, as wild animals, they are exposed to a panoply of potential pathological agents, which associated with prolonged time intervals between the death and the cadaver examination, make it difficult to objectively evaluate macro- and microscopic *post mortem* findings.

The results of this study showed that the liver and lung are the organs most affected. Indeed, literature considers hepatic lipidosis, as well as other causes of liver failure, such as primary and metastatic neoplasms, and respiratory diseases are very common in hedgehogs (Ivey & Carpenter, 2012; Johnson, 2011).

As with other small mammals, extramedullary hematopoiesis is frequent in hedgehogs and its etiology is undetermined, although in some cases, it may be related

with anemia and infection (Juan-Sallés & Garner, 2007). It usually involves the spleen causing splenomegaly; however, the herein images of megakaryocytes suggestive of extramedullary hematopoiesis were also found in the liver.

Concomitant disease is routinely found in *post mortem* examination of hedgehogs (Keeble & Koterwas, 2020), which is in line with the results of this study. Microscopically, all studied hedgehogs revealed alterations in one or more systems. Nevertheless, the discussion of this study will focus on uncommon or less common pathologies reported to date in these species. Thus, case 1 (diagnosed with *Mycobacterium* spp. and WHS), case 3 (diagnosed with oral SCC and thyroid carcinoma) and case 11 (diagnosed with hepatic CC, lipoid pneumonia and subcutaneous MCT) will be thoroughly addressed.

5.1 Case 1: *Mycobacterium* spp.; Wobbly hedgehog syndrome

Case 1 was a 2-year-old African pygmy hedgehog with a clinical history of ataxia lasting 6 months and lameness of the hind limbs. External examination of the cadaver revealed malnutrition and an ulcerated wound on the right forelimb. Macro- and microscopic findings consisted of multiple and white foci of necrotizing granulomatous lesions in the lungs, kidneys, liver and spleen, having the Ziehl-Neelsen stain allowed the recognition of multiple and intracytoplasmic bacilli compatible with *Mycobacterium* spp.

There are only single reports of *Mycobacterium tuberculosis* var. *bovis* which cause typical tuberculosis (Lugton et al., 1995) and *Mycobaterium avium* ssp. *paratuberculosis* a causative agent of paratuberculosis in ruminants, both in European hedgehogs (Nugent et al., 2011). Although *M. bovis* mainly affects the respiratory tract, small granulomas can also develop in the liver, kidneys and spleen. In 2014, a study conducted to determine the prevalence of *M. bovis* in Portugal did not detect the infection in hedgehogs (Matos et al., 2014).

Mycobacterium marinum is classified as an atypical non-tuberculous mycobacterium (NTM) and there are three reports of this mycobacteriosis: a cutaneous form is described in an African pygmy hedgehog (Blume et al.,2021) and two cases of systemic infection in a European and an African pygmy hedgehog (Nakamura et al., 2020; Tappe et al, 1983). *M. marinum* is ubiquitous in aquatic environments and causes a chronic progressive disease in various freshwater and saltwater fishes. In humans, it also causes cutaneous infection ("fish tank granuloma") through traumatic injuries associated with contaminated fish, fish tanks or swimming pools, but more severe lesions, including disseminated infection, particularly in immunosuppressed patients, have been reported

(Nakamura et al., 2020). In the reported case, the European hedgehog became infected with *M. marinum* through contact with water from a nearby fish tank, located in the same pet store (Tappe et al, 1983). In the herein case, molecular analysis would be indispensable for the specific etiologic agent species identification.

Although WHS can occur at any age, it affects most frequently hedgehogs with less than 2 years of age, as in this case (Graesser et al., 2006). Additionally, the neurological clinical signs displayed by this animal (ataxia and lameness of the hind limbs), along with the histopathological alterations detected in the CNS were compatible with WHS.

The bilateral and symmetrical vacuolization of the CNS white matter, associated with myelin degeneration and loss were confirmed through Luxol fast blue histochemical, which has proven to be an excellent method to aid this diagnosis. In this case, the neurodegenerative processes were more pronounced in the cerebellum and medulla oblongata. These alterations can be the cause of the lack of the animal's mobility and can further explain the cutaneous wound found in its right forelimb, a common finding in hedgehogs with WHS, as the result of the animal dragging its limbs due to the inability to fully extend them and loss of full range of motion (Graesser et al., 2006).

Hepatic and renal pathology seem to be incidental findings in hedgehogs with WHS (Graesser et al, 2006). In this case, these organs were severely affected with granulomatous lesions associated with *Mycobacterium* spp.

Some authors claim that WHS lesions resemble hepatic and renal encephalopathy lesions (Díaz-Delgado et al., 2018). Thus, a potential link between the granulomatous infection, liver and kidney failure, that could have led to encephalopathy and a histological image similar to WHS should also be considered.

The lack of knowledge of the specific *Mycobacterium* species herein prevents further inferences regarding the source and the pathogenic route of this infection. This hedgehog was kept as a pet, which suggests a controlled environment. However, when referring to zoonoses, a "controlled environment" is a highly critical concept. The ulcerated wound present in the right forelimb should also be considered a potential bacteria entrance point, with consequent dissemination to other organs. However, in transcutaneous infection, extensive necrosis of the inoculated skin areas is usually observed, with consequent edema of the respective limb and enlargement of regional lymph nodes, which was not observed in this case (Blume et al.,2021; Nakamura et al., 2020). On the other hand, WHS is a common pathology in African pygmy hedgehogs and a genetic predisposition to this disease is recognized in these animals (Díaz-Delgado et al., 2018). Thus, WHS disease progression may have led, to a certain point

and extension, to an immunodepression state that favored secondary *Mycobacterium* spp. infection and dissemination.

5.2 Case 3: Oral squamous cell carcinoma; Thyroid carcinoma

Case 3 was an African pygmy hedgehog with more than 3-years of age diagnosed with an oral SCC and thyroid carcinoma with splenic metastasis. Although both neoplasms are relatively common in hedgehogs, what makes this case interesting is the presence of two different concomitant malignant neoplasms.

The prevalence and clinical significance of masses within the thyroid gland vary widely across species. Humans usually have benign thyroid nodules. Likewise, they are also relatively common in older cats, most consisting in functional adenomatous hyperplasia, but malignant tumors are occasionally recognized. In contrast, thyroid neoplasms are rare in dogs and, when seen, they are likely malignant (Barber, 2007).

Thyroid carcinomas are usually large, solid, firm, irregular and non-painful masses that commonly invade adjacent structures. Case-reports describing these neoplasms in hedgehogs, often report associated clinical signs such as dysphagia, weight loss, polydipsia and tetraparesis (LaRue et al., 2016; Miller et al., 2002). In this case, the animal presented severe tachypnea, which could be due to compression of adjacent structures, given the large size of the mass.

Commonly, thyroid carcinomas originate from two distinct endocrine cell types (Scott-Moncrieff, 2015). Thyroid tumors of follicular cell origin arise from epithelial cells that line the colloid follicles. These cells are able to concentrate iodine and are involved in thyroid hormone production (Barber, 2007). Depending on their pattern of growth, thyroid tumors may be classified into follicular, compact (solid), papillary, compact-follicular, or undifferentiated (anaplastic) carcinomas (Scott-Moncrieff, 2015).

In addition, medullary thyroid carcinomas arise from the parafollicular C-cells, which produce calcitonin and are part of the amine precursor uptake decarboxylation (APUD) system (Scott-Moncrieff, 2015). Most thyroid tumors in humans, dogs and cats arise from the follicular epithelium. Medullary thyroid tumors are relatively rare, typically occurring in less than 10% of thyroid tumors in these species (Barber, 2007). In hedgehogs, the literature and reported cases of thyroid carcinomas is scarce. However, follicular adenocarcinoma and C-cell carcinoma have been previously reported in African pygmy hedgehogs (LaRue et al., 2016; Miller et al., 2002; Raymond & Garner, 2001).

In this case, the histological image is compatible with a solid carcinoma originating from thyroid follicular cells. However, the distinction between follicular or

medullary thyroid carcinoma through conventional microscopy is not clear (Carver et al., 1995). Thyroid tumors that arise from parafollicular C-cells often have a compact cell growth pattern, making it difficult to distinguish solid follicular tumors with similar histological appearance by routine light microscopy alone (Scott-Moncrieff, 2015).

The presence of amyloid interspersed with the tumor cells is a variable finding among species affected with C-cell carcinoma. In hedgehogs, amyloid material was not a major feature in the tumor (Miller et al., 2002), as in the present case, where no substance was detected using Congo red staining through polarized light.

Thyroid tumors derived from follicular cells routinely stain positively for thyroglobulin. In contrast, C-cell tumors have demonstrated strong immunoreactivity to calcitonin and more variable staining for synaptophysin (Barber, 2007). According to the immunohistochemical study performed, despite the heterogeneous labeling, a large part of the tumor was immunopositive for thyroglobulin. Regions with a higher number of mitosis presented lower immunoreactivity for this antibody, however, this may be due to loss of expression. Labeling for synaptophysin and TPO was weak and synaptophysin cover only a small percentage of the tumor. Nevertheless, the controls for NSE and synaptophysin immunomarkers are from canine tissue, requiring a period of validation and optimization, so the interpretation of results must be prudent.

Given the positive results for these cells' immunomarkers, along with the weak or non-existent immunoreactivity for the parafollicular cell markers, the results of this immunohistochemical panel suggest that the neoplastic lesion has a possible follicular origin.

Usually, these tumors are located close to the typical location of the normal thyroid and may be unilateral or as in this case, bilateral. Distant metastasis is common and may affect regional lymph nodes, lungs, jugular vein, liver, adrenal gland, kidneys, heart base, spleen, bone and bone marrow, prostate, brain, skeleton, and spinal cord (Scott-Moncrieff, 2015).

Macro- and microscopic examination of the spleen allowed the identification of multiple nodular lesions, with well-defined borders. The epithelial immunophenotype of the neoplastic cells that make up these splenic nodules, later confirmed through AE1/AE3 immunopositivity, makes it quite plausible to hypothesize that they constitute metastases of a primary neoplasm, which in this specific case include SCC and thyroid carcinoma. Primary spleen epithelial neoplasms are rare. In addition, the spleen is not a common site of metastases for SCC, which usually tends to metastasize to the lungs (Juan-Sallés & Garner, 2007). Histologically, splenic nodular lesions were composed of neoplastic cells displaying a phenotype identical to those constituting the thyroid

carcinoma. Additionally, thyroglobulin-immunoreactivity of these lesions is very suggestive of the thyroid origin of these cells.

Pesticide and environmental contaminants have been reported as endocrine disrupting chemicals provoking thyroid-disrupting effects in several vertebrate species (Bicho et al., 2012). It is known that some chemicals can interfere with the regulation of thyroid hormones by the hypothalamus and pituitary gland, affecting their synthesis. In addition, several histological biomarkers to assess the increase or decrease in thyroid activity have been developed (Bicho et al., 2012). Hedgehogs, like animals that inhabit areas with these environmental contaminants, are subject to a greater risk of exposure and consequent negative effects that can have repercussions for their health. Thus, further investigationshould be carried out to assess to what extent thyroid pathologies can predict the level of environmental contamination of the habitat (Bicho et al., 2012).

5.3 Case 11: Lipoid pneumonia; Cholangiocarcinoma; Subcutaneous mast cell tumor; Ovarian fibroma

Case 11 was a 5-year-old and obese African pygmy hedgehog diagnosed with lipoid pneumonia and CC, and to date, it is thought to be the first report of these pathologies in hedgehogs. The same animal was diagnosed with two other concomitant pathologies: a subcutaneous MCT and an ovarian fibroma. To the latter, as it is considered a common and incidental finding, minor emphasis will be given during this discussion.

Lipoid pneumonia is an uncommon, non-infectious, inflammatory lung disease histologically characterized by intra-alveolar lipid and lipid-laden macrophages in the alveoli (Costa et al., 2013; Pérez-Accino et al., 2020). Consolidation of the lung parenchyma, accumulation of acellular acidophilic substance and fibrin exudation were histological findings in this animal, consistent with previous reports of this pathology in other species (Costa et al., 2013).

Depending on the lipid source, this condition is classified as exogenous or endogenous. Exogenous lipoid pneumonia has been widely described in human and veterinary medicine and is caused by a chronic foreign body reaction to fatty substances in the alveoli after inhalation or aspiration of laxative mineral oils (Pérez-Accino etal., 2020). Although the pathophysiology of endogenous lipoid pneumonia (EnLP) is not completely understood, it is probably related with pneumocyte injury, leading to alveolar lipid deposition (Costa et al., 2013; Pérez-Accino et al., 2020).

EnLP has been associated with infectious agents (bacteria and parasites), obstructive pulmonary diseases (e.g., due to exudative fluid or cellular debris, bronchoconstriction, anomalous bronchi or tumors) and reduced airway clearance. In

addition, it has been associated with atherosclerosis and hepatopathy (Costa et al., 2013). In this case, bacterial and parasitic infections were ruled out by the non-histological observation associated with the set of negative histochemical stains (Grocott, PAS and Gram). Less commonly, other processes may be involved in the appearance of endogenous lipids in the lung: fat embolism, pulmonary alveolar proteinosis, Wegener's granulomatosis or lipid storage diseases (Berghaus et al., 2007). In human medicine, EnLP is known to be associated with pulmonary neoplasia. Indeed, both concomitant pathologies were already described in cats (Pérez-Accino et al., 2020).

In addition to this pathology, this animal was also diagnosed with CC. CC is a primarymalignant tumor of the bile duct epithelium. Other tumors of the biliary system include cholangioma, biliary cystadenoma, adenomas, and carcinomas of the gall bladder. This tumor is relatively uncommon in domestic animals, and previous reports focus on dogs and cats, but seldom in sheep, cattle, camel, horse, goat and avian species (Azizi et al., 2016).

The etiology of bile duct tumors remains poorly understood, with various factors involved in animals and humans. In humans, liver flukes are associated with CC development and a causative link with some parasites, namely *Clonorchis sinensis (C. sinensis)* in Southeast Asia and *Opisthorchis viverrine* in Thailand have been proposed. In animals, the association between bile duct parasites and bile duct carcinoma is unclear, however, *C. sinensis* and *Platynosomum fastosum* have been associated with CC in dogs and cats. Cholelithiasis and sclerotic cholangitis are other risk factors (Azizi et al., 2016).

In addition, prolonged exposure to insecticides containing sulfur appears to be related to the occurrence of CC (Azizi et al., 2016). This is an important point because as wild animals, hedgehogs are often in contact with sources of pesticides in the environment. Furthermore, as opportunists, they are indirectly exposed to pesticides through contaminated baits or prey (Taucher et al., 2020). In humans, recent studies suggest that metabolic disorders caused by obesity might be involved in the progression and poor prognosis of CC (Yugawa et al., 2021). Steatosis and its chronic inflammation could be a predictor of liver neoplasms (Schulz et al., 2015).

To date, there have been many studies reporting the metastasis of CC into the peritoneum, lungs, lymph nodes, diaphragm, spleen, kidneys, heart, adrenals and the bone marrow (Aslan et al., 2014). In the case of this animal, no metastases were detected.

Histological distinction among CC, hepatocellular carcinoma (HCC) and metastatic tumors is quite difficult and immunohistochemical staining methods are widely used to

make this differential diagnosis (Aslan et al., 2014). In dogs and cats, AE1/AE3 marker has been reported to be reliable for making CC cells and studies proved that human CC extensively expressed MUC-1 apomucin (Aslan et al., 2014; Mall et al., 2010). In this case, we failed the optimization of MUC-1 antibody in hedgehog tissues. The immunoreactivity validation may not have been achieved due to the main fact that most of these antibodies are produced for human use only and also, to the variability between mucin interspecies sequencies, that usually do not cross react with tissues from other animals. The CC in this animal was diagnosed based on histological findings, histomorphology (tubular and acinar pattern, with proliferation essentially around the portal spaces and bile ducts), PAS-positive amorphous material and IHC labeling for AE1/AE3. For future investigations, the combined analysis of CK7, CK19 and Hep Par 1, already reported to be useful to differentiate HCC and CC, should be exploited (Aslan et al., 2014).

The liver is a major regulator of body metabolism and plays a central role in the synthesis, storage and degradation of lipids. Hepatic tumors are frequently associated with rearrangements of metabolic pathways, leading to the dysregulation of metabolism (Satriano et al., 2019). In the same way, lungs are actively involved in lipid metabolism, and macrophages help to clear lipids from the lungs. The mechanisms involved in lipoid pneumonia are unclear but may involve an inflammatory response that is linked to increased lipid uptake by alveolar macrophages (Berghaus et al., 2007). In this way, perhaps a dysregulation in lipid metabolism derived from a bile duct neoplasm may be at the origin of the lipoid pneumonia presented in this case. From another point of view, we cannot exclude the hypothesis that the primordial pathological process is an alteration/rearrangement in the lipid metabolism. This is in line with the lipid vacuoles observed in the lung and with the hepatic steatosis presented in this animal, whose chronic character may have contributed to the development of a neoplastic process (Satriano et al., 2019).

MCT in hedgehogs are rare in veterinary literature, with only four previous reports (Heatley, 2009; Juan-Sallés & Garner, 2007; Kandefer-Gola et al., 2020; Raymond et al., 1997). However, in this species, different kinds of mast cell diseases are reported. The cutaneous form is classified into a solitary, localized tumor of the skin and diffuse cutaneous mastocytosis, and systemic mastocytosis is characterized by the proliferation of mast cells in the internal organs (Kandefer-Gola et al., 2020). The cutaneous form often affects the head, neck and axillary region. In these species MCT can metastasize to regional lymph nodes (Juan-Sallés & Garner, 2007). There is also a case in which single mast cells were reported in the spleen, however, in insectivorous animals, their

presence is quite common in this organ (Raymond et al., 1997). In the case of this animal, no metastasis was observed.

On the contrary to dogs, hedgehogs have no strict standards for determining the malignancy grade of tumors. Thus, to evaluate the MCT malignancy grade, the classical criteria of cellular morphology, amount of cytoplasmic granules, number of cell nuclei, number of mitotic figures per HPF and infiltration of deeper tissues are considered relevant points (Kandefer-Gola et al., 2020).

6. Conclusion

This study provided us with new insights about pathological diseases that can be found in *post mortem* examinations of hedgehogs. With only twelve cases, a significant and diversified number of pathological conditions was obtained, with considerable value from the comparative pathology point of view.

To date, this constitutes the first report of *Mycobacterium* spp. in hedgehogs in Portugal. Although already reported, this remains the second report of follicular thyroid carcinoma in hedgehogs. In addition, this study describes a CC and lipoid pneumonia for the first time in this species.

These animals' high propensity to develop neoplasia seems to be consensual worldwide, and this study is in agreement with these results. It is speculated that the life span increase in captivity leads to an increase in the onset of neoplastic conditions verified in this species (Keeble & Koterwas, 2020). Despite the presence of several concomitant pathologies affecting multiple systems, these are often incidental findings. This reinforces that they are strong and resilient animals, often not showing clinical signs. This panoply of lesions highlights the importance of this small mammal in the eco-epidemiological context of disease, given its potential to carry zoonotic diseases and its relevant role in the 'One Health' concept. Furthermore, these species can act as 'sentinels' for the surrounding environment, since they are used as biomarkers for heavy metal bioaccumulation and other persistent compounds, such as organochlorine and organobromine (Saengtienchai et al., 2016).

Therefore, for the correct diagnosis of pathologies in hedgehogs, it is essential to optimize and validate the complementary diagnostic methods commonly used in pathology laboratories, since the existing skills are not directed to this species.

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Attachments

Table A1. Some reported diseases in hedgehogs.

Diseases of infectious, noninfectious, unknown, degenerative or multiple etiologies	
Organ system	
Integument	Maggot, flea and tick (Ixodida) infestation (Ivey & Carpenter, 2012) Acariasis (*Caparinia spp.*, *Chorioptes* spp., *Notoedres* spp.) (Ivey & Carpenter, 2012) Dermatophytosis (*Trichophyton mentagrophytes* var *erinacei, Microsporum* spp., *Arthroderma benhamiae)* (Ivey & Carpenter, 2012) Viral papillomas (Ivey & Carpenter, 2012) Contact dermatitis (Ivey & Carpenter, 2012)
Cardiovascular and hematologic	Dilated cardiomyopathy (Juan-Sallés & Garner, 2017) Myocardial mineralization (Ivey & Carpenter, 2012) Splenic extramedullary hematopoiesis (Juan-Sallés & Garner, 2017)
Gastrointestinal and hepatic	Enteritis (*Salmonella* spp.); Alimentary candidiasis (*Candida albicans*); Cryptosporidiosis (Ivey& Carpenter, 2012) Pyloric and intestinal obstructions (Ivey & Carpenter, 2012) Hepatic lipidosis (Juan-Sallés & Garner, 2017)
Respiratory	Pneumonia and respiratory infections (*Pasteurella* spp., *Bordetella bronchiseptica*) (Johnson, 2011) Lungworms (*Crenosoma striatum* and *Capillaria aerophila*) (Johnson, 2011)
Reproductive and Urinary	Endometrial polyps, pyometra and metritis (Ivey& Carpenter, 2012) Cystitis and urolithiasis (Ivey & Carpenter, 2012) Renal disease (nephritis, tubular necrosis, nephrocalcinosis, renal infarcts, polycystic kidneys,glomerulonephrophaties) (Ivey & Carpenter, 2012)
Neurologic	Intervertebral disc disease (Ivey & Carpenter, 2012) Hypocalcemia (Ivey & Carpenter, 2012) Vestibular signs (otitis media/internal or central neurologic disease) (Ivey & Carpenter, 2012) Demyelinating paralysis (wobbly hedgehog syndrome) (Díaz-Delgado et al., 2018)
Musculoskeletal	Myositis secondary to cellulitis (Ivey & Carpenter, 2012) Osteoarthritis (Ivey & Carpenter, 2012) Bone cysts (Ivey & Carpenter, 2012) Fractures and lameness (ingrown toenails, arthritis, nutritional deficiencies, pododermatitis, neurologic disease) (Ivey & Carpenter, 2012)

Oral and Dental	Periodontal abscesses, gingivitis and periodontitis (Del Aguila et al., 2019)
Otic	Pinnal dermatitis (dermatophytes and acariasis) (Ivey & Carpenter, 2012) Ear mites (*Notoedres cati*) (Ivey & Carpenter, 2012) Otitis media/interna (Ivey & Carpenter, 2012)
Ocular	Corneal ulcers and ocular proptosis (Ivey & Carpenter, 2012)

Neoplasia

Organ system		**Organ affected**
Integument	Mammary tumors* (Raymond & Garner, 2001)	Mammary gland
	Oral squamous cell carcinoma* (Del Aguila et al., 2019)	Junction of quilled and unquilled skin, Oral, Lung
	Mast cell tumor (Raymond & Garner, 2001), (Juan-Sallés& Garner, 2017)	Skin
Hemolymphatic	Lymphosarcoma* (Raymond & Garner, 2001), (Juan-Sallés & Garner, 2017)	Multicentric or Gastrointestinal
	Fibrous histiocytoma (Heatley, 2009)	Skin
Neuroendocrine	C-cell carcinoma (Juan-Sallés & Garner, 2017)	Thyroid
	Islet cell tumor (Raymond & Garner, 2001)	Pancreas
	Adenoma (Ivey & Carpenter, 2012)	Pituitary
	Schwannoma (Ivey & Carpenter, 2012)	Nerve Sheath
Musculoskeletal	Osteosarcoma (Ivey & Carpenter, 2012)	Bone
Gastrointestinal	Fibrosarcoma plasmacytoma (Raymond & Garner, 2001)	Oral
	Acinic cell carcinoma (Heatley, 2009)	Eye, retrobulbar
	Adenocarcinoma (Heatley, 2009)	Liver, Stomach, Colon
Reproductive	Leiomyoma/leiomyosarcoma (Raymond & Garner, 2001)	Uterus
	Adenocarcinoma (Raymond & Garner, 2001), (Juan-Sallés & Garner, 2017)	Uterus
	Spindle cell tumor (Ivey & Carpenter, 2012)	Vagina, uterus

*Most common tumors