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Title

Combined diagnostic approaches for the diagnosis of canine splenic neoplasm: Contrast-Enhanced Ultrasonography, ultrasound guided cytology, histology and immunohistochemistry in selected lesions.

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1. Introduction

The canine spleen is important for a range of haematopoietic and immunological functions. Many of these functions can be appropriated by other tissues in the event that the spleen is removed because of a diagnosed disorder.[21] However, in splenectomised dogs there is inevitably some reduction of functional immune surveillance, which has negative consequences, such as increased susceptibility to microbial infection and erythrocyte parasitism.[39,105] Splenectomy in humans has the risk of pneumococcal sepsis within the first year after surgery with prevention by vaccination.[78]

Splenic parenchyma can be affected by a variety of neoplastic and non neoplastic diseases and their clinical presentation may vary according to their nature and dimensions. The largest case load concerning splenic disease in veterinary literature by Spangler et al. in 1992 highlighted a total of 1372 (1,3%) cases of splenic canine diseases on approximately 124000 total accessions from all species of animal during a 50 months period. Considering that splenic lesion can be often asymptomatic, the real prevalence could be far higher. Presenting complaints vary and can range from vague, nonspecific illness, asymptomatic swelling of the abdomen to acute death secondary to hypotensive shock.[18]

2. Diagnostic techniques

There are several ways to determine if a dog has a splenic mass. The most common is by physical examination but this technique may lack of sensitivity since small lesion or lesion located into the visceral surface of the organ can be missed. Abdominal radiographs and ultrastrasonography are also useful routine diagnostic procedure for the detection of splenic lesions. The former being a good test for screening but lacking in specificity.

2.1 Ultrasound (US)

The ability of US to depict focal or multiple splenic lesions depends on several factors such as the size of the spleen and the focal lesions, the location and echotexture of these pathologies as well as US findings involving the adjacent organs. The patient's clinical history and symptoms can definitely help the clinicians to formulate a correct differential diagnosis.[8]

In human medicine, in the early 1980s, it was said that US examination of the spleen only served to differentiate a solid lesion from a cystic lesion because focal benign and malignant splenic lesions could present the same US pattern. Furthermore malignant lesions of the same histology could present different US patterns.[87]

In the 1990s, it was not considered possible to formulate a diagnosis on the basis of US imaging only, although in these years some epidemiologic US characteristics of splenic diseases were defined showing that benign lesions accounted for 57–60 % that malignant lesions were hypo-echoic in 97 % of cases and that 83.33 % of these were lymphomas. These studies also demontrated that metastases often have an echoic halo or a target pattern, that high-grade non-Hodgkin lymphomas are often solitary lesions exceeding 3 cm in diameter, whereas low-grade non-Hodgkin

lymphomas and Hodgkin's disease are multiple lesions less than 3 cm in diameter or diffuse, with echostructure almost always hypo-echoic and very rarely hyperechoic.[27]
Around the year 2000, the ability to study focal splenic pathologies was further improved and it was reported that US imaging could identify 88 % of splenic diseases detected at computed tomography (CT), but US was unable to detect iso-echoic subdiaphragmatic and subcapsular lesions and particularly 50 % of infarctions, 33.3 % of metastases, and 16.6 % of lymphomas.[8] The presence of solitary focal splenic lesions, anechoic masses or nodules with increased echogenicity due to gas or calcification were interpreted as benign lesions with a positive predictive value (PPV) of 85, 100, or 100 %, respectively, whereas complex or echoic, diffuse or multiple lesions, target lesions or associated with abdominal lesions were suspected of malignancy with a PPV of 70, 70, 100, 100 %, respectively.
Recent studies highlightes a diagnostic accuracy of US reached 87.2 %, although it was demonstrated that different splenic diseases may present a similar echostructure.[8]
However, in the last decade due to experience acquired and the introduction of second-generation contrast agents, this technique has been re-evaluated as contrast-enhanced US (CEUS) allows detection and characterization of most focal lesions of the spleen with a high sensitivity and a good specificity.
CEUS is a new diagnostic imaging procedure to investigate tissue vascularity and perfusion dynamics.[53] A major advance of contrastenhanced ultrasonography is the development of contrast-specific software allowing pulse subtraction imaging, and the development of second-generation contrast agents, which allow low-mechanical index (MI) ultrasonography. First-generation contrast agents have contrast effect only at high MI leading to microbubble destruction.[4] Therefore, continuous or high-frame

rate observation cannot be performed with first-generation contrast agents. On the contrary, second-generation contrast agents are of diagnostic value even with a very low MI because of their physical behavior during insonation. Second-generation contrast agent perflubutane microbubbles[a] produce stable contrast effect at low MI without destruction and allow continuous real-time imaging.[31]

In Europe, contrast-enhanced ultrasound is routinely used in humans to characterize liver lesions and the diag- nostic accuracy is similar to that of contrast-enhanced computed tomography (CT) and magnetic resonance (MR) imaging.[69,98] Recently, abdominal extrahepatic application has been proposed.[64,102] Initial studies suggest that contrastenhanced ultrasound is of diagnostic value in patients with splenic and perisplenic lesions,[64,97] but little information is available on the perfusion pattern of focal splenic nodules. Benign neoplasia is uncommon in humans and malignancy is extremely rare. Lymphoma and angiosarcoma are reported only occasionally,[35,86] nodular hyperplasia is a rare condition with a postmortem incidence ranging from 0.024% to 0.13%.[44]

In small animals, contrast-enhanced ultrasound has been used to study normal liver perfusion,[112] liver focal lesions,[57] portosystemic shunts,[77] and lymphadenomegaly caused by lymphoma.[76] This method can be used in small animals without adverse effects and it is useful in characterizing liver nodules and in identifying the vascular architecture of malignant lymph nodes.[75] However, to this day, cytology or histology are required for the final diagnosis.

2.2 Cytology

Cytopathological examination through fine-needle aspiration (FNA) is a widely available and cost-effective method of diagnosis for the dog and cat.[50,95] Although many clinicians are reluctant to perform FNA, because of the popular belief that

aspiration of a haemic organ is inherently dangerous. On the contrary, numerous companion animal studies have reported low risk associated with splenic FNA.[1,58] Furthermore, extensive human studies of splenic aspiration using a 22-gauge needle indicated that complications arose in approximately 5.3% of patients, and of these complications, less than 0.75% were considered severe.[13]

An additional benefit of cytopathological examination is that it often identifies the eziologic agent and because of the thinness of the smear there can be better assessment of cytoplasmic and nuclear detail.[50] Unfortunately, cytology do not permit the evaluation on the architectural changes within the affected tissue,[50,105] and may occasionally lead to non adequated or non diagnostic samples. However by current technology an ultrasound examination of a splenic mass lesion can provide a great deal of information on the character of the lesion and on more solid areas likely to yield cells of diagnostic value.

Despite the variety of splenic neoplastic diseases few studies had addressed the accuracy of splenic cytology in veterinary medicine.[1,12,19,58,109]

Some of them assessed the accuracy of cytology in larger case load study in which cytological sample are taken from different organs, but the exact accuracy of splenic cytology could not be gathered from these papers.[15,61,95]

Gross appearance of splenic lesions is extremely heterogeneous as long as their clinical behaviours. For example, when e focal splenic lesion is evidenced the differential diagnosis may range from benign lesions (e.g. lymphoid hyperplasia, focal extramedullary hematopoiesis, hematoma, inflammatory lesions) to malignant one (e.g. lymphoma, angiomatous tumors, non-angiomatous-non lymphomatous tumours). Therefore, it is important to ensure that the diagnosis of splenic disorders, which may lead to elective splenectomy, is as accurate as possible.

2.3 Histology

Histology is considered the “gold standard” test for the diagnosis of splenic lesion since is able to maintain the structural and architectural elements, thereby indicating cellular relationships.[50] This feature is very important when evaluating splenic lesions since the most frequent subtypes of lymphoma are mantle cell lymphoma (MCL) and marginal zone lymphoma (MZL).[105,107] Both of them are indolent types of lymphoma characterized by the neoplastic proliferation of small to medium sized lymphocytes with a low mitotic rate that alter the normal splenic follicle architecture. [105,107] On cytology they usually yield a mixed cellularity with a high prevalence of small lymphocytes which can be misdiagnosed as a normal to reactive lymphoid population.[105,107]

Another frequent splenic neoplasm in dogs in haemangiosarcoma. When haemangiosarcoma is suspected, chose a region for sampling can be challenging since on gross examination hemangiosarcoma and hemangioma could be identical and malignant neoplastic tissue can involve a small portion of the lesion. In these cases indication regarding perfusion patter observed on US can be helpful. Limitation of histology include the need of a surgical approach and anesthesia to achieve splenic samples.

Spleen

3.1 Embryology

The spleen is a hematopoietic organ that filters the blood through a system of sinusoids in contrast to the lymph nodes that filter lymph through a sinusoidal system.[105] Thus the spleen, which lacks an afferent or incoming lymphatic system, deals with antigen or foreign material being carried in the blood stream.[54] The immune functions of the spleen are accomplished by small sprigs of vessels that permeate the dendritic cell beds of the germinal centers, allowing these cells to react to blood-borne antigen[54]. The mechanical filtering function of the spleen is carried out after the blood leaves the arteriolar lymphoid sheaths and germinal centers and enters the meshlike sinusoidal system where macrophages line the narrow vascular spaces. In phylogenetic terms, the spleen appears in primitive vertebrates as a spiral fold in the midgut, with lymphoid sheaths encircling arterioles in cartilaginous fishes, and becomes a retroperitoneal structure in bony fishes.[70] In mammals, the spleen begins as a thickening of the dorsal mesogastrium containing a loose supporting stroma, which in mouse embryos becomes occupied by primitive mononuclear cells by 2 weeks of gestation and with erythroid and lymphocytic differentiation evident by day 16.[70] In rats and mice, there is active hematopoiesis in the spleen in the latter part of gestation. This organ becomes a major source of blood cells as hematopoiesis declines in the liver and before the fetal bones develop a medullary cavity. In larger mammals like domestic animals and humans, the fetal spleen does not contribute to myelopoiesis as in rodents.[70,79] In the latter species, the liver is much more involved in hematopoiesis during fetal development. This is especially apparent in the pig where neonatal animals are frequently seen in pathology

examinations due to various diseases causing death in sucklings.[79] Piglets up to about 1 week of age have scattered foci of erythropoiesis in liver that must be recognized as a normal finding. In health, there is a constant relationship between splenic mass and body weight, as there is for the liver and kidney. In beagles, the average weights of the spleen of 95 male and female pups at birth was 0.8 g, rising to 3.4 g at 1 month, 10.6g at 2 months, 46 g at 6 months, and 45g at 1 year.[79,105]

The spleen can be divide structurally into 3 compartment: the vascular structure, the lymphoid structure and the sinus structure.

3.2 Vascular Structure

The spleen has a complex arterial and venous system but only an efferent lymphatic system.[11,45] Thus, all antigen enters the spleen via the arterial system and must be captured in an appropriate rheological and cellular environment. The arborization of the arterial system ends in a series of capillaries that have been described as penicillary, where a series of small vessels all terminate in a fanlike projection reminiscent of a fungal fruiting body. [11,45] These small vessel terminations, referred to as *ellipsoids* and *sheathed capillaries*, terminate with the endothelium encircled by a series of concentric cells now recognized to be largely macrophages. [11,45] The “open” and “closed” concept of splenic circulation is based on these terminations occurring into germinal centers and splenic sinus interstitium (open mode) or directly into large bore veins with relatively direct return to the systemic circulation (closed mode). There are differences in the circulation through the spleens of mammals with those described as *sinusal* (including the dog) having relatively direct connections between the sheathed capillaries and the larger splenic sinuses.[80,81] In contrast, the cat spleen has no direct connections between the sheathed capillaries and veins and is termed

nonsinusal type.[81] These determinations were made by utilizing erosion casts of dilated and contracted spleens injected with polymer prior to fixation to avoid normal vascular contraction from obscuring vascular channels. Despite these differences, the blood return to the systemic circulation in the cat is as rapid as in the dog, apparently because the distance of sojourn in the interstitial areas in the cat are very short.[80,81] In all mammals, including human, the venule walls are constructed with the endothelial cells elongated in the long axis of the vessel. Endothelial cells are not joined laterally but are held in place by very thin reticulin fibers that periodically encircle the vessels like hoops around barrel staves. This architecture permits red cells to permeate between the membranes of adjacent endothelial cells; phagocytic cells remain able to pinch off intracellular Heinz or Howell-Jolly bodies from erythrocytes. This allows unwanted remnants of RBCs to remain in the interstitial area to be phagocytosed.[11] In the dog, there are 3–4 major branches of the hilar arterial system that make partial splenectomy possible should there be a need to do so.[80] The splenectomized dog is highly susceptible to red cell parasitisms and may re-present with anemia due to *Mycoplasma haemocanis* with a high level of red cells infected.[105]

3.3 Lymphoid Structure

The lymphoid structure of the spleen is similar in most mammals, but the rat and mouse have a definite structural containment of the germinal center that is not present in many domestic species or in humans.[42] Cats have a peripheral sinus that separates the mantle and marginal zone lymphoid compartments, is not present in dogs, and is not apparent histologically.[81] In the rat and mouse the germinal center and, to a lesser extent, the periarteriolar lymphoid sheath have a stromal encapsulation, but the marginal zone cells lie directly on these capsules and the

peripheral sinus lies outside the marginal zone cells regardless of the depth of this cuff. In the calf, cat, dog, and rodents, the peripheral sinus forms a band surrounding the germinal center outside the mantle cells and current level of marginal zone cells and appears as a layer with more red cells and fewer nucleated cells than the adjacent splenic sinus area.[42,105] It appears that this peripheral sinus may have a counterpart in other species but may be less well defined both anatomically and histologically. The major difference between human and animal spleens is that, in humans, the thymic-dependent T-cell cuff around the central arterioles is discontinuous and does not enter regions of germinal centers.[94] In the rat and mouse, the marginal zone cells may surround the periarteriolar lymphoid sheaths, but it does not in domestic animals or in human spleens.[94] In animals there is some degree of T-cell cuffing around all small arterioles in contrast to the human spleen. Small radicles that branch from the larger arterioles at right angles form the source of antigen for the lymphocytes and dendritic cells of the germinal centers.[105] In most sections, the arteriole is seen eccentric to the germinal center with the initiating small arterial branch seldom in the plane of section. The arteriole is surrounded by the "periarteriolar lymphoid sheath" (PALS).[105] An apparent continuation of this sheath extends around the periphery of the germinal center. The germinal center is surrounded by a narrow cuff of mantle cells that are morphologically small lymphocytes with round or minimally indented nuclei with minimal cytoplasm and immunologically are naive B-cells. The marginal zone cells are comprised of postgerminal center memory B-cells that lie outside the mantle cell cuff. Marginal zones are of variable thickness depending on the activity of the spleen.[105] In humans, but not in most domestic animals except the cat, the division between the mantle cell and marginal zone layers is a potential space (the perifollicular zone) that is rich in

sheathed capillaries and corresponds to the region in rats rich in metallophilic macrophages.[94] There are many T-cells within the largely B-cell mantle cell cuff of humans and domestic animals.[105] These T-cells are small and morphologically not distinguishable from B-cells of the mantle cell area. In follicular involution, these T-cells persist and identify former areas of germinal center activity in tissues stained with CD3.[105] In dogs and humans, and less so in cats, the marginal zone cells tend to be closely associated with splenic follicles and don't extend along the periarteriolar lymphoid sheaths. In the dog, marginal zone hyperplasia of the spleen is commonly seen in association with both follicular and PALS atrophy.[105] This process appears as cohesive clusters of large lymphocytes with prominent nucleoli adjacent to small arterioles and often hyalinized germinal centers. In splenic marginal zone lymphoma in the dog, the area of neoplastic proliferation is usually multicentric in a single region.[105] The region or perifollicular zone between the mantle cell cuff and the marginal zone cells is the area where the sheathed capillaries open for filtering of the arterial blood through the meshwork of the marginal zone cells. These marginal zone cells colonize stroma of very specific phenotype that express smooth muscle actin and myosin and specific adhesion molecules in humans.[42] There are numerous T-cells in this area, which suggests this as a T-cell area in quiescent spleens with little marginal zone cell proliferation. In inflammatory states, the area outside the perifollicular and marginal zone cells may appear reddened at an architectural level of histologic examination because this is the area where the cells exiting the sheathed capillaries into an "open" circulation accumulate as they are filtered back into the splenic red pulp sinuses. In animals like the cat and dog, which mount a strong neutrophilia in response to infectious and inflammatory diseases, this area will contain many mature neutrophils.[80,81] In contrast, cattle tend to develop neutropenia

during acute inflammation, so mature neutrophils may be absent from the perifollicular and marginal zones, but band and metamyelocyte neutrophils may be detected.[24] The splenic lymphatics are largely unrecognized and are located near the small arterioles where they may appear as slitlike structures with a thin lining membrane and an empty lumen.

3.4 Sinus Structure

The dendritic cell population of the spleen has been extensively studied in the mouse and rat where it is found that all splenic dendritic cells are of relatively direct lymphoid or myeloid origin.[2] This is in contrast to the other lymphoid tissues where some dendritic cells are derived first from myeloid cells that then form a secondary progeny of Langerhans cells.[105] The phagocytic cells of the spleen are found primarily in the sinus or red pulp areas but are also prominent in the perifollicular areas. Following intravenous injection of mice with India ink, the perifollicular zone was heavily laden with carbon-bearing phages within an hour, suggesting that many of these had arrived from the blood and entered the area via the sheathed capillaries.[2] Some of the labeled cells migrated into the adjacent germinal centers where they persisted for at least 6 months. Similarly, dendritic cells resident in the sinus or perifollicular areas migrate into the germinal centers following systemic immunization.[2] The spleen modulates the febrile response to various antigens. Splenectomized guinea pigs given lipopolysaccharide intravenously mounted a 1.2–1.8°C increase in temperature that did not occur in sham-operated intact controls.[2,20]

The sinus areas of the spleen are areas of recognition and removal of senescent blood cells by the combined effects of the lattice-like endothelial cells of the sinus veins and the extravascular phagocytic macrophages.[105] The littoral or lining cells of the splenic sinuses are themselves phagocytic as can be visualized microscopically

by the presence of hemosiderin in the cytoplasm of otherwise flattened lining cells. Anatomically, the littoral cells share function and staining characteristics of both endothelial cells and of phagocytes (lysozyme+).[45] Aging red cells contain altered hemoglobin in small focal areas of the cytoplasm. These areas are pinched off as they squeeze between the endothelial cells to reenter the systemic circulation. These vesiclelike structures are approximately 0.3 microns in diameter and can be seen in older red cells of normal individuals.[45] It is estimated that 20% of hemoglobin is lost from aging red cells in this manner. In asplenic individuals, these vesicles remain within the red cells in the absence of the "pitting" function of the spleen.[45,105]

3.5 Function

The major functions of the spleen include antibody production, hematopoiesis, and removal of aged blood cells and microorganisms.[10] The first of these two can be entirely managed by other areas of the body in asplenic animals.[10] However, the loss of the blood filtering is directly returned to the systemic circulation. About 3% of arterial blood enters the sinus filtering system with each passage through that organ.[40] The rate of flow through the spleen is sufficient that the total blood volume is filtered through the sinus system at least once each day.[40,105] In terms of function, as determined from examination of peripheral blood, there is a marked difference in the function of the spleen in animals such as the dog and horse with very muscular spleens and ruminants with more collagenous and less contractile splenic capsules.[105] The spleen of the dog is extremely efficient in removing senescent cells and nuclear fragments such as Howell-Jolly bodies from young red cells. If Howell-Jolly bodies are present in the blood of a dog without other clinical signs (such as regenerative anemia), some occult lesion in the spleen should be expected and defined.[65] Typical lesions include focal areas of peliosis or focal nodular lymphoid

hyperplasia flanked by abnormal areas of sinus dilation that defeats the efficiency of the sinus filter.[105] More serious causes include hemangiosarcoma or other diffuse splenic neoplasms.[85] Similarly the splenic filter may become overloaded by a very high leukocyte count that presents the sinuses with many aging leukocytes. This allows some aging cells to circulate with nuclear hypersegmentation or pyknotic nuclei. An example of this situation is seen in dogs with a high neutrophilia caused by pyometra or pyelonephritis.[10] If there is very brisk red cell destruction, as occurs in aggressive immune-mediated haemolytic anemia, the overload of sensitized red cells will result in the presence of peripheral blood Howell-Jolly bodies and senescent leukocytes of all types.[10] Leukemias capacity leaves the asplenic individual much more susceptible to coccal bacteremia and red cell parasitism.[105]

The amount of antibody production and hematopoiesis that occurs in the spleen varies between species, but the extent of splenic involvement for clearance of infectious agents is very similar in all species.[10] For example, mice have splenic trilineage hematopoiesis throughout life, which should be differentiated from myeloproliferative disease during histopathologic examination. Rodents do not have well-defined splenic germinal centers (which are found in domestic animals), and there are minor differences in the anatomy of the vascular sinus interface in species as noted above[106]. It is felt that in most normal mammals the spleen functions largely as a “closed” organ in that most of the blood will also result in splenic hypofunction; this is due partially to an increased burden of removing the effete neoplastic leukocytes but also because the spleen itself may be primarily involved with tumor that has resulted in altered patterns of flow.[105]

Proper function of the spleen requires that the organ is free of focal lesions that can distort vascular flow patterns. Thus a mass lesion as small as 1–2cm in diameter

may in itself be no hazard to the major function of cellular and microbial clearance, but it may create flanking areas of sinus dilation that permits pooling of platelets and clinically evident thrombocytopenia[105]. The cat seems less often affected in this manner, and lymphoid hyperplasia is more likely to be diffuse than focal as in the dog.[105] The horse, like the dog, has a very efficient system of controlling the age of cells in circulation and the horse does not release reticulocytes into the peripheral blood stream. Horses have fewer causes of focal splenic dysfunction than dogs but rarely permit aged leukocytes to circulate in non-neoplastic diseases. Ruminants, principally sheep and cattle, are much less reactive hematologically than horses or carnivores and have spleens that are much less capable of volume change over a short time span. The differences in splenic architecture with species are evident in the proportions of smooth muscle to collagen, but how this translates into splenic function is less clear.[105] Perhaps a reduced ability of ruminant spleens to contract permits larger sinus diameter and less efficient filtering in some disease states. In any case the latter animals are characterized by frequent neutropenia in acute septic diseases and more variability in the character of cells in circulation during wide fluctuations in levels of blood cells.[105]

The filtering actions of the spleen have been described in the vascular and sinus areas above. The phagocytic cells of the spleen include the macrophages and littoral cells of the sinuses. Both cell types are capable of ingesting injured and senescent cells, as evidenced by their frequent hemosiderin granulation.[10] The splenic macrophages are primarily derived from the marrow via the blood, but under demand there is undoubtedly local proliferation.[62] Blood flow through the sinus areas of the spleen is slow, with compression of blood cells by leakage of plasma into the efferent lymphatics facilitating sorting of blood cells by the macrophages and littoral cells. The

red cell membranes become less flexible as the cells age, and their decreased ability to deform to pass between endothelial membranes makes them more easily recognized by the phagocytic complement.[62] The storage function of the spleen is likely overstated but the surface charge of red cells causes them to adhere to splenic membranes when they are very young and again when senescent.[10] The pooling of young red cells in the spleen may be a form of storage in the delivery of immature cells.[10] The spleen may also contain a large number of platelets.[10]

4.Developmental and Degenerative Diseases of the Spleen

4.1 Developmental Diseases of the Spleen

Congenital anomalies of the spleen include complete absence or asplenia, as well as polysplenia or fusions during embryonic development with other organs including the kidney and gonads.[65] Asplenia occurs in a strain of inbred mice and rarely in humans. Human asplenia is associated with other malformations of the heart and great vessels. Nude mice have spleens that lack the thymic-dependent periarteriolar inner sheath lymphoid areas.[65]

A condition of hyposplenia occurs in humans in which the spleen may weigh less than a gram and has deficient development of the lymphoid areas, although thymus and nodes are not affected.[65]

An accessory spleen occurs in many species and consists of an additional small splenic mass (or masses) in the region of the hilar vessels, but it may occur in other organs including the pancreas and omentum.[9] Accessory spleen has also been applied to a condition more correctly termed *splenosis*, where there are multiple splenic implants throughout the abdomen.[9] This is usually as an acquired condition following splenic trauma.[9]

4.2 Degenerative Diseases of the Spleen

Age-associated atrophy occurs in the spleen as in other lymphoid organs in animals like dogs and horses that live to full maturity.[10,65] Atrophy of the lymphoid areas of the spleen occurs in autoimmune diseases treated by immunosuppression. The most marked atrophy is seen in animals that have had several courses of combination

chemotherapy, in which case destruction of cells is added to the atrophy usually associated with severe and chronic disease.[105] Acute lympholysis consisting of generalized apoptosis of germinal center cells may occur as a result of an endogenous steroid increase in acute stressing diseases. This change is seen in foals aborted at term due to equine herpesvirus type 1.[105] In animals that survive this initial reaction, there is complete removal, within 24 hours, of the apoptotic debris, and the germinal centers then appear hypocellular and consist of the dendritic cell bed and a few macrophages.[105] The presence but not the absence of the apoptotic debris can be used to estimate the onset of the disease prior to death. Hyalinized germinal centers with associated vascular changes occasionally occur in old sheep, goats, and dogs.[105] When encircled by concentric rings of small lymphocytes the lesion is referred to as *Castleman's disease*.[34] In animals this change is seen most often as a type of fading follicular hyperplasia representing a reaction that is in decline, but rarely lymphoid hyperplasias in the dog lymph node may closely resemble the human reaction.[105]

The spleen as a major lymphoid organ and a site of macrophage accumulation reflects systemic changes involving these cell systems. In systemic amyloidosis, the spleen is always involved, usually not to a major degree, with only the germinal centers affected and the sinuses are usually spared. Even in advanced involvement of the lymphoid follicles producing waxy nodules (*sago-spleen*) on the cut surface, the organ is not enlarged and hyperfunction is not present.[7]

The lysosomal storage diseases of animals like the gangliosidoses and globoid cell leukodystrophy have major effects in the nervous system with minor involvement of the liver and spleen.[30] In contrast, the glucosylceramidase deficiency (Gaucher's disease) is termed a *neurovisceral* storage disease with major involvement of the

liver and spleen. This rare disease has been reported in Sydney silky terrier dogs and in sheep.[30]

The most common pigment accumulation in the spleen consists of iron and calcium-protein complexes of various types that produce black to brown or yellow nodules in the capsular margins called *siderotic nodules* or *Gamna- Gandy bodies*. These elements deposit in fibrous and elastic tissues, are blue-black with hematoxylin, and in fibrillar form may be mistaken for fungal hyphae. These changes often occur in association with areas of yellow ceroid pigment and suggest a genesis in focal areas of haemorrhage.[105] Hemosiderin is present in sinus macrophages of mature animals in some degree and may be very extensive, but it is only significant if it is accompanied by fibrosis.[28] Hemosiderin iron is relatively inert and unavailable for short-term utilization in response to haemorrhage even if present in abundance. Hemosiderin accumulation occurs in anemias of chronic disease where there is enzymatic scavenging of iron from transport proteins to deny iron use by bacterial pathogens. In these conditions splenic macrophages contain large deeply stained aggregates of hemosiderin and very little fine diffuse iron on specific staining. In contrast, in haemolytic anemias where there is very rapid recycling of iron, the macrophages contain iron in very fine aggregates. These are smaller complexes more rapidly digested for iron release with iron present in combination with ferritin and some hemosiderin. Splenic infarction is a rare occurence in animals as compared to humans. In humans hypertension and vascular accidents are more common as well as thrombotic lesions associated with sickle cell anemia, all of which can lead to infarcts.[105] Dogs with myeloma may have splenomegaly and these may be associated with focal septic infarction due to the hyperviscosity of hyperglobulinemia causing impaired splenic circulation.[105] Animals presenting with

these signs (serum protein above 10 g) are highly vulnerable to hypotensive shock even with minor sedation, as required for marrow or splenic aspiration.[105]

4.3 Splenic Trauma and Splenic Torsion

Most frequently, trauma to the spleen occurs as a result of crushing injury or puncture wounds. These events result from a variety of causes that often involve falls, contact with moving vehicles, and gunshot wounds. Often, massive abdominal hemorrhage is the net result. If the animal survives to have the lesion identified later (as often occurs), the spleen may be completely bisected or appear twisted and with deep linear creases in areas of capsular rupture. These changes may be accompanied by several areas of extra splenic explants or splenosis consisting of red round masses 1–2cm in diameter resembling hemal nodes and usually attached to areas of omentum or mesentery.[9] Trauma sufficient to cause rupture of the splenic capsule will often also result in hepatic laceration. The essential functions of the spleen are not altered by previous trauma, and signs of splenic hypofunction with aged cells or Howell-Jolly bodies in peripheral blood cells have not been associated with healed trauma.[9]

An enlarged spleen of any cause is much more susceptible to tearing of the capsule with fatal haemorrhage resulting. Splenic rupture without apparent trauma is seen in dairy cattle with the adult type of bovine lymphoma. In these cases the spleen is massively enlarged and the capsule is greatly thinned. Similar events are rare in the dog or horse but do occur occasionally in the mouse and rat, likely associated with capture and restraint.[105]

Torsion of the spleen occurs rarely in large-breed dogs and in humans. The broad attachment of the spleen to the rumen makes torsion impossible in cattle, sheep, and goats. After torsion has occurred, the peripheral blood picture appears similar to

postsplenectomy cases with hypersegmented neutrophils, Howell-Jolly bodies in red cells, and a severe moderately responsive anemia. If the infarction occurred several days earlier, the plasma will be brown from blood pigments leaching from the splenic capsule and the urine will be dark brown. Torsion is usually of 180 degrees with complete occlusion of the veins but not the arteries, permitting slow distension of the spleen with ultimate blockage, thrombosis, and infarction. Less often, the spleen may be rotated on its long axis with congestion and infarction of the tail area.[105]

5. Hyperplastic and Dysplastic Changes of the Spleen

5.1 Splenic Hyperplasia

Hyperplasia of the spleen may involve the white pulp and lymphoid proliferation or may be primarily of sinus areas. These do not usually occur together and follicular hyperplasia is usually accompanied by compression of the sinuses; sinus distension or sinus hyperplasia is usually seen with follicular and even thymic-dependent atrophy.[105] Hypersplenism is a condition of increased blood destruction by the spleen accompanied by marrow hyperplasia and cytopenia of one or more elements of the blood. Splenomegaly may not be observed.[59]

Hyperfunction of the sinus areas of the spleen may occur in a spleen of normal size with histologic evidence of increased numbers of macrophages; often, hemosiderosis and sinuses may have irregular distension that is not perceived as peliosis.[59]

Hypersplenism may be accompanied by immune sensitization of any one of the cellular elements but may be entirely mechanical. An enlarged spleen of any cause results in slower passage of cells through the sinus areas that in itself is a hazard to cell survival. The environment of the sinus plasma is of lowered pH, cholesterol, and glucose as compared to the peripheral circulation. Under these circumstances red cells especially are prematurely aged in a process known as *conditioning*.[59]

Extramedullary hematopoiesis (EMH) is one of the most common types of sinus hyperplasia that occurs in all species and occurs normally in mice at all ages. EMH is usually of trilineage type, but any one cell line may predominate depending on the condition and the ability of the marrow to respond. In the dog with immunemediated hemolytic anemia, the EMH is largely erythroid but other cells are always present to

some degree. If there is concurrent thrombocytopenia, megakaryocytes may be prominent as well, particularly near the muscular trabeculae. In the dog with immune-mediated anemia it may not be possible to determine if the spleen is producing more cells than it is destroying without resorting to tracking isotopically labeled red cells. [105] Myeloid metaplasia is characterized by marked EMH with myeloid predominance and is dealt with under dysplastic changes.[49] In acute septicemia (as occurs in calves with Salmonellosis and rarely in canine pyometra), the germinal centers are outlined by a reddened ring consisting of a dilated peripheral sinus. The perifollicular sinus area contains the cells that are most predominant in the peripheral blood circulation; dogs will have many mature neutrophils present, whereas calves will have few mature neutrophils with immaturity to myelocytes. These changes are very dynamic and the animal either succumbs to disease at this stage or contains the infection. If the animal survives, the splenic reaction becomes more of a general sinus hyperplasia with a follicular reaction following in several days. [111]

Hyperplasia of the white pulp or lymphoid areas of the spleen is seen in chronic systemic infections as occurs in bovine trypanosomiasis, equine infectious anemia, feline leukemia virus, or pseudorabies virus infection of swine.[105,111] A very vibrant follicular hyperplasia will have large well-defined germinal centers that have complete cuffs of mantle cells and may have some marginal zone cells in eccentric layers outside the mantle cell cuffs. Marginal zone hyperplasia does not usually occur with complete mantle cell cuffs and more often is seen with follicular involution of some degree. Frequently there is also atrophy of the thymic-dependent periarteriolar lymphoid sheaths.[105]

5.2 Splenic Dysplastic Changes

Focal clusters of sinuses may become widely dilated forming multiple histological foci of 15–20 cross sections of dilated sinuses, each from about 0.5–1.0 mm in diameter. These structures are lined by littoral cells that have low, flat nuclei and narrow flattened layers of cytoplasm that contains fine basophilic granular debris and hemosiderin. In foci of this type with closely clustered small areas of venous ectasia, the endothelial lining cells react positively for CD31 and focally for lysozyme. Sinus cystic foci of this small type are seen with large cystic hematomas 2–5cm in diameter, which undergo pathologic examination because of removal for suspected hemangiosarcoma, which is often present. Cysts of this small clustered type are found in human splenic lesions, are known as littoral cell angiomas, and may become metastatic.[32] In the dog, the biological intent and importance of these lesions are largely unknown. Their association with haemangiosarcoma suggests that they be looked on as a precursor lesion as they become better recognized and studied. It would be logical to consider these lesions as dysplastic changes of sinus lining cells that focally may dedifferentiate to a benign or malignant vascular neoplasm.[26]

6. Neoplastic disease of the Spleen

Splenic parenchyma may be involved both by primary and metastatic neoplasms. Primary neoplasms arise from resident population of cells and may cause diffuse splenomegaly or give rise to focal or multifocal nodules.

Data regarding the prevalence of splenic neoplastic diseases are rather heterogeneous and may vary according to the study considered: 75% on 57 cases, 52% on 23 cases,[33] 23,6% on 1527 cases,[88] 44% on 87 cases,[16] 48,2% su 500 cases,[90] 54,8% on 31 cases,[1] 42,5% on 40 cases.[109]

Recently in a study by Eberle *et al.* (2012) on 249 Splenic masses, 47% (n=117) were diagnosed histologically as non-malignant diseaseand malignant splenic disease rapresented 53% (n=132) of the cases examinated. Hemangiosarcoma was the most common histological diagnosis (n=97; 73.5%). Other malignant tumors included sarcoma (n=14), fibrohistiocytic nodules (n=9) as well as lymphoma, blastoma and adenocarcinoma. The non-malignant masses consisted of nodular hyperplasia (n=60), splenic hematoma (n=41), and splenitis (n=6).

Like wise other studies have assessed a higher frequency of malignant tumours [16,18] and support the theory according to which 2/3 of splenic masses are malignant and the majority of them are hemangiosarcomas.

On the contrary, other studies highlighted a higher frequency of bening splenic diseases.[1,12,58,61,88,95]Christensen *et al.* (2009) evaluated 51 splenic histological samples and 69 cytological samples. Of these 43% and 20% had a histological and cytological diagnosis of malignant tumor respectively.

6.1 Splenic primary neoplasms

Primary splenic tumors can be classified into 3 majour groups: angiomatous tumors (angioma, angiosarcoma), lymphoma and non angiomatous- non lymphomatous sarcomas (NANLs).

6.1.1 Angiomatus neoplasia

6.1.1.1 Hemangioma

Hemangioma is well circumscribed benign tumor of well differentiate endothelial cells forming vascular channels. Common in dogs and cats, they can appear on gross examination as focal, dark red to blue lesion with a friable texture. Hemangiomas are tumors composed of variably sized vascular spaces filled with erythrocytes and lined by a single layer of uniform endothelial cells. Many tumors have organized thrombi with foci of hemosiderosis. Variants of these tumors have been calle cavernous or capillary, based on the size of vascular channel.[3]

6.1.1.2 Hemangiosarcoma

Hemangiosarcoma (HSA) is an aggressive malignant tumor originating from vascular endothelial cells and is the most common splenic tumor in dogs,[6,16,18] accounting for 51% to 66% of all splenic neoplasms.[74]

In wider terms, HSA has an estimated prevalence of 0,3-2%.[108 3,41,63]

HSA may arise from different tissues and the most common sites of origin in dogs are: spleen (28-63%),[3,5] right atrium and right auricle (3-50%),[5,41] skin and subcutaneous tissues (23,9%).[92]

German Shepherd dogs, Retrievers, and Poodles show some predisposition for AS,[3,5,16,18,67,88,90,92], whereas short-haired and light skinned breeds (eg, Boxers, American Staffordshire Terriers, Whippets) are at increased risk for developing cutaneous AS.[3,29] The tumor occurs predominantly in older dogs between 8 and 10

years of age; mean age at time of diagnosis is 9 to 12 years, and no gender predisposition has been noted.

Clinical signs may vary depending on where the tumor is located, if there is spontaneous rupture, and whether coagulopathies and cardiac arrhythmias are present.[83] More than half of the dogs with HSA are presented to clinics because of acute collapse after spontaneous rupture of a primary or metastatic lesion.[83] Some of these episodes of collapse are also due to ventricular arrhythmias, such as premature ventricular contractions and paroxysmal ventricular tachycardia.[83,85] Ventricular arrhythmias are relatively common with splenic and cardiac HSA, occurring in up to 39% of dogs with splenic HSA.[83]

Prognosis is usually poor because HAS are locally infiltrative and tend to metastasize rapidly through hematologic routes or via local seeding after tumor rupture. Only primary HSA confined to the cutis have a lower frequency of metastasis.[74]

Greater than 80% of cases are reported to have metastasized by the time of clinical diagnosis, with common sites of metastasis including the lung, liver, and omentum.[14,22,74] Up to 25% of the splenic tumors have a corresponding cardiac tumor (right atrial and auricular).[14,83]

Even though surgery is still the therapy of choice, many studies highlighted median survival times ranging from 19 to 143 days in splenectomized dogs.[67,90] Wood *et al.* (1998) demonstrated a median survival time of 86 days (mean, 116 days; range, 14 to 470 days), and the one-year survival rate was estimated to be 6.25%. Survival was not influenced by signalment, presenting signs, stage of disease, or clinicopathological findings.

Grossly, HSA may not be distinguished from other focal splenic lesions such as emangioma and hematoma.[88,99] It can be a single focal mass, can give rise to

multifocal masses or may involve diffusely the splenic parenchyma. If multifocal, nodules are usually smaller compared to hematoma end the majority of them are characterized by blood filled cavities with a honey comb appearance on cut surface.[88] HAS are usually characterized by a friable texture that may lead to pathologic ruptures and consequent hemoperitoneum. HSAs are also difficult to distinguish from other splenic lesions using ultrasound.[99] Bertazzolo *et al.* (2005) highlighted that cytologic appearance of HSA can be very heterogeneous, and additional criteria such as cellular cohesiveness, a bloody background, background neutrophilia or eosinophilia, erythrophagocytosis, EMH, vasoformative features, and cellular apoptosis can be used to support a suspect of HSA. Furthermore, epithelioid features and pseudoacinar forms may cause erroneous diagnosis as carcinoma or adenocarcinoma. Immunocytochemistry and IHC are therefore warranted in such cases.[3]

Histologically HSA is made up by plump and pleomorphic endothelial cells forming irregular vascular clefts and channels and cointainig a variable amounts of blood and fibrin (Pulley e Stannard, 1990). This neoplasm can have different patterns including vascular to solid pattern. HAS is an heterougeneous type of neoplasia and many patterns may cohexist within the same neoplastic lesion.[3]

6.1.2 Lymphomas

6.1.2.1 Marginal zone lymphoma

Indolent lymphoma is a subgroup of lymphomas with a low mitotic rate and a slow clinical course of progression.[56,107] Marginal zone lymphoma (MZL) is a form of indolent B-cell lymphoma in humans with 3 recognized subtypes, including splenic, nodal, and mucosal-associated lymphoid tissue forms.[38,51,101] Marginal zone

lymphoma originates from the marginal zone of lymphoid follicles and is characterized by a proliferating cuff outside of the mantle cell layer.[38,105,107]

In humans, splenic MZL is considered rare, representing approximately 1% of the cases of non-Hodgkin's lymphoma.[38,100] This lymphoma also is thought to be rare in dogs. Additionally, the diagnosis of MZL can be challenging for pathologists because it begins with marginal zone hyperplasia (MZH). However MZL is being recognized more because of increased awareness by veterinary pathologists and with the use of immunophenotyping and molecular clonality assessment.

The incidence and prognosis of MZL in dogs is largely unknown because only 2 reports of a limited number of cases are available. Valli et al reported 66 dogs with indolent lymphoma, of which 46 had MZL.[107] In this study, 33 dogs had nodal MZL and 13 dogs had splenic MZL.[107] The majority of splenic MZL cases were incidentally identified on routine abdominal ultrasound examination, and all dogs had multifocal areas of neoplastic proliferation located within a solitary splenic lesion.[107] Molecular clonality assessment and immunophenotyping were performed on the majority of cases in this study. All cases of splenic MZL were found to be CD79a positive and CD3 negative, and most had clonal rearrangement of immunoglobulin (Ig) heavy chain loci, consistent with B-cell lymphoma.[107]

In other domestic animals, many of the larger reviews were reported in the era of lymphoma classification by the NCI Working Formulation, which recognized follicular lymphoma but not mantle cell or marginal zone lymphomas.

The most characteristic presentation of MZL is in adult large breed dogs that present with a single enlarged lymph node, often submandibular.[105,107] Cases with splenic involvement usually present with no evidence of lymphoma in other tissues and, in the dog, as in humans, MZL is the type of lymphoma most likely to be primary in the

spleen.[107] Spread from the spleen is slow and likely to invade the hilar nodes and thus may be removed during routine splenectomy. In humans, splenic MZL is a diffuse disease, whereas in the dog, splenic MZL is not usually diffuse and occurs as a focal splenic mass often identified in asymptomatic dogs presented for other reasons.[107]

Both nodal and splenic MZL progress slowly, but only the nodal type tends to become generalized, after 18 months to 2 years of initial lymphadenopathy. Dogs with late stage MZL still tend to feel well even with bulky adenopathy.[105]

MZL has not been reported to induce leukemia in animals. In most cases, the only blood manifestations of MZL are in animals with splenic MZL that may present with thrombocytopenia due to sequestration of platelets in stagnant areas of sinus dilatation surrounding and within focal areas of tumor.[105] In human MZL some of the splenic cases become leukemic and of these a particular subset known as *hairy cell leukemia,* which is a type of B-cell CLL, is a consideration for differential diagnosis.[103] Human cases of splenic MZL that become leukemic tend to have “polar villi” in the peripheral blood and may appear as a unipolar hairy cell.[103]

Currently, little is known about clinical outcome in dogs with splenic MZL. Only 3 dogs with splenic MZL in Valli's study had follow-up data available, and none of these dogs died from MZL in the follow-up period (7–19 months).[107] In another study by Stefanello et al., outcome was described in 5 dogs with splenic MZL, of which 4 of 5 received adjuvant single-agent chemotherapy with doxorubicin.[93] Results of that study suggested a possible survival benefit with the addition of adjuvant chemotherapy.[93] On the contrary, a recent study by D. O'Brien et al, suggested that adjuvant chemotherapy may not prolong survival time in dogs with MZL, indicating that splenectomy may be the treatment of choice, which is similar to the human

counterpart of this lymphoma type. The finding of lack of survival benefit with the use of adjuvant chemotherapy in this study is in contrast with the publication by Stefanello et al.[93] The lack of survival benefit observed may have been related to the type of adjuvant chemotherapy used. In humans, splenectomy is the standard approach for MZL for patients with symptomatic splenomegaly and progressive hematological abnormalities such as lymphocytosis and cytopenias.[46,100] Splenectomy is not curative in humans, but yields excellent control of the disease with a median time to progression of 4–5 years.[56,101] The role of chemotherapy in humans remains largely unknown, but chemotherapy is used when splenectomy is contraindicated.[38,46]

The study by D. O'Brien et al., also concluded that a definitive diagnosis of MZL requires architectural assessment and hence histopathology since MZL cannot be diagnosed by cytology. Thirteen of the 29 dogs that underwent splenectomy had fine-needle aspiration cytology performed on a splenic mass. A diagnosis of lymphoma was made in only 3 of these dogs. On cytology, MZL is described as having an immature cell morphology with rare mitoses observed.[105] Cytological diagnosis may be confused with concurrent reactive hyperplasia.[56] Lack of awareness of this lymphoma type also may hamper the correct diagnosis.[56]

In benign proliferations, the MZH cells are of mixed type, including lymphocytes of variable size along with those of MZH type that are distinctive, because of the broad rim, of lightly stained cytoplasm.[56] In contrast, in MZL the rim of proliferation is equal to the diameter of the germinal center, making the overall diameter about three times normal size.[105,107] There is less coalescence of the proliferative areas of splenic MZL because the origin of the germinal centers on end arterioles insures their separation in a more even pattern than in nodal MZL.[105] In the cat, MZL is more like the human

form with a uniform involvement of all follicular areas, while in the dog, MZL appears invariably to be a focal and locally extensive disease.[107] It may be that in the cat, a splenic lesion with three cellular layers in the germinal centers (follicular center cells, mantle cells, and marginal zone cells, in a bulls-eye configuration is already a malignant neoplasm.[107] In humans, splenic MZL is always a diffuse disease involving all areas of the spleen, and a three laminar lesion in one area of a diffusely enlarged spleen is considered diagnostic of MZL.[101,107] The cat may be different from the dog in that involvement of the spleen, plus other tissues, may be more common but the cell type is very similar.

6.1.2.2 Mantle cell lymphoma

Mantle cell lymphoma (MCL) is a distinct neoplastic disease of humans and animals characterized architecturally by multifocal origin around fading germinal centers and composed of small round to lightly cleaved B cells that lack nucleoli and mitoses and are characterized biologically by an indolent course.[107] MCL is of recent recognition in human pathology and had been previously termed as *centrocytic lymphoma* in Europe and *lymphoma of intermediate cell type* in the U.S. It was then called *mantle zone lymphoma*, and finally *MCL,* identified as a distinct identity arising from the presence of a specific translocation t(11;14) and characterized by the overexpression of the cyclin D1 protein.[37]

Mantle cell lymphoma constitutes about 5% of human lymphoma cases in North America and about twice that level in Europe.[17] There is a 3:1 male predominance in humans, and the disease is seen in elderly patients with a median age of 65–75 years.[17]MCL became recognized in the 1980s and fully characterized in the early 1990s. With the further definition of marginal and mantle type lymphomas, the adjunct term of *zone* has been reserved for the marginal layer of perifollicular

lymphoma, with the mantle layer lymphomas identified as mantle cell type.[17] Valli reported an incidence in the dog of about 2% (11 cases of canine MCL have been diagnosed out of 461 total cases). The gender was known in 8 of the 11 cases of MCL, and of these 5 were male and 3 female. The ages on 10 of these dogs ranged from 1.5 to 15 years with a mean of just under 8.0 years. In the comparable period, 150 cases of cat lymphoma have been diagnosed, with only 1 case of MCL. No cases of MCL have been recognized in the horse, with one in each of a pig and spectacled bear.[105] One of the more remarkable features of MCL is that in the dog, 7 of the 11 cases were found only in the spleen.[106] Like marginal zone lymphoma, and unlike follicular lymphoma, MCL in the spleen presents as a locally extensive mass and not as a diffuse involvement. Like splenic marginal zone lymphoma, MCL may be associated with other lesions, including plasmacytoma, myelolipoma, fibrosis, and focal hemorrhagic infarction.[106] In human cases of MCL, most are advanced at diagnosis with generalized lymphadenopathy with splenomegaly in about half of cases and extra nodal involvement usually including bone marrow in most cases.[107] Other areas frequently involved include lymphomatous polyposis of the intestine and, less commonly, the skin, lung, breast, and other soft tissues.[105] The central nervous system is involved in 5–20% of relapsed cases.[17] The extent of involvement on both humans and animals appears to depend largely on the extent of the staging procedures.[105] In all of the cases in the dog where there was peripheral node lymphadenopathy, the submandibular node was involved, and 3 of the 4 cases that presented with nodal involvement had generalized lymphadenopathy.[105] The extent of extrasplenic spread in the cases that came to clinical attention because of a focal splenic mass is not known, but many of these cases were examined in oncology practices and presumably were staged as splenic only.[17] In human pathology a

"blastoid variant" of MCL has been identified that has slightly larger nuclei and nucleoli and tends to become leukemic and have extensive marrow involvement.[17] Remarkably, this variant type is also seen in the dog, with a characteristic splenic lesion consisting of tumor foci that surround blood-filled cavities.[105]

In both the dog and cat, MCL is likely to be encountered in a splenic mass that is multifocal and locally extensive and not diffuse as in the more aggressive types of lymphoma.[105]

When primary in the spleen, MCL is subsequently found in many other tissues. Two forms of splenic MCL occur, with the most common type representing still a rare finding and is characterized by multifocal areas of round solid foci of lymphoid proliferation that can occasionally be found associated with an end arteriole. These foci are larger than germinal centers and may be sharply defined against a background of intense congestion or hemorrhagic necrosis that has brought the lesion to clinical attention. Like MZL, the internodular areas may have diffuse sheets of plasma cells. There may be coalescing areas of lymphoid proliferation and the splenic capsule may be focally thinned or invaded. The only indication of germinal centers associated with these foci is an occasional pale area in the center of a nodular proliferation that is the residue of the larger dendritic cells or of an area of follicular hyalinosis. There is a complete absence of the residual normal mantle cells and none of the fading clusters of small benign cells seen at the center of splenic MZL.[105]

The second form of splenic MCL is of the blastic or blastoid type, which has a unique architecture and genesis.[84] A major difference of the blastoid MCL as seen in the dog is that the lesion is diffuse with multifocal nodules of lymphoid tumor throughout a more uniformly enlarged organ. The lymphoid nodules are roughly twice the size of

germinal centers, and in the well- advanced neoplasm many or most of these will appear as a narrow rim of lymphoid tissue surrounding a central lake of red blood cells.[105] In viewing multiple areas and neoplastic foci the progression of lesions can be deduced to arise as proliferative nodules, apparently overgrow their blood supply, and undergo central ischemic necrosis. These necrotic cells rapidly disappear and the central area of the nodule becomes filled with blood, presumably as a result of having arisen on an end arteriole. Animals with this form of MCL often have recurrent febrile episodes and a gammopathy, both of which might arise as a result of the continuing areas of tumor necrosis.[105] Both of these changes regress or disappear following splenectomy, which suggests that the gammopathy is not a constituitive product of the tumor cells but of host response to the tumor and tissue necrosis.[105] Cytologically, the cells of the blastoid type of MCL have nuclei that are 1.5 red cells in diameter and may be round, oval, or triangular and have shallow nuclear indentations. The chromatin pattern is coarse granular with a regular deposition highlighting the nuclear membrane and mild parachromatin clearing. There are 1–2 small but prominent nucleoli and 4–6 mitoses/400× field. Within the individual neoplastic foci there may a fading dendritic area or a central area of smaller benign mantle cells with round and densely stained nuclei. Most of the foci have numerous tingible body macrophages and a number of apoptotic nuclei.[105]

The surrounding areas of sinus have a mixture of lymphocytes and atypical plasmacytoid cells, and the muscular veins have endothelial colonization by the same cells as the blastoid follicles. The architectural pattern of blastoid MCL is not specific to the diagnosis but to the nature of the canine splenic circulation and may occur in other types of neoplasms including plasmacytoma and mast cell tumor.[105]

6.1.3 Non Angiomatous- non Lymphomatous neoplasms

Angiosarcoma and lymphoma are the most common primary canine splenic tumors,[9] but splenic neoplasms can arise from a variety of intrinsic tissues such as smooth muscle, fibrous or nervous tissues. This type of neoplasms have been grouped under the term Non Angiomatous-Non Lymphomatous sarcomas (NANLs). NANLs are uncommon neoplasms with uncertain characterization[16,88,89,110] and account for 23% to 34% of primary splenic neoplasms[16,89].

The largest case load study made by Splangler et al. (1994) evalulated 87 NANLs and morphologic classification of these lesions in standard H&E preparations yielded the following neoplastic groups: fibrosarcoma (1 9/87), undifferentiated sarcoma (1 9/87), leiomyosarcoma (1 4/87), osteosarcoma (8/87), mesenchymoma (7/87), myxosarcoma (6/87), histiocytic sarcoma (6/87), leiomyoma (3/87), lipoma-myelolipoma (2/87), liposarcoma (2/87), and malignant fibrous histiocytoma (1/87). Among 83/ 87 cases in which the breed was reported, 30 distinct breeds were represented. The most prevalent breeds were: Golden Retriever (10/83), Labrador Retriever (9/83), German Shepherd (6/83), Cocker Spaniel, Cockapoo, and Poodle (5/83), Sheltie, Schnauzer, and Doberman Pincer (3/83). The remaining 19 breeds were represented by one or two cases each.[89]

Female dogs appear to be overrepresented among splenic sarcomas (66%, 53/82). Morphologic classification of each of the splenic tumours was based of gross and microscopic features:

- Fibrosarcoma was characterized by the presence of spindle cells embedded in an abundant amount of extracellular collagen
- Leiomyosarcoma was composed of elongated spindle-shaped cells containing blunt-ended nuclei and arranged in long interlacing bundles.

- Undifferentiated sarcoma was characterized by no anatomic clues regarding tissue of origin. These tumors demonstrated a high level of cellular pleomorphism, lacked evidence of differentiation in any location, and had no diagnostic cytological features or extracellular matrix
- Osteosarcoma was characterized by the production of neoplastic osseous matrix with entrapment of individual neoplastic cells. Mesenchymoma as an anatomic class of lesions was characterized by the presence of two or more distinct cell types. Among those seen in this study, adipose tissue was a consistent feature. Myxomatous, osseous, and/or chrondroid matrix was also consistently present.
- Mixosarcoma was characterized grossly by mucinous stringy secretions exuded from the cut surfaces. Microscopically, a uniformly occurring basophilic mucinous intercellular matrix surrounding spindle or stellate fibroblastic type cells distinguished this group as a distinct morphologic entity.
- Histiocytic sarcoma was characterized by the lack of intercellular matrix and dissociation of pleomorphic polyhedral-shaped cells. These masses were composed of cells with extreme variability in nuclear size and shape. The lack of apparent intercellular association and cohesiveness distinguished this type of neoplasm anatomically from undifferentiated sarcomas.
- Leiomyoma was a discrete, expansile nodular splenic masses made up of well-differentiated myocytes with fibrillar eosinophilic cytoplasm and elongated, bluntended nuclei, typical of those found in mature smooth muscle.
- Lipoma occurred as solitary fatty, soft, pale, circumscribed nodules that sometimes contained aggregates of hematopoietic cells (myelolipoma).

- Liposarcoma consisted of pleomorphic, often polygonal cells with cytoplasmic lipid droplets of varying size and a scalloped nucleous.
- Malignant fibrous histiocytomas was characterized by a peculiar storiform pattern (Weinstein et al., 1989)

The aforementioned study divided neoplasms according to their biological behaviour into 3 cathegories:

(1) benign, noninvasive tumors (leiomyoma, lipoma) with prolonged survival intervals; (2) malignant tumors (fibrosarcoma, undifferentiated sarcoma, leiom yosarcoma, osteosarcoma, myxosarcoma, histiocytic sarcoma, and liposarcoma), showing severely truncated survival (median 4 months with 80-100% mortality after 12 months; (3) intermediate survival periods (median 12 months with 50% **1** year survival) attributed to a single group of neoplasm, the mesenchymomas.

The biological behavior of primary splenic nonangiomatous, nonlymphomatous sarcomas was most closely correlated with observed mitotic index. Splenic neoplasms of this type with a mitotic index < 9 showed significantly ($P < 0.000\ 1$) longer survival intervals than those with an index > 9.[89]

6.1.4 Fibrohistiocytic nodules

Nodular fibrohistiocytic proliferation in the canine spleen are characterized by a mixed population of histiocytoid and/or spindle cells in varying proportions intermixed with hematopoietic elements, plasma cells and lymphocytes.[90,105] This focal nodular proliferations of the canine spleen are commonly referred to as *nodular hyperplasia* or *fibrohistiocytic nodule.*[90] These lesions resemble what is referred to in the human spleen as *inflammatory pseudotumor*[43,72] and are differentiated from a similar lesion of spleen and other soft tissues known as *inflammatory myofibroblastic tumor* by being negative for the ALK tyrosine kinase that is found in anaplastic large cell

lymphoma of T-cell type.[43,72] Little is known of these lesions in animals, but they appear to form a continuum between splenic lymphoid nodular hyperplasia, defined as lesions of >70% lymphocytes with interspersed fibrohistiocytic cells (grade 1 SFHN); to malignant splenic stromal neoplasms, which are primarily fibrohistiocytic cells with <40% lymphocytes (grade 3 SFHN).[90]

A study by Spangler e Kass (1998) evaluated 98 canine splenic samples with focal lesions characterized by a combine proliferation of lymphoid and fibrohistiocytic cells. Among the 93/98 dogs with complete (12 months) follow-up information, 48% (45/93) were alive and 52% (48/93) were dead. Dogs that died or were euthanized during the follow-up period had a median survival of 5 and 5.5 month respectively (range 0-15 months). Forty-four percent (21/48) died from causes linked to their splenic disease, and 35% (17/48) died from competing causes. This study highlight tha lymphoid: fibrohistiocytic proportion and mitotic index in the nodules were anatomic features most predictive of postplenectomy mortality. A higer proportion of lymphoid to fibrohistiocytic type cells was associated with ncreased long-term survivl, whereas lower lymphoid:fibrohistiocytic proportions and higher mitotic index indicated a probability of higher short term mortality.

Recently a study by Moore et al. (2012) retrospectively evaluated the clinical course in 32 dogs with splenic fibrohistiocytic nodules following splenectomy, and retrospectively subjected available samples to immunohistochemistry and reclassification in an attempt to identify prognostic factors for survival. After immunohistochemistry they re-classified 31 avaliable samples as nodular hyperplasia (13; 8 complex, 5 lymphoid including 2 marginal zone), lymphoma (4;2 marginal one lymphoma, 1 high grade B cell lymphoma and 1 marginal zone transitional to high grade B-cell lymphoma), 8 stroma sarcoma, and 6 histiocytic sarcomas. Dogs with

histiocytic sarcoma had a worse survival (median 74 days)than dogs with other diseases.[48,49] According to these results they felt that the definition of disease has evolved beyond the usefulness of the original description, and therefore the term splenic fibrohistiocytic nodules is no more warranted to describe a specific disease entity.[48] Careful morphological assessment coupled using immunohistochemistry reveals a plethora of other diseases with widely different outcomes and requiring different treatment strategies.[48]

6.2 Splenic Metastatic Neoplasms

The spleen is often involved in metastatic neoplastic disease. In humans, when the spleen is involved in metastatic carcinoma there typically are already widespread areas of tumor in other tissues. In animals, the spleen is most often involved in metastatic sarcoma rather than carcinoma.[105] The reason generally expressed for the low incidence of metastatic carcinoma in spleen is not that the spleen is not unexposed, but the sinus system is relatively efficient in preventing colonization.[105] Even the cat with more carcinomas of the oral cavity and mammary gland than the dog, has few metastatic carcinomas in the spleen as compared to lung and regional nodes.[105]

7. AIM

This study has been divided into a prospective and in retrospective part.

The aim of the prospective part was to evaluate whether contrast-enhanced ultrasound can be used to more accurately characterize the perfusion of splenic focal abnormalities in small animals. Moreover, we attempted to establish criteria that can be used to distinguish benign from malignant splenic lesions and different types of malignancies.

The aim of the retrospective study is to evaluate the role of cytology in the diagnosis of primary and metastatic splenic tumours using histology as the gold standard.

8. Materials and methods

8.1 Prospective study

The prospective study included:

1. dogs and cats with one or multiple focal splenic lesions identified by gray-scale ultrasound
2. only nodules defined as a focal lesion not larger than 5 cm in diameter were included
3. a contrast enhanced ultrasound examination was performed to evaluate the perfusion of the lesion
4. the perfusion of the lesion was compared with the normal surrounding spleen
5. a presumptive diagnosis was attained by the radiologists
6. a definitive diagnosis for comparison was obtained by cytology, histopathology or both. Cytological samples by imprint were collected also from biopsies, splenectomized samples or post-mortal in order to compare cytology and histology.

1. Data collection:

The following data were collected:

- ID of the patient (name of the owner or ID number, species, breed, sex, age, weight)
- number and type (focal, multifocal, size) of lesions in the patient
- number of lesions analyzed by contrast ultrasound
- type of probe/s
- grey scale appearance of each studied lesion/s

- type of tissue sample collected (FANB cytology, core biopsy, surgical biopsy, splenectomy or post mortem sampling).
- type and Gauge of needle, length of sample.
- definitive diagnosis
- overall quality of the contrast study (poor – fair - good – excellent)
- any other comment

2. Contrast medium:

- Sonovue® (Bracco)
- dosage 0,03 ml/Kg
- a new vial of fresh-prepared contrast medium was used to perform the study.

3. Ultrasound equipment:

- an ultrasound system with dedicated contrast harmonic modality must was used.
- Two types of probes are desired:
    1. one linear median/high-frequency probe – (range 5-10 MHz) – for smaller and more superficial lesions
    2. one curvilinear or linear probe with lower frequency (range 2-5 MHz) – for larger and deeper lesions
- one or both probes were used to perform the contrast study.

4. Grey-scale examination:

Before any contrast study, grey-scale imaging was repeated to document the lesion/lesions in order to assess:

- number
- size
- margination (well or poorly defined)

- distribution
- echogenicity in comparison with the normal spleen
- echogenicity of the lesion (homogeneous, heterogeneous)

5. Setting for the contrast-study:

- low MI (< 0,1 – suggested 0,08)
- frame rate between 10 and 15 Hz
- high dynamic range (50 Db or higher)
- no persistence (setting on 0)
- use a single focal zone in the deepest part of the lesion
- select a grey or coloured map with progressive luminance
- if available, use the B-Mode side by side image mode.
- a contrast and B-Mode overlay was not used
- system post-processing such as “Maximum Intensity Projection”, “smoothing”, “Vascular Recognition” or other processing which may alter the pure rendering of the instantaneous concentration of contrast microbubbles were not used.

6. Contrast examination:

- in case of multiple nodules, one lesion should was chosen considering adequate image quality and accessibility for the following biopsy.
- if the lesion was heterogeneous in echogenicity on grey-scale ultrasound, the scan plane during the contrast examination represented the different parts of the nodule.
- the contrast medium was administered as a rapid intravenous bolus followed by injection of 5 ml of saline.
- the timer was started at the moment of the start of injection.

- the contrast examination was performed so that the lesion was evaluate during the injection keeping the same plane at least for 120 seconds. After this time, a complete scan of the lesions and of the surrounding spleen was done, to identify lesions with different perfusion in the late phase or previously undetected lesions.
- the entire examination is recorded, each video is saved as an DICOM file.
- All DICOM files should be submitted to an ECVDI diplomated.

7. Cytology:

- When possible, cytology was always performed initially.
- Cytology was air dried and stained with May Grünwald-Giemsa stain as follow:
    - Undiluted May Grünwald stain (10 minutes)
    - Rinse in de-ionized water
    - Giemsa stain (1:40 dilution in de-ionized water)
    - Rinse in de-ionized water
    - Dry in the heater
    - Covering slides with mounting media (Eukitt Bioptica©)

8. Biopsy samples

- the decision to sample was performed based on the experience of the radiologist
- whenever possible, a core biopsy was taken. The gauge and type of biopsy device was recorded as part of the information of each case.
- Sample was also taken after splenectomy.
- All samples were fixed in 10% buffered formalin and sent for histopathology.
- Formalin fixed tissues were processed as follow:
    - Tissues were trimmed ant put into histology cassettes.

- Dehydration of samples through a series of alcohols (70% to 95% to 100%) and clearing with xylene (12 hours)
- Embedding in paraffin (1 hour)
- Sectioning with microtome (5 μm thick)
- Staining with Hematoxylin and Eosin (H&E) Staining Protocol
    - clearing with xylene (15 minutes)
    - Sections are brought to distilled water through a series of alcohols (100% to 95% to 70%)
    - Rinse in de-ionized water
    - Stain nuclei with haematoxylin (25 minutes)
    - Rinse in running tap water (5 minutes)
    - Stain with eosin (3 minutes)
    - Dehydration of samples through a series of alcohols (70% to 95% to 100%)
    - Clearing with xylene 2 times (15 minutes each)
    - Covering slides with mounting media (Eukitt Bioptica©)

8.2 Retrospective study

Correlation between cytology and histology

The cytology database of the department of veterinary pathology diagnostic service of the University of Milan, were examined for canine splenic samples using key words such as dog, spleen and splenic. In particular cytological and histological specimens obtained between 1998 and 2012 from splenic parenchyma were retrospectively evaluated.

Information collected from medical records:

- gender, age, breed of dogs
- type of sampling,
- gross appearance
- total number of cytological samples
- total number of histological samples
- cases with both cytology and histology available were reviewed
- Cytological diagnosis were compared with histopathological and results were grouped into 4 categories:
    - true positive (TP) included cytological diagnosis of neoplasia or suspected neoplasia that were exactly or almost exactly the same as the histopathological diagnosis.
    - false positive (FP) included all cytological neoplastic samples diagnosed as non neoplastic on histology
    - true negative (TN) was assigned if either the cytology or hystophatology reports indicated a non neoplastic diagnosis

- false negative (FN) included all cytological non neoplastic sample that was diagnosed as neoplastic on histology.

- Using the aforementioned categories we calculated
  - Accuracy. It indicate the closeness of agreement between cytological and histologic diagnosis in neoplastic diseases. It was calculated by the sum of TP and TN divided by the total number of cases.
    [(VP+VN)/(VP+VN+FP+FN)]·100
  - Specificity. It indicate the probability that use of cytology would detect the absence of disease and was calculated as the number of cases with agreement between cytologic and histopathologic diagnosis of no disease, divided by total number of cases with no disease.
    [VN/(VN+FP)]·100
  - Sensitivity. It was defined as the probability that use of cytology would detect disease and was calculated as the number of cases with agreement between cytologic and histopathologic diagnoses for a disease, divided by the total number of cases in which animals had that disease.
    [VP/(VP+FN)]·100
  - Positive predictive value. It indicates the likelihood that a dog with a diagnosis of neoplasia would actually have a neoplasia.
    [VP/(VP+FP)]·100
  - Negative predictive value. It indicates the probability that a dog with a negative diagnosis of neoplasia is really free of this condition
    [VN/(VN+FN)]·100

Primary Splenic Lymphomas

Canine cases of confirmed primary splenic lymphoma were identified. For the application of the WHO classification cell size, the number of mitoses and tumor cell phenotype was assessed.

- Cell size: lymphomas were classified as small (nuclei approximately 1 times the diameter of a red blood cell), intermediate (nuclei approximately 1.5 to < 2 times the diameter of a red blood cell) and large (nuclei approximately ≥2 times the diameter of a red blood cell).
- The number of mitoses was evaluated by counting mitoses per 10 representative, artefact-free, fields at 400× magnification
- Immunohistochemistry: 5 m tissue sections were glued onto polylysine coated glass slides and were dewaxed and rehydrated before immunostaining. Immunohistochemical staining was performed utilizing monoclonal anti-human CD79a- (Dako, Glostrup, Denmark) at 1:100 and polyclonal anti-human CD20 (Neomarkers, Freemont, CA) at 1:400 for B cells recognition. Polyclonal anti-CD3- (Dako, Glostrup, Denmark) was utilized at 1:900 dilution for the identification of the intracellular epsilon chain expressed mostly in T cells. Heat induced antigen retrieval was performed by incubation in citrate buffer (pH 6.4) and heated in a microwave oven for 1 min at 900 watt and for 3 min for two times at 750 watt and then cooled at room temperature. Slides with primary antibodies were incubated overnight in a humidified chamber at 4◦C. Secondary detection was performed with the Avidin-Biotin enzyme Complex (ABC kit, Vectastain®, Burlingame, CA, USA) for 30 min. The immunoreaction was visualized with amino-9-ethylcarbazole chromogen (AEC, Kit, Vector,

Burlingame, CA, USA). Smears were counterstained with Mayer's haematoxylin for 3 min and cover-slipped with an aqueous mounting media (Glycerine, Sigma–Aldrich®, St. Louis, MO, USA).

9. Results

9.1 Prospective study

A total of 26 cases of canine focal splenic masses analysed with contrast-enhanced ultrasound with the association with cytological and/or histological diagnoses was collected.

In one case contrast-enhanced ultrasound was performed twice, therefore 27 focal splenic lesions where characterized.

Eleven cases had both cytological and histological diagnoses, whereas in 12 and 6 cases only cytological or histological diagnoses were available respectively.

Regarding signalment, the majority of dogs were mixed breed (6 cases) followed by Labrador Retriever (4 cases), Dachshund (3 cases), Boxer (2 cases), English Setter (2 cases), Pomeranian (1 case), Breton (1 case), Golden Retriever (1 case), Pointer (1 case), Poodle (1 case), German Shepherd (1 case), Italian Hound (1 case), Rottweiler (1 case) and Bernese Mountain dog (1 case).

There were 14 males (53,8%) and 12 females (46,2%) with a M/f ratio of 1,16. Mean age was 9,7 years with a range from 4 to 14 years.

The mean weight of dogs was 20,8 with a range from 4 to 50 kilograms (Kg). When grouped into two categories (> or < 15 Kg), 19 dogs belonged to the > 15 Kg category whereas 9 dogs weighted less than 15 Kg.

Two dogs had two concurrent different neoplasms, in one dog concurrent fibrohistiocytic nodule in conjunction with a mantle cell lymphoma and in the other myeololipoma associated with an angioma. They were considered as a single case at contrast-enhanced ultrasound since both neoplasms where located within the same focal lesion in both dogs.

On gray-scale ultrasound examination, single (21 cases) or multiple parenchymal lesions (6 cases) of variable size and echogenicity were detected.

Histologically the definitive diagnosis included 11 benign and 15 malignant and splenic lesions, whereas in one case the sample was non diagnostic.

The 11 benign histological lesions included extramedullary hematopoiesis associated with reactive lymphoid hyperplasia (4 cases), examedullary hematopoiesis (2 cases), reactive lymphoid hyperplasia (2 cases), extramedullary hematopoiesis associated with hemosiderosis (1 case), myelolipoma (1 case), myelolipoma associated with angioma (1 case).

Malignancy was represented by the following diseases: hemangiosarcoma (7 cases), mantle cell lymphoma (3 cases), marginal lymphoma (2 cases), histiocytic sarcoma (1 case), fibrohistiocytic nodules associated with mantle cell lymphoma (1 case) and fibrohistiocytic nodules (1 case).

On B-mode ultrasound 19 lesions were hypoechoic, 6 were characterized by a mixed echogenicity and 2 were isoechogic. No lesions were hyperechoic on B-mode analysis.

Among benign lesions 9 were hypoechoic (4 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis, 2 cases of extramedullary hematopoiesis, 2 reactive lymphoid hyperplasia and 1 case of myelolipoma associated with angioma) and 2 focal lesions were characterized by a mixed echogenicity (1 case of extramedullary hematopoiesis associated with hemosiderosis and 1 case of myelolipoma).

Among malignant lesions 9 were hypoechoic (3 cases of haemangiosarcoma, 2 marginal zone lymphoma, 2 mantle cell lymphoma, one histiocytic sarcoma and one fibrohistiocytic nodule), 4 focal lesions were characterized by a mixed echogenicity (4

cases of haemangiosarcoma) and 2 focal lesions were isoechoic (1 fibrohistiocytic nodule and 1 mantle cell lymphoma).

No cavitations were found in 23 cases whereas 3 focal splenic lesions were characterized by the presence of cavitation at B-mode analysis (2 cases of haemangiosarcoma and 1 case of myelolipoma).

Histologicla type and Gray-Scale Appearance of Splenic Focal Lesions are summerized in Table 1.

All contrast studies were of diagnostic quality, although the signal-to-noise ratio was improved subjectively during the second contrast agent injection.

After contrast medium injection the enhancement pattern was homogeneous in 23 focal lesion and heterogeneous in 3 cases and all of them were malignant (1 marginal lymphoma,1 haemangiosarcoma and 1 fibrohistiocytic nodule).

In the wash-in phase 7 focal lesions (3 mantle cell lymphoma, 1 mantle cell lymphoma associated with a fibrohistiocytic nodules, 1 myelolipoma associated with an angioma, 1 reactive lymphoid proliferation and 1 extramedullary hematopoiesis associated with hemosiderosis) were characterized by an hyperechoic appearance, 9 by an isoechoic appearance (4 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis, 1 hystiocytic sarcoma, 1 fibrohistiocytic nodule, 1 marginal zone lymphoma, 1 reactive lymphoid hyperplasia an 1 extramedullary hematopoiesis), 7 by a mildly hypoechoic appearance (5 cases of haemangiosarcoma, 1 case of myeololipoma, 1 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis) 2 (1 hemangiosrcoma and 1 marginal cell lymphoma) by a moderately hypoechoic appearance and 1 hemangiosarcoma) had an extensively hypoechoic appearance.

At peak 7 focal lesions (3 mantle cell lymphoma, 1 mantle cell lymphoma associated with an fibrohistiocytic nodules, 1 myelolipoma, 1 reactive lymphoid proliferation and 1 extramedullary hematopoiesis associated with hemosiderosis) were characterized by an hyperechoic appearance, 8 (4 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis, 1 hystiocytic sarcoma, 1 fibrohistiocytic nodule, 1 reactive lymphoid hyperplasia an 1 extramedullary hematopoiesis) by an isoechoic appearance, 8 (5 cases of haemangiosarcoma, 1 case of myeololipoma associated with angioma, 1 case os marginal zone lymphoma and 1 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis) by a mildly hypoechoic appearance, 2 (1 hemangiosrcoma and 1 marginal cell lymphoma) by a moderately hypoechoic appearance and 1 hemangiosarcoma had an extensively hypoechoic appearance.

In the wash-out phase 1 myelolipoma associated with an angioma was characterized by an hyperechoic appearance, 4 (2 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis,1 extramedullary hematopoiesis and 1 extramedullary hematopoiesis associated with hemosiderosis) by an isoechoic appearance, 18 (5 cases of haemangiosarcoma, 3 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis, 3 mantle cell lymphoma, 3 reactive lymphoid hyperplasia,1 case of myeololipoma, 1 case of histiocytic sarcoma, 1 case of marginal zone lymphoma, 1 case of a fibrohistiocytic nodule associated with a marginal zone lymphoma, 1 case of extramedullary hematopoiesis) by a mildly hypoechoic appearance, 2 (1 hemangiosrcoma and 1 marginal cell lymphoma) by a moderately hypoechoic appearance and 1 hemangiosarcoma had an extensively hypoechoic appearance.

Fourteen cases (7 hemangiosarcomas, 2 mantle cell lymphoma, 1 fibrohistiocytic nodule, 1 fibrohystiocytic nodule associated with mantle cell lymphoma, 1 myelolipoma, 1 marginal zone lymphoma and 1 case of extramedullary hematopoiesis associated with hemosiderosis) had visible feeding vessels whereas 12 cases were not characterized by the presence of feeding vessels (4 cases of reactive lymphoid hyperplasia associated with extramedullary hematopoiesis, 2 cases of extramedullary hematopoiesis, 2 cases of reactive lymphoid hyperplasia, 1 case of myelolipoma associated with angioma, 1 mantle cell lymphoma, 1 histiocytic sarcoma and one marginal zone lymphoma).

HAS were all characterized by a persistent hypoechoic appearance during all phases of contrast enhanced ultrasound examination and 6 of them had an homogeneous enhancement pattern. All HAS were characterized by the presence of feeding vessels along with 2 among 3 mantle cells lymphoma and 1 of the two marginal zone lymphoma present in the current study.

All mantle cell lymphoma were hyperechoic during wash in and wash out phases, whereas they were mildly hypoechoic during the wash out phase. They were characterized by an homogeneous enhancement pattern.

Marginal zone lymphomas were hypoechoic in all phases, but in one case that was isoechoic in the wash in phase.

Feeding vessels were present in 11/15 cases of malignancy and in 3/11 cases of benign lesions.

Contrast enhanced ultrasound findings are summarized in Table 2

9.2 Retrospective study

Prevalence

Cytological samples:

A total of 22.979 cytological samples was retrieved from the electronic archives in 15 years (1998-2013). Among them, 653 cytological samples were obtained from canine splenic parenchyma and 214 were classified as neoplastic with prevalence of neoplastic diseases of 32,8%.

Histological samples

In the same time frame 21.329 histological samples were retrieved from the electronic archives, 428 of came from canine splenic parenchyma. Two hundred and nineteen splenic samples were classified as neoplastic highlighting a prevalence of 51,2 %.

Signalment

Cytological samples

Regarding signalment data the majority were mixed breed dogs (26% - 56 dogs) followed by German Shepherd (14% - 30 dogs), Rottweiler (9% - 19 dogs), Boxer (8% - 14 dogs), Golden and Labrador Retrievers (6% - 13 dogs), Doberman (4% - 8 dogs), Setter (4% - 8 dogs), Sennenhunde (3% - 6 dogs), Cocker (2% - 5 dogs) and other breeds (24% - 51 dogs).

For the cytopathological submission 53% (113 dogs) of the animals were male, 6% (7 dogs) of which were neutered, and 47% (101) of female, 16% (16 dogs) of which were neutered with a female to male ratio of 0,89.

Histological samples

Samples submitted for histopathological analysis included mixed breed dogs (36% - 79 cases), German Shepherd (12% - 26 cases), Boxer (8% - 17 cases), Rottweiler

(6% - 13 cases), Golden and Labrador Retriever (4% - 9 cases), Setter (4% - 9 cases), other Shepherds (3% - 6 cases), Cocker (3% - 6 cases), Doberman (1% - 2 cases) and other breeds (23% - 50 cases).

Regarding histopathological sample submissions, canine gender distribution was characterized by 54 % male (118 cases), 8% (17 cases) of which were neutered, and 46% (101 cases) were female, 22% (48 cases) of which were neutered with a female to male ratio of 0,85.

Diagnoses

Cytological samples

Of the 214 cytological cases extracted, the most common diagnoses were lymphoma (34 cases) and lymphoma/lymphoid leukemia (33 cases), followed by sarcoma (10 cases), hemangiosarcoma (9 cases), lymphocytic leukemia (5 cases), plasmocytoma (2 cases), mesenchymal neoplasia (2 cases), mielolipoma (2 case), liposarcoma (3 cases), lipoma (3 cases), fibrosarcoma (2 cases), myeloma (2 cases), malignant undifferentiated neoplasm (2 cases), histiocytic neoplasm (2 case) , plasmocytosis (1 case), mieloma (1 case), haemophagocytic syndrome (1 case), metastatic neoplasia (33 cases) and suspected neoplasms (77 cases).

Histological samples

Hemangiosarcoma (74 cases) and lymphoma (59 cases) were the most frequent tumors encountered representing the majority of neoplastic diseases and were followed by angioma (24 cases), sarcoma (10 cases), histiocytic sarcoma (7 cases), fibrohistiocytic nodules (5 cases), myelolipoma (3 case), liposarcoma (3 cases), lipoma (3 cases), fibrosarcoma (2 cases), myeloma (2 cases), malignant mesenchyma neoplasm (2 cases), systemic reactive histiocytosis (1 case), histiocytoma (1 case) , plasmocytoma (1 case), mieloma/plasmocytoma (1 case),

myxosarcoma (1 case), haemophagocytic sarcoma (1 case), haemophagocytic syndrome (1 case), metastatic neoplasia (14 cases) and suspected neoplasms (6 cases).

Sixty-six cases met the inclusion criteria since both cytology and histopathology were available (Table 4).

According to the aforementioned grouping criteria, 35 cytological samples were classified as true positive (TP), 12 samples as true negative (TN), 18 samples as false negative (FN) and only 1 false positive (FP) sample was present.

Sarcomas were the most frequent neoplasms diagnosed among the TP group with 13 cases, 7 of which were hemangiosarcomas. The second most frequent category was represented by lymphomas (9 cases), followed by metastatic neoplasms (7 cases) and histolytic neoplasms (6 cases). Regarding their distribution, we had 14 focal to multifocal lesions and in 5 cases the spleen was diffusely enlarged, but in 15 cases this datum was not determined.

Among 18 FN samples, lymphoma (7 cases) was the most frequent histological diagnosis followed by sarcomas (5 cases), angiomas (3 cases), lipoma/mielolipomas 2 cases) and metastatic neoplasms (1 case). Most of them (10 out of 18 cases) were characterized by a focal to multifocal distribution, in one case the spleen was diffusely enlarged and the type of distribution was unknown in 7 cases.

Samples belonging to the TN category were characterized by a mixed histological appearance including, white and red pulp atrophy, hemosiderosis, extramedullary hematopoiesis and, blood stasis. Furthermore, in one case a diagnosis of piroplasmosis was made on cytology and confirmed by histopathology. Regarding gross appearance, most of the lesions were characterized as diffuse distribution.

The only false positive sample was diagnosed on cytology as a well differentiated spindle cell neoplasm, since was characterized by the presence of occasional spindle cells with mild atypia. On the contrary, scar tissue was evidenced on histology. In this case the lesion distribution was not recorded.

Overall, there was 71.2% agreement between cytologic and histologic diagnoses. In diagnosing splenic neoplasia, cytology had a sensitivity of 66 %, a specificity of 71%, a positive predictive value of 97%, and a negative predictive value of 40%.[107]

Primary Splenic Lymphoma

A total of 29 primary splenic lymphomas were collected from the electronic archives of the Anatomical Pathology service of the School of Veterinary Medicine of the University of Milano, Italy. Grade was determined according to the WHO classification (cell size, diagnosis and mitotic index). In Table X the list of all the neoplastic samples with the corresponding mitotic index, phenotype and grade is reported.

The majority of dogs were mongrel (16 cases), followed by 3 Boxers, 1 Cairn Terrier, 1 Fox Terrier, 1 German Shepherd, 1 West Highland White Terrier, 1 Siberian Husky, 1 Pyrenean Mountain Dog, 1 Cocker Spaniel, 1 medium Schnauzer, 1 Rottweiler and 1 Lagotto. Thirteen dogs were female, 4 were spayed female and 12 were male. Age ranged from 5 to 14 years, with a mean age of 9 years. In 2 cases age was not recorded.

Eleven Marginal zone lymphomas (MZLs) were diagnosed, followed by 7 diffuse lymphomas, 3 Mantle cell lymphomas (MCLs), 2 follicular lymphomas, 2 lymphocytic lymphomas, 2 Peripheral T-cell lymphomas (PTCL), 1 hepatosplenic lymphoma and 1 nodular lymphoma NOS.

Nineteen were classified as low grade lymphomas, 7 classified as Intermediated grade, 2 were classified as high grade and 1 was classified as low-intermediated grade.

Regarding phenotype of lymphomas, twenty-three were classified as B cell lymphoma, 3 as T cell lymphomas and 3 as NK-like lymphomas.

Signalment, phenotype, grade, histological diagnosis of primary splenic lymphoma are listed in Table 5.

10. Tables

Table 1. Histologicla type and Gray-Scale Appearance of Splenic Focal Lesions							
Lesion	**Mean Size (Range) (cm)**	**Single (s) or Multiple (m)**	**Hypoechoic**	**Hyperechoic**	**Isoechoic**	**Mixed**	**Cavitations**
Malignancy (n= 15)							No
Hemagiosarcoma (*n*=7)	3,24 (1-6)	2m +1s	3			4	Yes (n=2) No (n=5)
Mantle cell Lymphoma (*n*=3)	1,73 (1-3,5)	1m +2s	2		1		No
Marginal Zone Lymphoma (*n*=2)	1,15 (1-2,3)	s	2				No
Histiocytic sarcoma (*n*=1)	n.d.	m	1				No
Fibrohistiocytic nodule (*n*=1)	3	s			1		No
Fibrohistiocytic nodule + Mantle cell Lymphoma (*n*=1)	6 + 3	m	1				No
Benignancy (*n*= 11)							No
EMH* + RLH** (*n*= 4)	0,83 (0,5-1)	s	4				No
EMH (*n*= 2)	1,75 (1,5-2)	s	2				No
RLH (*n*= 2)	1,25 (1-1,5)	1s+1m	2				No
EMH + hemosiderosis (*n*= 1)	3	s				1	No
Myelolipoma + angioma (*n*= 1)	1	s	1				No
Myelolipoma (*n*= 1)	7	s				1	Yes

* Extramedullary hemaopoiesis

** Reactive lymphoid hyperplasia

Table 2. Contrast Enhanced Ultrasound Diagnostic Findings/Criteria for Malignancy and Benignancy					
	Contrast enhanced ultrasound findings				
	Echogenicity (lesion vs spleen)				
Lesions	**Wash-in**	**Peak**	**Wash out**	**Enhancement pattern**	**Feeding vessels**
Malignancy (*n*= 15)					
Hemagiosarcoma (*n*=7)	Mildly hypo (n=5) Moderately hypo (n=1) Extensively hypo (n=1)	Mildly hypo (n=5) Moderately hypo (n=1) Extensively hypo (n=1)	Mildly hypo (n=5) Moderately hypo (n=1) Extensively hypo (n=1)	Homogeneous (n=6) Heterogeneous (n=1)	Yes
Mantle cell Lymphoma (*n*=3)	Hyper	Hyper	Mildly hypo	Homogeneous	Yes (n=2) No (n=1)
Marginal Zone Lymphoma (*n*=2)	Iso (n=1) Moderately hypo (n=1)	Mildly hypo (n=1) Moderately hypo (n=1)	Mildly hypo (n=1) Moderately hypo (n=1)	Homogeneous (n=1) Heterogeneous (n=1)	Yes (n=1) No (n=1)
Histiocytic sarcoma (*n*=1)	Iso	Iso	Mildly hypo	Homogeneous	No
Fibrohistiocytic nodule (*n*=1)	Iso	Iso	Iso	Heterogeneous	Yes
Fibrohistiocytic nodule + Mantle cell Lymphoma (*n*=1)	Hyper	Hyper	Mildly hypo	Homogeneous	No
Benignancy (*n*= 11)					
EMH* + RLH** (*n*= 4)	Iso (n=3) Mildly hypo (n=1)	Iso (n=3) Mildly hypo (n=1)	Iso (n=1) Mildly hypo (n=3)	Homogeneous	No
EMH (*n*= 2)	Iso	Iso	Iso (n=1) Mildly hypo (n=1)	Homogeneous	No
RLH (*n*= 2)	Iso (n=1) Hyper (n=1)	Iso (n=1) Hyper (n=1)	Mildly hypo (n=1)	Homogeneous	Yes (n=1) No (n=1)
EMH + hemosiderosis (*n*= 1)	Hyper	Hyper	Iso	Homogeneous	Yes
Myelolipoma + angioma (*n*= 2)	Hyper	Hyper	Hyper	Homogeneous	No
Myelolipoma (*n*= 1)	Mildly hypo	Mildly hypo	Mildly hypo	Homogeneous	Yes

* Extramedullary hemaopoiesis

** Reactive lymphoid hyperplasia

Table 3. Contrast Enhanced Ultrasound Finding in Lymphoma cases						
			Echogenicity (lesion vs. spleen)			
Diagnosis	**N° of cases**	**Cytology (C), Hystology (H)**	**Wash-in**	**Peak**	**Wash-out**	**Reference**
Lymphosarcoma	7		Hyper (n=2) Iso (n=5)	Hyper (n=2) Iso (n=5)	Hypo	Rossi F. et al., 2008
Lymphosarcoma	6	n.d	Iso	Iso	Mod hypo (n=1) Mildly hypo (n=1) Iso (n=4)	Ohlert S. et al., 2008
Lymphoma	3	C	Iso (n=2) Hetero (n=1)	Hypo	Hypo	Nakamura K. et al., 2010
Mantle cell Lymphoma	3	H	Hyper	Hyper	Mildy hypo	Current Study
Marginal zone Lymphoma	2	H	Iso (n=1) Moderately hypo (n=1)	Mildly hypo (n=1) Moderately hypo (n=1)	Mildly hypo (n=1) Moderately hypo (n=1)	

Tale 4: Cases with Cytological and Histological diagnosis							
N. cases	**N. samples**	**Breed**	**Sex**	**Age**	**Cytological diagnosis**	**Histological diagnosis**	**Diagnostic category**
1.	C 222/98 PN 58/98	Boxer	SF	8 years	Negative for Leishmania	Follicular splenic atrophy	True Negative
2.	C 890/98 PN 240/98	Rottweiler	M	2 months	Piroplasmosis	Piroplasmosis	True Negative
3.	C 1042/98 PN 271/98	Poodle	M	5 years	Suspected Lymphoma	Multifocal to coalescing follicular centrocytic centrobastic lymphoma (grade I)	True Positive
4.	C 1102/98 PN 298/98	ND	ND	ND	Lymphoid hyperplasia	Lymphoma	True Positive
5.	C 777/99 PP 861/99	Schnauzer	F	13 years	Sarcoma	Undifferentiated sarcoma with giant cells and locally extensve area of mixosarcoma.	True Positive
6.	C 799/99 PN 249/99	ND	M	5 years	Lymphoid hyperplasia associated with extramedullary hematopoiesis.	Linfoma. Extramedullary hematopoiesis	False Negative
7.	C 921/99 PN 287/99	ND	ND	ND	Mild extramedullary hematopoiesis	Hemosiderosis, extramedulary hematopoiesis, lymphoid atrophy and focal necrosis and fibrosis	True Negative
8.	C 849/99 PP 949/99	Mongrel	SF	11 years	Immunoblastic and centroblastic lymphoma	Diffuse large cell lymphoma (immunoblastic and centroblastic).	True Positive
9.	C 373/00 PN 90/00	Newfoundland	F	9 years	Suspected lymphoma	Hepato-spelnic lymphoma.	True Positive

10.	C 226/01 PN 73/01	German Shepherd	F	8 years	Suspected hemangiosarcoma	Well differentiated hemangiosarcoma	True Positive
11.	C 234/01 PN 77/01	French Bouledogue	M	3 years	Metastatic mast cell tumor	Mast cell tumor.	True Positive
12.	C 789/01 PP 1093/01	Rottweiler	M	8 years	Undifferentiated sarcoma (most likely histiocytic origin).	Histiocytic sarcoma	True Positive
13.	C 1284/01 PP 1591/01	Rottweiler	M	6 years	Hemangiosarcoma.	Hemangiosarcoma	True Positive
14.	C 322/01 PN 116/01	ND	ND	ND	Lymphoid hyperplasia and hemosiderosis	Diffuse stasis associated with hemosiderosis	True Negative
15.	C 959/01 PP 1328/01	ND	ND	ND	Hemodilution with rare reactive spindle cells without cytological atypia	Stasis with hemosiderosis	True Negative
16.	C 1284/01 PP 1591/01	Rottweiler	M	6 years	Hemangiosarcoma	Hemangiosarcoma	True Positive
17.	C 783/02 PP 586/02	Drahthaar	M	9 years	Suspected well differentiated spindle cell tumor	Hemosiderosis, mineralizations, Gamna-Gandy body	False Positive

18.	C 1059/02 PN 170/02	ND	ND	ND	Hemodilution	Metastatic pancreatic adenocarcinoma	FN
19.	C 1130/02 PN 180/02	ND	ND	ND	Suspected sarcoma Poor quality sample	Lymphoma. Poor quality sample	True Positive
20.	C 1476/02 PN 262/02	German Shepherd	NM	6 years	Sarcoma (most likely hemagiosarcoma)	Hemangiosarcoma	True Positive
21.	C 1748/02 PN 304/02	ND	ND	ND	Sarcoma	Undifferentiated malignant neoplasia (most likely histiocytic)	True Positive
22.	C 1992/02 PN 369/02	Mongrel	F	7years	Mastocytosis/ Mast cell leukemia	Mast cell leukemia	True Positive
23.	C 43//03 PN 12/03	ND	ND	ND	Extramedullary hematopoiesis	Multifocal infarcts associated with extramedullary haematopoiesis and lymphoid atrophy	True Negative
24.	C 1604/03 PP1 438/03	Fox terrier	SF	8 years	Lymphoid hyperplasia	Mantle cell lymphoma nodular	False Negative
25.	C 133/04 PP 147/04	ND	ND	ND	Hemangiosarcoma	Hemangiosarcoma solid	True Positive
26.	C 1074/04 PP 894/04	ND	ND	ND	Lymphoma	Lymphoma	True Positive
27.	C 920/04 PP 684/04	ND	ND	ND	Metastatic carcinoma	Metastatic adenocarcionoma	True Positive
28.	C 565/05	ND	ND	ND	Hemosiderosis and extramedullary	Lymphoid hyperplasia associated with	True

	PN 77/05				hematopoiesis	hemorrhages and extramedullary hematopoiesis	Negative
29.	C 705/05 PN 98/05	ND	ND	ND	Systemic mastocitosis	Mastocitoleucemia.	True Positive
30.	C 326/06 PN 83/06	Cocker Spaniel	NM	12 years	Sarcoma Poor quality sample	Metastatic carcinoma	True Positive
31.	C 617/06 PP 522/06	ND	ND	ND	Sarcoma	Well differentiated liposarcoma	True Positive
32.	C 738/06 PP 760/06	ND	ND	ND	Lymphoid hyperplasia and extramedullary hematopoiesis	Sarcoma	False Negative
33.	C ND/06 PP 759/06	Mongrel	ND	5aa	Lymphoid hyperplasia	Margina cell lymphoma	False Negative
34.	C 1148/06 PP 1219/06	ND	ND	ND	Sarcoma	Histiocytic neoplasm	True Positive
35.	C 1181/06 PP 1208/06	ND	ND	ND	Lymphoid hyperplasia	Marginal zone lymphoma.	False Negative
36.	C 327/07 PP 279/07	ND	ND	ND	Lymphoid hyperplasia and extramedullary hematopoiesis	Extramedullary hematopoiesis	True Negative
37.	C 645/09 PP 715/09	ND	ND	ND	Hemodiution	Follicular lymphoid atrophy	True Negative
38.	C 682/09 PP 759/09	German Shepherd	M	14 years	Sarcoma	Hemangiosarcoma	True Positive
39.	C 232/10 PP 230/10	German Shepherd	NM	6 years	Lymphoid hyperplasia	Margina zone lymphoma	False Negative
40.	C 374/10 PP 366/10	ND	ND	ND	Hemodilution	Hemangiosarcoma	False Negative

41.	C 530/10 PP 563/10	Schnauzer	F	10 years	Lymphoma	Follicular lymphoma	True Positive
42.	C 944/10 PP 1229/10	ND	ND	ND	Hemodilution	Atherosclerosis associated with Gamma gandy areas	True Negative
43.	C 982/10 PP 1089/10	German Shepherd	M	10 years	Sarcoma	Undifferentiated sarcoma	True Positive
44.	C 1270/10 PP 1425/10	Shitzu	M	10 years	Extramedullary hematopoiesis	Myelolipoma	False Negative
45.	C 116/11 PP 145/11	Mongrel	M	12 years	Hemangiosarcoma	Hemangiosarcoma.	True Positive
46.	C 329/11 PP 420/11	Cocker Spaniel	M	12 years	Sarcoma	Giant cell sarcoma	True Positive
47.	C 406/11 PP 505/11	Pomeranian	M	6 years	Lymphoid hyperplasia	Marginal zone lymphoma	False Negative
48.	C 724/11 PP 937/11	Norfolk terrier	SF	10 years	Extramedullary hematopoiesis and hemosiderosis	Hemangioma	False Negative
49.	C 725/11 PP 1001/11	Golden Retriever	F	2 years	High grade lymphoma	Large cell diffuse lymphoma	True Positive
50.	C 757/11 PP 961/11	Boxer	M	8 years	Suspected lymphoma	Mantle cell lymphoma	True Positive

51.	C 846/11 PP 1033/11	Mongrel	M	11 years	Lymphoid hyperplasia and extramedullary hematopoiesis	Fibrohistiocytic nodule and hemangioma	False Negative
52.	C 847/11 PP 1073/11	ND	ND	ND	Extramedullary hematopoiesis	Hemangiosarcoma	False Negative
53.	C 985/11 PP 1170/11	Bernese Mountain Dog	SF	8 years	Hemophagocytic syndrome	Hemophagocytic syndrome	True Positive
54.	C 1009/11 PP 38/12	Golden Retriever	F	12 years	Sarcoma (most likely hemagiosarcoma) B: Lymphoid hyperplasia	A: Fibrohistiocytic nodules with areas of mantle cell lymphoma and areas of histiocytic sarcoma B: Mantle cell lymphoma	True Positive False Negative
55.	C 1063/11 PP 10/12	Labrador Retriever	F	9 years	Extramedullary hematopoiesis	Lipoma	False Negative
56.	C 135/12 PP 155/12	German Shepherd	M	9 years	Hemangiosarcoma	Hemangiosarcoma	True Positive
57.	C 233/12 PP 257/12	Mongrel	F	10 years	Sarcoma	Hemangiosarcoma	True Positive
58.	C 238/12 PP 268/12	Mongrel	NM	12 years	Hemodilution	Hemangioma	False Negative
59.	C 446/12 PP 486/12	Mongrel	M	11 years	Hemangiosarcoma	Undifferentiated sarcoma	True Positive
60.	C 626/12 PP 667/12	Mongrel	SF	13 years	Extramedullary hematopoiesis and hemosiderosis	Hemangiosarcoma	False Negative
61.	C 850/14 RC 65/12	ND		ND	Hemodilution	Hemangiosarcoma	False Negative

62.	C 851/12 RC 64/12	Yorkshire Terrier	M	7 years	Hemodilution	Multifocal hematomas associated with a diffuse hemosiderosis and white pulp atrophy	True Negative
63.	C 762/12 RC 54/12	Belgian Shepherd	SF	10 years	Hemodilution	Multifocal hematomas associated with a diffuse hemosiderosis	True Negative
64.	C 760/12 RC 53/12	Dougue de Bordeaux	NM	8 years	Hemangiosarcoma	Metastatic adenocarcinoma	True Positive
65.	C 369/12 RC 25/12	Mongrel	NM	12 years	Epithelial metastatic neoplasia	Epithelial metastatic neoplasia	True Positive

Table 5. List of Primary Splenic Lymphoma						
	Breed	**Sex**	**Age**	**Histological Diagnoses**	**Grade**	**Phenotype**
1	Mongrel	Female	12 years	Marginal zone lymphoma	Low	B
2	Boxer	Female	5 years	Follicular lymphoma	Low	B
3	Cairn Terrier	Male	8 years	Marginal zone lymphoma	Low	B
4	Mongrel	Female	8 years	Marginal zone lymphoma	Low	B
5	Fox Terrier	Spayed Female	8 years	Mantle cell lymphoma	Low	B
6	Mongrel	Female	Adult	Marginal zone lymphoma	Low	B
7	Mongrel	Male	11 years	Marginal zone lymphoma	Low	B
8	Mongrel	Male	8 years	Marginal zone lymphoma	Low	B
9	Mongrel	Spayed Female	14 years	Marginal zone lymphoma	Low	B
10	Mongrel	Male	9 years	Marginal zone lymphoma	Low	B
11	Mongrel	Male	7 years	Marginal zone lymphoma	Low	B
12	Mongrel	Male	9 years	Mantle cell lymphoma	Low	B
13	Cocker	Spayed Female	Not Recorded	Lymphocytic lymphoma	Low	T
14	Medium Schnauzer	Female	10 years	Follicular lymphoma	Low	B
15	Boxer	Male	7 years	Lymphocytic lymphoma	Low	B
16	Rottweiler	Female	8 years	Nodular NOS lymphoma	Low	B
17	Mongrel	Female	6 years	Marginal zone lymphoma	Low	B
18	Mongrel	Female	10 years	Marginal zone lymphoma	Low	B
19	Mongrel	Male	10 years	Mantle cell lymphoma	Low	B
20	Lagotto	Male	9 years	Diffuse lymphoma	Intermediate	NK like
21	Mongrel	Spayed Female	11 years	Diffuse lymphoma	Intermediate	B
22	Boxer	Female	10 years	Peripheral T-cell lymphoma (PTCL)	Intermediate	T
23	Mongrel	Female	8 years	Diffuse lymphoma	Intermediate	B
24	German Shepherd	Female	10 years	Diffuse lymphoma	Intermediate	NK like
25	West Highland White Terrier	Female	7 years	Diffuse lymphoma	Intermediate	B
26	Mongrel	Male	8 years	Peripheral T-cell lymphoma (PTCL)	Intermediate	T
27	Boxer	Male	7 years	Hepatosplenic lymphoma	Intermediate/high	NK like
28	Siberian Husky	Male	11 years	Diffuse lymphoma	High	B
29	Pyrenean mountain dog	Female	11 years	Diffuse lymphoma	High	B

11.Plates

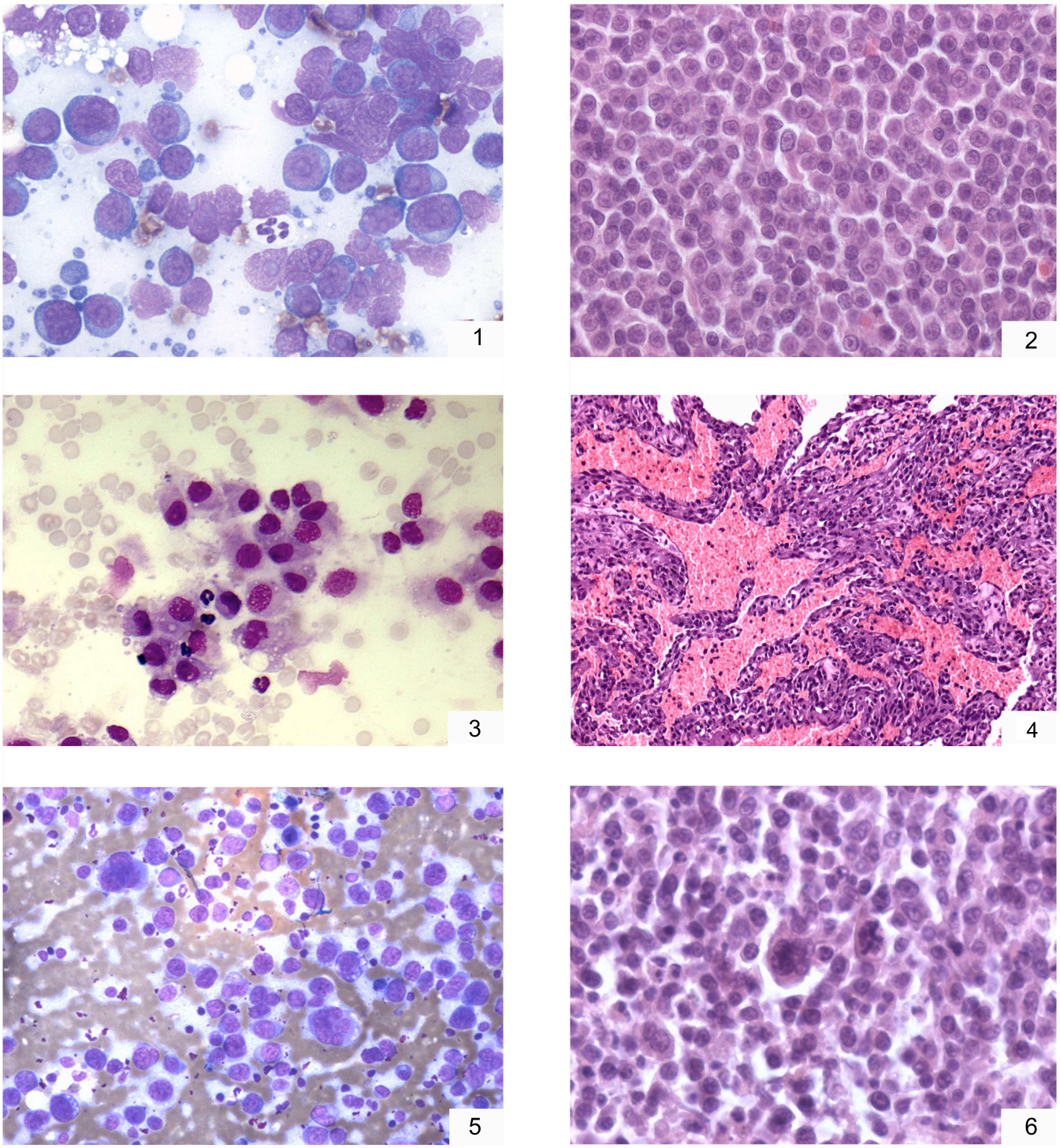
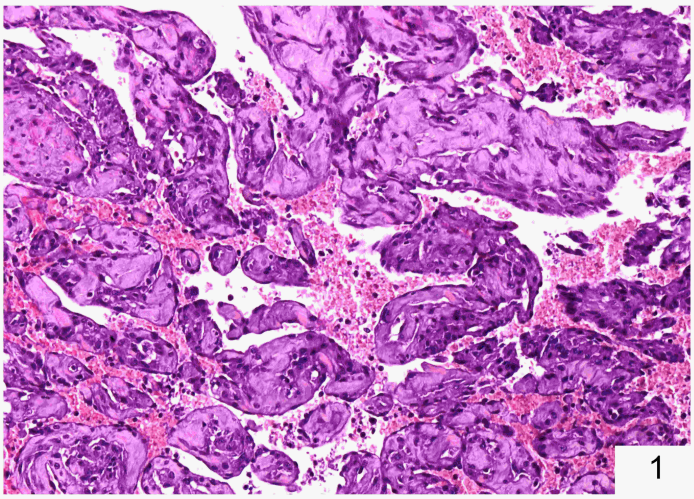
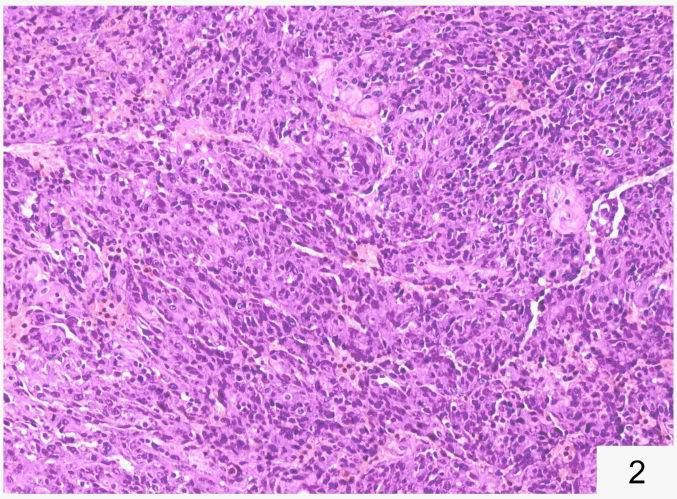


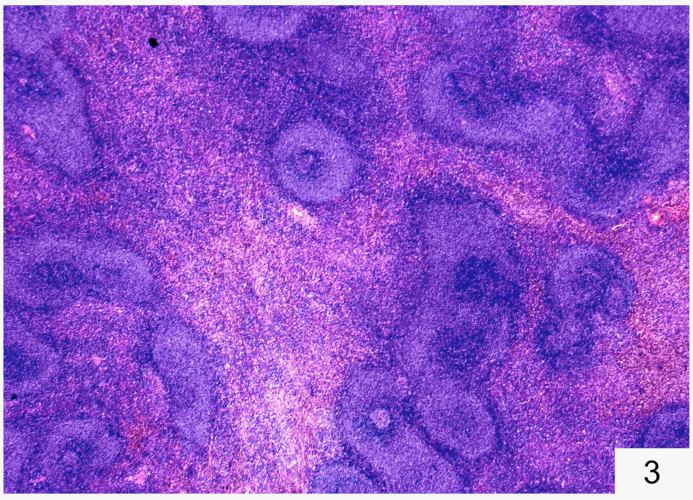
Fig. 1 and 2: Marginal zone lymphoma. Cytology is characterized by the presence of intermediated size with nuclei approximately 1,5 red cells in diameter with a deeply stained chromatin with a prominent nucleolus and a bluish cytoplasm. (Fig 1). On histology neoplastic cells had a complete cytoplasmic rim, moderate amount of eosinophilic cytoplasm, a round to oval central nucleus of intermediate size with peripheralization of chromatin and a single central prominent nucleolus (Fig. 2)

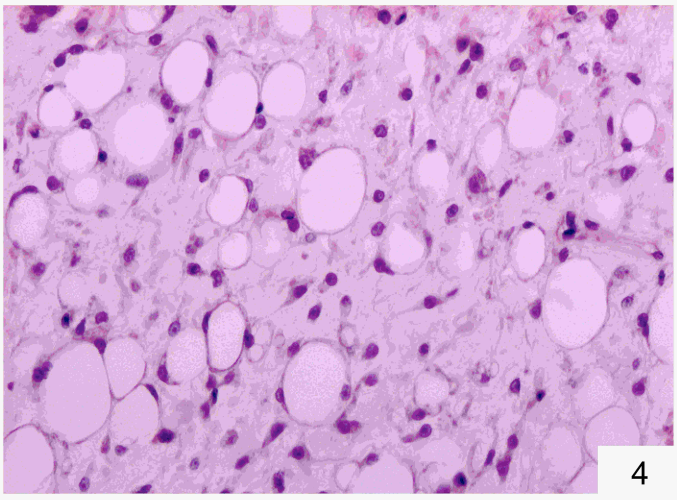
Fig. 3 and 4: Well-differentiated angiosarcoma. On cytology the sample was characterized by clusters of slightly cohesive spindle cells with and intermediate N:C ratio, a moderate amount of light blue cytoplasm and punctate cytoplasmic vacuoles. Fig 3). Histopathology from the same case: well-differentiated canine angiosarcoma (Fig. 4)

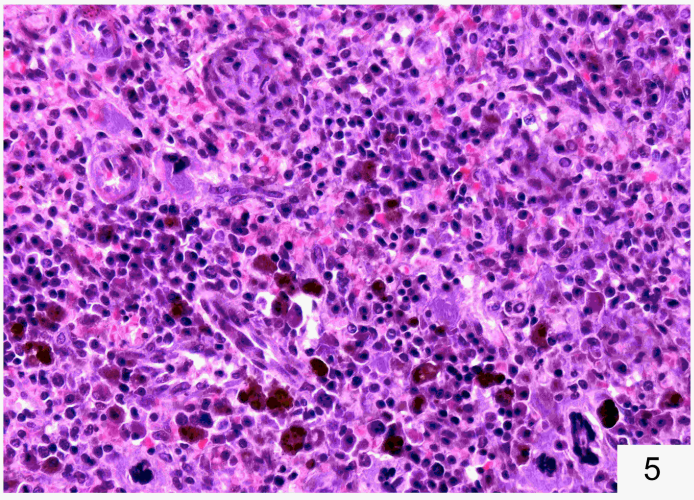
Fig. 5 and 6: Cytological features of a high grade lymphoma. Multinucleated cells, with pleomorphic nuclei, irregularly clumped chromatin and a moderate to abundant amount of cytoplasm were frequent (Fig 5). On histopathology, the neoplastic population had similar morphological features than in cytology. Atypical mitoses were common (Fig.6).











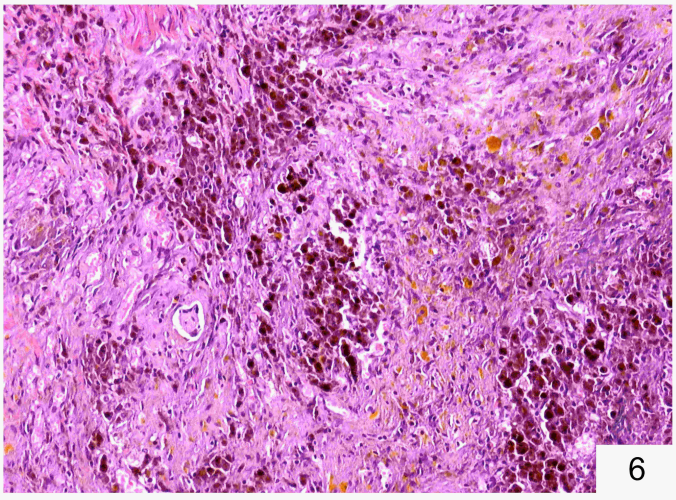


Fig 1. Dog: spleen, histopathologic section from a well differentiated canine angiosarcoma. The proliferating neoplastic cells form vascular lumina and papillary-like projection. Note the presence of a hyaline stroma between vascular clefts.

Fig 2. Dog: spleen, histopathologic section from a poorly differentiated epithelioid canine angiosarcoma. Epithelioid neoplastic cells are arranged in cords and nests but retain the ability to form vascular lumina.

Fig 3. Dog: spleen, marginal zone lymphoma centered on darkly stained areas of mantle cells surrounding splenic arterioles. The marginal zone layers are of lighter-stained cells of irregular width with focal areas of coalescence.

Fig 4. Dog: spleen, myxoid liposarcoma composed of highly pleomorphic spindle to stellate cells embedded in a myxoid matrix. Few cells characterized by occasional cytoplasmic clear vacuoles with distinct margins (lipid).

Fig 5. Dog: spleen, histopathologic section characterized by a diffuse and severe extramedullary hematopoiesis admixed with a moderate number of hemosiderin laden macrophages (hemosiderosis).

Fig 6. Dog: spleen, hemosiderosis with iron staining of smooth muscle fibers and focal areas of yellow ceroid pigment due to extensive local red cell destruction admixed with numerous fibrocytes, likely a result of focal interstitial hemorrhages.

12. Discussion

12.1 Prospective study

Sonovue® is a second-generation ultrasound contrast medium containing a central gaseous nucleus (sulfur hexafluoride) stabilized by an external elastic phospholipidic shell. When exposed to an ultrasound wave, microbubbles have significantly higher reflectivity than blood and soft tissues,[47] so they enhance the acoustic signal in the blood circulation. When imaged with an ultrasound wave of low acoustic power (mechanical index lower than 0.1 or 45kPa), the microbubbles are minimally disrupted and oscillate continuously, allowing real-time imaging of tissue perfusion. When compared with the incident wave, the reflected ultrasound is modified in two ways.[27] First, it contains nonlinear components, as a consequence of the asymmetric oscillation of the microbubbles. Second, it includes harmonic frequencies. To improve the contrast-to-tissue ratio and maximize the distinction between blood derived signal and tissue-derived signal, contrast-specific derived imagin modalities were developed. Because the difference in microbubble tissue backscatter is much higher for harmonic frequencies, harmonic imagin can be used a contrast-specific method to clearly depict differences in the perfusion tissue. The flow of contrast medium can be assessed by coded harmonic imaging by suppressing the contribution of the fundamental frequency in the image construction. With this modality, multi-pulse sequences with precise changes in transmitted inter-pulse amplitude and phases are used to reject the linear fundamental tissue signal and retain the nonlinear signal from the microbubbles. If precise, this modulation produces very good contrast-to-tissue ratio and has high sensibility in the detection of the contrast medium signal.[104]

In this study, coded harmonic imaging was used to evaluate the perfusion of focal splenic lesions in a population of 26 dogs of various ages.

Focal or multi-focal splenic lesions were examined with contrast-enhanced ultrasound and, sonographic findings were compared and grouped according to the final diagnosis, with the aim of establishing criteria that could be associated with benign and malignant splenic lesions.

In this series, most benign lesions (7/11) had a perfusion pattern similar to the adjacent parenchyma, so that the lesions were isoechoic or mildly hypoechoic compared to the surrounding normal spleen in the wash in and peak phases. Five of them were characterized by a mild decrease in their echogenicity during the wash out phase. The similar echogenicity of bening lesions can be explained by the similar architecture of the vascular network associated with benign hyperplastic conditions and normal spleen. This finding parallel previous study, were bening lesions are compared with the normal surrounding splenic parenchyma.[52,75] Furthermore Nakamura et al. (2010) characterized 8 cases of nodular hyperplasia and highlighted that in 2 of them, the nodules become hypoechoic in the late vascular phase, and this is in agreement with our findings.

The perfusion pattern of malignant lesions was different than that observed in the surrounding normal parenchyma. After variable wash-in and peak phases, all malignant lesions became completely or extensively hypoperfused during the wash-out phase. Thirty seconds after injection, these hypoechoic lesions were easily detected, as they were surrounded by hyperechoic normal splenic parenchyma. These results confirm the observations reported for splenic malignancy in humans[64] and dogs.[75] Furthermore, other have found that hypoechogenicity trough all perfusion

phases was always associated with malignancy,[60] and this is in agreement with our data if considering HAS alone. Hemangiosarcomas are neoplasm of vascular endothelial origin. Hemorrhage, infarction, thrombosis, necrosis, and fibrosis are frequently observed. Furthermore, neoplastic tissue may cause vascular encasement and subsequentely congestion with decreased blood flow, and hypoperfusion may occur.[60] However, differentiation between haemangiosarcoma and hematoma should be done cautiously. A previous study with the contrast agent perlutren lipid microsphere by Ivancic et al. (2009) demonstrated that HSA and hematoma were characterized by similar heteroechoic patterns during the peak enhancement. Moreover, it was demonstrated that some case of hematoma exhibited a hypoechoic pattern with sulphur hexafluoride microbubbles.[60,75] Although the exact reasons for this differences are uncertain it has been speculated that they might be because of the difference of contrast agents or patients population in each study. Further studies are needed to clarify the criteria for discrimination between haemangiosarcoma and hematoma.

Considering all malignant lesions Nakamura et al. (2010) found that in the early vascular phase, a hypoechoic pattern was significantly associated with malignancy with sensitivity of 38% and specificity of 100% along with a hypoechoic pattern in the late vascular phase with a sensitivity of 81% and specificity of 85% with no significant differences during the parenchymal phase. Moreover, other studies found that vascular phase imaging with sulphur xafloride microbubble could differentiate benign and malignant focal splenic lesions based on the finding that malignant tumors were hypoechoic to the surrounding normal spleen parenchyma in the wash out phase.[60,75] The vascular phase imaging with sulphur hexafluoride microbubbles could differentiate benign and malignant focal splenic lesions based on the finding that

malignant tumors were hypoechoic to the surrounding normal splenic parenchyma in the wash-out phase. The slightly different results among studies could be explained by the heterogeneity of splenic malignant lesions found in the aforementioned studies since when compared HAS results they are all consistent.

Lymphomas are the second most frequent malignant neoplastic lesions in dogs. However, their appearance on contrast enhanced ultrasound have been evaluated in few cases and have been always named lymphoma without further subtyping.[52,60,75] Moreover, the aforementioned diagnoses have been achieved both by histology and cytology (Table 5). MZL and MCL are the most common primary lymphoma of the spleen and, due to their architectural features, are difficult to diagnose by cytology, for these tumors histology is mandatory for their diagnoses. Therefore, no comparison between our results and the literature can be made. However, we can highlight that in all studies, including ours, most lymphosarcoma were characterized by a hypoechoic appearance in the wash out phase. This finding is in agreement with the literature in which all malignant tumors were hypoechoic to the surrounding normal spleen parenchyma in the wash out phase.[52,60,75]

In our study, the presence of feeding vessels was associated with malignancy and this is in agreement with the literature.[96] In a study by Taeymans and Penninck (2011) the presence of tortuous feeding vessels in the arterial phase, together with the persistence of feeding vessels in the parenchymal phase was an indicator of malignancy, evidence that resulted in accuracy of 100%.

Limitations of this study are the heterogeneity of the lesions and the lack of other common types of neoplastic and non neoplastic splenic conditions. Many types of sarcomas (mixo-, leiomio-, fibro-, and osteosarcoma) were not observed in this study,

therefore, it is not known if they meet the criteria for malignancy. Other neoplastic conditions (liposarcoma, mast cell tumor, metastatic disease) were seen only in isolated patients, so it is not possible to verify whether they have specific perfusion patterns and thus make any conclusions.

In humans, contrast-enhanced CT and MR imaging are gold standard diagnostic imaging methods for diagnosing and characterizing focal lesions of the spleen, and specific perfusion patterns are known.[64,71,73] Compared with CT and MR imaging, contrast-enhanced ultrasound has similar diagnostic performance with some advantages, being less expensive, portable, and rapidly performed, without ionizing radiation exposure.[102] Therefore, contrast-enhanced ultrasound is considered to be a valid alternative to contrast CT and MR imaging.

In conclusion, contrast-enhanced ultrasound can be useful in the differentiation of focal splenic lesions. A larger number of patients, including all types of conditions, are needed to confirm our findings and to increase knowledge in this complex field. Traumatic splenic injuries and vascular diseases are lesions were contrast-enhanced ultrasound studies are indicated in humans,[11–15] and these could also become applications in veterinary medicine. In animals with thromboembolism, infarction due to partial splenic torsion or posttraumatic splenic rupture, contrast enhanced ultrasound could be useful in confirming the diagnosis and assisting in the correct therapeutic or surgical approach.

12.2 Retrospective study

In this retrospective study 428 splenic biopsies and 653 cytological samples were included allowing for the evaluation of the prevalence of canine splenic neoplastic disorders and the accuracy of cytology in the diagnosis of splenic neoplasms.

The current study highlighted a cytological and an histological prevalence of 32,8% and 51.2% respectively. From the data obtained, the more accurate evaluation of prevalence was considered the one obtained from the histopathological analysis since FNA cytology is frequently performed as an initial diagnostic investigative technique for any splenic lesions but results often in a blood contaminated non diagnostic sample not always followed by a histopathological evaluation or diagnosis. Furthermore, studies investigating the prevalence of canine splenic disorders have been based on histopathological examination alone since it considered the "gold standard" for the diagnosis of splenic disorders.[1,12,18,58,88]

The histological prevalence obtained from this word falls within the range reported in the veterinary literature[5,58] ranging from 23,6%[88] to 75%[25] and resembles to the prevalence data reported in the majority of papers with a range of 42,5%[109] to 54,8%.[1]

Our histopathological prevalence data indicate that malignant primary neoplasm (116 cases) have a higher prevalence compared to neoplastic benign neoplasms (36 cases). These data are in agreement with some authors[16,18,36] whereas several previous studies have highlighted a higher frequency of benign splenic lesions.[1,12,58,61,88,95] However, these studies included all benign splenic diseases (neoplastic and non neoplastic) thus this datum is not comparable to our results since we did not included benign non neoplastic splenic lesions in the "benign" category.

Regarding diagnostic distribution of different histological tumor types o, the most frequent diagnosed neoplasms were angiosarcoma (33%), followed by lymphoma (27%). The high number of haemangiosarcoma (HSA) in our study parallel previous data that consider HAS the most frequent canine splenic neoplasm[36,66,82,88,92] accounting for 45%,[16] 51%[91] or 73,5% [18] of all splenic malignant neoplasms. Furthermore, some authors considered HSA as the most frequent canine splenic lesion overall.[5,16,18,25,33,36]

On the contrary, data concerning the prevalence of splenic lymphomas are fragmentary making comparison with our data difficult since, in studies on splenic neoplasms, lymphomas are frequently grouped into the hematopoietic category, thus the exact incidence of lymphoma alone can not be extrapolated from these works.[18,25,33] However, our prevalence data is higher compared to the ones highlighted by Frey e Betts in 1977 (2 cases out of 43 splenic tumors) and by Hosgood in 1987 (1 case out of 10 splenic tumors).

Our results are similar only to one study evaluating the prevalence and type of splenic lesions, that reports lymphosarcoma as the second most frequent splenic tumour (38 of 287 cases - 13%) after HAS.[88]

Although MZL seems one of the most frequent primary splenic lymphomas in dogs, Its' true prevalence is unknown.[107] MCL seems rare in dogs, and it is primarily a nodular indolent splenic lymphoma characterized by slow progression.[23,105]

Although canine primary follicular lymphoma (FL) has been reported in lymph nodes, it seems to have been rarely diagnosed in the spleen and no prognostic data on canine primary splenic FL are available.[105]

In this study, a comprehensive grading of 29 canine lymphoma samples was performed according to the WHO classification.[106] All cases of MZL, MCL and

follicular lymphoma with a nodular pattern of growth included in this study were of low grade. On the contrary, diffuse lymphomas, including marginal lymphomas progressing to the blastic diffuse form, large B cell and PTCL lymphomas were all intermediate to high grade.

In our study, the most common breeds were mixed breed, followed by German shepherd Rottweiler and Boxer. No enough data were available regarding the breed predisposition of splenic neoplasms in general. On the other hand, the breed predisposition of splenic HSA has been well documents and partially parallels our finding. HSA was most frequently observed in mongrel dogs, immediately followed by German Shepherds. The latter is well known to be predisposed to splenic HAS.[5,67,92,16,88,91,3,18,82] On the contrary, an unusual high frequency of mongrel dogs, Dachshunds and Cockers was documented in this work. Surprisingly, Boxers seem not predisposed to HAS even though is a breed prone to tumor development.[16,67,88] Concerning other tumor histotypes, no comparison regarding signalment can be done since no data are available in literature.

Splenic parenchyma can be affected by a wide variety of neoplastic diseases however, there are limited reports describing the accuracy of splenic cytology and none describing its' sensitivity, specificity, positive and negative predictive diagnostic values. The overall accuracy of cytology, utilizing histology as the gold standard, in the diagnosis of splenic neoplastic versus non-neoplastic conditions in our study was 71,2%. This result falls within the range reported in the literature spanning form 51,4% reported by Watson and coworkers to 100%[58] and is similar to accuracy rates previously reported in veterinary medicine.[1,12,58,109] However, all the previous reports have evaluated the accuracy of cytology in the diagnosis of neoplastic and non neoplastic diseases.[1,12,58,109] Specifically, O'Keefe and Couto (1987) compared

cytological and histological splenic samples from 13 dogs and 2 cats evaluating 6 hematopoietic neoplasms (lymphomas and mast cell tumors) and found that all cytological diagnoses correlated well with the definitive histological diagnosis (100% agreement) concluding that results from this diagnostic procedure are highly reliable. Whereas, the accuracy was reduced if considering non hematopoietic neoplasms. Interestingly, most of the diagnosed neoplasia were of haematopoietic origin and characterized by a diffuse distribution in the splenic parenchyma.[58] On the contrary, Ballegeer et al (2007) and Watson et al (2011) reported lower accuracy values of cytology. In the report from Ballegeer et al (2007) the overall agreement between cytology and histology was 47%. Watson et al (2011) evaluated 35 cases and 17 were diagnosed as neoplastic with a cytohistological agreement in 18/35 dogs (51%). In both studies, results that agreed with regard to lesion categorization (neoplasia, benign disorders), but differed in the specific disease diagnosis were included in the partial agreement category.[16] The value of partial agreements were 16,1 and 8,6 respectively.

Compared with these reports our accuracy rate was higher since, samples where a tumor was suspected by cytology was included in the TP category when neoplasia was confirmed by histology.

A study by Eich *et al.* (2000) evaluated the accuracy of cytology compared to histology in the diagnosis of 100 diseases that belonged to visceral organs. The overall agreement in the diagnosis of splenic diseases was 87% with a sensitivity and specificity of 89% and 100% respectively. Furthermore, this study highlighted that the accuracy rate for all kind of diseases (neoplastic and non-neoplastic) was 83% and increased to 90% by the exclusion of splenic masses. Even though the type of splenic lesions are not mentioned, the accuracy of splenic cytology was 38%. This

result is lower that our data and can be explained by the fact that in this study they considered a full agreement only when there was an exact cytpathological diagnosis. Other studies have evaluated splenic cytology diagnostic accuracy highlighting values ranging from 60%[109] to 88%.[12] However, these studies analysed the accuracy of cytology in neoplastic and non neoplastic diseases, thus their accuracy data cannot be compared to our work.

Limitations of splenic cytology are frequently related to non-diagnostic often hemodiluted samples[19,68] deriving especially from blind sample collection or as a consequence of the high incidence of focal cavernous splenic lesions that can derive either from hematomas or to HSA.[3,19,68] Consequently, higher values of accuracy have been reported in the diagnosis of diffuse splenic lesions than in focal leisons.[19,55] On the contrary O'Keefe and Couto (1987) did not found differences of cytological accuracy between focal and diffuse splenic lesions. In our study, most of the false negative diagnoses involved focal to multifocal splenic lesions. Specifically, most of the incorrect diagnoses were lymphomas and all of them were classified as marginal zone lymphomas. This subtype of lymphoma is characterized by the neoplastic proliferation of small to medium sized lymphocytes with a low mitotic rate and on cytology are difficult to be diagnosed since the architectural changes in splenic follicles are not evaluable.[105,107] Two HSA and 2 angiomas were also included in the false negative diagnoses. This result was expected since the cavernous and sinusoidal composition of splenic vascular neoplasms easily associates with an inconclusive cyopathological examination.[3]

In the true positive category most cases were classified as sarcoma, in particular 7 were HAS and 6 were classified as other types of sarcomas. Even though HAS are usually challenging to diagnose,[3] our result can be justified by the fact that we

included in the true positive category also cytological samples with a diagnosis of suspicious neoplastic disease, where the presence of few but severely atypical cells warranted a diagnosis of a suspected spindle cell neoplasia.

Our only false positive samples came from a diagnosis of a suspected spindle cell neoplasia. This error derived by the prevalent presence of plump reactive spindle cells with mild atypical features. The mistake can be explained by the fact that usually splenic samples of reactive lesions lead to non cellular and blood contaminated samples (thus are non diagnostic) and that reparative processes may bear spindle reactive fibroblasts and endothelioblasts that may bear some cytological atypia. However, based on this result, caution must be taken to diagnose sarcomas in the spleen unless cellular atypia is severe.

Concerning specificity, sensitivity, positive and negative predictive values no data were available in veterinary literature in order to compare these with our results. However, we can consider splenic cytology as a valuable diagnostic tool characterized by a high specificity and a good positive predictive value.

Even though cytology and histology should be considered complementary techniques, splenic cytology has inherent advantages. First is an easy and safe sampling technique since it does not need anaesthesia and has a very low complication rate as reported in literature.[1,58,95] It allows a rapid examination of sample and a preliminary lesion assessment. In addition, a greater level of individual cellular detail can be discerned in cytological preparations compared with formalin fixed tissue. According to the high specificity and a high positive predictive value highlighted in our study, along with the higher frequency of false negative results cytology should be always recommended prior to splenic biopsy procedures. Furthermore, in case of cytological sample negative for neoplasia, if the clinical

suspicious is still high, cytological examination should be repeated (especially with the aid of ultrasound to sample focal lesions).

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14. Appendix

14.1. Oral Presentations

National Congresses

1. Santagostino SF, Mortellaro CM, Avallone G, Caniatti M, Forlani A, Roccabianca P.
Title: “Il linfoma nasale nel gatto: valutazione citologica, istologica e immunoistochimica.”
Meeting: IX Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 25th-26th May 2012
City: Perugia, Italy.

2. Forlani A, Avallone G, Stefanello D, Palmieri C, Santagostino SF, Roccabianca P.
Title: “Liposarcoma mixoide splenico primario in 2 cani”
Meeting: IX Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 25th-26th May 2012
City: Perugia, Italy.

3. Forlani A, Avallone G, Santagostino SF, Roccabianca P.
Titolo: “Disordini neoplastici nel furetto domestico: classificazione e caratteristiche Epidemiologiche.”
Meeting: X Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 29th-31st Maggio 2013
City: Giulianova Lido (TE) – Italy.

4. Forlani A, Caruso M, Ghisleni G, Roccabianca P, Caniatti M.
Title: “Non-diagnostic fine needle aspiration biopsy (FNAb) of cutaneous masses in dogs and cats”
Meeting: XI Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 16th-18th June 2014
City: Pisa - Italy

International Congresses

1. Bielli M, Forlani A, Nardini G, Avallone G.
   Titolo: “Mucinous melanophoroma in a Northen Red Bellieid Cooter (Pseudemis Rubriventris)”
   Meeting: 1st International Conference on Avian, Herpetological and Exotic Mammal Medicine
   Date: 25th April 2013
   City: Wiesbaden, Germany.

2. Forlani A, Caniatti M, Santagostino SF, Rotondi B, Luraschi C, Roccabianca P.
   Titolo: “Correlation between cytology and histopathology in the diagnosis of splenic neoplasms in dogs.”
   Meeting: 31st Annual Congress of the European Society of Veterinary Pathology (ESVP)
   Date: 4th-7th September 2013
   City: London, United Kingdom.

3. A. Forlani, G. Zanna, M. Tecilla, E. Zini and P. Roccabianca
   Title: “Concomitant cutaneous neosporosis and toxoplasmosis in a Golden Retriever”
   Meeting: Second Joint European Congress of the European Society of Toxicologic Pathology, the European Society of Veterinary Pathologists and the European College of Veterinary Pathologists.
   Date: 27th-30th August 2014
   City: Berlin, Germany August 2014, 94.

14.2. Posters:

National Congresses

5. Forlani A, Santagostino SF, Queliti R, Roccabianca P.
Title: “Correlazione tra indagine citologica ed istologica nella diagnosi delle neoplasia spleniche del cane.”
Meeting: X Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 29th-31st Maggio 2013
City: Giulianova Lido (TE) – Italy.

6. Forlani A, Santagostino SF, Quieliti R, Roccabianca P.
Title: “Morte improvvisa causata da una grave lipoproteinosi alveolare polmonare in un Bulldog Inglese.”
Meeting: X Annual Congress of the Italian Society of Veterinary Pathology (AIPVet)
Date: 29th-31st Maggio 2013
City: Giulianova Lido (TE) – Italy.

International Congresses

1. Gabriele G, Ferrari MG, Santagostino SF, Forlani A.
Title: “NK-cell Large Granular Lymphocyte leukemic Lymphoma in a dog”
Meeting: 1st meeting of the European Canine Lymphoma Group
Date: 22nd June 2013
City: Lugano, Switzerland.

2. Forlani Annalisa, Avallone G, Santagostino SF, Roccabianca P.
Title: “Epidemiological survey of neoplasms in ferrets: 856 cases”
Metting: 31st Annual Congress of the European Society of Veterinary Pathology (ESVP)
Date: 4th-7th September 2013
City: London, United Kingdom.

3. Forlani A, Palmeri C, Santagostino SF, Queliti R, Roccabianca P.
Title: “Massive Pulmonary alveolar lipoproteinosis in a English Bulldog”
31st Annual Congress of the European Society of Veterinary Pathology (ESVP)
Date: 4th-7th September 2013
City: London, United Kingdom.

4. Santagostino SF, Mortellaro CM, Forlani A, Ghisleni G, Roccabianca P.
Title: “Primary angiocentric/angioinvasive T-cell lymphoma of the timpanic bulla in a Felv positive cat”

31st Annual Congress of the European Society of Veterinary Pathology (ESVP)
Date: 4th-7th September 2013
City: London, United Kingdom.

5. Santagostino SF, Forlani A, Roccabianca P, Fedrizzi G, Malandra R, Ranghieri V, Zaffra N, Ghisleni G.
Title: "Metal exposure and toxicology in selected fish species from the Mediterranean sea: risk assessment for human consumption"
Second Joint European Congress of the European Society of Toxicologic Pathology, the European Society of Veterinary Pathologists and the European College of Veterinary Pathologists.
Date: 27th-30th August 2014
City: Berlin, Germany August 2014, 94.

6. Ghisleni G, Forlani A, Caruso M, Roccabianca P, Caniatti M.
Title: Non-diagnostic fine needle aspiration cytology (fnac) of cutaneous masses in dogs.
16th European Society of Veterinary Clinical Pathologists (ESVCP) Annual Congress.
Date: 1st-4th October 2014.
City: Milan - Italy

Abstracts

IL LINFOMA NASALE DEL GATTO: VALUTAZIONE CITOLOGICA, ISTOLOGICA E IMMUNOISTOCHIMICA

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Il linfoma è il tumore primario nasale più comune nel gatto; tuttavia, i dati pubblicati sulle patologie nasali in generale ed in particolare sul linfoma nasale nei gatti, sono pochi. In questo studio sono state esaminate biopsie endoscopiche di 156 lesioni nasali feline. Le biopsie sono state fissate in formalina e processate routinariamente. Si evidenziavano 35 linfomi, 23 neoplasie epiteliali e 17 sarcomi, mentre in 79 casi si osservavano processi non neoplastici. In 26 casi, i linfomi erano classificati clinicamente come nasali primari, in 6 casi come nasali-nasofaringei e in 3 come rinofaringei. In 26/35 linfomi erano disponibili campioni citologici colorati con May Grünwald-Giemsa. Ai casi di linfoma veniva applicata la classificazione WHO. Si eseguivano inoltre colorazioni immunoistochimiche per CD20, CD3, FeLVp27, FeLVgp70 e Calicivirus. La maggioranza dei gatti era di razza Comune Europea (n=28), con età media di 10,5 anni (range 1-19) e di sesso maschile (F/M=0,54). I linfomi avevano crescita diffusa (31) o nodulare (4). In 6 casi si osservava epiteliotropismo. La distribuzione dei citotipi era di 13 linfomi a piccole cellule, 12 a grosse cellule, 6 a medie cellule, 1 centrocitico-centroblastico di I grado e 3 plasmocitomi. La concordanza diagnostica tra citologia ed istologica era del 50%. In 30 casi si evidenziava un fenotipo B. In 21 casi si osservava positività ad antigeni di FeLV. L'esame necroscopico era eseguito in 4 gatti; in un caso il tumore era limitato al rinofaringe, mentre negli altri era dimostrata una progressione con coinvolgimento degli organi interni. Nonostante la classificazione ed il fenotipo abbiano permesso di diagnosticare entità diverse, la maggioranza dei linfomi era caratterizzata da comportamento aggressivo e prognosi sfavorevole.

Parole chiave: linfoma, gatto, naso, fenotipo, FeLV.

FELINE NASAL LYMPHOMA: CYTOLOGICAL, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

Lymphomas are the most common primary nasal tumors in cats. Few reports on feline nasal diseases and nasal lymphomas are currently available. Nasal endoscopic biopsies of 156 cats were analyzed; all samples were formalin-fixed and routinely processed. Lymphomas (35), epithelial neoplasias (23), sarcomas (17) and non neoplastic process (79) were diagnosed. Lymphomas were primary nasal in 26 cats, nasal and nasopharyngeal in 6 and only nasopharyngeal in 3. In 26/35 cases cytology was also available. Lymphomas were classified according to the WHO criteria. Immunohistochemistry for CD20, CD3, FeLVp27, FeLVgp70 and Calicivirus was performed. Most cats were DSH (n=28), with a mean age of 10,5 years (range 1-19) and a male prevalence (F/M=0,54). Lymphomas were diffuse (31) or nodular (4). Epitheliotropism was observed in 6 cases. Cytotypes of lymphomas were 13 small cell lymphomas, 12 large cell types, 6 medium sized, 1 centrocitic-centroblastic grade I and 3 plasmacytomas. Cytology and histology were in agreement in 50% of cases. B cell phenotype was observed in 30 cases and a FeLV positivity was detected in 21 cases. Necropsy was performed in 4 cats; in one cat lymphoma was limited to the nasopharynx while internal organ invasion was evidenced in the other 3. Despite a variable cytotype and phenotype, most lymphomas demonstrated an aggressive clinical course and poor prognosis.

LIPOSARCOMA MIXOIDE SPLENICO PRIMARIO IN DUE CANI

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I sarcomi non vascolari/non linfoidi (SNVNL) rappresentano il 23-34% dei tumori primari splenici del cane. Il liposarcoma splenico è molto raro rappresentando il 2-6% dei SNVNL. Il liposarcoma è più frequentemente una neoplasia sottocutanea per la quale sono state riportate le varianti istologiche differenziata, mixoide e pleomorfa, mentre a livello splenico non sono riportate le varianti. In due cani, un incrocio maschio di 8 anni ed un Bouledogue francese femmina di 15 anni venivano evidenziate rispettivamente una massa splenica rotondeggiante di 15 cm e una massa bilobata di 7,5 x 6 cm. In sezione le neoplasie presentavano superficie traslucida, gelatinosa e mucoide. Si eseguivano istologia, colorazioni istochimiche, immunoistochimiche e microscopica elettronica. L'istologia evidenziava, in entrambi i casi, cellule neoplastiche organizzate in fasci, frammiste ad abbondante matrice extracellulare. Le cellule neoplastiche erano fusate con citoplasma debolmente eosinofilo, che occasionalmente conteneva vacuoli a margini netti ed otticamente vuoti. L'indice mitotico era di 0,1 e 1,6, rispettivamente. Sulla base della morfologia si poneva una diagnosi di mixosarcoma di II grado in entrambi i casi. La matrice extracellulare era Alcian-Blue positiva e PAS negativa. I vacuoli citoplasmatici erano Oil-red O positivi. Le cellule neoplastiche erano vimentina positive ed actina, desmina, fattore VIII ed S100 negative. In un caso la microscopia elettronica evidenziava cellule adipose a vari stadi maturativi. Due mesi post splenectomia il primo cane veniva soppresso e l'autopsia evidenziava metastasi epatiche disseminate e linfonodali del tumore primario. Solo le analisi aggiuntive permettevano la diagnosi di liposarcoma mixoide.

Parole chiave: cane; istochimica; microscopia elettronica; liposarcoma mixoide; milza.

PRIMARY SPLENIC MYXOID LIPOSARCOMA IN TWO DOGS

Nonvascular-nonlymphoid (NVNL) sarcomas represent 23-34% of canine primary splenic sarcomas. Splenic liposarcoma accounts for 2-6% of NVNL. Liposarcomas commonly raise in the subcutaneous fat and are classified in three histologic variants: differentiated, myxoid and pleomorphic. No histological subtyping has been described for splenic cases. Two dogs, a mongrel 8-year-old male and a Bouledogue 15-year-old female presented with a splenic round mass of 15 cm and a bilobed mass of 7,5 x 6 cm. On cut section both tumours had a translucent, gelatinous and mucous surface. Histology, histochemistry, immunohistochemistry and electron microscopy (EM) (1 case) were performed. Histology was characterized by neoplastic cells organized in loose bundles admixed with abundant extracellular matrix. Neoplastic cells were spindle, with lightly eosinophilic cytoplasm occasionally containing sharply demarcated clear vacuoles. Mitotic index was 0,1 and 1,6, respectively. Morphology was consistent with myxosarcoma of grade II in both cases. Matrix was Alcian-Blue positive and PAS negative. Vacuoles were Oil red O positive. Neoplastic cells were vimentin positive and actin, desmin, Factor VIII, S100 negative. In one case, EM evidenced adipose cells at different maturative stages. Two months post-splenectomy the first dog was euthanized and necropsy revealed disseminated hepatic metastases and lymph node involvement. Ancillary techniques were necessary for the diagnosis of myxoid liposarcoma.

Keywords: dog; histochemistry; electron-microscopy; myxoid liposarcoma; spleen.

MUCINOUS MELANOPHOROMA IN A NORTHERN RED BELLIEID COOTER (*PSEUDEMYS RUBRIVENTRIS*)

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ABSTRACT:

An adult, female, Northern red bellied cooter (Pseudemis Rubiventris) was presented with a 15 days history of anorexia and lethargy. On physical examination the animal was in critical condition and a prominent swelling of the proximal area of the left forelimb was evident. Laboratory analysis highlighted anemia, hyperuricaemia, iperuremia, low total protein, elevated aspartate aminotransferase and lactate dehydrogenase. X-ray evidenced a mass infiltrating the humerus and cortical osteolysis. Fine needle aspiration biopsy was performed and cytology was characterized by a prevalence of spindle to stellate cells frequently containing intracytoplasmatic brown black granules associated with a moderate amount of mucin. The tortoise died 24 hours later and only the affected forelimb was sent for histopathology. On gross examination the proximal humerus was encircled by a 3 cm in diameter, black, gelatinous mass with mucoid appearance on cut surface. Histology revealed a dermal, poorly demarcated, sparsely cellular, not capsulated neoplasm that infiltrates the underlying soft tissues and the bone and composed by stellate cells embedded in an abundant PAS positive myxoid stroma. Cells frequently contained intracytoplasmatic brown black granules. These findings were consistent with a mucinous type of melanophoromas. Melanophoromas are tumour of melanin producing cells rarely reported in reptiles, most commonly in snakes and bearded dragons, and rarely in chelonians. The mucinous variant have been described only in Beared Dragons and, to the best of our knowledge, this in the first report of a mucinous type of melanophorma in tortoise.

DISORDINI NEOPLASTICI NEL FURETTO DOMESTICO: CLASSIFICAZIONE E CARATTERISTICHE EPIDEMIOLOGICHE DI 856 CASI

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Riassunto

Il furetto rappresenta uno dei più diffusi animali da compagnia non convenzionali. Negli ultimi decenni, è stato registrato un aumento del numero delle neoplasie in questa specie. Lo scopo dello studio è di classificare ed analizzare la prevalenza delle neoplasie dei furetti in Italia.

Sono state rivalutate le biopsie e i campioni necroscopici raccolti tra il 2000 e il 2010 presso il servizio di patologia veterinaria dell'Università di Milano. I dati riguardanti segnalamento e reperti istopatologici sono stati ottenuti dall'archivio elettronico della sezione. Il numero totale dei campioni è stato utilizzato per calcolare la prevalenza delle neoplasie. Sono stati selezionati 908 campioni provenienti da differenti tessuti o organi. Di questi, in 688 casi era stato diagnosticato almeno un tumore (75.77%). Sono stati frequentemente riscontrati animali colpiti da neoplasie multiple pertanto la casistica finale comprendeva 856 tumori. La popolazione oggetto di studio era caratterizzata da un'età media di insorgenza di 5 anni (range 5 mesi-10 anni) e da un rapporto F/M=0.99.

Il sistema endocrino (63.8%), seguito dall'apparato tegumentario (14.7%) e dal sistema emolinfatico (8.9%) rappresentavano le sedi di insorgenza più comuni. In particolare, le neoplasie maggiormente diagnosticate sono state le neoplasie corticosurrenaliche (25.8%), le neoplasie pancreatiche insulari (24.9%) ed i mastocitomi cutanei (5.8%). I carcinomi squamosi cutanei (SCC) sono stati riscontrati solo in associazione a neoplasie di origine sebacea. Nel 2.6% dei casi sono stati diagnosticati tumori addominali fusati di origine non definibile. La prevalenza dei tumori dei furetti in Italia appare in accordo con la letteratura corrente. L'inusuale associazione tra SCC e neoplasie sebacee e l'esatta origine dei tumori intraddominali a cellule fusate necessitano di ulteriori indagini.

Parole chiave: Furetto, neoplasia, epidemiologia

NEOPLASTIC DISEASE IN THE DOMESTIC FERRET: CLASSIFICATION AND EPIDEMIOLOGICAL SURVEY OF 856 CASES

Summary

Ferrets are considered one of the most common non-conventional companion animals. Neoplastic diseases have been reported with increasing frequency over the last decades. The aim of this study is to classify and analyze the prevalence of neoplasms in Italian ferrets.

All the definitive diagnosis of bioptic and necroscopical specimens received between 2000 and 2010 at the pathology service of the University of Milan were reviewed. Data concerning signalment and histology were collected from the electronic archives. The total number of samples permitted to calculate the prevalence of neoplasms.

A total of 908 samples were received and processed. Of these, 688 cases included at least one tumor (75.77%). Ferrets with multiple neoplasms were commonly detected. Thus, a total of 856 tumors were collected. Our population was characterized by a median age of 5 years (range 5 months-10 years) with F/M ratio=0.99.

Endocrine (63.8%), integumentary (14.7%) and hemolymphatic (8.9%) systems were most commonly affected. A high frequency of adrenal gland (25.8%), pancreatic islet cell (24.9%), and mast cell tumors (5.8%) was evidenced. Cutaneous squamous cell carcinomas (SCC) occurred together with sebaceous gland tumors. In 2.6% of cases, abdominal spindle cell tumors with primary undefined origin were observed. The tumor prevalence recorded in this study paralleled previous findings. The unusual association between SCC and sebaceous gland neoplasms and the origin of intrabdominal spindle cell neoplasms should be further investigated.

Keywords: Ferret, neoplasia, epidemiology

MORTE IMPROVVISA CAUSATA DA UNA GRAVE LIPOPROTEINOSI ALVEOLARE POLMONARE IN UN BULLDOG INGLESE

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Riassunto
La proteinosi alveolare polmonare è una rara patologia caretterizzata dall'accumulo alveolare di fosfolipidi e proteine (lipoproteinosi). Molti farmaci anfofilici cationici sembrano essere convolti nell'insorgenza di tale patologia nell'uomo. Il presente studio descrive un caso di morte improvvisa in un Bulldog inglese, maschio di 7 anni, affetto da grave dispnea e sottoposto a terapia cronica con fenobarbitale e bromuro per epilessia idiopatica.

All'esame necroscopico si evidenziava una grave e diffusa splenomegalia associata a lieve endocardiosi a carico della valvola mitrale e diffuso, grave edema polmonare. A carico del polmone erano presenti lesioni disseminate di 1-8 mm di diametro, ombelicate, accompagnate da aumento di consistenza a carico di entrambi i lobi apicali, dei lobi accessori e del lobo principale destro.

L'istologia evidenziava la presenza nel lume alveolare di un materiale debolmente eosinofilo da amorfo a granulare, PAS-positivo, von Kossa- e Rosso Congo-negativo frammisto ad occasionali neutrofili e macrofagi. Erano presenti anche una moderata fibrosi e mineralizzazioni interstiziali. Il miocardio presentava una moderata infiltrazione adiposa prevalentemente a carico del setto e del ventricolo destro. La microscopia elettronica evidenziava all'interno del lume alveolare corti fasci irregolari composti da lamelle elettrondense parallele di spessore pari a 3.125 nm e disposte ad una distanza di 6.25 nm tra loro. Le indagini effettuate permettevano una diagnosi di lipoproteinosi/fosfolipidosi alveolare. Si ipotizza che la somministrazione cronica di bromuro possa avere svolto un ruolo importante nella patogenesi di tale lesione.

Parole chiave: brumuro, proteinosi alvolare, istologia, microscopia elettronica, cane.

SUDDEN DEATH CAUSED BY MASSIVE PULMONARY ALVEOLAR LIPOPROTEINOSIS IN A ENGLISH BULL DOG

Summary
Pulmonary alveolar proteinosis (PAP) is a rare disease characterized by alveolar accumulation of phospholipids and proteins (lipoproteinosis), that may be induced by many cationic amphophilic drugs. We describe a case of a 7-year-old, male, English Bulldog with a history of juvenile idiopathic epilepsy treated with phenobarbital and bromide and developing sudden severe dyspnoea with respiratory arrest 12 hours post-admission. Necropsy revealed severe hepatomegaly, mild endocardiosis and a diffuse pulmonary edema with disseminated 1-8 mm firm, umbilicated lesions in apical, accessory and right cranial lobes. Microscopically, alveoli (60-80%) were filled by a pale eosinophilic, amorphous to granular PAS-positive, von Kossa- and Congo red-negative material associated with macrophages and neutrophils. Interstitial fibrosis and mineralization were moderate. Myocardial septal and right ventricle fatty infiltration was present. TEM revealed short lamellar electrondense haphazardly arranged 3.125 nm fascicles at 6.25 nm periodic distance compatible with accumulation of abnormal surfactant. These findings were suggestive of an alveolar lipoproteinosis/phospholipidosis. The chronic administration of bromide may be involved in the pathogenesis of this lesions.

Keywords: bromide, alveolar proteinosis, histology, electron microscopy, dog.

CORRELAZIONE TRA INDAGINE CITOLOGICA ED ISTOLOGICA NELLA DIAGNOSI DELLE NEOPLASIE SPLENICHE DEL CANE

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Riassunto

L'esame citologico della milza rappresenta un'importante ed utile tecnica nella diagnosi delle patologie che possono coinvolgere il parenchima splenico ed, in particolare, nei disordini neoplastici. Tuttavia, in letteratura esistono pochi studi che ne valutano l'attendibilità. Lo scopo di questo studio è quello di valutare la correlazione cito-istologica nella diagnosi delle neoplasie spleniche del cane. E' stata effettuata una ricerca retrospettiva di prelievi citologici ed istologici di lesioni spleniche per un periodo che va dal 1998 al 2013. Sono stati valutati l'accuratezza, la sensibilità, la specificità dell'indagine citologica ed i valori predittivi dei risultati positivi e negativi utilizzando l'esame istologico come gold standard. Venivano raccolti un totale di 67 casi. Trentuno casi venivano diagnosticati come non neoplastici alla citologia (12 veri negativi e 19 falsi negativi), mentre in trentasei casi è stata emessa una diagnosi citologica definitiva di neoplasia (33 veri positivi e 3 falsi positivi). La diagnosi citologica ed istologica concordavano nel 67.2% dei casi (45/67). L'esame citologico della milza ha dimostrato una sensibilità pari all'89%, una specificità pari all'80%, un valore predittivo positivo pari al 91.2% ed un valore predittivo negativo pari al 38.7%. Alla citologia la maggior parte dei casi venivano diagnosticati come non neoplastici (31/67). I sarcomi erano i tumori più frequentemente diagnosticati (20/67), seguiti dai linfomi (7/67).

Sebbene l'esame citologico e istologico debbano essere ritenuti complementari nell'iter diagnostico delle patologie spleniche, in base ai risultati ottenuti, la citologia può essere considerata una tecnica diagnostica utile e sensibile nella diagnosi delle neoplasie spleniche.

Parole chiave: Cane, Citologia, Istologia, Milza

CORRELATION BETWEEN CYTOLOGY AND HISTOPATHOLOGY IN THE DIAGNOSIS OF SPLENIC NEOPLASMS IN DOGS

Summary

Spleen can be affected by a variety of diseases and cytology represents a useful diagnostic technique. However, few studies have addressed the accuracy of cytology in the evaluation of splenic lesions and specifically of neoplasms. The aim of the study is to evaluate the cyto-histological correlation in the diagnosis of canine splenic tumors. Splenic cytological and corresponding histopathological samples obtained between 1998 and 2013 were retrospectively evaluated. Concordance between cytology and histology was determined. Accuracy, sensibility, specificity, positive and negative predictive value of cytology for the diagnosis of splenic neoplasias was determined considering histopathology as the gold standard. Sixtyseven cases were collected. Thirty-one cytological samples were classified as nonneoplastic (12 true negatives, 19 false negatives compared with histopathology). Cytological diagnosis of neoplasia was obtained in 36 cases (33 true positives and 3 false positive). Cytological diagnosis was in agreement with the histopathological diagnosis in 67.2% (45/67) of cases. Cytology had a sensitivity of 89.3%, a specificity of 80%, a positive predictive value of 91.2%, and a negative predictive value of 38.7% in the diagnosis of splenic neoplasms. The majority of cases were non neoplastic (31/67). The most common tumors were sarcomas (20/67) followed by lymphoma (7/67). Although cytopathology and histopathology should be considered complementary techniques in the diagnosis of splenic lesions, cytology demonstrated to be a useful and sensitive tool for the diagnosis of splenic neoplasias.

Keywords: Cytology, Dog, Histology, Spleen

PRIMARY ANGIOCENTRIC/ANGIOINVASIVE T-CELL LYMPHOMA OF THE TYMPANIC BULLA IN A FeLV- POSITIVE CAT

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Introduction: Reports of primary middle ear lymphoma are rare in cats and their extension to internal organs is exceptional. A 5-yearold, neutered female, FeLV-positive domestic shorthair cat was referred for stertor, dyspnoea and head-tilt. CT scan revealed soft tissue opacity inside the right tympanic bulla with bone lysis and concurrent nasopharyngeal and intracranial invasion.

Materials and Methods: Endoscopic-guided biopsy samples were collected for histology and immunohistochemistry. A full necropsy examination was performed.

Results: Grossly, the lesion was poorly demarcated, white and soft. Cytology identified round, large, plasmacytoid neoplastic cells. Histology revealed dense sheets of round neoplastic cells often surrounding or invading vascular walls (angiocentric/angiodestructive pattern). Neoplastic cells expressed CD3 (T cell phenotype) and FeLV p27 and gp70 antigens. A middle ear angiocentric angioinvasive T-cell lymphoma was diagnosed. Following radiation therapy, clinical conditions improved, but dysphagia recurred and the cat died suddenly. At necropsy examination, a soft, red mass filled the ventromedial compartment of the tympanic bulla. Extension to the base of the skull with right piriform lobe compression was recorded. Hepatic and splenic metastases were present.

Conclusions: Diagnosis of primary middle ear tumors is often delayed since clinical signs mimic more common otological conditions. Multiple biopsy specimens and immunohistochemistry were pivotal for the diagnosis in this case. FeLV might have been involved in tympanic lymphoma development.

NK-CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIC LYMPHOMA IN A DOG

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Abstract

Large granular lymphocytes (LGL) are defined as lymphoid subset that constitute 1-10% of the circulating pool of lymphocytes in dogs and are characterized either by CD3 positivity (in>90% of the cases) or negativity for both T- and B- cell markers (in <10% of cases, natural killer origin). A 11-year-old, male, mongrel dog was submitted to the referring veterinarian following a 2-month history of progressive generalized weakness, vomiting, weight loss and anorexia. Physical examination revealed severe jaundice and hepatomegaly. Results of complete blood count (CBC) were within reference intervals (RI). Serum biochemical analysis revealed increases in ALT (175U/L; RI, 20-150 U/L), AST (295 U/L; RI, <80U/L) and total bilirubin (6,3 mg/dL; RI, 0,1-0,6 mg/dL). Ultrasound fine needle aspiration of the liver was also performed. Cytological examination of hepatic and blood smears revealed the presence of a large number of lymphoid cells characterized by an abundant amount of pale bluish cytoplasm containing numerous distinct magenta granules variably in sized and shape (LGL), with densely stained reinform nuclei and without apparent nucleoli. On immunocytochemistry, neoplastic cells were negative for both T- and B- cell markers. A final cytological diagnosis of LGL leukemic lymphoma of NK origin was made. Due to the poor clinical condition of the animal the owner did not allow any chemotherapeutic approach and the dog died spontaneously few days later. Large granular lymphocytes leukemias of NK-type are surface CD3-negative disorders with variable clinical behaviour in dogs, are reported in humans.

METAL EXPOSURE AND TOXICOLOGY IN SELECTED FISH SPECIES FROM THE MEDITERRANEAN SEA: RISK ASSESSMENT FOR HUMAN CONSUMPTION

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Introduction

Myriad of toxic substances are released into our environment daily, either deliberately manufactured or accidentally produced. Aquatic systems throughout the world are increasingly under a wide array of anthropogenic stressors. However, some of aquatic environments can sustain fish populations, indicating that they are able to tolerate toxic levels of metals. The presence of twenty-seven heavy metals and minerals (Hg, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sn, Sb, Ba, Tl, Pb, Th, U) was investigated in muscles from *Pagellus bogaraveo*, *Dentex dentex*, and *Thunnus thynnus* from the Mediterranean sea.

Materials and Methods

Fish samples were collected monthly from April 2013 to September 2013. No abnormalities were evidenced on macroscopical examination. Aliquots of muscle ranging from 50 to 100 mg were examined with inductively coupled plasma mass spectrometry (ICP/MS). The mean concentration of each element was calculated. A comparison to the provisional tolerable weekly intake established by the Joint FAO/WHO Expert Committee on Food Additives and the EFSA was assessed. The results were expressed in mg/kg (μg/g) on a wet weight basis, as required by Reg. CE 1881/2006 and 333/2007. The limit of quantification was 0.005 mg/kg. The maximum safe consumption (MSC) for adult intakes was achieved for each element with an established safety limit.

Results

The mean concentration of Hg, Al, Cr, Ni, Cu, Zn, Se, Cd, Sn, and Pb was compared to each PTWI (mg/kg). Hg, Ni, Zn, Se, and Cd were present at higher level within muscular samples of the 3 different fish species. In particular, the levels of Hg and Ni in all species, Zn and Cd in *Thunnus thynnus*, and Se in muscular samples from *Thunnus thynnus* and *Dentex dentex*, exceeded the PTWI established by law. The MSC calculated for mercury in all the tested fish species leads to a limited recommended weekly intake for both men and women for all the tested fish species.

Conclusions

Mercury concentration in the edible parts of *Pagellus bogaraveo*, *Dentex dentex*, *Thunnus thynnus* exhibits a high risk for human consumption.

CONCOMITANT CUTANEOUS NEOSPOROSIS AND TOXOPLASMOSIS IN A GOLDEN RETRIEVER

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Introduction: This report describes a case of concomitant cutaneous Neosporosis (CN) and cutaneous Toxoplasmosis (CT) in a dog. .

Materials and Methods: A 10 years old, intact female Golden Retriever under treatment with cyclosporine for an autoimmune disorder had sudden development of multifocal cutaneous nodules. Imprints, fine-needle aspirates and skin punch biopsies were submitted. Immunofluorescence antibody test (IFAT) on serum samples was performed twice at a one month interval. Unstained smears and deparaffinized sections of skin were immunochemically stained with polyclonal anti-*Toxoplasma gondii (TG)* and anti-*Neospora caninum (NC)* primary antibodies. PCR assay with pimers for *TG* and *NC* were conducted using DNA extracted from cutaneous formalin-fixed, paraffin-embedded tissue.

Results: Cytology demonstrated a prevalence of degenerated neutrophils admixed with fewer reactive macrophages containing numerous intracytoplasmic crescent shaped, 4-6 µm microrganisms, with a light basophilic cytoplasm and a central nucleous (tachyzoites). Histology revealed diffuse and severe neutrophilic, histiocytic, eosinophilic dermatitis and panniculitis associated with necrotizing vasculitis. Elevated numbers of free and cytoplasmic thachizoites within macrophages and epiderma and follicular keratinocytes were present. IFAT was positive for both *TG* and *NC,* with increased *TG* antibody titers after one month. Immunochemistry and PCR confirmed a concomitant *TG* and *NC* cutaneous infection. Clindamycin administration (11 mg/kg PO every 12 hours) and withdrawal of immunosuppressive medication resulted in clinical remission.

Discussion (and/or Conclusions): CN and CT are rare manifestation of both diseases. To the best of our knowledge this is the first report of a simultaneous infection of TG and NC with cutaneous anatomical location.

NON-DIAGNOSTIC FINE NEEDLE ASPIRATION CYTOLOGY (FNAC) OF CUTANEOUS MASSES IN DOGS AND CATS

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Objective: The causes of non-diagnostic FNAC samples in a 12 years (2002-2013) operational span are reviewed.

Design and methods: 138 cases of non-diagnostic FNACs from cutaneous masses in dogs and cats were reviewed. Only cases with concurrent histology were included. Signalment, site, size and type of lesion were included. The number of submitted slides per case was recorded and grouped in three categories ("1-3", "4-6", and >6 slides). A chi-square test was used (p values <0,05 were considered significant).

Results: On histology, cases were classified as: neoplastic (95), inflammatory (19), and 24 non-neoplastic/noninflammatory. Non-diagnostic results were associated with hemodilution (96 cases), poor cellularity (39 cases) and artifacts (3 cases). Site of the lesions were: trunk (52 cases), limbs (38 cases), head/neck (33 cases), mammary gland (14 cases) and not-otherwise-specified (1 case). Size of lesions was <2cm (39 cases), 2-5cm (33 cases) and >5cm (19 cases). Large size of the lesion (>5cm) was statistically associated with a common non-diagnostic cytologic result (p=0.000). Mesenchymal tumors were frequently associated to non-diagnostic results (p=0.031). Compared to a control group of 100 diagnostic cases, the number of submitted smears was higher and statistically significant in the category "4-6 slides" of inconclusive cases.

Conclusions: Large mesenchymal tumors represented one of the major causes of non-diagnostic results. Lesions on the head may provide more non-diagnostic cytology compared to other sites probably due to the patient compliance. Hemodilution is a common cause of non-diagnostic samples since poorly cellular samples not contaminated by blood can be easily esteemed grossly.

14.3. Full Papers

1. Fantinato E, Pravettoni D, Forlani A, Riccaboni P, Binanti D.
"Severe thymic hyperplasia in a newborn calf associated with impaired T-cell differentiation"
J Vet Diagn Invest, 2013; 25(2):603-607.

2. Santagostino SF, Mortellaro CM, Boracchi P, Avallone G, Caniatti M, Forlani A, Roccabianca P.
"Feline Upper Respiratory Tract Lymphoma: Site, Cyto-histology, Phenotype, FeLV Expression, and Prognosis."
Veterinary Pathology, 2014, Jun 5. Online first.

3. Forlani A, Palmieri C, Santagostino SF, Queliti R, Roccabianca P.
"Pulmonary alveolar proteinosis/phospholipidosis in a English Bulldog"
JAVMA. Accepted for publication.

4. Bielli M, Forlani A, Nardini G, Avallone G.
Mucinous melanophoroma in a Northern Red Bellied Cooter (Pseudemys Rubriventris)
Journal of Exotic Pet Medicine
In press.

14.3 Published Papers

Severe thymic hyperplasia in a newborn calf associated with impaired T-cell differentiation

Eleonora Fantinato, Davide Pravettoni, Annalisa Forlani, Pietro Riccaboni and Diana Binanti

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Severe thymic hyperplasia in a newborn calf associated with impaired T-cell differentiation

Eleonora Fantinato, Davide Pravettoni, Annalisa Forlani, Pietro Riccaboni, Diana Binanti[1]

Abstract. A 1-day-old female Holstein–Friesian calf was presented for severe dyspnea. Physical examination revealed respiratory distress, moderate edema of the ventral neck, and swollen jugular veins. The calf died and was submitted for necropsy. A severely enlarged thymus (40 cm × 20 cm × 10 cm) weighing 1.37 kg was detected on gross examination. Histomorphology was normal but no tingible body macrophages were observed in the medullary areas. Immunohistochemistry was characterized by the lack of thymic cluster of differentiation 3 and major histocompatibility complex class II expression compared to age-matched controls. The findings were consistent with severe thymic hyperplasia, a rare congenital condition that is also described in children. Immunohistochemical findings were suggestive of impaired T-cell development and selection associated with lack of apoptosis of thymic cells (lack of tingible body macrophages). Thymic hyperplasia in juvenile animals should be considered among the differential diagnoses of mediastinal masses as a rare cause of respiratory distress in newborn calves.

Key words: Calves; immunohistochemistry; T-cell differentiation; thymic hyperplasia.

Thymic hyperplasia is a rare condition reported mostly in young animals. The disease is characterized by the finding of a histologically normal, severely enlarged thymus, extending from the neck to the cranial mediastinum. The lesion may compress and embrace adjacent organs such as the veins and heart, resulting in heart failure.[19] The lesion has been reported in several domestic animals such as cats, tortoises, rabbits, birds, and cattle.[3,6,19] It has previously been reported that repeated immunizations in calves, rabbits, and birds can lead to diffuse thymic hyperplasia.[19] In calves, a case of enlarged thymus has been described in a stillborn malformed calf, but no information on histological or immunohistochemical findings was reported.[2] The following report describes gross, histological, and immunohistochemical findings of massive thymic hyperplasia in a newborn calf.

A 1-day-old female, 60-kg, Holstein–Friesian calf was referred to the Clinic for Ruminants and Pigs of the Veterinary University Hospital of Lodi (Università degli Studi di Milano, Italy) because of severe dyspnea. The calf had been delivered spontaneously with a few days delay by a heifer. The latter was immunized for *Bovine herpesvirus 1* and *Bovine viral diarrhea virus*. The farmer reported that the placenta was expelled in a few hours. The calf was fed 1 liter of colostrum and treated with corticosteroids. At physical examination, the subject was macrosomic and unable to stand. Rectal temperature was 34.4°C, mucous membranes were cyanotic, and episcleral veins were severely hyperemic and dilated. The pulse was rhythmic but reduced in frequency (72 beats per minute); breathing was abdominal (60 breaths/min), superficial, and characterized by inspiratory effort. At lung auscultation, a reinforced inspiratory murmur associated with diffuse crackling sounds was present. The examination of the neck revealed moderate edema of the ventral part with swollen jugular veins. Auricular and menace reflexes were absent, and suckling reflex was weak. Venous blood gas analysis showed the following: uncompensated respiratory acidosis with pH 7.29, partial pressure of carbon dioxide (pCO_2) at 71 mmHg, partial pressure of oxygen (pO_2) at 30 mmHg, base excess at 4.8 mmol/l, HCO_3 at 33.2 mmol/l, and anion gap at 11.8 mmol/l. On clinical examination, an initial diagnosis of right heart failure associated with severe respiratory distress was made. Although the calf was placed under a heat lamp and infused with saline solution, it died spontaneously and was submitted to necropsy.

Gross examination was performed, and tissues samples were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin wax. Serial sections (3–5 μm) were stained with hematoxylin and eosin. Immunohistochemistry was performed on tissue samples from lymphoid organs (thymus, spleen, lymph nodes) by means of polyclonal

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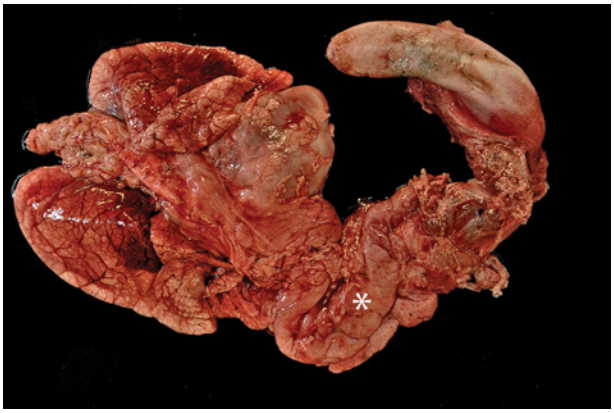

Figure 1. The thoracic organs of a bovine calf diagnosed with severe thymic hyperplasia. Note the severe thymic enlargement, extending from the larynx to the heart base (asterisk). Severe diffuse edema and lung atelectasis are also present.

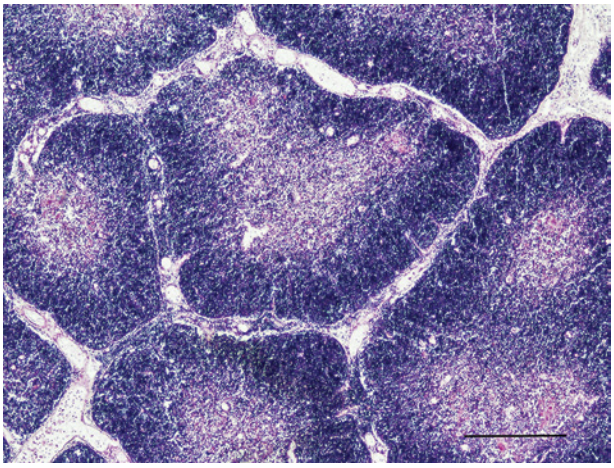

Figure 2. The thymus of a bovine calf with severe thymic hyperplasia. Note the normal distribution of lymphocytes in cortical and medullary areas. Hematoxylin and eosin. Bar = 750 μ.

antihuman cluster of differentiation (CD)20[a] at 1:400 for B-cell recognition, polyclonal anti-CD3[b] at 1:900 dilution for T-cell identification, and monoclonal antihuman HLA-DR[c] at 1:300 dilution for the identification of antigen-presenting cells. These antibodies recognize epitopes conserved in many species, including bovids.[14,18,20] As positive control, a normal thymus from an age-matched newborn calf was utilized. Negative controls consisted of substitution of specific antibodies with an isotype-matched, irrelevant monoclonal antibody or omission of the primary antibody. Briefly, heat-induced antigen retrieval was performed for CD3 and CD20 antibodies by incubation in citrate buffer (pH 6.4) and heated in a microwave oven at maximum power for 1 min and twice at 750 watt for 3 min, then cooled for 20 min. Primary antibodies were incubated overnight in a humidified chamber at 4°C. Secondary detection was performed with the avidin–biotin enzyme complex[d] for 30 min. The reaction was developed with the peroxidase 9-ethylcarbazol-3-amine (AEC) substrate kit for CD3 and CD20 antibodies and with the diaminobenzidine substrate kit for major histocompatibility complex class II (MHCII).[e] Slides were counterstained with Mayer hematoxylin for 3 min and then coverslipped.[f]

Postmortem examination identified a severely enlarged thymus that extended from the retromandibular area to the cranial mediastinum, reaching the heart. The typical cranial "V-form" of the young bovine thymus was lost. The organ measured 40 cm × 20 cm × 10 cm and weighed 1.37 kg. The thymus was lobulated, pale pink, soft, and homogeneous (Fig. 1). The intermandibular region and the visceral space of the neck were characterized by diffuse severe edema. The dorsocranial part of the thorax revealed mild subcutaneous petechial hemorrhages. Additional congenital anomalies included interatrial septal defect, patent ductus arteriosus, mild right ventricle dilation, and complete duplication of the gallbladder. Lymph nodes were unremarkable, and no other macroscopic abnormalities were found.

Histological examination of the thymus revealed a normal corticomedullar ratio with well-demarcated lobules (Fig. 2). Hassall corpuscles were normal in number, morphology, and distribution, but neither tingible body macrophages (TBM) nor apoptotic bodies were identifiable in medullary areas. Lymph nodes and spleen were characterized by diffuse moderate lymphoid depletion. All other organs were microscopically normal.

Immunohistochemical examination of the thymus utilized as positive control was characterized by diffuse CD3 immunolabeling (Fig. 3A), with major intensity in the medulla. Malformed thymus showed very rare CD3-positive cells, both in the medulla and in the cortex (Fig. 3B). Similar immunohistochemical findings were recorded in lymph nodes and spleen, where only rare CD3-positive T cells were present (data not shown). A moderate number of lymphocytes were positive for CD20 in the control thymus (Fig. 3C), while the malformed calf evidenced rare scattered CD20-positive B cells in the thymic medulla (Fig. 3D).

Tissue expression of the MHCII molecule was observed in the thymus of both control and affected calves. Numerous MHCII-positive cells, with strong labeling, were detected in the thymic medulla of the control animal (Fig. 3E); most of the positive cells presented a stellate shape consistent with dendritic cells, which are very important in both positive and negative selection during T-cell development. A moderate number of MHCII-positive cells were polygonal in shape, compatible with macrophages.

In the malformed animal, immunolabeling against MHCII was restricted to the thymic medulla, where rare and weak immunoreactivity was observed (Fig. 3F). Positive cells had a dendritic morphology; macrophages were not observed in the sections examined.

Immunohistochemical results were suggestive of impaired T-cell development. The presence of a severe, diffuse enlarged

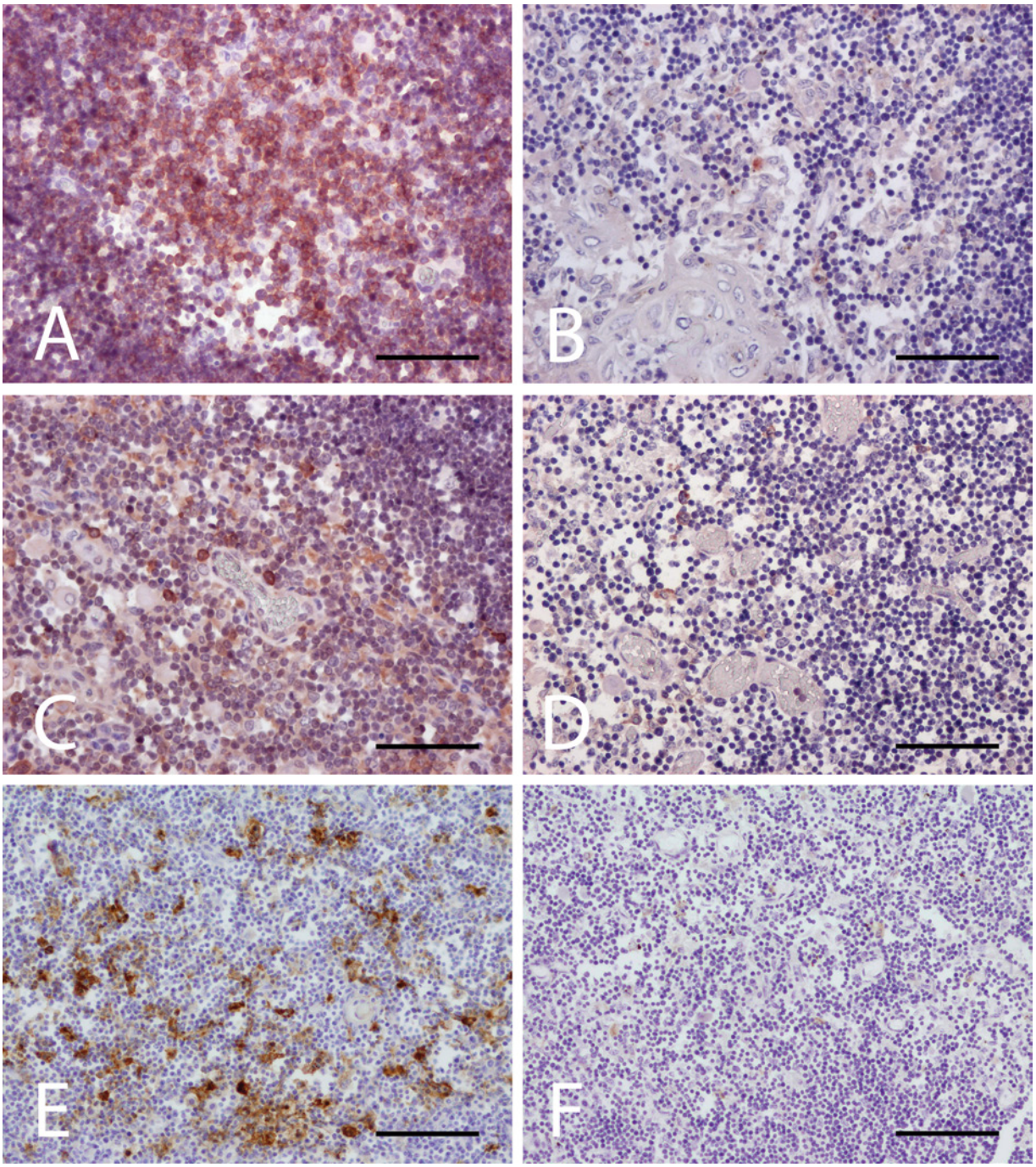

Figure 3. The thymus of a bovine calf with severe thymic hyperplasia compared to the thymus of a normal bovine calf of the same age. **A**, control sample: diffuse cluster of differentiation (CD)3 immunolabeling. Bar = 150 μm. **B**, malformed sample: very rare CD3-positive cells. Bar = 150 μm. **C**, control sample: moderate number of CD20-positive cells. Bar = 150 μm. **D**, malformed sample: rare scattered CD20-positive cells. Immunohistochemistry (IHC), 9-ethylcarbazol-3-amine (AEC), hematoxylin counterstain. Bar = 150 μm. **E**, control sample: numerous major histocompatibility complex class II (MHCII)-positive cells. Bar = 300 μm. **F**, malformed sample: rare and weak MHCII-positive cells. IHC, diaminobenzidine, hematoxylin counterstain. Bar = 300 μm.

thymus with a histologically normal architecture was consistent with a diagnosis of a congenital thymic hyperplasia.

The normal bovine thymus is situated in the anterior mediastinum, and organ involution occurs physiologically and progressively from the fourth to sixth year of life. A typical newborn calf thymus weighs approximately 92 g.[15] The thymus weight of a calf 1–3 weeks of age ranges from 100 g to 200 g, with an increase to 400–600 g at 4–6 weeks of age. At slaughter, a normal calf thymus weighs 600–800 g.[7] In the current case, the organ weighed 1.37 kg just after birth,

which is 14 times heavier than the thymus of a normal newborn calf.

In children, occasional congenital thymic enlargement has been reported. Two entities are well recognized: true thymic hyperplasia (TTH)[5,9,12] and massive thymic hyperplasia (MTH).[10,11,13,16] In human medicine, TTH and MTH are characterized by a variably severe enlargement of the thymus (beyond the normal age range) with the standard microscopic structure.[5,9,12] The main difference between TTH and MTH is represented by the severity of thymus hyperplasia. In MTH, thymus weight represents over 2% of body mass, with preservation of normal thymic architecture.[10,11,13,16] According to a previous study, the organ weight relative to body weight in a newborn calf should be approximately 0.2%.[15] In the present case, the calf weighed 60 kg, and the thymus represented more than 2% of body mass.

The etiology and prognostic significance of human MTH is unknown. This abnormality occurs in young infants with no apparent cause, such as preexisting pathologies, and in the absence of immunologic diseases or previous systemic stressors.[5,10,12] Histological examination is required to differentiate this kind of thymic hyperplasia from other thymic disorders such as thymic follicular hyperplasia of myasthenia gravis, thymoma, and thymic lymphoma.[10] In calves, the most common cause of congenital thymic enlargement is *Bovine leukemia virus*.[1] A second possible differential diagnosis includes thymoma; however, this is an extremely rare neoplasm in juvenile cattle.[4] In the current case, gross and microscopic examination enabled the exclusion of both tumors. Congenital myasthenia gravis-like syndrome has been described in calves, but no pathological thymic alterations have been reported previously (Thompson PN: 2006, Congenital myasthenic syndrome of Brahman cattle. PhD Thesis, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, The Netherlands).[22]

In human fetal thymic development, CD3-positive cells are present from week 9.5 of gestation.[8] Immunohistochemical investigation revealed the lack of CD3 expression in T cells of calf thymus, spleen, and lymph nodes in the current study. This anomalous expression could be related to an altered T-lymphocyte development leading to a putative lack of negative and positive selection. Macrophages in thymus are involved in lymphocyte selection; negatively selected T cells will physiologically undergo apoptosis with apoptotic bodies phagocytized by reactive macrophages termed TBM. In the present case, the severe increase of thymus size could have been related to an altered T-lymphocyte selection with no removal of autoreactive or nonreactive T cells. The lack of TBM supports this hypothesis, with lack of apoptosis leading to accumulation of T cells leading to thymic hyperplasia. The absence of CD3 expression identified in other lymphoid organs may derive from the disorder evidenced in the thymus. In human medicine, there are no immunohistochemically comparable data, and a pathogenic hypothesis on the origin of the severe hyperplasia has not been reported to the authors' knowledge.

Human thymic hyperplasia is often asymptomatic, but the most common clinical sign is, as in the present case, respiratory distress.[5,10,11,13,16] Moreover, the calf showed congenital neck swelling, as in a reported case of thymic hyperplasia in a Galapagos giant tortoise (*Chelonoidis nigra*; syn. *Geochelona nigra*),[6] with edema of the ventral part of the neck, and turgor of jugular veins due to the mass compression effect. In the present case, in addition to thymic enlargement, the calf had a combination of severe congenital lesions including interatrial septal defect, patent ductus arteriosus, and mild right ventricular dilation. In human medicine, concurrent congenital heart defects are reported in conjunction with thymic abnormalities.[17,21] To the best of the authors' knowledge no case of severe thymic hyperplasia has been described in calves before; therefore the possibility that heart and thymic malformations represent a more complex congenital syndrome cannot be ruled out.

In conclusion, the thymic lesion observed in this calf closely resembled cases of human MTH. Severe thymic hyperplasia should be considered among the differential diagnosis of mediastinal masses as a rare cause of respiratory distress in newborn calves; this condition in veterinary medicine should be further characterized by observing multiple cases.

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Sources and manufacturers

a. NeoMarkers Inc., Freemont, CA.
b. Dako Denmark A/S, Glostrup, Denmark.
c. Dako Denmark A/S, Glostrup, Denmark.
d. Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA.
e. Dako Denmark A/S, Glostrup, Denmark.
f. Glicerine, Sigma-Aldrich, St. Louis, MO.

Declaration of conflicting interests

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Feline Upper Respiratory Tract Lymphoma: Site, Cyto-histology, Phenotype, FeLV Expression, and Prognosis

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Feline Upper Respiratory Tract Lymphoma: Site, Cyto-histology, Phenotype, FeLV Expression, and Prognosis

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Abstract

Lymphoma is the most common feline upper respiratory tract (URT) tumor. Primary nasal and nasopharyngeal lymphomas have been evaluated as distinct pathological entities; however, data on their differing clinical behavior are missing. A total of 164 endoscopic- guided URT pinch biopsies were formalin fixed and routinely processed. Imprint cytological specimens were stained with May Grünwald-Giemsa. Immunohistochemistry for anti-CD20, CD3, FeLVp27, and FeLVgp70 was performed. Prognostic significance of clinicopathological variables was investigated by univariate and multivariate analysis. Lymphoma was diagnosed in 39 cats (24%). Most cats with lymphoma were domestic shorthair (32 [82%]), were male (F/M = 0.56), and had a mean age of 10.3 years (range, 1–16 years). Lymphomas were primary nasal in 26 cats (67%), nasopharyngeal in 6 (15%), and in both locations (combined lymphomas) in 7 cats (18%). Neoplastic growth pattern was diffuse in 35 cases (90%) and nodular in 4 (10%). Epitheliotropism was observed in 10 cases (26%). Tumor cells were large in 15 cases, were small and medium in 11 cases each, and 2 had mixed cell size. Submucosal lymphoplasmacytic inflammation was observed in 23 cases (59%). Cytology was diagnostic for lymphoma in 12 of 25 cases (48%). A B-cell origin prevailed (34 [87%]). Feline leukemia virus (FeLV) p27or gp70 antigen was detected in 21 lymphomas (54%). URT lymphomas were aggressive, with survival varying from 0 to 301 days (mean, 53 days). Epitheliotropism in 8 B-cell lymphomas (80%) and in 2 T-cell lymphomas (20%) correlated with prolonged survival. Age younger or older than 10 years had a negative prognostic value. Lymphoplasmacytic inflammation and FeLV infection may represent favoring factors for URT lymphoma development.

Keywords

feline, FeLV, lymphoma, nasal, nasopharyngeal, phenotype, prognosis, respiratory

Lymphomas are among the most common feline malignancies, representing more than 50% of all tumors in cats.[18] Feline lymphoma prevalence rates are approximately 1.6% of cats in the general population and 4.7% of hospitalized sick cats.[18]

The most common sites of occurrence are intestinal and mediastinal,[18] while primary nasal or nasopharyngeal lymphomas represent a rare manifestation,[18] accounting for less than 1% of all feline tumors. Nasal lymphomas seem more common in male cats,[12,14,25] presumably because of behavioral characteristics that make transmission of feline leukemia virus (FeLV) more efficient. The upper respiratory tract (URT) is considered a relatively rare site of lymphoma development; however, lymphoma represents still approximately 50% of primary URT mesenchymal tumors[1,14,16,18,25] and is the most common primary feline nasal tumor.[1,6,14,16,22] URT lymphomas originate more frequently in the solitary nasal location,[1,6,14,16,22] while 10% are found in the nasopharynx and 8% affect both anatomical compartments.[22] In previous studies, microscopic, immunophenotypical features[5,14,25] and the role of cytology in the diagnosis of feline primary URT lymphomas have been assessed.[22] However, the evaluation of the 3 distinct presentations of URT lymphoma (nasal, nasopharyngeal, or lymphoma simultaneously developing in both locations [combined lymphomas])[22] as distinct clinicopathologic and prognostic entities seems unavailable. On the contrary, in human medicine, primary nasal and nasopharyngeal

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Table 1. Panel of Primary Antibodies Used to Characterize Primary Feline Upper Respiratory Tract Lymphomas.

Antibody	Clone/Species Specificity	Dilution	Source	Supplier	Main Cell Reactivity
CD3ε	CD3-12 human[a]	1:10	Rabbit polyclonal	Serotec, Oxford, UK	T cells
CD20	Human[a]	1:400	Rabbit polyclonal	Neomarkers, Freemont, CA	B cells
FeLV gp70	C11D8 feline	1:100	Mouse monoclonal	Custom Monoclonals International, Sacramento, CA	FeLV envelope glycoprotein 85/70
FeLV p27	PF12J-10A feline	1:100	Mouse monoclonal	Custom Monoclonals International, Sacramento, CA	FeLV capsidic protein

[a]Cross-reactive with feline tissues.

lymphomas are considered 2 clinically distinct diseases since nasal lymphoma bears a more frequent T-cell phenotype with poor response to chemotherapy and higher relapse rates.[21]

The aim of this study was the evaluation of cytological, histological, phenotypical FeLV tissue antigen expression and follow-up data of 3 different URT lymphoma presentations—nasal, nasopharyngeal, and combined tumors—to find a possible significant prognostic role of the different clinical and pathological variables.

Materials and Methods

Case Selection

Pinch biopsies from all cases of nasal or nasopharyngeal feline disease were retrospectively collected from the surgical pathology archives of the Anatomical Pathology Section of the School of Veterinary Medicine of Milan between 2000 and 2012. All tissue samples were obtained by anterograde or retrograde endoscopic biopsy. The retrograde endoscopic technique was applied for nasopharyngeal tissue samples. A total of 39 (24%) of 164 cats undergoing nasal biopsies between 2000 and 2012 were diagnosed with lymphoma, representing 50% of nasal tumors diagnosed. Other tumors were carcinomas in 25 cats (15%) and mesenchymal tumors in 14 cats (9%).

For each cat with lymphoma, signalment, presenting complaints, clinical signs, diagnostic imaging, rhinoscopic appearance, multiple endoscopic pinch biopsies, histopathology, and, when available, cytology were collected.

Histological and Cytological Evaluation

All biopsy samples were fixed in 10% neutral-buffered formalin, routinely processed, and stained with hematoxylin and eosin (HE).

In cats with URT lymphomas, a number of biopsies varying from 4 to 14 (mean, 6) were collected by endoscopy. For primary nasal and for combined lymphomas, biopsies were performed from both (left and right) nasal cavities, even when a space-occupying lesion was not detected during endoscopic examination. Biopsy sampling was repeated to check for disease progression or to confirm the preceding diagnosis in selected cases. During scheduled clinical check-ups, the cat Nos. 5, 31, and 32 were reevaluated clinically and by endoscopy. Biopsies were taken after 3 and 9 months in cat No. 5 and after 1 and 3 months in cat Nos. 31 and 32.

For lymphomas, cytological samples from impression smears, fine-needle aspirates, and brushings were air dried and stained with May-Grünwald Giemsa.

To reduce inappropriate diagnostic interpretation, all the cytological and histological slides were blindly and independently submitted to pathologists with a different degree of experience, including 1 resident (S.S.F.) and 2 board-certified pathologists, with 1 more experienced in histopathology (R.P.) and 1 more experienced in cytopathology (C.M.). Only those cases that were classified in the same way by the 3 readings were included in the caseload. URT lesions were grouped in the following diagnostic categories: lymphomas, other neoplasms, inflammatory or degenerative lesions, and miscellaneous lesions. Cases of lymphomas were selected and subsequently classified on the basis of their anatomical location (nasal/nasopharyngeal/combined), growth pattern (follicular, nodular, diffuse, epitheliotropic), and cell type and grade, applying the modified World Health Organization (WHO) classification for lymphomas.[33] Mitotic index was evaluated counting the number of mitoses at a 400× magnification in 10 fields using the same microscope.

Immunohistochemical Staining

For all cases of lymphoma, cell phenotype and FeLV antigen expression were assessed. Unstained 5-µm sections from paraffin-embedded biopsy specimens were mounted onto Superfrost Plus Slides (Menzel Glasser; Gerhard Menzel, Glasbearbeitungswerk GmbH & Co, Braunschweig, Germany). For phenotypic and FeLV antigen expression evaluation, samples were stained with primary antibodies listed in Table 1. For all antibodies, antigen retrieval was achieved by heating slides in citrate buffer at pH 6.0 in a commercial pressure cooker Decloaker (Biocare Medical, Walnut Creek, CA) for 10 minutes. Endogenous peroxidase was quenched for 30 minutes with 3% hydrogen peroxide. Sections were stained using an avidin-biotin-peroxidase technique as previously described.[15] Omission of the primary monoclonal antibody or application of an isotype-matched nonspecific monoclonal antibody anticanine CDH5R was used as a negative control. Formalin-fixed specimens from a reactive lymph node and from a

FeLV-positive cat were used as positive controls. The immunoreaction was visualized with amino-9-ethyl-carbazole chromogen (AEC; Vector, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin and mounted with glycerine.

Follow-up

Follow-up was performed when possible by the oncologists at the same institution and consisted of a clinical examination every 3 months. When this was not feasible, follow-up consisted of telephone interview with the owner or the referring veterinarian to collect clinical information, type of treatment, and time and cause of death.

Statistical Analysis

Statistical analysis was performed following when possible the guidelines of the American College of Veterinary Pathologists' Oncology Committee.[37] The end point considered for each cat with lymphoma was time to death, calculated as the time elapsed from the date of diagnosis to the date of death for any cause, or to the date of last clinical information for cats that were alive at the study closing date (right-censored times).[37] Median follow-up was estimated by the reverse Kaplan-Meier method.[29] Overall survival probability curve was estimated by the Kaplan-Meier method. First, the putative prognostic role of the considered variables (age, sex, breed, anatomical site, size of lymphomatous cells, neoplastic phenotype, FeLV status, mitotic index, and epitheliotropism) was investigated, including each single variable in a Cox regression model (univariate analysis). Aiming to evaluate the adjusted prognostic role in Cox multivariable regression models, the maximum number of variables that can be included to avoid unreliable results depends on the number of observed deaths. According to the caseload included in the study, a ratio of 1:5 was considered, as previously suggested.[36] Since it was not possible to include all the above-mentioned variables, only a subset of variables were jointly analyzed in multivariable regression models (mitotic index, age, FeLV status, phenotype, and epitheliotropism). These variables were selected according to the specific clinical experience of the authors. A model containing only variables with the greatest prognostic role was then obtained by a backward selection procedure based on the Akaike information criterion (AIC).[35] Continuous variables (age and mitotic index) were analyzed according to their original measurement scale in such a way as to maintain their maximum possible prognostic information. Potential nonlinear relationships between continuous variables and logarithm of the hazard in the Cox model were evaluated by restricted cubic spline transforms.[13]

Results are reported in terms of estimated hazard ratios (HRs) with 95% confidence intervals (CIs). For categorical variables, one of the modalities was chosen as reference. Thus, for each of the remaining modalities, the HR is the ratio between the hazard of death for this modality and the hazard of death of the reference. The null hypothesis of each regression coefficient equal to zero was evaluated by the Wald test, and the prognostic contribution of the variable to the model was evaluated by likelihood ratio test. For continuous variables, model estimates in terms of HRs cannot be easily interpreted in the case of a nonlinear relationship between the variable and model response. Thus, after identifying the value of the variable corresponding to the minimum hazard of death (reference), selected hazard ratios for values of the variable greater and lower than reference were provided to describe the prognostic behavior.

Results

URT Lymphomas: Signalment and Clinical Findings

Signalment and major clinical complaints divided for the 3 different URT lymphoma presentations are summarized in Supplemental Table S1. In cats with URT lymphoma, median age was 10.3 years (range, 1–16 years) with a female/male ratio of 0.56 and a majority of domestic short-haired (DSH) cats (32/39 [82%]). Other breeds included 3 Siamese, 2 Chartreux, 1 Persian, and 1 Devon Rex. FeLV and feline immunodeficiency virus (FIV) serology were available in 7 cats (18%); all except 1 (cat No. 29) were FeLV negative, while two cats were FIV positive (cat Nos. 1, 27). Major clinical complaints for cats with lymphoma were nasal discharge (30/39 [77%]), sneezing (26/39 [67%]), stertor (18/39 [46%]), dyspnea (9/39 [23%]), epiphora (8/39 [21%]) or ocular discharge (4/39 [10%]), epistaxis (8/39 [21%]), and anorexia (7/39 [18%]).

Skull radiographs (11 [28%]) or computed tomography (CT) scans (8 [21%]) were performed prior to rhinoscopy in some cats with URT lymphoma. Based on diagnostic imaging and rhinoscopy, 26 of 39 (67%) lymphomas were primary nasal, 6 of 39 (15%) were nasopharyngeal, and 7 of 39 (18%) had both a nasal and nasopharyngeal location and were termed *combined lymphomas* (CLs).

By rhinoscopy, lymphomas were described as exophytic, pale pink to whitish, cerebroid, and friable space-occupying lesions in 28 of 39 (72%) cases. No masses were evident in 11 cats (28%), where a rough thickened mucosa was documented.

Cytological Findings in URT Lymphomas

Cytological specimens were available in 25 of 39 cases (64%) and were obtained by impression (16 [64%]), brushing (6 [24%]), or fine-needle aspiration (3 [12%]). A diagnosis of lymphoma was attained by cytology in 12 of 25 (48%) cases, while for 11 cases (44%), a false-negative diagnosis of lymphoplasmacytic rhinitis was achieved. An inconclusive cytology was recorded in 2 cases (8%). All cytological samples, with the exception of the 2 inconclusive cases, exhibited good cellularity; small lymphocytes admixed with plasma cells, neutrophils, or macrophages were often observed in association with the neoplastic population (Fig. 1). Less frequent findings included

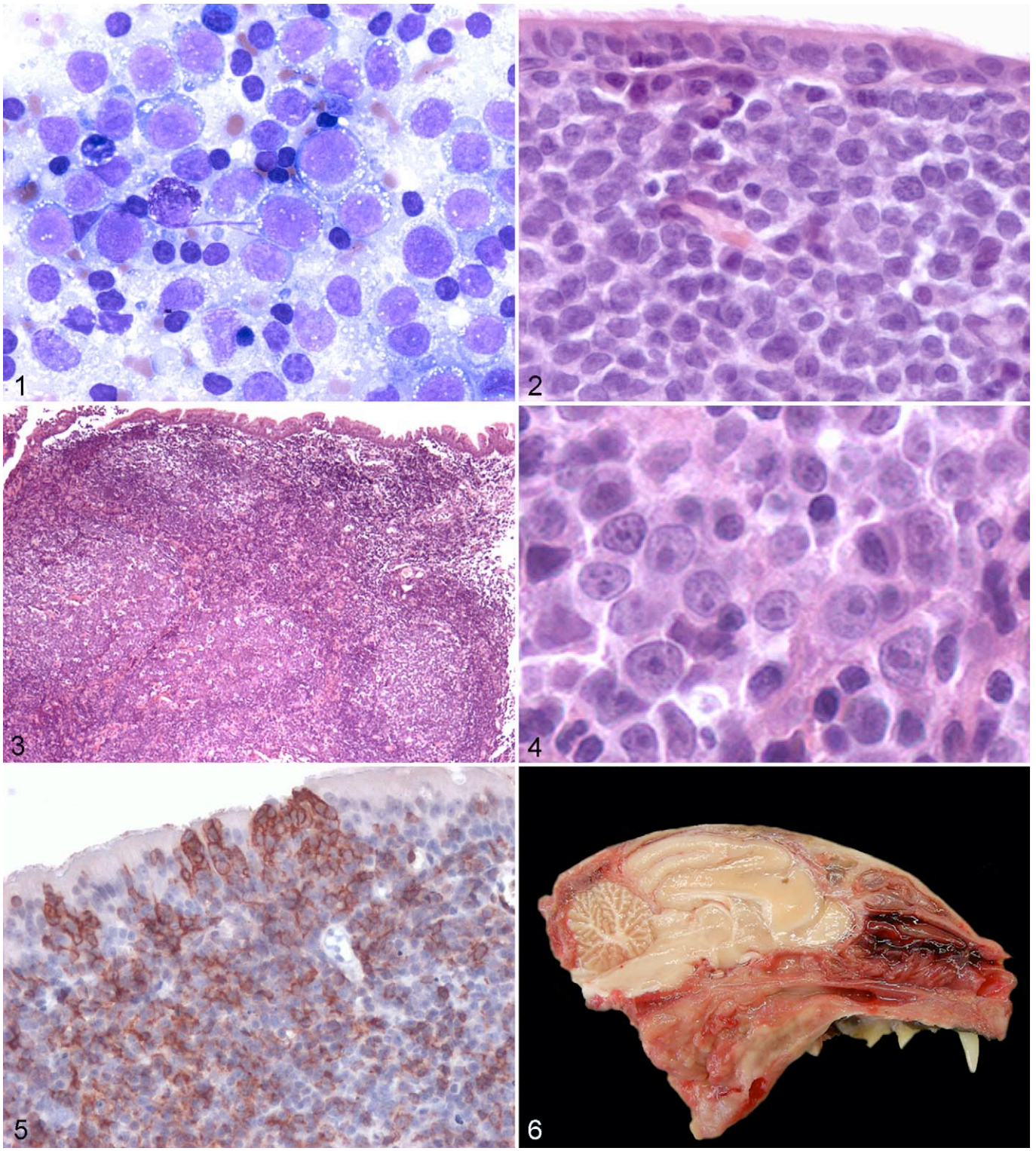

Figure 1. Nasopharynx, diffuse large B-cell lymphoma, immunoblastic morphology; cat No. 28. Nasopharyngeal brushing. Large, discrete lymphoid cells with abundant blue vacuolated cytoplasm, round nucleus, and 1 to 2 prominent nucleoli. Scattered small mature lymphocytes are visible. May Grünwald-Giemsa. **Figure 2.** Nasal cavities and nasopharynx, combined lymphoma; cat No. 34. Diffuse growth pattern of predominantly large neoplastic round cells obscuring the respiratory lamina propria. Nuclear pleomorphism is evident. Occasional plasma cells are present. Hematoxylin and eosin (HE). **Figure 3.** Nasal cavities and nasopharynx, combined lymphoma, follicular center cell lymphoma grade I; cat No. 35. Nodular growth with expansion of follicular center; loss of mantle zone and initial coalescence of follicles is evident. HE. **Figure 4.** Nasopharynx, diffuse large B-cell lymphoma, immunoblastic morphology; cat No. 28. Neoplastic cells are large with generally indistinct cell boundaries and characterized by a finely stippled chromatin pattern and a single prominent central basophilic nucleolus. Small mature

Table 2. Incidence of Cell Type Based on Tumor Location.

Location	Small Cell, No. (%)	Medium Cell, No. (%)	Large Cell, No. (%)	Mixed Cell, No. (%)	Total, No.
Nasal	8 (31)	7 (27)	11 (42)		26
Nasopharyngeal		3 (50)	2 (33)	1 (17)	6
Combined	3 (43)	1 (14)	2 (29)	1 (14)	7

hyperplastic to dysplastic respiratory epithelium, necrosis, and presence of occasional osteoclasts.

Pathology and Phenotype of URT Lymphomas

Microscopic features, lymphoma classification, and phenotype are summarized in Supplemental Table S2. The incidence of lymphoma cell type based on tumor location is summarized in Table 2.

Histological growth pattern was diffuse (Fig. 2) in 35 URT lymphomas (90%) and nodular (Fig. 3) to follicular in 4 cats (10%). Comparing the differing anatomical distributions, primary nasal lymphomas were diffuse in 25 cases (96%) and nodular in 1 cat (4%). Five cases (83%) of nasopharyngeal lymphoma had diffuse cell growth, and 1 (17%) was nodular. Combined lymphomas were observed in 7 cats (18%) and were characterized by diffuse growth in 5 cats (71%) and nodular growth in 2 cats (29%).

Comparing the cell type, 11 cases (28%) were diagnosed as small cell lymphoma, including lymphocytic (6 [55%]), prolymphocytic (4 [36%]), and plasmacytic (1 [9%]). Medium-sized cell lymphomas were diagnosed in 11 cases (28%); 8 of 11 (73%) were medium sized, not otherwise specified (NOS); 2 were plasmacytomas (18%); and 1 was a peripheral T-cell (PTCL)–like lymphoma (9%). Two follicular center cell lymphomas were observed, one grade I and one grade II. Large cell lymphoma (Fig. 4) was diagnosed in 15 cats (38%), with 13 cases having an immunoblastic morphology (87%). Of the primary nasal lymphomas, 11 were large (42%), 7 were intermediate (27%), and 8 (31%) were small cell cases. Nasopharyngeal lymphomas were large immunoblastic in 2 cats (33%), intermediate (including 1 plasmacytoma) in 3 cases (50%), and 1 case of mixed cell size (17%) diagnosed as follicular center cell lymphoma grade II. Combined lymphomas were large cell in 2 cases (29%), intermediate in 1 case (14%), and small cell in 3 cases (43%), and 1 mixed cellularity (14%) was diagnosed as a follicular center cell lymphoma grade I. Regardless of the anatomical location, in 23 cases (59%) of URT lymphomas, variable degrees (mild to severe) of submucosal plasmacytic to lymphoplasmacytic inflammation were evident.

Additional histopathological findings included multifocal mucosal ulceration with secondary neutrophilic and catarrhal inflammation, cystic dilation of mucous glands, edema, fibroplasia, and bone remodeling. Concurrent hyperemia, edema, and abundant catarrhal to purulent exudate were detected in 24 of 39 cats (61.5%).

A B-cell phenotype was identified in 34 of 39 URT lymphomas (87%). Of these, the largest number was represented by diffuse large cell lymphomas (15 [44%]), followed by small cell lymphomas (8 [24%]), intermediate cell NOS (7 [21%]), plasmacytomas (2 [6%]), and follicular lymphomas (2 [6%]). In 3 of 39 cases (8%), a T-cell phenotype was identified, comprising 2 diffuse small cell lymphomas and 1 PTCL-like lymphoma. In 2 of 39 cases (5%), 1 small cell lymphoma and 1 intermediate cell lymphoma, neoplastic cells were negative for T-cell and B-cell markers. Epitheliotropism (Fig. 5) was observed in 10 cases (26%), specifically in 8 cases of B-cell lymphomas (80%) and in 2 T-cell lymphomas (20%).

Phenotypic distribution in nasal lymphomas was characterized by a predominance of B-cell tumors (22 [85%]), with 2 cases (8%) of T-cell and 2 cases (8%) of the non B–non T-cell phenotype. All nasopharyngeal lymphomas displayed a B-cell origin. Combined lymphomas were B cell in 6 cases (86%) and T cell in 1 cat (14%).

Immunohistochemical Expression of Viral Antigens in URT Lymphomas

FeLV antigen expression in conjunction with anatomical distribution of URT lymphomas is listed in Supplemental Table S2. FeLV p27 and FeLV gp70 antigen expression either singly or in conjunction was detected by immunohistochemistry in a total of 21 of 39 cases (54%). Expression of FeLV p27 was observed only in 17 of 39 (44%), while gp70 only was observed in 12 of 39 cases (31%). Concurrent positivity for FeLV antigens was detected in 8 of 39 cats (21%). These cats were considered productively FeLV infected. Expression of either antigen in conjunction with lymphoplasmacytic inflammation was observed in 12 of 39 cats (31%).

Therapy and Follow-up

Five cats were lost to follow-up at 7, 7, 10, 43, and 54 days after diagnosis. Combination chemotherapy and/or radiation therapy were always offered but refused by owners in all but 1 cat that received radiation therapy (cat No. 5).

Figure 4. (Continued) lymphocytes are also present. HE. **Figure 5.** Nasal cavities and nasopharynx, combined lymphoma, follicular center cell lymphoma grade I; cat No. 35. Accumulation of neoplastic CD20-positive B cells (epitheliotropism) in the nasal mucosa. CD20 immunohistochemical stain, AEC chromogen, hematoxylin counterstain. **Figure 6.** Longitudinal section of head; cat No. 28. Presence of a whitish, cerebroid mass obliterating the nasopharyngeal cavity.

Table 3. Univariate Analysis.

Variable	HR	95% CI	z	P	χ^2	P
Age					**3.25**	**.1972**
Linear term	0.8264	0.6800–1.004	−1.916	.0553		
Nonlinear term	1.2323	0.9709–1.564	1.717	.0859		
Sex					**0.65**	**.4205**
Female [reference]						
Male	0.7214	0.3285–1.584	−0.813	.416		
Breed					**0.08**	**.7709**
DSH [reference]						
Other	0.8794	0.3672–2.106	−0.288	.773		
Site					**0.19**	**.909**
Nasal cavity [reference]						
Nasopharynx	1.2795	0.4258–3.844	0.439	.661		
Combined	0.9959	0.3900–2.543	−0.009	.993		
FeLV gp70					**0.62**	**.4309**
Negative [reference]						
Positive	0.7238	0.3182–1.646	−0.771	.441		
FeLV p27					**0.06**	**.8064**
Negative [reference]						
Positive	0.7238	0.3182–1.646	0.245	.806		
FeLv gp70 and FeLv p27					**0.13**	**.7153**
Negative-negative [reference]						
At least 1 positive	1.152	0.537–2.471	0.363	.717		
Cell size					**1.32**	**.5166**
Small [reference]						
Medium	1.078	0.3894–2.984	0.144	.885		
Large	1.613	0.6409–4.058	1.015	.310		
Mitotic index					**1.49**	**.2225**
One-unit increase	1.018	0.9911–1.046	1.309	.191		
Phenotype					**0.03**	**.8568**
B [reference]						
Other than B	0.8962	0.268–2.996	−0.178	.859		
Epitheliotropism					**2.9**	**.08831**
Negative [reference]						
Positive	0.4642	0.1817–1.186	−1.604	.109		

Cox model results: HR = hazard ratio, 95% C.I.= 95% confidence interval.
Z= Wald statistic and corresponding P value are referred to the test for each HR.
X^2= likelihood ratio test and corresponding P value are referred to the test for the contribution of the variable to the model.

According to the reverse Kaplan-Meier method, median follow-up was 301 days, the first quartile (25%) was 60 days, and the third quartile (75%) was 381 days. The survival probability at 30, 60, and 300 days after surgery was 0.793 (95% CI, 0.6751–0.932), 0.375 (95% CI, 0.2422–0.581), and 0.153 (95% CI, 0.0595–0.396), respectively.

Major causes of death were euthanasia (21 cats) due to the severity of respiratory obstruction caused by the disease. At the end of the study, 6 cats were still alive, with 1 cat affected by a small B-cell lymphocytic lymphoma treated with radiation therapy surviving for 1293 days (cat No. 5).

In 4 cats, lymphoma was confirmed by full necropsy (cat Nos. 1, 27, 28, 33). Multiorgan involvement was found in 3 cases (cat Nos. 1, 27, 33), with renal (cat Nos. 27, 33) and myocardial infiltration (cat No. 27) and contiguous central nervous system (CNS) involvement by ethmoidal bone lysis (cat Nos. 1, 33). In 1 case (cat No. 28), lymphoma was limited to the nasopharyngeal location (Fig. 6). In this cat, severe monolateral dysplasia of the bronchial walls was also evident.

Statistical Analysis

In univariate analysis, concerning age, a nonlinear relationship was considered because the fit of a 3-knots spline function was better than the fitting by only a linear term (likelihood ratio test for the model with spline = 3.25, $P = .1972$; likelihood ratio test for the model with only the linear term = 0.55, $P = .4578$; likelihood ratio test for the contribution of the nonlinear term = 2.6958, $P = .10$). No variables showed a significant prognostic value based on a significance level of .05 (Table 3).

When mitotic index, age, FeLV positivity, phenotype, and epitheliotropism were jointly considered in a multiple regression model (Table 4), a significant prognostic role was obtained only for epitheliotropism (likelihood ratio test

Table 4. Multivariate Analysis.

Variable	HR	95% CI	z	P	χ^2	P
Initial model						
Age					**3.6975**	**.1574**
Linear term	0.8108	0.6529–1.0069	–1.898	.0577		
Nonlinear term	1.2949	1.0002–1.6765	1.961	.0498		
Mitotic index					**0.3463**	**.5562**
One-unit increase	1.0108	0.9765–1.0463	0.611	.5412		
FeLv gp70 and FeLv p27					**0.5404**	**.4623**
Negative-negative [reference]						
At least 1 positive	0.7063	0.2807–1.7775	–0.738	.4603		
Phenotype					**0.4546**	**.5001**
B [reference]						
Other than B	1.6127	0.4239–6.1352	0.701	.4833		
Epitheliotropism					**4.5316**	**.03327**
Negative [reference]						
Positive	0.3115	0.1019–0.9523	–2.046	.0408		
Final model (backward selection)						
Age					**4.2817**	**.1176**
Linear term	0.8106	0.6709–0.9795	–2.175	.0296		
Nonlinear term	1.2863	1.0109–1.6366	2.048	.0405		
Epitheliotropism					**3.9396**	**.04716**
Negative [reference]						
Positive	0.3981	0.1505–1.0533	–1.855	.0636		

Cox model results: HR = hazard ratio, 95% C.I.= 95% confidence interval.
Z= Wald statistic and corresponding P value are referred to the test for each HR.
X^2= likelihood ratio test and corresponding P value are referred to the test for the contribution of the variable to the model.

= 4.5316, P = .03327), where the subjects with negative epitheliotropism showed a greater risk of death than did subjects with positive epitheliotropism (hazard ratio = 0.3115). After applying the backward selection procedure to the above-mentioned model, only age and epitheliotropism were maintained as putative prognostic factors.

Considering 10 years as the reference, a U-shaped behavior of the hazard was observed as a function of age. Thus, the estimated hazards of death of 3-, 5-, and 7-year-old cats are about 1.47, 2.22, and 3.38 times greater than the hazard of death of a cat aged 10 years, respectively, and the estimated hazards of death of 12-, 13-, and 15-year-old cats are about 1.18, 1.47, and 2.46 times greater than the hazard of death of a cat aged 10 years. The hazard of death of subjects with negative epitheliotropism is about 2.5 times greater than the hazard of death of subjects with positive epitheliotropism.

Discussion

This report describes and compares pathological findings, FeLV antigen expression, and survival of feline URT lymphomas by their division into nasal, nasopharyngeal, or combined primary anatomical locations. Nasal or nasopharyngeal tumors have been mostly evaluated separately,[5,12] and lymphomas in the 3 distinct presentations seldom have been compared.[22] Histopathology in conjunction with diagnostic imaging (radiographs and CT scans) resulted in the classification of URT lymphomas into 26 nasal, 6 nasopharyngeal, and 7 combined nasal-nasopharyngeal lymphomas. The predominance of primary nasal lymphomas paralleled the frequency reported previously.[22] No major differences in phenotype, classification, and prognosis were evident. Of particular note were that nasopharyngeal lymphomas always had a B-cell origin and that combined lymphomas did not express FeLV antigens. Also, epitheliotropism was a positive prognostic factor, while age correlated with a poor prognosis.

The 39 cats with lymphoma were drawn from a case series of 164 cats biopsied for nasal lesions. Of these, lymphomas were 24% of all lesions, accounting for 50% of tumors. This distribution resembled frequencies previously reported, with lymphoma being the most common URT tumor.[1,6,14,16,25] Our findings closely paralleled a retrospective study of nasopharyngeal disease in 53 cats reporting that 49% had lymphoma and 28% had polyps.[1]

Of 164 nasal biopsies, 163 were diagnostic. The result of the diagnostic accuracy of histopathology was not comparable with other veterinary reports since studies on the diagnostic precision of endoscopic nasal biopsy evaluation seem not to be available. However, this result seems to represent a high success rate compared with what has been reported for the diagnosis of primary nasal carcinoma in humans, in whom 5% percent of cancers are missed at endoscopic biopsy.[20] The diagnostic success rate observed herein may derive from the expertise of the surgeon providing samples of large size and an adequate number of biopsies that ranged from 4 to 14 per single case. In humans, for nasal carcinoma diagnosis, an average of 6 biopsies from both diseased and adjacent apparently healthy nasal tissue are suggested as the minimum number for the correct diagnosis.[20] Also, in this study, depth of biopsies was pivotal

since the inclusion of the deep lamina propria and turbinates allowed the evaluation of the distribution and of the infiltrative behavior of tumors. Besides, endoscopic-guided biopsy permitted the visualization of nasal and nasopharyngeal mucosa, assisting in the selection of lesional areas to sample.[20] The success rate of histology contrasted with the poor results of cytology, which was diagnostic only in 50% of lymphomas. The main problem encountered was sample contamination by inflammation, leading to misdiagnosis. Our results parallel the observation that many of the cytological sampling techniques have an unacceptably low yield of diagnostic material for nasal specimens.[2,23] In concordance with previous reports,[2,6] impression smears from biopsies and fine-needle aspiration biopsies detected lymphoma more accurately compared with brushing. Brushing is a technique performed blindly,[2,6] and thus small tumors can be missed and inflammatory findings may be misleading. Also, most lymphomas of this caseload developed in the mid to deep lamina propria, which is not sampled by brushing. Similar problems have been reported for nasopharyngeal masses,[1] since cytology is undertaken without direct visualization of the nasopharyngeal area, leading to an inaccurate diagnosis.[1] Crush cytological specimens have demonstrated a better diagnostic yield.[6] Unfortunately, contrary to imprints, crush sample biopsies need to be sacrificed, reducing the subsequent diagnostic capability of histopathology. Despite a low diagnostic potential, in this report, cytology was extremely useful for lymphoma classification thanks to a better assessment of cell morphology. The correct WHO cytological classification of lymphomas is essential for their prognostication. Thus, for lymphoma diagnosis, cytology should be performed when possible and evaluated in conjunction with histopathology.

Cats developing URT lymphomas were mostly adult to aged males, similar to previous reports,[12,25] but contrasting with findings in other caseloads.[14] The predominance of male cats has been hypothesized to derive from the male territorial behavior that makes transmission of FeLV more efficient.[18] In our caseload, FeLV antigen positivity demonstrated by immunohistochemistry was distributed almost equally among sexes, contrasting with this hypothesis. FeLV serological positivity has been correlated to the anatomical form of lymphoma, with most positive cats having mediastinal lymphoma (90%) and multicentric lymphoma (80%) and less than 10% having cutaneous lymphoma.[18] FeLV serology has been seldom evaluated in URT lymphomas. In this study, most cats were not tested for FeLV, likely owing to their old age; however, 6 of 7 cats tested were negative. FeLV p27 capsid and gp70 envelope immunohistochemical protein expression by neoplastic cells was documented in 54% of our cases, but combined URT lymphomas were always negative. A comparison with other URT lymphoma caseloads was not possible due to the lack of data regarding FeLV tissue antigen expression. The old age of cats developing URT was considered incongruent with a causal role of FeLV since it is well established that FeLV-positive cats generally develop lymphomas at a young age;[17] thus, the finding of FeLV protein expression by neoplastic cells was surprising. Significantly, old seronegative cats may still bear the virus at the genomic level and can transcribe FeLV upon reactivation or neoplastic transformation of infected cells.[7] Noteworthy, FeLV gp70 expression was observed in 12 cats. Expression of FeLV gp70 denotes viral particle assembly, confirming a productive infection.[30] Thus, FeLV might still be involved in feline URT lymphoma development.

A concurrent plasmacytic inflammation was observed in 59% of URT lymphomas. Chronic rhinitis-rhinopharyngitis is considered a consequence of prior infection with either feline herpesvirus type 1 or feline calicivirus.[34] Up to 80% of cats that recover from acute viral URT infections may become chronic carriers, which can lead to persistent clinical signs and inflammation.[10] In addition, viral infection causes injuries that predispose to secondary bacterial disease.[14] Chronic inflammation is considered a risk factor for the development of a variety of cancers, including hematopoietic malignancies in humans.[8,11,28] In humans, B-cell lymphomas developing after longstanding chronic inflammation[4,26,31] seem common, and the association between bacterial and viral infections with lymphoma development is well documented for mucosa-associated lymphoid tissue (MALT) B-cell lymphomas.[8,11,28] Notably, in our caseload, inflammation and FeLV tissue antigen positivity were concurrently documented in 31% of cats. Specifically, chronic persistent rhinitis may favor expansion of virally transfomed B-lymphoid cells, explaining the higher frequency of URT lymphomas with a B-cell phenotype observed in this and other caseloads.[5,22,25]

The histological diagnosis of lymphoma was performed using the modified WHO classification.[32,33] In other studies of feline URT lymphomas, the Revised European American Lymphoma (REAL)/ revised WHO classification[5] and the National Cancer Institute working formula classification[22] have been applied. Large cell lymphomas were mostly identified in our series (39%), with a prevalence of immunoblastic morphology, paralleling other studies.[5,22] Interestingly, small cell lymphomas were also frequent (28%). As for these cases, rare lymphoma variants such as plasmacytoid/plasmacytic[22] follicular grade II,[5] as well as anaplastic large and small cell lymphomas,[14] have rarely been described.

Epitheliotropism was unexpectedly identified in B-cell lymphomas of the 3 URT anatomical types. In cats, epitheliotropism usually has been described in cutaneous and gastrointestinal T-cell lymphomas;[3,9,24,27] however, this growth pattern has also been occasionally reported in feline URT lymphomas.[22,25] Human studies have documented epitheliotropism of B cells in tonsillar hyperplasia and in MALT lymphomas.[19] Interestingly, in our study, epitheliotropism was statistically associated with a prolonged survival time of diseased cats. Considering other histological variables, mitotic index, cell size, phenotype, and FeLV antigen expression had no significant correlation with mortality. Epitheliotropism and age, evaluated on a continuous scale, were the only prognostic factors identified; however, lack of statistical evidence of other variables having a significant prognostic impact should be considered with caution. In fact, power of statistical tests may be low, and the reduced sample size did not permit a full evaluation of the joint

prognostic role of the considered variables. For a more reliable evaluation, a case series with at least 100 deaths would be needed. Also, an increased number of combined and nasopharyngeal lymphomas are necessary to obtain a representative selection of cases providing a suitable cohort for statistical analysis of potentially significant prognostic variables.

In conclusion, URT lymphomas are aggressive tumors with a prevailing primary nasal B-cell origin. Lymphoplasmacytic inflammation is frequently associated with URT lymphomas and may represent alone, or in conjunction with FeLV infection, a possible favoring factor in lymphoma development.

Declaration of Conflicting Interests

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Poster presentation at the 10th Annual Congress of the Italian Association of Veterinary Pathology (Giulianova Lido, Teramo, Italy, May 2013) and the 31st Annual Congress of the European College of Veterinary Pathology and European Society of Veterinary Pathology (London, UK, September 2013).

History: A 7-year-old, male, English bulldog (31 Kg [68 lb]) was referred for severe expiratory dyspnoea unresponsive to furosemide. The dog had a history of idiopathic juvenile epilepsy treated with phenobarbital (100 mg, PO, q 12h) and bromide (400 mg, PO, q 12h). The emergency care included administration of cephalexin (30 mg/kg [66 mg/lb],IV, q 12h), enrofloxacin (5 mg/kg [11 mg/lb], IV, q 12h), beclomethasone dipropionate (aerosol, q 8h), buthorphanol (0,2 mg/Kg [4,4 mg/lb], IM), oxygen therapy (65 ml/Kg [143 mg/lb], nasal probe) and intravenous fluids (65 ml/die + 2% of dehydration). Despite therapy the animal underwent a respiratory arrest twelve hours after admission.

Clinical and gross findings: Respiratory signs persisted in the dog from admission till decease. Thoracic radiographs evidenced an alveolar to interstitial pattern. The cell blood count (CBC) and serum biochemistry revealed mild leucocytosis (17.9 $x10^3$ leukocytes/µL; reference interval, 6 to 17 x 10^3 leukocytes/ml) with neutrophilia (13.96 $x10^3$ neutrophils/µL; reference interval, 3 to 11.5 x 10^3 neutrophils/ml) and monocytosis (2.5 $x10^3$ monocytes/µL; reference interval, 0.15 to 1.35 x 10^3 monocytes/ml), numerous platelets aggregates, and increased level of AST (34 IU/L; reference interval, 0 to 32 IU/L), ALT (208 IU/L; reference interval, 15 to 90 IU/L), ALP (356 IU/L; reference interval, 0 to 85 IU/L), GGT (49 IU/L; reference interval 0 to 10 IU/L). At necropsy, the liver was severely and diffusely enlarged (weight: 1850 g), soft and friable with enhanced lobular pattern, associated with severe dilation of the abdominal vena cava over three times the normal diameter interpreted as secondary to hypertension. The apical, accessory and right principal lobes of the lung contained multifocal to coalescing, up to 0.6 cm in diameter, round, firm umbilicated lesions. A moderate right ventricular dilation along with minimal endocardiosis of the mitral valve was also evident.

Histopathological findings: Tissues samples were fixed in 10% buffered formalin for 5 days, routinely processed and stained with Haematoxylin and Eosin (H&E). Histochemical

special stains (Periodic acid-Schiff (PAS), von Kossa and Congo red) of selected pulmonary sections were also performed and lung samples were submitted for transmission electron microscopy (TEM).

The hepatic lesions consisted of diffuse and severe congestion, severe multifocal to coalescing vacuolar degeneration and centrilobular haemosiderosis. Most of the alveoli were filled by abundant, pale eosinophilic, homogenous, amorphous PAS-positive and von Kossa, Congo red-negative material. Within the alveolar lumen multifocal oedema associated with increased numbers of alveolar macrophages and occasional multinucleated giant cells and fewer neutrophils were present. The pulmonary interstitium was expanded by moderate, multifocal to coalescing deposit of collagen (fibrosis). Anthracosis was observed in the regions of interstitial fibrosis. Multifocal mineralization, confirmed by the von Kossa staining, was observed in the interstitium and the alveolar septa. In the interventricular septum and, to a lesser extent, in the right ventricle, myofibers were multifocally replaced by adipose tissue. Multifocal areas of fibrosis were also dissecting the myofibers of the right ventricle.

TEM of the material accumulating within the alveoli identified short lamellar 3.125 nm fascicles at 6.25 nm periodic distance, a morphology consistent with abnormal surfactant.

Morphological diagnosis and case summary:

Morphological diagnosis: Severe, diffuse, pulmonary alveolar proteinosis/phopholipidosis and histiocytosis with multifocal to coalescing mild pyogranulomatous pneumonia, multifocal interstitial fibrosis, mineralization.

Case summary: Pulmonary alveolar proteinosis/phospholipidosis in a English Bulldog.

Comments

Pulmonary alveolar proteinosis (PAP) is a rare disease described in humans, dogs, rats, hamsters, guinea pigs and goats.[1,2,3] Although PAP has only been reported in three dogs of

different breed (Cocker Spaniel, Shih Tzu, Golden Retriever),[2,4,5] it should be considered one of the differential diagnoses for canine interstitial lung disease.

PAP is characterized by abnormal surfactant homeostasis with alveolar accumulation of PAS-positive proteinaceous material.[1] Mice lacking surfactant protein SP-D develop alveolar lipoproteinosis,[6] since SP-D is able to form tubular myelin-like structures from surfactant phospholipids and enhance surface activity.[7] Three types of PAP have been described in man: autoimmune (previously named primary or idiopathic), secondary (acquired) and genetic.[8] Genetic PAP is most commonly observed in children and the severity of presentation depends on the type of mutation.[3] Autoimmune and secondary PAP are the most frequent forms.[3] Automimmune PAP is caused by anti-granulocyte macrophage colony stimulating factor (GM-CSF) antibodies that inhibit macrophage function and, therefore, clearance of surfactant,[3] as demonstrated in mice homozygous for a disrupted GM-CSF.[9] Secondary PAP is associated with exposure to toxics, drugs n as well as systemic inflammatory disorders and malignancies.[3] PAP is a subacute and subtle disease that is generally diagnosed months or years after its initiation.[1] The accumulation of abnormal material within the alveolar spaces leads to left to right shunt, impaired diffusion, and ventilation-perfusion mismatch.[10] Clinical signs in affected dogs and humans are non-productive cough, fever, and progressive exercise intolerance.[1,2] Haematological findings include neutrophilia, eosinophilia, or both,[2] most likely secondary to bacterial infections favoured by the accumulation of abnormal material in the alveoli and impairment of the alveolar defence mechanisms.[2] Thoracic radiographs commonly reveal a fine, diffuse peri-hilar alveolar or interstitial pattern.[2] A crazy-paving appearance of the lungs (defined as scattered or diffuse ground-glass attenuation superimposed on a network of interlobular septal thickening and intralobular lines) on a high resolution computed tomography (CT) is a characteristic but not diagnostic feature of this disease, since it is also reported in a variety of interstitial and airspace pulmonary disorders.[1,3]

Bronchoalveolar lavage (BAL) is considered an additional useful diagnostic aid.[1,2,3] Grossly, BAL fluid may be milky and opaque, comprising a thick sediment and a translucent supernatant.[1,2] In dogs, globules of mucus, protein and cholesterol clefts with small numbers of inflammatory cells are also cytologically detectable.[2] Numerous lamellar bodies structurally similar to myelin have been observed in human BAL fluid.[3] BAL examination data are not available in our case since it was not collected during cardiopulmonary resuscitation. Lung biopsies represent the gold standard test for PAP diagnosis.[1] Histopathology demonstrates alveoli filled with PAS-positive granular eosinophilic material in an otherwise intact alveolar architecture.[1,2,3] This material corresponds to the tubular myelin-like multilamellar structures observed by electron microscopy.[11] Multilamellar structures appear to be abnormal forms of tubular myelin lacking the regular architecture found in normal lungs of humans and animals.[11] Small tubular myelin-like structures may be seen in normal lungs, but never in such large amounts and bizarre arrangement as occurring in PAP.[11] Whole-lung lavage is commonly used as therapy for patients with symptomatic alveolar proteinosis and severe hypoxaemia.[1,3] Numerous therapies aiming to an enhanced surfactant clearance have been attempted such as administration of exogenous GM-CSF or plasmapheresis to reduce anti-GM-CSF antibody levels.[1,3] Silvestrein et al. (2000) have described the therapeutic application of lung lavage in a symptomatic dog and, although follow up was not available, results seemed encouraging (absence of clinical respiratory signs 20 month after lung lavage).[2]

The accumulation of phospholipids in the lung induced by cationic amphophilic therapeutic agents (CAD) is generally referred as phospholipidosis, although the term “alveolar proteinosis” has also been used to describe this condition.[11] Bromide-induced lower airway diseases have been described in cats and specifically endogenous lipid pneumonia with suppurative inflammation[12] and bronchitis resulting from a hypersensitivity reaction.[13]

Alterations of bronchial secretions, mucociliary function and cytokine stimulation have been described in humans[14] although the pathophysiology of this drug-induced reaction is still unclear. In dogs, adverse effects associated with bromide administration are usually mild and self-limiting, with polyphagia, vomiting, polyuria, ataxia and sedation as the most frequently observed.[15,16] Respiratory lesions associated with bromide-treatment in dogs have not been reported. In our case histology, histochemistry and TEM warranted a diagnosis of pulmonary proteinosis/phopholipidosis. Potassium Bromide and phenobarbital may be used in combination therapy for idiopathic canine epilepsy.[17] Despite no data on the possible association between epilepsy therapy and alveolar proteinosis/lipoproteinosis seems available for dogs, the prolonged administration of both drugs from an early age may have contributed to the development of alveolar proteinosis/lipoproteinosis in this dog. The two drugs have mechanisms of action similar to active principles that have been correlated with abnormal accumulation of alveolar surfactant in human beings.[11]

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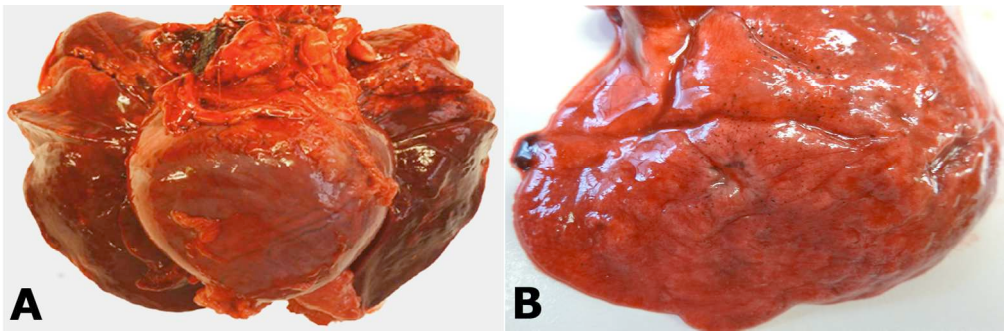
Figure legends

Figure 1-Photographs of lung and heart from a 7 years old, male, English bulldog referred for sudden dyspnoea and died 12 hour after admission. A- Note the diffuse severe oedema and hyperaemia with disseminated focal lesions more severe in the left cranial and caudal lobe. A rounded heart outline due to moderate right ventricular dilation was also present. B-Photograph of a closer view of the cranial left pulmonary lobe. Note the presence of multifocal umbilicated lesions.

Figure 3- A-Photomicrograph of a section from the lung. Alveoli are filled with pale blue-grey amorphous homogeneous material associated with increased numbers of alveolar macrophages and occasional multinucleated giant cells. H&E stain; bar = 120 µm. B-Photomicrograph of a section from the lung. The material within the alveolar lumen is variably PAS positive. PAS stain, haematoxylin counterstain; bar=121 µm. C-Photomicrograph of a section from the lung. The substance in the alveolar lumens is composed of short lamellar 3.125 nm fascicles at 6.25 nm periodic distance compatible with accumulation of abnormal surfactant. Lead citrate and uranyl acetate staining; bar=500nm.



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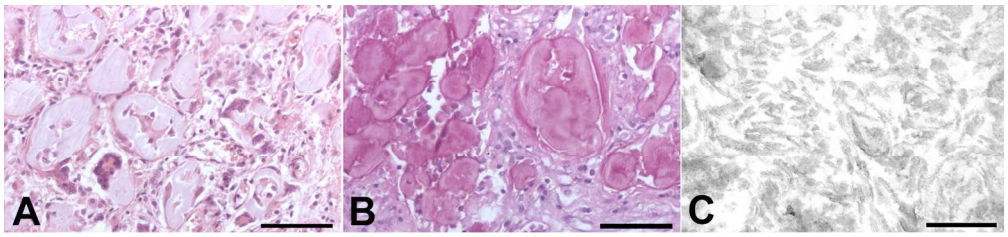

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Mucinous Melanophoroma in a Northern Red Bellied Cooter (*Pseudemys rubriventris*)

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Case Report

Mucinous Melanophoroma in a Northern Red Bellied Cooter (*Pseudemys rubriventris*)

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Abstract

An adult female Northern red-bellied cooter (*Pseudemys rubiventris*) was presented with a 15 day history of anorexia and lethargy. On physical examination the animal was considered to be in critical condition with a prominent swelling of the proximal aspect of the left forelimb. Laboratory analysis highlighted anemia, hyperuricaemia, hyperuremia, low total protein, and elevated aspartate aminotransferase and lactate dehydrogenase. Radiographic images revealed a soft tissue mass infiltrating the humerus with boney involvement. Fine needle aspiration was performed and cytological evaluation indicated the sample was characterized by a prevalence of spindle to stellate cells frequently containing intracytoplasmatic brown to black granules associated with a moderate amount of mucin. The terrapin died 24 hours later with only the affected forelimb being submitted for histopathological evaluation. On gross examination the proximal humerus was encircled by a 3 cm diameter, black, gelatinous mass with a mucoid, black appearance on cut surface. The histological description of the mass was that it was a poorly demarcated, sparsely cellular, nonencapsulated neoplasm expanding from the dermis and infiltrating into the underlying soft tissues and bone. The neoplasm was composed of stellate cells embedded in an abundant Periodic Acid-Schiff positive myxoid stroma. The cells frequently contained intracytoplasmic brown- black granules. These findings are consistent with a mucinous type of melanophoroma. Melanophoromas are tumors of melanin producing cells and are well known in reptiles, most commonly in snakes and bearded dragons (*Pogona vitticeps*), but rarely reported in chelonians. The mucinous variant has only been described in the bearded dragon.

Key words: Chelonian; cytology; histology; melanophoroma; Northern red-bellied cooter; *Pseudemys rubriventris.*

An adult female Northern red-bellied cooter (*Pseudemys rubriventris*) was presented for clinical evaluation with a 15 day history of anorexia and lethargy. The chelonian was purchased as a juvenile from a pet shop and initially maintained in a small tank inside the owner's house. The animal's tank had been replaced by larger aquaria over the years as the cooter grew, but no filtration was ever provided. Moreover, throughout the life of the animal there was no ultraviolet light source provided, although natural sunlight could enter from a window in front of the tank allowing for seasonal (spring and summer) basking in direct sunlight. Excluding the infrequent periods allowed outside, the cooter's environment was room temperature as no external heat source was provided. The animal's diet consisted of dried *Gammarus* spp., commercial pellets for aquatic terrapins, and, occasionally, bits of various meat and fish. The inadequate husbandry and diet provided for this terrapin over its lifetime were thought to have a significant influence on the poor body condition of the animal upon presentation to the veterinary hospital.

PHYSICAL EXAMINATION AND DIAGNOSTICS

The cooter weighed 624 grams and was lethargic, dehydrated, and hyporeactive to external stimuli. Irregular petechiae were evident on the skin of the forelimbs and rear limbs. Dyspnea, with repeated extensions of the neck and open mouth breathing, was also occasionally observed. In addition, a prominent fluctuant swelling was evident at the base of the left forelimb (Fig. 1). Radiographic examination of the affected limb showed increased density of soft tissues and evidence of osteolysis with cortical erosion in the proximal portion of the humerus (Fig. 2).

Blood was collected from the patient and submitted for a complete blood count (CBC) and plasma chemistry panel. Abnormal results from the CBC were anemia, leukocytosis, heterophilia, toxic heterophils, eosinophilia, and monocytosis (Table 1). The plasma chemistry panel revealed

hyperuricaemia (UA), hyperuremia (BUN), low total proteins (TP), elevated aspartate aminotransferase (AST), lactate dehydrogenasis (LDH), and an altered calcium (Ca) to phosphorus (PHO) ratio, and an increased solubility index (Table 2). The hematologic findings were consistent with severe chronic inflammation, while the plasma chemistry results were suggestive of both kidney and hepatic failure.

In the authors' experience, such clinical and laboratory findings are common in mature or geriatric terrapins maintained in poor husbandry conditions and fed an improper diet.

Fine needle aspiration of the tissue mass at the base of the left forelimb was performed. Smears of the collected samples were air dried and stained with Hemacolor©(). Cytologic samples were characterized by adequate cellularity and moderate hemorrhage. Multifocal aggregates of amorphous bluish material, consistent with urate crystals, were evident along with moderate to abundant amounts of violet fibrillar material consistent with a mucinous extracellular matrix. The predominant cell population was composed of 20 to 25 μm spindle to stellate cells with indistinct cell borders and moderate amounts of blue cytoplasm, frequently vacuolated or containing brown-black granules. The nuclei of the spindle to stellate cells were oval, centrally located, 12 to 14 μm in diameter, and were characterized by a fine granular chromatin without prominent nucleoli. Anisocytosis and anisokaryosis with rare mitotic figures were observed. Additionally, numerous foamy, reactive macrophages containing brown-black pigment were observed. The cytological findings were consistent with a chromatophoroma, most likely a melanophoroma (Fig. 3).

The cooter died 24 hours following presentation to the veterinary hospital and the owner did not consent to a full necropsy examination. Consequently, only the affected limb was resected and fixed in 10% neutral buffered formalin for a histopathological examination.

DIAGNOSIS

The forelimb was 3 cm in diameter and had a poorly demarcated tissue mass encircling its proximal portion and extending from the shoulder to the midpoint of the humerus. The tissue mass was soft,

black, and had a mucoid consistency on its cut surface. The sample was decalcified in 5% trichloroacetic acid and then embedded in paraffin. A complete transverse section of the tissue mass was routinely processed, sectioned at 5 μm, and then stained with hematoxylin and eosin. Additional sections of the tissue mass were stained with Periodic Acid Schiff (PAS). Histological examination of the stained sections of the tissue mass revealed a 3 cm in diameter, sparsely cellular, poorly demarcated neoplasm that was expanding from the dermis and infiltrated the underlying humerus. The overlying skin had multifocal ulcerations. The cells within the neoplasm were arranged in short interlacing bundles interspersed in an abundant fibrillar, pale, eosinophilic extracellular matrix. Cells were spindle to stellate, 25-30 μm in diameter, with variably distinct cell borders, long cytoplasmic processes, and a moderate amount of pale cytoplasm containing variable numbers of brown-black granules or optically empty vacuoles. The nuclei were oval, 10-15 μm in diameter, and centrally placed with 1-2 nucleoli. Anisocytosis and anisokaryosis were moderate and mitoses ranged from 0 to 1 per high power field (HPF). The degree of pigmentation of the cells was variable within the sections. In most fields, amelanotic cells were admixed with markedly pigmented cells. A delicate plexiform capillary network produced by thin-walled normal capillaries branching at 40 to 90 degree angles was evident (Fig. 4). The tumor was characterized by proteoglycan–rich, PAS positive mucinous interstitial matrix along with the presence of urate crystals within neoplastic tissue encircling and involving the shoulder joint.

The gross appearance, cytology, histology, and histochemistry of the cutaneous pigmented tumor from this adult Northern red-bellied cooter was consistent with a mucinous variant of melanophoroma.

DISCUSSION

In reptiles, tumors of the pigment-producing cells are collectively referred to as chromatophoromas and usually develop in the subcutis and dermis.[1] Chromatophoromas may be subclassified according to the specific pigment-producing cells (chromatophores) found in the neoplasm. In

particular, melanophoromas originate from melanin-producing cells, xanthophoromas from carotenoid or pteridine-producing cells, and iridophoromas from crystalline purine-producing cells containing reflecting granules of guanine, adenine, hypoxanthine, or uric acid.[2-4] Although in previous retrospective works chelonians showed a low prevalence of neoplastic disease,[5,6] in recent years there has been an increasing number of neoplasia reports in this reptile order.[1,3,7-12] Moreover, melanophoromas in reptiles were considered rare in the early literature[4,13] but today appears to occur in a number of reptile orders, especially squamata.[5,14-20] A sub-type of melanophoroma, the mucinous form, has only been described in bearded dragons (*Pogona vitticeps*).[16] Despite this increased knowledge, to date there is only one report describing a melanophoroma in a chelonian species, a Hermann's tortoise, *Testudo hermanni*.[3]

Cellular material collected from fine needle aspirates of tissue masses is often difficult to interpret and frequently unrewarding.[21] However, in this case, the cytologic evaluation of the samples collected from the mass allowed for a presumptive diagnosis with findings very similar to those confirmed later by histopathology. Furthermore, the cytomorphologic description of the cooter's tissue mass was consistent with that found in a report describing a melanophoroma in a green iguana (*Iguana iguana*) and Hermann's tortoise.[3,19] In addition, optically empty vacuoles in the neoplastic cells and scattered melanomacrophages were observed in the cytological evaluation of the fine needle aspirate smears collected from the cooter's tissue mass. The presence of uric acid deposits is unlikely associated with the neoplasm but rather a result of the patient's hyperuricemia. Furthermore, even though iridophoromas arise from cells that may contain granules of uric acid, they are usually characterized by a white color on gross examination and the presence of golden-brown to olive-green pigment granules histologically.[16] Jacobson et al. have described a case of both an iridophoroma and a melanophoroma diagnosed in an adult male pine snake (*Pituophis melanoleucus*) which was characterized by a mixed (black and white) gross appearance.[22]

The histologic appearance of the neoplastic tissue collected from the Northern red-bellied cooter in this case is similar to previous reports of melanophoromas in reptiles.[16] The common

histologic findings of melanophoromas between reptile species include infiltrative growth, spindle-shaped cells, mild to moderate anisokaryosis, 1-2 nucleoli, few mitotic figures per HPF, and variable pigmentation.[16] Moreover, histochemical staining confirmed a proteoglycan-rich, PAS-positive mucinous interstitial matrix similar to the mucinous variant described in a bearded dragon.[16] Conversely, the aforementioned abundant fibrillar extracellular matrix was not reported in a melanophoroma found in a Hermann's tortoise.[3] Unfortunately, many reptile patients are presented in advanced stage of tumor disease,[21] and the terrapin described in this article was no exception with blood values consistent with multi organ failure.

Melanophoromas in reptiles are reported to be highly malignant and readily metastasize.[5,16] In reptilian melanophoromas, lymphatic and/or vascular invasion and metastases are the best indicators of malignancy, in addition to a mitotic activity equal or greater than 2 mitosis per HPF, cellular pleomorphism, and increased numbers of nucleoli.[16] Unfortunately, a full necropsy examination was not allowed on the cooter; therefore, no conclusive cause of death could be determined, including the possibility of a metastatic spread of the tumor. However, blood chemistry values were suggestive of a systemic impairment through major organ failure (e.g., renal) that might have contributed to its death.

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Table 1. Hematological values of the Northern red-bellied cooter at presentation

Parameters	Value	Reference values 1[22]	Reference values 2[23]
Red blood cells (cells/µl)	120,000		558,000
Hematocrit (%)	9	26	22.9
Hemoglobin (g/dl)	3.2		
White blood cells (cells/µl)	18,400	12,903	6,660
Heterophils (cells/µl)	10,700	4,911	
Lymphocytes (cells/µl)	3,120	3,015	
Monocytes (cells/µl)	280	50	
Eosinophils (cells/µl)	1,100	644	
Basophils (cells/µl)	2,800	4,282	

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Table 2. Chemistry values of the Northern red-bellied cooter at presentation.

Parameters	Value	Reference values[22]
Total protein (mg/dl)	1.7	2.7
Aspartate aminotransferase (U/l)	284	66
Lactate dehydrogenase (U/l)	>2,800	
Blood urea nitrogen (mg/dl)	72	34.6
Uric acid (mg/dl)	8.4	0.9
Calcium (mg/dl)	7.4	9.6
Phosphorus (mg/dl)	8.7	3.5
Calcium-phosphorus ratio	0.85	2.8
Calcium-phosphorus product	64.4	

FIGURE LEGENDS

Figure 1. The Northern red-bellied cooter at presentation. Note the dehydrated status, petechiae on the skin and the prominent swelling at the base of left forearm.

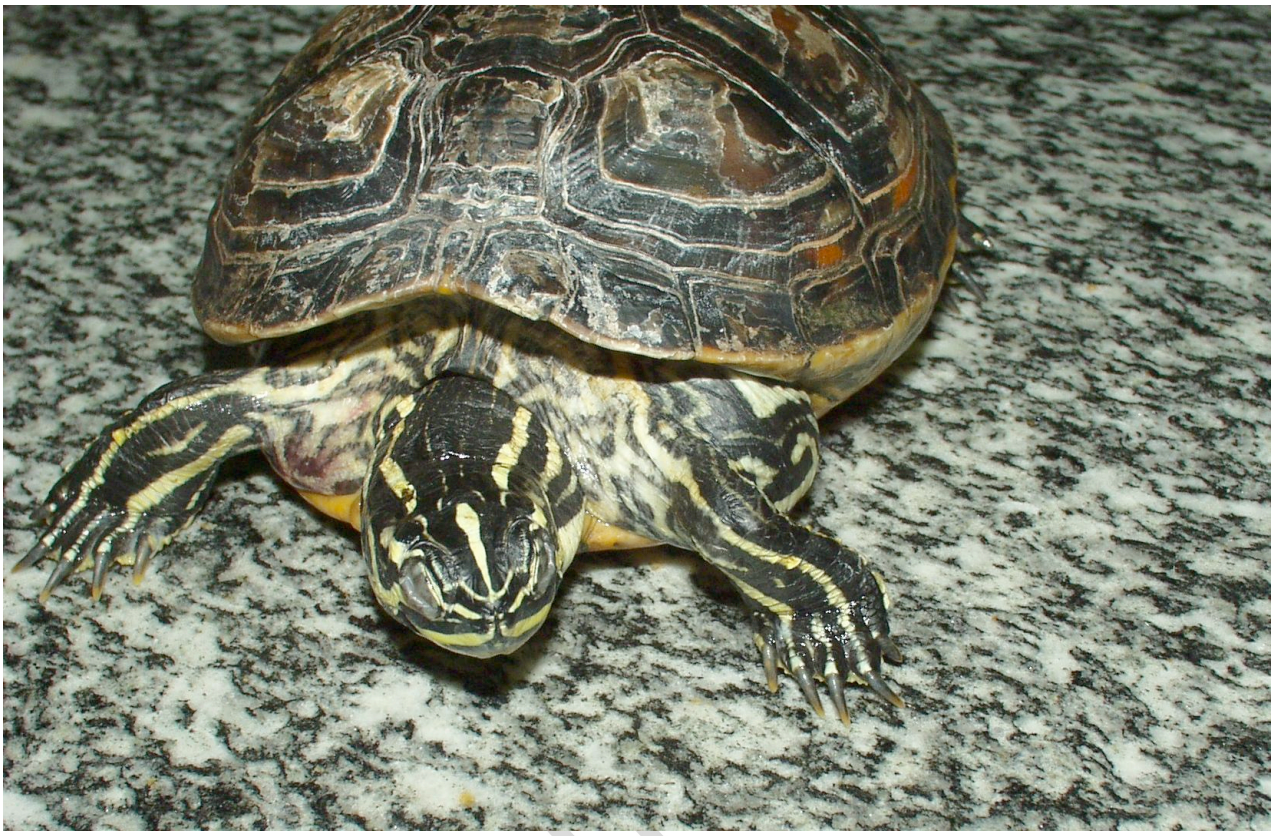

Figure 2. Radiographic image of the affected limb. Note the osteolytic changes in the proximal portion of the humerus

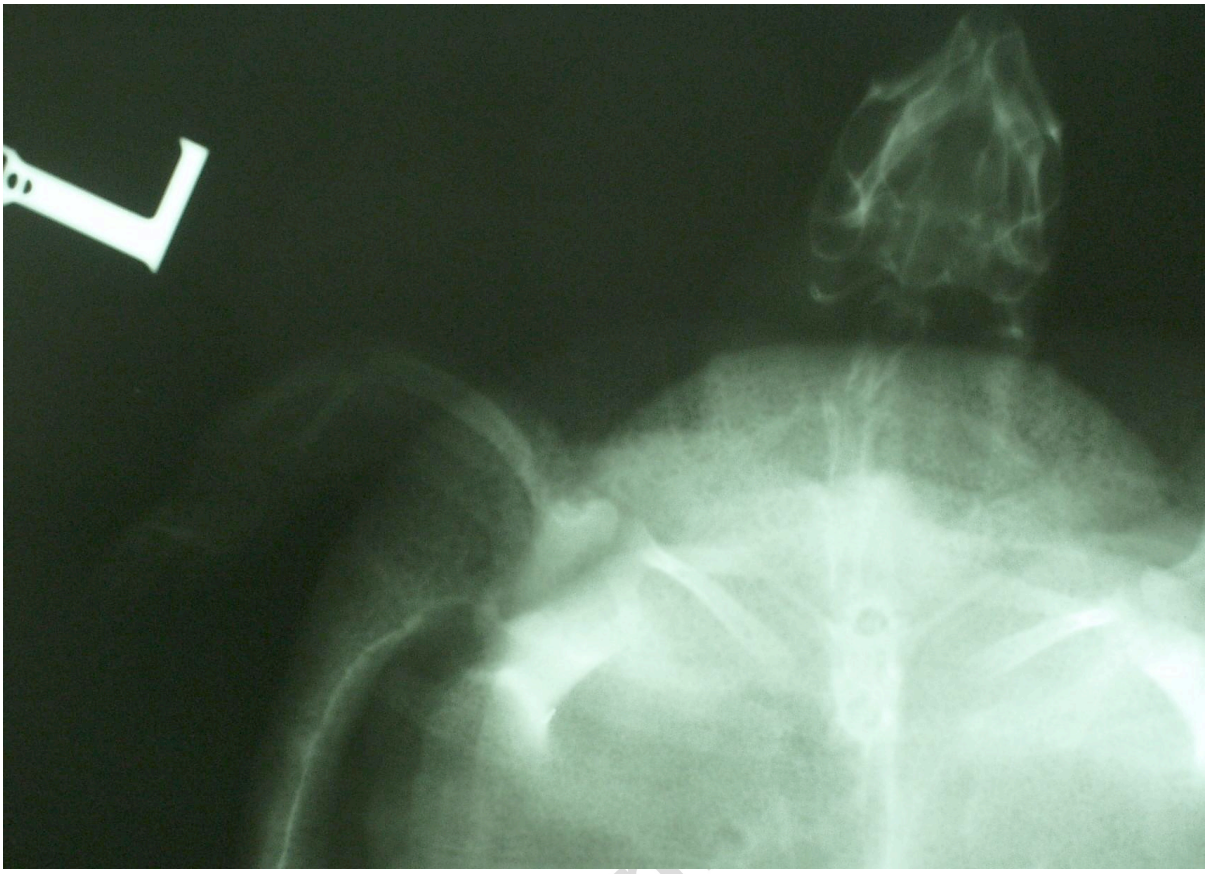

Figure 3. Cytology, fine needle aspirate, tumor mass: Spindle to stellate cells characterized by long cytoplasmic processes and a moderate amount of pale cytoplasm containing black-brown granules or optically empty vacuoles. Hemacolor stain, 400x magnification.

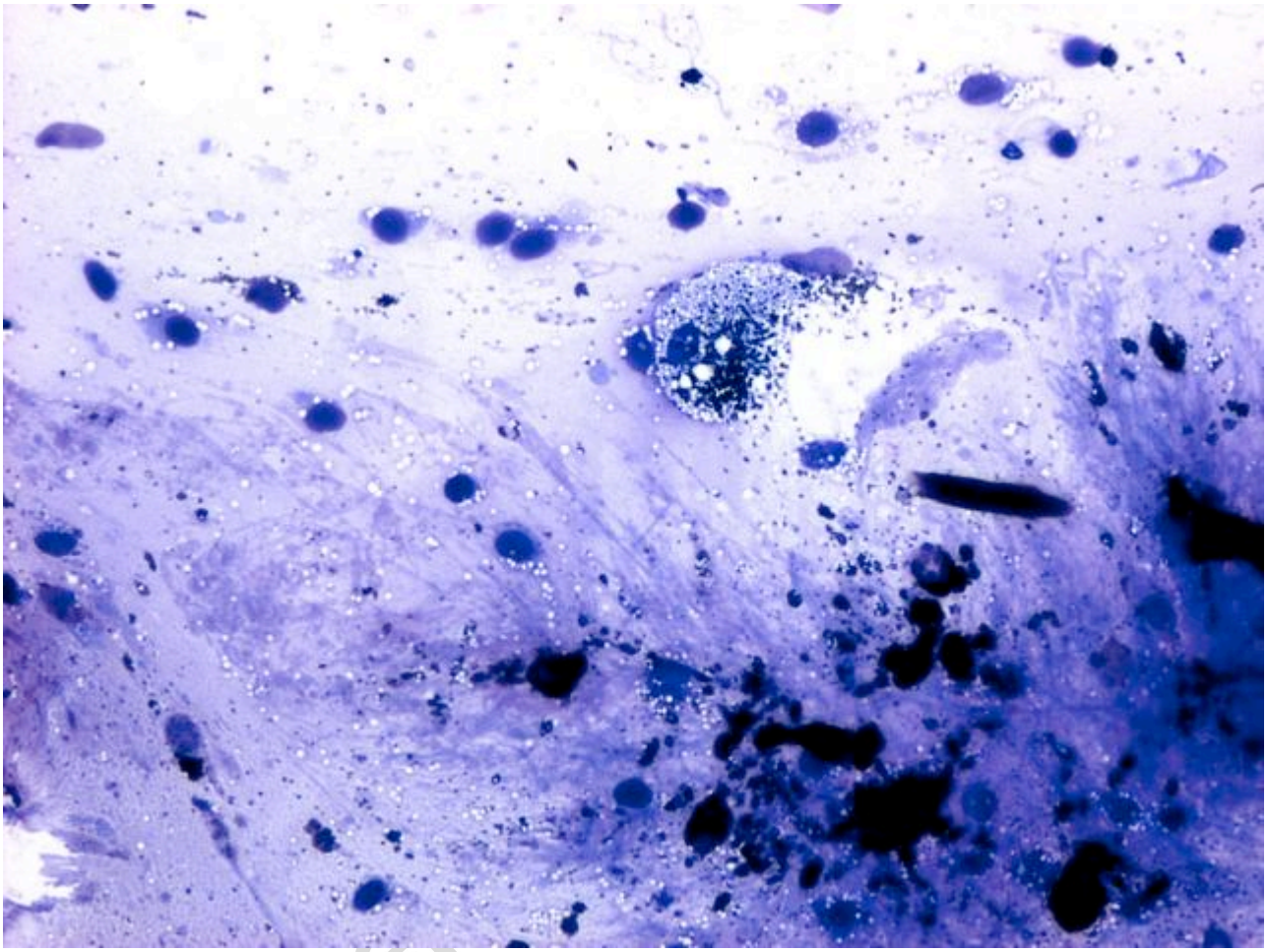

Figure 4. Histopathology, tumor mass: The neoplasm is composed of spindle to stellate cells with variable pigment that are infiltrating the underlying bone. Hematoxilyn and eosin, 100x magnification.