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Cutaneous lymphoma at injection site: pathological, immunophenotypical, and molecular characterization in 17 cats

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ABSTRACT

Feline primary cutaneous lymphomas (FPCL) account for 0.2-3% of all lymphomas in cats and are more frequently dermal non-epitheliotropic small T cell tumors. Emergence of FPCL seems unrelated to FeLV serological positivity or to skin inflammation. A total of 17 cutaneous lymphomas with a history of vaccine injection at site of tumor development were selected from 47 FPCL. Clinical presentation, histology, immunophenotype, FeLV p27 and gp70 expression and clonality were assessed. A majority of male (12/17), DSH (13/17) cats with a mean age of 11.29 years was reported. Post-injection time of development ranged from 15 days to approximately 9 years in 5 cats. At diagnosis, 11/17 cats had no evidence of internal disease. Lymphomas developed in interscapular (8/17), thoracic (8/17) and flank (1/17) cutaneous regions, lacked epitheliotropism and were characterized by necrosis (16/17), angiocentrism (13/17), angioinvasion (9/17), angiodestruction (8/17), and peripheral inflammation composed of lymphoid aggregates (14/17). FeLV gp70 and/or p27 proteins were expressed in 10/17 tumors. By means of WHO classification, immunophenotype and clonality the lesions were categorized as large B-cell lymphoma (11/17), anaplastic large T-cell **(**3/17) or NK- cell like (1/17) lymphoma, peripheral T cell lymphoma (1/17). Lineage remained uncertain in 1 case. Cutaneous lymphomas at injection sites (CLIS) shared some clinical and pathological features with feline injection-site sarcomas and with lymphomas developing in the setting of subacute to chronic inflammation reported in human beings. Persistent inflammation induced by the injection and reactivation of FeLV expression may have contributed to emergence of CLIS.

Key Words:

Cat, skin, lymphoma, FeLV, injection, immunohistochemistry, molecular biology, clonality

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INTRODUCTION

Lymphomas represent more than 50% of all tumors in cats, with a prevalence of approximately 1.6% of the general feline population and 4.7% of hospitalized sick cats.[34] Most common sites include the intestine and mediastinum. [34] Primary cutaneous lymphomas account for 0.2 to 3%[25,29,54,71] of all feline lymphomas.

While epitheliotropic lymphomas of the skin account for up to 44% of primary cutaneous lymphomas in dogs and humans,[21,27,31] epitheliotropism is rarely observed in cats. Non-epitheliotropic lymphomas are more frequent in cats[27,54] and include indolent T cell lymphoma, also referred to as cutaneous lymphocytosis,[23,24] diffuse T cell lymphoma, T cell rich large B cell lymphoma,[15,27] and lymphoplasmacytic lymphoma.[37] Similar to other species, cutaneous lymphomas mostly have a T cell origin[21,27] while B cell tumors are considered extremely rare.[14,27] In addition to viral infections, such as Epstein Barr virus (EBV) and human T-lymphotropic virus type 1 (HTLV-I),[32,73] chronic inflammation is generally accepted as a risk factor for the development of hematopoietic malignancies in humans[20,26,59] specifically cutaneous T and B cell lymphomas in humans.[6,20,59]

The overall incidence of feline leukemia virus (FeLV) in cats with lymphomas has decreased from 70%[33] to 15% in north America.[43] A similar drop in prevalence, from 59% to 13% between two consecutive 15-year periods has been reported in Germany.[51] FeLV prevalence in the '70s was estimated to less than 10% in cats with cutaneous lymphoma. In Italy, seroprevalence evaluated in high risk (stray cats) and sick cats has decreased from 18% to 3,8 %[4,64] while no data are available regarding cats with lymphoma. Therefore, this excludes a major role of progressive FeLV infection in feline cutaneous lymphoma development. Progression of chronic inflammation to lymphoma is not yet confirmed in cats. However, the development of sarcomas at injections sites (e.g. rabies vaccine, long acting antibiotics or steroids,[38] benzoylurea pesticide lufenuron,[18] sites of implanted foreign material (non-absorbable suture material,[5] microchip implants,[7,12] retained surgical sponges[28] or trauma[17,22,63] are well documented and thought to be

attributed to the chronic inflammation elicited..[39] Moreover, cutaneous lymphomas have arisen in areas of feline injection site sarcomas subsequent to chemotherapy or radiation therapy of the primary tumor, and lymphomagenesis in these cases was hypothesized to be due to the mutagenic action of chemotherapy or radiation treatment.[44]

In a subgroup of recently reported primary feline subcutaneous lymphomas, site of development (lateral thorax and interscapular region), clinical presentation as a single nodule, presence of necrosis leading to central cavitation and peripheral inflammation were considered clinically highly suggestive of feline injection site sarcomas.[52] The aim of this report is to describe pathological features of a series of primary feline cutaneous lymphomas, which developed in sites of previous injections and were not preceded by sarcomas.

MATERIALS AND METHODS

Tissue samples and clinical history

Forty-seven cases of feline primary cutaneous lymphomas were submitted to the diagnostic histopathology service at the School of Veterinary Medicine of Milan from 2001 to 2012. Samples were submitted by veterinary practitioners or as second opinion cases from other veterinary schools and private diagnostic laboratories throughout Italy. Punch or excisional biopsies of skin nodules or formalin fixed paraffin embedded tissue blocks were submitted. Clinical data including signalment, tumor site, previous history of injection at the tumor site, clinical staging, therapy and follow up information were received upon submission or collected by contacting the referring veterinarian after the case selection according with histomorpholgy suggestive of injection/vaccination or in cases where injection/vaccination was suspected by the referring veterinarian. Based on histopathological features and clinical information, 17/47 primary cutaneous lymphomas were included in this study.

Histopathology

Tissue specimens received fixed in 10% neutral buffered formalin were routinely processed and tissue blocks (from the diagnostic service or from other institutions) were utilized to obtain multiple 4–6-µm thick section. Morphologic features were evaluated on tissue sections stained with Haematoxylin and eosin stain. The following parameters were recorded: site and extension of the neoplastic lesion, presence of angiocentric, angioinvasive and angiodestructive behavior, cell morphology and cell size, number of mitoses, necrosis, mineralization and inflammation including reactive lymphoid nodular aggregates. The mean Mitotic Index (MI) per high power field (400x magnification) was evaluated dividing by 10 the mitotic count obtained in 10 consecutive HPF in areas having the highest number of mitotic figures. The MI was calculated independently by three pathologists (PR, GA, CG) utilizing the same microscope and a mean of the three readings was used to calculate the mitotic index per filed. According with MI, low grade was assigned to cases with mean of 0-6 mitoses, medium grade to cases with a mean of mitoses of 6-10 and high grade with a mean over ≥11.[70] Definitive diagnoses were obtained according to the veterinary WHO classification of lymphomas.[70,72]

Immunohistochemistry

Detailed information about the panel of primary antibodies is listed in Supplemental Table 1. Cross-reactivity of anti-human CD3-ε, CD79a, CD45RA and CD20 with feline tissues has been previously demonstrated.[1,13,23,36,48] Paraffin sections (4-6 µm) were mounted onto Poly-L-Lysine-coated slides, deparaffinized in xylene and hydrated through graded ethanol solutions. Endogenous peroxidase was quenched with hydrogen peroxide (0.3%) and sodium azide (0.1%) in Tris buffer (0.1-M solution, pH 7.5) for 30 minutes and rinsed in 3 changes of Tris buffer for 5 minutes each. For heat induced antigen retrieval, slides were immersed in 10-mM Citrate buffer, pH 6.0 (DAKO, Carpinteria, CA) and microwaved at maximum power for 1 minute and twice at 750 watt for 3 minutes. After cooling the

slides down to room temperature for 20 minutes, unspecific staining was blocked with 10% heat inactivated horse serum in Tris buffer

for 30 minutes. Primary antibodies were applied at the specific working concentrations (Supplemental Table 1) diluted in Tris buffer containing 10% inactivated serum and were incubated in a humidified chamber at 37°C for one hour. Secondary detection was performed with the avidin-biotin enzyme Complex (ABC kit, Vectastain®, Burlingame, CA, USA) for 30 minutes. The reaction was developed with the peroxidase Amino-9-ethyl-carbazole (AEC) substrate kit (Dako®, Glostrup, Denmark). Smears were counterstained with Mayer's hematoxylin for 3 minutes and cover-slipped with an aqueous mounting medium (Glycerol, Sigma-Aldrich®, St. Louis, MO, U.S.A.). Negative controls consisted of substitution of specific antibodies with an isotype-matched, irrelevant monoclonal antibody or omission of the primary antibody. Sections of feline reactive peripheral submandibular lymph node were used as positive controls. For FeLV antibodies, formalin-fixed sections from a lymph nodal lymphoma with FeLV p27 and gp70 positive cells from a FeLV ELISA p27 serologically positive cat were used as positive controls.

Clonality assessment

For DNA extraction, four 25µm paraffin sections were collected in an Eppendorf tube. Blades were changed and microtome was cleaned after each case to avoid DNA cross-contamination. The sections were deparaffinized in xylene and washed twice in 100% Ethanol. Genomic DNA was extracted using the DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The concentration of genomic DNA was measured using an Introspect 2100 pro spectrophotometer UV/Visible spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). Polymerase chain reaction (PCR) analysis for T cell and B cell clonality assessment was performed as previously described. [55,74] A single

primer pair was used for assessment of T-cell receptor gamma (TCRG), as previously designed and described.[55] An approximately 110 base pair (bp) segment (±20 bp) of the T cell receptor–gamma (TCRG) locus was amplified, using the forward primer in the variable region (5'-3': GAA GAG CGA YGA GGG MGT GT, Y: C or T; M: A or C) and the reverse primer in the joining region (5'-3': CTG AGC AGT GTG CCA GSA CC, S: C or G). Two segments of the immunoglobulin heavy chain (IgH) locus were amplified to assess B-cell clonality. [23,74] One upper (forward) primer (5'-3': CCA GGC TCC AGG GAA GGG) was paired with two lower primers (5'-3': TGA GGA CAC TGT GAC TAT GGT TCC and GGA CAC CGT CAC YAK GVY TC, Y: C or T; K: G or T; V: A, C or G;) to amplify framework 2 (an approximately 250 bp segment). The same lower primers were paired with upper primer (5'-3': CTC CGT GAA GGG CCG ATT) to amplify framework 3 (an approximately 180 bp segment). Each reaction was performed with 100 ng of genomic DNA. A reaction mixture was used as previously described, with 2-step touch down amplification conditions applied.[23,74] The polyclonal control was composed of genomic DNA extracted from feline peripheral blood mononuclear cells; DNA extracted from feline lymph nodes with confirmed T-cell lymphoma or B-cell lymphoma were used as clonal controls.

For six samples (cases Nos. 1-6) PCR reactions (prior to summer of 2007) were run in duplicates to confirm results. Native samples were used directly for gel electrophoresis. In addition, heteroduplex analysis, previously described to assist separation of true clonal from false-positive results, was performed for all samples.[23,74] Native PCR products (10µL) were denatured at 95°C for 10 min, then allowed to re-anneal at 4°C for 1 h prior to gel electrophoresis. Duplicate PCR samples (10µL) of each native and heteroduplex sample, including controls (negative, polyclonal and clonal) were analyzed by gel electrophoresis using pre-cast 10% non-denaturating polyacrylamide Tris-Borate-EDTA (TBE) gels (Criterion Pre-cast gels; Bio-Rad, Hercules, CA, USA). Gels were subsequently stained with Gel Star nucleic acid

stain (Cambrex Bioscience Rockland Inc., Rockland, ME, USA) as previously described. Bands and smears were visualized on a UV transilluminator and photographed.

For eleven samples (cases Nos. 7-17) PCR products (after summer 2007) were processed using the HDA-G12 Genetic Analyzer by eGene, Inc. Triplicate native samples (10µl), including controls (negative, polyclonal and clonal) were analyzed using BioCalculator software and eGene's DNA Gel Cartridge Kit. BioCalculator analysis method OL700 was utilized for 2-5 base pair resolution between products of 100-500 base pairs.

RESULTS

Clinical history and signalment

Based on histopathological features and clinical information, 17/47 primary cutaneous lymphomas were included in this study. All 17 cases were confirmed to have arisen at sites of previous vaccine injection by the referring veterinarian and presented either as a single nodule or localized disease. Of the 30 cases not included, 24 developed at non injection cutaneous sites while in six cases, site and history were not retrieved, only one of these had microscopic features resembling those described in this caseload but was not included All cats were vaccinated with a combination vaccine including Panleukopenia Virus, feline Herpesvirus-1 and feline Calicivirus (FHV-1/FCV). The vaccines came from six different manufacturers. FeLV vaccination was administered to 5/17 cats. No rabies vaccines were administered, as Italy is a country free of rabies. Exact time span between vaccination and development of lymphoma was available for 5/17 cats: lesions developed 15 days after last FeLV vaccination in cats No.s 3, 6, one year after the last annual routine vaccination in cat No.17, approximately five years after last vaccination in cat No.14 (no additional injections were given) and approximately 9 years after the last vaccination in cat No.16.

Signalment of cats and clinical information including size, site, number and microanatomical extension (evaluated histologically) of lesions (when available) are summarized in Supplemental Table 2. The majority were domestic short haired cats (13/17). Mean age was 11.29 years; 14/17 cats (81.35%) were 10 years or older. Male neutered cats were 12/17 (70.5%) and 5/17 cats (29.5%) were female spayed and the male to female ratio was 2.4. Serology for FIV was negative for 6/17 cats, and 8/17 cats tested negative for FeLV. At time of diagnosis, none of the cats had systemic clinical signs. Most cats (14/17) had a solitary nodular skin lesion. The remaining 3/17 cats had either two nodules connected by a linear mass or regional disease characterized by multiple nodules limited to a 5 cm area or two adjacent nodules. Pruritus, ulceration and pain were absent with the exception of 2/17 cats, which both presented with ulcers at first surgery.

Initial clinical staging by thoracic radiographs, abdominal ultrasound, or total body CT scan and blood analysis was available in 11/17 cats, all of which were confirmed negative for internal disease. In 3/17 cats (cases Nos. 2, 6, and 13) the presence of lymphoma limited to the skin was confirmed at necropsy.

Microscopical features

Incisional biopsies were available for all 17 cases included in the study. Main microscopic findings are listed in Table 1. In 4/17 cats cytological specimens were also available and contributed to the diagnosis and classification of tumors. Compared with the other primary cutaneous lymphomas (not included in the study), morphology of the lesions was distinctive and consisted of a poorly circumscribed, non-encapsulated, infiltrative mass that variably extended from the superficial to the deep dermis to the subcutis (Supplemental Table 2). A Grenz zone was always present, epidermal and adnexal tropism was never observed. Typically, cells were organized in dense sheets and in perivascular aggregates, commonly associated with extensive areas of necrosis (16/17 cats). Neoplastic lymphoid cells tightly surrounded vessel walls

(angiocentrism) in 13/17 cases (Figure 1). In 10/17 cats angioinvasion was observed (Figure 2). Angiodestruction, a feature associated with vascular wall disruption and variable mural hemorrhages, fibrin deposition and luminal occlusion was recorded in 8/17 cats (Figure 3). Central microcavitation with fibrin accumulation and presence of occasional reactive macrophages and non-degenerated neutrophils was present in most cases. Foreign material was not found in the necrotic cavities nor in macrophages.

Inflammatory cells (lymphocytes and reactive macrophages) were frequently located at the periphery of the lesions or admixed with neoplastic cells as well as within and surrounding the central cavity. Perivascular aggregates of small mature lymphocytes were observed at the margins of the main tumor in 14/17 cats.

Complete excision was observed in 7 cases. Of the 7 cases that were completely excised, 3 recurred (cases Nos.1, 2,14), 1 did not recur (case No. 15) while no information was available for 3 cases.

Cytological features of neoplastic cells including cell size, nuclear shape, chromatin pattern, number and evidence of nucleoli, and mitotic index are summarized in Supplemental Table 3. According to the MI, in 2/17 cats, large cell lymphomas were of intermediate grade. In the remaining 15/17 cats lymphomas were low grade.

Immunohistochemistry

Detailed immunohistochemical results are listed in Table 2. Expression of CD18 confirmed leukocyte origin of all 17 lesions examined. B cell lymphoma was considered in 10/17 cats based on expression of CD20 (Figure 4) and CD79a. Strong CD3 expression (Figure 5) by neoplastic cells in 5/17 cats was consistent with T cell lymphoma. Neither CD3 nor CD79a and CD20 were expressed in 2/17 cats. Nodular and follicular lymphoid aggregates of small lymphocytes were mixed B cells (CD79a) and T cells (CD3) and perivascular reactive small mature lymphocytes were consistently CD3 positive, interpreted as reactive lymphoid infiltrates.

The neoplastic cells expressed FeLV gp 70 and p27 antigens (5 cats), FeLV gp 70 only (4 cats), or FeLV p27 only (1 cat). No expression was observed in tumor cells of 7 cats (Table 2).
The quantity and intensity of expression of both proteins varied. Expression was cytoplasmic in all but one cat in which cell membrane expression of gp 70 (Figure 6) was observed; case No 2 had predominately membranous expressions of gp70.
Of the 8 FeLV serologically negative cats, 5 had FeLV gp 70 and/or p27positive neoplastic cells.

Clonality

Results of clonality testing are listed in Table 2. Clonal rearrangement of IgH was consistent with a B cell origin in 8/17 cats. Despite clonal IgH rearrangement, Cat No. 4 lacked expression of CD79a and CD20. In 3/17 cats no amplification of B cell or T cell loci was obtained and 1/17 cats had a pseudoclonal IgH rearrangement; immunophenotype of all 4 cats was consistent with B cell origin of the lesion.
Clonal rearrangement of TCRG along with strong expression of CD3 by tumor cells confirmed T cell origin in 3/17 cats. Polyclonal TCRG arrangement was seen in 1/17 cats, although tumor cell morphology and immunophenotype were highly consistent with T cell lymphoma.

Definitive Diagnosis

Definitive diagnoses based on histology, cytology, immunohistochemistry and, clonality are listed in Table 2. The majority of cases were diffuse large B cell lymphoma (11 cases) with a predominance of immunoblastic morphology (Figure 7) observed in 7 cases. Rare types of diffuse large B cell lymphoma were plasmacytoid (Figure 8) and anaplastic cell variants observed in two cases each.
Anaplastic large cell lymphomas were diagnosed in 4 cats when a T cell (3 cases) or NK-like cell (1 case) phenotype was associated with large size, severe pleomorphism with presence of C shaped nuclei (Hallmark cells) and frequent bi- to multinucleated atypical cells (Figure 9). In

Cat No. 3 neither immunophenotyping on paraffin embedded material, nor clonality testing assisted in the identification of cell origin as cells lacked expression of B and T cell markers and a pseudoclonal IgH and a polyclonal TCRG rearrangement were observed. Hence, lineage was considered uncertain. However, multiple fresh cytological specimens of the primary cutaneous tumor were available for case No. 3. Extensive additional immunophenotyping (data not shown) revealed lack of expression of CD21 (B cells), CD3, CD4 and CD8 (T cells), myeloperoxidase, CD1 and the β2-integrin CD11d. Weak expression of CD11b was seen in approximately 20% of cells. Morphology and immunophenotype were consistent with a Natural Killer (NK) cell origin. Cat No12 had an unusual immunophenotype; only 30% of the cytologically atypical cells expressed CD3 , and 10% of neoplastic cells were CD20 positive while CD79 was negative in all cells. In this case, in addition to a clonal IgH rearrangement, pseudoclonality was observed for TCRG. Since, CD20 is not considered a lineage specific B cell marker while, CD3ε-cytoplasmic expression can be found in T cells and in subpopulations of NK cells immunohistochemistry did not provide a definitive result. In this case, discordance between clonality and marker expression may be explained by antigen loss or lack of primers covering all DNA segments utilized by the tumor population could explain this result. Thus a definitive lineage was not attributed to this case

Clinical follow-up

Clinical follow up was available in 15/17 cats and is listed in Supplemental Table 4. Two cats were lost to follow up. Date to death after diagnosis in 15/17 cats ranged from 15 to 761 days. Local recurrence after was reported in 9/15 cats; one cat relapsed twice. Due to recurrence and progression of cutaneous disease 6/16 cats were euthanized while 9/16 died spontaneously, cause of death was not retrieved in 2 cats (Cases Nos 9, 11)

Two cats had cytologically confirmed progression of disease via fine needle aspirate to the spleen and liver (cat No 5) and to pleural effusion (cat No 14). Three cats were submitted for necropsy (cases No.s 2, 6, 13) and no internal disease was observed.

DISCUSSION

Primary feline cutaneous injection-site lymphomas (CLIS) described in this study were extracted from a caseload of 47 cutaneous lymphomas on the basis of their development at sites of previous injection. Relevant traits, included lesion development at injection sites, high recurrence rate and microscopic features of necrosis, central cavitation and inflammation at the tumor periphery. These microscopic features have been consistently described in feline injection-site sarcomas (FIS).[11,41,47] A direct connection between injection and the development of a primary cutaneous lymphoma has not been previously confirmed. Madewell et al.[44] reported cutaneous lymphoma development following FIS. These lymphomas, which emerged at the same location as FIS following an interval of 3-45 months after treatment, were interpreted as the consequence of the mutagenic action of locally applied chemotherapy or radiation therapy.[44] A subset of cats with indolent cutaneous lymphomas, also referred to as cutaneous lymphocytosis, developed lesions on the lateral thorax, shoulder, dorsal neck, flank and rear legs, all common injection sites in cats. Hence, vaccines were discussed as a possible causation for these lesions.[24] As an additional support to our findings, Meichner et al.[52] have voiced the suspicion of injection-site reactions inducing lymphoma for a subgroup of recently described feline subcutaneous lymphomas.[52]

Sites of chronic inflammation, surgery, trauma, metallic implants and viral infections have been well documented as a potential nidus for B cell lymphomas in humans.[8-10,56,57] The WHO classification lists DLBCL associated with chronic inflammation (DLBCL-ACI) as a specific entity developing in the context of long standing inflammation frequently in association with EBV.[8,10,56] DLBLC-ACI is often angiocentric[2] and occurs more frequently in middle aged to old male

patients. It develops from terminally differentiated B cells after a long latency period of over 10 years.[8,56] Paralleling DLBCL-ACI, angiocentrism was common in CLIS and was variably associated with angioinvasion, angiodestruction and tissue necrosis. The latter is considered rare in dermal lymphomas,[27,69,71] but has been recently reported as a common feature of feline subcutaneous lymphomas.[52] Likewise, the majority of CLIS of this study were diffuse large B-cell lymphomas (DLBCL). This is in contrast to previous reports for feline primary cutaneous lymphomas, which are predominantly of T cell origin with a small cell morphology.[14,21,24,27,71] However, our data parallel recent findings of a predominance of a diffuse large cell morphology and a prevalent B cell phenotype for feline subcutaneous lymphomas.[52] According with mitotic index, only two DLBCL were medium grade while 15 resulted low grade. This was an unexpected finding especially since DLBCL, PTCL and ALCL tend to be intermediate to high grade. Grade did not correlate with clinical progression since, in most cases where clinical history was available tumors were aggressive. Thus, for this type of cutaneous lymphomas grading according to mitotic index may not be prognostically useful. Unfortunately, due to the low number of cases and the fragmentary clinical information retrieved, a statistical analysis could not be performed and a higher number of cases should be evaluated to substantiate this hypothesis.

Noteworthy, primary cutaneous human DLBCL have a relatively poor prognosis compared with other B cell cutaneous lymphomas, with a 5-year survival rate of 20-55%.[42,65] Similar to our cases, primary human cutaneous DLBCL manifest as a solitary nodule or as multiple tumors restricted to one anatomic area (regional disease). The most common morphological variants of human DLBCL are centroblastic, immunoblastic and anaplastic.[16,65] In this study, a predominance of immunoblastic lymphomas followed by anaplastic and plasmacytoid forms was documented. Immunoblastic morphology has been correlated with a poorer prognosis in man[16,35] although, this correlation is still considered debatable.[65] Cutaneous immunoblastic lymphomas of this caseload demonstrated highly variable survival times ranging

from 15 to 124 days, two were lost to follow up and one was still alive. Thus, a conclusion on their biological behavior could not be clearly drawn. In the human WHO classification, B cell ALCL have been excluded from the "true ALCL category"[50] and are now grouped with DLBCL[65] while the term ALCL is utilized for cutaneous or systemic T/NK lymphomas expressing CD30 and differentiated prognostically by ALK (anaplastic lymphoma kinase) expression.[50] Unfortunately, specific markers for ALCL characterization and prognostication are not available in veterinary medicine and the diagnosis is made only on morphological grounds.

Results from extensive clonality testing in this work emphasize the importance of interpreting data in association with morphologic features as well as immunophenotypic characteristics of the lesions. Assessment of clonal rearrangement of IgH for B cells and TCRG for T cells supported the immunohistochemistry findings in 11/17 cats. These included cutaneous B cell lymphoma in 8/17 cats and cutaneous T cell lymphoma in 3/17 cats. However, one lesion with T cell immunophenotype and one lesion with B cell immunophenotype revealed a pseudoclonal and a polyclonal result respectively. A robust reactive lymphoid population may not allow detection of a neoplastic clone within a polyclonal background, the latter originating from the reactive lymphocytes.

Also, DNA amplification failed in three lesions with a B cell immunophenotype. This may occur if rearrangement is not accounted for by the primers used for the amplification or if somatic hypermutation of primer-binding sequences occurs and impedes the binding of oligonucleotide primers, thus preventing the detection of the rearranged sequences. Impaired sensitivity of B cell clonality analysis has previously been attributed to somatic hypermutation.[53] False negative results have also been attributed to extended time of tissue fixation prior to embedding, inhibition of DNA amplification due to the presence of substances such as formalin, hemoglobin or residues of the extraction process contaminating the nucleic acid extract.[45] Despite the fact that clonality was not able to support the diagnosis in some cases, morphologic features of the

lesions as well as the tumor cell immunophenotype were consistent with a neoplastic lymphoid process of B cell origin in the 4 cats and T cell origin in 1 cat.

Initially, the cell of origin remained undetermined in 2 cases, as immunophenotype and clonality were contradictory or inconclusive. The possibility of a natural killer (NK) cell proliferation was considered for case No. 3. The tumor cells were of leukocytic origin with a morphology consistent with large lymphocytes. However, neoplastic cells lacked expression of B cell or T cell markers and rearrangement of IgH or TCRG was not clonal. Immunocytochemistry on fresh cytological samples further supported an NK-like origin that was finally attributed to this tumor. However, at this point specific markers for feline NK cells are not available to definitely confirm this hypothesis. In cat 12 only a very small subpopulation of tumor cells expressed CD20 (but were CD79 negative) while a slightly larger portion expressed CD3; clonality testing revealed a clonal IgH rearrangement and a pseudoclonal TCRG rearrangement. Based on marker expression and clonality a clear origin was not attributed since the tumor might have undergone antigen loss, a common event in B and T cell lymphomas or, primers might not have covered the gene segments used by tumor cells. However, a T cell origin was suspected since the case closely resembled previous lymphomas described in humans.[3,46] Indeed, CD20 positive T cell lymphomas are frequently reported in humans .[3,49,58] These results further heighten that CD20 should not be considered a lineage specific B cell marker and should not be utilized alone to identify neoplastic B cells.

It is important to emphasize that regardless of the variable clonal and phenotypic results, in all these cases a rapid relapse and the aggressive clinical course confirmed the diagnosis of lymphoma.

As an adjunctive unexpected immunohistochemical finding, at least one of the FeLV antigens investigated in this study were expressed by neoplastic cells in 5 FeLV serologically negative cats. Also, in 5 cases (3 serologically negative and two unknown), discordance of FeLV p27 and gp70 staining was documented**.** Similar results have been previously

reported.[33,40,62] FeLV positive cats developing FeLV enteritis with gp70 strong expression and concurrent p27 negativity have been reported.[40] Interestingly, FeLV positive non diseased cats show intense p27 expression in normal intestinal crypts with concurrent negativity to gp70. Moreover, in cats with FeLV focal infection, p27 expression may be variable, discordant and positive and negative results may alternate.[30] Thus, p27 capsid protein cell positivity indicates that viral infection has occurred at a certain point, but does not imply productive and pathogenic viral assembly and hence, does not indicate infection nor disease in progress.[30,66]

Kipar et al.[40] document a correlation between gp70 intensity and presence and severity of intestinal lesions supporting the main pathogenic role of envelope proteins. Thus, FeLV gp70 envelope protein expression denotes viral assembly confirming viral replication and productive infection.[30,66] Previously, FeLV positive serology has been reported as low as 10% in feline cutaneous lymphomas.[34] However, a negative FeLV serology does not necessarily contradict the involvement of FeLV in cats with CLIS. Noteworthy, FeLV provirus integration has been demonstrated in two FeLV serologically negative cats with primary cutaneous epitheliotropic[67] and non-epitheliotropic lymphomas.[19] During regressive and focal FeLV infection, cats may be seronegative, but FeLV provirus can be demonstrated in peripheral blood and bone marrow cells by PCR.[30,68]. Therefore, seronegative cats may bear the virus insert, but transcription and reactivation may occur only upon neoplastic transformation of infected cells.[19]

To further complicate the correlation between serology and FeLV specific protein expression, disorderly and abnormal nonfunctional translation and assembly of FeLV has been described[30] Moreover, most FeLV tests evaluate for the presence of p27 antibodies or antigens and FeLV negative serology may derive from the lack of translation of p27 while gp70 may still be produced. Last but not least, differences in glycosylation and abnormal protein synthesis with structural changes have been described especially for gp70.[40] These changes may alter the monoclonal antibody binding with immunohistochemical false negative results.

Chronic inflammation following the local delivery of a persistent immunogenic stimulus may have contributed to reactivation of FeLV transcription and development of CLIS in selected cases. This hypothesis is supported by FeLV protein expression by neoplastic cells in cats with CLIS, with some cases expressing both FeLV p27 and gp70. To support this theory qRT-CR for FeLV will be necessary. Similarly, inflammation has been implicated in the reactivation and proliferation of EBV transformed B cells and seems to be the most accredited pathogenesis for DLBCL-ACI in humans.[8] Chronic inflammation enables virally transformed B cells to escape from host immune surveillance through production of IL-10 and providing autocrine and paracrine cell growth stimuli via IL-6 production. Furthermore, the finding of angiocentrism may derive from antigen persistence or immune complex deposition in vascular walls secondary to antigen challenge, followed by the expression of specific homing and tethering molecules by endothelial cells in the setting of the vaccine immune stimulation.

Conclusions

In summary, cutaneous lymphomas arising in areas commonly used for injection resembled previously reported cases in cats [52] and that of human DLBL-ACI[60,61] in which persistent chronic inflammation and immune stimulation in association with a favorable cytokine milieu leads to neoplastic transformation of lesional lymphocytes, facilitated in some instances by viral transformation. In conclusion, CLIS are angiocentric, angioinvasive, primary cutaneous, and predominantly large cell lymphomas with aggressive clinical behavior. They represent a distinct clinicopathological entity among feline cutaneous lymphomas that arise in areas commonly used for injections. Samples from severely necrotizing panniculitis in areas of injections need to be carefully screened in view of an underlying angiocentric lymphoma.

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Table 1. Histopathological features of feline primary cutaneous lymphomas at injection site.

Case	Angio centric	Angio invasive	Angio destructive	Central cavity	Necrosis %	Mineralized fibers	Hemorrhage	Reactive Lymphocytes	Other Inflammation (reactive)
1	+	-	-	+	15	-	+	Perivascular Follicles Admixed	M[1]
2	+	+	+	+	40	+	+	Perivascular Follicles Admixed	M (rare)
3	+	+	+	-	40	-	+	Perivaslular Follicles Admixed	M (rare)
4	+	-	-	+	15	+	+	Perivascular Follicles	M
5	+	+	+	+	30	+	+	Perivascular Follicles Admixed	M
6	+	+	+	-	40	+	+	-	-

7	+	+	+	-	60	-	+	Perivascular Follicles	M, PMN[2]
8	-	-	-	+	60	-	-	Perivascular Follicles	-
9	-	-	-	+	70	-	+	Perivascular Follicles	-
10	+	+	-	+	40	-	+	Perivascular	M
11	+	-	-	+	80	+	-	-	M, PMN
12	-	-	-	+	10	-	+	Perivascular	M, PMN
13	+	+	-	-	40	+	+	-	M
14	-	-	-	+	-	-	-	Perivascular	M
15	+	+	+	+	60	-	+	Perivascular Admixed	M, PMN
16	+	+	+	+	40	+	-	Perivascular	M (rare)
17	+	+	+	+	50	+	+	Perivascul	M

	ar
	Follicles

[1] M= macrophages, [2] PMNs= neutrophils

Table 2. Immunohistochemistry, clonality and definitive diagnoses of feline primary cutaneous lymphomas at injection site.

Case	Definitive diagnosis	Phenotype					FeLV[1]		Clonality	
		CD45RA	CD18	CD79a	CD20	CD3	gp70	p27	B cell loci[2]	T cell locus[3]
1	DLBCL[4] Immunoblastic	+	+	+	+	-	-	+	clonal	-
2	DLBCL Immunoblastic	+	+	+	+	-	++	++	clonal	-
3	ALCL[5] NK like*	+	+	-	-	-	+	+	pseudoclonal	polyclonal
4	DLBCL Immunoblastic	+	+	-	-	-	-	-	clonal	-
5	DLBCL Plasmacytoid	+	+	+	+20%	-	-	-	clonal	-
6	ALTCL[6]	-	+	-	-	+	+	-	-	clonal
7	ALTCL	+	+	-	-	+	-	-	-	clonal
8	ALTCL	-	+	-	-	+	-	-	-	polyclonal
9	DLBCL Immunobla	+	+	+	+	-	-	-	no amplif	-

	stic									
10	DLBCL Anaplastic	+	+	+	+	-	+	+	no amplif	-
11	DLBCL Immunoblastic	-	+	+	+	-	+	-	clonal	-
12	Uncertain Intermediate size	-	+	-	+10 %	+30 %	-	-	clonal	pseudoclonal
13	PTCL[7]	+	+	-	-	+	+++	+++	-	clonal
14	DLBCL Plasmacytoid	+	+	+	+	-	-	-	no amplif	-
15	DLBCL Immunoblastic	+	+	+	+	-	+	-	clonal	-
16	DLBCL Anaplastic	+	+	+	+	-	+	-	pseudoclonal	-
17	DLBCL Immunoblastic	+	+	+	+	-	+++	+++	clonal	-

[1] FeLV antigens - = negative, + = 1≤25%, ++: >25-≥50%, +++>51%; [2] B cell loci: IgH, frame work 2 and 3; [3] T cell locus: T cell receptor gamma; [4] DLBCL: diffuse large B cell lymphoma; [5] ALCL NK: anaplastic large cell lymphoma, potential NK cell origin: additional immunocytochemistry and smears: CD1c -, CD4 -, CD8-, CD11c-, CD11d-, CD21- , CD11b + weak; [6] ALTC: anaplastic large T cell lymphoma; [7] PTCL: peripheral T cell lymphoma unspecified.

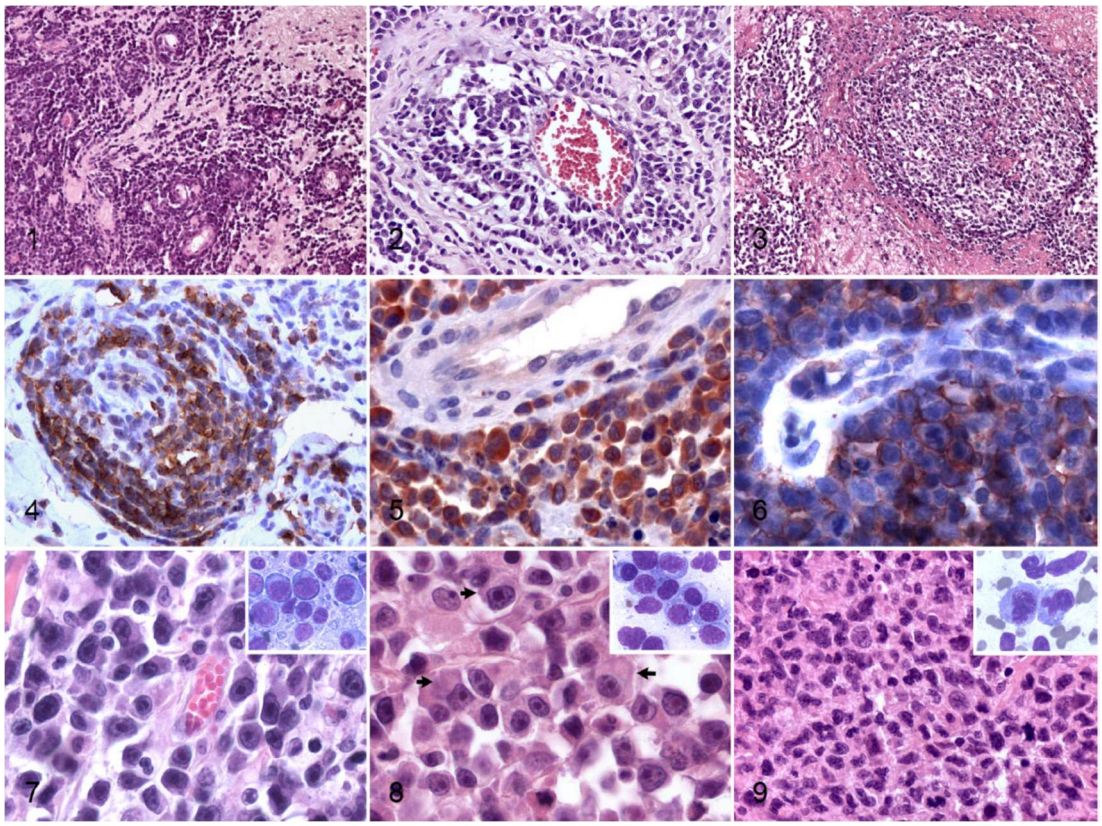

Figure Legends

Figures 1-9. Feline cutaneous lymphoma. Figure 1. Diffuse anaplastic large B cell lymphoma, case 10. Prominent angiocentrism: Neoplastic cells tightly surrounding vessels in the deep dermis. Haematoxylin and eosin (HE). Figure 2. Diffuse immunoblastic large B cell lymphoma, case 2. Angioinvasion: neoplastic cells infiltrating and expanding the wall of a medium sized vessel. HE. Figure 3. Diffuse immunoblastic large B cell lymphoma, case 15. Angiodestruction: mural vascular infiltration of

neoplastic cells is associated with endothelial necrosis, fibrin leakage and luminal occlusion. Adjacent tissue necrosis is prominent. HE. Figure 4. Diffuse immunoblastic large B cell lymphoma, case 2. Angiocentric accumulation of CD 20 positive neoplastic B cells. Immunohistochemistry (IHC) for CD20, Carbazole chromogen, Mayer's haematoxylin counterstain. Figure 5. Peripheral T cell lymphoma, case13. Perivascular accumulation and mural infiltration by CD3 positive neoplastic T cells. IHC for CD3, Carbazole chromogen, Mayer's haematoxylin counterstain. Figure 6. Diffuse immunoblastic large B cell lymphoma, case 2. FeLV gp 70 cell membrane expression by neoplastic cells. IHC for gp70, Carbazole chromogen, Mayer's haematoxylin counterstain. Figure 7. Diffuse immunoblastic large B cell lymphoma, case 1. Large cells (nuclei > 2 red blood cells in diameter) characterized by abundant cytoplasm, variably incomplete rim of cytoplasm and a large, round to oval nucleus with a central prominent large basophilic nucleolus. HE. Inset: corresponding cytological specimen demonstrating immunoblastic morphology. May-Grünwald Giemsa (MGG).Figure 8. Diffuse large B cell lymphoma, plasmacytoid variant of immunoblastic lymphoma, case 14. Large cells (nuclei > 2 red blood cells in diameter) characterized by abundant eosinophilic cytoplasm, variably incomplete rim of cytoplasm and a round to oval nucleus with variably prominent 1-2 nucleoli. An occasional cytoplasmic halo (arrows) can be seen. HE. Inset: corresponding cytological specimen with neoplastic cells characterized by a prominent clear cytoplasmic halo. MGG. Figure 9. Anaplastic large T cell lymphoma, case 7. Neoplastic cells are characterized by severe plemorphism, abundant cytoplasm and variably shaped nuclei. Mitoses are frequent. Typical Hallmark

cells can be seen (arrows). HE. Inset: corresponding cytological specimen illustrating cytological features of anaplastic lymphoma. MGG.

Supplemental Table 1. Panel of primary antibodies utilized for the characterization of feline primary cutaneous lymphomas at injection site

Antibody	Clone/ Species Specificity	Dilution	Source	House	Main cell reactivity
CD3-ε	CD3-12 Human†	1:10	Rabbit polyclonal	Serotec, Oxford, UK	T cells
CD18	Fe3.9F2 Feline	1:10	Mouse monoclonal	Leukocyte Antigen Biology Laboratory, UC Davis, CA, U.S.A.	All Leukocytes
CD20	Human†	1:40	Rabbit Polyclonal	Neomarkers, Freemont, CA, U.S.A.	B cells
CD45RA	B220 Mouse†	1:10	Mouse monoclonal	Biosciences Pharmingen, San Diego, CA, U.S.A.	B cells, T cell subpopulations
CD79a	HM57 Human†	1:100	Mouse monoclonal	Santa Cruz Biotechnology Inc.	B cells
FeLV gp70	C11D8 Feline	1:200	Mouse monoclonal	Custom Monoclonals Int., Sacramento, CA, U.S.A.	FeLV capsid glycoproteins 85/70
FeLV p27	PF12J-10A Feline	1:100	Mouse monoclonal	Custom Monoclonals Int., Sacramento, CA, U.S.A.	FeLV core protein 27

† Cross reactive with feline tissues (referenced in the materials and methods).

Supplemental Table 2. Signalment, serology and presentation of cases of feline primary cutaneous lymphomas at injection site

	Breed	Sex	Age (years)	FIV/FeLV serology	Site of lesion	Number of nodules	Size (cm)	Extension
1	Devon rex	Mc	15	-/-	Interscapular	Single	1	Panniculus to deep muscles
2	Siamese	Mc	9	U/U	Thorax laterodorsal	Two regional nodules	U	Panniculus to deep muscles
3	DSH	Mc	15	U/U	Interscapular	Single	10x3	Superficial dermis to panniculus
4	DSH	Fs	10	-/-	Dorsal thorax	Single	U	Deep dermis to muscles
5	DSH	Fs	15	-/-	Left lateral thorax to lumbar area	Single	4x2.5	Superficial dermis to panniculus
6	DSH	Mc	13	U/-	Interscapular	Single	U	Mid dermis to panniculus
7	DSH	Fs	10	U/U	Lateral thorax	Multiple regional	U	Deep dermis to panniculus
8	DSH	Mc	11	U/U	Left lateral thorax	Single	8	Panniculus to deep muscles
9	DSH	Fs	10	U/U	Lateral thorax	Single	2.5x1.3	Mid dermis to panniculus
10	DSH	Fs	3	U/U	Thorax	Single	2	Deep dermis, to panniculus
11	DSH	Mc	10	U/U	Interscapular	Single	U	Mid dermis to panniculus
12	Persian	Mc	15	U/U	Interscapular	Single	U	Panniculus
13	DSH	Mc	7	-/-	Dorsal	Single	U	Superficial dermis to panniculus
14	DSH	Mc	10	-/-	Intescapular	Single	U	Panniculus
15	DSH	Mc	12	U/U	Left flank	Single	5.5x2 ulcerated	Superficial dermis to panniculus

16	DSH	Mc	16	-/-	Interscapular	Two regional nodules	3.5; 0.8	Panniculus
17	Norwegian forest	Mc	11	U/-	Interscapular	Single	4 x 2 x 6	Mid dermis to deep muscles

U= unknown
†+ dimensions are listed as provided in the history of the submitted sample

Supplemental Table 3. Cytological features of feline primary cutaneous lymphomas at injection site.

Case	**Cell size**	**Nuclear Shape**	**Chromatin**	**Nucleoli**	**Multinucleated neoplastic giant cells**	**TBMs**	**MI[3]**	**Pleomorphism**
1	Large[1]	Round to indented	Finely granular	Single prominent	+	+	5.5	Moderate
2	Large	Round to indented	Finely granular	Single prominent	+	-	1.3	-
3	Large	Round to Pleomorphic C-shaped	Dense	Rarely evident	+	-	0.8	Severe
4	Large	Round to oval	Finely granular	Single prominent	+	+	4.4	-
5	Intermediate[2] to large	Round to indented	Coarsely granular to clumped	Multiple	+	+	4.9	Moderate
6	Large	Round to Pleomorphic C-shaped	Granular	Rarely evident	-	+	7.3	Severe
7	Large	Round to Pleomorphic C-shaped	Granular	Variably evident	-	-	3.5	Severe
8	Large	Round to Pleomorphic C-shaped	Granular	Multiple	-	+	3.4	Severe
9	Large	Round	Finely granular to diffuse	Single prominent	-	-	3.2	Severe
10	Large	Round to C-shaped	Granular	No evidence	-	+	8.4	Severe

11	Large	Round to indented	Diffuse to vesicular	Single prominent	-	-	0.1	-
12	Intermediate	Round to indented	Granular	Single, variably evident	-	-	4.7	Mild
13	Large	Round to oval	Granular to vesicular	Single prominent	-	-	3.9	-
14	Intermediate to Large	Round	Coarsely granular to clumped	Single multiple central	-	-	0.5	-
15	Large	Round to oval	Granular	Single prominent	+	-	2.1	-
16	Large	Round to pleomorphic	Granular	Multiple prominent	+	-	5.1	Severe
17	Large	Round	Granular to vesicular	Multiple prominent	-	-	4.3	-

[1] large cell: nuclei > 2 red blood cells in diameter; [2] intermediate: nuclei of 1.5-2 red blood cells in diameter; [3] TBMs: tingible body macrophages; MI: mitotic index;

Supplemental Table 4. Staging, therapy and clinical follow-up in cats with primary cutaneous lymphomas at injection site.

Case	Staging	Therapy after excision	History and Follow up	Survival Time (days)
1	Negative [1]	COP	No therapy response. Recurrence 98 days after local excision. Euthanasia.	124
2	NP[2]	NP	Previous diagnosis of panniculitis. Recurred twice. Euthanasia after diagnosis. Necropsy negative for internal organ involvement.	30
3	Negative	Vincristine and prednisone	Developed 15 days after injection. Recurrence 16 days after surgery with ulceration. No therapy response. Euthanasia.	127
4	NP	NP	Previous diagnosis of panniculitis. Spontaneous death.	15
5	Negative	NP	Recurred and progressed rapidly. Internal organ invasion (spleen, liver) after 200 days confirmed by cytology. Spontaneous death.	214
6	Negative	NP	Developed 15 days after FeLV vaccine injection. Rapid progression. Euthanasia. Necropsy negative for internal organ involvement.	18
7	Negative	NP	Spontaneous death due to car accident 4 months after diagnosis.	122

8	NP	Owners refused chemotherapy	Lesion developed 1 month prior to diagnosis. Recurred rapidly and ulcerated. Renal failure and spontaneous death.	60
9	NP	NP	Previous diagnosis of panniculitis.	LFU[4]
10	Negative	Owners refused chemotherapy. Prednisone	Recurred 30 days post-surgery. Rapid progression with involvement of entire dorsal area. Anorexia, pain and ulceration. Euthanasia.	61
11	NP	NP	NA[3]	LFU
12	Negative	Owners refused chemotherapy	Spontaneous death.	21
13	NP	Owners refused chemotherapy. Prednisone	Recurred, rapid progression, pain and ulceration. Euthanasia. Necropsy negative for internal organ involvement.	35
14	Negative	NP	Last vaccine injection 5 years before decease. Recurrence after 1,5 years. Incomplete excision. Spontaneous death with thoracic effusion of lymphoma confirmed by cytology after approx. 700 days from initial diagnosis.	761
15	Negative	Owners refused chemotherapy. Prednisone.	No recurrence of skin lesions. One month prior to decease bilateral renal cortical hyperechogenicity. Spontaneous death of renal failure.	124
16	Negative	Owners refused chemotherapy. Prednisone 3 months after diagnosis.	Developed approx. 9 years after last vaccine injection Development of second nodule 10 days after first evaluation. Two months after surgery the cat was in good body conditions, no recurrence. After one month anorexia, rapid worsening of body conditions. Staging negative for internal disease. Spontaneous death.	105
17	Negative	Owners refused chemotherapy.	Developed approx. 1 year after vaccine injection. Mass grown rapidly in 20 days. Surgery with wide margins, second surgery to close wound. Recurrence of cutaneous lymphoma 6 months after diagnosis. Surgery was performed again. Spontaneous death (tumor recurrence-progression) 4 months later.	304

[1] Negative: no evidence of internal organ involvement; [2] NP=Not performed; [3] NA=Not available; COP= Cyclophosphamide, Oncovin, Prednisone chemotherapy