3rd Meeting of the European Canine Lymphoma Group

New Developments in Canine Lymphoma

Workshop Proceedings

Palazzo dei Congressi,
Piazza Indipendenza 4
CH-Lugano, June 17th 2017

Edited by
Franco Guscetti

A satellite workshop to the 14-ICML
European Canine Lymphoma Network
EU-CAN-LYMPH.NET

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Zurich, June 2017
Acknowledgements

We acknowledge the kind and continuous support of the 14-ICML which again invited us to organize this workshop as a satellite of the 14-ICML meeting; the kind financial support of the University of Milano, Dept. of Veterinary Medicine; the kind financial and logistic support of Solaris Healthcare.

The abstracts of the poster session have been accepted after peer-review by a scientific committee composed by F. Guscetti, L. Marconato, S. Comazzi and L. Aresu under strict avoidance of conflicts of interests.
Welcome from the Organizing Committee

Lugano, June 17th, 2017

Dear colleagues,

the meeting of the European Canine Lymphoma Network as a satellite workshop to the International Conference on Malignant Lymphoma (ICML) in Lugano has reached its 3rd edition. We wish to acknowledge the kind invitation of the 14-ICML organizers to join again. Indeed, canine lymphoma has continued to arouse interest for its potential as a comparative model. The European Canine Lymphoma Network (www.eu-can-lymph.net), born in 2010 and now linking more than 70 researchers from 25 European institutions involved in research and cure of canine lymphoma, constantly strive towards this goal.

The workshop is traditionally divided in three sessions with plenary lectures on actual topics held by internationally recognized experts and will cover this year several topics of great comparative interest. In the first session, focused on pathogenesis, K. Richards (Cornell University, Ithaca NY) will present an overview on gene expression profiling in canine lymphoma and L. Aresu (University of Padova, I) will convey present knowledge on methylation profiles of these tumors. Novel genomic-wide approaches to study dog lymphomas have contributed to unveil many similarities but also differences between human and canine lymphoma. Eventually this kind of approach will allow for a better, more precise use of the dog model. In the session dedicated to diagnostics, S. Keller (University of Guelph, CDN) will give an overview on the advantages and limits of clonality testing in canine lymphoma and emphasize the need for a better standardization. Subsequently, S. Comazzi will briefly present current and prospective projects of the European Canine Lymphoma Network focused on pathogenesis and diagnostics. During the following coffee break there will be the chance to discuss the posters which have been peer-reviewed by the scientific committee and present interesting novel aspects of canine lymphoma research. In the last session, focused on therapy, we will explore a novel comparative formula. A. Stathis (Oncology Institute of Southern Switzerland, CH-Bellinzona) on one side, and L. Marconato (Centro Oncologico Veterinario, I-Bologna) and G. Polton (North Downs Specialist Referrals, UK-Bletchingley,) on the other side will discuss the State of the Art in human and canine indolent lymphoma and refer to practical issues related to the organization of clinical trials. In particular, a proposal for a multi-institutional clinical trial on canine Marginal Zone Lymphoma will be presented.

The variety of the programme topics and of the common activities of the network members eu-can-lymph network that will be presented provide evidence that canine lymphoma research is indeed getting momentum. We wish that our initiative will contribute to foster further interest in this topic in the future.

Enjoy the workshop!

The organizing committee,
Stefano Comazzi, Laura Marconato, Luca Aresu and Franco Guscetti
Programme

13:00 -13:30  Registration

13:30-13:50  Welcome and update of European Canine Lymphoma Network activities
Stefano Comazzi, Laura Marconato

SESSION ON PATHOGENESIS

13:50-14:30  Gene Expression Profiles in Canine Lymphoma
Kristy L. Richards, Cornell University, US

14:30-15:10  Methylation Profile in Canine Lymphoma
Luca Aresu, University of Padova, I

SESSION ON DIAGNOSTICS

15:10-15:50  Clonality Testing in the Diagnosis of Canine Lymphoma
Stefan Keller, University of Guelph, CDN

15:50-16:05  Presentation of research projects on pathogenesis and diagnostics
S. Comazzi, University of Milano, I

16:05 – 16:30  Coffee-break and poster viewing

SESSION ON THERAPY

16:30-17:00  Indolent B-Cell Lymphoma - the State of the Art in Human Patients
A. Stathis, Oncology Institute of Southern Switzerland, Bellinzona, CH

17:00-17:15  Indolent Lymphoma - the State of the Art in Dogs
L. Marconato, Centro Oncologico Veterinario, Bologna, I, and
G. Polton, North Downs Specialist Referrals, Bletchingley, UK

17:15-17:45  How to Organize a Clinical Trial - the Experience of the International Extranodal Lymphoma Study Group (IELGS)
A. Stathis, Oncology Institute of Southern Switzerland, Bellinzona, CH

17:45-18:05  Canine MZL: proposal for a multi-institutional clinical trial
L. Marconato, Centro Oncologico Veterinario, Bologna, I, and
G. Polton, North Downs Specialist Referrals, Bletchingley, UK

18:05-18:15  End of works and take home message
Abstracts of invited lectures

SESSION ON PATHOGENESIS

Gene Expression Profiles in Canine Lymphoma

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Canine B-cell lymphoma mimics the human disease in several fundamental ways, including histology, B-cell specific markers (e.g., CD20, PAX5, and CD79a), responsiveness to CHOP-based chemotherapy, and gene expression profiles that mirror activated B-cell (ABC) and germinal center B-cell (GCB) cell-of-origin subtypes in human DLBCL. However, in other important ways, canine B-cell lymphoma differs from human DLBCL. These include mutation spectra from exome sequencing studies, prognostically important immunohistochemistry markers like BCL2 and c-MYC (“double expressor phenotype”), and less frequent cure rates after CHOP-based chemotherapy. These differences highlight the importance of credentialing each pathway that is targeted by new therapeutics, to be sure that it accurately mirrors the human disease.

We have developed a program for experimental therapeutic development involving more accurate animal models, including canine clinical trials with pet dogs under veterinary oncology care. We call this program PATH (Progressive Assessment of Therapeutics) and it engages researchers from across the Cornell campus. As an example of our program, we are developing a new therapeutic agent with activity against fascin, an actin bundling protein. Fascin overexpression has been shown to be a poor prognostic factor both in human and in canine cancers. Cancer cells often upregulate fascin, and in many cancer types, this is associated with poor prognosis, due to increased invasive and metastatic potential. We have developed fascin inhibitors that prevent actin bundling and block cancer metastasis in mice. We have also shown that fascin is upregulated in approximately half of canine B-cell and T-cell lymphoma cases. We have further shown that a fascin inhibitor, NP-G2-044, kills human and canine lymphoma cells in vitro, and in vivo in mice bearing canine diffuse large B-cell lymphoma (DLBCL) xenografts. We are currently working on a canine clinical pilot trial to treat 10 dogs with lymphoma to measure clinical benefit. We are also using RNAseq data from canine B-cell lymphomas to understand what genes are differentially expressed in high-fascin vs. low-fascin individuals.

Together, our studies will further validate the pet dog as a useful addition to the therapeutic development pipeline. We intend to advance human and canine health by using clinical trials, in areas where dogs and human overlap in their molecular characteristics. This strategy should advance both canine and human health.

Selected References


3. Curran KM, Schaffer PA, Frank CB, Lana SE, Hamil LE, Burton JH, Labadie J, Elrhart EJ, Avery PR. BCL2 and MYC are expressed at high levels in canine diffuse large B-cell lymphoma but are not predictive for outcome in dogs treated with CHOP chemotherapy. Vet Comp Oncol. 2016.


**Methylation Profile in Canine Lymphoma**

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Canine B-cell lymphoma (BCL) is the most common hematopoietic cancer in dog, being considered a curable disease in less than 10% of dogs. In the remaining 90% of dogs the disease is rapidly fatal if left untreated. Dogs diagnosed with BCL may respond differently to traditional chemotherapeutic and chemo-immunotherapeutic protocols, but responsiveness remains scarce, with a significant number of dogs succumbing to lymphoma early in the disease course. Studies on gene expression profiling and chromosomal aberrations in cBCL have significantly extended the understanding of pathogenesis and clinical outcomes. Recently, mechanisms controlling gene expression in DLBCL have been described. However, the biology of this tumor is still not entirely explained by genomic events and transcriptional programs, and much less is known about epigenetic changes. Epigenetic mechanisms are fundamental for normal development and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. In this contest, DNA methylation is perhaps the most extensively studied epigenetic mechanism in mammals and evidence shows its role in the development and progression of various human cancers. By definition, DNA methylation is a heritable epigenetic modification characterized by the transfer of a methyl group to the cytosine residue of CpG dinucleotides by DNA-methyltransferases (DNMTs). In mammals, plants and other organism, most CpG dinucleotides are methylated on cytosine residues; conversely CpG
dinucleotides within gene promoters tend to be protected from methylation. Physiologically, methylation is extremely important in numerous cellular processes, including embryonic development, genomic imprinting, X-chromosome inactivation and chromosomal stability. In contrast, defects in DNA methylation are closely associated with cancer and two forms of aberrant DNA methylation are found in cancer: 1) the overall loss of 5-methyl-cytosine (global hypomethylation) and 2) gene promoter-associated (CpG island-specific) hypermethylation. While the precise consequences of genome-wide hypomethylation are still debated (activation of cellular proto-oncogenes, induction of chromosome instability), hypermethylation of gene promoters is associated with gene inactivation. The biological consequence of DNA methylation is a double-edged sword: the neoplastic process is promoted by local hypermethylation resulting in silencing of tumour suppressor genes and in parallel by global hypomethylation triggering reactivation of cellular proto-oncogenes. A plethora of studies reported that silencing of tumour suppressor genes and other cancer-related genes may occur through hypermethylation in the absence of obvious genetic change.

So far, different techniques for methylation analysis have now been published. The bisulfite conversion of DNA results probably one of the most efficient to study methylation and is based on the selective chemical conversion of the unmethylated cytosines into uracil by treatment with sodium bisulfite, which is then amplified as thymine during PCR. In contrast, the methylated cytosines are not converted, such that, in the final sequencing result, the 5-methylcytosine will be still detected as cytosine. A limited number of reports in canine lymphoma are available and only investigating DNA methylation aberrations at single gene level. Methylation-specific PCR and pyrosequencing have shown significative results in these studies but it's now evident that perturbation of methylation should be analysed at the whole genome level, also to identify gene-interactions that orchestrate tumor transcriptome and phenotype.

In the recent years, the research in our laboratory has aimed at providing a better understanding of the epigenetic mechanisms underlying the pathogenesis of B-cell lymphoma in dog. Given the previous considerations for the first time we analysed the genome-wide DNA methylome by means of a canine DNA CpG microarray. The platform was developed and probe design was carried out by the Agilent bioinformatic support team using proprietary prediction algorithms to locate CpG Islands on the C. familiaris draft genome as deposited on Ensembl database (CanFam 3.1) and to design high quality oligo-probes. Microarray probes were selected in order to provide the highest possible coverage of dog genome. Coding DNA sequence (CDS) regions and CpG islands were given top priority. The X chromosome was excluded from analysis and probe design. A total of 170,000 probes (60mers, sense orientation) were designed on both CpG and CDS regions. In details, 102,000 probes were designed targeting a total of 36,807 CpG regions while 68,000 probes were directed against 672 CDS; average base pair tiling was 90 bp. The array was used for the first time in veterinary medicine and a bioinformatic pipeline was constructed ad hoc.

In the experimental design we compared tissues obtained from 40 dogs affected by DLBCL with lymph nodal tissue obtained by 8 germ-free dogs used as control. Results showed that DLBCLs are characterized by a widespread aberrant methylation affecting 1,194 regions, corresponding to 823 genes. The hypermethylated sequences were enriched in neighbourhood (upstream ≤ 10kb) or promoter regions while the hypomethylated sequences were preferentially located in gene bodies and downstream regions. This was quite expected considering that CpGIs are highly susceptible to DNA methyltransferases in cancer determining gene silencing. Conversely, CpG-poor regions undergo a global decrease of genomic DNA methylation affecting genome stability, transcriptional elongation, and RNA splicing. Functional analysis of differentially methylated genes identified 22 Biological Process (BP), 5 Molecular Function (MF), 2 Cellular Component (CC) Gene Ontology (GO) terms and 3 KEGG pathways as significantly enriched. Overall, 19 out of 22 GO_BP enriched terms were
involved in the development/morphogenesis of anatomical structures, including 10 terms directly linked to embryogenesis and 9 related to specific tissues development. All these terms were mainly represented by genes playing key roles in regulating organogenesis (SHH, BMPs, GREM1), body patterning (HOX gene family) and tissues differentiation (FGFR2, FGF18, SOX9). Clustering analysis based on the methylation levels of a subset of methylated CpGs identified three different subgroups of DLBCL that were significantly associated to overall survival.

Next generation sequencing is rapidly becoming a more affordable option, and permits to overtake limits of microarray analysis such as requirement of species- or transcript-specific probes and legacy technology. Also, in the last years the costs of sequencing have dramatically decreased. Considering this innovation, in 2016, in our lab we profiled 50 DLBCLs for whole genome methylation profiles by a Methyl Binding Protein (MBD)-seq approach. MBPs are able to capture methylated DNA regions that are then sequenced through high-throughput technology. On the same samples we performed RNA-seq for transcriptomic profiling and array comparative genomic hybridization (ACGH) for identification of chromosomal aberrations. The opportunity to investigate the three omics on the same tissue has no precedents in veterinary medicine and the results have delineated the correlation of gene-expression with methylation in canine DLBCL. More specifically, it’s evident how the reduction of expression of about 400 tumor-suppressor genes is significantly correlated to the increase of methylation at the promoter level. Conversely, the up-regulation of genes that are involved in DLBCL pathogenesis seems to be driven by copy number aberrations, most likely gains. In the next future, the major challenge will be to identify hypomethylating agents acting as specific inhibitors of DNA methylation and targeting down-expressed genes (i.e. tumor suppressor genes) to reactivate their expression. Few studies have now validated these molecules in veterinary medicine but more robust data are needed to consider their use in the clinic.

Selected References

Clonality Testing in the Diagnosis of Canine Lymphoma

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Clonality testing is a molecular method that can differentiate reactive from neoplastic lymphoid proliferations if microscopic assessment is inconclusive. The scope of this talk is to highlight the principle, workflow, pitfalls and future directions of clonality testing in veterinary medicine.

**Principle.** Lymphocytes recognize antigen by means of a highly variable receptor, the lymphocyte antigen receptor (LAR). The enormous diversity of LAR genes is created by shuffling of gene segments and random insertion and deletion of nucleotides. As a result, every lymphocyte has a unique LAR gene sequence and ligand specificity. Clonality testing exploits the genetic diversity of lymphocytes to visualize the clonality of lymphocytes within a given sample. Lymphocyte antigen receptor genes are amplified using conventional PCR and amplicons are size separated by electrophoresis. The resulting size distribution of LAR genes is used to infer whether lesional lymphocytes are reactive or neoplastic. In reactive processes, lymphocytes are derived from multiple precursor cells that differ in CDR3 length resulting in amplicons of variable size. In neoplastic processes, lymphocytes are derived from a single precursor cell and hence have identical CDR3s resulting in amplicons of equal size.

**Workflow.** DNA is extracted from fresh or formalin-fixed and paraffin embedded scrolls or sections, cytological preparations, blood or fluids. Depending on the lineage of presumed neoplastic cells (B cell vs. T cell), pertinent LAR gene loci are amplified by conventional PCR. PCR products are then size separated by gel electrophoresis. In general, reactive proliferations are associated with a polyclonal profile and neoplastic proliferations are associated with a clonal profile. However, false positive and negative as well as equivocal results occur and might present as diagnostic challenge. The consideration of clinical, morphological and immunophenotypical data is pertinent to minimize misdiagnoses.

**Pitfalls.** Clonality testing has inherent pitfalls that need to be considered on a ‘per lab’ basis but also across the veterinary clonality community as a whole. First, LAR genes are currently amplified by PCR, which makes the electrophoresis results dependent on the quality of the primer set. Genes that are not covered by primers are a potential source of false negative results. Differences in primer efficiency and multiplexing strategy might skew clonal proportions. Second, the electrophoresis profiles are highly dependent on the clinical context, i.e. a given profile can indicate a reactive or a neoplastic process. It is hence pertinent to contrast the electrophoresis profile with clinical data, histopathology/cytology and immunophenotyping. Over the years, different primer sets and interpretational practices have been developed in different laboratories. Today, there is no agreement on which primer sets to use and how to interpret electrophoresis results. In addition to the lack of standardization, the methodological details of the laboratory workflow as well as the rationale behind
the interpretation of a given electrophoresis profile are not routinely communicated to the client. As a result, clonality testing is currently conducted in a black box that faces little scrutiny and that is generally not amenable to independent review by third parties. If clonality testing in veterinary medicine is to evolve into a broadly accepted, standardized, reproducible and transparent method analogous to the situation in human medicine, a multi-institutional effort is required to develop commonly agreed up on methods, standards and guidelines.

Future directions. In addition to standardizing existing practices in veterinary clonality testing across institutions, these efforts need to be applied to emerging technologies alike. Next generation sequencing is revolutionizing the field of lymphocyte antigen receptor sequencing in human medicine and will eventually become the gold standard in veterinary medicine as well. Along with great benefits, this method poses a whole new set of challenges in regards to library preparation, data analysis and interpretation. Since this is mostly uncharted territory in veterinary medicine, it also provides a unique opportunity to standardize practices early on and to minimize the divergence of methodology and interpretational approaches that has occurred with traditional electrophoresis-based clonality testing in veterinary medicine.

Selected References


SESSION ON THERAPY

Indolent B-cell Lymphoma - the State of the Art in Human Patients

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Marginal zone lymphomas
Marginal zone lymphomas (MZL) represent a group of lymphomas that originate from B lymphocytes normally present in the “marginal zone,” which is the external part of the secondary lymphoid follicles. In the most recent WHO classification, the MZL category comprises three different subtypes: the extranodal MZL of mucosa-associated lymphoid tissue type (MALT lymphoma), the splenic MZL with or without villous lymphocytes (SMZL), and the nodal MZL (NMZL). Together they account for approximately 10% of the total number of cases of non-Hodgkin lymphoma: primary splenic and nodal MZLs comprise 1% to 2% each and MALT lymphomas 8% of the total number of non-Hodgkin lymphoma cases.

Marginal zone B cell lymphomas are indolent lymphomas. First line treatment including rituximab ± chemotherapy results in long-lasting remissions in the majority of patients. However, despite high remission rates following first-line treatment, most patients will experience a relapse of their disease and will require additional treatments. Marginal zone lymphoma is in fact considered an incurable lymphoma.

For MALT lymphoma, the most frequent of these 3 entities, the combination of the anti-CD20 monoclonal antibody rituximab with chlorambucil has been reported in a phase III trial to be the most efficient treatment compared to rituximab alone or chlorambucil alone. The combination rituximab-bendamustine has been recently reported as extremely active combination in a phase II trial evaluating 57 patients, with a complete remission rate at 95% if disseminated and 100% in localized disease, and a 88%- event-free survival (EFS) at 4 years. This combination is currently being evaluated in SMZL by the International extranodal lymphoma study group (IELSG). In NMZL, there is no consensus of treatment and patients are usually managed as those with follicular lymphoma with the combination of rituximab and bendamustine representing a valid option of treatment.

For patients that relapse after first line treatment, despite abundant literature on histological, clinical and biologic features, there is a lack of controlled trials to define the optimal treatment. Progression-free-survival is usually short, responses to single-alkylating agents or to rituximab monotherapy are of short duration and therefore novel systemic treatment strategies are needed. Recently, Ibrutinib, a small molecule targeting the Bruton tyrosine kinase (BTK) has shown single-agent activity in patients with relapsed-refractory MZL inducing durable responses with a favorable benefit-risk profile, confirming the role of the B-cell receptor (BCR) signaling in this malignancy. Based on its activity observed in a phase II study, ibrutinib gained FDA approval and provides a treatment option without chemotherapy for MZL.
Selected References


Indolent Lymphoma - the State of the Art in Dogs

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In humans as well as in dogs, indolent lymphomas represent a group of incurable slow growing tumors, characterized by a relatively long natural history with a continuous pattern of relapse, for which there are no defined first-line therapies. In general, the biologic behavior is extremely variable, with some patients having an extremely aggressive course and death within a few months despite intense treatment, and others living for years and never requiring therapy.5-7

In dogs, indolent lymphomas are not rare; however, their true incidence remains unknown. According to one study, indolent lymphomas represented about 30% of all lymphoma subtypes. The different histological subtypes that constitute the group of disorders known as indolent lymphomas include marginal zone lymphoma (MZL), follicular lymphoma (FL), B-cell small lymphocytic lymphoma (B-SLL), B-cell lymphoplasmacytic lymphoma (LPL), T-zone lymphoma (TZL), and T-cell small lymphocytic lymphoma (TSSL).5-7
While most of these lymphomas are easily identified by histology and immunohistochemistry, MZL is challenging to diagnose. Indeed, primary nodal MZL has morphologic, immunophenotypic and molecular similarities with DLBCL, rendering these two entities difficult to discriminate. Nevertheless, the distinction may have clinical relevance.

To date, indolent lymphomas have not proven curable with traditional chemotherapy. A course of ‘watchful waiting’ may be proposed for human patients without disease-related symptoms or adverse prognostic factors. An advanced clinical stage and the presence of symptoms typically speak in favor of undertaking treatment. Chlorambucil has been used as first line therapy, followed by CHOP-based protocols +/- rituximab in case of relapse or refractory disease; however, no consensus has been achieved on the optimal first-line or relapse treatment. Unfortunately, although chemotherapy is believed to improve the duration of remission and survival, the disease is essentially incurable. Indeed, indolent lymphomas grow too slowly to be selectively targeted by chemotherapy.

In line with human oncology, and in contrast to the rapidly expanding insights into the biology of the lymphatic system and the pathogenesis of canine lymphoproliferative disorders, comparatively little progress has been achieved in the treatment of dogs with indolent lymphoma, highlighting a lack of knowledge and of randomized studies. In frontline treatment, a watchful waiting policy remains a good option if the dog has no risk criteria or symptoms. In dogs needing therapy, chemotherapy is the best option, although it has not been established which strategy works best (dose-intense versus metronomic). It also remains to be determined whether after front-line treatment, maintenance treatment plays a role instead of observation only.

While achieving complete remission translates into prolonged survival for dogs with aggressive lymphoma, the same does not hold true for indolent lymphomas, as outcome cannot be anticipated by treatment response.

It appears obvious that studies aimed at carefully allocating treatment according to disease presentation are warranted. Indeed, eligibility criteria for clinical trial enrollment are extremely important if results of studies performed internationally are to be compared. A literature search identified 3 studies focusing on canine indolent nodal lymphomas. Unfortunately, enrollment criteria, staging work-up, treatment and response assessment varied among these studies, impeding meaningful comparisons. It may be possible that the fairly good prognosis attributed to indolent lymphomas by some studies only reflects a less advanced stage, thereby challenging the belief that dogs with indolent lymphoma are uniformly long-term survivors.

Selected References


How to Organize a Clinical Trial - the Experience of the International Extranodal Lymphoma Study Group (IELGS)

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The IELSG is a unique cooperative group. By bringing together numerous scientists from different institutions, it has become possible to amass the data from a sufficient number of patients to study specific extranodal sites of involvement by a lymphoma. Although extranodal lymphomas are not rare, the frequency of involvement of any particular site is not high enough for a single institution to answer the major questions.

The idea of setting up an international group of investigators to study the extranodal lymphomas was first launched in 1997 during a mid-summer meeting of the Lymphoma Group at the Oncology Institute of Southern Switzerland IOSI. The background was the successful task of joining clinicians and pathologists from several Italian and Swiss Oncology Institutes to take part to the international randomized study of gastric MALT lymphomas initially designed by the United Kingdom Lymphoma Group (UKLG/BNLI LY03 trial). The idea gathered enthusiastic followers and that mid-summer dream soon became a reality: the International Extranodal Lymphoma Study Group (IELSG) was created in Ascona, Switzerland, on January 23-24, 1998 during what we now remember as the First IELSG Annual Meeting.

The first decision was to start a clinicopathologic retrospective review of non-gastric MALT lymphomas from the Italian and Swiss centers that were already participating to the above-mentioned gastric MALT lymphoma LY03 trial. Since then, the group expanded very rapidly to include active members from many other countries such as France, the U.K., Spain and Germany.
The second meeting was held at the end of February 1999 again in Ascona. There were for the first time participants from Canada and the USA and new protocols in the fields of testicular, CNS and intestinal lymphomas were accepted and activated. This success underscores the fact that only a cooperative group can accrue enough cases of extranodal lymphoma to carry out meaningful basic and clinical research. In fact, not only do the different extranodal lymphoma sites have different biologies, but very often have different treatment criteria to be applied, and no single institution will ever be able to accumulate enough cases.

The outstanding dedication and commitment of all participants to the IELSG has been very productive. Results of many IELSG studies have already been presented at major international oncohematology congresses (including ASCO, ASH and the Lugano Conference on malignant Lymphoma) and several manuscripts have been and will be published in the near future in peer-reviewed scientific journals.

Currently, standard treatments for different extranodal lymphomas, including primary central nervous system lymphoma, testicular lymphoma and MALT lymphoma are based on prospective studies conducted by the IELSG. In addition, a large international prospective trial is assessing the role of radiotherapy in patients with primary mediastinal lymphoma that become PET negative following induction chemoinmunotherapy.

To deal with the administrative problems related to its expanding activity, in 2005 the IELSG has been registered as a non-profit society according to the Swiss law. A Board of directors was subsequently designated and an Operation Office was created and is located in Bellinzona, Switzerland. The aim of this initiative is to coordinate the activity of the group between the annual meetings and to explore new possibilities for funding the IELSG programs.

A quick summary of the IELSG achievements:
- About 260 institutions from 5 continents are participating within the IELSG
- ~ 6000 patients enrolled in the so far conducted studies (>1000 in prospective, randomized trials)
- 27 completed studies and 10 studies ongoing
- Regular pathology review sessions, chaired by professor Pileri are organized
- 35 peer-reviewed publications in the principal journals (Lancet, JCO, Blood)
- The operating office at IOSI (Bellinzona) coordinates the activities (Clinical Trial Unit)
**Poster abstracts**

**P01 Results of flow-cytometric immunophenotyping in canine multicentric lymphoma - 73 cases**

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**Introduction.** Low invasiveness, timely availability and multicolour staining, the latter providing more comprehensive information than immunohistochemistry, make flow cytometric immunophenotyping (FCM) an indispensable complementary tool in the characterisation of different types of canine lymphoma. Objective of this study was to summarize the results of FCM based classification with an extended standardized antibody panel over 6 years.

**Materials and Methods.** Results of FCM immunophenotyping of canine peripheral lymph node aspirates with lymphoma submitted from 2010 - 2016 were epitomized. Multicolour staining with canine species-specific and human cross-reactive monoclonal antibodies against CD3, CD4, CD5, CD8, CD11a, CD11d, CD14, CD21, CD34, CD45, CD45RA, CD79 α cy, CD3 ε, MHCII, Thy-1 and the corresponding isotype controls was used. The cut off for positivity was set at 80% for the population gated as lymphocytes.

**Results.** Out of 73 samples, 41 (56.2%) were, based on their FCM expression patterns, classified as compatible with Large B-cell Lymphoma (LBCL), 7 (9.6%) as T-helper-cell lymphoma, 1 (1.4%) as cytotoxic T-cell lymphoma and 1 (1.4%) as NK-cell like lymphoma. Five cases (6.8%) showed a mixed phenotype with expression of B- and T- cell markers being CD3+CD21-CD79+ and 7 (9.6%) showed a mixed phenotype expressing B- and T- cell markers being CD3+CD21+CD79+. Three cases (4%) were T-zone lymphomas and 3 (4%) peripheral T-cell lymphomas. One (1.4%) LBCL showed positivity for CD34 and 4 cases (5.6%) showed different aberrant expression.

**Conclusion.** Overall the results, regarding B and T cell differentiation, correspond well with published data that show comparable distribution patterns. In total 15.2% of the cases showed aberrant expression, comprising 9.6% with a CD3+CD21+CD79+ phenotype and 5.6% showing different aberrant expression. Standardization and continuous refinement of FCM protocols are pivotal to identify these rare disease entities and to enable research on these cases by multicenter studies.
P02 Monoclonal antibodies recognizing DLA-DR dimers and their use in rapid screening and experimental therapy of canine lymphoma

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Introduction. Rapid screening methods allowing straightforward diagnostics of canine lymphoma (CL) are missing. Moreover therapy of CL, unlike human non-Hodgkin’s lymphoma, lacks modern biological medicines that are able to significantly prolong the lifespan of patients. Here, we evaluate two proprietary mAbs to dog leukocyte antigen DR (DLA-DR) as candidate tools for development of rapid screening tests and experimental therapy of CL.

Material and methods. Two murine mAbs (B5 and E11) that recognize conformational, monomorphic and non-overlapping epitopes present uniquely on the DLA-DR dimers were produced. These mAbs were analyzed by flow cytometry, ELISA and lateral flow tests (LFT) using a panel of established canine lymphoma/leukemia cell lines (n=5), biopsies from lymph nodes of healthy dogs (n=8) or dogs with confirmed CL (n=33) or enlarged for other reasons (n=2). The ELISA and LTF methods were evaluated for sensitivity and specificity of CL detection. The mAbs were also tested for their cytotoxicity towards CL cell lines.

Results. By flow cytometry, B5 and E11 mAbs both strongly and specifically labeled DLA-DR antigens on the surface of cell lines and neoplastic cells of both B, T and mixed B/T immunophenotypes but not of normal peripheral blood leukocytes. The ELISA test displayed over 76% sensitivity and 100% specificity for CL detection (n=43). LTFs were performed on a total of 11 fine needle aspirates from enlarged lymph nodes with an overall apparent sensitivity and specificity of 100%. The mAbs B5 and E11 exhibited direct and complement mediated cytotoxicity against CL cell lines in vitro.

Conclusions. This work presents preliminary evidence that the B5 and E11 mAbs are promising tools for development of rapid screening tests and experimental therapy of primary CL, and possibly other conditions manifesting an increase in the expression of DLA-DR antigen.
Identification and validation of novel and annotated LncRNAs in canine B-cell lymphoma by RNA-Seq

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Introduction. Molecular mechanisms of protein-coding genes leading to lymphoma development and driving the clinical outcome have partially explained the biology of canine B-cell lymphoma (cBCL). To expand the knowledge of noncoding molecules involved in cBCL, we performed an analysis to uncover and characterize both novel unannotated and annotated long non-coding RNAs (LncRNAs).

Materials and Methods. Using RNA-seq data obtained from cBCLs (50 DLBCLs, 7 FLs and 5 MZLs) and 11 normal lymph nodes (nLNs), we implemented a customized pipeline to detect and GO-functionally enrich novel LncRNAs. Different bioinformatics tools such as STAR for reads-alignment, StringTie for assembly, CuffCompare to generate the set of LncRNA candidates, FEELnc to assess the coding-potential (CPs) were included. Candidates were filtered based on the exon content and differential expression (Limma/EdgeR). Validation through a public available human (phs000235.v6.p1, SRP021509) and a cBCL transcriptome datasets (SRA059558) was performed.

Results. 1666 novel and 884 already annotated LncRNAs were identified as expressed in the 62 cBCLs and 11 nLNs. Interestingly, a total of 839 novel and 439 annotated LncRNAs were differentially expressed in DLBCL compared to nLNs. Only one LncRNA was differentially expressed when comparing DLBCL and MZL. To identify a possible mechanism causing this aberrant expression we compared LncRNAs with data obtained from DNA profiling. In DLBCL, the expression of 47 LncRNAs resulted to be correlated to a genomic gain whereas only one LncRNA was associated with genomic loss (FDR<0.05). Deeper investigations including subtyping of DLBCLs, co-expression and survival analysis were also performed. Validation showed that approx. 67% and 43% of the novel LncRNAs were found in common in the canine or human public dataset, respectively.

Conclusions. Our work, so far the most comprehensive analyses for LncRNAs in cBCL, provides the foundation for future investigations on biological functions and molecular mechanisms of the most significant LncRNAs and their application to the clinic.

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Introduction. Despite B-cell Lymphoma (BCL) is the most common canine hematological cancer, there is still a lack of knowledge on the molecular events contributing to its development and progression. Aim of this work was to investigate the epigenetic regulation of HOXD10, FGFR2 and ITIH5 genes, whose promoters were recently shown to be methylated, by genome-wide methylation profiling, in canine diffuse large BCL. To understand the role of methylation in silencing the aforementioned genes, the CLBL-1 cell line was treated with two hypomethylating drugs (HDs). Owing to the complexity of epigenetic mechanisms, histone deacetylase inhibitors (HDACis), alone or in combination with HDs, were also tested.

Materials and Methods. CLBL-1 cells were incubated with two HDs (azacytidine and decitabine), alone or in combination with HDACis (valproic acid, trichostatin and vorinostat), at concentrations corresponding to their IC₅₀ and IC₂₀ values (Alamar blue test), respectively. Then, gene methylation status and mRNA levels were measured using methyl sensitive PCR (MSP) and quantitative Real Time PCR (qPCR).

Results. MSP showed an overall decrease of gene methylation status following the incubation with both HDs; meantime, qPCR highlighted a reversion of gene expression, confirming a methylation-dependent gene silencing mechanism. Interestingly, a higher pattern of gene re-expression was observed following the exposure to HDs combined with HDACis (and, mostly, with valproic acid). Moreover, HDACis alone increased the expression of FGFR2, demonstrating the important contribution of histone deacetylation, above methylation, in the regulation of this gene.

Conclusions. Overall, this work shows the involvement of epigenetic mechanisms in the regulation of HOXD10, FGFR2 and ITIH5 gene expression in an in vitro model of canine BCL. In the future, functional studies will be performed to unveil the methylation-dependent mechanisms of gene silencing.

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Identification of possible candidates for myeloid-derived suppressor cells in canine lymphoma

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Introduction. Myeloid derived suppressor cells (MDSC) represent a heterogeneous population of not fully differentiated cells derived from myeloid progenitors which are generally divided into monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC). MDSC expand in the blood as well as tumour tissue of cancer patients. By exerting immunosuppressive effects, they are implicated in cancer immune evasion. The aim of the study was to identify and evaluate the expansion of putative MDSC in the blood of dogs with lymphoma using flow cytometry.

Materials and methods. Peripheral blood of dogs diagnosed with lymphoma (n=10) and healthy controls (n=10) were collected into K2-EDTA tubes during standard diagnostic procedures. For flow cytometry immunophenotyping, samples were disposed into flow tubes and labelled with a panel of monoclonal antibodies comprising anti-dog CD11b, MHC class II and anti-human CD14 (clone TÜK4). Controls included isotype controls when available or omission of the primary antibody. CD11b⁺CD14⁺MHCII⁻ cells were measured as the percentage of all CD11b⁺ cells gated.

Results. Dogs with lymphoma had a higher median value of CD11b⁺CD14⁺MHCII⁻ cells percentage in the blood comparing to healthy dogs (6.575%; SD=1.71, versus 1.84%; SD=0.5 respectively). The difference was statistically significant (Mann-Whitney U = 0; P = 0.0001).

Conclusions. While in humans and mice MDSC are extensively studied, there are only few reports on canine MDSC. The percentage of different MDSC subtypes in the blood may be significantly increased depending on the tumour type. CD11b⁺CD14⁺MHCII⁻ cell population may represent possible candidates for canine M-MDSC that expand in canine lymphoma, however their presence within the neoplastic tissues remains to be demonstrated. The phenotype might be consistent with that of the human M-MDSC subset (CD11b⁺CD14⁺HLA-DR⁻/loCD15⁻). However, as MDSC do not display any unique markers, their identity need to be confirmed basing on their suppressive activity towards T-cells.

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P06 Prevalence of Golden retrievers among dogs with lymphoma: preliminary data from a European study


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Introduction. Canine breeds are genetic clusters and thus provide good models for studies on disease genetic predisposition. Golden retrievers (GR) have been reported to have a high overall incidence of lymphomas (19%) and T zone lymphoma (TZL, 40%) with differences among geographical areas in the US (1, 2). Similar findings have been reported from Japan but not in European (EU) case series even though specific studies are still lacking. The aim of the present study was to investigate the prevalence of GR in a large case series of canine lymphomas from different EU countries, and to compare the prevalence of different subtypes with studies in extra-EU countries to explore the possibility of a different genetic predisposition in European GR.

Materials and Methods. In the context of the European Canine Lymphoma Network, signalment data on 1734 consecutive canine lymphomas collected from 9 European countries were retrospectively analysed. When subtypes were available, cases were further separated into three subgroups: 1) B-cell lymphoma, 2) T-cell lymphoma-high grade, 3) TZL. Odds ratios for the different lymphoma subgroups were calculated in comparison with the mixed breed population serving as control.

Results. The overall prevalence of GR in the lymphoma sample was 5.19% (range 1.59-7.32%). The prevalence of GR slightly varied among EU countries. No subgroup predilection was found in GR when compared with the mixed breed population.

Conclusions. Both the overall prevalence of GR and the lymphoma specific subgroup prevalence seem much lower in the EU lymphoma population studied than reported from the US and Japan. More studies are necessary to define if this may be related to a different genetic predisposition of EU GR breed.

References


Development and characterization of a novel anti-canine CD20 monoclonal antibody

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Introduction. Lymphomas are among the most common malignant tumors of dog and most of the canine lymphomas originate from transformed B-cells. In humans, immunotherapy with anti-CD20 monoclonal antibodies has improved outcomes of patients with B-cell malignancies. However, rituximab and other currently available anti-human or anti-mouse CD20 antibodies do not bind native canine CD20, and thus cannot be used as tools for immunotherapy in dogs. Therefore, dog-specific antibodies are needed to develop immunotherapy approaches to treat canine B-cell lymphoma.

Materials and Methods. Anti-canine CD20 antibody was developed and provided by Moravian Biotechnology (Czech Republic). The peptide from canine CD20 extracellular domain was used for immunization to generate mouse monoclonal antibodies. The canine B-cell lymphoma cell line CLBL1, human diffuse large B-cell lymphoma cell line SUDHL4 and primary canine B-cells were used for antibody characterization with immunoblotting, flow cytometry, phage display and cell viability assays.

Results. We developed a novel anti-canine CD20 monoclonal antibody designated as NCD-1.2. This antibody recognizes the extracellular domain of canine CD20 and shows high-affinity binding to denatured canine CD20 protein on the membrane as well as to its native conformation on canine B-cells and canine lymphoma cells. NCD-1.2 uniquely binds to canine B-cells and this specificity was confirmed with epitope mapping using phage display. NCD-1.2 did not induce direct cytotoxicity or complement dependent cytotoxicity in vitro.

Conclusions. In summary, we have established a novel anti-canine CD20 monoclonal antibody that might be useful as a diagnostic tool to phenotype B-cells. Whether NCD-1.2 has a therapeutic potential (for example as an antibody-drug conjugate) to treat dogs with B-cell disorders still needs to be determined.

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P08 Archival biopsies of canine lymphoma may be used for biologically meaningful molecular classification

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Introduction. The prognostic relevance of the activated B-cell-like (ABC) and germinal centre B-cell-like (GBC) subtype classification of human diffuse large B-cell lymphoma (BCL) was established by multiple gene expression profiling (GEP) studies involving hundreds of surgical lymph node biopsies. Recent investigations that demonstrated the prognostic significance of canine lymphoma GEP subtypes, and the existence of canine BCL ABC and GCB-like subtypes, featured ‘fresh’/‘freshly frozen’ biopsies. Large-scale studies necessary to validate the prognostic relevance of the ABC/GCB classification, or other GEP classifiers, are hindered by the scarcity of surgical BCL biopsies. In this preliminary investigation we tested the hypothesis that global GEP of formalin-fixed, paraffin-embedded (FFPE) canine lymphoma biopsies will yield biologically meaningful information by evaluating whether canine BCL and T-cell lymphoma (TCL) could be distinguished by FFPE biopsy GEP.

Materials and Methods. Total RNA was isolated from FFPE biopsies of 35 BCL and 7 TCL (confirmed by CD79a and CD3 immunohistochemistry), collected for histopathology (2008-2013), and processed for hybridisation to Canine Gene 1.0 ST Arrays (Affymetrix). Probe-level background correction, quantile normalisation, log transformation and signal summarisation were performed using RMA, and outlier arrays identified by ‘consensus’ using 2 Bioconductor packages and ‘sample quality’ metrics.

Results. One outlier TCL was excluded, and unsupervised hierarchical clustering (HC) of 41 tumours performed using probe set data selected by various criteria. HC using 1000 probe sets with the highest variance stratified the tumours into 3 groups, the smallest comprising 5 of 6 TCL. Genes statistically significantly differentially expressed between 2 BCL clusters (comprising 34/35 BCL) were enriched for members of the B-cell receptor signalling pathway, the expression of which delineates human diffuse BCL ABC/GCB subtypes.

Conclusions. GEP of FFPE canine lymphoma biopsies supports the existence of ABC and GCB-like BCL subtypes, and demonstrates that archival biopsies may be used for biologically-relevant GEP-based lymphoma classification.
**P09 Significance of cell size evaluated by flow cytometry in canine lymphoma**

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**Introduction.** Description of lymphoma cell size represents an important feature in flow cytometry (FC). Nevertheless, this parameter does not seem to have clinical relevance in dogs. We hypothesized that FC identification of large cells is indicative of an aggressive or high-grade subtype of lymphoma.

**Materials and Methods.** Fifty-eight canine lymphomas diagnosed by cytology, histology, immunohistochemistry and FC were retrospectively evaluated. Cases were identified as indolent or aggressive and as low or high-grade based on the WHO histological and Kiel updated classifications, respectively. FC plots were reviewed and a marker was set on forward scatter using circulating lymphocytes as reference. Neoplastic cells were described as small or large if >50% of the events were located at the left or right side of the marker, respectively. Association between cell size (small vs. large) and histological (indolent vs. aggressive) or cytological (low vs. high-grade) categories was evaluated.

**Results.** Thirty-four of 46 (74%) large cell lymphomas were classified as aggressive, whereas 8/12 (67%) small cell lymphomas were indolent. The association between FC size and WHO subtype was significant (p=0.008; Pearson’s χ²). Four aggressive lymphomas showed FC small size. Ten of 11 indolent marginal zone lymphomas (MZL) had FC large size and were classified as late stage MZL by histology. Nine of 11 MZLs were described as centroblastic or immunoblastic (high-grade) by cytology. The association between cell size and category was higher for Kiel subtype (p=0.005; Pearson’s χ²) than for histotype.

**Conclusions.** FC description of large and small lymphoma cell size is indicative of aggressive and indolent WHO subtype, and high and low-grade Kiel subtype, respectively. However, concomitant FC and histological evaluation should be encouraged considering the relative high number of discordant cases. Large cell size MZL might reflect a late stage of the disease and its classification as aggressive lymphoma should be considered. Prospective clinical studies comparing outcome are warranted.
If you are interested to join the European Canine Lymphoma Network you are invited to register to the European Canine Lymphoma Network. Thank you!

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3rd Meeting of the European Canine Lymphoma Group
New Developments in Canine Lymphoma

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A satellite workshop to the 14-ICML