IMMUNE PROFILING OF DOGS WITH BIOLOGICALLY AGGRESSIVE HEMANGIOSARCOMA IN THE AGE OF IMMUNOTHERAPY

Scientific background

Cancer is the leading cause of death in dogs worldwide. Every year approximately 4 million dogs will be diagnosed with some form of cancer, and 50% of all dogs over the age of 10 will die as a result of developing the disease.
Canine hemangiosarcoma (HSA) is a malignant tumor arising from endothelial cells and it is ultimately fatal. Despite advances in treatment, dogs with visceral or cardiac HSA still have a poor prognosis and the disease is almost always incurable. Even in dogs with apparently localized disease, HSA cannot be cured by local therapy and adjuvant chemotherapy, and disseminated relapses inevitably occur after treatment. Reported median survival times after surgery and adjuvant doxorubicin-based chemotherapy range from 3 to 12 months, depending on the metastatic status at admission.

In human beings and dogs with cancer, immunosuppressive CD4+CD25(hi)FoxP3+ T cells (T(reg) cells) have been found overrepresented in the peripheral blood and/or tumor environment of various tumor types, being capable of suppressing protective antitumor T-cell immune responses.
As a consequence, promoting the expansion of Treg cells appears to be a mechanism by which tumor cells restrain the function of T-cells and escape autologous immune responses.
Canine HSA is thought to derive from a bone marrow (BM) pluripotent cell of origin expressing markers shared by endothelial and hematopoietic progenitor cells (CD34, CD117, and CD133), thereby providing the rationale to identify their presence in BM and peripheral blood (PB) and distinguish them from normal white blood cells.
Due to the poor prognosis of dogs with HSA and the high metastatic rate, it is plausible that small numbers of circulating tumor cells must be present at diagnosis even in dogs with early and localized stages of the disease.
It is well documented that the control of tumor development and growth by the immune system has been shown to be orchestrated by the elimination, equilibrium, and escape phases. Advances in the knowledge of cancer immunology have led to an unprecedented clinical development of immunotherapy with, for the first time, a documented improvement for survival in human beings with various cancer. The ultimate scope of immunotherapy is to re-educate the patient's immune system to fight the disease.
Recently, immunotherapy has been advocated as a novel and promising treatment in dogs with HSA with a lower risk for adverse events compared with chemotherapy. In fact, HSAs have a number of characteristics that render them amenable to novel immune-based therapeutic strategies.
Unfortunately, only a few clinical trials have been published so far, putting this new treatment approach at an embryonic stage, mainly due to financial issues.

The current research accomplishes three goals:

1) To give a better insight into the immune milieu of dogs with biologically aggressive HSA
2) To document tumor perfusion changes during treatment
3) To explore the therapeutic role of active immunotherapy, possibly having translational relevance

To develop the project, the aforementioned activities will be split among three Units at the Department of Veterinary Medical Sciences (DIMEVET), *Alma Mater Studiorum*- University of Bologna. All Units will work in strict collaboration. Specific details are described below.

UNIT 1: Clinical pathology service (CLINLAB)

The principal aim of Unit 1 will be devoted to investigate the immunological profile of dogs with biologically aggressive HSA.
In human patients with various cancers, including solid tumors, tumor-reactive memory CD8+ T-cells have been found to be enriched in their BM. Cytotoxic effector T-cells coexisting with Treg cells in the BM micromilieu may shift the balance toward antitumor immune control. Because of the metastatic properties of BM-resident tumor cell subsets and the possible preferential location of long-term persisting tumor cells in this compartment, these T cells may have special relevance for the control of disseminated minimal residual disease.
The first phase of the current research is aimed at 1) identifying by means of flow cytometry the HSA progenitor cells expressing CD34, CD117, and CD133 in PB and BM, and correlating them to clinical aggressiveness, and 2) investigating by means of flow cytometry the pattern of immune cell subset distribution including NK cells, gammadelta T cells, memory CD8+ and CD4+ T cells as well as T cells with regulatory phenotype (T(reg) cells) in PB and BM obtained at diagnosis, at the end of treatment, and at relapse from dogs with visceral or cardiac HSA receiving treatment, and to associate them with outcome data.
We hypothesize that BM-resident T-reg cells may contribute to a permissive milieu for immune escape of HSA, possibly leading to the development of metastatic disease. Conversely, the existence of a CD8+ memory T-cell compartment may be critically important in preventing tumor progression and metastasis.
We also hypothesize that dogs responding to antitumoral treatment will show a reduction of BM and PB T-reg cells, and an increase of CD8+ memory T-cells, thereby offering a minimally invasive procedure to monitor treatment response and to possibly predict early cancer recurrence.

UNIT 2: Diagnostic imaging/radiology service (SDIMM)

The principal aim of Unit 2 will be devoted to investigate the tumor perfusion changes during treatment.
The recently introduced contrast-enhanced ultrasound (CEUS) demonstrated to be an extremely valuable tool in oncology. In the case of HSA, CEUS is particularly useful in identifying both vascular and parenchymatous tumoral patterns. Nevertheless, vascular changes produced by the treatment have never been documented in dogs with HSA.
In general, response assessment after therapy in solid cancer has always used radiological imaging techniques, with tumor size reduction representing a presumed therapeutic efficacy. However, with the introduction of immunotherapy, the evaluation of tumor size has become unsuitable because some tumors, under treatment, show only tumor perfusion changes rather than lesion shrinkage.
We hypothesize that CEUS may be used to noninvasively dynamically, and safely detect the microcirculation patterns of canine HSA and to monitor therapeutic response after treatment.
Since new treatment approaches are becoming available to canine cancer patients, a proper evaluation of tumor response is very important in the achievement of therapeutic decisions.

UNIT 3: Internal Medicine/Oncology Unit (SMI-Oncology)

The principal aim of Unit 3 will be devoted to the clinical management of dogs with biologically aggressive HSA, including enrolment, staging, treatment and follow-up, as delineated below.
Newly-diagnosed dogs with biologically aggressive HSA will be completely staged by means of total body CT and the vascular pattern of the primary and metastatic cancer evaluated by CEUS. At admission, peripheral blood and marrow samples will be collected for the flow cytometric analysis.

One week after surgical removal of the tumor, dogs will be treated with 4 cycles of IV doxorubicin (@ 30 mg/m2 every 21 days). One week after the third chemotherapy administration, dogs will be restaged and, in case of no metastatic disease, they will receive a heterologous vaccine. Otherwise, they will be switched to metronomic therapy (thalidomide, cyclophosphamide, piroxicam).
The vaccine production will be carried out in Humanitas (Milan) following the procedure also described in the manuscript currently under review "Identification of a new class of immunogenic peptides derived from unconventional ER stress responses (ERStrePs)"; submitted to Immunity. Briefly, primary laboratory-grown canine hemangiosarcoma cells are expanded and infected with a Salmonella Thyphi (Ty21a) vaccine strain to increase the expression of membrane proteins Cx43 and secrete immunogenic peptides into their culture medium via hemi- channels. The cell medium thus enriched in peptides is subjected to a 0.22 μm filter which guarantees its sterility and finally freeze-dried. Each single dose of the vaccine corresponds to the peptides derived from 2x106 cells. From the data obtained from the clinical protocol performed on canine osteosarcoma and sarcoma patients, heterologous vaccinations with vaccine derived from cells of a comparable tumor donor have been shown to be effective in inducing an antitumor immune response, which in some cases has also proved to be higher than that induced by autologous vaccines. For this reason, the vaccine for all hemangiosarcoma patients who will be enrolled will be derived from the cells of a single donor. This choice has two other advantages. The first is that the vaccine is already accessible to all patients without waiting; the second is that the uniformity of the vaccine source will allow to make more reliable considerations on the efficacy or otherwise of the proposed immunotherapeutic strategy without introducing variability in the protocol.
This vaccine has already received the ethical approvement from the COBA at the University of Bologna for the treatment of dogs with another type on cancer, specifically melanoma (Prot. 259495, 9/11/2020).
At the end of treatment and at relapse and/or disease progression, peripheral blood and marrow samples will be sampled again for flow cytometry analysis.
Treatment response will be documented by means of thoracic radiographs, abdominal ultrasound and CEUS carried out on a monthly basis during treatment.
Follow-up re-staging consisting of thoracic radiographs and abdominal ultrasound will be performed 1 month after the end of the protocol for 3 months and every 3 months afterwards.
Toxicity resulting from treatment will be assessed based on the dog's history, physical examination, complete blood count, biochemical profile and urinalysis performed at each treatment session, as stated by the Veterinary Co-operative Oncology Group.
Endpoints of the current research are time to metastasis, time to progression, overall survival, hemangiosarcoma specific survival, and safety. Outcome data will be correlated to the dogs' immunologic profile and CEUS vascular pattern.

Possible application potentialities and scientific and/or technological and/or social and/or economic impact of the project

Angiosarcoma is an extremely aggressive cancer in human beings, although rare, accounting for 0.01%–0.1% of all cancers. Conversely, HSA is common in dogs, yet inexorably fatal regardless of treatment.
The current research represents an opportunity to investigate new methods of stratification, apply immune-system biomarkers, and evaluate serial vascular pattern that have never been considered before in veterinary oncology. Additionally, it will help obtaining new data on the immune system interference in HSA to better tailor therapy protocols. Likewise, it will explore a new treatment approach (active immunotherapy), possibly improving outcome.
If this goal is achieved, then future immunotherapeutic strategies will have to adequately consider the regulatory milieu within areas of HSA-immune interactions by relying on efficient in vivo depletion of Treg cells.

Finally, the results of the present project will definitely consolidate the role of the dog both in veterinary and comparative oncology to test new therapies.

Project time frame (3 years)

	1st semester	2nd semester	3rd semester	4th semester	5th semester	6th semester
UNIT 1 CLINLAB	Validation panel (flow cytometry)	Sample analysis (flow cytometry)	Sample analysis (flow cytometry)	Sample analysis (flow cytometry)		Integration and interpretation of results
UNIT 2 SDIMM	Validation panel (CEUS)	Serial monitoring of enrolled patients	Serial monitoring of enrolled patients	Serial monitoring of enrolled patients	Serial monitoring of enrolled patients	Integration and interpretation of results
UNIT 3 SMI-Oncology		Enrolment, sample collection, treatment	Enrolment, sample collection, treatment, follow-up of already enrolled cases	Enrolment, sample collection, treatment, follow-up of already enrolled cases	Follow-up of enrolled cases	Integration and interpretation of results

Selected references

- Baxter PJ. The British hepatic angiosarcoma register. Environ Health Perspect. 1981 Oct;41:115-6.
- Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004 Sep;10(9):942-9.
- Del Prete M, Di Sarno A, Modica R, Lassandro F, Giorgio A, Bianco A, Muto M, Gasperi M, Del Prete F, Colao A, Montesarchio V, Faggiano A; ENETS Centre of Excellence Multidisciplinary Group for Neuroendocrine Tumors in Naples (Italy). Role of contrast-enhanced ultrasound to define prognosis and predict response to biotherapy in pancreatic neuroendocrine tumors. J Endocrinol Invest. 2017 Dec;40(12):1373-1380.
- Falk H, Herbert J, Crowley S, Ishak KG, Thomas LB, Popper H, Caldwell GG. Epidemiology of hepatic angiosarcoma in the United States: 1964-1974. Environ Health Perspect. 1981 Oct;41:107-13.
- Finotello R, Stefanello D, Zini E, Marconato L. Comparison of doxorubicin-cyclophosphamide with doxorubicin-dacarbazine for the adjuvant treatment of canine hemangiosarcoma. Vet Comp Oncol. 2017 Mar;15(1):25-35.
- Finotello R, Henriques J, Sabattini S, Stefanello D, Felisberto R, Pizzoni S, Ferrari R, Marconato L. A retrospective analysis of chemotherapy switch suggests improved outcome in surgically removed, biologically aggressive canine haemangiosarcoma. Vet Comp Oncol. 2017 Jun;15(2):493-503.
- Gorden BH, Kim JH, Sarver AL, Frantz AM, Breen M, Lindblad-Toh K, O'Brien TD, Sharkey LC, Modiano JF, Dickerson EB. Identification of three molecular and functional subtypes in canine hemangiosarcoma through gene expression profiling and progenitor cell characterization. Am J Pathol. 2014 Apr;184(4):985-995.
- Konduri V, Halpert MM, Baig YC, Coronado R, Rodgers JR, Levitt JM, Cerroni B, Piscoya S, Wilson N, DiBernardi L, Omarbekov Z, Seelhoff L, Ravi V, Douglass L, Decker WK. Dendritic cell vaccination plus low-dose doxorubicin for the treatment of spontaneous canine hemangiosarcoma. Cancer Gene Ther. 2019 Sep;26(9-10):282-291.
- Lamerato-Kozicki AR, Helm KM, Jubala CM, Cutter GC, Modiano JF. Canine hemangiosarcoma originates from hematopoietic precursors with potential for endothelial differentiation. Exp Hematol. 2006 Jul;34(7):870-8.
- Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol. 2002 Sep 1;169(5):2756-61.
- Lucroy MD, Clauson RM, Suckow MA, El-Tayyeb F, Kalinauskas A. Evaluation of an autologous cancer vaccine for the treatment of metastatic canine hemangiosarcoma: a preliminary study. BMC Vet Res. 2020 Nov 18;16(1):447.
- Marconato L, Chalfon C, Finotello R, Polton G, Vasconi ME, Annoni M, Stefanello D, Mesto P, Capitani O, Agnoli C, Amati M, Sabattini S. Adjuvant anthracycline-based vs metronomic chemotherapy vs no medical treatment for dogs with metastatic splenic hemangiosarcoma: A multi-institutional retrospective study of the Italian Society of Veterinary Oncology. Vet Comp Oncol. 2019 Dec;17(4):537-544.
- O'Neill K, Guth A, Biller B, Elmslie R, Dow S. Changes in regulatory T cells in dogs with cancer and associations with tumor type. J Vet Intern Med. 2009 Jul-Aug;23(4):875-81.
- Pinheiro DY, Garden OA, Lara A, Stell A. Investigating the pathogenic role of regulatory T cells in canine B cell lymphoma. Vet Rec. 2011 Feb 19;168(7):196.
- Pinheiro D, Chang YM, Bryant H, Szladovits B, Dalessandri T, Davison LJ, Yallop E, Mills E, Leo C, Lara A, Stell A, Polton G, Garden OA. Dissecting the regulatory microenvironment of a large animal model of non-Hodgkin lymphoma: evidence of a negative prognostic impact of FOXP3+ T cells in canine B cell lymphoma. PLoS One. 2014 Aug 13;9(8):e105027.
- Rissetto KC, Rindt H, Selting KA, Villamil JA, Henry CJ, Reinero CR. Cloning and expression of canine CD25 for validation of an anti-human CD25 antibody to compare T regulatory lymphocytes

in healthy dogs and dogs with osteosarcoma. Vet Immunol Immunopathol. 2010 May 15;135(1-2):137-145.
- U'Ren LW, Biller BJ, Elmslie RE, Thamm DH, Dow SW. Evaluation of a novel tumor vaccine in dogs with hemangiosarcoma. J Vet Intern Med. 2007 Jan-Feb;21(1):113-20.
- Vail DM, MacEwen EG, Kurzman ID, Dubielzig RR, Helfand SC, Kisseberth WC, London CA, Obradovich JE, Madewell BR, Rodriguez CO Jr, et al. Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine adjuvant immunotherapy for splenic hemangiosarcoma in the dog: a randomized multi-institutional clinical trial. Clin Cancer Res. 1995 Oct;1(10):1165-70.
- Viguier M, Lemaître F, Verola O, Cho MS, Gorochov G, Dubertret L, Bachelez H, Kourilsky P, Ferradini L. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol. 2004 Jul 15;173(2):1444-53.