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TITLE: A Novel Approach Combining Oncolytic Virotherapy and Dual Immune Checkpoint Blockade for Metastatic Osteosarcoma

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14. ABSTRACT Osteosarcoma is one of the most common cancers of children and adolescents and young adults (AYAs). The outcomes for with metastatic osteosarcoma are especially dismal because there are no effective options to treat this condition. Our objectives are to tilt the immune landscape of metastatic osteosarcoma from immunosuppression to immunoreactivity, and to generate pre-clinical support to translate this innovative immunotherapy for the treatment of children and AYAs with metastatic bone cancer. To achieve this, we propose to combine an oncolytic virus, which will kill tumor cells and activate the immune system in the tumor environment, with a drug that blocks the myeloid checkpoint to enhance phagocytosis and antigen presentation as well as the immune exhaustion checkpoint to enhance tumor killing by T cells and NK cells. We will define optimal combinations in lab animal models and subsequently use these data to translate the approach into a therapy for dogs with naturally occurring metastatic osteosarcoma, which will serve as a clinically realistic model in the pathway towards applying this therapy to treat children and AYAs with metastatic osteosarcoma.					
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1. Introduction

Osteosarcoma is one of the most common cancers of children and adolescents and young adults (AYAs). In particular, the outcomes for patients with metastatic disease are dismal because there are no effective options for this condition. We have recognized that the mutational profiles in osteosarcoma create fertile environments for immunotherapy, and that the immune response is an important determinant of disease progression. These discoveries are foundational components of this project. In particular, immunotherapy is an appealing modality to treat metastatic cancer not only because tumors that are resistant to conventional treatment, such as radiation and chemotherapy, can be attacked with immunologic approaches, but also because this modality can reach tumors that are inaccessible for conventional treatments. For osteosarcoma, the inaccessibility of metastasis is a major factor that influences prognosis. Our recent work shows that osteosarcomas segregate into distinct groups with “immunologically silent” and “immunologically aware” tumors¹. Patients with immunologically aware tumors have significantly better outcomes than patients with immunologically silent tumors. However, rationally designed immunotherapies have yet to be tested in the setting of metastatic osteosarcoma.

Immunotherapies, particularly checkpoint inhibitors (CPIs), designed to reactivate suppressed immune cells against tumors has revolutionized the management of patients with advanced melanoma and lung cancer, and they represent promising treatment approaches for many other cancers. But despite the success in these areas, many cancers are unresponsive to immune checkpoint blockade. Even among patients with cancers that are approved indications for immune checkpoint blockade, many patients are unresponsive to - or relapse after treatment. Immunotherapy resistance has been attributed to a exclusion or dearth of immune cells in the tumor microenvironment (TME). Oncolytic viruses are an emerging form of immunotherapy designed to selectively kill tumor cells and induce an inflammatory immune response in tumors. Preclinical studies using the oncolytic Vesicular stomatitis virus (VSV) that we are employing in this project can be administered systemically to reach and selectively replicate in tumor tissues leading to their destruction and consequent recruitment and activation of immune cells to the TME^{2,3}. We hypothesize that combining systemic VSV therapy with CPI therapy can induce a potent and durable antitumor immune response that delivers clinical benefit in the treatment of metastatic osteosarcoma.

There are many challenges associated with the investigation of novel immunotherapies, and particularly combinatorial approaches. These difficulties are compounded in childhood cancers, and in particular in osteosarcoma which is a very rare tumor. We have thus learned about pathogenesis of this disease using conventional laboratory animal models (mice), but these models have limitations for therapy development. Studying spontaneous dog cancers as models to develop new therapies has gained favor due to the similarities in incidence and natural history of these diseases. Dogs are treated with similar protocols and dosing schedules as humans, providing a clinically realistic setting for drug development in a compressed timeline. Furthermore, canine clinical trials will almost always include animals of both sexes, allowing sex to be considered among the important biological variables. Biological samples to monitor safety, clinical toxicities, and local and systemic immune responses can be obtained from veterinary patients, so in addition to potential improvements in treatment modalities and dose schedules, veterinary trials also create opportunities to identify companion biomarkers to predict patient responses⁴.

This project is designed to begin to address the potential to reach and manage metastatic osteosarcoma lesions by combining oncolytic VSV with ONIx, a novel CPI dually targeted to activate the CD47:SIRP α and PD-1:PD-L1 pathways. Therapies that block the interaction of CD47 and SIRP α stimulate tumor cell phagocytosis, while inhibitors of the PD-1:PD-L1 pathway stimulate T-cell activation that work in concert to induce anti-tumor immune responses⁵. This combination therapy is being studied in dogs with metastatic osteosarcoma with correlative studies to identify and investigator biomarkers of response.

2. Keywords

Osteosarcoma, metastasis, tumor immunity, oncolytic virotherapy, vesicular stomatitis virus, immune checkpoints, CD47, PD-1, PD-L1, drug development, animal models.

3. Accomplishments

NOTE REGARDING NO COST-EXTENSION: ON 4/February/2021, the PIs received an email notice from DOD describing the concern shared by CDMRP about the impacts of COVID-19 on Service Members and the public, and specifically on the challenges it posed to ongoing research. In that email, we were asked to disclose significant COVID-19-related developments that would impact performance of this project.

On 25/May/2021, Dr. Modiano (contact PI) notified Dr. Senkevitch (Program Officer) that our laboratories had been operating at less than full capacity due to the COVID-19 responses adopted by our institutions, which were magnified by supply chain disruptions, which continue to this day, and by personnel departures.

On 7/March/2022, Dr. Modiano confirmed in emails to Dr. Senkevitch and to Jodi Cardoza (Grants Specialist) that a no-cost extension would be needed to complete the objectives of the project. Both responded with assurance that a no-cost extension would likely be awarded. On 23/June, Dr. Senkevitch notified the PI that a Year 2 Annual Report was due 9/30/22. At this time a DD882 would not be needed.

On 8/August/2022, the University of Minnesota Sponsored Programs Administration Officer (Brett Carlson) submitted a formal request on the PI's behalf to request a 1-year no-cost extension. The request was acknowledged by Ms. Cardoza, and the no-cost extension was subsequently approved.

This Technical Report is submitted to document progress from the previous 2-years following the milestones in the approved SOW.

- **What are the major goals of the project?**

The project has three specific aims:

Specific Aim 1: Optimize dose and schedule for combination VSV-IFN β -NIS and P-DIC (now called Onco-Immune Accelerator or ONIx) in mouse models of metastatic osteosarcoma.

Milestone: Project Specific Approvals by Local IACUC and IBC. Completed.

Milestone: Project Specific Approvals by ACURO. Completed.

Milestone: Production of VSV-IFN β - NIS for mouse studies. Completed.

Milestone: Production of P-DIC (ONIx). Completed.

Milestone: Cell authentication. Ongoing.

Milestone: Define toxicity of combination VSV-IFN β -NIS and P-DIC (ONIx). Ongoing.

Milestone: Define optimal dose for combination VSV-IFN β -NIS and P-DIC (ONIx). Ongoing.

Milestone: Complete necropsies. Ongoing.

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Ongoing.

Specific Aim 2: Define immunological effects and mechanism of action for combination VSV IFN β -NIS and P-DIC

Milestone: Assessment of immune response induced by VSV-IFN β -NIS and P-DIC (ONIx) in mice with metastatic osteosarcoma. Ongoing.

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Ongoing.

Specific Aim 3: Characterize safety, efficacy, and immunomodulatory effects of VSV-IFN β - NIS and P-DIC in dogs with naturally occurring metastatic osteosarcoma

Milestone: Production of in vivo grade VSV-IFN β -NIS for use in canine studies. Completed.

- Note, a new clinical-grade batch of VSV-IFN β -NIS was produced, and QC testing was completed in 2022 to ensure the test article being used in these studies was active.

Milestone: Production of in vivo grade P-DIC (ONIx). Completed.

Milestone: Identify safe dose to guide clinical development. Ongoing.

Milestone: Assessment of immune response induced by VSV-IFN β -NIS and P-DIC (ONIx) in dogs with metastatic osteosarcoma. Ongoing .

Milestone: Data analysis; organization and conclusions. Ongoing.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Ongoing.

Milestone: Planning for pediatric clinical trial planning (translation to humans). Ongoing.

- **What was accomplished under these goals?**

Milestone: Project Specific Approvals by Local IACUC and IBC. Completed.

University of Minnesota IACUC protocol 2004-38033A was approved on July 20, 2020. The three-year renewal for University of Minnesota IBC protocol (new number assigned, 2106-39146H) was approved on October 22, 2021 (previous IBC approval date 1805-35967H).

Mayo Clinic IACUC protocol A00005309-20 was approved on September 30, 2020

Milestone: Project Specific Approvals by ACURO. Completed.

ACURO approval for Proposal Number CA190276, Award Number W81XWH-20-1-0682 was obtained on November 20, 2020.

Protocols have been renewed and updated yearly and as needed to remain in compliance with animal welfare and proposed protocol modifications.

Milestone: Production of VSV-IFN β - NIS. Completed.

Production of a new batch of recombinant VSV-cIFN β -NIS for veterinary studies was completed in March 2022 using processes similar to those defined for GMP production of clinical grade VSV. Briefly, recombinant VSV-cIFN β -NIS was produced in a human suspension cell line and purified by tangential flow filtration. Following production, the new virus batch was subject to quality control (QC) testing including assays to confirm potency, purity, sterility, functional activity, and sequence identity. This new virus preparation is being used canine studies.

Table 1. testing and validation of VSV-cIFNβ-NIS batch 2022-01	
<i>Date of production</i>	03/25/2022
<i>Titer</i>	4.8x10 ¹⁰ TCID ₅₀ units/ml
<i>Total protein concentration</i>	6115 ug/ml
<i>Total DNA concentration</i>	8035 ng/ml
<i>Benzonase concentration</i>	<0.4ng/ml
<i>Endotoxin level</i>	2.6 EU/ml
<i>Sequence analysis</i>	completed
<i>VSV N gene</i>	4.23x10 ¹¹ copies/ml
<i>Sterility testing</i>	No growth after 14 days

Milestone: Production of P-DIC (ONIx). Completed.

We had a pre-existing batch of P-DIC (now called Oncoimmunology accelerator, or ONIx) that was sufficient for pilot studies (see below for experimental data using ONIx in pilot mouse studies). This product had been previously tested for sterility and potency, and the data were provided in the original grant application.

Production of ONIx for this project was delayed because of the response to the COVID-19 pandemic. The University of Minnesota maintained minimal operations from March 2020 until approx. May of 2021, primarily focused on development of COVID-19 tests, ventilators, and other procedures that aided the national response to this public health crisis. Laboratories and facilities, including the Molecular Cell Therapy Center, where drug production for ONIx was to take place, were closed as part of the need to avoid having people in close contact, as well as to divert all physical resources (pipettes, glassware, etc.) to COVID-related research, development, diagnosis, treatment, and response operations.

In May of 2021, PIs at the University of Minnesota were directed to submit individual plans to resume work under the umbrella of a University Health Sciences-wide “Sunrise Plan.” With few exceptions, the individual plans required staff to work in staggered shifts with a maximum occupancy in labs of 50%. As part of the implementation of that plan, Dr. Vallera was able to resume synthesis of ONIx and complete the batch for pre-clinical work (in mice and dogs). The production run was completed on May 27, 2021 and vialled for use in 3-mL vials at a concentration of 9 mg/mL (total yield = 3.1g). Purity of the product was confirmed by SDS-PAGE (Figure 1).

Sterility testing for ONIx was completed through IDEXX laboratories (See report as an appendix). Potency testing (measured as displacement of anti-CD47 and anti-PD-L1 antibodies from human A549 and canine CLBL-1 cells) was completed. We confirmed that ONIx binds canine CLBL-1 cells with comparable affinity to human A549 cells (Figure 2). We are using CD47-driven phagocytosis assays with mouse J774 cells as effectors and Raji lymphoma cells as targets to examine the impact of CD47 blockade by ONIx^{6,7}.

We requested a cell line from Dr. Lin at AstraZeneca that will allow us to measure the effect of ONIx to inhibit PD-1 signaling in a Jurkat indicator cell line *in vitro*, although we have not received a response⁸.

Reminder emails were sent to Dr. Lin and to the Oncology-Partnering group at AstraZeneca on September 12 and September 24, 2022. Samples from the canine study are being banked to evaluate ONIx pharmacokinetics using binding assays, phagocytosis and PD-1 signaling bioassays.

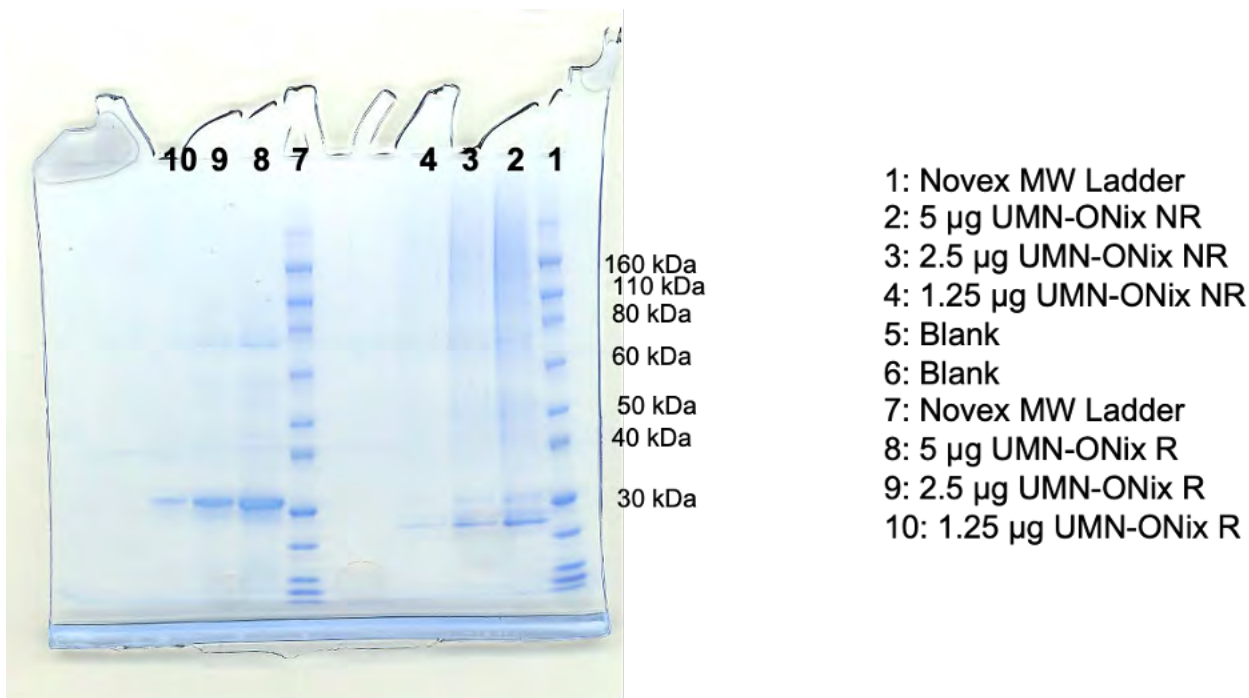


Figure 1. Purity of ONIx determined by gel electrophoresis. Coomassie blue-stained gel showing a titration of the final ONIx peptide under non-reducing

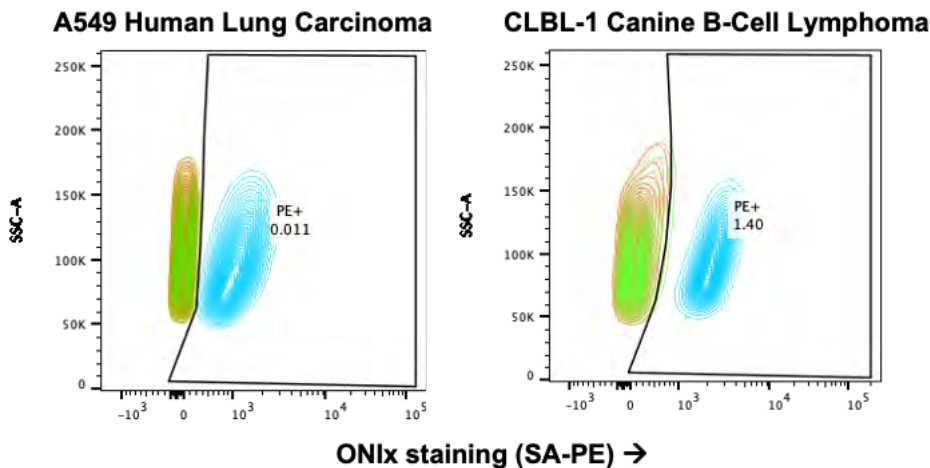


Figure 2. ONIx binds canine cells with approximately equal affinity as human cells. A549 human lung carcinoma cells and canine CLBL-2 B-cell lymphoma cells were stained with biotinylated ONIx alone (brown), streptavidin (SA) conjugated to phycoerythrin (PE) alone (green), or a combination of biotinylated ONIx and SA-PE (blue) and analyzed by flow cytometry. Two-dimensional contour plots show that ONIx staining is similar for both cell lines.

Milestone: Cell authentication. Ongoing.

The PI labs follow rigorous SOPs for cell authentication. Authentication of cell lines, including newly obtained K7M2 and F420 cell lines, has proceeded in approximately quarterly schedules and/or according to SOPs since labwork resumed for this project.

Milestone: Define toxicity of combination VSV-IFN β -NIS and P-DIC (ONIx). Ongoing.

To confirm the safety of VSV and ONIx combination therapy, a pilot study was carried out in the well-established A20 (Balb/c) murine lymphoma model. Mice with established A20 tumors were treated with

either PBS, VSV (1×10^8 TCID₅₀/mouse), ONIx (5mg/kg for 6 doses), or combination of VSV and ONIx. Mice were monitored for adverse events, including weight loss. The results show that all treatments, including combination therapy were well tolerated, with minimal transient weight loss and resumption of weight gain in mice at approximately the same rate as the controls (Figure 3.).

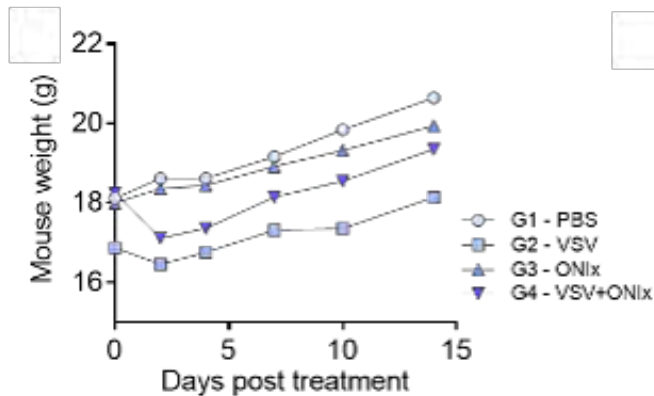


Figure 3. VSV and ONIx combination therapy is well tolerated in tumor bearing mice.

Mice were treated as described in the text above. ONIx treatment alone did not alter the course of weight gain in the mice. The VSV treatment group included smaller mice (see weight at Day 0) and the trend for weight gain was comparable to the controls. Mice treated with the combination showed transient weight loss, but then resumed weight gain at the same rate as the controls, despite continued treatment with ONIx.

Milestone: Define optimal dose for combination VSV-IFN β -NIS and P-DIC (ONIx). Ongoing.

We have learned that, despite published data, the K7M2 model (and its non-metastatic counterpart, K12) are both challenging to work with. Previous reports describe the models as reliable transplantable tumors in the orthotopic and experimental metastasis (intravenous, or iv) settings⁹⁻¹². As we have progressed through the project, however, we have tested K12 and K7M2 cells from various sources with limited success in establishing reproducible models of tumor growth and metastasis. Data regarding the success (or failure) rate for tumor take of these cells are largely unpublished, but investigators from two independent institutions have relayed to us (confidentially) that failure to form tumors is not a rare event in this model. Our efforts to develop the model are detailed below; but given the inconsistent performance of the K12 and K7M2 models, we have started working on alternative models to address this milestone.

We obtained K12 and K7M2 cells from the National Cancer Institute (NCI) for use in this project under a multi-institutional Materials Transfer Agreement. Neither cell line was modified with reporters for *in vivo* imaging or with a known antigen for immunological studies. Thus, our first step was to genetically edit the cells to express luciferase for *in vivo* tracking and ovalbumin to test antigen-specific immune responses. We generated a genetically edited K12 cell line incorporating chicken ovalbumin (OVA), as a target antigen for immune cells and Gaussia luciferase (Gluc) as a reporter for *in vivo* imaging using CRISPR-Cas9. Integration of the Gluc-OVA cassette in frame into the *Rosa26* locus was confirmed by sequencing, however, unlike MC17 cells which we previously modified using the same methodology¹³, the cells did not show significant luciferase activity *in vitro*, and certainly not enough for *in vivo* experiments (Figure 4).

The K12-17 cells also failed to form subcutaneous (sq) tumors in syngeneic Balb/c recipient mice when injected at 2×10^6 cells per mouse. Our attempts to introduce exogenous genes into K7M2 cells failed repeatedly. Thus, we obtained a K7M2 cell line that is stably transfected with firefly luciferase (K7M2-Luc) from Dr. Brock Lindsey at West Virginia University¹². It took several months to obtain these cells due to the challenges associated with the COVID-19 pandemic, as Dr. Lindsey's lab had to wait until they could return to the laboratory in-person to expand cells and ship them. We received K7M2 cells on February 10, 2021, expanded them, and submitted them for authentication and *Mycoplasma* testing to IDEXX BioResearch. The cells passed QC (authenticated as K7M2, were negative for all *Mycoplasma sp.*, and had robust luciferase activity). They also expanded *in vitro* with an estimated doubling time of 22-24 hours and had robust luciferase activity (Figure 5).

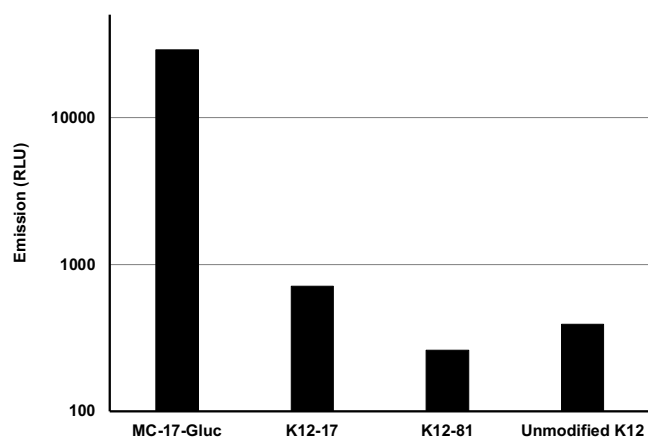


Figure 4. Gaussia Luciferase Activity in MC17 fibrosarcoma and K12 mouse osteosarcoma cells genetically edited to express the *Gluc* gene in the *Rosa 26* locus using CRISPR-Cas9.

Culture supernatants were harvested to analyze light emission upon the addition of coelenterazine substrate (Gaussia luciferase is secreted). Similar results were obtained from cell lysates, to ensure that lack of detectable activity in K12-Gluc clones 17 and 81 was not due to failure of secretion, as well as in live cultured cells imaged on an IVIS spectrum instrument. RLU = relative light units.

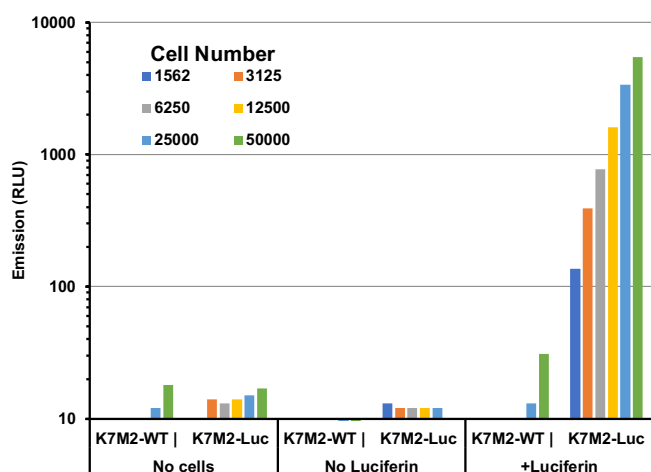


Figure 5. Firefly Luciferase Activity in K7M2-Luc cells transfected with the *Fluc* gene.

Wild type K7M2 cells (obtained from the NCI) and K7M2 cells transfected to express firefly luciferase (*Fluc*) were grown in culture and cell-associated luciferase expression was measured in lysates from 2-fold serial dilutions of 50,000 to 1,562 cells. Only the two highest concentrations were used for wild type cells. Controls included omitting cells (media + luciferin substrate) and omitting substrate (media + cell lysate, no luciferin). RLU = relative light units.

We had to wait several months to start the *in vivo* experiments, as the animal facilities at the University of Minnesota were also experiencing a severe shortage of caretaker staff during the COVID pandemic and PIs were asked to reduce the number and frequency of animal experiments, or not to start any new ones until further notice when staffing could be restored.

We finally were able to initiate up a titration experiment on May 10, 2022. Groups of three female Balb/c mice received 2.5×10^5 , 5×10^5 , or 1×10^6 K7M2-Luc cells by tail vein injection. One group of three Balb/c mice received 2×10^6 K7M2-Luc cells as a sq injection.

Viable cells were identified using *in vivo* imaging 24 hours after the injection (Figure 6). However, most of the activity in the mice receiving tail vein injections was seen in the spleen, with no luciferin conversion above background noted in the lung fields for any mouse. Viable cells were seen at the site of tumor implantation in all of the mice that received sq injections. Unlike what has been previously reported (see for example, reference¹⁰), we did not see any significant tracking of tumor cells to the lungs based on imaging over a 12-week experimental period. Rather, the luciferin-converting activity remained in the abdomen (anatomically, in the area where the spleen is located), showing peak activity at 24 hours, then waning progressively over the next eight weeks, and increasing again slightly in some mice between weeks 8-12 (Figures 6 and 7).

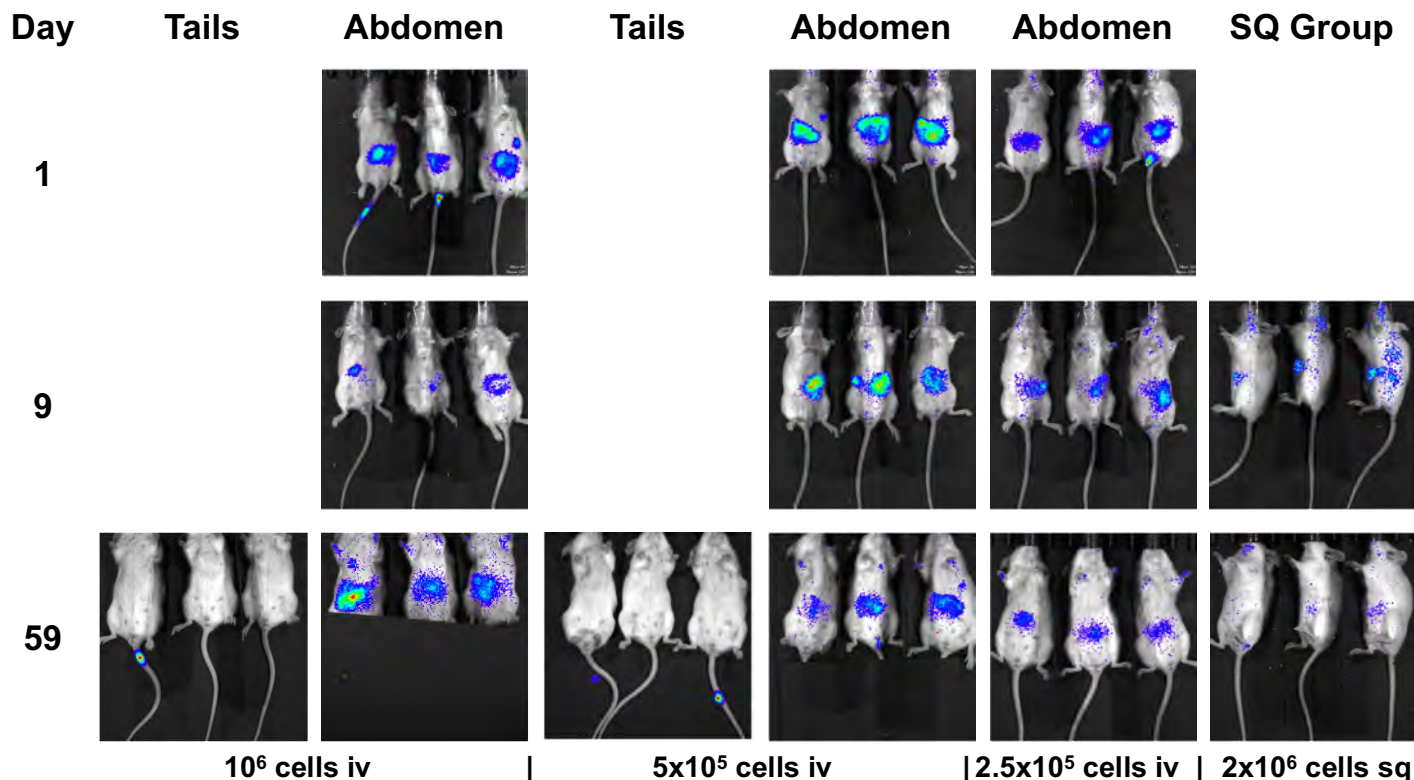


Figure 6. *In vivo* imaging by light emission in mice receiving tail vein or subcutaneous injections of K7M2-Luc cells.

Groups of three mice were injected with K7M2-Luc cells in the tail vein in 2-fold decrements (1×10^6 cells, 5×10^5 cells, and 2.5×10^5 cells). In this model, it is reported that iv injection of 1×10^6 cells results in 100% lung colonization and death (reaching endpoint) of the mice by approximately 2-3 weeks and 5×10^5 cells results in 100% lung colonization and death by approximately 3-4 weeks. Here, the data indicate that after iv injection, the cells distributed to the abdomen (almost certainly the spleen, based on the anatomical location of the luminescent signal) regardless of cell dose, and they remained in the spleen, where the signal eroded over the course of two months. Two mice (one each in the groups that received 1×10^6 and 5×10^5 cells) developed tumors in the tail vertebra near the site of injection, so imaging of the abdomen was also done masking the tail signal to appreciate the presence of cells in the spleen. The abdomens were similarly masked to image the lung fields (images not shown), but signals above background were not apparent without sensitive quantification using the instrument detector (see Figure 7). One group of three mice received 2×10^6 K7M2 cells subcutaneously (sq). A signal was detectable at the site of injection for approximately one week, but the signal eventually dissipated and returned to baseline.

Two mice that received K7M2 cells in the tail vein (one at the 1×10^6 cells dose and one at the 5×10^5 cells dose) developed tumors at the injection site, which were grossly evident approximately 11 weeks after inoculation. These tumors, which probably arose from cells that extravasated at the time of injection, grew slowly, did not impair the quality of life of the mice, and were readily apparent in the *in vivo* imaging studies. The potential extravasation did not impede systemic distribution of the cells through the vascular system, as there were no obvious differences in the whole body imaging for these mice compared to the other two mice in each of their groups. We terminated the experiment for futility after 12 weeks. At the end of the experiment, the mice were humanely sacrificed, and a complete necropsy was performed on each mouse. Necropsies were done systematically, starting with a complete examination of the abdomen for evidence of metastasis and collection of the spleen with gross examination for evidence of tumor growth. No other abdominal organs were collected, as there was no evidence of gross metastasis or tumor growth (luciferin conversion *in vivo*) in the abdomen outside of the spleen for any animal. The chest cavity was then carefully opened without perforating

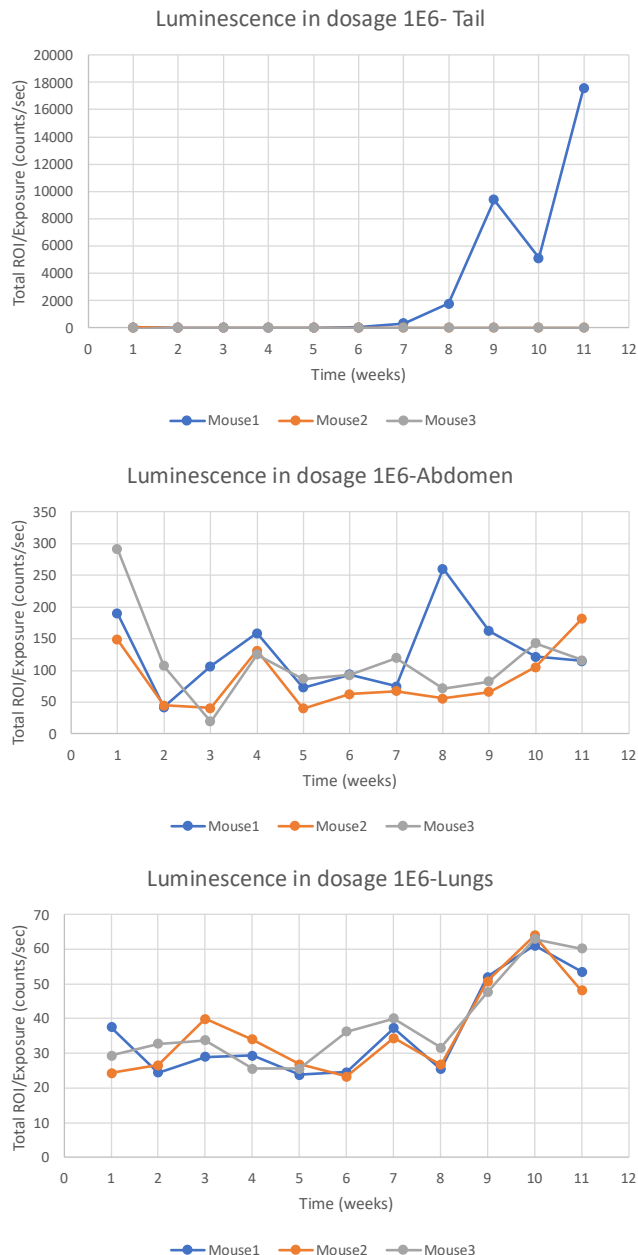


Figure 7. Illustration of quantitative light emission signals from Figure 6.

Signal quantification was determined using the *Living Image* software (Perkin Elmer). Graphs show luminescence data as counts per second over time for individual mice in the group receiving 1×10^6 cells iv. Note the relative scale of signals in the single mouse that developed a tail tumor (top), as compared to the signals in the abdomen (splens), which mostly eroded over time, and the signals in the lung, which increased slightly over background after week 8, but were still about two to three times lower than the signals in the abdomen. The data for mice receiving fewer cells showed similar patterns, with lower (although not proportionately lower) light emission values.

the diaphragm by removing the skin and carefully transecting the ribs at the left costochondral junctions. The lungs were allowed to deflate to examine the chest cavity for gross metastasis (none was noted). The trachea was transected above the mediastinal entry, and the lungs were gently inflated *in situ* by infusing 10% neutral buffered formalin through the tracheal transection. The inflated lungs were then removed from the chest cavity and examined for evidence of gross metastasis (a putative tumor measuring approximately 3 mm in diameter was identified in the single mouse that received 5×10^5 cells and developed a tumor in the tail, Figures 6 and 8). The tail tumors were collected from both mice in which they were grossly apparent (Figure 6).

No gross tumors were seen in any of the mice that received sq K7M2 cells, consistent with the observation that the luciferase activity in those mice had waned. A prominent sq fat pad was collected from one mouse to assess if there was evidence of a tumor remnant. The tissue collected consisted of skin, subcutis, multiple small quiescent mammary, glands and ducts, abundant adipose tissue and a lymph node. There were no significant abnormalities detected.

Histopathologic examination of the lungs, spleens, and tails showed that three mice (one in the group receiving 1×10^6 cells and two in the group receiving 5×10^5 cells) had microscopic evidence of tumors in the lungs (Figure 9). The tumors were similar in all cases (in the lungs, and in the two mice that had tumors in the tail), consisting of unencapsulated, moderately well-demarcated,

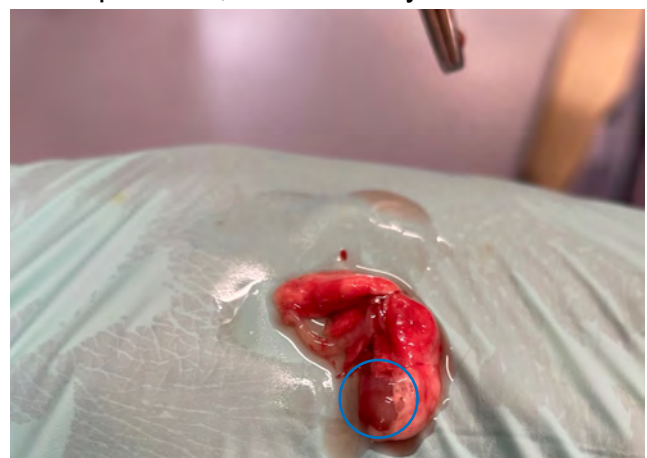


Figure 8. Image of mass in the lungs from mouse #4.

The approx. 3 mm mass is highlighted by the blue oval in the image.

densely cellular masses that were consistent with a diagnosis of sarcoma. The neoplastic cells were arranged into ill-defined broad sheets to poorly formed bundles and streams, and they were supported by a fine fibrovascular stroma. Rarely, between neoplastic cells, there were scant amounts of wispy, fibrillary, eosinophilic material (possible osteoid). The neoplastic cells were plump, spindle shaped, with indistinct cell borders, and they had large amounts of eosinophilic to amphophilic cytoplasm. The nuclei were round to oval with vesicular chromatin, and they had up to three prominent, magenta nucleoli. There was marked anisocytosis and anisokaryosis throughout the tumors.

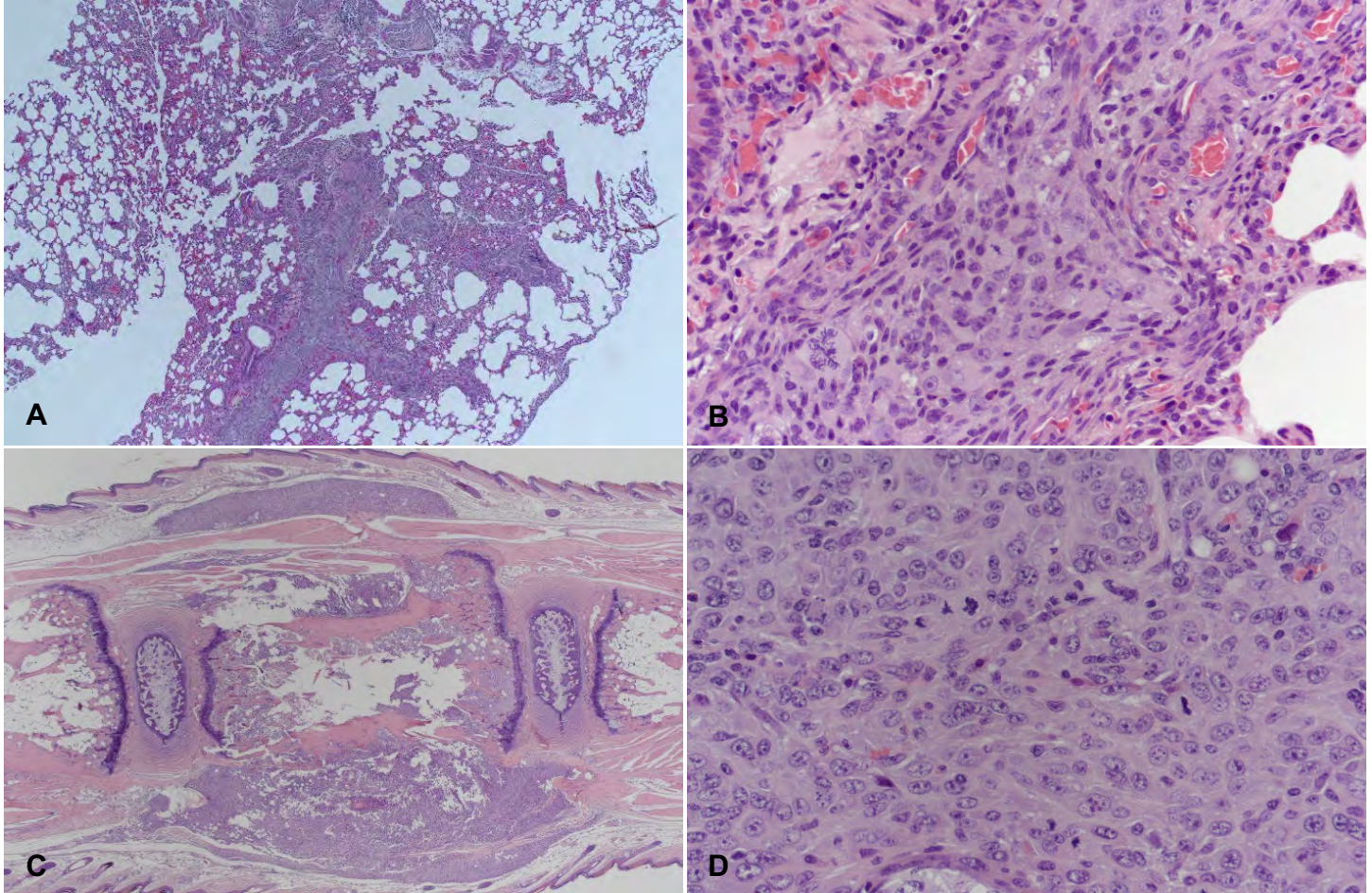


Figure 9. Photomicrographs showing histopathology of tumors in representative sections of the lungs and tail from mouse 3 (injected with 1×10^6 K7M2 cells iv).

A. Lungs, 40x (H&E). Multiple cross sections of vessels have tumor emboli. B. Lungs, 400x (H&E). Higher magnification of tumor emboli. Note multiple mitotic figures, some of which are bizarre. C. Tail, 20x (H&E). Surrounding and infiltrating a vertebra is a sarcoma. D. Tail, 400x (H&E). Higher magnification of the sarcoma. There are numerous mitotic figures, many of which are atypical.

The lungs of every mouse in the experiment also showed evidence of inflammation: there was diffuse, mild, alveolar vascular congestion, and mild increased cellularity of the alveolar septa, owing to increased large round mononuclear cells (macrophages) and small lymphocytes. Particularly at the edge of the sections, there were foci of atelectasis. The spleens showed diffuse, moderate congestion and extramedullary hematopoiesis, although a component of inflammation could not be excluded. The lung pathology was atypical and suggestive of an inflammatory or immune response which possibly eliminated the tumor cells or rejected an incipient tumor. The spleen pathology could be within the confines of what is normally observed for this strain of mouse (Balb/c), but it also could reflect inflammation or immune stimulation in response to the tumor cells that were retained in the spleen (presumably within the reticuloendothelial system). In particular, the fact that cells tracked to the spleen

first could be the cause of why a strong anti-tumor immune response might have been generated, as the spleen is a major immune organ in mice. It is interesting that two of the three mice that developed lung metastasis had tumors in the tail, as these tumors might have provided a source of metastatic cells (outside of the spleen). This would be consistent with the observed metastasis of K7M2 cells injected intratibially¹⁰⁻¹². A copy of the full pathology report is included as an appendix.

Nonetheless, it was clear from these experiments that the tail vein model of K7M2-Luc cells (at least using the cells from West Virginia University) will not be useful for our experiments. We thus obtained K7M2-Luc cells from a different source (Dr. Steve Dow, Colorado State University). We received these cells in August of 2022, but the viability was poor, and we have had limited success expanding them.

Upon our consultation with other osteosarcoma experts who have used the K7M2 model, we learned that it is not unusual for K7M2 cells to track to the spleen. But under conditions when the experiment works as intended, the cells will then appear in the lungs (either cells trapped in the lungs will grow out or cells growing in the spleen will migrate to the lungs). As noted above, published data state that when the experiment is successful, this should happen within about 2 weeks after a 1×10^6 cell injection into the tail vein, and within about 3 weeks after a 5×10^5 cell injection into the tail vein^{10,11}. The possible reasons for the unexpected results in this experiment, and observations to support or refute them, include:

1. *Poor cell viability*: this is unlikely. The cells were >99% viable when they were prepared for injection (based on trypan blue exclusion) and viable cells were detectable in the mice for several weeks based on luciferase expression (luciferin conversion).
2. *Aged or senescent cells*: Previous work indicates cells were used for *in vivo* experiments between passages 1 and 10^{10,11}. The cells we obtained from NCI, West Virginia University, and Colorado State University did not have information about passage number, so it is impossible for us to know the length of time cells were cultured before we received them. In our hands, cells were only passaged three to five times after thawing before mouse injections.
3. *Poor injection technique with cells exiting the vasculature*: This is possible, albeit unlikely. Two mice did show local growth of tumor cells at the injection site, suggesting at least some cells might have been deposited outside the vein. However, all the mice showed evidence of cells tracking to the spleen (Figures 6 and 7), and the luciferase activity was consistent for every mouse in each group with a predictable dose response (Figure 7), suggesting that all of the mice received approximately the intended dose of cells into the vasculature.
4. *The cells were rejected by the mouse hosts*: we believe this is the most likely possibility. As described in the results of the microscopic pathology examination above, there was evidence of inflammation in the lungs of the mice, also showing a dose response, and the spleens showed evidence of extramedullary hematopoiesis, or possibly inflammation. In addition, three mice had evidence of micrometastasis, indicating that the cells did have the potential to colonize the lungs. They just did not do so with the expected efficiency or frequency.

While we cannot be certain about the reason why the K7M2 cells might have been rejected by most of the mice in this experiment (both those receiving tail vein injections and those receiving sq injections), there are at least three possible explanations. One explanation is that there are subtle differences in Balb/c mice from different vendors. The tumor microbiome of mice purchased from different vendors has been shown to have important differences that mediate the baseline of inflammation and impact systemic immune responses^{14,15}. There are also subtle differences in genetic drift among colonies maintained at different locations and under different husbandry conditions, which, for example, significantly impact susceptibility to conditions such as epilepsy¹⁶. We obtained our mice from Jackson Labs. Other investigators report obtaining mice from Envigo or from the NCI. Another explanation is

that the luciferase transgene can act as a neoantigen in immunocompetent animals, enhancing the likelihood of immune rejection^{17,18}. Although this is not a universal phenomenon¹⁹, it seems to be an area of increasing concern among investigators using syngeneic, transplantable cancer models, to the extent that groups have developed mice that are tolerant to the immunodominant epitopes of common reporters such as green fluorescent protein (GFP) and luciferase²⁰. Finally, the K7M2 model also has been shown to express high levels of endogenous retrovirus sequences, such as gp70, which may initiate or enhance anti-tumor responses in this model²¹.

As noted above, the K12 and K7M2 tumors are syngeneic with Balb/c mice. In addition to their inconsistent growth *in vivo*, immunological reagents for Balb/c mice are more limited than those for other strains, such as C57B/6 (B6), creating yet another obstacle for use of these models. With this in mind, we obtained the F420 and the F331 murine osteosarcoma cell lines from Dr. Jason Yustein (Baylor University; now at Emory University). These cells were derived from tumors isolated from genetically engineered B6 mice, and they grow in syngeneic B6 hosts as local sq tumors as well as in the lungs after intraosseous or iv injection²². These tumor models appear to have the advantage of being amenable to genetic modification, while retaining their tumor-forming capacity.

We received cells on February 1, 2022, and so far, we have focused on growing the F420 model. A microsatellite profile (single tandem repeats) was generated for the cells at IDEXX BioResearch and the cells were shown to be negative for *Mycoplasma sp.* The cells grow reliably *in vitro*, and we have shown that they can be modified to express green fluorescent protein (GFP) and Luc transgenes via lentivirus transduction (approximately 5% efficiency in our initial experiments). We have obtained high-titer lentivirus encoding GFP-Luc to optimize the transduction conditions and sort for GFP-positive cells for *in vivo* inoculation into immunocompetent B6 hosts (experiments in progress). Unmodified F420 cells (injected sq) and GFP-Luc-positive cells (injected intravenously) will be the focus of our experiments to define the effects and optimal combinations of VSV-IFN β -NIS and ONIx in the mice.

Another approach we have taken is to develop the methylcholanthrene-17 (MC17) murine sarcoma model as an alternative to intravenous or sq osteosarcoma models. Although MC17 represents a fibrosarcoma, it is a reliable model for drug development, and it is especially recalcitrant to conventional immunotherapy (MC17 is a prototypical “cold tumor”). Yet, MC17 can respond to unconventional immunotherapies. For example, we have developed a molecule called eBAT, which consists of full-length human EGF linked to the amino terminal fragment (ATF) of human urokinase plasminogen-type activator (uPA) and to a *Pseudomonas* exotoxin (PE) modified to enhance its cytotoxicity and reduce its immunogenicity.

As part of our experiments to develop this model, we have shown that the drug, eBAT, has activity against a wide variety of sarcomas, effectively targeting sarcoma stem cells²³⁻²⁵. eBAT is a polypeptide ligand targeted toxin (LTT) that consists of full-length human epidermal growth factor (EGF) linked to the amino terminal fragment (ATF) of human urokinase plasminogen-type activator (uPA) and to a genetically modified *Pseudomonas* exotoxin (PE). eBAT also kills activated endothelial cells, potentially depleting or normalizing tumor vasculature²⁶. But most importantly for this discussion, eBAT remodels the immune and inflammatory TME by targeting urokinase receptor (uPAR)-expressing, immunosuppressive myeloid cells, potentially enhancing the immune response against tumors. We tested this premise using MC17 and a uPAR-knockout (KO) derivative of MC17 called D10, and a mouse-specific version of the drug eBAT, called meBAT that incorporates mouse uPA¹³. The binding affinity of meBAT to wild type (WT) MC17 cells, as determined by fluorescence intensity and the proportion of bound cells, was higher than that of eBAT, and binding of both eBAT and meBAT was reduced, although not fully abrogated in D10 cells (Figure 10). This suggests that eBAT and meBAT can likely bind cells by interacting with either or both uPAR and EGF receptors (EGFR).

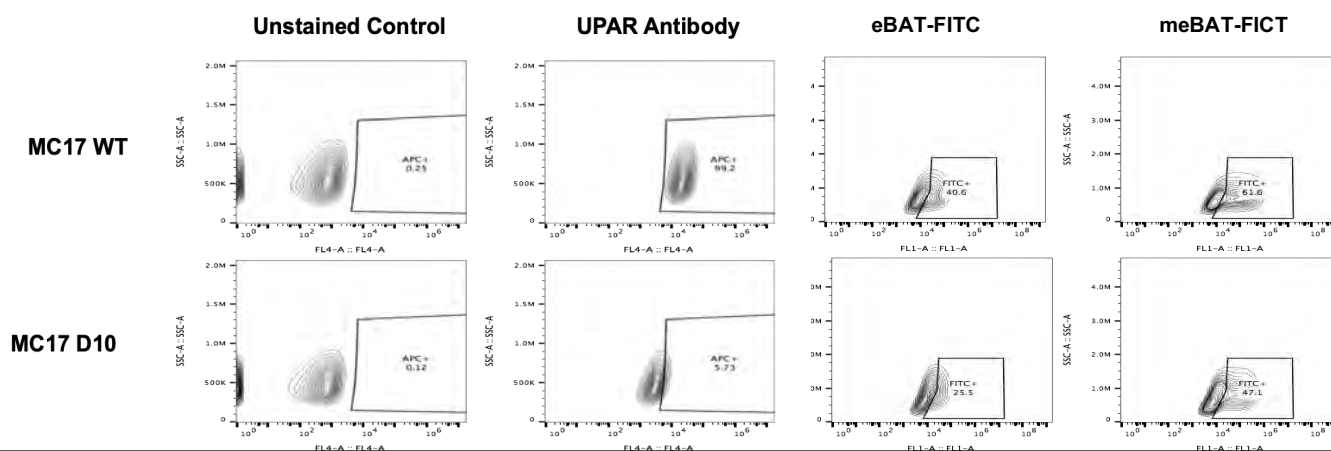


Figure 10. Binding of eBAT and meBAT to MC17 mouse fibrosarcoma cells and to D10 uPAR-KO mouse fibrosarcoma cells.

D10 cells were derived from MC17 by CRISPR-Cas9-mediated knockout of uPAR (introduction of a missense mutation in exon 2). The two-dimensional flow cytometry contour plots show fluorescence (x-axis) as a function of cellular complexity (90° light side scatter, y-axis). The top panels represent WT MC17 cells, and the bottom panels represent D10 (uPAR-KO) cells. The panels in the first column from the left are unstained controls. The panels in the second column show uPAR expression based on staining with anti-mouse uPAR. The panels in the third column show staining with eBAT-FITC. The panels in the fourth column show staining with meBAT-FITC. The reduced binding of eBAT and meBAT to D10 cells is attributable to lack of uPAR.

Both WT MC17 cells and D10 cells were resistant to eBAT cytotoxicity *in vitro* (Figure 11). Resistance to bacterial toxins that target protein synthesis has been attributed to loss of the pro-apoptotic BH3-only protein, Bim²⁷. But for our purpose, the MC17/D10 model has been exceptionally useful because the resistance to meBAT and eBAT has allowed us to *focus our attention on the impact of these drugs on remodeling the TME* (comparable to our plan with VSV and ONIx). Our data show that uPAR expression is not necessary for tumor formation, as both MC17 and D10 achieved log-growth and

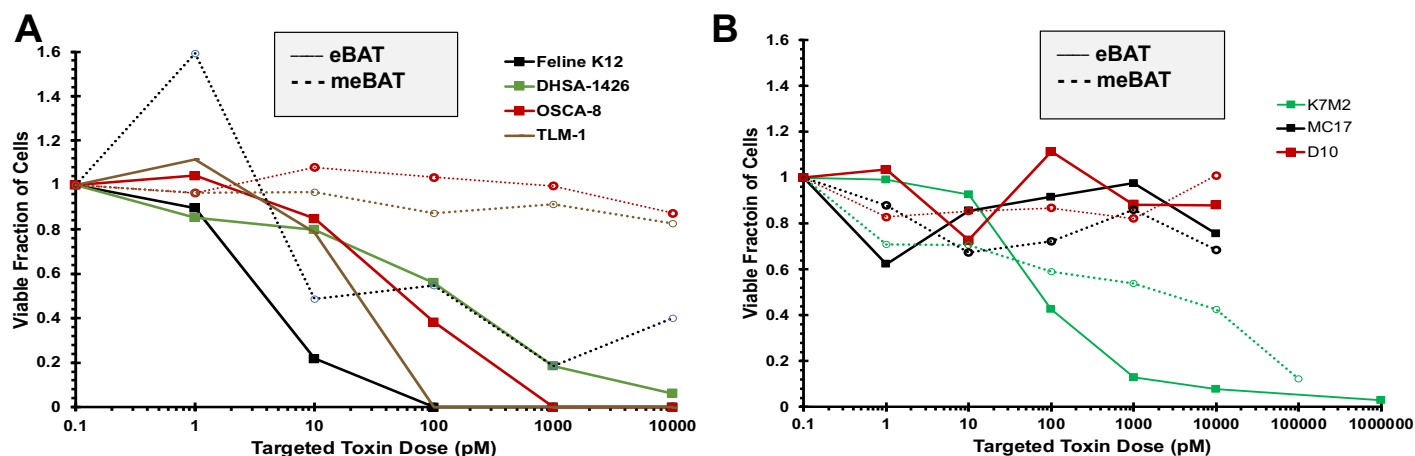


Figure 11. *In vitro* cytotoxicity of eBAT and meBAT against non-human cells.

(A) Graph representing viability from MTS assays as a function of drug dose for feline K12 mammary cancer cells (black lines), DHSA-1426 canine hemangiosarcoma cells (green line), TLM-1 canine melanoma cells (brown lines), and OSCA-8 canine osteosarcoma cells to illustrate the cytotoxic capacity of eBAT (solid lines) and meBAT (dashed lines). Note the reduced capacity of meBAT to kill feline and canine cancer cell lines. (B) Graph representing viability from MTS assays as a function of drug dose for mouse K7M2 osteosarcoma cells (green lines), mouse MC17 fibrosarcoma cells (black line), and mouse D10 uPAR-KO fibrosarcoma cells to illustrate the cytotoxic capacity of eBAT (solid lines) and meBAT (dashed lines). Note the remarkable resistance of MC17 and D10 cells to both eBAT and meBAT.

generated tumors that reached endpoint (maximum diameter of 1.5 cm or any ill health effects on the mouse host). But uPAR deficiency retarded tumor growth *in vivo*: the median time to endpoint was 24 days for MC17 cells and 47 days for D10 cells (Figure 12, inset). On the other hand, systemic uPAR deficiency in the TME (*i.e.*, germ line uPAR KO hosts) did not impair tumor growth (Figure 12).

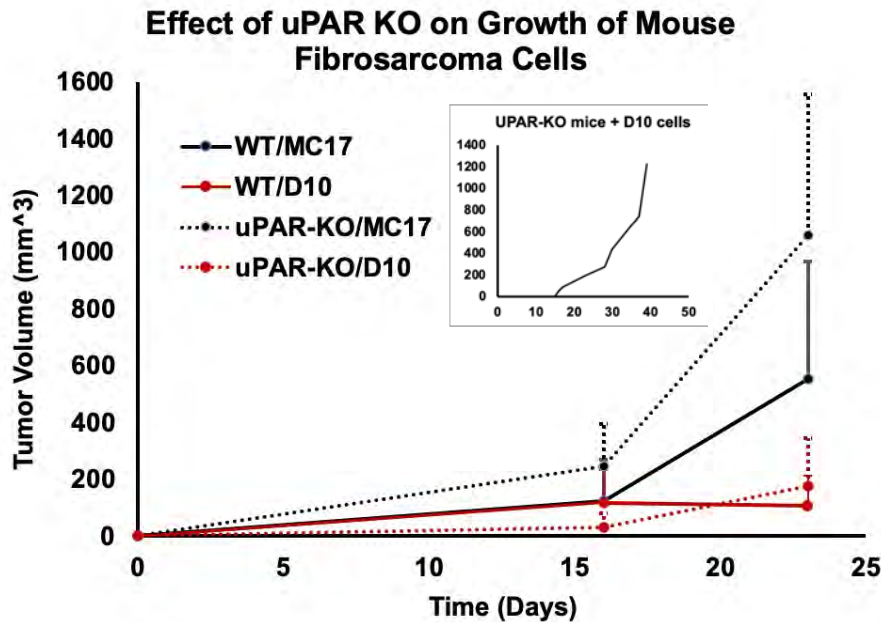


Figure 12. uPAR-KO in tumor cells delays progression but does not abrogate fibrosarcoma formation in WT or uPAR-KO mice.

WT MC17 or uPAR-KO D10 cells (3×10^5 per mouse) were injected subcutaneously into WT B6 hosts or germ line uPAR-KO B6 hosts. Tumor growth was followed over time. Log growth of the D10 cells was noted starting at Day-28, with tumors, on average, achieving volume of approx. 700-1,200 mm³ by day 40-47 as shown for uPAR-KO mice (inset).

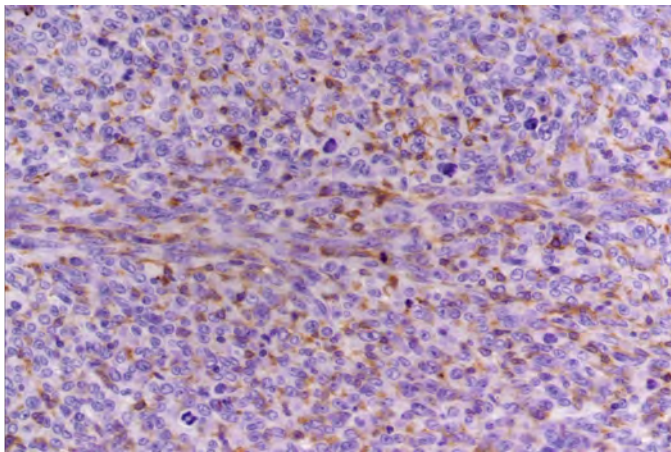


Figure 13. MC17 tumors are extensively infiltrated by TAMs.

Representative image of a sq MC17 tumor in a B6 recipient mouse. 3×10^5 cells were implanted subcutaneously and allowed to grow for 17 days. At the endpoint, the animals were humanely sacrificed, and tumors were immunostained using the mouse macrophage specific F4/80 antibody. Brown staining in this tumor shows the extent of TAM infiltration into MC17 tumors. (Magnification 400x).

As is common for sarcomas²⁸, MC17 tumors attracted large numbers of TAMs into the TME. In this case, we documented that by immunostaining with the mouse macrophage specific antibody, F4/80 (Figure 13). We then examined whether eBAT could deplete TAMs by targeting uPARs, which they express in abundance. WT MC17 tumors from WT B6 mice and uPAR-deficient (D10) tumors from uPAR-KO mice were immunostained with F480. The data indicate that eBAT or meBAT treatment had no effect on TAMs from D10 (uPAR-KO) tumors growing in uPAR-KO mice, whereas they were almost completely ablated in WT MC17 tumors grown in WT B6 mice (Figure 14). This conclusively shows that macrophage depletion by eBAT was dependent on the expression of uPAR in the tumor and/or in the TME.

To further define the impact of remodeling the MC17 immune landscape, we created uPAR-WT and uPAR-KO bone marrow chimeras (see Figure 15 for an illustration of the experiment). Engraftment efficiency was approx. 75-85% for both groups, providing WT controls and mice with only hematopoietic (+/- vascular) cells lacking uPARs. Mice were injected with mCherry-labeled MC17 cells and treated

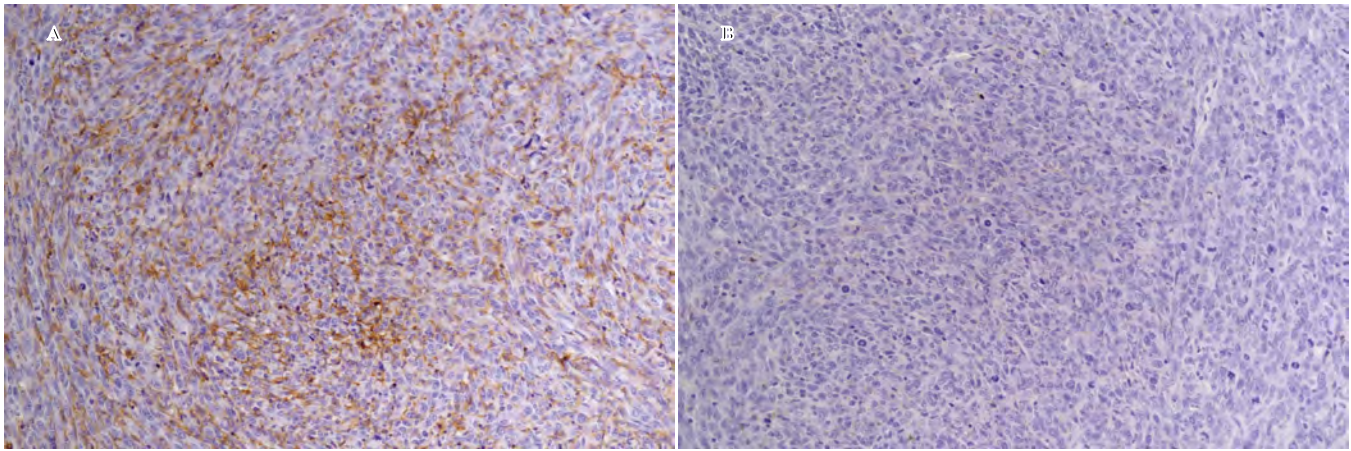


Figure 14. Depletion of F4/80⁺ Immunosuppressive TAMs in mouse sarcoma treated with meBAT.

Representative IHC to document the presence of F4/80⁺ TAMs (brown staining) in mouse tumors. Panel A: uPAR KO mouse inoculated with D10 (uPAR KO) cells and treated with two cycles of meBAT starting on day-16. Panel B: WT mouse inoculated with WT MC17 cells and treated with two cycles of meBAT starting on day-16. Tumors were harvested at day-28 and day-30, respectively. Note the depletion of TAMs in panel (B) as compared to panel (A). Even though staining in the tumors was variable, decreased F4/80⁺ TAM density was reproducibly observed only in WT mice harboring WT tumors treated with meBAT or eBAT. (Magnification 200x).

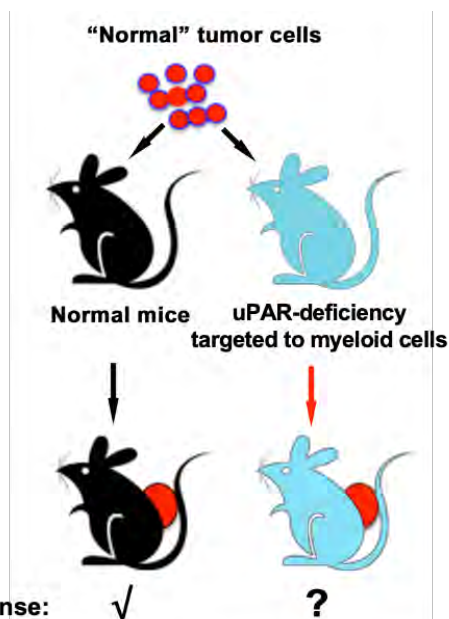


Figure 15. Bone marrow chimeras to test the impact of reshaping the TME.

B6 mice that were lethally irradiated and rescued by bone marrow transplantation from WT B6 mice (black) or from B6 uPAR-KO donors (blue) received subcutaneous MC17 implants. Five days after implantation, mice were treated with eBAT, meBAT, or PBS. Tumors (when present) were collected from animals at the endpoint and divided in half for (a) routine pathology and immunohistochemistry, and (b) flow cytometry analysis after disaggregation into single cell suspensions.

with eBAT or meBAT for two consecutive Mon-Wed-Fri cycles. Figure 16A shows that, despite the fact that MC17 cells are resistant to eBAT and meBAT, both drugs reduced the tumor burden in mice whose hematopoietic cells expressed WT uPAR. The therapeutic benefit was completely ablated in mice whose hematopoietic cells had uPAR knocked out. Consistent with what we observed in the WT and germline uPAR KO mice, eBAT and meBAT remodeled the myeloid landscape of the MC17 sarcomas. The total numbers of CD11b⁺ myeloid cells were similar in the WT and uPAR-KO bone marrow recipients. However, TAMs were significantly reduced in mice treated with meBAT (Table 1,) and a significant number of CD11b⁺ cells in the WT bone marrow recipients were also positive for mCherry (Figure 16B), suggesting these myeloid cells had been reprogrammed from an immunosuppressive phenotype to a pro-immune phenotype and had almost certainly engulfed mCherry⁺ MC17 tumor cells (doublet conjugate cells would have been excluded by the staining protocol). The increased myeloid cell phagocytosis was not seen in the uPAR-KO bone marrow recipients. This pro-immune myeloid remodeling appeared to be functionally significant: we observed a reproducible increase of CD3⁺ T cells infiltrating the TME in the WT bone marrow recipients (Figure 16C), whereas virtually no T cells were present in the TME of the

uPAR-KO bone marrow recipients, and the overall survival time (based on time to a tumor endpoint) was also improved in the WT bone marrow recipients (Figure 16D) as compared with the uPAR-KO bone marrow recipients.

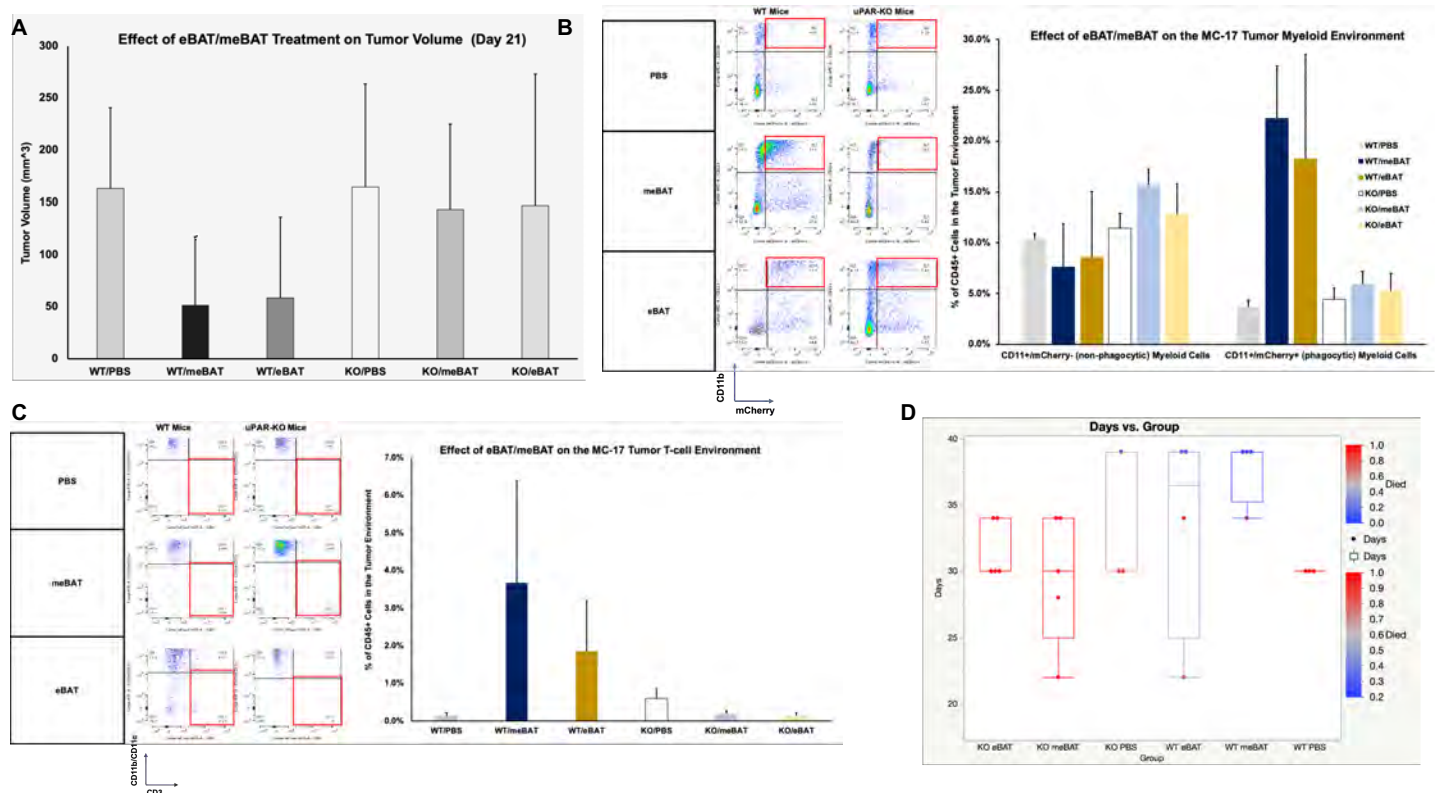


Figure 16. Remodeling of the immune landscape by eBAT or meBAT treatment leads to improved outcomes in the MC17 sarcoma model.

Five-week old, WT B6 (CD45.1) mice were lethally irradiated and rescued from death by bone marrow reconstitution using WT (WT) or germline uPAR-KO (KO) B6 (CD45.2) donors. The proportion of CD45.2+ cells in the reconstituted mice was, on average, approx. 75-80%. Three weeks after bone marrow reconstitution, mice were injected subcutaneously with mCherry-labeled MC17 cells, and five days later the mice were treated with phosphate buffered saline (PBS), or with two cycles of meBAT or eBAT. Tumor cells did not engraft in one mouse of the KO/PBS group, but this mouse was still included in the analyses of tumor size and survival. Panel (A) shows tumor volume in mice from the three groups at Day-21 after tumor inoculation. Panel (B) shows representative 2-dimensional flow cytometry dot plots of myeloid cells stained with anti-CD11b and cells expressing mCherry. The red boxes delineate putative phagocytic myeloid cells (CD11b+/mCherry+). Panel (C) shows representative 2-dimensional flow cytometry dot plots of myeloid cells stained with anti-CD11b and anti-CD11c and T cells stained with anti-CD3. The red boxes delineate T cells found in the TME. The bars in (B) and (C) are color coded to assist in identification of groups: WT recipients treated with PBS are colored in gray; WT recipients treated with meBAT are colored in dark blue; WT recipients treated with eBAT are colored in gold; KO recipients treated with PBS are colored in white; WT recipients treated with meBAT are colored in light blue; and KO recipients treated with eBAT are colored in yellow. Panel (D) shows a survival box and whisker plot for all groups, with individual animals denoted by red dots (dead) or blue dots (alive at the end of the experiment). Only the mouse that did not engraft with tumor in the KO/PBS group and mice in the WT/meBAT and WT/eBAT groups survived longer than 34 days. The mice were sacrificed at Day-40 to maximize the probability that changes in the TME attributable to meBAT or eBAT treatment would still be detectable.

The alterations in myeloid populations induced by eBAT and meBAT were specific to the tumor environment, as *neither depletion nor alterations in phenotypes were observed in peripheral blood from*

the same mice. Our preliminary assessments indicate that there were no differences in the number of microvessels (based on CD31 immunostaining in the tumor), but there appeared to be differences in the distribution of vessels in mice treated with eBAT and meBAT, suggesting that targeting uPAR+ vessels might lead to vascular normalization. Here, we will extend these observations to examine whether eBAT and meBAT normalize tumor vasculature and alter the immune landscape in the TME, and whether these effects enhance infiltration and activity of cytotoxic T cells and NK cells.

Table 1. meBAT Ablates F480+ TAMs.

Group/Treatment	% TAMs in TME (range across all of the tumor)
WT BM/PBS	21.8 (17.7 - 27.7)
WT BM/meBAT	5.1* (1.4 - 19.6)
uPAR-KO BM/PBS	11.4 (7.4 - 21.0)
uPAR-KO BM/meBAT	17.8 (1.6 - 34.1)

WT = wild type; BM = bone marrow; PBS = phosphate buffered saline; KO = knockout. uPAR-KO BM are the chimeric mice with targeted uPAR deficiency in bone marrow cells; *=statistically different from other conditions, with a p value <0.05.

In the interim, while the MC17 model was being developed to Mayo Clinic, Dr. Naik carried out a pilot study in the well-established A20 murine lymphoma model to confirm the safety of combination VSV and P-DIC combination therapy. This was done concurrently with the tolerability experiment described in Figure 2. The results show that mice that received both VSV and combination therapy had transient tumor remissions (including formation of scabs on the tumor site), with a significant survival benefit compared to PBS treatment (Figure 17). These data support further analysis of this combination therapy in relevant murine sarcoma models.

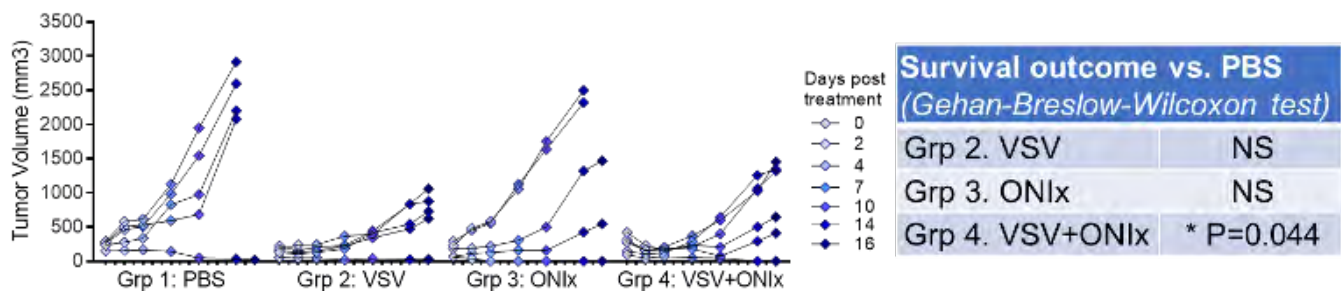


Figure 17. VSV and P-DIC combination treatment induces tumor remission in a Balb/c tumor model. Mice were injected with A20 lymphoma cells, and once visible tumors arose, they were treated with PBC (control), VSV alone, ONIx alone, or VSV + ONIx. The spider plots show tumor growth for individual mice in each group.

Milestone: Complete necropsies. Ongoing.

Dr. Modiano, Ms. Schulte, and Dr. Kim performed necropsies for all the experiments using MC17 at the University of Minnesota. As described above, Dr. Modiano and the lab staff completed necropsies on the mice inoculated with K7M2-Luc cells and with K12 cells. Necropsies will continue to be performed for all experimental animals.

Dr. Naik is developing this aspect of the project as described above and in the Milestones for Aim 2.

Milestone: Data analysis; organization and conclusions. Ongoing.

The project investigators have been holding regular meetings to update the status of the project.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Ongoing.

QC and QA for data are performed on a continuous basis. We have presented the MC17 data at national and international meetings and continue to accrue data to prepare a manuscript for the osteosarcoma experiments, especially if we can form a consensus around publication of negative data from the K12/K7M2 model.

Specific Aim 2: Define immunological effects and mechanism of action for combination VSV IFN β -NIS and P-DIC (ONIx)

Milestone: Assessment of immune response induced by VSV-IFN β -NIS and P-DIC in mice with metastatic osteosarcoma. Ongoing.

Milestone: Assessment of immune response induced by VSV-IFN β -NIS and P-DIC in mice with metastatic osteosarcoma. Ongoing.

Pilot data were generated for this milestone, which were highly informative. The effect of VSV and ONIx combination therapy in murine tumors especially on the immune landscape has not been previously established.

To fill this gap in knowledge, Dr. Naik's group initially conducted two pilot studies: the first to establish murine sarcoma models in the lab and the second to perform a detailed immune assessment of VSV and ONIx combination therapy in the well-established A20 murine lymphoma model.

To establish sarcoma models in the lab, we initially did a pilot study by implanting 5×10^6 K7M2 osteosarcoma cells (from NCI) sq in syngeneic Balb/c mice. Tumor growth was quite slow in this model with about ~50% of mice developing tumors (N=4). Tumor bearing mice were treated with PBS or 1×10^8 TCID₅₀ VSV (per mouse) given intravenously. Mice were sacrificed on study day 4 to

optimize methods to measure intra-tumoral immune infiltration and antitumor immunity.

Preliminary assessment of CD8 T-cells and macrophages in peripheral blood mononuclear cells (PBMCs), spleen and tumor (Figure 18) show (a) a notable increase of CD8 T-cells in PBMCs but not in tumor and spleen; and (b) an increase in macrophages in PBMCs, spleen, and tumor. Additional and larger experiments will be needed to confirm these findings. Addition of checkpoint inhibition dually targeted to macrophages (CD47) and T-cells (PD-1), *i.e.*, ONIx, may potentially activate antitumor responses mediated by these immune cell populations. PD-1 expression was increased on CD4 and CD8 T-cells in the blood and spleen (Figure 19) suggesting addition of a PD-1 inhibitor, *i.e.*, ONIx, would be a potent activator of antitumor immune responses. We are currently conducting a pilot study to establish the MC17 sarcoma model in Dr. Naik's lab.

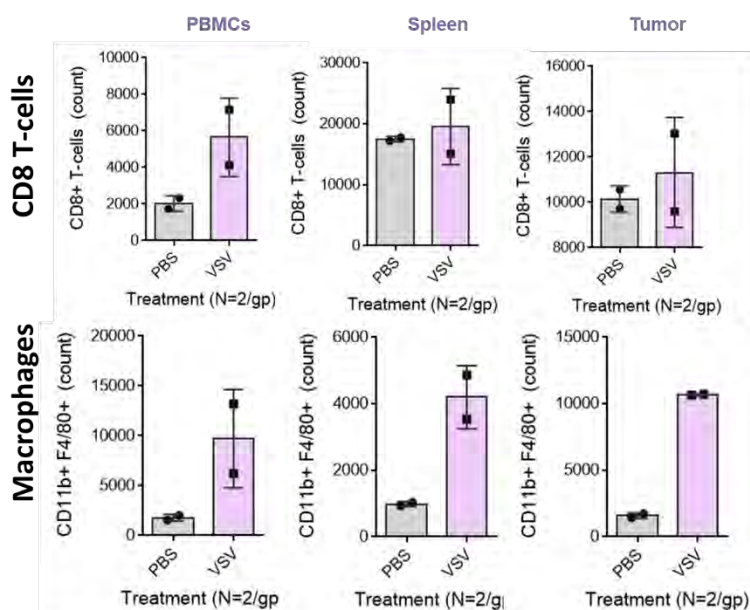


Figure 18. VSV treatment induces and increases presence of immune cells in tumors and systemically.

PBMCs, spleens, and tumors were harvested from the mice at the time of sacrifice and the presence of CD8 T-cells and tumor-associated macrophages was established using flow cytometry.

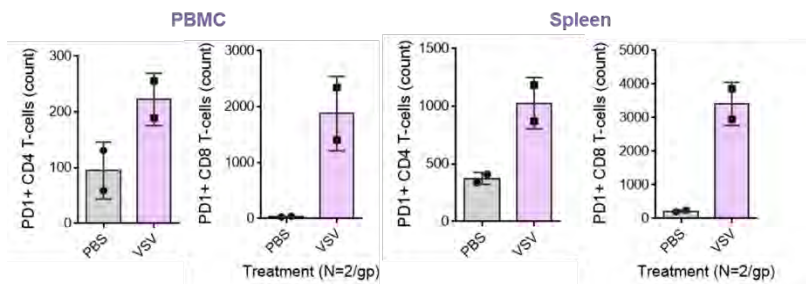


Figure 19. Increased PD-1 expression on T-cells in tumor and spleen following VSV treatment.

PBMCs and spleens obtained as in Figure 4 were evaluated for CD4 T-cell activation and potential exhaustion based on expression of PD-1 using flow cytometry.

A detailed assessment of the efficacy of combination therapy with VSV and either ONIx or PD-1 inhibitor in the A20 tumor model was carried out: A20 tumor bearing mice were treated with PBS, VSV, or combination therapy VSV + ONIx or VSV + α PD-1. In this study, a higher dose of VSV was utilized and toxicity was observed following systemic VSV administration. Most of the mice that received systemic VSV monotherapy were euthanized due to excessive weight loss. Interestingly combining systemic VSV therapy with either ONIx or an α PD-1 antibody had reduced toxicity and most mice recovered following transient weight loss. These data

suggest that addition of CPI can mitigate potential toxicity of oncolytic virotherapy, and they show that combination therapy with both VSV+ONIx and VSV+ α PD-1 induce durable tumor remission in a subset of tumor bearing mice (Figure 20A) compared to rapidly growing tumors in the majority of PBS treated mice (though there was spontaneous tumor remission observed in some PBS treated mice). Near the end of the study, mice were euthanized and splenocytes were analyzed by ELISPOT assay to detect immune responses against PHA (positive control), VSV antigen, and MHC-I specific tumor associated AH1 antigen²⁹. These data indicate show that VSV treatment increases overall activated T-cell responses, VSV specific T-cell responses, and detectable responses against the AH1 tumor associated

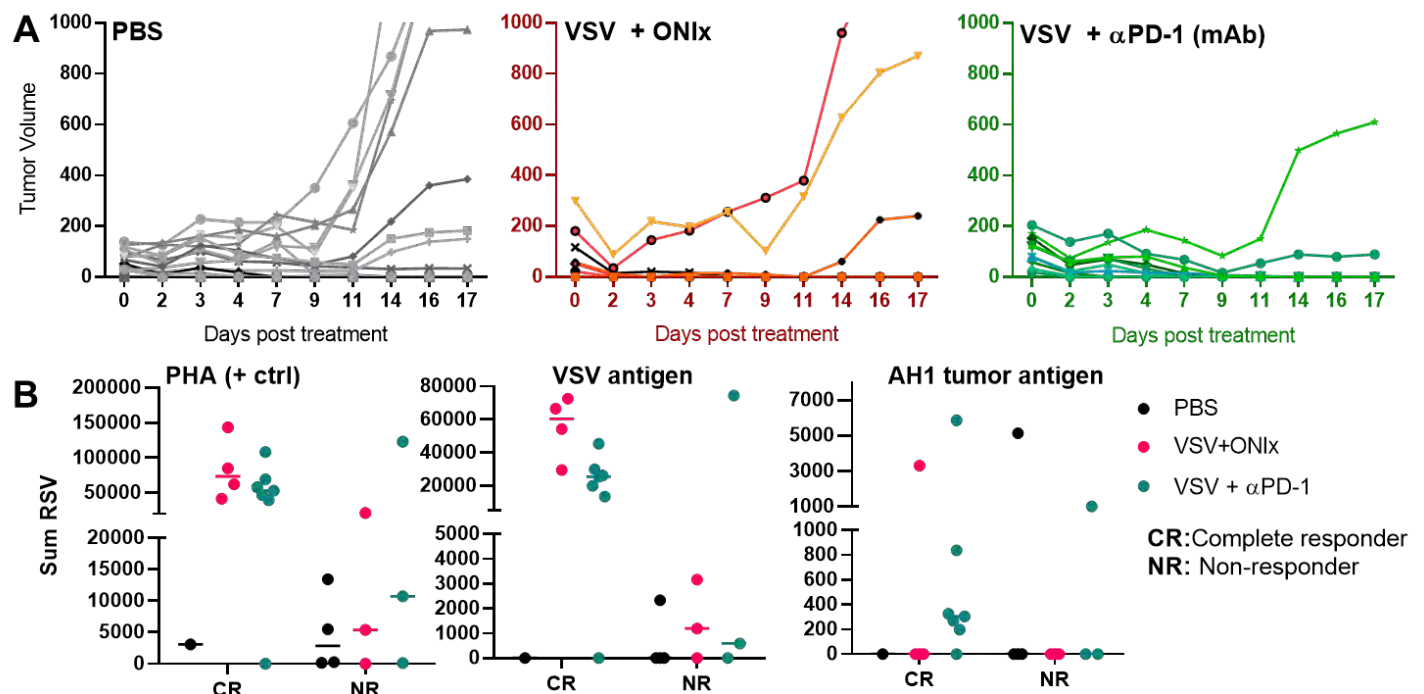


Figure 20. Immunomodulatory effects of VSV and CPI combination therapy in the A20 murine lymphoma model.

Mice with established subcutaneous A20 murine lymphoma tumors were treated with 5×10^8 VSV-mIFN-NIS in combination with 6 doses of 5mg/kg ONIx or 200ug α PD-1 antibody. (A) Therapeutic response was assessed by measurement of tumor volume by serial caliper measurements. (B) Mice were monitored to designate complete responders (CR) and non-responders (NR). Mice were euthanized and spleens were collected. Splenocytes were analyzed by ELISPOT to detect T-cell response against assessed PHA (Lectin, positive control), VSV antigen peptides, and the MHC-I specific AH1 tumor associate antigen peptide.

antigen (Figure 20B). VSV and tumor specific T-cell responses were generally higher in mice that had complete response to combination therapy compared to mice that did not respond, suggesting that development of virus and tumor specific T-cell responses may correlate with therapeutic response.

Milestone: Data analysis; organization and conclusions. Ongoing.

Emerging data support the hypothesis of synergy between VSV-IFN β -NIS and ONIx. Data analysis is ongoing in real time and the investigators meet regularly to discuss data, reach consensus for the conclusions, and decide on next steps for experiments and reporting.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. Ongoing.

QC and QA for data are performed on a continuous basis in real time.

Specific Aim 3: Characterize safety, efficacy, and immunomodulatory effects of VSV-IFN β -NIS and P-DIC (ONIx) in dogs with naturally occurring metastatic osteosarcoma

Milestone: Production of *in vivo* grade VSV-IFN β -NIS. Completed.

Please see milestones for specific aim 1. A new batch of *in vivo* grade VSV-IFN β -NIS for the canine clinical trial was produced by the Mayo Clinic investigators.

Milestone: Production of *in vivo* grade P-DIC (ONIx). Completed.

Please see milestones for specific aim 1. Release assays for sterility and potency have been completed and assays for stability are scheduled semiannually throughout the project period. This milestone was accelerated and ONIx was produced in a single batch, which should be sufficient to assess if there is a biological effect at 2 mg/kg dose after VSV administration. If we fail to see a response in the first dog treated, we will consider dose escalation.

Milestone: Identify safe dose to guide clinical development. Ongoing.

The mouse experiments shown as part of Aims 1 and 2 show that ONIx at a dose of 5 mg/kg is safe. This dose equivalent is considerably higher than the maximal dose we will be able to administer to dogs in this project (manufacturing limits). The data also suggest that the dose of ONIx required to achieve a therapeutic effect in dogs might be no higher than 1-2 mg/kg (1/6 of the effective mouse dose).

Milestone: Assessment of immune response induced by VSV-IFN β -NIS and P-DIC (ONIx) in dogs with metastatic osteosarcoma.

The study parameters were extensively reviewed, and the METEOR (metastatic experimental osteosarcoma research) study opened in August of 2022. A Data Safety Monitoring Board comprised by Dr. Antonella Borgatti (board certified veterinary oncologist and Director of the Clinical Investigation Center of the University of Minnesota College of Veterinary Medicine), Dr. Michael Conzemius (board certified veterinary surgeon and former Director of the Clinical Investigation Center of the University of Minnesota College of Veterinary Medicine), and Dr. Aaron Rendahl (biostatistician) has been assembled to oversee the canine clinical study and ensure safety criteria are rigorously followed.

The enrollment web page and study details are available at z.umn.edu/meteor. The first eligible dog whose owners applied for the study was screened on the week of September 5, 2022. The dog is a 1.75 years-old spayed female spaniel that was diagnosed with a primary osteosarcoma of the left distal femur on May 3, 2022. The family decided to pursue standard of care (amputation followed by adjuvant chemotherapy). The dog's affected limb was amputated on May 12, 2022, and adjuvant chemotherapy was started with carboplatin (300mg/m²) three weeks later on June 2, 2022, with the intent to administer between four and six cycles once every three weeks. On the same date when the initial chemotherapy was administered, two pulmonary nodules were visible radiographically, although they did not meet the

complete criteria for definitive diagnosis of metastatic disease. A second dose of carboplatin was administered on June 23, 2022. Mild lymphocytosis and eosinophilia were the only abnormalities noted in the laboratory testing done on that date. On July 14, 2022, the dog had neutropenia, so chemotherapy was delayed. Imaging showed evidence of progressive disease (increased size of the pulmonary nodules), so the chemotherapy protocol was switched to doxorubicin (30 mg/m²), with the intent to administer between four and six cycles once every three weeks. The neutropenia had resolved by July 21, 2022, which allowed for the first dose of doxorubicin to be administered. A second dose was given on August 4, 2022, but by August 18, 2022, there was evidence of further disease progression and chemotherapy was discontinued. The family was given options to discontinue treatment and switch to palliative care, begin an experimental rescue protocol using Losartan and toceranib (+/- propranolol), or screen for eligibility for the METEOR study. They elected the latter.

The dog completed screening for METEOR on September 6, 2022 and was deemed to be eligible to enroll. The dog was hospitalized on July 12 and released from the hospital and back to the family on September 16, 2022. Two consecutive doses of VSV-clFN β -NIS (5x10⁹ TCID₅₀/kg) were administered on September 12 and 13, 2022. A mild (not clinically significant) decrease in platelet count and a mild (not clinically significant) increase in circulating levels of fibrinogen were noted on September 14, 2022, about 24 hours after the second dose of VSV-clFN β -NIS. Given that these laboratory changes were deemed to be non-clinically significant, the first dose of ONIx (2 mg/kg) was administered on September 14, 2022, as per the protocol. On September 15, 2022, the dog showed grade-1, gastrointestinal adverse events (inappetence and vomiting). The signs were managed medically and a second dose of ONIx was given on September 16, 2022. Laboratory testing revealed recovery of platelets and fibrinogen back to baseline levels and a slight decrease in red cell mass, probably attributable to the blood obtained for laboratory sampling over the experimental treatment period. It should be noted that the dog was not anemic. The dog was discharged on September 16, 2022, and returned on September 19, 2022, to receive a third dose of ONIx in the outpatient setting. Laboratory testing showed that all hematology and serum biochemistry values were within the specified reference ranges for the clinical laboratory, with resolution of all prior mild abnormalities. The family reported that the dog had persistent inappetence and nausea (determined to remain as a grade-1 adverse event), although without vomiting. These gastrointestinal signs continued to be managed medically and the dog improved and resumed eating.

Blood, serum, urine, and feces obtained prior to and during the 24 hours after VSV administration were banked for viral shedding and VSV pharmacokinetic studies. Serum samples obtained prior to any treatment, after VSV, and for the first hour after ONIx were also banked for ONIx pharmacokinetic studies. PBMCs for immunological analysis have been collected at time points before treatment, after the administration of the test articles, and will continue to be collected periodically for 6 months or until the dog relapses and goes off study. This will be repeated for all enrolled dogs. If three dogs complete the study without severe adverse events attributable to ONIx, we will consider a dose escalation.

In humans receiving PD-1/PD-L1 immune checkpoint inhibition therapy, the gastrointestinal system is one of the most commonly affected organ systems and is responsible for the most frequent emergency visits resulting from immune-related adverse events³⁰, so the observed adverse gastrointestinal events in this dog were not unexpected. Mild (grade-1 and grade-2) gastrointestinal adverse events, including diarrhea, nausea, vomiting, constipation, and dyspepsia were also observed in clinical trials of CD47 blockade³¹, so the signs in this first dog treated by ONIx could be due to either the effects of PD-1 blockade, CD47 blockade, or both. Optimistically, the observed, mild gastrointestinal toxicity suggests that ONIx is indeed biologically active at the dose given. The dog will continue to be monitored according to the study schedule, and additional dogs will be recruited to continue data gathering for this aim during the no-cost extension period.

Additional experiments that were supported by this project, which are related to our overall objectives, although not explicitly stated in the specific aims, are described in detail below under the heading, “Other Related Work”.

Milestone: Data analysis; organization and conclusions. This is ongoing, as described above.

Milestone: Quality control and quality assurance for data – manuscript preparation and submission. This is ongoing, as described above.

Milestone: Planning for pediatric clinical trial planning (translation to humans). Ongoing.

At Mayo Clinic, Dr. Naik and Dr. Steven Robinson, a medical oncologist with a focus on sarcoma treatment have worked together to develop a clinical trial outline to evaluate systemic oncolytic VSV therapy in combination with checkpoint blockade to be given in the neoadjuvant setting in sarcoma patients scheduled to undergo metastasectomy. They have currently gathered data from a retrospective analysis of sarcoma patients undergoing metastasectomy at Mayo Clinic between 2010-2021 to better understand treatment outcomes with standard of care.

At the University of Minnesota, Dr. Modiano has initiated discussions with Drs. Brenda Weigel, Emily Greengard, and Andrea Espejo-Freire to develop plans for translating this therapy into adult patients as well as children and AYAs with osteosarcoma and/or other sarcomas.

- **Other related work**

In addition to the work described here, the COVID-19-related laboratory closures gave us the opportunity to make progress on various other related fronts that are highly pertinent to this project.

First, being confined to working from home provided an opportunity to refine our understanding of how spontaneous canine cancers should be used in the context of “models” for human disease. In particular, we know that there are important similarities in the biology and natural history of osteosarcoma in humans and dogs³², but we wished to address the persistent questions regarding the major difference in age of susceptibility for osteosarcoma in humans, where the age distribution is bimodal with the major peak in adolescence, and in dogs, where the age distribution is also bimodal but has its major peak in older adults.

These efforts culminated in a peer reviewed publication (perspective) in the journal *Aging and Cancer*³³, where we propose that the excess cancer risk seen in humans and in domestic dogs is driven in large part by the technological gains that have extended lifespans beyond the evolutionarily adapted limits for both species. Notably, this has only happened in humans and in dogs (to a lesser extent, perhaps, in domestic cats and in pet/laboratory mice bred and kept in captivity), and suggests that, even as the relative frequency of tumor types diagnosed in humans and dogs is different, the overall lifetime risk of cancer is comparable because both species exceed their evolutionarily adapted cancer protective mechanisms. A copy of the manuscript is included as an appendix.

It follows from this perspective that causality of osteosarcoma might be different for pediatric human cases where the major influence seems to come from genetic predisposition (*i.e.*, syndromic) or from a confluence of rare, random events leading to malignancy³⁴ and for older canine cases where the major influence is from risk associated with aging. As such, human pediatric osteosarcoma and canine osteosarcoma could be considered convergent (rather than homologous) diseases with potentially different causation but comparable tissue organization. We have followed this line of investigation with additional experiments using whole exome sequencing analysis, whole transcriptome sequencing analysis, and methylome analysis. Some of these experiments were supported in part by DOD grant CA170218 (*i.e.*, methylome sequencing), and by DOD grant CA190276, which is the subject of this

report (*i.e.*, whole transcriptome sequencing for one canine osteosarcoma cohort as described below). In addition to our previously published work in this field^{1,35,36}, we are preparing additional manuscripts from these data, which will be forwarded to our program officers once they are published in the peer reviewed literature and/or deposited in pre-print servers. One such manuscript shows results from our parallel analysis of the mutational landscapes of canine and human osteosarcoma. The data indicate that there are some conserved events in both species, but these are immersed among many critical differences of the genes that undergo recurrent mutation, copy number gains, and copy number losses. One of the most significant differences was the distribution of COSMIC signature imprints, suggesting that distinct mechanisms are responsible for creating the mutational landscape and the genomic chaos in osteosarcoma from both species (Figure 21).

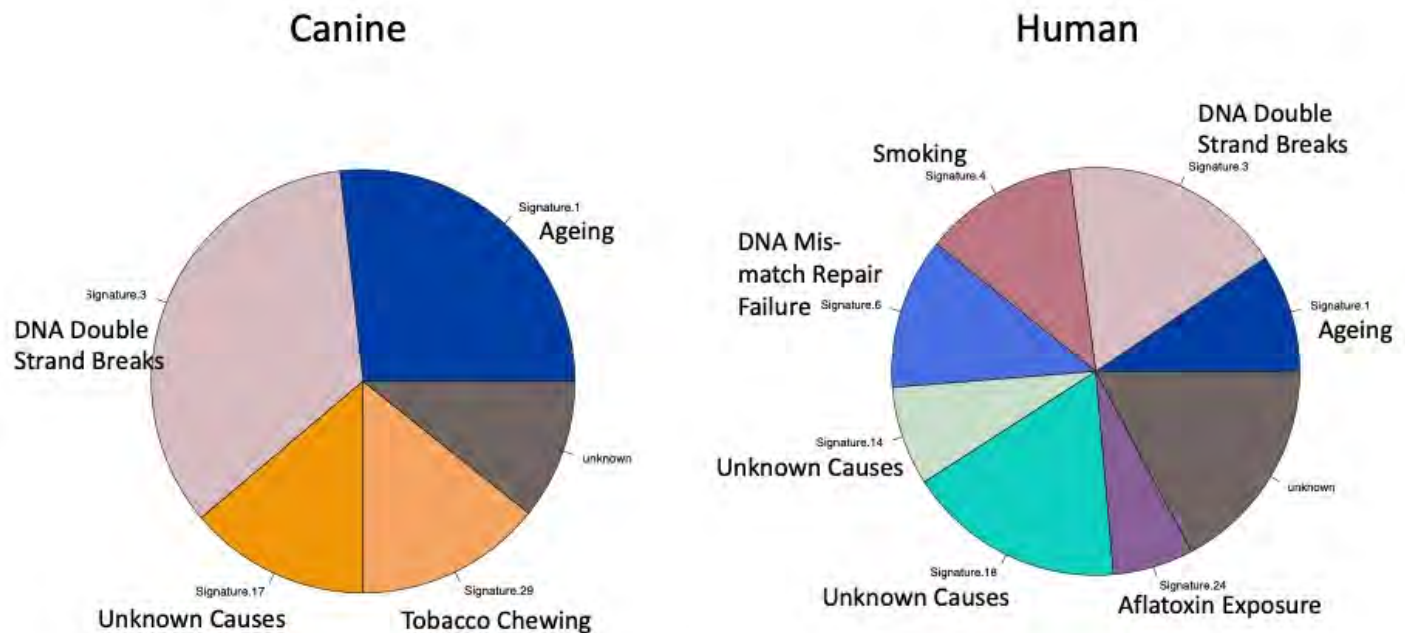


Figure 21. Cosmic signatures describing inferred mutational mechanisms in human and canine osteosarcoma. Whole exome sequencing data were analyzed to uncover the context of recurrent mutations and infer potential mechanisms leading to such mutations in osteosarcoma samples from dogs and humans. The data show that COSMIC signatures 1 (cellular “aging”) and 3 (DNA double strand breaks) putatively account for more than half of the mutations seen in canine osteosarcoma, but they only account for about 25% of the mutations in human osteosarcoma. These are also the only COSMIC signatures shared between both species, suggesting that independent, likely unrelated events are responsible for the mutational patterns seen in 75% of human osteosarcomas and about one third of canine osteosarcomas.

Another manuscript describing different mechanisms through which the *PTEN* gene is inactivated in canine and human osteosarcoma has been submitted for peer review and publication. The data show that in dogs, the genomic localization of the *PTEN* gene near the distal terminus of the q arm of canine chromosome 26 (CFA26) appears to create a unique vulnerability under conditions of genomic instability (Figure 22A and B). So, in canine osteosarcoma, which is characterized by loss of function of *TP53* and genomic chaos, *PTEN* seems to be recurrently deleted, and the transcript levels (and hence, presumably, the functional protein) are directly related to the *PTEN* copy number in the tumors (Figure 22C-E). In humans, however, the *PTEN* locus is located near the middle of chromosome 10 (Figure 22A and B). This region seems to be less susceptible to deletion (Figure 22C); and yet, *PTEN* transcripts in human osteosarcomas seem to be universally silenced, independent of their stable copy number status (Figure 22D and F). We found that a portion of the *PTEN* promoter that seems to be required for inducible expression of the gene is heavily methylated in virtually all of the human

osteosarcomas analyzed, leading to silencing and loss of inducibility. Thus, *PTEN* loss seems to favor development of aggressive osteosarcomas in dogs, whereas *PTEN* silencing by methylation seems to be almost a requisite event for the development or progression of osteosarcomas in humans. We conclude from these data that *PTEN* inactivation is achieved as a terminal event in both canine and human osteosarcomas. But this occurs via independent mechanisms, and it is not shared by all of the tumors. That is, more than half of canine osteosarcomas retain one, and more often two copies of the *PTEN* gene, and the gene is expressed robustly in those tumors.

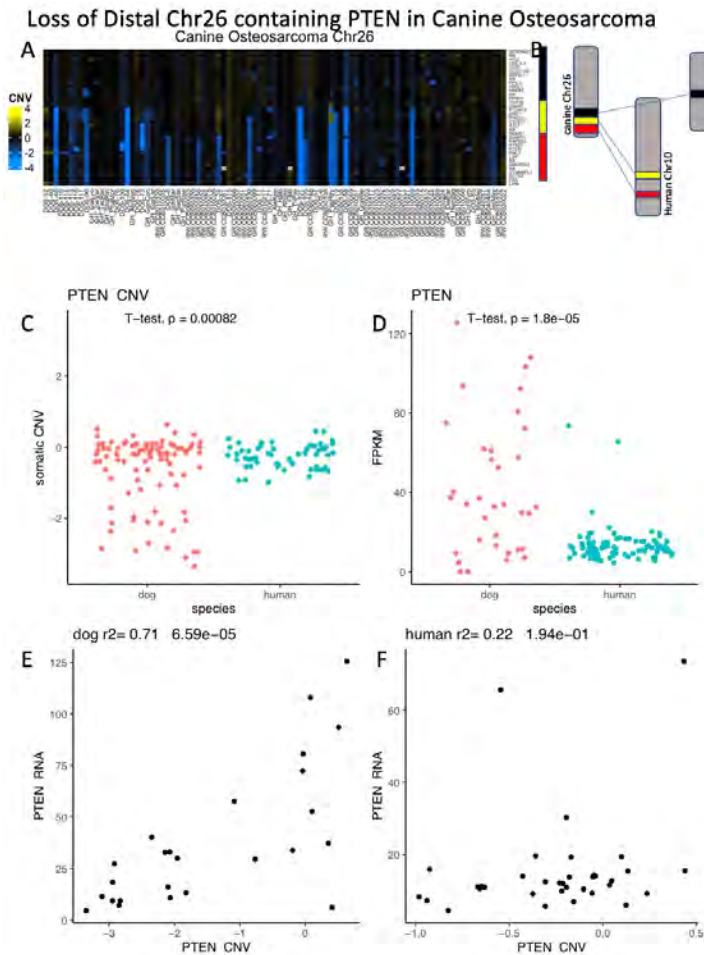


Figure 22. Loss of Distal Chr26 region containing *PTEN* in canine osteosarcoma.

Somatic changes observed between normal tissue and canine osteosarcoma tumors are shown for the distal end of canine chromosome 26. The data are log base₂ transformed, and genes shown in bright blue represent somatic loss, genes shown in black are unchanged between tumor and control, and genes that show somatic gain are shown in yellow. B) The syntenic alignment between canine chromosome 26 and the human genome is shown. The canine lost region is orthologous to two separate regions on human chr10 and one region on human chr22. C) Values for *PTEN* copy number are shown for canine samples in orange and human samples shown in blue. D) The FPKM transcript level of *PTEN* observed in RNA-Seq samples derived from canine osteosarcoma is shown in orange and from human osteosarcoma is shown in blue. E-F) For 25 canine- and 34 human exome samples, we also had RNA-Seq data derived from sections of the same tumor. The CNV for *PTEN* is plotted on the X-axis and the FPKM level for *PTEN* is plotted on the y axis. A high and significant correlation was observed for the canine samples which was not observed the human samples.

Knowing this, we would argue that canine osteosarcoma is not a good model, for example, to test strategies that reverse *PTEN* methylation, and more generally to study risk, susceptibility, or causation of human osteosarcoma, or even to test therapies targeted at certain mutations or genome abnormalities that might be present in one species, but not the other. On the other hand, the observation that transcriptional programs, global patterns of methylation, and inferred cellular distribution are conserved across species^{1,35} suggests that canine osteosarcoma may be a very suitable “model” to study how disrupting the organization of the tumor tissue by attacking the tumor microenvironment could improve outcomes. This is especially relevant to this project.

One intriguing finding in this study was that the gene encoding the Fas death receptor (*FAS* or CD95) is closely linked to *PTEN* in both species. We previously showed that neoadjuvant therapy using a non-replicating adenovirus encoding Fas ligand stimulated antitumor immunity in dogs, but only when the malignant tumor cells themselves did not express Fas (*i.e.* when Fas expression was confined to cells in the tumor microenvironment)⁴. Like *PTEN*, copy number loss of *FAS* in dogs was associated with decreased expression in the tumors, making this therapy appropriate for only a subset of the dogs. In

humans, *FAS* seems to be silenced by methylation, so the adenovirus-Fas ligand therapy would have had much greater potential benefit.

To follow on these data, we have started to analyze the chromatin landscape of osteosarcoma. Thus far, we have used the assay for transposase-accessible chromatin using sequencing (ATAC-seq) to evaluate regions of accessible (open) and inaccessible (closed) chromatin in canine T cells and in canine osteosarcoma cells. Our initial experiments show that we are able to define distinct regions of accessible chromatin in canine resting and activated CD4 and CD8 T cells (Figure 23). Additional experiments to contrast these findings with the chromatin landscape of osteosarcoma cells will provide guides to further establish the heterogeneous composition of osteosarcoma tumor tissues. We are complementing these experiments using single cell sequencing and spatial genomics of canine and human osteosarcomas, which will further refine our understanding of the tumor microenvironment and highlight opportunities to address this disease using immunomodulation.

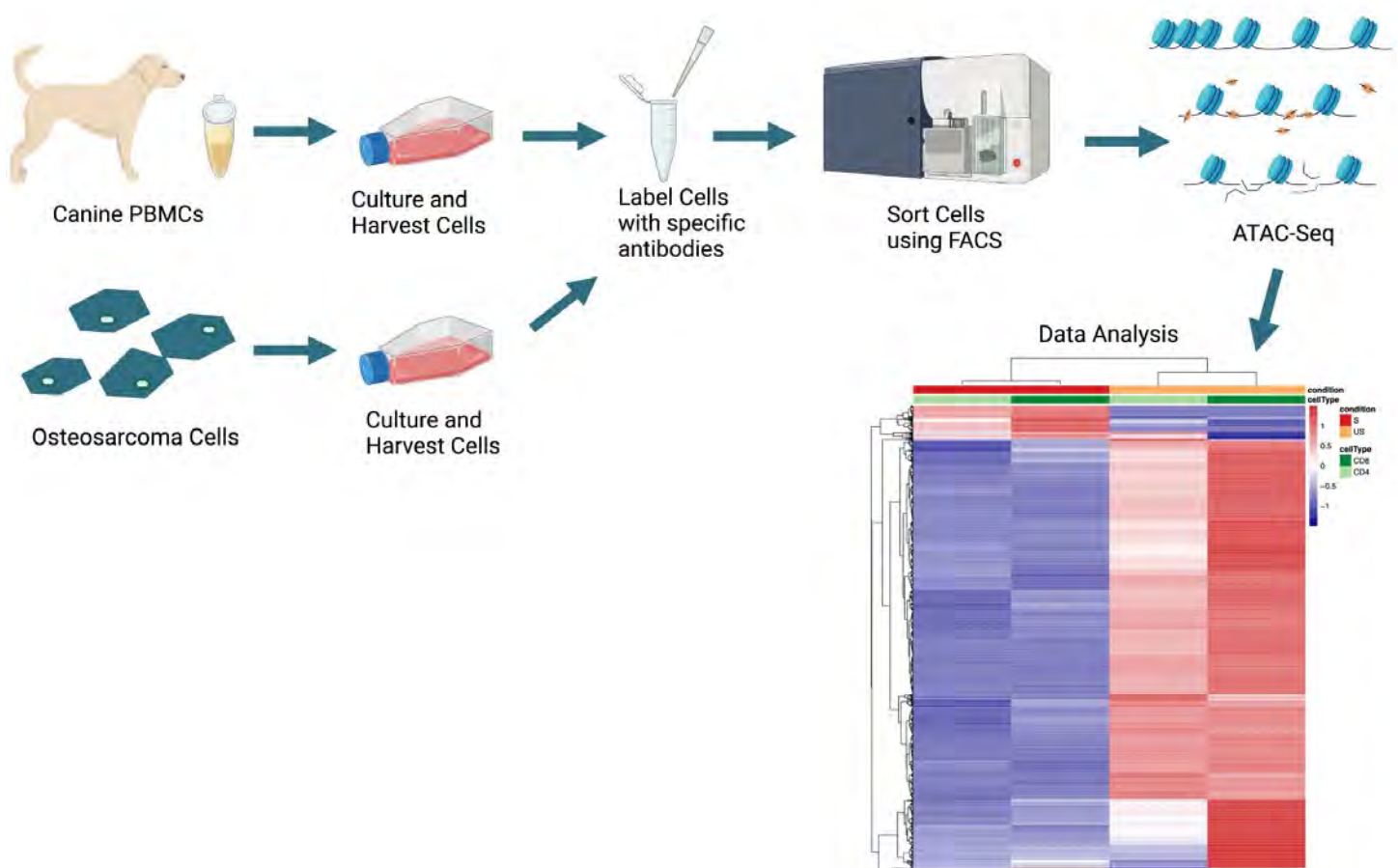


Figure 23. Graphical illustration of experiments designed to evaluate chromatin accessibility in canine T cells and canine osteosarcoma cells.

Canine peripheral blood mononuclear cells (PBMCs) and canine osteosarcoma cell lines are being used for these experiments. PBMCs are enriched from peripheral blood, set up in parallel cultures with no stimulation or stimulated by mitogens, and at the peak of activation, CD4 and CD8 T cells are further enriched by fluorescence activated cell sorting (cell purity >98%). These cells, and cultured osteosarcoma cells are subjected to ATAC-seq to assess chromatin accessibility. The heat map illustrates data from our initial experiment, showing that unique patterns of open chromatin are found in both cell types under resting and stimulated conditions.

We have also continued to analyze data from our previously completed VSV clinical trial in dogs with naïve osteosarcoma (the VIGOR study). Concurrent with the preparation of this report, we are finalizing

the manuscript describing this study for submission to a peer reviewed journal. The summary that follows includes the most recent work, which was done in part during the COVID shutdown while the PI and staff salaries were supported in part by this grant, and which is especially pertinent to this project.

We evaluated event free survival (EFS) and overall survival from dogs in the VIGOR study and compared them to two contemporary cohorts of dogs with appendicular osteosarcoma with no evidence of metastasis. The first cohort (n = 57) included dogs seen at the U of M Veterinary Medical Center (VMC) between July 2011 and July 2018, where there was intent to treat with standard-of-care surgery and adjuvant carboplatin chemotherapy, and that had a successful limb amputation and completed at least one cycle of adjuvant chemotherapy. The second control cohort was from a study recently published by the National Cancer Institute’s Comparative Oncology Trials Consortium (COTC). This cohort included 157 dogs with primary appendicular, non-metastatic osteosarcoma enrolled between November 2015 and February 2018 that were treated with standard-of-care surgery and adjuvant carboplatin chemotherapy³⁷. The COTC cohort was made available as a control dataset for evaluation of novel neoadjuvant or adjuvant treatments for osteosarcoma.

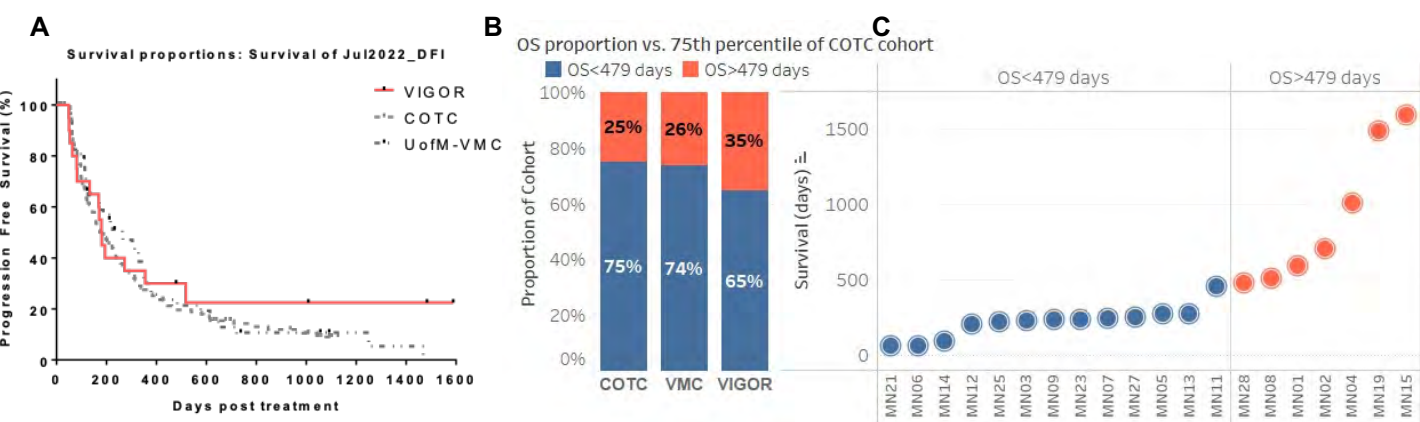


Figure 24. Overall survival of dogs treated with neoadjuvant stomatitis virus (VSV) followed by standard of care. (A) Kaplan-Meier survival curve with progression free survival (PFS) of dogs treated with systemic VSV in the VIGOR study, compared to two contemporary control cohorts. (B) The VIGOR study had a larger percentage of dogs considered long term responders (overall survival > 479 days) than the two control cohorts. (C) Distribution of dogs treated with VSV in the VIGOR study into groups based on length of overall survival.

Overall survival for dogs showed a “tail” of long-term survivors, including 4 dogs that were still alive at the time of this report. Three dogs were excluded from survival analysis: two dogs that were eventually diagnosed with an indication other than osteosarcoma (one rhabdomyosarcoma of bone and one hemangiosarcoma of bone) and one dog that died immediately after surgery due to hypovolemic shock, yielding a cohort of 20 evaluable VSV-clFNβ-NIS treated dogs. When compared to the control cohorts, neoadjuvant VSV-clFNβ-NIS therapy did not significantly improve EFS or overall survival (Figure 24A), but importantly it did not worsen survival outcomes. We analyzed long-term survivorship of evaluable VSV-clFNβ-NIS treated dogs by tabulating the proportion of dogs with survival that exceeded the 75th percentile value of the COTC cohorts (479 days). In this analysis, the VMC cohort showed that 26% of dogs exceeded the overall survival benchmark of 479 days, virtually identical to the proportion of dogs in the COTC cohort. In contrast, the VIGOR study cohort had a higher-than-expected proportion of long-term survivors, with 35% of dogs exceeding the benchmark of 479 days (Figure 24B and C). This potential efficacy signal was intriguing and noteworthy, although the sample size was insufficient to predict with high certainty whether this finding will be reproducible in a larger sample set.

To begin to establish a mechanism for this response, we completed next generation RNA sequencing for tumor samples from dogs in the VIGOR study. Tumor biopsies were obtained from dogs prior to

treatment and 10 days after administration of neoadjuvant VSV (at the time of amputation and prior to chemotherapy. mRNA was extracted from the biopsies and subjected to next-generation RNA sequencing. Tumor samples for sequencing were not available for dogs in either of the clinical control cohorts, but they were available for a fourth independent cohort of dogs, obtained from the Canine Comparative Oncology and Genomics Consortium, Inc. (CCOGC). Dogs from the CCOGC cohort were selected based on having received standard of care therapy (amputation plus adjuvant chemotherapy) with relevant survival metadata. The overall survival for dogs in this group was comparable to that of the two control cohorts (VMC and COTC). Sample processing and next-generation RNA sequencing for this group were supported, in part, with funds from this project.

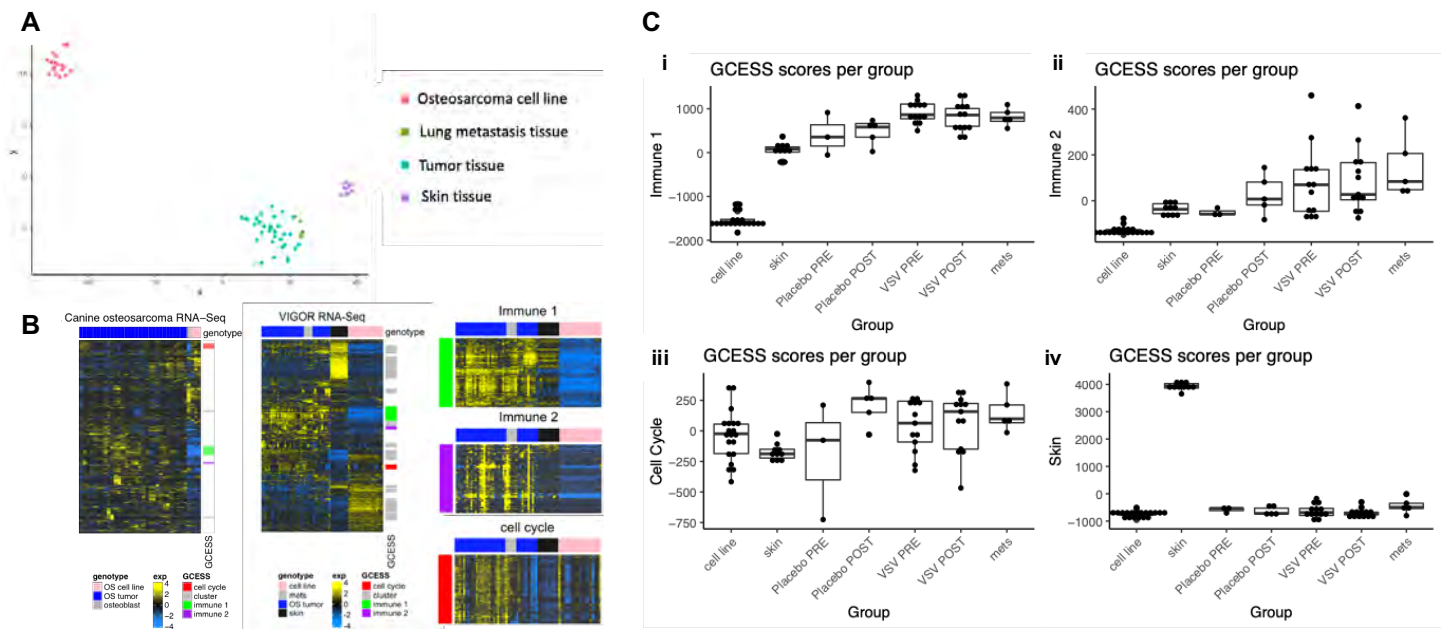


Figure 25. Gene cluster expression summary scores (GCESS) in dogs with osteosarcoma treated with neoadjuvant VSV followed by standard of care (the VIGOR trial).

Twenty-eight (28) dogs were enrolled in the VIGOR trial evaluating safety and efficacy of neoadjuvant VSV-IFN β in dogs with treatment naïve osteosarcoma. The study began as an open label trial to evaluate safety, as well as whether mitigation practices would be needed to control environmental shedding. Safety and absence of environmental shedding were addressed in the first 15 dogs, after which the trial was modified to a double-blinded, placebo controlled study. Thirteen additional dogs were enrolled into this second phase of the study. Pre-treatment needle core biopsies were collected from each dog prior to VSV treatment. Tumor samples were collected 10-days later at the time of definitive surgical management (amputation). Each sample was divided into sections for diagnosis (FFPE), RNAseq, and single cell disruption to establish cell lines. RNAseq was completed for 16 pre-treatment samples that passed quality control metrics (those for which the needle core biopsies were able to obtain high quality, viable tumor material). Panel (A) shows the distribution of primary tumors, metastatic tumors, skin samples, and tumor-derived cell lines on a t-distributed Stochastic Neighbor Embedding (t-SNE) plot. Panel (B) shows heat maps for a prior experiment characterizing the GCESS method and for the VIGOR samples, highlighting the two immune clusters and the cell cycle cluster. Panel (C) shows the distribution of GCESS for each sample group across the two immune clusters, the cell cycle cluster, and a skin cluster identified when we included the control skin samples from dogs in the VIGOR study.

We looked for evidence of an immune component in the tumor response using the gene cluster expression summary score (GCESS) method to summarize co-regulated gene clusters in tumor samples and their association with outcome¹. Pre- and post-treatment samples from primary osteosarcoma and metastatic osteosarcoma tumors segregated together, separate from both the cell

lines derived from the pre-treatment tumors as well as from normal skin biopsy samples obtained from the same dogs at the time of amputation (Figure 25A). This suggested that the composition of the microenvironment was a stronger driver of clustering than any traits associated with malignant transformation. Fourteen gene clusters were identified and were apparent in unsupervised hierarchical clustering heatmaps (Figure 25B), including the immune and cell cycle gene clusters previously identified in canine and human osteosarcoma¹. The distribution of cell cycle and immune GCESS across tumor samples, cell lines, and skin biopsy samples was predictable, with no immune transcripts identified in the osteosarcoma cell lines. VSV treatment did not significantly impact the GCESS score of pre- versus post-treatment tumor specimens (Figure 25C).

When we analyzed the association between GCESS and survival, we observed that the dogs with the highest CD8-related immune GCESS had longer survival times (Figure 26). This relationship was not observed for the CD37-positive monocyte-related GCESS, and somewhat surprisingly, it was also not evident for the cell cycle GCESS (Figure 26A and B). To assess whether neoadjuvant VSV was associated with the improved survival of dogs with higher CD-8 positive GCESS scores, we examined the relationship between GCESS and survival in the 23 dogs from the CCOGC cohort. When comparing the VIGOR study dogs and the CCOGC cohorts, we noted that a higher immune GCESS was associated with a longer survival in both groups (Figures 26 and 27) but the effect was larger in the VSV-treated group. Specifically, all the dogs with longer survival had higher CD8 GCESS, but not all the dogs with higher CD8 GCESS had longer survival in the CCOGC group (Figure 27 – see overlapping box plots), whereas in the VIGOR group, all of the dogs with higher CD8 GCESS had longer survival (Figure 26 – see non-overlapping VSV high and VSV low box plots).

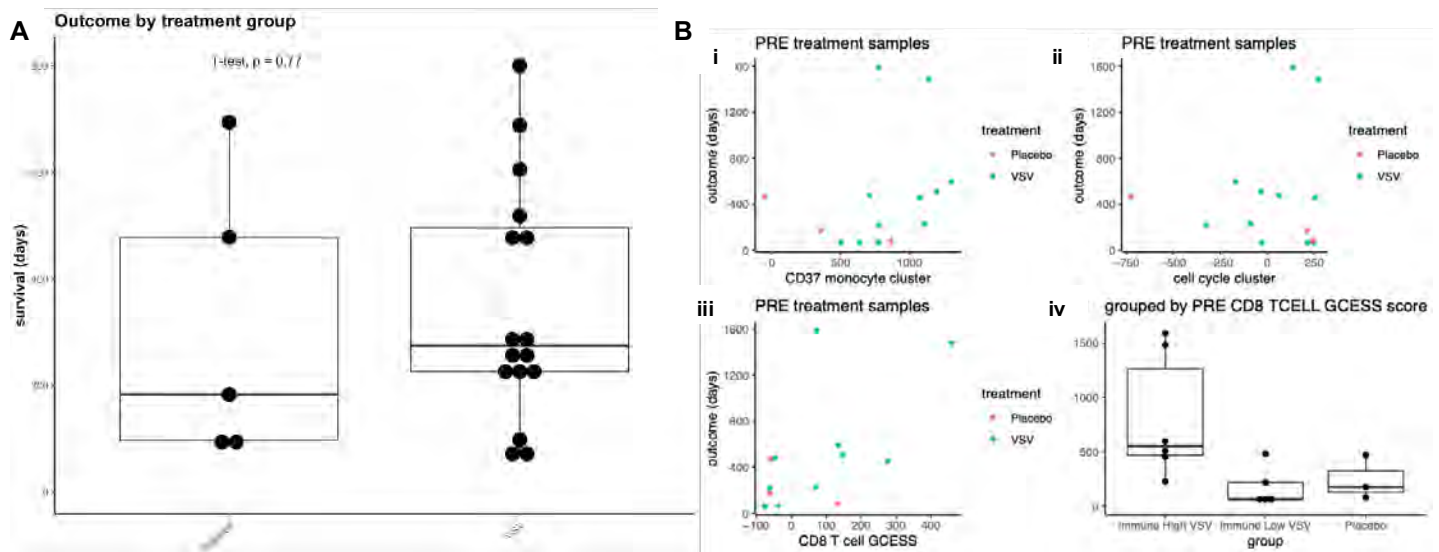


Figure 26. High immune gene cluster expression summary scores (GCESS) are associated with longer overall survival in dogs with hemangiosarcoma treated with neoadjuvant VSV followed by standard of care (the VIGOR trial).

Panel (A) shows the survival outcomes for dogs in the VIGOR trial receiving neoadjuvant VSV or placebo. Panel (B) shows survival of individual dogs as a function of the pre-treatment CD37 (innate immune) GCESS (panel i), pre-treatment cell cycle GCESS (panel ii), and pre-treatment CD8 (adaptive immune) GCESS (panel iii). Panel (B.iv) is a box and whisker plot showing the outcome for dogs with high CD8 GCESS treated with VSV, low CD8 GCESS treated with VSV, and dogs treated with placebo. The dogs with high CD8 GCESS treated with VSV survived longer than either of the other two groups ($p=0.04$ and $p=0.06$, respectively). There was no difference in survival between the dogs in the low CD8 GCESS group treated with VSV and the dogs in the group treated with placebo ($p=0.55$). Similar results were seen when comparing dogs where post-VSV treatment biopsy samples had high vs low CD8 GCESS.

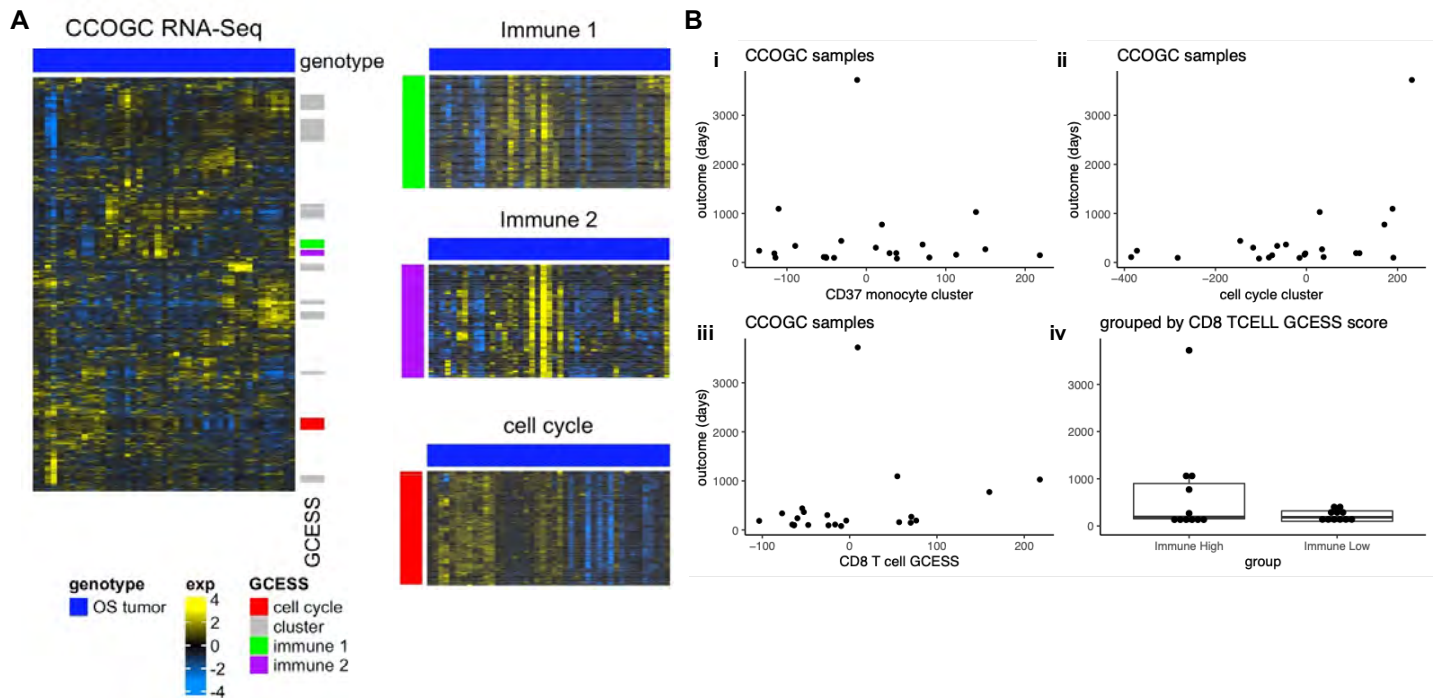


Figure 27. Gene cluster expression summary scores (GCESS) in dogs with osteosarcoma treated with the standard of care (CCOGC cohort).

Twenty-three (23) dogs were included in this analysis. Panel (A) shows heat maps for osteosarcoma samples from the CCOGC cohort highlighting the two immune clusters and the cell cycle cluster. Panel (B) shows survival of individual dogs as a function of the pre-treatment CD37 (innate immune) GCESS (panel i), pre-treatment cell cycle GCESS (panel ii), and pre-treatment CD8 (adaptive immune) GCESS (panel iii). Panel (B.iv) is a box and whisker plot showing the outcome for dogs with high CD8 GCESS.

- **What opportunities for training and professional development has the project provided?**

In Dr. Modiano's lab, one of the technicians involved in the project (Ms. Taylor DePauw) was accepted into graduate school starting in the Fall of 2021. This is a great achievement for a young person who wishes to eventually contribute to the research enterprise as a principal investigator.

Dr. Makielski continues her progression towards independence, having been promoted from a post-doctoral position to a Researcher-5 position, which is in the professional academic and administrative track. Dr. Makielski is committed to developing an independent research program focused on developing more refined and accurate diagnostic methods and more effective and safer immunological therapies for osteosarcoma. She has recently received notification that her Special Emphasis Research Career Award (SERCA, K01) in Pathology and Comparative Medicine got a fundable score and is expected to be awarded during this cycle (Fall 2022). Dr. Makielski is currently applying for an Assistant Professor position at the University of Minnesota.

Ms. Sonam Varnasi (Sonam) is a student enrolled in the Masters in Stem Cell Biology program at the University of Minnesota. Sonam has completed her master's thesis work developing the mouse osteosarcoma models and the phagocytosis assays to quantify ONIx potency. Sonam is writing her thesis with a defense date expected in December 2022. She aims to secure a job working as a scientist in industry.

Ms. Lauren Kreager joined Dr. Modiano's laboratory as a Researcher-2 (technician) after 3 years working in the lab as an undergraduate student. She aims to apply to graduate school for the 2023 academic year.

Dr. Aishwarya Sathyanarayan was involved in execution in the preliminary pilot studies in the K7M2, MC17, and A20 mouse models. During COVID-19 she became involved in the institutional response to the pandemic and shifted roles to gain training in viral vector production. She leveraged these skills and decided to take a new position in industry working in a GMP viral vector production role in November 2020. Recruitment to replace Dr. Sathyanarayan's role was challenging during the pandemic. Dr. Naik has recently hired and is training a research technologist (see below) and is recruiting an additional post-doctoral fellow to continue work on this project.

Caitlin Preusser recently completed her Bachelor of Science from St. Mary's University. She was recruited into a research technologist position in Dr. Naik's lab and has received extensive training in methods in virology, molecular biology, immunology, and executing preclinical in vivo studies in mice.

Finally, Dr. Naik, the Co-PI for this award was promoted to an Associate Consultant and attained her Assistant Professor academic rank at the Department of Molecular Medicine at the Mayo Clinic. She was also the recipient of a U01 award (September 2022) to investigate the immunomodulatory effects of novel oncolytic and immunotherapy combinations in relapsed or refractory lymphoma.

- **How were the results disseminated to communities of interest?**

Emerging data from this project have been disseminated through publication of peer reviewed manuscripts and abstracts/posters at scientific meetings (listed below).

- **What do you plan to do during the next reporting period (no-cost extension period) to accomplish the goals?**

We plan to continue pre-clinical development, studies to characterize the mechanisms of action of VSV and ONIx, alone and in combination, and to complete the canine clinical trial.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

We published a thought-provoking perspective challenging current notions of cancer risk and highlighting novel advantages, as well as limitations of how companion dogs can inform cancer risk in humans.

1. Sarver AL, Makielski KM, DePauw TA, Schulte AJ, Modiano JF. (2022). Increased risk of cancer in dogs and humans: a consequence of recent extension of lifespan beyond evolutionarily determined limitations? (Perspective). *Aging and Cancer*, 3(1), 3-19. PMID: 35993010. PMCID: PMC9387675

We documented that canine and human osteosarcoma represent convergent diseases, requiring a deliberate and cautious approach to advancing and/or using canine osteosarcoma as a model for human osteosarcoma. The manuscript is undergoing peer review in consideration for publication.

1. Sarver AL, Mills LJ, Makielski MK, Temiz NA, Wang J, Spector L, Subramanian S, Modiano JF. Distinct mechanisms of PTEN inactivation in dogs and humans highlight convergent molecular events that drive cell division in the pathogenesis of osteosarcoma

Our data describing the safety and immunomodulatory activity of systemic VSV therapy administered in the neoadjuvant setting in dogs undergoing standard of care amputation and chemotherapy following

diagnosis of osteosarcoma has been compiled into a manuscript that will be submitted for publication in October 2022.

We disseminated our findings through abstract and poster presentations at national and international meetings.

1. Schulte AJ, Durose W, Nolan E, Taghizadeh L, Todhunter D, O'Sullivan MG, Seelig D, Vallera DA, Lund TC, Modiano JF. (2022). Therapeutic effect of eBAT is mediated by modulating the tumor microenvironment. Proceedings of the Mid-Year Meeting of the Veterinary Cancer Society
2. Callaghan CM, Sarver AL, Kim JH, Makielski KM, Modiano JF, and Medland JE. (2022). The Immune and Molecular Landscape of Canine Osteosarcoma. Proceedings of the National Veterinary Scholars Symposium, St Paul, MN, August 5, 2022

- **What was the impact on other disciplines?**

We have raised awareness and attention of how dogs and humans share a recent history of breaking through their evolutionarily adapted lifespan barriers through technological achievements. Dr. Modiano has been invited to present our research, "Identification and Characterization of the Cancer Permissive Environment in Companion Dogs," as an oral platform talk at the AACR Aging and Cancer Meeting in San Diego in November of 2022.

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Our work is highlighting a new opportunity to expand the role of dogs in society, not only as companions and service animals, but as research subjects that can inform methods and strategies to treat, manage, and prevent cancer and to generally help us understand how we can maintain our health through graceful aging. This is especially relevant to military personnel after they leave active service.

5. Changes/Problems

- **Changes in approach and reasons for change**

As described in the Accomplishments section, the K12 and K7M2 mouse osteosarcoma models have not proved to be as reliable as we expected, and so we are developing alternative models to investigate our hypotheses and address the goals of this project, including new cell lines in the C57Bl/6 background and the MC17 sarcoma model.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

As we described above, the COVID-19 pandemic and the responses to the pandemic caused significant disruptions to the project. From the departure of a key person (postdoc) at the Mayo site to lab closures that lasted more than a year, to persistent problems sourcing reagents and materials and a very strong

and direct request from the U of M administration to consider reducing animal activities due to staffing problems.

Fortunately, as the severity of the pandemic has abated, we have been able to resume work and make progress. We are grateful that the program officers and the agency approved a no-cost extension that will allow us to complete the proposed experiments to achieve our aims.

- **Changes that had a significant impact on expenditures**

The COVID-19 pandemic and the resultant requirement for remote work meant that personnel effort (salary) had to be dedicated to “soft” tasks, including data analysis (see for example the data presented in Figures 3, 4, and 8 of this report), and instead of “hard” tasks (experiments in the lab). This does not appear to have created an imbalance in the budget categories. If salary support needs us to consider re-budgeting for the no-cost extension period, we will contact our program officer to discuss.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None

- **Significant changes in use or care of human subjects**

None

- **Significant changes in use or care of vertebrate animals.**

None

- **Significant changes in use of biohazards and/or select agents**

None

6. Products

Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	<i>Jaime Modiano</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	0000-0001-6398-7648
Nearest person month worked:	2

Contribution to Project:	<i>Dr. Modiano is the contact PI. He has overseen the work and contributed to the experiments conducted at the University of Minnesota, as well as to the analysis of all project data.</i>
Funding Support:	<i>Institutional funds (endowed Chair)</i>

Name:	<i>Shruthi Naik</i>
Project Role:	<i>Co-PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	<i>Dr. Naik is the Mayo site PI. She has contributed to all the experiments performed at the Mayo Clinic, including supervision of personnel (post-doc) and data analysis.</i>
Funding Support:	

Name:	<i>Michael Henson</i>
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Henson is the clinical lead for the canine clinical study (METEOR). He was involved in study design, organization, and was the attending clinician for the case enrolled in the study.</i>
Funding Support:	

Name:	<i>Kelly Makieslki</i>
Project Role:	<i>Postdoctoral research associate</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	<i>Dr. Makielski contributed to the experiments performed at the University of Minnesota (in vitro validation of ONIx, MC17 model experiments, and analysis of the VIGOR trial).</i>
Funding Support:	

Name:	<i>Lauren Mills</i>
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Project Role:	<i>Research Associate</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	<i>Dr. Mills has been involved in data storage and data management, especially for sequencing data, and she performed the ATAC-seq analysis.</i>
Funding Support:	

Name:	<i>Andy Sicheneder</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Mr. Sicheneder was responsible for production of ONIx.</i>
Funding Support:	

Name:	<i>Mitzi Lewellen</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Ms. Lewellen is involved with managing inventories and the animal colonies in the Modiano lab. For this project, she has kept experimental inventories and managed the mouse colonies for the K7M2 and MC17 experiments.</i>
Funding Support:	

Name:	<i>Ashley Schulte</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	<i>Ms. Schulte was the lead scientist in the development of the MC17 model. She also generated the genome-edited mouse</i>

	<i>osteosarcoma cell lines with assistance from the GESR at the University of Minnesota. She has contributed to QC testing and potency assays for ONIx and has been the lab manager and safety officer for the Modiano lab throughout the project.</i>
Funding Support:	<i>Karen Wyckoff Rein in Sarcoma Foundation; Morris Animal Foundation</i>

Name:	<i>Taylor DePauw</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Ms. DePauw was responsible for leading the ONIx validation studies (in vitro) during year 1. She also was responsible for all purchasing activity and equipment maintenance in the Modiano lab during year 1. Ms. DePauw left the lab to start graduate school at the end of year 1.</i>
Funding Support:	

Name:	<i>Lauren Kreager</i>
Project Role:	<i>Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Ms. Kreager joined the lab during year-2 of this project. She assisted with the experiments to develop the mouse models of osteosarcoma and with the experiments for single cell sequencing. She was the lead scientist in the experiments to assess the chromatin landscape in lymphocytes.</i>
Funding Support:	<i>Institutional Funds</i>

Name:	<i>Caitlin Preusser</i>
Project Role:	<i>Research technologist</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Ms. Preusser joined the lab during year-2 of this project. She assisted with the experiments to evaluate the impact of VSV and ONIx in the mouse models and the correlative experiments. Ms.</i>

	<i>Preusser is the lab manager and safety officer for the Naik lab, where she is also responsible for purchasing activity and equipment maintenance.</i>
Funding Support:	

Name:	<i>Kathy Stuebner</i>
Project Role:	<i>Certified Veterinary Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Ms. Stuebner was the coordinator for the Clinical Investigation Center of the University of Minnesota College of Veterinary Medicine. She assisted with completion of all the documentation needed to file IACUC and IBC approvals as well as ACURO approvals, as well as with amendments. She prepared the enrollment site for the canine clinical study and was responsible for coordinating release assays for ONIx.</i>
Funding Support:	

Name:	<i>Amber Winter</i>
Project Role:	<i>Certified Veterinary Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Ms. Winter worked with Dr. Modiano and Ms. Stuebner to complete the documentation for IACUC and ACURO approval during year 1.</i>
Funding Support:	

Name:	<i>Andrea Chehadeh</i>
Project Role:	<i>Certified Veterinary Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	<i>Ms. Chehade worked with Drs. Modiano and Henson and with Ms. Stuebner to complete preparations for the canine clinical study (METEOR) and is the lead nurse for the study.</i>

Funding Support:	
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Name:	<i>Aishwariya Sathyanarayan</i>
Project Role:	<i>Postdoctoral research associate</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	<i>Dr. Aishwariya initiated the mouse experiments at Mayo Clinic before COVID-related events forced her relocation from Dr. Naik's lab.</i>
Funding Support:	

Name:	<i>Aaron Sarver</i>
Project Role:	<i>Assistant Professor</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Sarver was responsible for analysis of the RNAseq data shown in Figure 8.</i>
Funding Support:	<i>NIH grant R50 CA211249 (year-1) and Masonic Cancer Center – Cancer Center Core Support Grant P30 CA077598</i>

Name:	<i>Davis Seelig</i>
Project Role:	<i>Associate Professor</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Seelig was responsible for histopathological analysis of mouse necropsy tissues from the K7M2 study. Dr. Seelig took over as retired as Director of the Masonic Cancer Center Comparative Pathology Shared Resource Core at the beginning of year 2 of this project.</i>
Funding Support:	<i>Masonic Cancer Center – Cancer Center Core Support Grant P30 CA077598</i>

Name:	<i>Jong Hyuk Kim</i>
Project Role:	<i>Assistant Professor</i>

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Kim assisted with the animal experiments and histopathological analysis of mouse necropsy tissues for the MC17 experiments.</i>
Funding Support:	

Name:	<i>M. Gerard O'Sullivan</i>
Project Role:	<i>Professor</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. O'Sullivan was responsible for histopathological analysis of mouse necropsy tissues from the MC17 experiments. Dr. O'Sullivan retired as Director of the Masonic Cancer Center Comparative Pathology Shared Resource Core at the end of year 1 of this project.</i>
Funding Support:	<i>Masonic Cancer Center – Cancer Center Core Support Grant P30 CA077598</i>

Name:	<i>Sonam Varnasi</i>
Project Role:	<i>Master's in Science graduate student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	<i>In vivo model development; ONIx/eBAT validation studies.</i>
Funding Support:	<i>Institutional funds</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Several grants held by the PI at the start of this project have ended (DOD IDEA Award, K01 mentored transition to independence award where the PI was a mentor, T32 support for a postdoc, two fixed cost contracts from Boston Scientific, a contract from Vyriad, and grants from the AKC Canine Health Foundation and Morris Animal Foundation).

New grants awarded to the PI since the start of this project include a grant from Venn Foundation intended to pursue commercialization of inventions in the PI's lab, a competitive renewal from the AKC Canine Health Foundation, two new grants from the AKC Canine Health Foundation, a U01 contract from the NCI, and an expected K01 mentored transition to independence award from the NIH Office of the Director where the PI is a mentor.

The PI's effort on this grant has not changed.

- **What other organizations were involved as partners?**

The only two organizations involved, University of Minnesota and Mayo Clinic, were listed in the original application.

8. Special Reporting Requirements

None

9. Appendices

a. Literature Cited

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b. Abbreviations Used in this Report

ACURO: animal care and use review office
ATF: amino terminal fragment
AYAs: adolescents and young adults
CD: cluster designation
CDMRP: congressionally directed medical research program
CPIs: checkpoint inhibitors
CRISPR: clustered regularly interspaced short palindromic repeats
COVID: (sudden acute respiratory syndrome) coronavirus-2 (2019) induced disease
DOD: (U.S.) Department of Defense
eBAT: EGF bispecific angiotoxin
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
FITC: fluorescein isocyanate
GFP: green fluorescent protein
EU: endotoxin units
Fluc: firefly luciferase
Gluc: Gaussia luciferase
IACUC: institutional animal care and use committee
IBC: institutional biosafety committee
IFN β : interferon-beta
iv: intravenous
KO: knockout
LTT: ligand targeted toxin
Luc: luciferase
MDSC: myeloid derived suppressor cells
NCI: National Cancer Institute (of the National Institutes of Health)
NIS: sodium iodide symporter
ONIx: onco-immune accelerator
PD-1: programmed death (receptor)-1
PD-L1: programmed death ligand-1
P-DIC: peptide for dual immune checkpoint blockade (this nomenclature has been replaced with the term onco-immune accelerator or ONIx)
PE: *Pseudomonas* exotoxin
PRCRP: peer reviewed cancer research program
PRMRP: peer reviewed medical research program
QA: quality assurance
QC: quality control
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRP: signal regulatory protein
SOP: standard operating protocol
SOW: statement of work
sq: subcutaneous
TAMs: tumor associated macrophages
TCID₅₀: half maximal tissue culture infective dose
TME: tumor microenvironment
uPA: urokinase type plasminogen activator
uPAR: urokinase type plasminogen activator receptor
VSV: vesicular stomatitis virus
WT: wild type

c. Other Reports

- i. **Pathology Report for Balb/c Mice Injected with K7M2 Cells**
(next page)

**University of Minnesota Masonic Cancer Center
Comparative Pathology Shared Resource**

CPSR Study Number:

22-237

Study Title

Histopathologic Analysis of BALB/c Mice Injected with K7M2 Mouse Osteosarcoma Cells

Author:

Davis M. Seelig, DVM, PhD
Diplomate, A.C.V.P.

&

Emma Torii, BVSc, MANZCVS (Pathobiology),
Postdoctoral associate

Comparative Pathology Shared Resource
Masonic Cancer Center
University of Minnesota
1352 Boyd Ave, St. Paul, MN 55108

Study Sponsor:

N/A

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1. Regulatory Compliance Statement and Data Integrity

This non-GLP study was conducted for research and development purposes. Sample receipt, processing, analysis and data reporting are performed in alignment with the best practices of The Society of Toxicologic Pathology. Histopathological evaluation was done in the spirit of FDA regulations on good laboratory practices (GLP) for nonclinical laboratory studies CFR title 21 part 58. The chain of custody for tissues analyzed and slides reported was verified by the study pathologist. The undersigned signifies that they approve all pathology-related data within this report, including tables, narrative, and overall summary.

Pathologist's Signature

Study Title: Histopathologic Analysis of BALB/c Mice Injected with K7M2 Mouse Osteosarcoma Cells

CPSR Study Number: 22-237

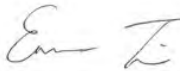
Sponsor Study Number: N/A

Study Pathologist(s)

Signature: 

Davis M. Seelig, DVM, PhD
Diplomate, A.C.V.P.

Date: September 6, 2022

Signature: 

Emma Torii, BVSc,
MANZCVS (Pathobiology),

Date: September 06, 2022

2. Study background and experimental objectives (from submitter)

- a. Female BALB/c mice injected with K7M2 mouse osteosarcoma cells on May 10 in different dosages – IV injection of 1E6 K7Me cells in mice numbered 1, 2 and 3; IV injection of 0.5E6 K7Me cells in mice numbered 4,5 and 6; IV injections of 0.25E6 K7Me cells in mice numbered 7, 8 and 9. 1 sample included from the mice numbered 14, received SQ injection of 1E6 K7Me cells.

3. Gross Necropsy Findings:

- a. None Provided

4. Clinical Pathology Data:

- a. None Provided

5. Methods

- a. Received tissues were paraffin-embedded, sectioned, mounted onto slides and stained with Hematoxylin and Eosin (H&E) using standard techniques¹. See Table 1 (below) for slide annotation information
- b. H&E-stained sections were evaluated using light microscopy according to STP guidelines². H&E-stained sections were evaluated.

Table 1. Study groups in histopathologic analysis

CPSR ID	Investigator Animal ID	Genotype	Sex	Treatment	List of tissues
22-237-1	M10K7M2-IV1-1	BALB/c	Female	1E6 K7M2 cells IV	Lung, Spleen
22-237-2	M10K7M2-IV1-2	BALB/c	Female	1E6 K7M2 cells IV	Lung, Spleen
22-237-3	M10K7M2-IV1-3	BALB/c	Female	1E6 K7M2 cells IV	Lung, Spleen, Tail
22-237-4	M10K7M2-IV0.5-4	BALB/c	Female	0.5E6 K7M2 cells IV	Lung, Spleen, Tail
22-237-5	M10K7M2-IV0.5-5	BALB/c	Female	0.5E6 K7M2 cells IV	Lung, Spleen
22-237-6	M10K7M2-IV0.5-6	BALB/c	Female	0.5E6 K7M2 cells IV	Lung, Spleen
22-237-7	M10K7M2-IV0.25-7	BALB/c	Female	0.25E6 K7M2 cells IV	Lung, Spleen
22-237-8	M10K7M2-IV0.25-8	BALB/c	Female	0.25E6 K7M2 cells IV	Lung, Spleen
22-237-9	M10K7M2-IV0.25-9	BALB/c	Female	0.25E6 K7M2 cells IV	Lung, Spleen
22-237-10	M10K7M2-SQ1-14	BALB/c	Female	1E6 K7M2 cells SQ	Skin and subcutaneous fat with possible tumor remnant

6. Results:**a. Descriptive histopathologic analysis of submitted tissues****Table 2. Summary histopathologic findings from submitted tissues.**

CPSR ID	Tissue	H&E Findings
22-237-1	Spleen	There is diffuse moderate congestion and extramedullary hematopoiesis (EMH; considered appropriate for species).
	Lung	There is diffuse mild alveolar vascular congestion, and mild increased cellularity of the alveolar septa by owing to increased few large round mononuclear cells (presumed to be macrophages) and few small lymphocytes. Particularly at the edge of the sections, there are small foci of atelectasis.
22-237-2	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Changes are similar to that described in the lungs of 22-237-1.
22-237-3	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Affecting approximately <5% of the total surface area, predominantly perivascularly and intravascularly, and less frequently randomly, there are small clusters of neoplastic cells similar to that described in the tail below (22-273-3). In a deeper section, multiple cross and tangential sections of large-caliber blood vessels contain tumor emboli. The remainder of the lungs have changes similar to that described in the lungs of 22-237-1.
	Tail	Surrounding, infiltrating, and effacing a vertebra and its marrow cavity and associated skeletal muscle, and elevating the overlying dermis and epidermis is an unencapsulated, moderately well-demarcated, densely cellular mass consistent with a sarcoma. The neoplastic cells are arranged into ill-defined broad sheets to poorly-formed bundles and streams, and are supported by a fine fibrovascular stroma. Rarely, between neoplastic cells are scant amounts of wispy, fibrillary, eosinophilic material (possible osteoid, and/or fibrous tissue). The neoplastic cells are plump spindle shaped with indistinct cell borders, and have a large amount of eosinophilic to amphophilic cytoplasm. The nuclei are round to oval with vesicular chromatin, and have up to 3 prominent, magenta nucleoli. There is marked anisocytosis and anisokaryosis. The mitotic count is 39 in 2.37mm ² (equivalent to 10 HPF), with frequent atypical mitotic figures. Throughout the mass are individual cell necrosis and/or apoptosis, as well as scattered, low numbers of lymphocytes, and rarely eosinophils.
22-237-4	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Within the right caudal lung lobe is an approximately 4 x 3 mm, unencapsulated, well-demarcated sarcoma that moderately compresses the surrounding lungs. The mass is similar to that described in the tail of 22-237-3, although with increased pleomorphism, including increased anisocytosis, anisokaryosis and irregularly indented nuclei. In addition, there are occasional multinucleated cells, and cells with bizarre, karyomegalic nuclei. There are a few medium-sized foci of lytic necrosis. The mitotic count is 17 in 2.37mm ² (equivalent to 10 HPF) with

		frequent atypical mitotic figures. The remainder of the lung lobes have changes similar to that described in the lungs of 22-237-1.
	Tail	Within the subcutis and dermis, and elevating the overlying epidermis is a moderately well-demarcated, unencapsulated, oval mass composed of neoplastic cells similar to that described in the lungs of 22-237-4, although with fewer cells with bizarre nuclei. The mitotic count is 18 in 2.37mm ² (equivalent to 10 HPF) with frequent atypical mitotic figures.
22-237-5	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	In one lung lobe, there is a small subpleural mass consisting of neoplastic cells similar to that described in the tail of 22-237-3, although with increased anisocytosis, anisokaryosis and rare multinucleated cells. The mitotic count is 14 in 2.37mm ² (equivalent to 10 HPF) with frequent atypical mitotic figures. The remainder of the lung lobes have changes are similar to that described in the lungs of 22-237-1.
22-237-6	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Changes are similar to that described in the lungs of 22-237-1.
22-237-7	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Changes are similar to that described in the lungs of 22-237-1.
22-237-8	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Changes are similar to that described in the lungs of 22-237-1, although the alveolar septal thickening is mild. There are rare medium-caliber blood vessels that are surrounded by moderate numbers of macrophages, eosinophils and fibroblasts. On deeper section, the perivascular inflammation was absent.
22-237-9	Spleen	Changes are similar to that described in the spleen of 22-237-1.
	Lung	Changes are similar to that described in the lungs of 22-237-1, although there is reduced alveolar septal thickening.
22-237-10	Skin and subcutaneous Tissue	The tissue consists of skin, subcutis, multiple small quiescent mammary glands and ducts, abundant adipose tissue and a lymph node. There are no significant abnormalities detected.

Representative Images

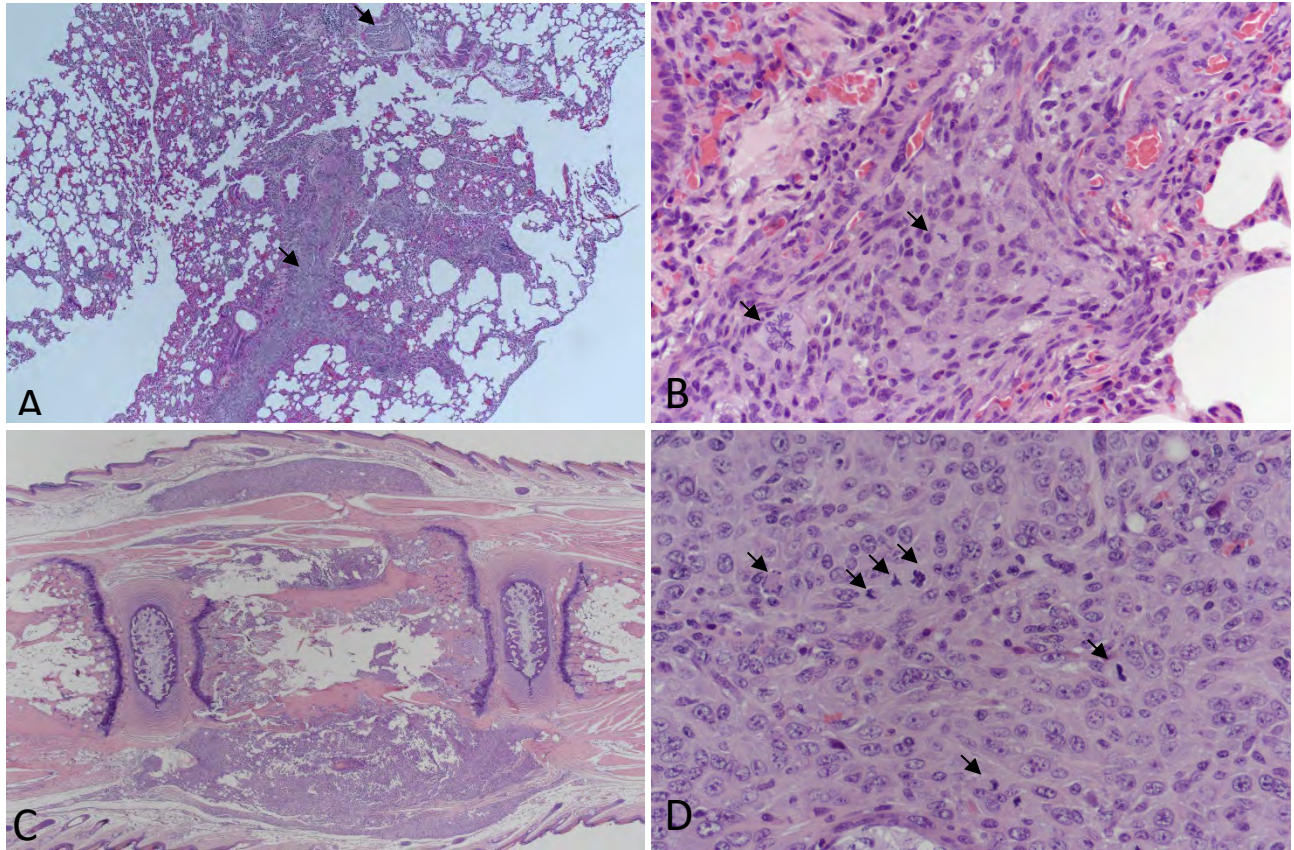


Figure 1 – Mouse 3, lungs (A-B) and tail (C-D), HE. A. Lungs, 40x. Multiple cross sections of vessels have tumor emboli (arrows). **B. Lungs, 400x.** Higher magnification of tumor emboli. Note multiple mitotic figures (arrows), some of which are bizarre (left arrow). **C. Tail, 20x.** Surrounding and infiltrating a vertebra is a sarcoma. **D. Tail, 400x.** Higher magnification of the sarcoma. There are numerous mitotic figures (arrows), many of which are atypical.

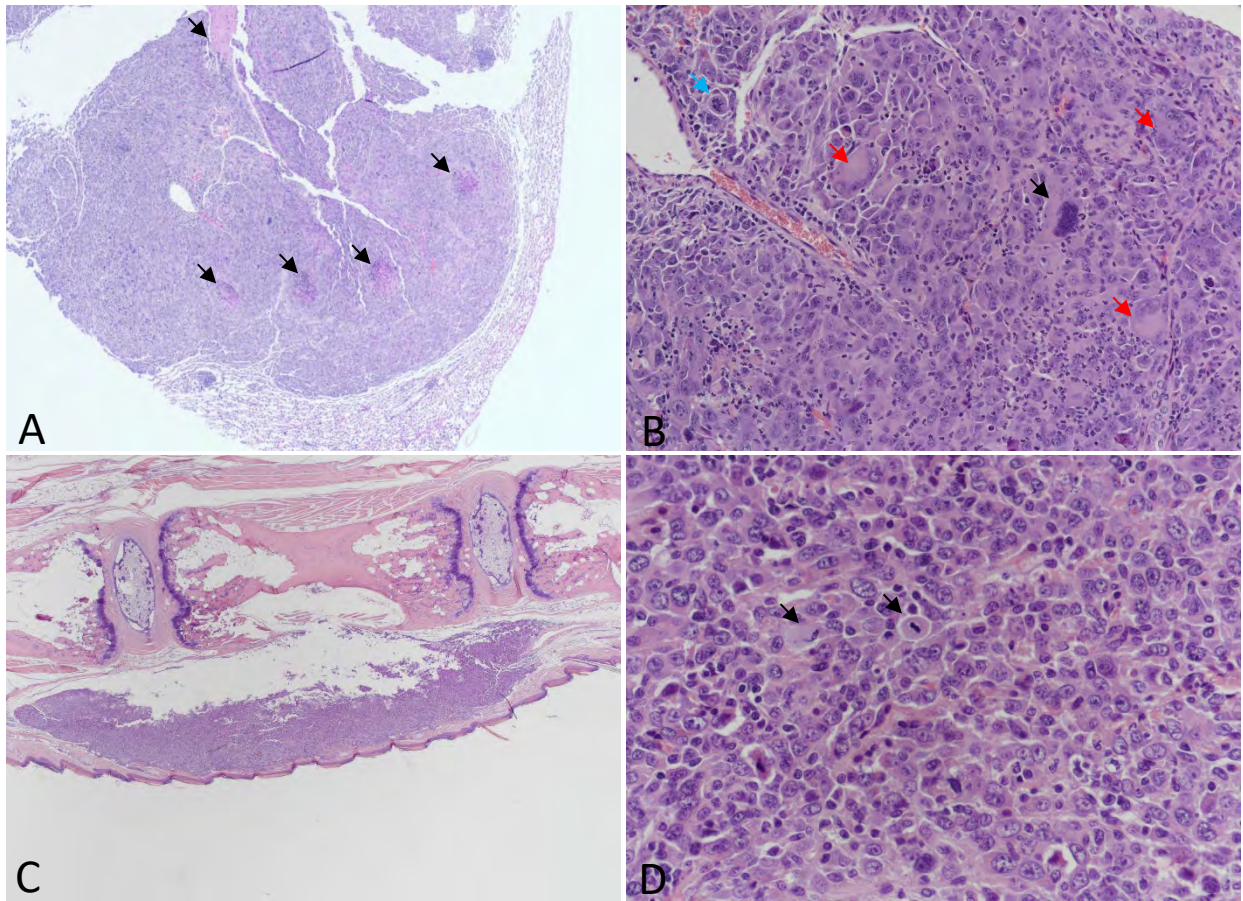


Figure 2 - Mouse 4, lungs (A-B) and tail (C-D), HE. **A. Lungs (right caudal lobe), 40x.** Well-demarcated sarcoma metastasis that moderately compresses the adjacent lung. Throughout the tumor are multiple necrosis (arrows). **B. Lungs, 200x.** Higher magnification of the mass. Compared to mouse 3, there is increased pleomorphism with occasional large cells with atypical, large, bizarre nuclei (black arrow) and multinucleated cells (red arrows). There is an atypical mitotic figure (blue arrow). **C. Tail, 20x.** Surrounding a vertebra is a sarcoma. **D. Tail, 400x.** Higher magnification of the sarcoma. There are numerous mitotic figures (arrows), many of which are atypical.

7. Project Summary:

- a. Three of nine mice (22-237-3, 22-237-4, and 22-237-5) had sarcoma within the lungs
 - i. Two of these mice (22-237-3 and 22-237-4) each had a mass in the submitted tails with similar histological appearance.
 - ii. There were rare, scant amounts of wispy material that somewhat resembled osteoid, however pre-existing stroma and/or fibrous tissue could not be excluded. Despite lack of convincing osteoid, osteosarcoma was considered most likely given the treatment history.
 - iii. All the masses across the affected mice had similar morphology, albeit 22-237-4 and 22-237-5 were more anaplastic. Differences in morphology of the K7M2 cells have been reported.⁴
 - iv. No other mice had evidence of neoplastic cells.
 - v. The prevalence of pulmonary metastasis in this study is in contrast to a previous publication, where intravenous injection of K7M2 osteosarcoma cell lines resulted in pulmonary metastasis and subsequent death in all treated mice.³
- b. Lungs:
 - i. All mice had mild diffuse increased cellularity of the alveolar septa.
 - ii. Subjectively, this change seemed less severe in the mice given the lower dosages.
 - iii. To our knowledge, previous publications using K7M2 cell lines did not provide any descriptions of lung pathology aside from metastasis.^{3,4} If further investigation is warranted, examination of age and sex-matched control mice is recommended.
- c. Spleen:
 - i. None of the mice had gross or histologic evidence of neoplastic cells.

8. Recommendations

- a. There was a lower than expected number of mice with pulmonary metastasis compared to previous publications. It is possible that small metastases may not have been evident in the examined section. Hence, if further investigation is warranted, deeper sections of each blocks can be requested at an additional cost.
 - i. However, it is possible that the differences between previously published work and this study may reflect: different sacrifice time-points, different age at tumor cell injection, and different tumor cell dosages (the cited publication above describes an inoculation dosage of 1×10^6 cells).
- b. Submission of control animals may be warranted to compare the pulmonary alveolar changes. In addition, adequate inflation of the lungs at the time of fixation will reduce the atelectasis, allowing for improved interpretation of the cellularity.

9. References

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ii. **ONIx Sterility Report**
(next page)

FINAL REPORT OF LABORATORY EXAMINATION

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IDEXX BioAnalytics Case # 114232-2022

Client Case # n/a

Submitted By

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Received Date: 5/20/2022

Completed: 6/3/2022

Specimen Description

Species: unspecified

Description: Culture Swab

Number of Specimens/Animals: 12

Building/Facility: VMC

Study: eBAT

Purchase Order #: 000225971

ID	Client ID	Room #	Species
1	Vial1-O	D317	unspecified
2	Vial1-G	D317	unspecified
3	Vial1-R	D317	unspecified
4	Vial1-DR	D317	unspecified
5	Vial2-O	D317	unspecified
6	Vial2-G	D317	unspecified
7	Vial2-R	D317	unspecified
8	Vial2-DR	D317	unspecified
9	Vial3-O	D317	unspecified
10	Vial3-G	D317	unspecified
11	Vial3-R	D317	unspecified
12	Vial3-DR	D317	unspecified

Services/Tests Performed: Aerobic and Anaerobic Culture and ID - bacteria and fungi (1-12)**Microbiologic evaluation for:** Aerobic bacterial growth, Anaerobic bacterial growth, Fungal growth

General Comments: Culture Swab - O=outside, G=gray cap, R=inside rim, DR=drug

Summary: All test results were negative.

MICROBIOLOGY

Specimen/Source: culture

Isolate	1	2	3	4	5	6	7	8	9	10
Aerobic bacterial growth	-	-	-	-	-	-	-	-	-	-
Anaerobic bacterial growth	-	-	-	-	-	-	-	-	-	-
Fungal growth	-	-	-	-	-	-	-	-	-	-

Specimen/Source: culture

Isolate	11	12
Aerobic bacterial growth	-	-
Anaerobic bacterial growth	-	-
Fungal growth	-	-

Legend: + = agent recovered - = agent not recovered blank = test not performed n = no growth X = Preliminary

d. Publications

- *Aging and Cancer* manuscript
(next page)

PERSPECTIVE

Increased risk of cancer in dogs and humans: A consequence of recent extension of lifespan beyond evolutionarily determined limitations?

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Abstract

Cancer is among the most common causes of death for dogs (and cats) and humans in the developed world, even though it is uncommon in wildlife and other domestic animals. We provide a rationale for this observation based on recent advances in our understanding of the evolutionary basis of cancer. Over the course of evolutionary time, species have acquired and fine-tuned adaptive cancer-protective mechanisms that are intrinsically related to their energy demands, reproductive strategies, and expected lifespan. These cancer-protective mechanisms are general across species and/or specific to each species and their niche, and they do not seem to be limited in diversity. The evolutionarily acquired cancer-free longevity that defines a species' life history can explain why the relative cancer risk, rate, and incidence are largely similar across most species in the animal kingdom despite differences in body size and life expectancy. The molecular, cellular, and metabolic events that promote malignant transformation and cancerous growth can overcome these adaptive, species-specific protective mechanisms in a small proportion of individuals, while independently, some individuals in the population might achieve exceptional longevity. In dogs and humans, recent dramatic alterations in healthcare and social structures have allowed increasing numbers of individuals in both species to far exceed their species-adapted longevities (by two to four times) without allowing the time necessary for compensatory natural selection. In other words, the cancer-protective

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in Sarcoma Foundation; Zach Sobiech Osteosarcoma Fund; Team Nat Fund of the Children's Cancer Research Fund; Animal Cancer Care and Research Program of the University of Minnesota

mechanisms that restrain risk at comparable levels to other species for their adapted lifespan are incapable of providing cancer protection over this recent, drastic, and widespread increase in longevity.

KEYWORDS

cancer-protective mechanisms, cancer risk, evolution, longevity

1 | INTRODUCTION: THE EVOLUTIONARY LEGACY OF CANCER

According to the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) Program, cancer describes a group of over 100 pathological conditions that are characterized by uncontrolled cellular proliferation.¹ Even including deaths due to COVID-19 in 2020, cancer is the most common or the second most common cause of death for humans in Europe² and in the United States,^{3–5} and it is believed that cancer is also the leading cause of disease-related death for older companion and working dogs in the developed world.^{6–12}

In a remarkable thesis of how cancer was transformed from an obscure and mystical condition to a medical problem,¹³ Koblenz notes that the death rate from cancer increased by almost 20-fold from the years 1850 to 2009, although it is apparent that a major portion of the cancer epidemic of the 20th century was driven by tobacco.¹⁴ For humans living in the United States in 2020, the lifetime probability of being diagnosed with an invasive cancer is approximately 40% (an approximate lifetime risk of 1 in 2.5).¹⁵ Tumors of the prostate, lung, and colon account for more than 40% of all cases in men, and tumors of the breast, lung, and colon account for 50% of all cases in women. Cancer also claims a large number of domestic dogs' and cats' lives.^{7,11,12,16–20} Even while the frequent occurrence of cancer in both of these domestic species is well recognized, the precise incidence of invasive cancers remains to be established.²¹ Registries that systematically account for cancer incidence and mortality of dogs and cats are not standardized, making comparisons among them challenging.²² Furthermore, many (perhaps most) cases of suspected cancers in animals are not definitively diagnosed histopathologically, and there are considerable referral biases in the hospital databases used to obtain estimates of cancer incidence and cancer mortality.²³

Despite these limitations, the Veterinary Cancer Society estimates that one in four dogs will be diagnosed with cancer in their lifetime, and that cancer is the leading cause of death in pets that are beyond middle age.²⁴

As hard as it is to obtain actuarial estimates in domestic animals, assessment of disease and cause-specific mor-

tality rates in wild animal populations is even more challenging.²⁵ The available data suggest that animals in the wild have variable incidence rates of specific cancers, although mortality is often due to loss of fitness, competition, predation, accidents, or human-related causes.²⁶ It is also clear that the overall rate of cancer among species in captivity is variable,²⁷ but none are as high as those seen in domestic dogs and cats.²⁸ Cancers in wild animals where the etiology can be determined are often due to infectious agents^{28,29} or the tumors themselves are transmissible,^{29–31} and it is notable that such infectious and transmissible cancers represent exceptions to the low rates of cancer observed in wild animals. Conversely, cancers associated with infectious agents (such as feline leukemia virus-associated tumors³²) and transmissible cancers (such as canine transmissible venereal tumors³³) account for only a small fraction of the rather large number of cancers seen in dogs and cats in the developed world.

Comparative biologists have questioned why cancer is so prevalent in domestic dogs and in humans, what accounts for the difference in cancer risk and incidence seen in dogs and humans as compared to wild populations, and what we can do about it. In this perspective, we propose that the apparent excess incidence of cancer in both of these species is due to the fact that dogs and humans are two species that have benefited from social, medical, and technological advances that allow them to live beyond the age that nature intended, as species-specific cancer-protective mechanisms that evolved over millions of years³⁴ cannot address the cancer vulnerabilities acquired through the rapid increase in longevity experienced by modern dogs and humans. The observation that there are substantial differences in the incidence of specific cancer types that afflict domestic pet dogs and humans, despite a closely shared environment, suggests that there are fundamental biological risk factors present in both species that are distinct from environmental exposures.^{15,21} This different prevalence of cancer types and the rather poor conservation of genomic driver events in histologically similar cancers of both species should raise a note of caution about "the dog as a model" of cancer causation.³⁵ On the other hand, the shared sensitivity of dogs and humans to age-associated cancers provides unique opportunities to understand the

relationships between organismal aging and cancer risk.³⁶ Finally, we advance the concept of risk assessment coupled with strategic prevention as a means to reduce cancer mortality in dogs, and eventually, perhaps also in humans.

1.1 | Cancer is an ancient disease

To understand the apparent cancer epidemic, it is essential to understand the foundational pathogenesis of cancer and its relationship to evolution across the animal kingdom. Cancer arises from the malignant transformation of a single cell that undergoes uncontrolled proliferation, but the disease is confined to multicellular animals.^{37,38} In single-cell organisms such as yeast, uncontrolled proliferation might lead to exhaustion of resources that will cause some individuals to die, but others can remain dormant until the environment is once again favorable for growth and reproduction.^{39,40} In contrast, because multicellular animals rely on specialization of tissues and organs, they cannot tolerate the damage and dysfunction created by excessive, uncontrolled cellular proliferation even in a single organ or tissue. Thus, cancer is not a modern physiological defect, but rather it is a vulnerability that is rooted deeply in vertebrate evolutionary history⁴¹ and nearly every multicellular animal is at risk for developing cancer.^{26,37,38,42} The ancient origins of cancer are supported by the increasing examples of cancers identified in the fossil record.^{43–47} While a precise estimate of cancer incidence and prevalence in antiquity is impossible, it has mostly been assumed that it was rare.^{48,49} This is partly due to the fact that the fossil record is biased toward tumors of bone (primary and metastatic), which overall represent only a fraction of the cancer burden in modern humans.⁵⁰ Historians and scientists have offered different interpretations for this assumed rarity, ranging from lack of exposure to carcinogens that are ubiquitous in modern societies to support it, to the technical and methodological pitfalls in the analysis of paleopathological samples to refute it,^{48,51} to the focus on literature and paleopathological specimens originating primarily from ancient Egyptian and Greek societies and neglecting the literature from Chinese and other significant ancient societies.⁵²

Ewald has also proposed that the rarity of cancer in antiquity (and in wildlife) could be due to increased mortality of individuals with cancer where such evidence of cancer-related mortality or cancer-related morbidity contributing to mortality would disappear due to predation (consumption of the tumorous tissue).⁵³ The evidence of cancer could also be erased by environmental effects on the cancerous tissues, directly related to the fragility caused by the cancer, or to the lack of specificity of the pathological

changes caused by the cancer.⁵³ Ewald also suggested that parasites that cause cancer (where parasites are defined broadly to include multicellular, cellular, and subcellular replicative agents that live in or on a host organism and negatively affect the evolutionary fitness of the host) have existed in human and animal populations for millennia, but their impact may be underappreciated by the emphasis of mutations as the root cause of cancer.⁵³ It is interesting that dogs appear to be a privileged species in this regard: with the exception of canine papillomaviruses that cause benign warts, neither DNA nor RNA oncoviruses that fulfill Koch's postulates have been definitively identified in canids, despite sporadic reports of retrovirus or herpes virus particles in dog lymphomas, leukemias, and peripheral blood lymphocytes.

1.2 | Cellular replication and the creation of aged (permissive) microenvironments are major risk factors for cancer

Malignant transformation, an essential step in the pathogenesis of cancer, is caused by mutations that alter the sequence or structure of DNA.⁵⁴ Cancer, thus, results in part from a collection of somatic changes leading to inappropriate cell division or survival, coupled with the failure of tumor-suppressive mechanisms, which together provide cells with a clonal growth advantage. Examples of clonal somatic changes include mutations, copy number changes, and epigenetic structural DNA changes that lead to oncogenic activation. A consequence of this paradigm is that anything that increases somatic changes can also lead to increased cancer risk. A further requirement for cancer formation is that the transformed cells must have a permissive environment where they can grow and develop the complex anatomical structures that create tumors.^{54–56} Recently, Laconi and colleagues proposed that, “the tumor-suppressive potential of youth is more potent than previously realized, limiting cancers through half a century of human life even in the face of increased DNA mutations and highly perturbed tissue environments,”⁵⁷ and much additional work suggests that organismal aging and the associated development of cellular senescence, proinflammation, and waning immunity appear to be major mechanisms that allow for the development of these permissive environments.^{58–64}

Pathologically significant mutations are propagated during the process of DNA replication, as nondividing cells will not pass on the cancer phenotype to progeny cells. The earliest examples that cemented the association between mutations, proliferation, and cancer included the increased cancer risk observed in individuals who had occupational exposures to mutagens,^{65,66} habitual

tobacco users,⁶⁷ or individuals who had mutations in genes involved in DNA damage repair.⁶⁸ Similarly, elevated cancer rates can be found in individuals with germline mutations in DNA polymerases (*POLE/POLD1*).⁶⁹

Companion dogs generally do not have the same level of exposures to industrial (occupational) or social (tobacco) mutagens as humans, their hair coat protects them from cancer-causing ultraviolet radiation from the sun, and controlled breeding practices help to significantly reduce the likelihood for true heritable cancer syndromes to become established. In a singular situation where a syndromic cancer (canine renal cystadenoma and nodular dermatofibrosis, associated with a mutation in the folliculin gene) arose in a canine pedigree, it was rapidly identified and characterized,^{70,71} providing tools to eliminate it from the breeding population. Some studies have identified associations between certain animal cancers and environmental exposures. For example, studies done by Reif and colleagues 30–40 years ago reported associations between exposure to environmental tobacco smoke and cancers of the respiratory tract in dogs.^{72,73} However, these findings have not been replicated in more recent studies.⁷⁴ Similar lack of replication has confounded definitive associations between other environmental exposures and dog cancers.^{75–77}

On the other hand, consistent associations between certain cancer types and dog breeds suggest enrichment of risk alleles that can inform heritable risks associated with cancer.^{21,78–87} While there are multiple examples of genomic regions and of coding and regulatory variants associated with canine tumors in specific breeds,^{85,86,88–90} virtually all of these cancers occur in dogs that are past middle age, accounting for variations in breed-specific aging. Furthermore, many factors in the germline seem to contribute to breed-specific risk for cancer in dogs,^{91,92} underscoring the complexity of the disease, and/or the incomplete penetrance of the putative risk alleles.

Importantly, the association between cellular replication and cancer is not a new concept. As far back as the early 1900s, Theodor Boveri's work on cell division had led to the hypothesis that cell division created cancer risk.⁹³ Even to this day, one of the most universal prognostic indicators within tumor tissue is the level of Ki-67 protein, which is often used to gauge the proliferation rate, and by extension the aggressiveness of a tumor.⁹⁴

1.3 | Peto's paradox

An implication of the association between replication and cancer is that increased longevity (more cell divisions) and increased size (more cells) should result in elevated cancer risk and greater cancer incidence. From this

paradigm, Sir Richard Peto, a statistician well known for helping to uncover the association between lung cancer and smoking, identified a paradox⁹⁵ and articulated the problem,⁹⁶ which was subsequently named “Peto's paradox” by Nunney.⁹⁷ Peto's paradox states that at the species level, the incidence of cancer does not appear to correlate with the number of cells in an organism. In other words, the predicted linear association between large size of an organism (which is partly due to greater numbers of cells, attained by increased cell division) and cancer is inapparent across the multitude of species in the animal kingdom²⁷; extremely large animals, especially those with long lifespans, which undergo far more replications (and therefore would be expected to have an excessively high replicative cancer risk) *do not* have higher cancer rates than smaller animals, which need less replication to attain their adult size and that generally have shorter lifespans (Figure 1). As detailed below, various investigators have since proposed that cancer-protective mechanisms acquired over the course of evolution provide satisfactory answers to Peto's paradox.

2 | CANCER-PROTECTIVE MECHANISMS IN LONG-LIVED SPECIES

Several decades passed between Peto's original observation and the initial formulation of a potential solution, which came to light through the study of large and/or long-lived species of animals that have evolved mechanisms allowing for the minimization of cancer risk (see references^{26,34,37,58,97–105} for previous, outstanding discussions on this subject).

As species have evolved, unique adaptations that protect them from cancer have arisen multiple times, and often, independently from each other. These cancer-protective mechanisms address different aspects of vulnerability and they become part of each species' evolutionary history. As illustrated below, different species have “attacked” different aspects of tumor formation, including reducing the likelihood that detrimental mutations are incorporated into their genomes, altering their metabolism, making the tissue microenvironment inhospitable to tumor growth, and probably others. These changes have occurred stochastically over millions of years, but they must have been subject to strong positive selection,¹⁰⁶ with the consequent effect of giving rise to adaptive traits that protect species from cancer⁹⁷ while allowing them to occupy niches where they can invest energy to become large, have a long lifespan, or both.¹⁰⁷ It is especially intriguing that in their evolutionary history, several tumor suppressor genes in hominoids have been subject to evolutionary selection such that the wild type (reference) alleles are distinct from variants

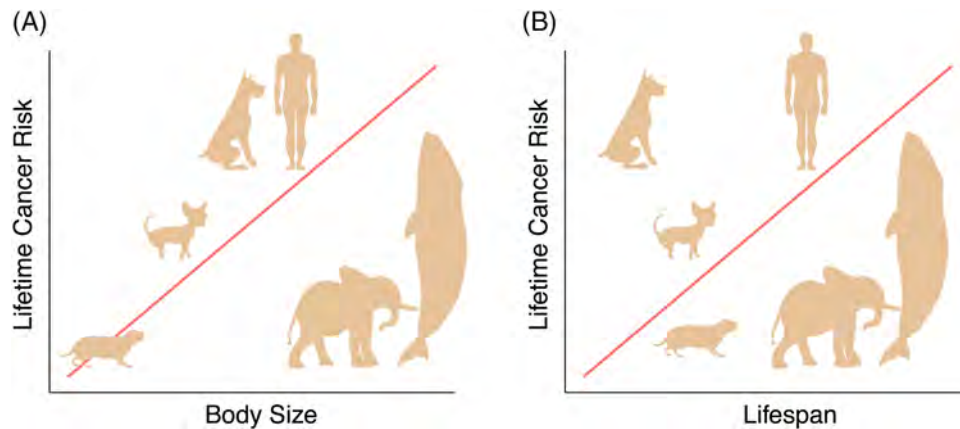


FIGURE 1 Cancer risk and Peto's paradox. Illustration of Peto's paradox, which describes the absence of the predicted relationship between animals' (A) size and (B) longevity relative to their cancer risk

found in apes, and presumably in the common ancestor to these primate species.¹⁰⁶ Indeed, the ancestral wild type alleles for some of these genes such as *APC* and *TP53* (as found in some modern apes), or *BRAF* and *TNFAIP3* (as found in Neanderthals and Denisovans, respectively) are found to be deleterious in modern humans, exclusively as somatic variants, and closely associated with cancer risk.¹⁰⁶

It is reassuring that many tumor-suppressive mechanisms are highly conserved across vertebrate (and often-times invertebrate) species. For example, the products of the *TP53* and the *PTEN* genes, and the enzymes that maintain telomere length, are highly conserved mechanisms that suppress tumor formation across many different species.^{108–116} Replicative senescence is another widespread, primordial mechanism that seems to protect animal species against longevity-associated somatic modifications.^{117,118} In most cells, senescence programs are activated upon reaching a predetermined number of replication events, as well as by somatic mutation events, and many genes involved in regulation of senescence have been directly implicated in tumor suppression.¹¹⁹

Specific examples of species-specific cancer-protective mechanisms have been described in the elephant family, bowhead and other whales, and in different families of long-lived mole rats,³⁴ while more are being deduced from recently sequenced genomes of other long-lived species such as Amazon parrots,¹²⁰ white sharks,¹²¹ and whale sharks.¹²² Each of these species appears to have acquired or “fine-tuned” unique mechanisms that allow them to have long and relatively cancer-free lives. For example, the *TP53* gene underwent a series of retrotranspositions in the elephant lineage, after the split from its last common ancestors that in the extreme resulted in more than 20 copies of this gene in modern African elephants.^{123,124} While not all of the elephant *TP53* genes are likely to be active, the

orthologous elephant p53 protein was shown to be a more potent inducer of the p21 cell cycle kinase inhibitor and to have greater pro-apoptotic activity than the human p53 protein. The specific mechanisms of the elephant p53's enhanced apoptotic ability, including it to be delivered as a novel therapeutic for cancer in dogs and humans remains a subject of intense investigation (Dr. Joshua Schiffman, personal communication). The function of the elephant p53 retrogenes remains unclear, but when transfected into human cells, the retrogenes were upregulated and translated upon treatment with chemotherapy and radiation,¹²³ and in separate experiments, Vazquez and colleagues showed that one or more of the retrotransposons may induce expression of at least one pseudogene (*LIF-6*) that seems to have been refunctionalized to promote apoptosis in elephant cells, but not in those of other mammalian species.¹²⁵ And yet, duplication of *TP53* is not unique to elephants, nor does it guarantee long life. Rats, wallabies, and some small bats also have duplicated this gene over the course of evolution without consequent gains in size or longevity.¹²⁴

The bowhead whale genome hints at unique metabolic adaptations, including mutations in *UCP1*, which encodes the mitochondrial uncoupling protein-1 of brown fat, as well as altered insulin signaling. Additionally, the bowhead whale genome includes cancer-protective genes under positive selection, including *ERCC1* (excision repair cross-complementing rodent repair deficiency, complementation group-1; a member of the nucleotide excision repair pathway) and duplication of *PCNA* (proliferating cell nuclear antigen; a gene involved in DNA repair).¹²⁶ Other whales, including humpbacks, minke, and toothed whales seem to have developed segmental duplications in tumor suppressor genes that are species-specific and are also under positive selection,^{104,127} consistent with the interpretation that these events occur over long

evolutionary periods and are unique to each species and dictated by their niche.

Rodents show extreme variation in lifespan, which can be used to identify potential cancer-protective mechanisms. Across multiple short- and long-lived rodent species, five amino acid substitutions in *SIRT6* account for most of the variation in DNA double-strand break repair efficacy, with increased *SIRT6* activity also showing a direct correlation with lifespan among these species.¹²⁸ Another example comes from mole rats, the longest lived rodents alive today, which can reach 20 to more than 30 years of age. African mole rats of the family Heterocephalidae have developed a series of distinct cancer-protective mechanisms that modify the organization of stromal matrix proteins, making the microenvironment inhospitable to cancer,¹²⁹ while the distantly related blind Middle Eastern mole rats of the family Spalacidae have developed a hypersensitive cell-death response to mitigate the excessive drive to proliferate that is associated with events that lead to malignant transformation.¹³⁰ Perhaps the most widely recognized cancer-protective mechanism in mole rats is the abundance of high molecular weight hyaluronic acid in the African naked mole rat, possibly due to increased production of hyaluronan by the *HAS2* gene product with concomitant reduced activity of the enzymatic machinery that degrades hyaluronic acid.^{129,131} This stands in sharp contrast to the hyaluronic acid content in, for example, human tumors, where hyaluronidases cleave hyaluronic acid into small molecular weight species that promote inflammation and tumor growth.¹³²

Together, these examples indicate that large and long-lived animals have evolved diverse and sometimes convergent mechanisms that have enabled large investments in reproduction and high energy requirements over long and relatively noncancer-prone lifespans.⁹⁷ These varied solutions to the cancer problem, in turn, create species-specific barriers with limitations on cancer protection determined by the idealized lifespan and body size acquired through these millions of years-long evolutionary processes. And thus, exceeding these barriers through technological innovation to achieve increased lifespans, as is the case with “caninity” and humanity, or via artificial selection (selection for size in the absence of selection for compensatory protective mechanisms), as is the case in dogs, generates conditions that are cancer prone.

We should note that Ujvari and colleagues proposed an alternative hypothesis that, while selective breeding (and inbreeding) and the consequent attenuation of genetic diversity result in the accumulation of deleterious genetic variants in domestic animals, the absence of natural selection could theoretically allow these animals to invest more resources to support existing anticancer mechanisms and potentially develop new ones.¹³³ While this is an intriguing

premise, humans have not significantly increased the lifespan of domestic animals other than dogs and cats, and in our opinion, rare anecdotes of spontaneous cancer regression, for example, in dogs with bone cancer, fail to support strong maintenance or widespread development of novel cancer-protective mechanisms in the accelerated time scale of domestication. On the contrary, the excess of cancers seen in modern dogs (and in humans), and the patterns of their association with age, would strongly suggest that there has been neither sufficient time nor selective pressure to allow for evolution of adaptive mechanisms to reduce the risk of cancer that comes with the creation of aged cellular environments, increased exposures, and the accumulation of somatic mutational events in these populations.

2.1 | Breaking through the evolutionary lifespan adaptation is a major contributor to cancer risk in humans and in dogs

One theme that has been underappreciated, in our opinion, is the dramatic gains in expected lifespan that have occurred in the past 150–200 years in humans and over the past ~50 years in dogs, and the associated risk of cancer that is attributable to aging. According to Finch,¹³⁴ life expectancy from birth in humans doubled in the 5–7 million years from the time the hominid lineage split from the common ancestor shared with chimpanzees until modern hunter-gatherers. It is worth noting that the process of speciation in apes and hominoids (presumably similar to the process of speciation in other animals) included unique adaptations in sequence, and perhaps in function of tumor suppressor genes.¹⁰⁶ In their study of 120 oncogenes and tumor suppressor genes in seven hominoid species, including two extinct species, Neanderthal and Denisovan, Kang and Michalak also found opposing selection pressures operating on these two classes of genes, with tumor suppressor genes being under weaker purifying selection than oncogenes.¹⁰⁶ The authors hypothesize that the selective pressures accounting for these differences could come from the activity of oncogenes, where a single variant can be detrimental to the individual, in contrast to tumor suppressor genes, which generally maintain activity even in a state of heterozygosity, and where loss of both alleles is usually necessary for cancer-prone phenotypes to arise.¹⁰⁶

The precise lifespans of intermediate species during human evolution are unknown, although it is apparent that early modern humans and Neanderthals had a larger proportion of older adults than prior Homo species and Australopithecines.¹³⁴ Human life expectancy doubled again in the 150 years between 1860 and 2010 during

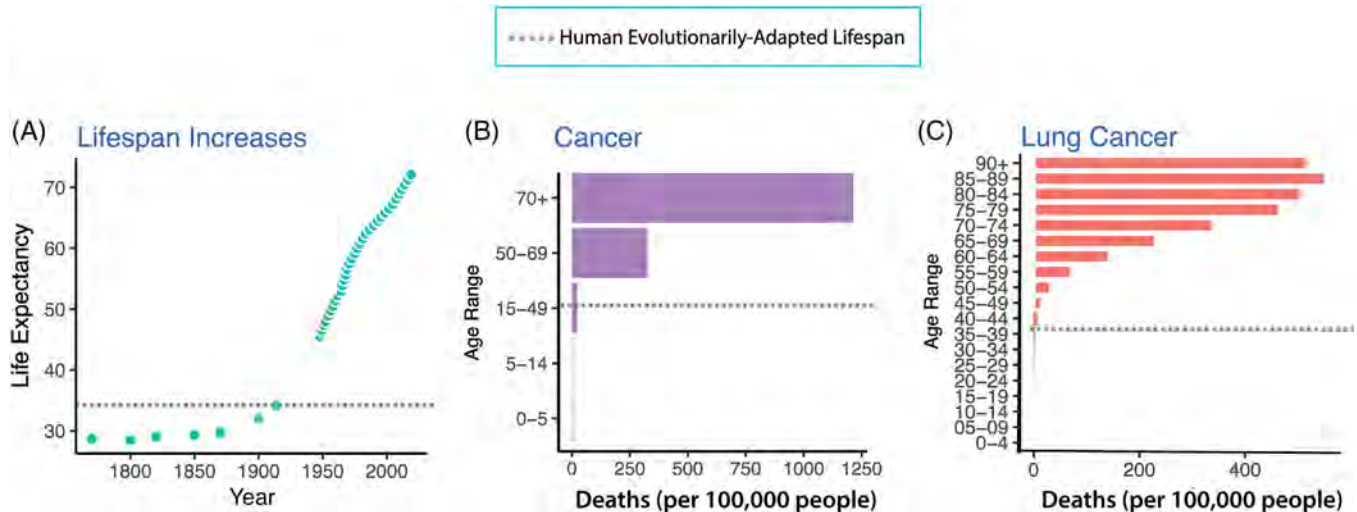


FIGURE 2 Increases in human lifespan overlap with the majority of human cancer deaths. (A) Humans had a relatively stable life expectancy of 30–40 years until the industrial revolution. The upper boundary of this life expectancy represents the ancestral evolved cancer barrier. However, in the 100 years between 1850 and 1950, human life expectancy increased by approximately 50% across the world, and it did so again in the 60 years between 1950 and 2010. That trend continued through the present (latest measurements shown for 2017). (B) The overall cancer death rates per 100,000 people do not have a proportional relationship with age in the United States. Instead, cancer death rates are vanishingly rare, proportionately, in individuals under 15 years of age, they become apparent with a marginal increase between the ages of 40 and 50 after individuals cross the cancer barrier, and then increase dramatically thereafter. Individuals over 60 years of age account for the vast majority of cancer deaths in the United States (United States data from 2017). This is representative for most of the developed world. (C) Lung cancer death rates per 100,000 men also do not have a proportional relationship with age. Despite decades of persistent exposure to myriad potent carcinogens in tobacco products, lung cancer is extremely rare in smokers under the age of 40. Lung cancer becomes more apparent in smokers by the age of 40 after individuals cross the cancer barrier and continues to increase geometrically until the age of 80–85 (United Kingdom data from 2015–2017). This is also representative for most of the developed world

industrialization, and it increased further in the contemporary era with increasing access to hygiene, medical care, and other advantages of complex societies¹³⁵ (Figure 2A).

Thus, human lifespan was molded to a niche over a 5–7-million-year period where there were strong evolutionary selective pressures, including selection for cancer-protective mechanisms.¹⁰⁶ Because the niche occupied by our hominid ancestors was different from that occupied by other great apes, we cannot infer their lifespan from that of modern nonhuman great apes. But we can estimate it was shorter than pre-industrial modern humans or 20th century foragers, whose life expectancy was 30–40 years,^{134,136} although this was heavily influenced by high infant mortality. Nevertheless, even if we accept an age somewhere between 35 and 45 years as the evolutionarily determined lifespan for humans, fewer than 5% of cancer cases are diagnosed in humans from birth to 35 and less than 10% from birth to 45, making the expected cancer incidence in this population comparable to that seen in apes and monkeys¹³⁴ (Figure 2B,C).

Like humans, the lifespan of canids in the wolf family was molded to a niche over a 5–7-million-year period under strong evolutionary selective pressures. We cannot be certain of the expected lifespan of the canid wolf ances-

tor, but an estimate of approximately 3.5–5 years is possible based on studies of modern wild wolves and feral dog or village dog populations.^{106,137,138} And yet, in contrast to what is observed in wolves and in feral dogs and village dogs even today, the expected lifespan of companion pet dogs in much of the developed world has increased by a factor of two to four (to an average of 9–14 years, depending on breed, according to information available from the American Kennel Club¹³⁹) in the ~50 years since dogs were brought into the household and integrated as members of the family in the latter part of the 20th century. Interestingly, the eventual selective breeding for form and function that occurred over the past 15,000 years, and the purposeful derivation of modern dog breeds over the past 100–400 years, might have influenced the canine lifespan through enrichment of alleles that encode for small size.^{140,141} But, following the human example, if we accept 3–5 years as the evolutionarily determined lifespan for primordial dogs evolving over a period of *ca.* 6 million years, the available data reproducibly indicate that fewer than 5%–10% of cancer cases are diagnosed in modern dogs from birth to 3–5 years,^{12,18,142,143} making the expected cancer incidence in this population comparable to that seen in captive wolves under 5 years of

age¹⁴⁴ (no estimates of cancer exist for wolves or other related canid species in the wild) and to that which has been estimated for most animals in the absence of human influences.¹⁰⁷ It is also worth noting that wolves and other wild canids that are maintained in captivity and that reach advanced ages also exhibit increased susceptibility to some of the most common cancers seen in modern domestic dogs.^{144,145}

Hence, we advance the premise that a major component of the elevated cancer risk seen in domestic dogs and in humans when compared to other animals is due to the shattering of the life-expectancy barrier that was evolutionarily determined: essentially “living longer than nature intended.” The longevity that both species enjoy at the present time has dramatically increased (two to four times), compared to the lifespan to which each species had adapted over its evolutionary history (an “evolutionarily determined lifespan”), through social, medical, and technological advancements, over very short periods of time, and with artificial selection playing an increasingly important role, especially in the case of dogs.^{58,98}

A recent study of cancer in nondomestic mammals kept in zoological collections is consistent with the interpretation that living longer than nature intended is a major risk factor for cancer. While captivity might introduce its own set of risks for cancer or premature mortality, such as exposure to anthropogenic pollutants, stress, etc., it also removes common causes of mortality for many species, such as competition, predation, accidents, or human-related causes. Consequently, animals in zoos would be expected to outlive some of their counterparts in the wild. Vincze and colleagues did not specifically address gains in longevity in their study, but cancer-related mortality was still lower than 10% for almost 80% of the species in this study (and it was zero for almost 25%).²⁷ As a group, cancer mortality was highest in carnivores. The authors propose that this might be due to dietary factors (consumption of meat), but it is possible that carnivores are also more amenable than noncarnivores to greater life extension in captivity.

Returning to the dog example, canine osteosarcoma provides a good example to illustrate the association of size and cancer susceptibility, and a solution to Peto’s paradox.⁹⁸ This disease occurs only rarely in humans, but it is quite common in dogs.¹⁴⁶ Because the natural history of the disease in both species is similar, canine osteosarcoma has been long proposed as a surrogate disease model to better understand its human counterpart.^{147,148} In addition to an anatomical predilection for the long bones and shared patterns of metastasis, a few molecular traits are conserved between canine and human osteosarcoma. An initiating event, such as *TP53* mutation, seems to be required, which allows replication of chaotic genomes and clonal

outgrowth of tumors with highly disrupted and heterogeneous genomes.¹⁴⁹

However, important epidemiologic differences coexist with the similarities between human and canine bone tumors. For example, the reported age-adjusted incidence of osteosarcoma in humans is between 0.2 and 4.5 per million, with most affected patients being children, adolescents, and young adults,¹⁵⁰ whereas in dogs, this disease is most commonly observed in adult and elderly individuals from large and giant breeds, with a lifetime risk of approximately one in five at the extreme.^{146,148,151–153} The strong association between size and risk of osteosarcoma in dogs illustrates a similar, albeit weaker, association between risk of osteosarcoma and size in humans, where it is more common in males than in females, and in children who are taller and have high birthweights.^{154–156}

Of additional interest, larger dogs age more rapidly than smaller dogs and have shorter lifespans.¹⁵⁷ This pattern of rapid aging and shorter lifespan also aligns well with the risk for bone cancer (Figure 3), where a high rate of cellular replication is necessary to form large bones (larger than the norm for the species as it evolved), where aged tissues might provide more permissive environments for the development of cancer, and where the protections afforded by modern veterinary healthcare, vaccination against lethal pathogens, appropriate nutrition, and shelter have more than doubled life expectancy for dogs living as companions in the human environment.

The association between increased body size and increased cancer risk is observed, not only with osteosarcoma where the effect manifests in the extreme, but also with other cancers.^{7,12,17,19,158} And while the major variant alleles associated with size determination in the dog (including *GHR*, *HMG2*, *IGF1*, *IGF1R*, *SMAD2*, *STC2*, *LCORL*, and *CDK4*)¹⁵⁹ have not been found to confer independent cancer risk, this may be due to the fact that variants are fixed in purebred dogs. To understand the potential significance of genomic traits that regulate size in dog aging and cancer, future studies should include multiple breeds and/or mixed breed dogs to unmask the potential contribution of fixed alleles.^{159,160}

Altogether, these observations suggest that Peto’s paradox is no paradox at all, when applied within a single species, and removing the impact of evolution within a niche. That is, Peto’s principle (as opposed to Peto’s paradox) would predict that increased body size should be associated with increased cancer risk in dogs. Thus, extension of this concept leads to the notion that *longevity is intricately associated with cancer risk, but only when such longevity exceeds the evolutionarily adapted lifespan for that species*. The observation that small dogs live longer than large dogs and have lower cancer rates, thus conforms with Peto’s principle.^{12,98,161,162}



FIGURE 3 Canine size, lifespan, and osteosarcoma risk. Larger dogs have shorter life expectancy and increased risk for osteosarcoma than smaller dogs. Specifically, using the German shepherd dog breed as the reference, great danes have an odds ratio (OR) of 5 for development of osteosarcoma, whereas miniature schnauzers have an OR of 0.2 for development of osteosarcoma.¹⁵³ Overall, the highest risk breed has an OR >200-fold greater than the lowest risk breeds for development of this disease (reproduced from Makielski et al. Risk factors for development of canine and human osteosarcoma: a comparative review. *Vet Sci.* 2019;6(2):48. Copyright: the authors)

Furthermore, these relationships are not completely static, so if replicative senescence is a cancer-protective mechanism, the earlier onset of senescence (as would occur in animals with shorter lifespans) that is observed in large dogs could be related to their increased cancer risk over the same timescale as their smaller counterparts. Untangling the connections between senescence, cancer risk, and lifespan in different dog breeds should generate important insights into how better to understand cancer in humans and identify people with increased risk earlier in their lifetime.^{21,36,58,84,162,163}

After considering these concepts, a “simple model” is that, within a given species over the course of a lifetime, cancer-preventive mechanisms may change from protective states ensuring evolutionary fitness to less-protective states once they no longer provide advantage. In other words, anticancer defenses in old animals will be under less selective pressure than in younger animals, and costly defenses are likely to be maintained at a lower level. In reality, this is likely to be far more complicated as progenitor cells need extreme protection from somatic damage, while damage might be tolerated (and is observed) in terminally differentiated cells.¹⁶⁴ This threshold of age-related vulnerability,⁵⁸ which is more readily observed after individuals cross cancer-protective barriers for their species, can also explain the prolonged latency seen in humans between carcinogen exposures and the onset of cancer. For example, habitual use of tobacco products usually starts in early adolescence, but the onset of tobacco-related cancers, on average, occurs after the age of 60,¹⁶⁵ which is beyond the predicted cancer-protective barriers for humans (Figure 2C). Intriguingly, the apparent evolution of a permissive environment in the aged lungs may be independent of exposure to tobacco-derived toxicants,

although there does appear to be an incontrovertible association between the overall increased frequency of lung cancer and increased mutagenesis mediated by these compounds.¹⁶⁶

The impact of these ideas is more profound among species. Extension of this concept indicates that small species that are highly vulnerable to predation and rely on explosive growth strategies, such as wild mice, have short lives, and the cost of cancer-protective mechanisms might decrease their overall fitness.¹⁶⁷ The elevated incidence of cancer in laboratory mice, which far exceed the evolutionarily determined lifespan for this species, is consistent with this observation and argues that exceeding this lifespan barrier in any species will probably result in excess cancer when compared to the same population inhabiting within its evolutionary niche. And the opposite may also be true: “ancient” species or species that are less vulnerable to predation and that have had relatively stable body sizes over “long periods” of evolutionary time such as turtles and alligators should have, and likely did evolve, stronger cancer-protective mechanisms.^{34,a} These examples support the ideas that cancer is influenced by evolutionary pressures⁵⁸ and that increased risk associated with modern increases in lifespans may be responsible for the observed increased incidence of cancer in human and canine populations. Large dogs are dually affected as artificial selection for large size seems to have had the unintended consequence of increasing cancer risk, perhaps in part by increasing the replicative risk as large stature requires

^a This concept was also attributed to Dr. Jay Olshansky (University of Chicago) in a Vox article, authored by Joseph Stromberg and published on June 16, 2014 (<https://www.vox.com/2014/6/16/5796732/do-naked-mole-rats-have-the-secret-to-long-healthy-lives>)

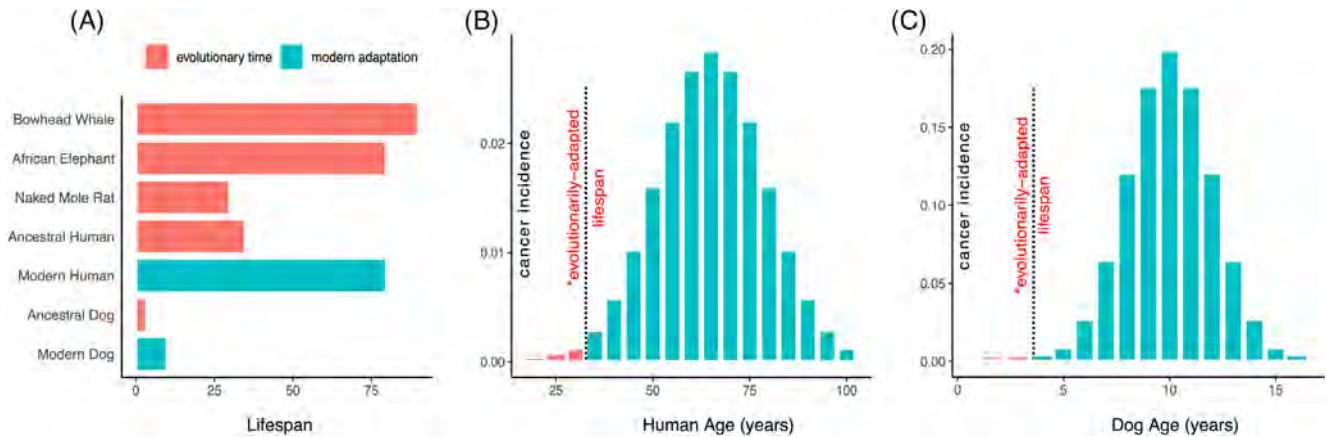


FIGURE 4 Increasing canine and human risk due to surpassing the species' respective evolutionarily determined lifespan barriers. (A) Multicellular species, represented here by bowhead whales, African elephants, African naked mole rats, ancestral humans, modern humans, ancestral dogs, and modern dogs, have achieved an evolutionarily derived balance between tumor-suppressive mechanisms and senescence mechanisms that have selected for lifespans that are consistent with their respective evolutionary pressures. Recent technological advances have led to lifespans in humans and dogs that are more than twice as long as those to which each species had adapted over millions of years of evolution. The vast majority of cancers occur after individuals cross this evolutionarily adapted lifespan (dashed line) for (B) humans and (C) dogs. Neither species has had the time, nor has it been subjected to selective pressures, to establish a new evolutionary balance that resets its cancer barrier

more cells.^{12,162} Figure 4 illustrates this concept using bowhead whales, African elephants, African naked mole rats, ancestral humans, modern humans, ancestral dogs, and modern dogs. Each of these species has achieved an evolutionarily derived balance between tumor-suppressive mechanisms and senescence mechanisms that have selected for lifespans that are consistent with their respective evolutionary pressures. Modern humans and modern domestic dogs are outliers in that their expected lifespan now is more than twice as long as that to which each species had adapted over millions of years of evolution, and for both species, the vast majority of cancers occur in individuals that have crossed this evolutionarily adapted lifespan. Neither species has had the time, nor has it been subjected to selective pressures, to establish a new evolutionary balance that resets its cancer barrier.

2.2 | If cancer is virtually inevitable, what is the solution

Cancer is virtually, but not entirely, inevitable. Even with the risks of replication-induced mutations, size, and longevity, only about 25%–40% of all humans and dogs will develop cancer in their lifetime. Incredible breakthroughs have been made in treatments for advanced human cancers, all of which raise hope and might find future applications in dogs.

Nevertheless, the best solution to cancer is to prevent it entirely. Decreasing environmental exposure to carcinogenic agents has been extensively described and pursued

in humans.¹⁴ It may be possible to engineer cancer risk out of the genome by increasing endogenous anticancer mechanisms, or potentially by transferring protective strategies utilized by large and long-lived organisms,¹⁶⁸ although this could raise serious ethical dilemmas. In dogs, strategies to decrease environmental exposure to carcinogenic agents are less likely to be an effective method to reduce cancer, as the exposure of dogs to such environmental carcinogens is generally quite limited, and at the same time, such exposures might be unavoidable.

In humans, early identification of cancer via screening of at-risk populations has led to effective treatment and decreases in mortality. To extend this approach to dogs, we are developing diagnostic tests to identify canine cancer as early as possible.¹⁶⁹ However, testing for early detection and for risk assessment alone is insufficient. In order to be ethical and valuable, the information from such testing must be actionable. So, our goal is to pair each test with a potential preventive solution that can eliminate the cancer at its earliest stage. One example is the Shine-On project^{169,170} (Figure 5), where we have developed a blood test to detect rare events associated with the presence (or risk) of hemangiosarcoma (a malignant tumor of blood vessel-forming cells that is relatively common in dogs), and which is meant to be used once dogs reach an age where risk for this cancer is meaningful (i.e., older than 6 years).^{12,142,171,172} Another example from our group is a serum exosomal mRNA signature associated with the presence of osteosarcoma¹⁷³ that is currently being investigated for early detection in at-risk breeds of dogs. By design, these early detection and risk assessment tests would be

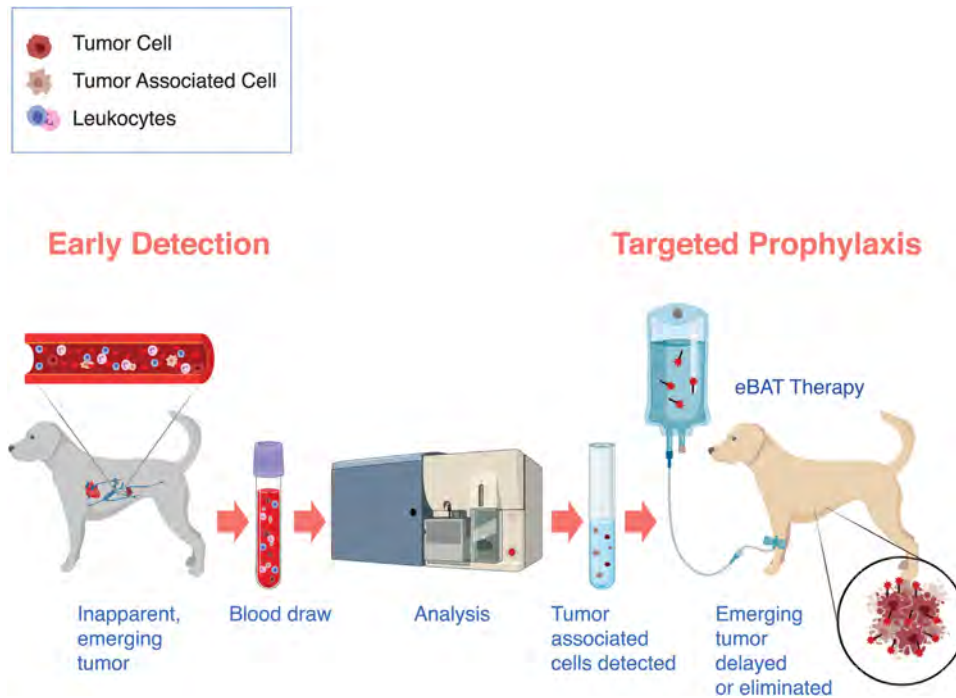


FIGURE 5 The Shine-On concept of paired early detection and strategic prevention. Shine-On illustrates the approach of early detection and strategic cancer prevention. In this case, dogs that reach the age of increased cancer susceptibility are screened for the presence of incipient or emerging hemangiosarcoma (a terminal tumor of blood vessel-forming cells) using the Shine-On suspicion (SOS) test. The SOS test uses flow cytometry and artificial intelligence to enumerate rare hemangiosarcoma-associated cells in blood and to assign a level of risk. Dogs at high risk would be eligible for targeted prophylaxis, for example, using the drug eBAT, with the intent to prevent or delay the development of clinical hemangiosarcoma

paired with a preventive strategy, for example, the targeted drug EGF bispecific angiotoxin (eBAT).^{174–176} This preventive solution is based on eBAT's safety profile, which justifies its use in otherwise healthy animals, and on its proposed mechanisms of action to simultaneously eliminate cancer stem cells and disrupt the tumor niche.^{174–177} In this way, eBAT may be effective in preventing or delaying the emergence of cancer in healthy dogs determined to be at high risk by eliminating emerging tumors before they ever have a chance to develop. This proof-of-concept in dogs would open the door to develop similar approaches for people.¹⁷⁸ We recognize that these approaches may not be the only or the definitive answer to cancer. But we are convinced that these approaches, and others like them, will be part of the solution. As we tilt the balance toward successful prevention, though, we must remember that it is our duty to support graceful aging. We must resist the siren's song of "longevity at any cost." It is not wise, and it is not evolutionarily sound.

3 | CONCLUSIONS

Our summation of the literature supports the concept that the increased risk of cancer in humans and dogs is a con-

sequence of recent extensions of lifespan and body size beyond evolutionarily determined cancer barriers. In the short-term, we contend that the inevitability of cancer that accompanies these extensions can and should be managed by early detection and risk assessment. For example, screening tests to detect cancer at its inception, before it has become a clinical entity, could be implemented, and paired with rational, strategic interventions such as eBAT that can eliminate the cancer-initiating cell populations and/or make the environment inhospitable for tumor growth. In the long-term, rather than wait for evolutionary innovation, we should carefully consider whether practical and ethical norms would allow engineering of extended cancer-protective mechanisms into at-risk genomes, at least in dog populations that have been rendered highly vulnerable to cancer by artificial selection.

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CONFLICT OF INTEREST

Jaime F. Modiano is an inventor on U.S. patent 7910315, "Early Detection of Hemangiosarcoma and Angiosarcoma," assigned to the Regents of the University of Colorado. Jaime F. Modiano is an inventor on U.S. patent application, "Reduction of EGFR Therapeutic Toxicity," filed on behalf of the Regents of the University of Minnesota. Jaime F. Modiano and Taylor A. DePauw are inventors on U.S. patent application, "Artificial Intelligence for Early Cancer Detection," filed on behalf of the Regents of the University of Minnesota.

AUTHOR CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization: Aaron L. Sarver and Jaime F. Modiano. Methodology: Aaron L. Sarver, Kelly M. Makielski, Taylor A. DePauw, Ashley J. Schulte, and Jaime F. Modiano. Data curation: Aaron L. Sarver and Jaime F. Modiano. Investigation: Aaron L. Sarver, Kelly M. Makielski, Taylor A. DePauw, Ashley J. Schulte, and Jaime F. Modiano. Formal analysis: Aaron L. Sarver and Jaime F. Modiano. Resources: Aaron L. Sarver, Kelly M. Makielski, Taylor A. DePauw, Ashley J. Schulte, and Jaime F. Modiano. Writing the original draft: Aaron L. Sarver and Jaime F. Modiano. Writing the review and editing: Aaron L. Sarver, Kelly M. Makielski, Taylor A. DePauw, Ashley J. Schulte, and Jaime F. Modiano. Visualization: Aaron

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DATA AVAILABILITY STATEMENT

The data used to illustrate the premises of this study were derived from the following resources available in the public domain: Life expectancy data (1770–2017) in Figure 2A are from Our World in Data and are available at <https://ourworldindata.org/life-expectancy>. Cancer death rates by age group in Figure 2B are from Our World in Data and are available at <https://ourworldindata.org/cancer>. Lung cancer incidence statistics in Figure 2C are from Cancer Research UK and are available at <https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/lung-cancer/incidence#ref-1>. Age and cancer risk data in Figure 4B are modeled from the NCI Surveillance Epidemiology and End Results (SEER) Program.¹ Cancer attributed deaths in dogs in Figure 4C are modeled from ref.¹⁴²

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