PRECISION MEDICINE IN VETERINARY ONCOLOGY

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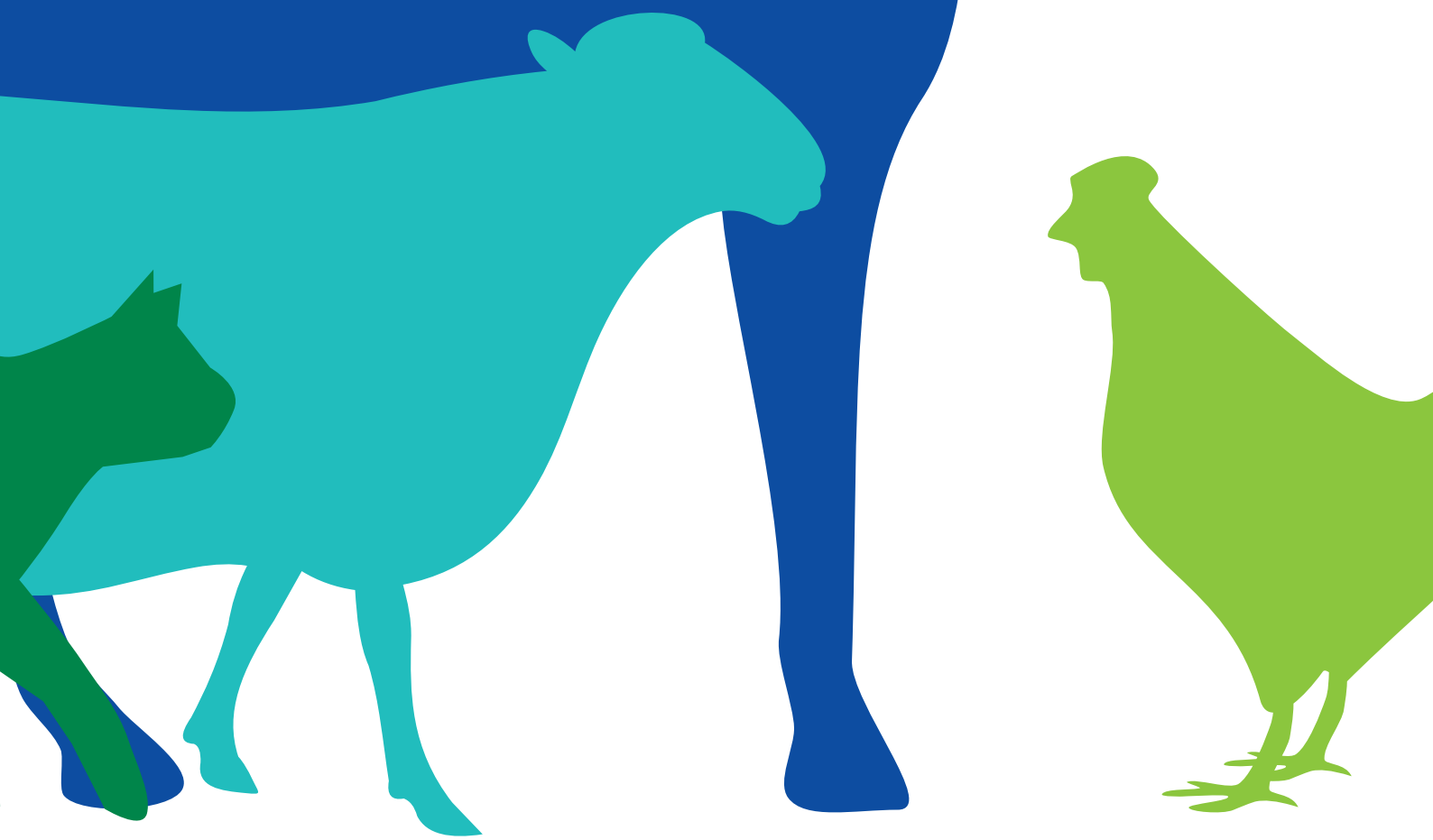

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PRECISION MEDICINE IN VETERINARY ONCOLOGY

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EDITORIAL
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Editorial: Precision Medicine in Veterinary Oncology

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Editorial on the Research Topic

Precision Medicine in Veterinary Oncology

Cancer is one of the most important diseases worldwide, with a global estimate of 10 million cancer-related deaths in 2020 (1). Although cancer prevention, diagnosis, and treatment have significantly improved in recent years, several cancers still represent a therapeutic challenge (2). The identification of tumor-specific characteristics has allowed for the use of monoclonal antibodies and tyrosine kinase receptor antagonist in cancer management and treatment. However, there is a growing need for models to study the efficacy of these new therapies. In this scenario, dogs and cats represent a unique opportunity for comparative oncology initiatives.

The Comparative oncology research has been strengthened in the last decade and uses animals as models to study human cancers. Initially, this concept was developed from the use of laboratory animals in cancer research and then re-shaped according to the new concept of One Health (3, 4). Since dogs develop spontaneous cancers with similarities to their human counterparts, they have been used as a model for new therapeutic interventions (5). Among the new therapeutic interventions, precision medicine has experienced unprecedented growth in recent years.

Research investigating and advancing precision medicine aims to identify individual tumor characteristics and provide treatment recommendations according to specific druggable target identification. Precision medicine covers different areas, including the identification of genomic markers, drug discovery, and health communication, supporting decision-making and providing evidence to choose the best treatment for patients (6). Although precision medicine has grown and matured tremendously in human cancer research, this concept is still new in veterinary oncology.

To fill this gap in veterinary oncology, this Research Topic is a compilation of the original and review articles that provide the most recent progress in precision oncology applied to veterinary cancer research. To instigate readers' interest in this new subject of veterinary oncology, we introduce here the main findings reported by different authors in their 10 manuscripts that form the bulk of this Research Topic.

Chibuk et al. reviewed the fundamentals of cancer genomics and provided a detailed explanation of the applications of liquid biopsies for the detection, characterization, and management of cancers in dogs. Gray et al. showed the importance of the tumor microenvironment and hypoxia as a tool for integration in a precision medicine approach. Overall, the authors reviewed several markers and technologies to be applied in precision oncology, including polymerase chain reaction assays to monitor tumor resistance and new targets for generating tyrosine kinase inhibitors. The third review paper provided a more specific direction for investigating the role of precision medicine in

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the diagnosis and treatment of canine mammary tumors. Valdivia et al. performed a comprehensive literature review on canine mammary tumors (CMTs), providing the most recent advances in the field of precision medicine, including the use of vasculogenic mimicry as a prognostic marker.

Interestingly, among the seven original manuscripts, three manuscripts introduced a new therapeutic perspective for canine prostate cancer. Schille et al. investigated the anti-tumor effects of PDA-66 and PDA-377 indolylmaleimides in canine prostate cancer cell lines. The authors demonstrated an interesting modification of prostate cancer cells after PDA-66 treatment and identified its anti-tumor effect through mitotic death. Another study by Kobayashi et al. investigated the antitumor effect of toceranib phosphate on two different canine prostate cancer cell lines, complemented by the cell line transcriptome after treatment. These authors identified both toceranib phosphate-sensitive and -resistant cancer cell lines. In particular, the cell line susceptible to toceranib phosphate displayed several transcriptome alterations, including dysregulation of the platelet-derived growth factor receptor pathway. Brito et al. evaluated the antitumor effect of a natural plant extract (*Synadenium grantii*) on two canine prostate cancer cell lines. These authors characterized the active principles of the plant extract using high-resolution mass spectrophotometry and demonstrated *in vitro* cytotoxicity in both prostate cancer cell lines.

Canine mammary gland tumors (CMTs) were also investigated in two original studies. Biondi et al. quantified global DNA methylation in CMTs in correlation with clinicopathological factors. Interestingly, the authors identified a specific pattern of global DNA methylation according to the tumor behavior, with hypomethylation being the most common in aggressive histological subtypes. Nakagaki et al. provided a morphological and phenotypic description of a new set of CMTs with neuroendocrine differentiation. Finally, Gambim et al. performed a meta-analysis and *in silico* analysis of dysregulated genes and proteins in canine bladder tumors, describing several genes with prognostic value in both human and canine bladder cancer.

Altogether, these manuscripts present new knowledge on the application of precision medicine in veterinary oncology that may drive a broader use of this strategy in veterinary oncology practice. The investigation of precision medicine approaches in veterinary oncology thus continues to be essential for a better strategy for cancer management and treatment in animals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2021) 71:209–49. doi: 10.3322/caac.21660
2. Zaorsky NG, Churilla TM, Egleston BL, Fisher SG, Ridge JA, Horwitz EM, et al. Causes of death among cancer patients. *Ann Oncol.* (2017) 28:400–7. doi: 10.1093/annonc/mdw604
3. Garden OA, Volk SW, Mason NJ, Perry JA. Companion animals in comparative oncology: one medicine in action. *Vet J.* (2018) 240:6–13. doi: 10.1016/j.tvjl.2018.08.008
4. LeBlanc AK, Mazcko CN. Improving human cancer therapy through the evaluation of pet dogs. *Nat Rev Cancer.* (2020) 20:727–42. doi: 10.1038/s41568-020-0297-3
5. Leis-Filho AF, Lainetti PdF, Franzoni MS, Palmieri C, Kobayshi PE, Laufer-Amorim R, et al. A comparative *in silico* analysis of CD24's prognostic value in human and canine prostate cancer. *J Personal Med.* (2021) 11:232. doi: 10.3390/jpm11030232
6. Kosorok MR, Laber EB. Precision medicine. *Annu Rev Stat Appl.* (2019) 6:263–86. doi: 10.1146/annurev-statistics-030718-105251

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REVIEW
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The Importance of the Tumor Microenvironment and Hypoxia in Delivering a Precision Medicine Approach to Veterinary Oncology

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Treating individual patients on the basis of specific factors, such as biomarkers, molecular signatures, phenotypes, environment, and lifestyle is what differentiates the precision medicine initiative from standard treatment regimens. Although precision medicine can be applied to almost any branch of medicine, it is perhaps most easily applied to the field of oncology. Cancer is a heterogeneous disease, meaning that even though patients may be histologically diagnosed with the same cancer type, their tumors may have different molecular characteristics, genetic mutations or tumor microenvironments that can influence prognosis or treatment response. In this review, we describe what methods are currently available to clinicians that allow them to monitor key tumor microenvironmental parameters in a way that could be used to achieve precision medicine for cancer patients. We further describe exciting novel research involving the use of implantable medical devices for precision medicine, including those developed for mapping tumor microenvironment parameters (e.g., O_2, pH, and cancer biomarkers), delivering local drug treatments, assessing treatment responses, and monitoring for recurrence and metastasis. Although these research studies have predominantly focused on and were tailored to humans, the results and concepts are equally applicable to veterinary patients. While veterinary clinical studies that have adopted a precision medicine approach are still in their infancy, there have been some exciting success stories. These have included the development of a receptor tyrosine kinase inhibitor for canine mast cell tumors and the production of a PCR assay to monitor the chemotherapeutic response of canine high-grade B-cell lymphomas. Although precision medicine is an exciting area of research, it currently has failed to gain significant translation into human and veterinary healthcare practices. In order to begin to address this issue, there is increasing awareness that cross-disciplinary approaches involving human and veterinary clinicians, engineers and chemists may be needed to help advance precision medicine toward its full integration into human and veterinary clinical practices.

Keywords: precision medicine, tumor microenviroenment, implantable technologies, genomics, one-health

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INTRODUCTION

Precision or personalized medicine endeavors to enhance patient outcomes by treating individuals based on certain factors. These factors can include disease biomarkers and molecular signatures at the cellular level but also the phenotype, environment, and lifestyle of the individual (1). While precision and personalized medicine are similar concepts, there are some differences in their definitions. Precision medicine greatly depends on data collection, data analysis and information whereas personalized medicine is a healthcare model that takes into account patient genetics with consideration of patient preferences, beliefs, attitudes, and social background. However, as a result of concerns that the phrase "personalized" might be misinterpreted, possibly leading patients to believe that unique treatments and/or drugs were being developed particularly for themselves, "personalized medicine" has now largely been replaced with the term "precision medicine" (2, 3).

The National Research Council in America (4) adopted the definition of precision medicine from the President's Council of Advisors on Science and Technology in 2008 as: "The tailoring of medical treatment to the individual characteristics of each patient. . . to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment. Preventative or therapeutic interventions can then be concentrated on those who will benefit, sparing expense and side effects for those who will not." As the definition implies, the overall aim of precision medicine is to provide the most effective treatment for a patient (5), enhancing the quality of care whilst also decreasing the use of unnecessary diagnostic tests and therapies, thereby reducing costs and side effects (6).

The Precision Medicine Initiative

Precision medicine has obtained increased awareness in recent years within both human and veterinary research and clinical communities. The announcement of the "Precision Medicine Initiative" by Barack Obama during the State of the Union Address in 2015 led to increased media coverage and awareness within the lay community. This initiative sought to encourage research in the field of precision medicine in an effort "to bring us closer to curing diseases like cancer and diabetes—and to give us all access to the personalized information we need to keep ourselves and our families healthier" (7). The Precision Medicine Initiative, now termed "All of US," plans to register over 1 million participants (8). Those enrolled are expected to disclose any data produced from sequencing, digital healthcare technologies and electronic medical records over a 10 year period. These data will then be examined to increase our understanding of disease biology and pathogenesis whilst providing data to enable a precision-led healthcare approach for both individuals and the population as a whole.

The former Prime Minister of the UK, David Cameron, launched the 100,000 Genomes Project in 2012 (9). This project was led by Genomics England and the National Health Service and aimed to use whole-genome sequencing to improve the management of patients diagnosed with cancer and rare inherited diseases. 13 Genomic Medicine Centers were created to carry out this programme and in 2018 this initiative reached its goal of sequencing 100,000 whole genomes. This project not only provided whole-genome sequencing data for patient treatment selection or enrolment into clinical trials, but also enabled researchers to have access to anonymous clinical and genomic data sets. In veterinary medicine in the UK, "Dogslife" was the first national longitudinal canine health program to be set up. Launched in 2010, the study aims to identify genetic and environmental risk factors for canine diseases and use the information to generate disease risk reduction approaches (10).

The feasibility of implementing the precision medicine initiative has improved in recent years largely due to decreased costs associated with high-throughput DNA sequencing, the implementation of electronic medical records across the country and the utilization of advanced imaging systems that have the ability to assess the tumor microenvironment (TME). Additional genome-based technologies are also progressively being employed as either diagnostic assays to categorize disease, or as prognostic or predictive tests. Collectively, these approaches have been seen as the foundation for a new molecular disease classification system which will deliver a more accurate means by which clinicians can screen for and discover disease at its earliest stages. This ultimately will enable the selection of drugs and/or treatments directed by individual patient genetics. Since disease evolution from baseline risk to clinical symptoms frequently takes many years, intermittent molecular and digital profiling is expected to advance healthcare approaches from acute intervention and disease control toward a greater emphasis on pro-active management of disease risks and eventually disease prevention (8).

Oncology is perhaps one field that precision medicine can be most readily applied to. Cancer is a heterogeneous disease and although patients may be diagnosed with the same histological cancer type, their tumors may have varying genetic mutations or TMEs that lead to different responses between patients treated in the same way (11). Distinct treatment approaches for individual patients may therefore lead to improved outcomes (12). Within the field of oncology there is increasing awareness that cross-disciplinary approaches involving human and veterinary clinicians, engineers, and chemists may be needed to make significant progress in the field of precision medicine. In this review we discuss precision medicine with particular emphasis on the TME and describe how multidisciplinary groups are investigating the use of novel implantable technologies to achieve precision medicine. Finally, we describe a range of veterinary clinical studies that have used a precision medicine approach in a range of cancers in an attempt to improve patient outcome (**Figure 1**).

The Tumor Microenvironment

Assessing the TME to improve patient treatment can be considered an important aspect of precision medicine. Stephen Paget initially put forward the concept of the TME in his seed and soil theory, which postulated that cancer development and progression are regulated by the interaction of cancer cells with the external environment of the tumor (13). The TME is a complicated network, comprising of not only the

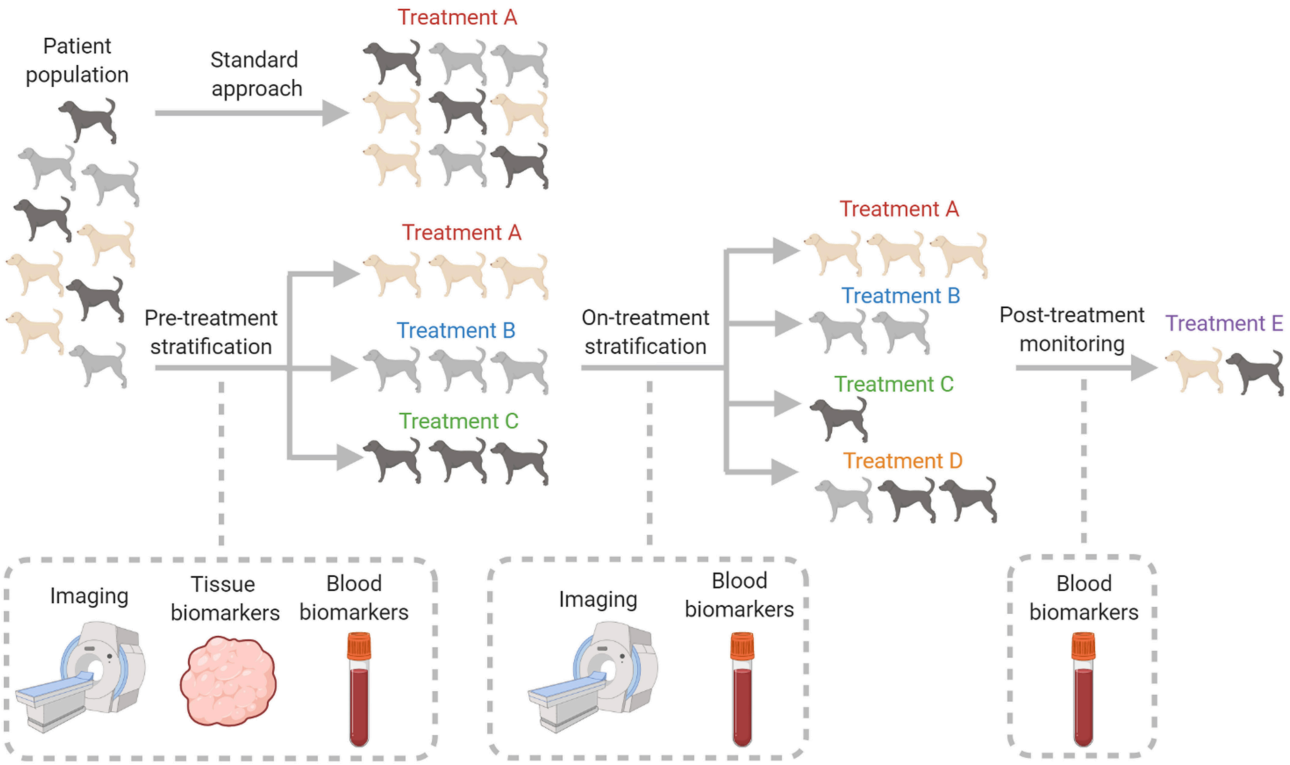

FIGURE 1 | Comparison of the currently employed traditional healthcare practices with prospective precision medicine approaches in veterinary oncology. Veterinary patients presenting with the same tumor type are currently classified by their clinical stage and histological grade. This usually leads to a dichotomic decision to treat with standard specific treatment protocols, or not to treat at all. The vision of precision medicine is to develop diagnostic and monitoring techniques, applied to tumors of the same histological grade and clinical stage, to distinguish an optimized treatment strategy for each individual patient. Figure created with BioRender.com.

tumor cells themselves but also various tumor-associated cells. These associated cells include cancer stem cells, cancer-associated fibroblasts, mesenchymal stem cells, pluripotent stromal cells, cancer-associated adipocytes, endothelial cells, pericytes, and tumor-associated immune and inflammatory cells. These tumor-associated cells secrete soluble molecules and microvesicles that control the interactions of cancer cells with other cell types. This can influence how the tumor cells proliferate, oppose apoptosis, avoid elimination from the immune system, preserve stemness, invade, and metastasize (14).

The physiology of cancers differs greatly from that of normal tissues within the body; this situation results from the tumor needing to develop its own blood supply as it begins to outgrow the accessible vasculature from the organ in which it originates. However, the tumor neovasculature is crude and disordered, suffering from many functional and structural irregularities. This tumor vasculature system is therefore unable to satisfy the metabolic needs of the developing cancer (15). This leads to areas within a tumor that have differing O_2 tensions (anoxia, hypoxia, and normoxia) in combination with glucose deprivation, interstitial hypertension, and extra-cellular acidosis (16, 17). The differing compositions of TME between patients is thought to impact the effectiveness of different cancer therapies. If TME parameters such as biomarkers, cancer metabolites, chemotherapeutic drug concentrations or intra-tumoural pH and O_2 could be analyzed over the course of a patient's treatment schedule, then this information could influence the management of the patient. Treatment could be tailored on the basis of the tumor's response to the initial therapy or through the identification of early markers of tumor recurrence or metastasis (18).

Tumor Hypoxia and pH

Low O_2 levels, or hypoxia, is a regular feature of many tumors. Approximately 60% of solid cancers contain hypoxic areas heterogeneously dispersed throughout the tumor (15); this makes O_2 a very relevant TME parameter to investigate. The vast majority of non-cancerous mammalian tissues operate in O_2 levels of 2–9%. Hypoxic and severely hypoxic states, defined as $\leq$2% O_2 and $\leq$0.02% O_2, respectively (19), can occur within tumors via 4 main mechanisms:

1) Diffusion-limited hypoxia. Mainly leading to chronic hypoxia, diffusion-limited hypoxia is caused by viable aerobic cancer cells positioned close to blood vessels using the O_2 transported in red blood cells (RBC). O_2 levels decrease as the distance from the vasculature increases, resulting in hypoxia (20, 21). Even though O_2 can diffuse up to 150–180 μm from a blood vessel, the distance at which hypoxia arises is dependent on the ability of RBC to carry O_2, in addition to the metabolic needs of the cancer cells situated closest to the blood vessels (22).

2) Perfusion-limited hypoxia. This mostly leads to acute and transient hypoxia, and is caused by interruptions to blood flow (23, 24) resulting from structural/functional defects of micro-vessels within a cancer, which can lead to micro-vessel collapse/blockade (25). This may lead to complete ischemic hypoxia, or hypoxia that stops RBC flow but permits plasma flow to continue the provision of nutrients to the cancer cells.
3) Anemic hypoxia. Resulting from therapy-induced and/or tumor-associated anemia that causes decreased RBC O_2 transport capabilities (20, 26).
4) Macroscopic regional hypoxia. Occurs due to the production of an O_2 gradient over the length of a blood vessel. Blood present at the arterial extremity has the highest O_2 levels, which is quickly consumed by tumor cells present in this area. O_2 levels in the blood decrease as distance from the arterial end increases. As a result, cancer cells positioned at the distal end of the blood vessel may be hypoxic, even if they are close to the vessel (27, 28).

While it seems reasonable that low O_2 levels would produce an adverse environment for cells to survive, tumor cells possess the ability to create a hypoxic response through alterations in gene expression that shield them from cell death mechanisms (29). Cancer cells that are incapable of adjusting to low O_2 concentrations will perish, resulting in the selection of tumor cells with hypoxic-induced, genetically-fixed characteristics, known as a "hypoxic phenotype" (30). This hypoxia-driven malignant progression can lead to tumor cells becoming increasingly metastatic and therapy-resistant (31). There are a variety of crucial inducible transcription factors that participate in this hypoxic response, with hypoxia inducible factor-1 (HIF-1) being the one that has been most comprehensively studied so far (32–35). In areas where O_2 levels are approximately 2% or less, these transcription factors are stabilized and transported to the cell nucleus, where they then bind hypoxia-response elements present in the promoter regions of target genes, leading to transcription (36). These hypoxia-regulated genes are involved in processes such as erythropoiesis, angiogenesis, apoptosis, autophagy, proliferation, glucose metabolism, intra-cellular acidosis, and metastasis. These adaptations enhance the capacity of cancer cells to endure these hostile low O_2 TME regions (37).

In order to counteract the reduced mitochondrial ATP production that occurs due to low O_2 levels, hypoxic cells can instead get their energy through anaerobic glycolysis, resulting in the generation of lactic acid, which can cause substantial acidosis within solid cancers (38). Even in normoxic conditions, some cancer cells still rely on glycolysis for energy production. This phenomenon, known as the Warburg effect, can lead to discrepancies in the spatial/temporal distribution of areas with low O_2 concentrations and increased amounts of acidosis (39–41).

The Tumor Microenvironment and Its Effect on Treatment Responses

The heterogeneity that occurs within the TME can influence how tumors respond to frequently-employed cancer treatments and can subsequently have a negative impact on patient outcome. The abnormal cancer vasculature can inhibit the delivery of systemic drugs to the tumor, leading to heterogeneous drug dissemination within cancer tissue (42). In immunotherapy, high molecular weight drugs access cancer cells by the interstitial space rather than through blood vessels (43). Interstitial hypertension that can occur within tumors pushes fluid out of the interstitial space, thus restricting drug delivery (44, 45).

O_2 levels within tissues can also affect tumor radiosensitivity and the efficacy of chemotherapeutic drugs (46). Radiotherapy works through damaging the DNA of cancer cells, either via the direct route (the ionizing radiation directly produces double or single strand breaks in the DNA) or the indirect route (ionizing radiation produces free radicals within the cancer cells, which subsequently cause DNA damage). It has been shown that tumor cells exposed to low O_2 concentrations can tolerate radiation doses 2–3 times higher than that which can be withstood by normoxic cancer cells. The O_2 fixation hypothesis has been suggested to explain the role of O_2 in radiotherapy (19, 47). O_2 present in cancer tissues reacts with DNA radicals produced as a result of radiation treatment, leading to the production of irreparable peroxy radicals. However, this reaction does not take place when O_2 is not present; in these instances, damaged DNA strands can be repaired by free-radical scavengers such as endogenous thiols, granting these hypoxic cancer cells a considerable survival advantage (48). The effect that O_2 has on radiotherapy was emphasized in a significant global clinical study which showed that pre-treatment O_2 levels within head and neck tumors was a prognostic factor for survival post-radiotherapy (+/– chemotherapy, surgical intervention, or radiosensitizer) (49). While the form of hypoxia (i.e., acute or chronic) is unrelated to the initial radioprotective effect, cancer cells under chronic hypoxic conditions are usually more deprived of nutrients in comparison to acutely hypoxic cells. This nutrient deprivation could have a part to play in the cell's capacity to repair radiation-induced DNA damage; as such, acute hypoxic cancer cells may be more radioresistant than chronic hypoxic cells.

Reduced O_2 levels and low pH conditions can also decrease the proliferation rates of tumor cells (50); this can in turn inhibit the efficacy of cytotoxic drugs that target rapidly-dividing cancer cells (51). Chemotherapeutic drugs such as doxorubicin (DOX) have also been shown to have increased efficacy in normoxic conditions; the intra-cellular metabolite of DOX reacts with O_2 present in the TME, leading to the generation of reactive oxygen species (ROS). These ROS damage major components of the cell, leading to cellular death (52). This reaction does not take place in areas of the tumor where O_2 levels are reduced ($\leq$0.33%), leading to a reduction in the efficacy of DOX (53–55).

Therapeutic Strategies to Target Hypoxic Tumors

Different treatment approaches have been assessed for their capacity to surmount hypoxia-related resistance to radiation. These strategies include hyperbaric O_2 treatment, the administration of agents that target the hypoxic areas of cancers, or drugs that are activated in these low O_2 regions.

Hyperbaric O_2 therapy, which uses high ambient air pressures to increase the amount of O_2 carried in the patient's blood, has been found to have a beneficial effect in patients suffering from head and neck squamous cell carcinomas (HNSCC) (56); in spite of this, varying data produced from clinical trials in other cancer types, in addition to the logistical issues associated with its use, have hindered its general utility. Additionally, this form of therapy is not appropriate in combination with some chemotherapeutic drugs, including DOX, because it increases the likelihood of systemic ROS-mediated toxicities (57). A different method that has been used to increase O_2 levels within tumors is the administration of carbogen with nicotinamide. Carbogen (also named Meduna's Mixture after its inventor) is a mixture of CO_2 and O_2 and has the ability to diminish diffusion-limited hypoxia, while nicotinamide is a vasoactive agent that counteracts acute hypoxia resulting from reduced perfusion. Accelerated radiotherapy in combination with carbogen and nicotinamide (ARCON) has been assessed in several clinical trials, demonstrating enhanced locoregional control and disease-free survival (58, 59). Likewise, clinical trials using nitroimidazole derivatives (e.g., nimorazole and doranidazole) that mimic the effects of O_2, have shown that these drugs can produce survival benefits when combined with chemoradiotherapy in HNSCC (60) and non-small cell lung cancer (NSCLC) patients (61), or when used with radiotherapy alone (62, 63). In both Norway and Denmark, nimorazole combined with radiotherapy is the standard of care treatment for head and neck cancers (60, 62, 64). Additionally, the combination of radiotherapy with nicotinamide and carbogen has exhibited encouraging results in bladder and laryngeal cancers (65).

The identification of hypoxic tumor areas can also allow more effective delivery of radiation. Tumors that have large regions of hypoxia could be given increased radiation doses over the gross tumor volume; however, this method may lead to an escalated risk of damaging surrounding normal tissues. A superior technique would be to establish a biological target volume based on low O_2 levels. With this approach the overall dose given to the gross tumor volume stays the same but it is redistributed to specific tumor regions; increasing the dose delivered to the low O_2 areas, whilst decreasing that given to the regions with higher O_2 concentrations. The biological target volume could also be given boost doses, either applied homogeneously over the hypoxic sub-volume, or altered in accordance with the local O_2 levels. Improvements in radiation-delivery technology, including intensity-modulated and image-guided radiotherapy, enable highly conformal and accurate delivery of radiation; these developments mean that the use of sculpturing techniques or dose painting by numbers are becoming increasingly attainable (66). These new treatments are substantiated by modeling studies, which indicate that a boost dose of 10 Gy to low O_2 regions in HNSCC patients could lead to a 17% increase in tumor control probability, without escalating the risk of complications (67). A different study has demonstrated that dose escalation, employing dose painting by numbers to the biological target volume, could also result in improved tumor control probability compared to that achieved with uniform dose escalation (68).

Case selection is a crucial feature of clinical trials designed to assess hypoxia-modifying therapies. Unfortunately, many previous studies enrolled patients into these clinical trials without initially determining the hypoxic nature of the tumors. Failure to specifically detect hypoxic tumors would inevitably lead to treating patients who were unlikely to gain any benefit from hypoxic modification. This issue leaves us with a crucial question: what is the best method to analyse O_2 levels so clinicians can effectively decide which patients will benefit from therapeutic strategies designed to target hypoxic cancer cells?

Tumor Functional Assessment

There are various methods by which hypoxic areas within tumors can be detected, but none are used routinely either due to their invasive nature or difficulties in incorporating them into clinical practice (**Table 1**). One of the first approaches for directly measuring intra-tumoral O_2 was with the Eppendorf O_2 electrode (70). This technique showed associations between hypoxia and treatment responses in numerous cancer types (71–73, 73–75) and that O_2 concentrations within breast cancer tissue can be lower than that of normal breast tissue (70). However, this technique is invasive and only applicable to readily accessible tumors and is not used routinely in the clinic.

While the Eppendorf O_2 electrode represents a direct method of measuring O_2 concentrations within cancer tissues, there are also various indirect methods that can be utilized. The use of cancer tissue biopsies to identify molecular reporters of O_2 is one such indirect method. Nitroimidazole-based agents, such as pimonidazole, are chemicals that produce adducts with intra-cellular macromolecules in reduced O_2 concentrations (76); pimonidazole has been utilized successfully in clinical trials to choose patients that would be suited for treatment with hypoxia-modifying drugs alongside accelerated radiotherapy (58, 59). To exploit the cellular response of cancer cells to low O_2 levels, the expression levels of hypoxia-induced genes, mRNAs, and proteins have also been employed as biomarkers of hypoxia (77–80). In dogs with mammary tumors, high *VEGFA* gene expression has been associated with poor outcome, with one study suggesting that it could be used as a prognostic marker to identify dogs at risk of disease progression (81). However, markers including HIF-1, CA9, VEGF and GLUT1, analyzed either at the mRNA or protein level, or through the use of nitroimidazole, have frequently brought about contradictory results. This is probably because these genes and proteins can be regulated by factors other than O_2, such as glucose levels or extracellular pH (82–84), or their expression is induced at O_2 concentrations not low enough to have a significant radiobiological effect. For nitroimidazole to bind to macromolecules it requires cells to be hypoxic for long periods of time. Therefore, this can result in an underestimation of the levels of acute hypoxia within the TME.

These problems led to the production of hypoxic signatures that were created by pinpointing a variety of genes that were upregulated in low O_2 conditions; these signatures were produced from either cell lines or clinical tissues (85–92). The Toustrup15-gene-classifier is one such signature that was generated from a panel of genes ascertained using HNSCC cell

TABLE 1 | Methods used for measuring tumor hypoxia.

Microelectrode
Technique: O_2 electrodes are put into solid tumors
Benefits: Provides direct readings of O_2 levels. Simple to use. Ability to acquire real-time readings
Drawbacks: Invasive procedure. May only be utilized in tumors that are accessible. Incapable of differentiating viable hypoxic tumor regions from necrotic areas
Tissue based biomarkers
Technique: Nitroimidazole compounds, such as pimonidazole, are given systemically and are changed into protein adducts in hypoxic cells. Detectable in biopsies through IHC
Benefits: Provides an estimate of hypoxia heterogeneity in different cancer regions
Drawbacks: Indirect measurement of O_2 concentrations. Invasive procedure (biopsy required). May not distinguish intermediate hypoxic phenotypes (HIF-1 stabilization occurs at higher O_2 levels than that at which adducts are formed). Real-time measurements are not possible
Tissue based biomarkers
Technique: Assessing the expression of proteins induced in hypoxic conditions (GLUT1, CA9)
Benefits: The location of protein expression and data on functional status can be provided through IHC. Gives an estimate of hypoxia heterogeneity within different cancer regions
Drawbacks: Indirect measurement of O_2 concentrations. Invasive procedure (biopsy required). Protein expression can be manipulated by factors other than hypoxia. Method limited to a small number of biomarkers due to antibody sensitivity and specificity issues. Real-time measurements are not possible
Tissue based biomarkers
Technique: Assessing gene expression levels
Benefits: Prognostic/predictive of response to hypoxic radiosensitizers
Drawbacks: Indirect measurement of O_2 concentrations. Invasive procedure (biopsy required). Gene expression can be manipulated by factors other than hypoxia. Real-time measurements are not possible
Serological based biomarkers
Technique: Analysis of biomarkers present within the serum (osteopontin)
Benefits: Non-invasive. Real-time measurements are possible
Drawbacks: Indirect measurement of O_2 concentrations. Biomarker levels can be manipulated by factors other than hypoxia. No information provided on hypoxic heterogeneity in different cancer regions.
Positron emission tomography
Technique: Copper-complexed dithiosemicarbazone or nitroimidazole agents, given systemically, are taken up by hypoxic cancer cells. Agents are attached to radiotracers that can be distinguished through PET imaging
Benefits: Non-invasive procedure. Capable of acquiring real-time data. Can provide an estimation of hypoxic heterogeneity in different cancer regions
Drawbacks: Indirect measurement of O_2 concentrations. May not distinguish intermediate hypoxic phenotypes (HIF-1 stabilization occurs at higher O_2 levels than that at which agents form adducts). Safety issues with the use of radioisotopes
Magnetic resonance imaging
Technique: Either blood oxygen level-dependent (BOLD) imaging, dynamic contrast-enhanced MRI (DCE-MRI) or oxygen-enhanced MRI (OE-MRI)
Benefits: Non-invasive procedure. Real-time measurements can be obtained. Can provide an estimation of hypoxic heterogeneity in different cancer regions. Radiation exposure avoided
Drawbacks: Possibility of artifacts. The parameters used to reflect O_2 levels and their link with hypoxia is yet to be determined

Advantages and disadvantages of each method used to measure tumor hypoxia (69).

lines; these genes were found to be upregulated by hypoxia, independent of pH (85). This gene signature was further developed in a training cohort of 58 patients suffering from HNSCC, with O_2 levels analyzed with an electrode. The classifier was validated in a Danish study where patients were randomly selected to be treated with radiotherapy combined with either the hypoxic radiosensitizer nimorazole or a placebo. The classifier was shown to be prognostic and also possessed predictive power for hypoxic modification (85, 93, 94). The 26-gene signature generated by Eustace et al. (91) is another classifier based on a metagene signature generated from patients suffering from lung, breast and head and neck cancers. The ARCON trial carried out in the Netherlands assessed this signature, evaluating radiotherapy combined with nicotinamide and carbogen compared to radiotherapy alone in patients with laryngeal cancer. The patients that were classed as "more hypoxic" with the 26-gene signature exhibited significantly better locoregional control when treated with hypoxia-modifying compounds (44, 59).

Indirect assessments of tumor hypoxia rely on the ability to obtain a tissue sample. Unfortunately, these can be extremely challenging or even impossible to acquire due to tumor location or the patient's condition. Treatment response monitoring through repeat biopsies is also difficult to clinically justify. The measuring of hypoxic-related biomarkers in blood samples has been investigated as a means of overcoming these issues. In humans, high levels of plasma osteopontin have been associated with a poor prognosis in head and neck cancers, which could be improved with O_2-mimicking drugs (95). Serum concentrations of HIF-1α and VEGF in dogs with mammary tumors have also been shown to have prognostic merit; high VEGF levels were associated with lymph node and distant metastasis, tumor vascularization and decreased survival times, whereas high HIF-1α levels were related to local recurrences or metastatic lesions

(81). The major disadvantage of using blood to evaluate the hypoxic status of tumors is that the sample, in effect, does not analyse the tumor at all. This means that specific hypoxic tumor areas cannot be identified and alterations to the O_2 distribution within the tumor cannot be assessed. Tumor hypoxia assessment through non-invasive advanced imaging techniques, such as positron emission tomography (PET) and magnetic resonance imaging (MRI), offer the ability to evaluate the whole tumor volume whilst providing a means for repeat measurement.

Dynamic contrast-enhanced MRI (DCE-MRI) typically uses an intravenous injection of a gadolinium-based contrast agent. Signal changes identified by MRI as the agent passes through the tumor's blood supply provide an indication of intra-tumoral perfusion. Blood O_2 level-dependent (BOLD) MRI imaging detects signal alterations caused by changes in deoxygenated hemoglobin levels. Patients are required to undergo dynamic challenges to alter deoxygenated and oxygenated hemoglobin ratios in order to map hypoxic tumor regions (69). Over the years, PET imaging has been developed to assess specific tumor characteristic such as glycolysis (2-deoxy-2-[^{18}F]fluoro-D-glucose [^{18}F-FDG]), hypoxia ([^{18}F]fluoromisonidazole [FMISO], CA9, copper-complexed dithiosemicarbazone [^{64}Cu-ATSM]), and proliferation (3′-deoxy-3′-[^{18}F]fluorothymidine [^{18}F-FLT]). ^{18}F-FDG, ^{64}Cu-ATSM, and ^{18}F-FLT have all shown promising results for monitoring treatment responses in numerous human and canine cancer types (96–100).

One canine study demonstrated the feasibility of using multiple PET tracers to simultaneously evaluate glycolysis (^{18}F-FDG), hypoxia (^{64}Cu-ATSM), and proliferation (^{18}F-FLT) in a dog diagnosed with a tibial fibrosarcoma. Serial imaging performed before, during and after 10 fractions of 4.5 Gy demonstrated a heterogeneous spatial distribution of the 3 tracers. ^{64}Cu-ATSM uptake progressively decreased during and after radiotherapy, suggesting either tumor reoxygenation (in areas where ^{18}F-FDG uptake was maintained) or the development of dead tumor tissue (in areas of reduced ^{18}F-FDG uptake). Although ^{18}F-FDG and ^{18}F-FLT uptake significantly decreased following the completion of radiotherapy, fluctuations were seen throughout the treatment course. ^{18}F-FLT fluctuations could be indicative of accelerated tumor cell repopulation, whereas ^{18}F-FDG fluctuations might suggest the presence of inflammation or tissue remodeling. The authors put forward the suggestion that each tracer provided distinct information about the TME, including heterogeneity, phenotype and response to treatment, which could be used to tailor therapies to individual patients (101). Other canine studies have used ^{64}Cu-ATSM and ^{18}F-FDG to identify biological target volumes for dose painting radiotherapy (102), with ^{18}F-FLT and ^{18}F-FDG also having been investigated as a means of monitoring radiotherapy responses and identifying disease progression (103). Pre-treatment ^{18}F-FDG, ^{18}F-FLT, and ^{61}Cu-ATSM scans could also be used to predict the response to radiotherapy (104).

Unfortunately, these advanced imaging techniques are not without limitations; in particular, the resolution (i.e., voxel size; a 3D representation of a pixel as determined by slice thickness and pixel size) offered by these techniques can be greater than the hypoxic tumor areas themselves. This means that small hypoxic "hotspots" may be classified as normoxic if the mean value for the area falls below a specific cut-off point. In clinical terms, PET imaging may not be able to accurately characterize intra-tumoral hypoxic heterogeneity at microregional levels.

Currently, regardless of whether tumor hypoxia, glycolysis, or proliferation are assessed using tissue or blood samples or through advanced imaging, the results only provide a static indication of the TME at the time of analysis. This is a significant limitation of any technique, as the TME is a dynamic landscape with potentially significant spatial and temporal changes occurring throughout the tumor at any given time. Therefore, despite technological advances, clinical techniques that can acquire continuous real-time data in order to generate an accurate 3D map of the TME, including its hypoxic status, within the entire tumor volume are still not available. A potential solution to this unmet clinical need is the use of an implantable sensor designed to continually measure key TME parameters. This technique has the potential to provide information on the spatial and temporal changes that occur within the tumor during a patient's treatment course.

Implantable Technology and Cancer

Developments in the fields of electronics, mechanical engineering, and microfabrication have resulted in increased interest in the clinical applications of implantable medical devices for precision medicine. In line with this, studies have begun investigating whether implantable devices could be used to map the TME for parameters such as O_2, deliver local drug treatments, assess treatment responses, and monitor for tumor recurrence and metastasis (**Figure 2**).

Implantable Sensors and Monitoring of Tumor Oxygenation and pH

Due to the limitations associated with current technologies designed for measuring intra-tumoral O_2 levels, researchers are investigating novel ways to address this unmet clinical need. Two recent studies have developed implantable sensors that are able to provide O_2 and pH measurements within a tumor.

The Implantable Microsystems for Personalized Anti-Cancer Therapy (IMPACT) project is developing both miniaturized Clark-type electrochemical O_2 sensors, methylene blue-based electrochemical pH sensors and ISFET pH sensors (105). The purpose of the project is to manufacture implantable wireless sensors for the real-time monitoring of intra-tumoral O_2 levels and pH, thus allowing radiotherapy to be delivered at the most effective location and time. *In vivo* mouse xenograft tumor studies using a human breast cancer cell line were used to evaluate biofouling and the foreign body response that occurred when constituent materials of the IMPACT sensors were implanted into a solid tumor. This study was the first to assess the effects of modern implantable materials within a TME. The authors concluded that none of the evaluated materials had any detrimental effect on tumor growth or body weight of the murine host. Up to 14 days post-implantation, immunohistochemistry (IHC) showed no significant changes in hypoxic cell number, tumor necrosis, apoptosis, proliferation, collagen deposition, or immune cell

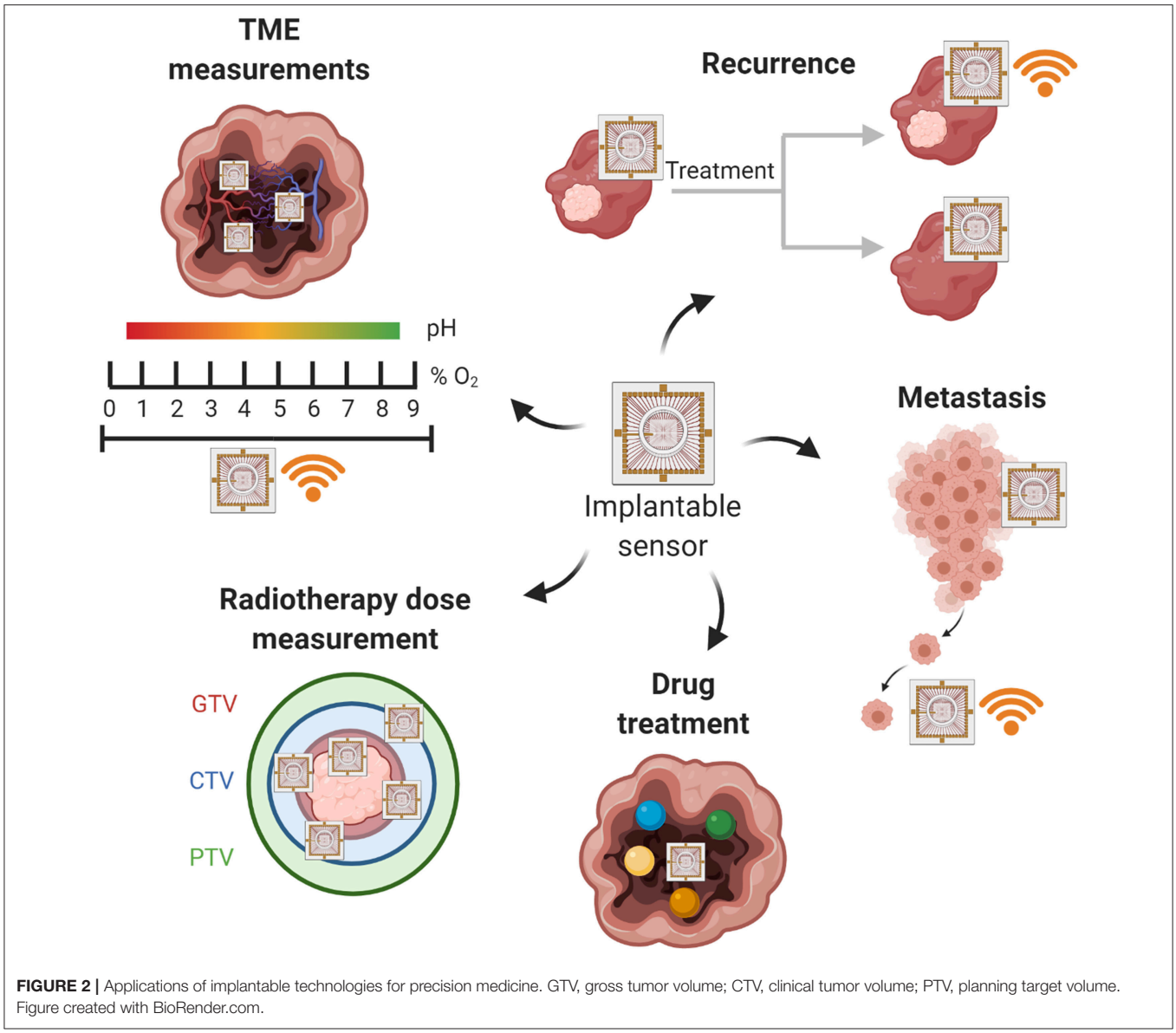

FIGURE 2 | Applications of implantable technologies for precision medicine. GTV, gross tumor volume; CTV, clinical tumor volume; PTV, planning target volume. Figure created with BioRender.com.

infiltration. The authors suggested that the absence of biofouling supports the use of these materials in medical devices designed for implantation within solid tumors (106). Functional O_2 sensors were subsequently evaluated in a translational large animal lung cancer model (107). Following CT-guided implantation into lung tumors, initial results have demonstrated that these sensors remained functional and were sufficiently sensitive to monitor acute changes in oxygenation within tumor tissue (108).

Other researchers have taken a different approach to monitoring TME parameters. One such study has designed a miniaturized implantable nuclear magnetic resonance (NMR) sensor. This incorporates a responsive NMR contrast agent that can be assessed wirelessly via magnetic coupling to an external reader without the need of an MRI scanner. This study demonstrated the feasibility of the implantable NMR sensor to separately detect pH and O_2 using *in vitro* and *in vivo* model systems. The *in vivo* experiments consisted of monitoring intra-tumoral pH in a xenograft mouse model and assessing O_2 levels in a rat hind limb constriction model. The authors suggested that this sensor could be implanted at the time of tumor biopsy and could remain within the tumor providing repeat O_2 and pH measurements from the same tumor area. They further suggested that the sensor could be used for other NMR applications, such as low-resolution spectroscopy to identify soluble biomarkers (109).

Although further engineering development of both sensor types is required, the results from these exciting pre-clinical studies have shown that intra-tumoral O_2 and pH can be monitored in real-time. If they progress toward testing in clinical trials, they could eventually prove to be formidable tools to identify and target treatment-resistant tumor areas.

Implantable Sensors and the Clinical Monitoring of Cancer Progression

The ability to monitor treatment responses and identify early indicators of tumor recurrence may be achieved through the use of implantable medical devices. Sensors that detect cancer-related biomarkers could be implanted either within the tumor at the time of biopsy or within the tumor bed at the time of surgery following tumor excision (110, 111). This application would be highly useful in disease conditions where advanced imaging cannot reliably distinguish between necrosis/fibrosis resulting from previous surgery/chemotherapy/radiotherapy and tumor recurrence (e.g., glioblastomas) (112).

The first *in vivo* report to support the use of implantable devices for this application was designed to detect the β subunit of human chorionic gonadotrophin (hcG-β) (111), a soluble cancer-related biomarker known to be secreted in high levels in ovarian and testicular cancers. The sensor was designed using nanoparticle magnetic relaxation switches (MRSw) conjugated with hcG-β antibodies and was evaluated using a mouse xenograft model. Sensors were implanted into tissue surrounding hcG-β-producing human choriocarcinoma xenograft tumors (JEG-3). The results demonstrated that MRI was able to identify MRSw aggregation that occurred with hcG-β antibody binding, which provided evidence that the implanted device could successfully detect hcG-β in peri-tumoral tissue. The authors suggested that, as MRSw can be modified to detect various molecules, they might provide a means of detecting and monitoring a variety of cancer-related biomarkers (113).

Another study has investigated the use of implantable devices designed to identify early indicators of metastasis through the evaluation of localized immune responses. This study developed a synthetic microporous polymer scaffold that promotes tissue ingrowth through vascularization, cellular infiltration, and immune cell recruitment. This engineered microenvironment was shown to have characteristics of a metastatic niche that could capture circulating tumor cells. *In vivo* evaluation of the immune responses that developed within subcutaneously-implanted scaffolds was performed using an orthotopic breast cancer murine xenograft model. Phenotypes and gene expression profiles of immune cells were determined from sequential scaffold biopsies obtained up to 21 days post-primary tumor inoculation. These results suggested that an immunosuppressive microenvironment developed which had characteristics of metastatic diseased lung. Following excision of the orthotopic breast tumor, sequential analysis of immune phenotypes within the scaffold showed an initial response to surgery, which could differentiate mice that suffered a tumor recurrence from those that survived. The authors concluded that the microenvironment that developed within the scaffold reflected the immunosuppressive events that contribute to the development of metastatic disease. Furthermore, monitoring these immune responses identified animals which had disease progression from those that responded to surgery (114). Technologies such as this may provide a means of identifying patients at risk of developing metastatic disease or those who have early metastatic disease that is clinically undetectable by imaging. Patient outcomes have the potential to be improved if the earliest stages of metastatic disease can be identified and treatment intensification initiated.

Implantable devices can not only be designed to monitor specific tumor-related factors but may be utilized to assess the accuracy of delivering a desired radiation dose to a specific tissue/tumor area, an issue which is fundamental to the clinical effectiveness and success of radiotherapy. To address this, implantable dosimeters have been developed to assess what radiation dose has been delivered per treatment fraction to the planned target volume. This dose-verification system has gained FDA approval for use in prostate and breast cancers, and has the potential to allow radiotherapy treatment regimens to become optimized for individual patients (115).

Cancer Treatment and Implantable Technology

Although many cancer types can be treated with a variety of clinically-approved drugs, clinicians are often faced with the difficult decision of which drug would be most effective in individual patients. Sometimes this decision can be made based on published evidence, but occasionally the choice will be empirical and based solely upon the clinician's personal experiences. To overcome this issue, studies have investigated whether multiple drugs can be simultaneously evaluated in the tumor itself to provide information on which is most effective. These types of studies have the ability to account for tumor heterogeneity, as drug effects can be analyzed within spatially distinct TME regions.

One study developed a medical device that was able to deliver 8 drugs concurrently into mapped and defined regions within a tumor. This research initially used lymphoma and NSCLC murine xenograft models for validation of the device. Following intra-tumoral injection, drug distribution was assessed through radiolabelling imaging. Tumors were excised up to 72 h after drug delivery and cytotoxic drug responses were evaluated through IHC. Results demonstrated that not only could local tumor responses predict response to systemically-delivered drugs, but that the most effective compound for a chemoresistant lymphoma xenograft tumor could be identified by screening multiple drugs simultaneously. This work was followed by a small clinical study designed to assess patient satisfaction and procedural complications. Using the device, combined with ultrasound guidance, human and canine lymphoma patients received microinjections of vincristine into enlarged lymph nodes. Results suggested that localized tumor responses can be tested in a toxicity-sparing manner (116). Employing a similar approach, a further study developed a short-term implantable device composed of multiple reservoirs. Following intra-tumoral implantation, each reservoir was capable of delivering drugs into spatially distinct TME areas at concentrations equivalent to systemically-achievable doses (117). 24 h after implantation, the device and the immediately adjacent surrounding tumor tissue was removed with IHC performed to assess each drugs' cytotoxic effect (117).

These studies demonstrate that implantable devices can be used to conduct *in vivo* drug testing through local delivery directly within the TME. The ability to evaluate spatially-defined tumor responses to multiple drugs provides an opportunity

to identify a patient's optimal drug treatment before definitive systemic chemotherapy begins.

A further consideration for determining the most appropriate chemotherapy regimen for a patient is the issue of multidrug resistance (MDR). MDR occurs when cancer cells develop cross-resistance to multiple functionally and structurally unrelated chemotherapeutic agents. One of the most common MDR mechanisms seen in various cancer types is related to the expression of phosphoglycoprotein multidrug resistance protein 1 (MRP1), part of the ATP-binding cassette. High MRP1 expression has been correlated with reduced tumor responses to many chemotherapeutic agents, including 5-fluorouracil (5-FU), along with reduced overall survival times. In order to address this issue, a study has developed a bio-responsive hydrogel-nanoprobe comprised of a 5-FU-intercalated DNA hairpin. This nanoprobe was designed to locally detect and bind to a complementary *MRP1* target sequence within the TME, resulting in *MRP1* silencing and inhibition of protein expression. Binding of the DNA hairpin to the *MRP1* sequence also caused a conformational change to the nanoprobe, which resulted in release of 5-FU. A 5-FU resistant breast cancer murine xenograft model was used to evaluate the ability of these nanoprobes to detect and overcome MDR. *In vivo* imaging was performed for 14 days following the implantation of a hydrogel disc loaded with nanoparticles into a xenografted tumor. Luciferase expression was utilized to assess tumor inhibition, while FITC fluorescence emission could identify nanoprobes before and after hybridization to MRP1 mRNA. Results indicated that, despite 5-FU resistance, more than 90% tumor reduction was achieved following 80% MRP1 silencing. The authors suggested that this approach could not only be used to reverse 5-FU resistance, but that it could also be used to reverse resistance to other chemotherapeutic agents and improve treatment responses (118).

As opposed to targeting specific resistance mechanisms, other studies have investigated ways in which the cytotoxicity of chemotherapeutic agents can be enhanced. A novel solution to this is through the use of implantable O_2-generating depots; these are designed to improve DOX cytotoxicity by promoting ROS production through their ability to increase intra-tumoral O_2 concentrations, without affecting systemic levels. Calcium peroxide (CaO_2) alginate microencapsulated pellets have been developed to react with interstitial fluid, forming calcium hydroxide [$Ca(OH)_2$] and hydrogen peroxide (H_2O_2); it is this H_2O_2 which then decomposes to release O_2. These pellets have been shown to successfully reduce the hypoxic TME of Hep3B xenograft tumors and increase the chemotherapeutic effect of DOX following their implantation into peri-tumoral tissue. The results from this study demonstrated the feasibility of using this O_2-generating system to locally enhance the cytotoxic effects of DOX and overcome hypoxia-induced DOX resistance (53).

Precision Medicine in Veterinary Clinics

Although veterinary precision medicine has yet to be incorporated into routine clinical practices, there are encouraging results from pre-clinical and clinical research studies that provide examples of how it could be used in the treatment of veterinary patients.

The development of the first canine-specific anti-cancer drug, toceranib (Palladia^{TM}), is the current closest example of veterinary precision medicine routinely used in the clinics. Toceranib is a novel multi-receptor tyrosine kinase inhibitor (119). Following pre-clinical studies demonstrating its anti-proliferative (*in vitro* cell line models) and anti-angiogenic activity (*in vivo* murine xenograft models), phase I trials were undertaken (120). These trials were conducted in dogs diagnosed with various tumor types; all patients had a guarded prognosis as they had either failed standard treatment regimens or there was no available therapeutic alternative. Results from this trial and subsequent studies showed toceranib had the greatest tumor response rates in mast cell tumors (MCT) (121). Toceranib subsequently gained clinical approval for the treatment of grade II and III cutaneous MCT. Although this drug was not developed as part of a precision medicine approach, further investigation of its mode of action demonstrated that MCT with a specific *c-KIT* mutation had better response rates compared to those that had no mutation (60 vs. 31% response). This *c-KIT* mutation was identified as an internal tandem duplication that alters *KIT* expression and causes constitutive receptor phosphorylation. Unlike clinically-approved kinase inhibitors in human medicine, the approval granted for toceranib did not stipulate its use solely for MCT with specific *c-KIT* mutations largely because some dogs without mutations still responded to treatment. The situation is also complicated by the issue that there is no clinically-approved canine genetic test for *c-KIT* mutations. Therefore, while toceranib is the closest, evidence-based, precision medicine approach in veterinary medicine, it currently fails to meet important considerations in which human-targeted therapies are based (122).

The use of circulating tumor cells (CTC) and transcriptomics to provide precision veterinary medicine through the identification of prognostic, predictive or treatment response information has also been investigated in multiple studies. CTC from canine mammary tumors have been shown to correlate with metastatic disease development and provide prognostic information that can be used to determine more aggressive treatment regimens for high-risk patients (123). Similar approaches have used transcriptomics to generate prognostic mRNA signatures from canine mammary tumors and lymphomas. These results demonstrated that the transcriptome could indicate malignancy and metastatic potential, which again could be used to identify high-risk patients requiring treatment intensification (124–127). Canine lymphoma mRNA expression signatures have also been associated with grade, immuno-phenotype and therapeutic response. The authors of this study successfully developed a real-time PCR-based test which they suggested could be easily adopted for use in clinical cases (128). A further exciting canine lymphoma study investigated the assessment of minimal residual disease and response to treatment (129). To monitor the response to chemotherapy, this study used a real-time PCR assay to detect immunoglobulin heavy chain gene fragment sequences in the blood of dogs diagnosed with high-grade B-cell lymphoma. Results showed

that the assay could predict the 25 week progression-free survival from as early as the 11th week of treatment. Translated into the clinics, these results would enable clinicians to identify non-responding patients early in their treatment; these patients could either be transferred onto a different treatment protocol or, at the very least, be spared the side effects and costs of continuing an ineffective chemotherapy regimen.

Another study has recently reported the use of a cross-species personalized medicine approach to identify new therapies for a dog diagnosed with multiple leiomyosarcomas. This study used tissue obtained from an excisional biopsy from 1 of the tumors to establish patient-derived xenograft (PDX) tumors. Using PDX samples, an *in vitro* cell line was subsequently established that was used for high-throughput drug screening. This *in vitro* work identified proteasome inhibitors as a potential therapy, which was then validated using the PDX model. Genomic profiling of mutations in the original tumors, PDX tumors and cell line was also performed. While these investigations were taking place, treatment with toceranib began. After 6 months, disease progression and local recurrence were detected; the dog was then treated with the proteasomal inhibitor bortezomib. Although an initial response was seen, tumor growth began again 6 weeks after the start of treatment and the dog was euthanized. While this study demonstrated that drug screening can be performed on patient-derived samples, the time scale that this occurred over was approximately 1 year, by which time that patient had developed metastatic disease and local recurrence. The major issue with this study is that these metastatic lesions may not have had the same oncogenic drivers as the original tumor, and therefore the use of bortezomib may not have been appropriate. This dog also had 6 original tumors, but cell lines and PDX tumors were only made from 1; tumor heterogeneity between the tumors should also have been evaluated. Although the study demonstrated what can be achieved through using patient derived samples, the technique is impractical in terms of expense and the time required to complete the work. These types of studies can only really be effective if treatment strategies can be individualized for the patient in order to treat the primary disease at the time of diagnosis (130).

In contrast to the traditional forms of cancer therapy such as surgery, radiotherapy and chemotherapy, research has shown the potential benefits of using immunotherapy in a variety of cancer types. Studies have investigated whether personalized immunotherapy can be achieved through the production of autologous therapeutic anti-tumor vaccines using hydroxyapatite (HA). HA is an appealing compound for this application, as it not only attracts antigen-presenting cells, but also presents tumor antigens to immune cells. Studies have shown that cell membrane and heat shock proteins extracted from tumor tissue can be combined with HA to produce a personalized vaccine. Using this approach, a clinical trial demonstrated that 15% of patients gained a partial response, while 25% obtained stable disease following vaccination. The authors suggested that the vaccine stimulated a T-cell response and that personalized vaccines using HA combined with self-antigens were safe to use in patients (131). This approach has subsequently been investigated in dogs diagnosed with diffuse large B-cell lymphomas (DLBCL) (132). Results from this clinical trial demonstrated that dogs which received chemo-immunotherapy had longer progression-free and lymphoma-specific survival times compared with dogs that received only chemotherapy. These results suggested that the personalized vaccine was safe in dogs and could be used to improve treatment outcomes in dogs with DLBCL. The authors proposed that this novel therapeutic strategy should be investigated in human patients diagnosed with DLBCL.

Other studies have shown that immunotherapies using chimeric antigen receptor (CAR) T cells may have value in treating human patients with B cell neoplasia; the FDA approved the use of CD19 CAR T cells for treating refractory/relapsed acute lymphoblastic leukemia and DLBCL (133). In line with these human studies, a recent first-in-species pilot trial investigated the use of CAR T cells in canine DLBCL patients. Using blood samples obtained from dogs diagnosed with DLBCL, CD20 CAR T cells were produced *ex vivo* using lentivectors, as had been previously done to manufacture human CAR T cells. Results showed that canine CAR T cells could be detected in blood samples post-infusion, and that these cells were antigen-specific, resulting in removal of $CD20^+$ target cells. Survival times also correlated with *ex vivo* CD20 CAR T cell expansion. Unfortunately, the induction of canine anti-mouse antibodies in the dogs resulted in CAR T cell loss. Furthermore, targeting $CD20^+$ cells eventually resulted in antigen escape and the emergence of $CD20^-$ disease. However, the study was able to show the successful lentivector production of functional canine CAR T cells, while also demonstrating that the challenges of effective CAR T cell therapy in animals were comparable to those seen in humans trials (134).

Osteosarcoma is another human and canine tumor type that may benefit from immunotherapies. Results from both canine (135–137) and human (138, 139) studies indicate that osteosarcoma patients which develop a surgical infection following limb sparing surgery have improved survival times. Although the immune mechanisms that lead to higher survival rates after infection are not yet clear these results suggest an immunogenic component to osteosarcoma pathogenesis. Studies using a mouse model of chronic bacterial osteomyelitis suggest that the innate immune system may be involved in the suppression of osteosarcoma growth (140). Immune modulation has produced positive effects on patient outcome in both dogs and humans; further studies are currently underway to ascertain the most effective immunotherapy or combination of immunotherapies that will lead to additional improvements in clinical response. With a better understanding of the methods required to redirect the immune system toward osteosarcoma, there is now ample opportunity to change the therapeutic landscape and improve osteosarcoma treatment in both humans and dogs (141, 142).

Other veterinary research projects have characterized the genotypes of several canine cancers including hemangiosarcoma (143), melanoma (144), and osteosarcoma (145), with the aim of identifying potential "actionable" targets. Although somatic mutations in these cancer types (in genes including *TP53*, *PIK3CA*, *NRAS*, *PTEN*, and *BRAF*) have similarities to those oncogenic mutations found in human cancers, veterinary clinical trials still need to be performed to confirm if they respond to targeted inhibition. Clinical trials that merge information gained

from sequencing individual canine tumors with survival data after targeted treatment could in the future influence the selection of treatment regimens for canine cancer patients. These types of trials have the potential to provide evidence to clinicians and clients that cancer genome sequencing for their pets could lead to improved outcomes though the adoption of a precision medicine approach.

Current Issues Limiting the Use of Precision Medicine

The translation of precision medicine approaches into accepted healthcare practices and policies has undoubtedly lagged behind the significant discoveries made through pre-clinical research. It is to be expected that, as with all novel innovations, it will take years to achieve this successful translation. Precision medicine is complicated by the fact that it not only represents a significant paradigm shift in how we treat cancer patients, but is also associated with complex legal, financial, social and ethical issues. The National Academy of Medicine outlined multiple challenges faced by the Precision Medicine Initiative, including the development of infrastructures that enable data sharing and easy access to extensive, highly-integrated clinical data sets. They also highlighted the absolute need for evidence that precision medicine can actually improve patient outcomes (146). To date, only a few clinical trials have assessed the adoption of a precision medicine approach for the treatment of cancer patients. The first of such precision medicine clinical trials involved refractory metastatic cancer patients. The results demonstrated that longer progression-free intervals could be achieved in patients that underwent molecular cancer profiling to guide their treatment (147). Unfortunately, other studies have suggested that there is limited evidence to support the use of genomic tests in healthcare practices (148, 149). The economic feasibility of adopting precision medicine policies is another significant challenge, as its successful implementation will ultimately depend on whether patients and healthcare providers are willing to pay for them. Currently, there is limited evidence that precision medicine provides sufficient cost-benefit advantages over standard treatment protocols. Some researchers even suggest that precision medicine could be a distraction from low-cost and effective population-wide interventions and that policies which focus on public health and prevention should be prioritized (150). Finally, precision medicine raises important questions regarding patient trust, including issues related to who actually owns the genetic data and how it can be securely stored.

To address some of these issues, centers such as The Personalized Medicine Coalition in the USA and The Center for Personalized Medicine in the UK have been established (151). Other on-going initiatives include the Clinical Sequencing Evidence-Generating Research center and the Implementing GeNomics In practice (IGNITE) project, which aim to support the integration of genome sequencing into healthcare practices (8). In veterinary medicine, following sequencing of the canine genome, the Canine Comparative Oncology and Genomics Consortium (CCOGC) was formed in 2004. This programme aims to generate an extensive archive (from 3,000 dogs) of clinical samples (neoplastic and non-neoplastic tissue, whole blood, plasma, serum and urine) from lymphoma, osteosarcoma, hemangiosarcoma, mast cell tumor, pulmonary, melanoma and soft tissue sarcomas. In 2013, the CCOGC biorepository was opened to the research community with the aim of improving the molecular characterization of canine cancers in order to help provide precision veterinary healthcare (152).

The challenges that limit the successful integration of precision medicine in human healthcare practices are equally applicable to veterinary medicine. As public awareness of the potential human benefits of precision medicine increases, veterinary clients will start to ask whether similar, precision-guided approaches can benefit their pets. This will become even more evident if pet owners themselves have received treatment as part of a precision medicine approach. In the future, the veterinary profession will need to adapt to the changes that are associated with precision medicine. Significant investment will be required to encourage openness for sharing research results and clinical data. Clinicians will also need to be trained in the use of molecular-based diagnostic assays and in performing high quality clinical trials using appropriately identified targeted treatments. Underpinning these ideas will ultimately require training and educational programmes for veterinary clinicians and veterinary healthcare professionals in subject areas such as tumor heterogeneity, genomic medicine, bioinformatics and targeted therapeutics, all of which are key components within human precision medicine.

CONCLUSION

Precision medicine has the potential to transform the way in which we treat human and veterinary cancer patients. Ongoing basic and clinical research is continuing to improve our understanding of tumor heterogeneity and is providing new information on how it influences disease prognosis and treatment responses. The challenge lies in the successful translation of these exciting results into novel diagnostic and therapeutic strategies for clinical use. Multidisciplinary projects that take on a one-health view to precision medicine, involving human and veterinary clinicians, engineers and chemists, are likely to become more important in the future if we are to ultimately advance the field of precision medicine to a stage where it can be fully integrated into clinical practices. Our review has highlighted this with studies investigating the TME and with research using novel implantable medical devices. Although precision medicine is still largely focused on human healthcare, we have shown that there have been exciting clinical research studies in the field of veterinary precision medicine, with promising results. These types of studies are likely to increase as more researchers realize that many of the cancers seen in veterinary patients share significant similarities with human equivalent cancers, including the molecular mechanisms of disease pathogenesis and the TME. It is these types of similarities that may prove to be clinically relevant and therapeutically actionable for both human and veterinary patients. Adopting a one-health approach, in which researchers and clinicians from both human and veterinary fields collaborate, will ultimately aid translational studies and improve the integration of precision medicine initiatives into healthcare practices for both human and veterinary cancer patients.

AUTHOR CONTRIBUTIONS

DA secured funding for this research. MG wrote the majority of the manuscript and composed the figures, with significant contributions from JM. Critical revisions were made by MG, JM, AT, CM-P, CK, LP and DA. All authors read and approved the final manuscript.

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REFERENCES

1. Ghasemi M, Nabipour I, Omrani A, Alipour Z, Assadi M. Precision medicine and molecular imaging: new targeted approaches toward cancer therapeutic and diagnosis. *Am J Nucl Med Mol Imaging*. (2016) 6:310–27.
2. Katsnelson A. Momentum grows to make personalized medicine more precise. *Nat Med*. (2013) 19:249–50. doi: 10.1038/nm0313-249
3. Biesecker L, Blaser MJ, Wylie Burke, Chute CG, Eddy S, Jaffe E, et al. *The National Academies Collection: Reports funded by National Institutes of Health, Toward Precision Medicine: Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease*. Washington, DC: National Academies Press (US). National Academy of Sciences (2011).
4. Council NR. Toward precision medicine: building a knowledge network for biomedical research and a new taxonomy of disease. Washington, DC: National Academies Press (2011).
5. Bu L-L, Yang K, Xiong W-X, Liu F-T, Anderson B, Wang Y, et al. Toward precision medicine in Parkinson disease. *Ann Transl Med*. (2016) 4:26.
6. Penet MF, Krishnamachary B, Chen Z, Jin J, Bhujwalla ZM. Molecular imaging of the tumor microenvironment for precision medicine and theranostics. *Adv Cancer Res*. (2014) 124:235–56. doi: 10.1016/B978-0-12-411638-2.00007-0
7. Collins FS, Varmus H. A new initiative on precision medicine. *N Engl J Med*. (2015) 372:793–5. doi: 10.1056/NEJMp1500523
8. Ginsburg GS, Phillips KA. Precision medicine: from science to value. *Health Affairs*. (2018) 37:694–701. doi: 10.1377/hlthaff.2017.1624
9. Turnbull C, Scott RH, Thomas E, Jones L, Murugaesu N, Pretty FB, et al. The 100 000 Genomes Project: bringing whole genome sequencing to the NHS. *BMJ*. (2018) 361:k1687. doi: 10.1136/bmj.k1687
10. Clements DN, Handel IG, Rose E, Querry D, Pugh CA, Ollier WER, et al. Dogslife: a web-based longitudinal study of Labrador Retriever health in the UK. *BMC Vet Res*. (2013) 9:13. doi: 10.1186/1746-6148-9-13
11. Kim Y-A, Cho D-Y, Przytycka TM. Understanding genotype-phenotype effects in cancer via network approaches. *PLoS Comput Biol*. (2016) 12:e1004747. doi: 10.1371/journal.pcbi.1004747
12. Katogiritis A, Khanna C. Towards the delivery of precision veterinary cancer medicine. *Vet Clin North Am*. (2019) 49:809–18. doi: 10.1016/j.cvsm.2019.04.011
13. Stephen P. The distribution of secondary growths in cancer of the breast. *Lancet*. (1889) 1:571. doi: 10.1016/S0140-6736(00)49915-0
14. Cheng YQ, Wang SB, Liu JH, Jin L, Liu Y, Li CY, et al. Modifying the tumour microenvironment and reverting tumour cells: new strategies for treating malignant tumours. *Cell Prolif*. (2020) 53:e12865. doi: 10.1111/cpr.12865
15. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol*. (2004) 14:198–206. doi: 10.1016/j.semradonc.2004.04.008
16. Najafi M, Goradel NH, Farhood B, Salehi E, Solhjoo S, Toolee H, et al. Tumor microenvironment: interactions and therapy. *J Cell Physiol*. (2019) 234:5700–21. doi: 10.1002/jcp.27425
17. Wu T, Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett*. (2017) 387:61–8. doi: 10.1016/j.canlet.2016.01.043
18. Vaddiraju S, Tomazos I, Burgess DJ, Jain FC, Papadimitrakopoulos F. Emerging synergy between nanotechnology and implantable biosensors: a review. *Biosens Bioelectron*. (2010) 25:1553–65. doi: 10.1016/j.bios.2009.12.001
19. Bertout JA, Patel SA, Simon MC. The impact of O2 availability on human cancer. *Nat Rev Cancer*. (2008) 8:967–75. doi: 10.1038/nrc2540
20. Vaupel P, Mayer A. Hypoxia in tumors: pathogenesis-related classification, characterization of hypoxia subtypes, associated biological clinical implications. In: Swartz HM, Bruley F, editors. *Oxygen Transport to Tissue XXXVI*. New York, NY: Springer (2014). p. 19–24. doi: 10.1007/978-1-4939-0620-8_3
21. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer*. (1955) 9:539–49. doi: 10.1038/bjc.1955.55
22. Bayer C, Shi K, Astner ST, Maftei C-A, Vaupel P. Acute versus chronic hypoxia: why a simplified classification is simply not enough. *Int J Radiat Oncol Biol Phys*. (2011) 80:965–8. doi: 10.1016/j.ijrobp.2011.02.049
23. Brown J. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *Br J Radiol*. (1979) 52:650–6. doi: 10.1259/0007-1285-52-620-650
24. Chaplin DJ, Olive PL, Durand RE. Intermittent blood flow in a murine tumor: radiobiological effects. *Cancer Res*. (1987) 47:597–601.
25. Horsman MR, Mortensen LS, Jørgen B, Petersen JB, Busk M, Overgaard J. Imaging hypoxia to improve radiotherapy outcome. *Nat Rev Clin Oncol*. (2012) 9:674–87. doi: 10.1038/nrclinonc.2012.171
26. Secomb TW, Dewhirst MW, Pries AR. Structural adaptation of normal and tumour vascular networks. *Basic Clin Pharmacol Toxicol*. (2012) 110:63–9. doi: 10.1111/j.1742-7843.2011.00815.x
27. Koch CJ, Jenkins WT, Jenkins KW, Yang XY, Shuman AL, Pickup S, et al. Mechanisms of blood flow and hypoxia production in rat 9L-epigastric tumors. *Tumor Microenviron Therapy*. (2013) 1:1–13. doi: 10.2478/tumor-2012-0001
28. Dewhirst M, Ong E, Braun R, Smith B, Klitzman B, Evans S, et al. Quantification of longitudinal tissue pO2 gradients in window chamber tumours: impact on tumour hypoxia. *Br J Cancer*. (1999) 79:1717. doi: 10.1038/sj.bjc.6690273
29. Bhandari V, Hoey C, Liu LY, Lalonde E, Ray J, Livingstone J, et al. Molecular landmarks of tumor hypoxia across cancer types. *Nat Genet*. (2019) 51:308–18. doi: 10.1038/s41588-018-0318-2
30. Leo C, Giaccia AJ, Denko NC. The hypoxic tumor microenvironment and gene expression. *Semin Radiat Oncol*. (2004) 14:207–14. doi: 10.1016/j.semradonc.2004.04.007
31. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*. (2003) 3:721–32. doi: 10.1038/nrc1187
32. Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, et al. Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem*. (1999) 274:15030–40. doi: 10.1074/jbc.274.21.15030
33. Koong AC, Chen EY, Mivechi NF, Denko NC, Stambrook P, Giaccia AJ. Hypoxic activation of nuclear factor-κB is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res*. (1994) 54:5273–9.
34. Bandyopadhyay RS, Phelan M, Faller DV. Hypoxia induces AP-1-regulated genes and AP-1 transcription factor binding in human endothelial and other cell types. *Biochim Biophys Acta*. (1995) 1264:72–8. doi: 10.1016/0167-4781(95)00116-X
35. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med*. (2002) 8:62–7. doi: 10.1016/S1471-4914(02)02317-1
36. Denko NC, Fontana LA, Hudson KM, Sutphin PD, Raychaudhuri S, Altman R, et al. Investigating hypoxic tumor physiology through gene expression patterns. *Oncogene*. (2003) 22:5907–14. doi: 10.1038/sj.onc.1206703

37. Semenza GL. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med*. (2001) 7:345–50. doi: 10.1016/S1471-4914(01)02090-1
38. Sørensen BS, Horsman MR. Tumor hypoxia: impact on radiation therapy and molecular pathways. *Front Oncol*. (2020) 10:562–73. doi: 10.3389/fonc.2020.00562
39. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer*. (2011) 11:325–37. doi: 10.1038/nrc3038
40. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res*. (1989) 49:6449–65.
41. Helmlinger G, Yuan F, Dellian M, Jain RK. Interstitial pH and pO 2 gradients in solid tumors *in vivo*: high-resolution measurements reveal a lack of correlation. *Nat Med*. (1997) 3:177–82. doi: 10.1038/nm0297-177
42. Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst*. (2007) 99:1441–54. doi: 10.1093/jnci/djm135
43. Rippe B, Haraldsson B. Transport of macromolecules across microvascular walls: the two-pore theory. *Physiol Rev*. (1994) 74:163–219. doi: 10.1152/physrev.1994.74.1.163
44. Harris BHL, Barberis A, West CML, Buffa FM. Gene expression signatures as biomarkers of tumour hypoxia. *Clin Oncol*. (2015) 27:547–60. doi: 10.1016/j.clon.2015.07.004
45. Heldin C-H, Rubin K, Pietras K, Östman A. High interstitial fluid pressure—an obstacle in cancer therapy. *Nat Rev Cancer*. (2004) 4:806–13. doi: 10.1038/nrc1456
46. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol*. (1953) 26:638–48. doi: 10.1259/0007-1285-26-312-638
47. Okunieff P, Fenton B, Chen Y (editors). Past, present, and future of oxygen in cancer research. *Oxygen Transport to Tissue XXVI*. New York, NY: Springer (2005). p. 213–22. doi: 10.1007/0-387-26206-7_29
48. Vos O. Role of endogenous thiols in protection. *Adv Space Res*. (1992) 12:201–7. doi: 10.1016/0273-1177(92)90109-B
49. Nordsmark M, Bentzen SM, Rudat V, Brizel D, Lartigau E, Stadler P, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiotherapy Oncol*. (2005) 77:18–24. doi: 10.1016/j.radonc.2005.06.038
50. Bedford JS, Mitchell JB. The effect of hypoxia on the growth and radiation response of mammalian cells in culture. *Br J Radiol*. (1974) 47:687–96. doi: 10.1259/0007-1285-47-562-687
51. Yoshiba S, Ito D, Nagumo T, Shirota T, Hatori M, Shintani S. Hypoxia induces resistance to 5-fluorouracil in oral cancer cells via G1 phase cell cycle arrest. *Oral Oncol*. (2009) 45:109–15. doi: 10.1016/j.oraloncology.2008.04.002
52. Deavall DG, Martin EA, Horner JM, Roberts R. Drug-induced oxidative stress and toxicity. *J Toxicol*. (2012) 2012:645460. doi: 10.1155/2012/645460
53. Huang C-C, Chia W-T, Chung M-F, Lin J-K, Hsiao C-W, Jin C, et al. An implantable depot that can generate oxygen *in situ* for overcoming hypoxia-induced resistance to anticancer drugs in chemotherapy. *J Am Chem Soc*. (2016) 138:5222–5. doi: 10.1021/jacs.6b01784
54. Wouters A, Pauwels B, Lardon F, Vermorken JB. Implications of *in vitro* research on the effect of radiotherapy and chemotherapy under hypoxic conditions. *Oncologist*. (2007) 12:690–712. doi: 10.1634/theoncologist.12-6-690
55. Karagoz B, Suleymanoglu S, Uzun G, Bilgi O, Aydinoz S, Haholu A, et al. Hyperbaric oxygen therapy does not potentiate doxorubicin-induced cardiotoxicity in rats. *Basic Clin Pharmacol Toxicol*. (2008) 102:287–92. doi: 10.1111/j.1742-7843.2007.00196.x
56. Henk JM, Kunkler PB, Smith CW. Radiotherapy and hyperbaric oxygen in head and neck cancer: final report of first controlled clinical trial. *Lancet*. (1977) 310:101–3. doi: 10.1016/S0140-6736(77)90116-7
57. Moen I, Stuhr LEB. Hyperbaric oxygen therapy and cancer—a review. *Target Oncol*. (2012) 7:233–42. doi: 10.1007/s11523-012-0233-x
58. Janssens GO, Rademakers SE, Terhaard CH, Doornaert PA, Bijl HP, van den Ende P, et al. Improved recurrence-free survival with ARCON for anemic patients with laryngeal cancer. *Clin Cancer Res*. (2014) 20:1345–54. doi: 10.1158/1078-0432.CCR-13-1730
59. Janssens GO, Rademakers SE, Terhaard CH, Doornaert PA, Bijl HP, van den Ende P, et al. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. *J Clin Oncol*. (2012) 30:1777–83. doi: 10.1200/JCO.2011.35.9315
60. Bentzen J, Toustrup K, Eriksen JG, Primdahl H, Andersen LJ, Overgaard J. Locally advanced head and neck cancer treated with accelerated radiotherapy, the hypoxic modifier nimorazole and weekly cisplatin. Results from the DAHANCA 18 phase II study. *Acta Oncol*. (2015) 54:1001–7. doi: 10.3109/0284186X.2014.992547
61. Nishimura Y, Nakagawa K, Takeda K, Tanaka M, Segawa Y, Tsujino K, et al. Phase I/II trial of sequential chemoradiotherapy using a novel hypoxic cell radiosensitizer, doranidazole (PR-350), in patients with locally advanced non–small-cell lung cancer (WJTOG-0002). *Int J Radiat Oncol Biol Phys*. (2007) 69:786–92. doi: 10.1016/j.ijrobp.2007.04.008
62. Overgaard J, Sand Hansen H, Overgaard M, Bastholt L, Berthelsen A, Specht L, et al. A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) protocol 5-85. *Radiother Oncol*. (1998) 46:135–46. doi: 10.1016/S0167-8140(97)00220-X
63. Hassan Metwally MA, Ali R, Kuddu M, Shouman T, Strojan P, Iqbal K, et al. A randomized multicenter study of the hypoxic radiosensitizer nimorazole concomitant with accelerated radiotherapy in head and neck squamous cell carcinoma. *Radiother Oncol*. (2015) 116:15–20. doi: 10.1016/j.radonc.2015.04.005
64. Horsman MR, Overgaard J. The impact of hypoxia and its modification of the outcome of radiotherapy. *J Radiat Res*. (2016) 57:90–8. doi: 10.1093/jrr/rrw007
65. Nikas IP, Paschou SA, Ryu HS. The role of nicotinamide in cancer chemoprevention and therapy. *Biomolecules*. (2020) 10:477. doi: 10.3390/biom10030477
66. Baumann M, Krause M, Overgaard J, Debus J, Bentzen SM, Daartz J, et al. Radiation oncology in the era of precision medicine. *Nat Rev Cancer*. (2016) 16:234–49. doi: 10.1038/nrc.2016.18
67. Hendrickson K, Phillips M, Smith W, Peterson L, Krohn K, Rajendran J. Hypoxia imaging with [F-18] FMISO-PET in head and neck cancer: potential for guiding intensity modulated radiation therapy in overcoming hypoxia-induced treatment resistance. *Radiother Oncol*. (2011) 101:369–75. doi: 10.1016/j.radonc.2011.07.029
68. Søvik Å, Malinen E, Bruland ØS, Bentzen SM, Olsen DR. Optimization of tumour control probability in hypoxic tumours by radiation dose redistribution: a modelling study. *Phys Med Biol*. (2006) 52:499–509. doi: 10.1088/0031-9155/52/2/013
69. Hammond EM, Asselin MC, Forster D, O'Connor JP, Senra JM, Williams KJ. The meaning, measurement and modification of hypoxia in the laboratory and the clinic. *Clin Oncol*. (2014) 26:277–88. doi: 10.1016/j.clon.2014.02.002
70. Vaupel P, Schlenger K, Knoop C, Höckel M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. *Cancer Res*. (1991) 51:3316–22.
71. Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phy*. (1997) 38:285–9. doi: 10.1016/S0360-3016(97)00101-6
72. Höckel M, Schlenger K, Aral B, Mitze M, Schäffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res*. (1996) 56:4509–15.
73. Okunieff P, Hoeckel M, Dunphy EP, Schlenger K, Knoop C, Vaupel P. Oxygen tension distributions are sufficient to explain the local response of human breast tumors treated with radiation alone. *Int J Radiat Oncol Biol Phy*. (1993) 26:631–6. doi: 10.1016/0360-3016(93)90280-9
74. Gatenby RA, Kessler HB, Rosenblum JS, Coia LR, Moldofsky PJ, Hartz WH, et al. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int J Radiat Oncol Biol Phy*. (1988) 14:831–8. doi: 10.1016/0360-3016(88)90002-8
75. Fyles AW, Milosevic M, Pintilie M, Hill RP. Cervix cancer oxygenation measured following external radiation therapy. *Int J Radiat Oncol Biol Phy*. (1998) 42:751–3. doi: 10.1016/S0360-3016(98)00307-1

76. Varia MA, Calkins-Adams DP, Rinker LH, Kennedy AS, Novotny DB, Fowler WC, et al. Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. *Gynecol Oncol*. (1998) 71:270–7. doi: 10.1006/gyno.1998.5163
77. Le Q-T, Courter D. Clinical biomarkers for hypoxia targeting. *Cancer Metastasis Rev*. (2008) 27:351–61. doi: 10.1007/s10555-008-9144-9
78. Meehan J, Ward C, Turnbull A, Bukowski-Wills J, Finch AJ, Jarman EJ, et al. Inhibition of pH regulation as a therapeutic strategy in hypoxic human breast cancer cells. *Oncotarget*. (2017) 8:42857–75. doi: 10.18632/oncotarget.17143
79. Gee HE, Ivan C, Calin GA, Ivan M. HypoxamiRs and cancer: from biology to targeted therapy. *Antioxid Redox Signal*. (2014) 21:1220–38. doi: 10.1089/ars.2013.5639
80. Choudhry H, Harris AL, McIntyre A. The tumour hypoxia induced non-coding transcriptome. *Mol Aspects Med*. (2016) 47:35–53. doi: 10.1016/j.mam.2016.01.003
81. Moschetta MG, Maschio LB, Jardim-Perassi BV, Gelaleti GB, Lopes JR, Leonel C, et al. Prognostic value of vascular endothelial growth factor and hypoxia-inducible factor 1α in canine malignant mammary tumors. *Oncol Rep*. (2015) 33:2345–53. doi: 10.3892/or.2015.3856
82. Sørensen BS, Hao J, Overgaard J, Vorum H, Honore B, Alsner J, et al. Influence of oxygen concentration and pH on expression of hypoxia induced genes. *Radiother Oncol*. (2005) 76:187–93. doi: 10.1016/j.radonc.2005.06.037
83. Sørensen BS, Alsner J, Overgaard J, Horsman MR. Hypoxia induced expression of endogenous markers *in vitro* is highly influenced by pH. *Radiother Oncol*. (2007) 83:362–6. doi: 10.1016/j.radonc.2007.04.028
84. Troost EG, Bussink J, Kaanders JH, van Eerd J, Peters JP, Rijken PF, et al. Comparison of different methods of CAIX quantification in relation to hypoxia in three human head and neck tumor lines, *Radiother Oncol*. (2005) 76:194–9. doi: 10.1016/j.radonc.2005.06.031
85. Toustrup K, Sørensen BS, Nordsmark M, Busk M, Wiuf C, Alsner J, et al. Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. *Cancer Res*. (2011) 71:5923–32. doi: 10.1158/0008-5472.CAN-11-1182
86. Seigneuric R, Starmans MH, Fung G, Krishnapuram B, Nuyten DS, van Erk A, et al. Impact of supervised gene signatures of early hypoxia on patient survival. *Radiother Oncol*. (2007) 83:374–82. doi: 10.1016/j.radonc.2007.05.002
87. Winter SC, Buffa FM, Silva P, Miller C, Valentine HR, Turley H, et al. Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. *Cancer Res*. (2007) 67:3441–9. doi: 10.1158/0008-5472.CAN-06-3322
88. Buffa F, Harris A, West C, Miller C. Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. *Br J Cancer*. (2010) 102:428–35. doi: 10.1038/sj.bjc.6605450
89. Ragnum H, Vlatkovic L, Lie A, Axcrona K, Julin C, Frikstad K, et al. The tumour hypoxia marker pimonidazole reflects a transcriptional programme associated with aggressive prostate cancer. *Br J Cancer*. (2015) 112:382–90. doi: 10.1038/bjc.2014.604
90. Yang L, Roberts D, Takhar M, Erho N, Bibby BA, Thiruthaneeswaran N, et al. Development and validation of a 28-gene hypoxia-related prognostic signature for localized prostate cancer. *EBioMedicine*. (2018) 31:182–9. doi: 10.1016/j.ebiom.2018.04.019
91. Eustace A, Mani N, Span PN, Irlam JJ, Taylor J, Betts GN, et al. A 26-gene hypoxia signature predicts benefit from hypoxia-modifying therapy in laryngeal cancer but not bladder cancer. *Clin Cancer Res*. (2013) 19:4879–88. doi: 10.1158/1078-0432.CCR-13-0542
92. Fjeldbo CS, Julin CH, Lando M, Forsberg MF, Aarnes E-K, Alsner J, et al. Integrative analysis of DCE-MRI and gene expression profiles in construction of a gene classifier for assessment of hypoxia-related risk of chemoradiotherapy failure in cervical cancer. *Clin Cancer Res*. (2016) 22:4067–76. doi: 10.1158/1078-0432.CCR-15-2322
93. Toustrup K, Sørensen BS, Metwally MAH, Tramm T, Mortensen LS, Overgaard J, et al. Validation of a 15-gene hypoxia classifier in head and neck cancer for prospective use in clinical trials. *Acta Oncol*. (2016) 55:1091–8. doi: 10.3109/0284186X.2016.1167959
94. Toustrup K, Sørensen BS, Lassen P, Wiuf C, Alsner J, Overgaard J. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. *Radiother Oncol*. (2012) 102:122–9. doi: 10.1016/j.radonc.2011.09.010
95. Overgaard J, Eriksen JG, Nordsmark M, Alsner J, Horsman MR. Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncol*. (2005) 6:757–64. doi: 10.1016/S1470-2045(05)70292-8
96. Weber WA, Wieder H. Monitoring chemotherapy and radiotherapy of solid tumors. *Eur J Nucl Med Mol Imaging*. (2006) 33:27–37. doi: 10.1007/s00259-006-0133-3
97. Hoeben BA, Troost EG, Span PN, van Herpen CM, Bussink J, Oyen WJ, et al. 18F-FLT PET during radiotherapy or chemoradiotherapy in head and neck squamous cell carcinoma is an early predictor of outcome. *J Nucl Med*. (2013) 54:532–40. doi: 10.2967/jnumed.112.105999
98. Troost EG, Bussink J, Hoffmann AL, Boerman OC, Oyen WJ, Kaanders JH. 18F-FLT PET/CT for early response monitoring and dose escalation in oropharyngeal tumors. *J Nucl Med*. (2010) 51:866–74. doi: 10.2967/jnumed.109.069310
99. Bradshaw TJ, Yip S, Jallow N, Forrest LJ, Jeraj R. Spatiotemporal stability of Cu-ATSM and FLT positron emission tomography distributions during radiation therapy. *J Radiat Oncol Biol Phys*. (2014) 89:399–405. doi: 10.1016/j.ijrobp.2014.02.016
100. Rødal J, Rusten E, Søvik Å, Skogmo HK, Malinen E. Functional imaging to monitor vascular and metabolic response in canine head and neck tumors during fractionated radiotherapy. *Acta Oncol*. (2013) 52:1293–9. doi: 10.3109/0284186X.2013.812800
101. Zornhagen KW, Clausen MM, Hansen AE, Law I, McEvoy FJ, Engelholm SA, et al. Use of molecular imaging markers of glycolysis, hypoxia and proliferation (18F-FDG, 64Cu-ATSM and 18F-FLT) in a dwith fibrosarcoma: the importance of individualized treatment planning and monitoring. *Diagnostics*. (2015) 5:372–82. doi: 10.3390/diagnostics5030372
102. Clausen MM, Hansen AE, Lundemann M, Hollensen C, Pommer T, Rosenschöld PMaf, et al. Dose painting based on tumor uptake of Cu-ATSM and FDG: a comparative study. *Radiat Oncol*. (2014) 9:228. doi: 10.1186/s13014-014-0228-0
103. Ballegeer EA, Forrest LJ, Jeraj R, Mackie TR, Nickles RJ. PET/CT following intensity-modulated radiation therapy for primary lung tumor in a dog. *Vet Radiol Ultrasound*. (2006) 47:228–33. doi: 10.1111/j.1740-8261.2006.00132.x
104. Bowen SR, Chappell RJ, Bentzen SM, Deveau MA, Forrest LJ, Jeraj R. Spatially resolved regression analysis of pre-treatment FDG, FLT and Cu-ATSM PET from post-treatment FDG PET: an exploratory study. *Radiother Oncol*. (2012) 105:41–8. doi: 10.1016/j.radonc.2012.05.002
105. Marland JRK, Blair EO, Flynn BW, González-Fernández E, Huang L, Kunkler IH, et al. Implantable microsystems for personalised anticancer therapy. In: Mitra S, Cumming DRS, editors. *CMOS Circuits for Biological Sensing and Processing*. Cham: Springer International Publishing (2018). p. 259–86. doi: 10.1007/978-3-319-67723-1_11
106. Gray ME, Meehan J, Blair EO, Ward C, Langdon SP, Morrison LR, et al. Biocompatibility of common implantable sensor materials in a tumor xenograft model. *J Biomed Mater Res B Appl Biomater*. (2018) 107:1620–33. doi: 10.1002/jbm.b.34254
107. Gray ME, Sullivan P, Marland JRK, Greenhalgh SN, Meehan J, Gregson R, et al. A novel translational ovine pulmonary adenocarcinoma model for human lung cancer. *Front Oncol*. (2019) 9:1–17. doi: 10.3389/fonc.2019.00534
108. Marland JRK, Gray ME, Dunare C, Blair EO, Tsiamis A, Sullivan P, et al., Real-time measurement of tumour hypoxia using an implantable microfabricated oxygen sensor. *Sensing Bio-Sensing Res*. (2020) 30:1–12. doi: 10.1016/j.sbsr.2020.100375
109. Vassiliou CC, Liu VH, Cima MJ. Miniaturized, biopsy-implantable chemical sensor with wireless, magnetic resonance readout. *Lab Chip*. (2015) 15:3465–72. doi: 10.1039/C5LC00546A
110. Sedlaczek P, Frydecka I, Gabryś M, Dalen Av, Einarsson R, Harłozińska A. Comparative analysis of CA125, tissue polypeptide specific antigen, and soluble interleukin-2 receptor α levels in sera, cyst, and ascitic fluids from patients with ovarian carcinoma. *Cancer*. (2002) 95:1886–93. doi: 10.1002/cncr.10917

111. Daniel KD, Kim GY, Vassiliou CC, Galindo M, Guimaraes AR, Weissleder R, et al. Implantable diagnostic device for cancer monitoring. *Biosens Bioelectron*. (2009) 24:3252–7. doi: 10.1016/j.bios.2009.04.010
112. Verma N, Cowperthwaite MC, Burnett MG, Markey MK. Differentiating tumor recurrence from treatment necrosis: a review of neuro-oncologic imaging strategies. *Neuro-Oncology*. (2013) 15:515–34. doi: 10.1093/neuonc/nos307
113. Yibo L, Terrence P, Christophoros CV, Paul LH, Michael JC. Implantable magnetic relaxation sensors measure cumulative exposure to cardiac biomarkers. *Nat Biotechnol*. (2011) 29:273–7. doi: 10.1038/nbt.1780
114. Oakes RS, Bushnell GG, Orbach SM, Kandagatla P, Zhang Y, Morris AH, et al. Metastatic conditioning of myeloid cells at a subcutaneous synthetic niche reflects disease progression and predicts therapeutic outcomes. *Cancer Res*. (2020) 80:602–12. doi: 10.1158/0008-5472.CAN-19-1932
115. Beyer GP, Mann GG, Pursley JA, Espenhahn ET, Fraisse C, Godfrey DJ, et al. An implantable MOSFET dosimeter for the measurement of radiation dose in tissue during cancer therapy. *IEEE Sens J*. (2008) 8:38–51. doi: 10.1109/JSEN.2007.912542
116. Klinghoffer RA, Bahrami SB, Hatton BA, Frazier JP, Moreno-Gonzalez A, Strand AD, et al. A technology platform to assess multiple cancer agents simultaneously within a patient's tumor. *Sci Transl Med*. (2015) 7:284ra58. doi: 10.1126/scitranslmed.aaa7489
117. Jonas O, Landry HM, Fuller JE, Santini JT, Baselga J, Tepper RI, et al. An implantable microdevice to perform high-throughput in vivo drug sensitivity testing in tumors. *Sci Transl Med*. (2015) 7:257–84. doi: 10.1126/scitranslmed.3010564
118. Conde J, Oliva N, Artzi N. Implantable hydrogel embedded dark-gold nanoswitch as a theranostic probe to sense and overcome cancer multidrug resistance. *Proc Natl Acad Sci*. (2015) 112:1278–87. doi: 10.1073/pnas.1421229112
119. London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin Cancer Res*. (2003) 9:2755–68.
120. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. *In vivo* antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res*. (2003) 9:327–37.
121. London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, et al. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clin Cancer Res*. (2009) 15:3856. doi: 10.1158/1078-0432.CCR-08-1860
122. Mealey KL, Martinez SE, Villarino NF. Personalized medicine: going to the dogs? *Human Genet*. (2019) 138:467–81. doi: 10.1007/s00439-019-02020-w
123. da Costa A, Kohn B, Gruber AD, Klopfleisch R. Multiple RT-PCR markers for the detection of circulating tumour cells of metastatic canine mammary tumours. *Vet J*. (2013) 196:34–9. doi: 10.1016/j.tvjl.2012.08.021
124. Klopfleisch R, Klose P, Gruber A. The combined expression pattern of BMP2, LTBP4, and DERL1 discriminates malignant from benign canine mammary tumors. *Vet Pathol*. (2010) 47:446–54. doi: 10.1177/0300985810363904
125. Klopfleisch R, Lenze D, Hummel M, Gruber AD. The metastatic cascade is reflected in the transcriptome of metastatic canine mammary carcinomas. *Vet J*. (2011) 190:236–43. doi: 10.1016/j.tvjl.2010.10.018
126. Mudaliar MA, Haggart RD, Miele G, Sellar G, Tan KA, Goodlad JR, et al. Comparative gene expression profiling identifies common molecular signatures of NF-κB activation in canine and human diffuse large B cell lymphoma (DLBCL). *PLoS ONE*. (2013) 8:e72591. doi: 10.1371/journal.pone.0072591
127. Richards KL, Motsinger-Reif AA, Chen H-W, Fedoriw Y, Fan C, Nielsen DM, et al. Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. *Cancer Res*. (2013) 73:5029–39. doi: 10.1158/0008-5472.CAN-12-3546
128. Frantz A, Sarver A, Ito D, Phang T, Karimpour-Fard A, Scott M, et al. Molecular profiling reveals prognostically significant subtypes of canine lymphoma. *Vet Pathol*. (2013) 50:693–703. doi: 10.1177/0300985812465325
129. Sato M, Yamzaki J, Goto-Koshino Y, Takahashi M, Fujino Y, Ohno K, et al. The prognostic significance of minimal residual disease in the early phases of chemotherapy in dogs with high-grade B-cell lymphoma. *Vet J*. (2013) 195:319–24. doi: 10.1016/j.tvjl.2012.07.013
130. Rao SR, Somarelli JA, Altunel E, Selmic LE, Byrum M, Sheth MU, et al. From the clinic to the bench and back again in one dog year: how a cross-species pipeline to identify new treatments for sarcoma illuminates the path forward in precision medicine. *Front Oncol*. (2020) 10:117–30. doi: 10.3389/fonc.2020.00117
131. Ciocca DR, Frayssinet P, Cuello-Carrión FD. A pilot study with a therapeutic vaccine based on hydroxyapatite ceramic particles and self-antigens in cancer patients. *Cell Stress Chaperones*. (2007) 12:33–44. doi: 10.1379/CSC-218R.1
132. Marconato L, Frayssinet P, Rouquet N, Comazzi S, Leone VF, Laganga P, et al. Randomized, placebo-controlled, double-blinded chemoimmunotherapy clinical trial in a pet dog model of diffuse large B-cell lymphoma. *Clin Cancer Res*. (2014) 20:668–77. doi: 10.1158/1078-0432.CCR-13-2283
133. Zheng P-P, Kros JM, Li J. Approved CAR T cell therapies: ice bucket challenges on glaring safety risks and long-term impacts. *Drug Discov Today*. (2018) 23:1175–82. doi: 10.1016/j.drudis.2018.02.012
134. Panjwani MK, Atherton MJ, MaloneyHuss MA, Haran KP, Xiong A, Gupta M, et al. Establishing a model system for evaluating CAR T cell therapy using dogs with spontaneous diffuse large B cell lymphoma. *Oncoimmunology*. (2020) 9:1676615. doi: 10.1080/2162402X.2019.1676615
135. Lascelles BDX, Dernell WS, Correa MT, Lafferty M, Devitt CM, Kuntz CA, et al. Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. *Ann Surg Oncol*. (2005) 12:1073–83. doi: 10.1245/ASO.2005.01.011
136. Liptak JM, Dernell WS, Ehrhart N, Lafferty MH, Monteith GJ, Withrow SJ. Cortical allograft and endoprosthesis for limb-sparing surgery in dogs with distal radial osteosarcoma: a prospective clinical comparison of two different limb-sparing techniques. *Vet Surg*. (2006) 35:518–33. doi: 10.1111/j.1532-950X.2006.00185.x
137. Culp WT, Olea-Popelka F, Sefton J, Aldridge CF, Withrow SJ, Lafferty MH. Evaluation of outcome and prognostic factors for dogs living greater than one year after diagnosis of osteosarcoma: 90 cases (1997–2008). *J Am Vet Med Assoc*. (2014) 245:1141–6. doi: 10.2460/javma.245.10.1141
138. Jeys L, Grimer R, Carter S, Tillman R, Abudu A. Post operative infection and increased survival in osteosarcoma patients: are they associated? *Ann Surg Oncol*. (2007) 14:2887–95. doi: 10.1245/s10434-007-9483-8
139. Lee JA, Kim MS, Kim DH, Lim JS, Park KD, Cho WH, et al. Postoperative infection and survival in osteosarcoma patients. *Ann Surg Oncol*. (2009) 16:147–51. doi: 10.1245/s10434-008-0184-8
140. Sottnik JL, U'Ren LW, Thamm DH, Withrow SJ, Dow SW. Chronic bacterial osteomyelitis suppression of tumor growth requires innate immune responses. *Cancer Immunol Immunother*. (2010) 59:367–78. doi: 10.1007/s00262-009-0755-y
141. Mason NJ. Comparative immunology and immunotherapy of canine osteosarcoma. *Curr Adv Sci Osteosarc*. (2020) 1258:199–221. doi: 10.1007/978-3-030-43085-6_14
142. Wycislo K, Fan T. The immunotherapy of canine osteosarcoma: a historical and systematic review. *J Vet Int Med*. (2015) 29:759–69. doi: 10.1111/jvim.12603
143. Wang G, Wu M, Maloneyhuss MA, Wojcik J, Durham AC, Mason NJ, et al. Actionable mutations in canine hemangiosarcoma. *PLoS ONE*. (2017) 12:e0188667. doi: 10.1371/journal.pone.0188667
144. Hernandez B, Adissu HA, Wei B-R, Michael HT, Merlino G, Simpson RM. Naturally occurring canine melanoma as a predictive comparative oncology model for human mucosal and other triple wild-type melanomas. *Int J Mol Sci*. (2018) 19:394. doi: 10.3390/ijms19020394
145. Monks NR, Cherba DM, Kamerling SG, Simpson H, Rusk AW, Carter D, et al. A multi-site feasibility study for personalized medicine in canines with Osteosarcoma. *J Transl Med*. (2013) 11:158. doi: 10.1186/1479-5876-11-158

146. Dzau VJ, Ginsburg GS. Realizing the full potential of precision medicine in health and health care. *Jama*. (2016) 316:1659–60. doi: 10.1001/jama.2016.14117
147. Von Hoff DD, Stephenson JJ Jr, Rosen P, Loesch DM, Borad MJ, Anthony S, et al. Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. *J Clin Oncol*. (2010) 28:4877–83. doi: 10.1200/JCO.2009.26.5983
148. Roberts MC, Kennedy AE, Chambers DA, Khoury MJ. The current state of implementation science in genomic medicine: opportunities for improvement. *Genet Med*. (2017) 19:858–63. doi: 10.1038/gim.2016.210
149. Phillips KA, Deverka PA, Sox HC, Khoury MJ, Sandy LG, Ginsburg GS, et al. Making genomic medicine evidence-based and patient-centered: a structured review and landscape analysis of comparative effectiveness research. *Genet Med*. (2017) 19:1081–91. doi: 10.1038/gim.2017.21
150. Coote JH, Joyner MJ. Is precision medicine the route to a healthy world? *Lancet*. (2015) 385:1617. doi: 10.1016/S0140-6736(15)60786-3
151. Carrasco-Ramiro F, Peiró-Pastor R, Aguado B. Human genomics projects and precision medicine. *Gene Therapy*. (2017) 24:551–61. doi: 10.1038/gt.2017.77
152. Mazcko C, Thomas R. The establishment of the Pfizer-canine comparative oncology and genomics consortium biospecimen repository. *Vet Sci*. (2015) 2:127–30. doi: 10.3390/vetsci2030127

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SYSTEMATIC REVIEW
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A Comparative Meta-Analysis and *in silico* Analysis of Differentially Expressed Genes and Proteins in Canine and Human Bladder Cancer

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Canine and human bladder cancer present similar anatomical, morphological, and molecular characteristics, and dogs can be considered a model for human bladder cancer. However, the veterinary literature lacks information regarding cross-validation analysis between human and canine large-scale data. Therefore, this research aimed to perform a meta-analysis of the canine literature on bladder cancer, identifying genes and proteins previously evaluated in these studies. In addition, we also performed a cross-validation of the canine transcriptome data and the human data from The Cancer Genome Atlas (TCGA) to identify potential markers for both species. The meta-analysis was performed using the following indexing terms: "bladder" AND "carcinoma" AND "dog" in different international databases, and 385 manuscripts were identified in our initial search. Then, several inclusion criteria were applied, and only 25 studies met these criteria. Among these studies, five presented transcriptome data, and 20 evaluated only isolated genes or proteins. Regarding the studies involving isolated protein analysis, the HER-2 protein was the most studied (3/20), followed by TAG-72 (2/20), COX-2 (2/20), survivin (2/20), and CK7 (2/20), and the remaining nine studies evaluated one isolated protein each. Regarding the cross-validation analysis of human and canine transcriptome data, we identified 35 dysregulated genes, including *ERBB2*, *TP53*, *EGFR*, and *E2F2*. Our results demonstrate that the canine literature on bladder cancer previously focused on the evaluation of isolated markers with no association with patient survival. This limitation may be related to the lack of a homogenous protocol for treating patients and the lack of follow-up during treatment. In addition, the lack of information regarding tumor muscle invasion can be considered an important limitation when comparing human and canine bladder tumors. Our *in silico* analysis involving canine and human transcriptome data provided several genes with the potential to be markers for both human and canine bladder tumors, and these genes should be considered for future studies on canine bladder cancer.

Keywords: transitional cell carcinoma, tyrosine kinase, gene ontology, dog, comparative oncology

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INTRODUCTION

Transitional cell carcinoma (TCC), also called urothelial carcinoma, is the most common bladder cancer in both humans and dogs, which share clinical, pathological, and molecular alterations (1–3). In the United States, 81,400 new cases and 17,980 bladder cancer-related deaths are expected in 2020 (4). The last global cancer statistics (GLOBOCAN) estimated 549,393 new cases and 199,922 bladder cancer-related deaths in 2018 (5). In dogs, urothelial carcinoma is the most common malignant tumor in the canine bladder, representing 1% of all neoplasms that affect dogs (6). In humans, TCC is a tumor associated with several factors, such as cigarette smoking, occupational exposure (7), arsenic, cyclophosphamide, arylamines, and polycyclic aromatic hydrocarbons (8). In pet dogs, a case–control study was previously performed to correlate cigarette smoke, obesity, and use of topical insecticides and chemicals used at home with canine bladder cancer development (9). The authors found a high risk of bladder cancer development in obese dogs and dogs that used topical insecticides (9). Since dogs and humans share the same environment, dogs can be considered a model system for humans (10).

Canine and human TCCs are usually locally infiltrative cancers that can extend throughout the entire bladder, including the submucosa and muscular layers (11). Usually, human bladder carcinomas are superficial tumors (70% of cases) and are classified as non-muscle-invasive bladder carcinomas (NMIBCs) (11). Though NMIBC presents a good prognosis, muscle-invasive bladder carcinoma is considered a therapeutic challenge (11, 12). Thus, in the human literature, muscle-invasive bladder carcinoma has been the focus of recent studies. In dogs, determining the degree of infiltration is not standardized, since in several cases, tissue samples come from cystoscopy (1, 13). Since during cystoscopy a superficial small piece of tissue is collected from different areas, it is not usually possible to have tumor specimens containing deep layers, such as the muscular layers. In addition, human and canine bladder carcinoma can invade adjacent tissues and organs such as the ureter, prostatic urethra, and prostate gland (12).

The molecular phenotype of human bladder cancer is widely studied, and some genomic subtyping was previously proposed (14, 15). The Cancer Genome Atlas (TCGA) database revealed 64 significantly mutated genes in human TCCs responsible for different cellular processes, such as evasion of DNA repair and apoptosis and cell proliferation. In addition, there are subtypes of human muscle-invasive bladder cancer: luminal-papillary, luminal-infiltrated, luminal, basal-squamous, and neuronal subtypes (16). However, few papers perform molecular characterization of canine bladder cancer, though it is considered a promising area, and the molecular characterization of canine bladder carcinoma can provide valuable information regarding the biological behavior of this tumor (17).

In dogs, some recent studies performing transcriptome analysis revealed several important molecular findings, such as different differentiation degrees of canine bladder cancer in molecular subtypes categorized according to the *BRAFV595E* somatic mutation (*BRAFV600E* in humans) (18, 19), the identification of therapeutic targets (*PTGER2*, *ERBB2*, *CCND1*, *VEGF*, and *EGFR*), and categories based on basal and luminal subtypes, as for human bladder cancers, enabling the comparison of muscular invasion potential between dogs and humans (1). Therefore, studies performing molecular comparisons between human and canine bladder carcinomas can provide a unique opportunity to study this cancer subtype in both species. In this regard, this manuscript aimed to perform a literature meta-analysis and extract all information regarding gene and protein expression in canine bladder cancer and perform an *in silico* analysis to identify common gene alterations among dogs and humans to select candidates for future studies regarding prognosis or treatment.

MATERIALS AND METHODS

Study Design

The study design is summarized in **Supplementary Figure 1**. We divided the study methods in three steps: (1) meta-analysis of the previous literature aiming to identify dysregulated genes and proteins in canine bladder cancer; (2) *in silico* analysis of dysregulated genes and proteins to identify their potential as prognostic and predictive markers in canine bladder cancer; and (3) selection of five previous studies with transcriptome data, extraction of common gene information from these studies and validation with The Cancer Genome Atlas (TCGA) data.

Meta-Analysis

To identify previously published papers to include in our meta-analysis, we performed a literature search in PubMed, MEDLINE, and Scielo databases using the indexing terms "bladder" AND "carcinoma" AND "dog" with no restriction regarding the year of publication. Then, we reviewed the reference section of the selected manuscripts and performed a manual search in the most relevant journals with oncology backgrounds to ensure that we included the highest number of available manuscripts.

Next, we selected manuscripts by title and abstract, including scientific articles that evaluated genes or proteins in canine bladder carcinomas. In this step, we excluded review manuscripts, case reports, and retrospective studies including only survival analysis. Then, we analyzed each included manuscript and selected scientific papers that evaluated genes or proteins in canine bladder samples and compared them with their counterparts in normal bladder tissues. In this step, we excluded manuscripts using only cell lines, manuscripts that compared bladder carcinomas with cystitis as a control (with no normal sample comparison), and manuscripts evaluating only bladder carcinomas with no comparison to normal bladder tissue. Our first search was performed on December 12, 2019, and it was last updated on April 2, 2020.

From the selected manuscripts, we retrieved information regarding each dysregulated gene or protein, the "*p*-value" for each gene or protein (comparison between bladder cancer and normal bladder tissues), and survival data.

TABLE 1 | Manuscripts ($N = 25$) meeting the inclusion criteria and from published studies of canine bladder carcinoma.

References	Manuscript title	Normal samples	Bladder cancer samples	Muscle invasion information	Gene or protein	Dysregulation	*P*-value
Maeda et al. (2)	Comprehensive gene expression analysis of canine invasive urothelial bladder carcinoma by RNA-Seq	$N = 5$	$N = 11$	Yes	Large-scale transcriptome analysis	No change	–
Tsuboi et al. (20)	Assessment of HER2 expression in canine urothelial carcinoma of the urinary bladder	$N = 8$	$N = 23$	No	HER2	Upregulated	$P = 0.0288$
Aupperle-Lellbach et al. (21)	Diagnostische aussagekraft der BRAF-mutation V595E in Urinproben, Ausstrichen, und Bioptaten Beim Kaninen ÜBergangszellkarzinom	$N = 3$	$N = 43$	No	BRAF V595E	Upregulated	N/R
Millanta et al. (22)	Overexpression of HER-2 via immunohistochemistry in canine urinary bladder transitional cell carcinoma—a marker of malignancy and possible therapeutic target	$N = 5$	$N = 23$	No	HER2	Upregulated	$P < 0.05$
Dhawan et al. (1)	Naturally occurring canine invasive urothelial carcinoma harbors luminal and basal transcriptional subtypes found in human muscle-invasive bladder cancer	$N = 4$	$N = 29$	No	Large-scale transcriptome analysis	No change	–
Walters et al. (23)	Expression of the receptor tyrosine kinase targets PDGFR-β, VEGFR2, and KIT in canine transitional cell carcinoma	$N = 10$	$N = 30$	No	PDGFR-β, VEGFR, c-KIT	Upregulated	$P \leq 0.001$, $P = 0.4268$, $P = 0.2453$
Mohammed et al. (24)	Prostaglandin E2 concentrations in naturally occurring canine cancer	$N = 10$	$N = 22$	No	PGE2	Upregulated	$P < 0.05$
Dhawan et al. (25)	DNMT1: an emerging target in the treatment of invasive urinary bladder cancer	$N = 6$	$N = 22$	No	DNMT1	Upregulated	$P < 0.02$
Suárez-Bonnet et al. (26)	Expression of cell cycle regulators, 14-3-3σ and p53 proteins, and vimentin in canine transitional cell carcinoma of the urinary bladder	$N = 5$	$N = 19$	No	14-3-3σ, p53, vimentin	Upregulated, Upregulated, Downregulated	$P = 0.0344$, $P = 0.044$, $P = 0.042$
Yamazaki et al. (27)	SiRNA knockdown of the DEK nuclear protein mRNA enhances apoptosis and chemosensitivity of canine transitional cell carcinoma cells	$N = 6$	$N = 14$	No	DEK	Upregulated	$P < 0.05$
Dhawan et al. (25)	Targeting folate receptors to treat invasive urinary bladder cancer	$N = 8$	$N = 74$	Yes	FRs (folate receptors)	Upregulated	$P < 0.0062$
Clemo et al. (28)	Immunohistochemical evaluation of canine carcinomas with monoclonal antibody B72.3	$N = 5$	$N = 13$	No	TAG-72	Upregulated	N/R
Clemo et al. (29)	Immunoreactivity of canine transitional cell carcinoma of the urinary bladder with monoclonal antibodies to tumor-associated glycoprotein 72	$N = 8$	$N = 51$	No	TAG-72	Upregulated	$P < 0.05$
Hanazono et al. (30)	Immunohistochemical expression of p63, Ki67, and beta-catenin in canine transitional cell carcinoma and polypoid cystitis	$N = 5$	$N = 25$	No	p63, beta-catenin, Ki67	Downregulated, Downregulated, Upregulated	$P < 0.01$, $P < 0.05$, $P < 0.01$
Hanazono et al. (30)	Epidermal growth factor receptor expression in canine transitional cell carcinoma	$N = 5$	$N = 25$	No	EGFR	Upregulated	$P < 0.01$
Khan et al. (31)	Expression of cyclooxygenase-2 in transitional cell carcinoma of the urinary bladder in dogs	$N = 8$	$N = 21$	No	COX-2, COX-1	Upregulated, No change	N/R

(Continued)

TABLE 1 | Continued

References	Manuscript title	Normal samples	Bladder cancer samples	Muscle invasion information	Gene or protein	Dysregulation	*P*-value
Finotello et al. (32)	Lipoxygenase-5 expression in canine urinary bladder: normal urothelium, cystitis, and transitional cell carcinoma	$N = 10$	$N = 29$	No	LOX-5, COX-2	Upregulated	$P < 0.01$
Rankin et al. (33)	Identification of survivin, an inhibitor of apoptosis, in canine urinary bladder transitional cell carcinoma	$N = 46$	$N = 41$	No	Survivin	Upregulated	$P < 0.001$
Rankin et al. (33)	Comparison of distributions of survivin among tissues from urinary bladders of dogs with cystitis, transitional cell carcinoma, or histologically normal urinary bladders	$N = 46$	$N = 41$	No	Survivin	Upregulated	$P = 0.07$
Espinosa de Los Monteros et al. (34)	Coordinate expression of cytokeratins 7 and 20 in feline and canine carcinomas	$N = 6$	$N = 14$	No	CK7, CK 20	Upregulated	N/R
LeRoy et al. (35)	Canine prostate carcinomas express markers of urothelial and prostatic differentiation	$N = 8$	$N = 19$	No	CK7	Upregulated	N/R
Parker et al. (18)	RNAseq expression patterns of canine invasive urothelial carcinoma reveal two distinct tumor clusters and shared regions of dysregulation with human bladder tumors	$N = 5$	$N = 15$	No	Large-scale transcriptome analysis	No change	–
Sakai et al. (36)	ErbB2 copy number aberration in canine urothelial carcinoma detected by a digital polymerase chain reaction assay	$N = 39$	$N = 36$	No	ERBB2	Upregulated	$P < 0.0001$
Dhawan et al. (13)	Comparative gene expression analyses identify luminal and basal subtypes of canine invasive urothelial carcinoma that mimic patterns in human invasive bladder cancer	$N = 4$	$N = 18$	No	Large-scale transcriptome analysis	No change	–
Ramsey et al. (3)	Cross-species analysis of the canine and human bladder cancer transcriptome and exome.	$N = 3$	$N = 6$	No	Large-scale transcriptome analysis	No change	–

In silico Analysis

The *in silico* analysis of each evaluated gene and protein was performed using free online tools. In the first step, we selected only the dysregulated or mutated genes and evaluated them with STRING (https://string-db.org/) to determine the proteins related to each gene of interest, using *Canis lupus familiaris* as a reference for comparisons. Then, we used only proteins for the subsequent analysis. We opted to evaluate only proteins in our *in silico* study due to the utility of proteins as prognostic and predictive markers.

The dysregulated proteins were together (upregulated and downregulated) and independently (upregulated or downregulated) using the online Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) to generate protein–protein interaction (PPI) networks. We considered only STRING interactions of high confidence (0.700), and we hid the disconnected nodes for better visualization. The interactions considered to generate the PPI networks were coexpression, co-occurrence, database, and neighborhood interactions.

Gene Ontology

Gene ontology (GO) analysis was performed to understand the biological role of proteins of interest among different species. The selected proteins were analyzed using Enrichr (https://amp.pharm.mssm.edu/Enrichr/). The analyzed information was retrieved from Enrichr and submitted to REVIGO (http://revigo.irb.hr/) to organize and visualize the enriched GO terms. We considered the three GO categories (biological process, cellular component, and molecular function) independently. However, since the biological process and molecular function terms have a higher chance of providing prognostic and predictive information, we focused on these two categories, and the enrichment analyses were performed using the same group of GO terms. For this analysis, we included only curated human annotations.

Transcriptome Data Retrieved From the Previous Literature

We selected three previous studies that evaluated the transcriptome (RNA-seq) of canine bladder carcinoma,

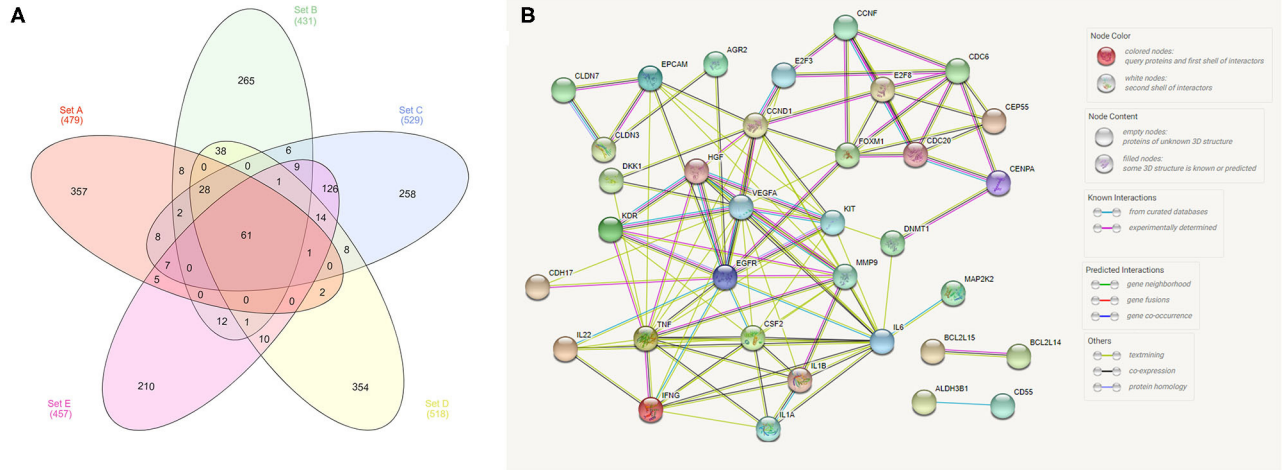

FIGURE 1 | Analysis of transcriptome data from the five previously published studies in canine bladder transitional cell carcinoma. **(A)** Venn diagram demonstrating the number of commonly dysregulated genes (61) among the five studies. **(B)** Protein–protein interaction (PPI) network of the 61 dysregulated genes. Several interactions were seen among genes, including *EGFR* and *VEGFA*, with high degrees of interaction. The Venn diagram was generated online (http://www.interactivenn.net/) using the five available manuscripts with transcriptome data, and the PPI was generated with the 61 genes commonly dysregulated among the five studies using STRING (https://string-db.org/).

with the datasets available online via the NCBI short-read archive (SRA) under BioProject ID PRJNA559406 (18), GEO database (ref: GSE24152) (1), and DDBJ Sequenced Read Archive repository (http://trace.ddbj.nig.ac.jp/dra/index_e.html) with accession number DRA005844. In addition, we also included one manuscript evaluating the transcriptome of canine bladder cancer using microarray data (13) and one manuscript that performed both mRNA-seq and exome-seq (3). For both studies (3, 13), mRNA data were obtained from the **Supplementary Materials**.

The genes differentially expressed between TCC and normal bladder were selected with the following criteria: $p < 0.05$ and a fold change of 2.5 or higher in either direction. A Venn diagram was generated using an online tool (http://www.interactivenn.net/) (37). Furthermore, the common differentially expressed genes among the five studies were validated using 344 bladder carcinoma samples from the TCGA database.

The Cancer Genome Atlas (TCGA) and The Cancer Proteome Atlas (TCPA) Cross-Validation

Due to the lack of a veterinary database with deposited information regarding the survival of canine patients with bladder carcinoma, we selected the most relevant proteins and validated them using 344 human samples from patients with muscle-invasive bladder carcinoma from TCGA (https://www.cancer.gov/tcga). The selected proteins were chosen based on the respective *p*-value and the biological function of the protein as previously described for other tumor subtypes. Then, the cross-validated proteins were evaluated via TCPA (https://tcpaportal.org/tcpa/index.html) (38). We considered proteins with a 5% interval of confidence or a *p*-value lower than 0.05.

In addition, we performed two different analyses using TCPA. First, we used the "visualization" tool to perform a global analysis to evaluate interactions among genes, including negative and positive interactions. In this way, we selected genes and pathways of human bladder cancer differentially implicated as possible markers to be used in canine bladder cancer. In the second analysis, we evaluated the overall survival of 344 human bladder cancer patients according to protein expression levels (high vs. low). In this analysis, we selected all genes in human bladder cancer with prognostic value. The Kaplan–Meier curves were generated using the TCPA online tool "individual cancer analysis" (https://tcpaportal.org/tcpa/analysis.html) (38, 39).

RESULTS

Meta-Analysis

A total of 385 manuscripts were identified in the initial search. Then, according to the inclusion criteria, after reading the title and abstract, we excluded 329 manuscripts, and after reading the full manuscript, 25 of them met our inclusion criteria (**Supplementary Figure 2**). Next, we divided the selected manuscripts into two categories: manuscripts with global transcriptome analysis ($N = 5$) and manuscripts with reported isolated genes or proteins ($N = 20$). A complete list of the selected manuscripts can be found in **Table 1**.

In the studies involving isolated protein analysis, HER-2 was the most studied protein (3/20), followed by TAG-72 (2/20), COX-2 (2/20), survivin (2/20), and CK7 (2/20). The remaining proteins were evaluated in only one previous study each (**Table 1**). In the protein–protein interaction analysis, we identified one interaction network with most interactions involving P53. After enrichment analysis using Enrichr, we evaluated the most common ontological processes associated

TABLE 2 | Dysregulated genes identified via cross-validation of data from the five veterinary studies with transcriptome data and data from 344 human samples from The Cancer Genome Atlas (TCGA).

Gene/protein	Chromosome	Gene ID	Regulation	Function
BRAF V595E	16	475526	Upregulated	Proto-oncogene, serine/threonine kinase
PTGER2	8	403797	Upregulated	Prostaglandin E receptor 2
ERBB2/HER-2	9	403883	Upregulated	Receptor tyrosine kinase 2
CHST4	5	489722	Upregulated	Carbohydrate sulfotransferase 4
PIGR	7	474357	Upregulated	Polymeric immunoglobulin receptor
S100A14	7	612322	Upregulated	Calcium binding protein A14
ADGRF1	12	474927	Upregulated	Adhesion G protein-coupled receptor F1
AGR2	14	482333	Upregulated	Anterior gradient 2, protein disulfide isomerase
Irgm 1	11	606863	Downregulated	Immunity-related GTPase family M protein-like
TP53	5	403869	Downregulated	Tumor protein
ZFP36	1	484510	Downregulated	Ring finger protein
E2F2	2	100855664	Upregulated	Transcription factor 2
IFI16	38	488622	Upregulated	Interferon-activatable protein 203
EGFR	18	404306	Upregulated	Epidermal growth factor receptor
ERK1/2	26	477575	Upregulated	Mitogen-activated protein kinase 1
NfkB-RelA	12	481711	Upregulated	NFKB inhibitor like 1
MAP2K3	5	489547	Upregulated	Mitogen-activated protein kinase kinase 3
IL1A	17	403782	Upregulated	Interleukin 1 alpha
RABL6	9	480675	Upregulated	RAS oncogene family like 6
CCND1	18	449028	Upregulated	Cyclin D1
FOXM1	27	486743	Upregulated	Forkhead box M1
E2F3	35	488239	Upregulated	Transcription factor 3
FOX01	6	609116	Upregulated	RNA-binding fox-1 homolog 1
IL6	14	403985	Upregulated	Interleukin 6
IL1B	17	403974	Upregulated	Interleukin 1 beta
CSF2	11	403923	Upregulated	Colony-stimulating factor 2
TNF	12	403922	Upregulated	Tumor necrosis factor
IFNG	10	403801	Upregulated	Interferon gamma
IL22	10	481153	Upregulated	Interleukin 22
HGF	18	403441	Upregulated	Hepatocyte growth factor
VEGF	12	403802	Upregulated	Vascular endothelial growth factor A
PDGFR-B	4	442985	Upregulated	Platelet-derived growth factor receptor beta
VEGFR2	13	482154	Upregulated	Kinase insert domain receptor
DNMT1	20	476715	Upregulated	DNA methyltransferase 1
SFN	2	487351	Upregulated	Stratifin

with each previously published protein, and we identified several processes related to tyrosine kinase regulation, cell communication and signaling, and the MAPK pathway (**Supplementary Figure 3** and **Supplementary Table 1**).

In silico Analysis of Canine Transcriptome Data

In our meta-analysis, we identified five previous studies containing transcriptome data, and in the most recent study (18), the authors cross-validated their findings with three other published manuscripts (1, 2, 13). Thus, we opted to analyze the transcriptome data from these five previous studies and cross-validate them with TCGA data. In our cross-validation analysis using the five previous veterinary studies, we identified 61 dysregulated genes (**Figure 1**), including CD55, IL17B, EGFR, CDH17, and CDH26. Moreover, we performed a PPI analysis among these genes and demonstrated a high interaction among them, with VEGFA, EGFR, TNF, and CCND1 being central genes in the interaction network (**Figure 1**).

We identified 35 dysregulated genes in the cross-validation analysis of the five veterinary studies and the TCGA data (**Table 2**).

Cross-Validation With Human Bladder Cancer

In the analysis of the human bladder cancer samples, we identified several positive and negative protein correlations. In the PPI, many proteins from the serine/threonine and tyrosine

TABLE 3 | Proteins in human bladder cancer associated with overall survival in the cohort of 344 patients.

Protein	Gene	Cox P	Log-rank P
ANNEXIN1	ANXA1	0.000039282	0.0000079633
TAZ	TAZ	0.012031	0.00016332
SRC	SRC	0.00058508	0.00037203
SF2	SRSF1	0.033481	0.00073484
ARID1A	ARID1A	0.021321	0.0008096
GATA3	GATA3	0.0015211	0.0012084
BAK	BAK1	0.068011	0.0012196
EGFR	EGFR	0.006267	0.0016964
CD20	MS4A1	0.049073	0.0071387
SCD1	SCD1	0.10015	0.0078273
CABL	ABL	0.028914	0.011089
GATA6	GATA6	0.18358	0.014776
BAP1C4	BAP1	0.41571	0.015214
RICTOR	RICTOR	0.010638	0.015853
AXL	AXL	0.15225	0.015863
SMAD3	SMAD3	0.0048029	0.017136
BRAF_pS445	BRAF	0.068071	0.017645
SMAC	DIABLO	0.0039151	0.017688
BECLIN	BECN1	0.027124	0.018469
PARPCLEAVED	PARP1	0.21592	0.018488
ADAR1	ADAR	0.050259	0.022731
SHC_pY317	SHC1	0.23036	0.027714
TRANSGLUTAMINASE	TGM2	0.53787	0.036741
ANNEXINVII	ANXA7	0.36004	0.045156
PEA15	PEA15	0.15168	0.046395
PKCALPHA	PRKCA	0.056178	0.047571
MEK1	MAP2K1	0.65034	0.051509
CAVEOLIN1	CAV1	0.03709	0.058778

kinase family, such as EGFR, ERK2, ERBB2, and BRAF, were observed. In addition, we identified 28 proteins with prognostic value in human bladder cancer (**Table 3**). Among these proteins, only two (EGFR and BRAF) were previously studied in canine bladder cancer (2/28). The top six proteins with prognostic value were Annexin 1, TAZ, SF2, SRC, ARID1A, and GATA3 (**Figure 2**).

DISCUSSION

Human bladder cancer molecular findings are widely described in the literature, making it possible to reanalyze these data to provide new insights for comparative oncology. Although dogs can be considered models of human bladder cancer, few studies have provided a full description of canine bladder cancer molecular data. Since most canine bladder cancer studies have published isolated assessments of different proteins, the present study extracted these data and evaluated them together to understand how these proteins interact with each other. However, it is important to consider the limitations of each study, including differences in terms of tumor stage and therapeutic protocols used in each publication. One limitation of our study is the use of online tools such as Enrichr that use Fisher's exact test, which is statistically more likely to identify larger pathways than smaller pathways as significant. Thus, we selected the upper size limit for the size of gene sets to avoid misinterpretation.

In our meta-analysis, after the first search, several studies were excluded because they had no matched normal tissue analysis ($N = 31/56$). The inclusion of normal tissue is important to establish a pattern of expression between normal and cancer tissue. During the carcinogenic process, cancer cells can change their expression profile with gains or losses of expression of several genes, and it is important to include normal samples to avoid bias (40). In addition, for some *in silico* analyses, it is necessary to have a *p*-value related to the protein expression in tumor compared to normal tissues.

The meta-analysis demonstrated that most of the veterinary studies did not evaluate muscle invasion (23/25) by the tumor or did not provide clear information regarding this topic. Thus, as a future direction for canine studies evaluating transitional cell carcinoma from the bladder, we strongly suggest that the authors evaluate muscle invasion to provide stronger evidence regarding dogs being models of human bladder cancer. In addition, this lack of information about muscle invasiveness may have introduced some confounding factors that influenced the results. Additionally, it is difficult to make cross-species validations and assumptions given the lack of information in veterinary studies.

Most of the published manuscripts that met the inclusion criteria evaluated one to three proteins or genes, and only five previous manuscripts performed large-scale analyses on canine bladder carcinomas. These large-scale transcriptome studies used RNA-seq technology of the tumor samples. On the other hand, new single-cell sequencing technologies have been used in recent years to provide more specific information regarding the transcriptome of human cancers (41). However, to the best of our knowledge, there is no information regarding single-cell sequencing in canine bladder cancer. The single-cell technology allows the evaluation of tumor transcriptome excluding other cell-type, such as stromal, endothelial, and inflammatory cells.

From the selected studies, we extracted protein or gene information from the manuscripts studying isolated proteins and evaluated these proteins together. All studies that reported isolated proteins were focused on the evaluation of oncogenes. Interestingly, most of them evaluated tyrosine kinase receptors, such as ERBB2, EGFR, VEGFR, and PDGFR (20, 23, 42). Our PPI analysis revealed a high number of interactions among these proteins, even though they were evaluated separately in each study. As such, future studies may benefit from our PPI analysis to evaluate the prognostic or predictive value of the identified proteins in canine bladder cancer. Interestingly, the ontology analysis of the studies with isolated proteins revealed several terms related to tyrosine kinase activity, phosphorylation, and alteration of the ERK1 and ERK2 cascade. Thus, previous studies have focused on the search for small-molecule inhibitor targets. However, no small-molecule inhibitors have yet been successfully proposed in the treatment of canine bladder cancer.

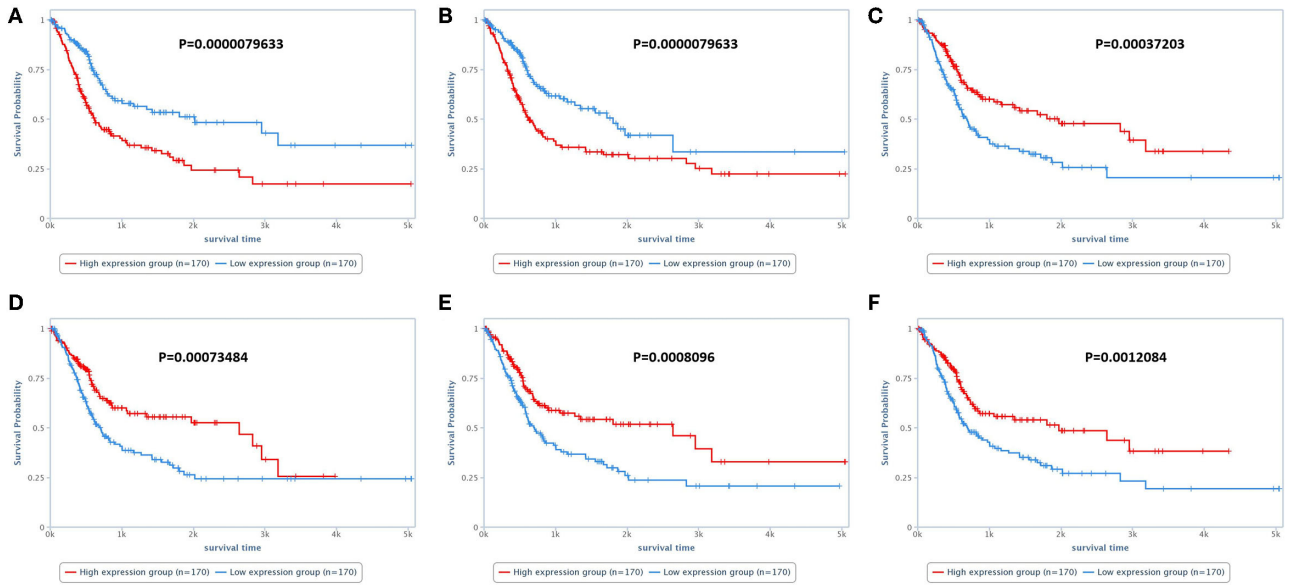

FIGURE 2 | Survival analysis of human patients with bladder cancer. **(A)** Survival of patients according to Annexin 1 expression. Patients presenting high Annexin 1 expression experienced a shorter survival time than patients with low Annexin 1 expression. **(B)** Overall survival of patients according to TAZ expression. Patients with high TAZ expression experienced a shorter survival time than patients with low TAZ expression. **(C)** Overall survival according to SRC expression. Patients with lower SRC expression experienced a shorter survival time than patients with low SRC expression. **(D–F)** Overall survival according to SF2, ARID1A, and GATA3 expression, respectively. For these three proteins, patients with lower expression experienced a shorter survival time than those with higher expression. The survival analysis was performed using the TCPA online tool (https://tcpaportal.org/tcpa/survival_analysis.html).

Thus, tyrosine kinase receptors could be overrepresented in our analysis, and this result should be interpreted with caution.

The cross-validation of canine transcriptome data with TCGA data revealed 35 genes with a high probability of presenting dysregulation in both human and canine bladder cancer. Since these data were obtained from five different canine studies and 344 human samples from TCGA, they are promising and could be used for further investigation. Though it has been difficult to identify potential markers to be tested in future studies, our list provides markers with strong potential that are upregulated or downregulated in both human and canine bladder cancer. One important limitation of our analysis was the absence of muscle invasion information/standardization in canine samples. Thus, we can lack data regarding important genes related to muscle invasiveness, which is known as a poor prognostic finding. Nevertheless, choosing a gene from our list for future studies could be more promising than a random search. In addition, we analyzed the data from 344 human bladder cancer patients with muscle-invasive patterns to identify genes related to overall survival. Since survival data are usually absent in published studies in veterinary medicine, human survival data represents a unique opportunity to identify candidates related to prognosis in veterinary oncology.

Among the 28 proteins with prognostic value, we identified Annexin 1, GATA-3, and EGFR. Annexin 1 overexpression was previously associated with tumor progression and was considered an independent marker for metastasis-free survival (43). In addition, Annexin 1 expression was also associated with chemotherapy relapse and resistance in human bladder cancer (44). In the present meta-analysis, studies evaluating Annexin 1 expression in canine bladder tumors were not found. GATA3 is widely used in human medicine as a diagnostic marker (45, 46). Interestingly, in addition to its use as a diagnostic marker, GATA3 has been shown to be an important prognostic marker in human bladder cancer (45). Decreased GATA3 expression is associated with low recurrence-free survival, a high frequency of muscle invasiveness, and a tumor progression. In veterinary medicine, one previous review mentioned GATA3 expression in canine bladder cancer and showed GATA3 expression in a sample of canine bladder carcinoma (10). However, since it was a review, these authors did not evaluate canine bladder carcinoma samples. The corresponding author was contacted and kindly provided information regarding the GATA3 antibody used for the data. Overall GATA3 has the potential to be a prognostic marker for canine bladder cancer.

EGFR is an important marker in human bladder cancer and is associated with overall survival, muscle invasiveness, and tumor recurrence (47). Thus, EGFR overexpression has been studied and is a target for anti-EGFR therapies (48). In dogs, EGFR has previously been evaluated in canine bladder cancer (42). However, the authors evaluated the EGFR gene and protein in samples but provided no association with clinicopathological findings. Regardless, based on meta-analyses and *in silico* analyses, EGFR shows promising potential in terms of both prognostic and predictive value in canine bladder cancer. (49) evaluated an anti-EGFR monoclonal antibody in

canine transitional carcinoma cells from the bladder *in vitro* and *in vivo*. The authors' findings suggested that this anti-EGFR monoclonal antibody could be promising for the treatment of dogs with bladder cancer. Thus, both humans and dogs can benefit from clinical trials involving anti-EGFR antibodies in dogs.

CONCLUSION

The canine literature on bladder cancer has been focused on the evaluation of isolated markers with no association with patient survival. In addition, the lack of information regarding tumor muscle invasion can be considered an important limitation when comparing human and canine bladder tumors. Our *in silico* analysis involving canine and human transcriptome data provided several genes with the potential to be markers for both human and canine bladder tumors, and these genes should be considered for future studies on canine bladder cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

VV and CF-A wrote the first manuscript draft. VV performed the meta-analysis and *in silico* analysis of the selected data. CF-A and RF checked the meta-analysis and the *in silico* data independently. RL-A and VG contributed constructive comments. CF-A supervised the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.558978/full#supplementary-material

REFERENCES

1. Dhawan D, Hahn NM, Ramos-Vara JÁ, Knapp DW. Naturally-occurring canine invasive urothelial carcinoma harbors luminal and basal transcriptional subtypes found in human muscle invasive bladder cancer. *PLoS Genet*. (2018) 14:e1007571. doi: 10.1371/journal.pgen.1007571
2. Maeda S, Tomiyasu H, Tsuboi M, Inoue A, Ishihara G, Uchikai T, et al. Comprehensive gene expression analysis of canine invasive urothelial bladder carcinoma by RNA-Seq. *BMC Cancer*. (2018) 18:472. doi: 10.1186/s12885-018-4409-3
3. Ramsey SA, Xu T, Goodall C, Rhodes AC, Kashyap A, He J, et al. Cross-species analysis of the canine and human bladder cancer transcriptome and exome. *Genes Chromosomes Cancer*. (2017) 56:328–43. doi: 10.1002/gcc.22441
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. (2020) 70:7–30. doi: 10.3322/caac.21590
5. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. (2018) 68:394–424. doi: 10.3322/caac.21492
6. Patrick D, Fitzgerald S, Sesterhenn I, Davis C, Kiupel M. Classification of Canine Urinary Bladder Urothelial Tumours Based on the World Health Organization/ International Society of Urological Pathology Consensus Classification. *J Comp Path*. (2006) 135:190–9. doi: 10.1016/j.jcpa.2006.07.002
7. Miyazaki J, Nishiyama H. Epidemiology of urothelial carcinoma. *Int J Urol*. (2017) 24:730–4. doi: 10.1111/iju.13376
8. Cha JD, Lourenço DB, Korkes F. Analysis of the association between bladder carcinoma and arsenic concentration in soil and water in southeast Brazil. *Int Braz J Urol*. (2018) 44:906–13. doi: 10.1590/s1677-5538.ibju.2017.0543
9. Glickman LT, Schofer FS, McKee LJ, Reif JS, Goldschmidt MH. Epidemiologic study of insecticide exposures, obesity, and risk of bladder cancer in household dogs. *J Toxicol Environ Health*. (1989) 28:407–14. doi: 10.1080/15287398909531360
10. Knapp DW, Ramos-Vara JA, Moore GE, Dhawan D, Bonney PL, Young KE. Urinary bladder cancer in dogs, a naturally occurring model for cancer biology and drug development. *ILAR, J*. (2014) 55:100–18. doi: 10.1093/ilar/ilu018
11. Grzegółkowski P, Kaczmarek K, Lemiński A, Soczawa M, Gołab A, Słojewski M. Assessment of the infiltrative character of bladder cancer at the time of transurethral resection: a single center study. *Cent European J Urol*. (2017) 70:22–6.
12. Hernández-Fernández C, Herranz-Amo F, Moralejo-Gárate M, Subirá-Ríos D, Caño-Velasco J, Barbas-Bernardos G. Infiltrating bladder cancer: prognostic factors, follow-up and treatment of relapses. *Actas Urol Esp*. (2017) 41:352–8. doi: 10.1016/j.acuroe.2016.07.010
13. Dhawan D, Paoloni M, Shukradas S, et al. Comparative Gene Expression Analyses Identify Luminal and Basal Subtypes of Canine Invasive Urothelial Carcinoma That Mimic Patterns in Human Invasive Bladder Cancer. *PLoS One*. (2015) 10:e0136688. doi: 10.1371/journal.pone.0136688
14. Jalanko T, de Jong J, Gibb EA, Seiler R, Black P. Genomic Subtyping in Bladder Cancer. *Curr Urol Rep*. (2020) 21:9. doi: 10.1007/s11934-020-0960-y
15. Inamura K. Bladder Cancer: New Insights into Its Molecular Pathology. *Cancers (Basel)*. (2018) 10:E100. doi: 10.3390/cancers10040100
16. Cancer Genome Atlas Research Network (2014). Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* 507, 315–322. doi: 10.1038/nature12965
17. Knapp DW, Dhawan D, Ramos-Vara JA, Ratliff TL, Cresswell GM, Utturkar S, et al. Naturally-Occurring Invasive Urothelial Carcinoma in Dogs, a Unique Model to Drive Advances in Managing Muscle Invasive Bladder Cancer in Humans. *Front Oncol*. (2020) 9:1493. doi: 10.3389/fonc.2019.01493
18. Parker HG, Dhawan D, Harris AC, et al. RNAseq expression patterns of canine invasive urothelial carcinoma reveal two distinct tumor clusters and shared regions of dysregulation with human bladder tumors. *BMC Cancer*. (2020) 20:251. doi: 10.1186/s12885-020-06737-0
19. Mochizuki H, Shapiro SG, Breen M. Detection of BRAF Mutation in Urine DNA as a Molecular Diagnostic for Canine

Urothelial and Prostatic Carcinoma. *PLoS One*. (2015) 10:e0144170. doi: 10.1371/journal.pone.0144170
20. Tsuboi M, Sakai K, Maeda S, Chambers JK, Yonezawa T, Matsuki N, et al. Assessment of HER2 expression in canine urothelial carcinoma of the urinary bladder. *Vet Pathol*. (2019) 56:369–76. doi: 10.1177/0300985818817024
21. Aupperle-Lellbach H, Grassinger J, Hohloch C, Kehl A, Pantke P. Diagnostische aussagekraft der BRAF-mutation V595E in urinproben, ausstrichen und bioptaten beim kaninen Übergangszellkarzinom [Diagnostic value of the BRAF variant V595E in urine samples, smears and biopsies from canine transitional cell carcinoma]. *Tierarztl Prax Ausg K Kleintiere Heimtiere*. (2018) 46:289–95. doi: 10.15654/TPK-180554
22. Millanta F, Impellizeri J, mcsherry L, Rocchigiani G, Aurisicchio L, Lubas G. Overexpression of HER-2 via immunohistochemistry in canine urinary bladder transitional cell carcinoma - a marker of malignancy and possible therapeutic target. *Vet Comp Oncol*. (2018) 16:297–300. doi: 10.1111/vco.12345
23. Walters L, Martin O, Price J, Sula MM. Expression of receptor tyrosine kinase targets PDGFR-β, VEGFR2 and KIT in canine transitional cell carcinoma. *Vet Comp Oncol*. (2018) 16:E117–22. doi: 10.1111/vco.12344
24. Mohammed SI, Coffman K, Glickman NW, Hayek MG, Waters DJ, Schlittler D, et al. Prostaglandin E2 concentrations in naturally occurring canine cancer. *Prostaglandins Leukot Essent Fatty Acids*. (2001) 64:1–4. doi: 10.1054/plef.2000.0231
25. Dhawan D, Ramos-Vara J, Hahn N, Waddell J, Olbricht G, Zheng R, et al. DNMT1: an emerging target in the treatment of invasive urinary bladder cancer. *Urol Oncol*. (2013) 31:1761–9. doi: 10.1016/j.urolonc.2012.03.015
26. Suárez-Bonnet A, Herráez P, Aguirre M, Suárez-Bonnet E, Andrada M, Rodríguez F, et al. Expression of cell cycle regulators, 14-3-3σ and p53 proteins, and vimentin in canine transitional cell carcinoma of the urinary bladder. *Urol Oncol*. (2015) 33:332.e1–7. doi: 10.1016/j.urolonc.2015.04.006
27. Yamazaki H, Iwano T, Otsuka S, Kagawa Y, Hoshino Y, Hosoya K, et al. Sirna knockdown of the DEK nuclear protein mrna enhances apoptosis and chemosensitivity of canine transitional cell carcinoma cells. *Vet J*. (2015) 204:60–5. doi: 10.1016/j.tvjl.2015.02.009
28. Clemo FA, denicola DB, Delaney LJ. Immunohistochemical evaluation of canine carcinomas with monoclonal antibody B72.3. *Vet Pathol*. (1993) 30:140–5. doi: 10.1177/030098589303000206
29. Clemo FA, denicola DB, Carlton WW, Walker E, Morrison WB. Immunoreactivity of canine transitional cell carcinoma of the urinary bladder with monoclonal antibodies to tumor-associated glycoprotein 72. *Vet Pathol*. (1995) 32:155–61. doi: 10.1177/030098589503200209
30. Hanazono K, Nishimori T, Fukumoto S, Kawamura Y, Endo Y, Kadosawa T, et al. Immunohistochemical expression of p63, Ki67 and β-catenin in canine transitional cell carcinoma and polypoid cystitis of the urinary bladder. *Vet Comp Oncol*. (2016) 14:263–9. doi: 10.1111/vco.12095
31. Khan KN, Knapp DW, Denicola DB, Harris RK. Expression of cyclooxygenase-2 in transitional cell carcinoma of the urinary bladder in dogs. *Am J Vet Res*. (2000) 61:478–81. doi: 10.2460/ajvr.2000.61.478
32. Finotello R, Schiavo L, Ressel L, Frohmader A, Silvestrini P, Verin R. Lipoxygenase-5 expression in canine urinary bladder: normal urothelium, cystitis and transitional cell carcinoma. *J Comp Pathol*. (2019) 170:1–9. doi: 10.1016/j.jcpa.2019.05.001
33. Rankin WV, Henry CJ, Turnquist SE, Turk JR, Beissenherz ME, Tyler JW, et al. Identification of survivin, an inhibitor of apoptosis, in canine urinary bladder transitional cell carcinoma. *Vet Comp Oncol*. (2008) 6:141–50. doi: 10.1111/j.1476-5829.2007.00150.x
34. Espinosa de los Monteros A, Fernández A, Millán MY, Rodríguez F, Herráez P, Martín de las Mulas J. Coordinate expression of cytokeratins 7 and 20 in feline and canine carcinomas. *Vet Pathol*. (1999) 36:179–90. doi: 10.1354/vp.36-3-179
35. leroy BE, Nadella MV, Toribio RE, Leav I, Rosol TJ. Canine prostate carcinomas express markers of urothelial and prostatic differentiation. *Vet Pathol*. (2004) 41:131–40. doi: 10.1354/vp.41-2-131
36. Sakai K, Maeda S, Saeki K, Yoshitake R, Goto-Koshino Y, Nakagawa T, et al. Erbb2 copy number aberration in canine urothelial carcinoma detected by a digital polymerase chain reaction assay. *Vet Pathol*. (2020) 57:56-65. doi: 10.1177/0300985819879445
37. Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics*. (2015) 16:169. doi: 10.1186/s12859-015-0611-3
38. Li J, Lu Y, Akbani R, Ju Z, Roebuck P, Liu W, et al. TCPA: a resource for cancer functional proteomics data. *Nature Methods*. (2013) 10:1046–7. doi: 10.1038/nmeth.2650
39. Li J, Akbani R, Zhao W, Lu Y, Weinstein J, Mills G, Liang H. Explore, visualize, and analyze functional cancer proteomic data using the cancer. *Proteome Atlas Cancer Res*. (2017) 77:e51–4. doi: 10.1158/0008-5472.CAN-17-0369
40. Kosti I, Jain N, Aran D, Butte A, Sirota M. Cross-tissue Analysis of Gene and Protein Expression in Normal and Cancer Tissues. *Sci Rep*. (2016) 6:24799. doi: 10.1038/srep24799
41. Cho B, Yoon SH, Lee D, et al. Single-cell transcriptome maps of myeloid blood cell lineages in Drosophila. *Nat Commun*. (2020) 11:4483. doi: 10.1038/s41467-020-18135-y
42. Hanazono K, Fukumoto S, Kawamura Y, et al. Epidermal growth factor receptor expression in canine transitional cell carcinoma. *J Vet Med Sci*. (2015) 77:1–6. doi: 10.1292/jvms.14-0032
43. Li C, Shen K, Huang L, Huang H, Wang Y, Wu TF. Annexin-I overexpression is associated with tumour progression and independently predicts inferior disease-specific and metastasis-free survival in urinary bladder urothelial carcinoma. *Pathology*. (2010) 42:43–9. doi: 10.3109/00313020903434405
44. Yu S, Meng Q, Hu H, Zhang M. Correlation of ANXA1 expression with drug resistance and relapse in bladder cancer. *Int J Clin Exp Pathol*. (2014) 7:5538–48.
45. Inoue S, Mizushima T, Fujita K, Meliti A, Ide H, Yamaguchi S, et al. GATA3 immunohistochemistry in urothelial carcinoma of the upper urinary tract as a urothelial marker and a prognosticator. *Hum Pathol*. (2017) 64:83–90. doi: 10.1016/j.humpath.2017.04.003
46. Agarwal H, Babu S, Rana C, Kumar M, Singhai A, Shankhwar S, et al. Diagnostic utility of GATA3 immunohistochemical expression in urothelial carcinoma. *Indian J Pathol Microbiol*. (2019) 62:244–50. doi: 10.4103/IJPM.IJPM_228_18
47. Hashmi A, Hussain Z, Irfan M, Khan E, Faridi N, Naqvi H, et al. Prognostic significance of epidermal growth factor receptor (EGFR) over expression in urothelial carcinoma of urinary bladder. *BMC Urol*. (2018) 18:59. doi: 10.1186/s12894-018-0373-0
48. Karyagina T, Ulasov A, Slastnikova T, Rosenkranz A, Lupanova T, Khramtsov Y, et al. Targeted Delivery of (111)In Into the Nuclei of EGFR Overexpressing Cells via Modular Nanotransporters With Anti-EGFR Affibody. *Front Pharmacol*. (2020) 11:176. doi: 10.3389/fphar.2020.00176
49. Nagaya T, Okuyama S, Ogata F, Maruoka Y, Knapp DW, Karagiannis SN, et al. Near infrared photoimmunotherapy targeting bladder cancer with a canine anti-epidermal growth factor receptor (EGFR) antibody. *Oncotarget*. (2018) 9:19026–38. doi: 10.18632/oncotarget.24876

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Transcriptome of Two Canine Prostate Cancer Cells Treated With Toceranib Phosphate Reveals Distinct Antitumor Profiles Associated With the PDGFR Pathway

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Canine prostate cancer (PC) presents a poor antitumor response, usually late diagnosis and prognosis. Toceranib phosphate (TP) is a nonspecific inhibitor of receptor tyrosine kinases (RTKs), including vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and c-KIT. This study aimed to evaluate VEGFR2, PDGFR-β, and c-KIT protein expression in two established canine PC cell lines (PC1 and PC2) and the transcriptome profile of the cells after treatment with TP. Immunofluorescence (IF) analysis revealed VEGFR2 and PDGFR-β protein expression and the absence of c-KIT protein expression in both cell lines. After TP treatment, only the viability of PC1 cells decreased in a dose-dependent manner. Transcriptome and enrichment analyses of treated PC1 cells revealed 181 upregulated genes, which were related to decreased angiogenesis and cell proliferation. In addition, we found upregulated *PDGFR-A*, *PDGFR-β*, and *PDGF-D* expression in PC1 cells, and the upregulation of *PDGFR-β* was also observed in treated PC1 cells by qPCR. PC2 cells had fewer protein-protein interactions (PPIs), with 18 upregulated and 22 downregulated genes; the upregulated genes were involved in the regulation of parallel pathways and mechanisms related to proliferation, which could be associated with the resistance observed after treatment. The canine PC1 cell line but not the PC2 cell line showed decreased viability after treatment with TP, although both cell lines expressed PDGFR and VEGFR receptors. Further studies could explain the mechanism of resistance in PC2 cells and provide a basis for personalized treatment for dogs with PC.

Keywords: dog, prostate, microarray, animal model, antitumor response

INTRODUCTION

Although the prevalence of prostate cancer (PC) in dogs is relatively low, canines are the only domestic species other than humans known to spontaneously develop PC (1, 2). Canine prostate carcinoma is a biologically aggressive neoplasm that exhibits a poor prognosis related to its late diagnosis, high metastatic rate (80% at death), and limited effective treatments (1, 3, 4). Some reported metastatic sites include lung, bone, lymph node, liver, spleen, and colon (4–8).

In contrast to PC in humans, PC in dogs is not androgen dependent, so androgen deprivation therapy is not effective (9). Due to some important differences between PC in men and dogs, many treatment modalities used successfully in human medicine cannot be applied in dogs (9). Therefore, there is a need for new therapies for canine PC, including targeted therapies, which are drugs that target specific proteins in neoplastic cells or tumor-associated antigens and exert less damage to normal cells (10).

Toceranib phosphate (TP), the veterinary counterpart to sunitinib (SU11248), works by preventing receptor tyrosine kinase (RTK) phosphorylation and consequent downstream signaling molecules (11–13). TP is a nonspecific RTK inhibitor with targets including VEGFR (vascular endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor), and c-KIT; this drug was approved for the treatment of dogs with cutaneous mast cell tumors and was originally developed as an antiangiogenic agent (14–16). Additionally, some phase I clinical trials in dogs suggest that TP treatment exhibits clinical antitumor activity against different cancers, including gastrointestinal stromal tumors, lymphomas, multiple myelomas, metastatic soft tissue sarcomas, and several carcinomas, such as metastatic mammary, head and neck, thyroid, and prostatic carcinomas (11, 17–19).

Although some studies have demonstrated the clinical anticancer effect of TP in dogs, the mechanisms of action have not been elucidated. We used global gene expression analysis in two canine PC cell lines to evaluate the genes involved in the treatment response to TP. To investigate the molecular mechanism underlying TP cytotoxicity in canine PC cell lines, we evaluated cell viability and gene expression alterations in response to TP.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Ethics Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of the São Paulo State University, Botucatu (Protocol: 0004/2017). Written informed consent was obtained from the owners for the participation of their animals in this study.

Canine Primary PC Cell Culture

Two characterized canine primary prostate cancer cell cultures (PC1 and PC2) from two different canine PCs collected during necropsy, were cultured as previously described (20). PC1 cell was stablished from prostate carcinoma of a 10-year- old, intact, mixed breed dog and PC2 from an 11-year-old, intact poodle dog. PC1 and PC2 cells were cultured at 37°C in 5% CO_2 in complete medium comprising DMEM/F12 (Lonza Inc., Allendale, NJ, USA), 10% inactivated fetal bovine serum (FBS, HYCLONE, Waltham, MA, USA), 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma, Portland, OR, USA).

Assessment of Protein Expression by Immunofluorescence (IF)

To evaluate the effects of TK receptors in these cell lines, we performed IF for VEGFR2, PDGFR-β, and c-KIT. PC1 and PC2 cells were seeded at 5×10^4 cells/well into 12-well chamber slides (SPL Life Sciences) and allowed to grow on coverslips at 37°C and 5% CO_2 until they reached ∼70% confluence. Cells were then washed using Dulbecco's phosphate-buffered saline (DPBS) at 4°C and fixed in methanol for 30 min at 27°C. Samples were permeabilized with 0.1% Triton X-100 in PBS for 10 min at 27°C and incubated with a commercial reagent (Protein Block Serum Free, Dako®) for 30 min to block nonspecific antibody binding. The cells were incubated for 18 h at 4°C in a humidified atmosphere with primary antibodies against VEGFR2 (Clone SC-6251, Santa Cruz Biotechnology), PDGFR-β (Clone 3162, Cell Signaling Technology®), and c-KIT (Clone A4502, Dako®), all of which were diluted 1:100. Next, the cells were incubated with secondary antibodies at 1:10,000 dilutions and conjugated to the following fluorophores: Alexa Fluor 594 (Clone Poly4053, BioLegend) for VEGFR and Alexa Fluor® 488 (Clone A11034, ThermoFisher Scientific) for PDGFR-β and c-KIT (CD117). The samples were then labeled with DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (D9542, Dako®) at a 1:10,000 dilution to stain the nuclei and examined and imaged under a TCS SP5 confocal microscope (Leica Biosystems, Wetzlar, Alemanha).

PC cell lines were considered to have positive or negative expression according to the immunofluorescent results.

MTT Assay and IC50 Detection

PC1 and PC2 cell lines were seeded into 96-well-plates at a concentration of 1×10^4 cells/well in 0.1 mL of complete medium and allowed to grow for 24 h in a 5% CO_2 incubator. The medium was removed and replaced with serum-free medium containing different concentrations of TP (Sigma-Aldrich; 3, 6, 9, and 12 μM; 12 wells per concentration) dissolved in dimethyl sulfoxide (DMSO—Hybri-Max™; Sigma). Two control groups—no treatment and 0.4% DMSO (vehicle control), were also established to confirm that the vehicle had no influence on cell viability. The wells were incubated for 24, 48, and 72 h after the addition of the treatments.

An MTT stock solution was prepared with 0.013 g of MTT (Invitrogen™, M6494) dissolved in PBS (2.5 mL); 1 mL of the stock solution was diluted 10-fold in serum-free medium to establish a final concentration of 0.5 mg/mL. After the addition of MTT, the plates were incubated at 37°C for 4 h. Next, the medium was removed from all the plates, and the precipitated MTT salts were dissolved with 200 μL of DMSO per well for 15 min. Absorbance values at 595 nm were recorded with a multiwell plate reader.

Cell viability was calculated as a percentage using the following formula: $(A_{treatment} - A_{blank})/(A_{DMSO} - A_{blank}) \times 100\%$ (21), with A = absorbance, DMSO = vehicle control, and blank = no cells. The IC50 values were calculated using Graph Pad Prism 8.0 from a log ([drug]) vs. normalized response curve fit.

Quantitative PCR

After establishing the IC_{50} value of TP for each cell line, we treated PC1 and PC2 cells with the IC_{50} (treated cells) for 24 h and extracted RNA for RT-qPCR and transcriptome analysis.

This assay was performed in duplicate, and, as a control, an equivalent volume of DMSO alone was added to cells (nontreated cells). Isolation and purification of total RNA were performed with a commercial kit according to the manufacturer's instructions (RNeasy mini kit, Qiagen, Hilden, Germany). The RNA concentration and purity were evaluated by spectrophotometry (NanoDrop™, ND-8000, Thermo Scientific, Waltham, MA, USA) whereas the RNA integrity was assessed by the Bioanalyzer 2100 and the Agilent RNA 6000 Nano Series kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

cDNA synthesis was carried out using 1 μg of total RNA treated with DNAse I (Life Technologies, Rockville, MD, USA), 200 U of *SuperScript* III Reverse Transcriptase enzyme (Life Technologies), 4 μL of SuperScript First-Strand Buffer 5X, 1 μL each of 10 mM dNTP (Life Technologies), 1 μL of Oligo-$(dT)_{18}$ (500 ng/μL) (Life Technologies), 1 μL of random hexamers (100 ng/μL) (Life Technologies), and 1 μL of 0.1 M DTT (Life Technologies). Reverse transcription was performed at 50°C for 60 minutes, and the reactions were inactivated at 70°C for 15 min. qPCR amplification for *VEGFR2*, *PDGFR-β*, and *KIT* as well as for reference genes (*GAPDH*, *HPRT*, *RPS19*, *RPS5*, and *RPL8)* was performed using QuantStudio 12k Flex Thermal Cycler equipment (Applied Biosystems; Foster City, CA, USA). The reactions were performed in duplicate in 384-well-plates using Power SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 1 μL of cDNA, and 0.3 μM of each primer. Relative gene quantification was calculated by the $2^{-\Delta\Delta CT}$ method (22).

Microarray

We generated a global gene expression profile (microarray) using GeneChip® Canine Gene 1.0 ST Arrays (Affymetrix, CA, EUA). cDNA labeling, hybridization, and detection were performed according to the manufacturer's instructions. Then, the chips were scanned in a Scanner 3000 7G series (Affymetrix, Santa Clara, CA, EUA). Affymetrix CEL files were downloaded and processed with Applied Biosystem™ Transcriptome Analysis Console (TAC, Affymetrix) software. The criteria for selecting differentially expressed genes (DEGs) were a 2.0-fold change cutoff and a $P < 0.05$. Hierarchical clustering heatmaps and Venn diagrams were generated using TAC software.

Gene Ontology (GO)

The DEGs between the groups were subjected to a GO enrichment analysis using Enrichr (https://amp.pharm.mssm.edu/Enrichr/). REVIGO (http://revigo.irb.hr/) was used to organize and visualize the enriched GO terms obtained from Enrichr. GO analysis was focused on two major categories: biological process and molecular function.

Protein-Protein Interaction (PPI) Networks

The upregulated and downregulated DEGs were independently submitted to the online Search Tool for the Retrieval of Interacting Genes—STRING (https://string-db.org/) to generate PPI networks. We considered only STRING interactions with high confidence (0.700), and active interactions were defined as databases, coexpression, neighborhood, and cooccurrence. To simplify the network, we hid the disconnected nodes.

Transcriptomic Analysis of Primary Canine Prostate Tumors

To evaluate the expression profile of PC1 and PC2 in primary tumors we downloaded the RNAseq data from GSE122916 study available at GEO (Gene Expression Omnibus) database (23). We then performed differential expression analysis using the NetworkAnalyst 3.0 software (24–26). Nine malignant were compared with nine non-malignant prostate tissues (biopsy) and two malignant were independently compared to five non-malignant prostate tissues (fine-needle-aspiration). Differentially expressed genes of prostate cancer were identified using EdgeR (27). The HTCounts were normalized using a trimmed mean of M-values (TMM). Genes were filtered out when presenting low abundance (less than four counts) and stable expression across conditions. We selected the genes with $|\log FC| > 1$ and adjusted $p < 0.05$ regulated in the same direction in both biopsy and fine-needle-aspiration tumor samples.

We used the Set Comparison Appyter v0.0.6 online tool (https://appyters.maayanlab.cloud/#/CompareSets) to determine whether the overlaps between PC1/PC2 with primary tumors are significant.

Statistical Analysis

Comparisons among the different doses in the treatment groups were made using the Tukey–Kramer test, and statistical significance was set at $p < 0.05$. Statistical analysis was performed, and graphs were generated using Graph Pad Prism 8.0 and Microsoft® Excel 2007.

RESULTS

Measurement of Protein Expression by IF

PC1 and PC2 cells showed VEGFR2 and PDGFR-β cytoplasmic expression. However, c-KIT protein was absent in both cell lines (**Figure 1**).

MTT Assay and IC50 Detection

TP reduced PC1 cell viability in a dose-dependent manner. After 24 h, 3, 6, 9, and 12 μM TP reduced the viability of PC1 cells to 91, 84, 59, and 0%, respectively; after 48 h, the cell viability was reduced to 93, 80, 50, and 2%; and after 72 h, the cell viability was reduced to 89, 73, 54, and 3% (**Figure 2**). Following treatment with TP for 24 (**Figure 2A**), 48 (**Figure 2B**), and 72 h (**Figure 2C**), a significant ($p < 0.05$) decrease in the number of viable cells

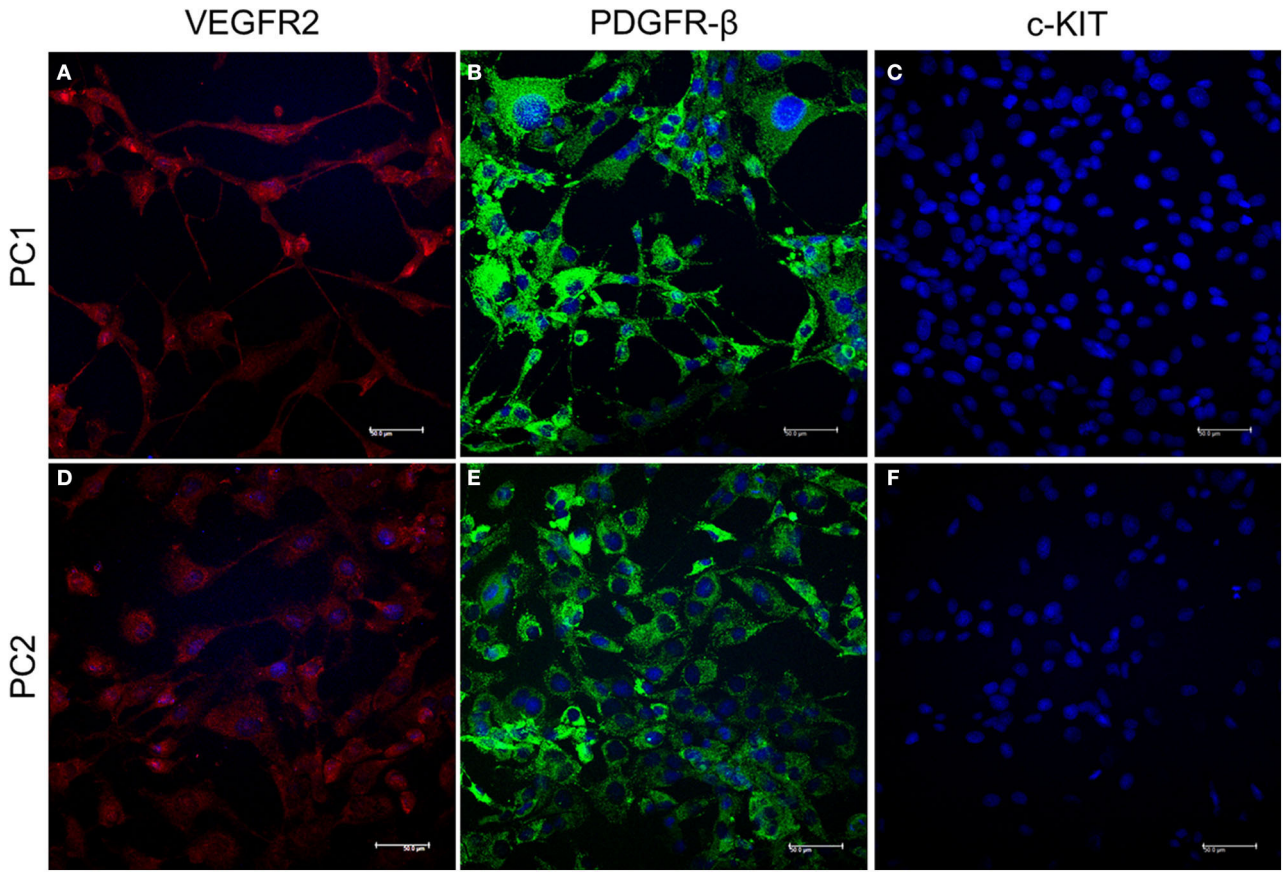

FIGURE 1 | Detection of VEGFR2, PDGFR-β, and c-KIT protein expression by IF. Cytoplasmic expression of VEGFR2 is indicated by red fluorescence **(A,B)** and PDGFR-β by green fluorescence **(C,D)** in PC1 and PC2 cells, respectively. No c-KIT protein expression **(E,F)** was observed in either cell line. The nuclei were counterstained with DAPI (blue).

was observed in PC1 cells compared to that of the control cells (DMSO). The IC_{50} values of TP in PC1 cells were 9.28, 9.13, and 8.95 μM at 24 (**Figure 2D**), 48 (**Figure 2E**), and 72 (**Figure 2F**) h, respectively.

On the other hand, TP did not reduce PC2 cell viability in a dose-dependent manner. After 24 h (**Figure 2G**), 3, 6, 9, and 12 μM TP reduced the viability of PC2 cells to 82, 90, 84, and 84%, respectively, compared to that of the control cells; after 48 h (**Figure 2H**), the cell viability was reduced to 70, 85, 72, and 65%; and after 72 h (**Figure 2I**), the cell viability was reduced to 86, 73, 78, and 74%. Thus, we considered the PC2 cell line to be more resistant to TP than the PC1 cell line. Lower toceranib phosphate doses concentrations (125, 250, 500nM, 1, 1.5 μM were tested in both cells (PC1 and PC2) with no cell viability alterations (data not shown).

qPCR

VEGFR2 transcript levels were statistically lower in treated PC1 cells (mean = 1.017) than in untreated cells (mean = 0.849; $p < 0.05$; **Figure 3A**); however, no significant differences were observed in *VEGFR2* gene expression between the untreated and treated PC2 cells (**Figure 3B**). There was a significant upregulation of *PDGFR-β* expression in treated PC1 cells (mean = 2.056) comparing to untreated PC1 cells (mean = 1.007) (**Figure 3C**). We observed downregulation of *PDGFR-β* expression in treated PC2 cells (mean = 0.549) compared with untreated PC2 cells (mean = 1.001; **Figure 3D**). However, treatment with TP for 24 h did not alter transcript *KIT* levels between untreated and treated cells for either PC1 or PC2 cells (**Figures 3E,F**, respectively).

Transcriptome Analysis

A total of 390 DEGs ($p < 0.05$) were observed between untreated and TP-treated PC1 cells (233 upregulated genes and 157 downregulated genes in treated cells). However, 122 DEGs were not annotated with gene names or gene symbols in TAC software, and 5 were microRNAs.

A total of 82 DEGs were observed ($p < 0.05$) in treated PC2 cells compared to control cells, including 42 upregulated genes and 40 downregulated genes. However, the DEG list showed that out of the 82, 42 genes had neither a gene name nor a symbol, and 1 gene was a microRNA. A heatmap of these DEGs is shown in

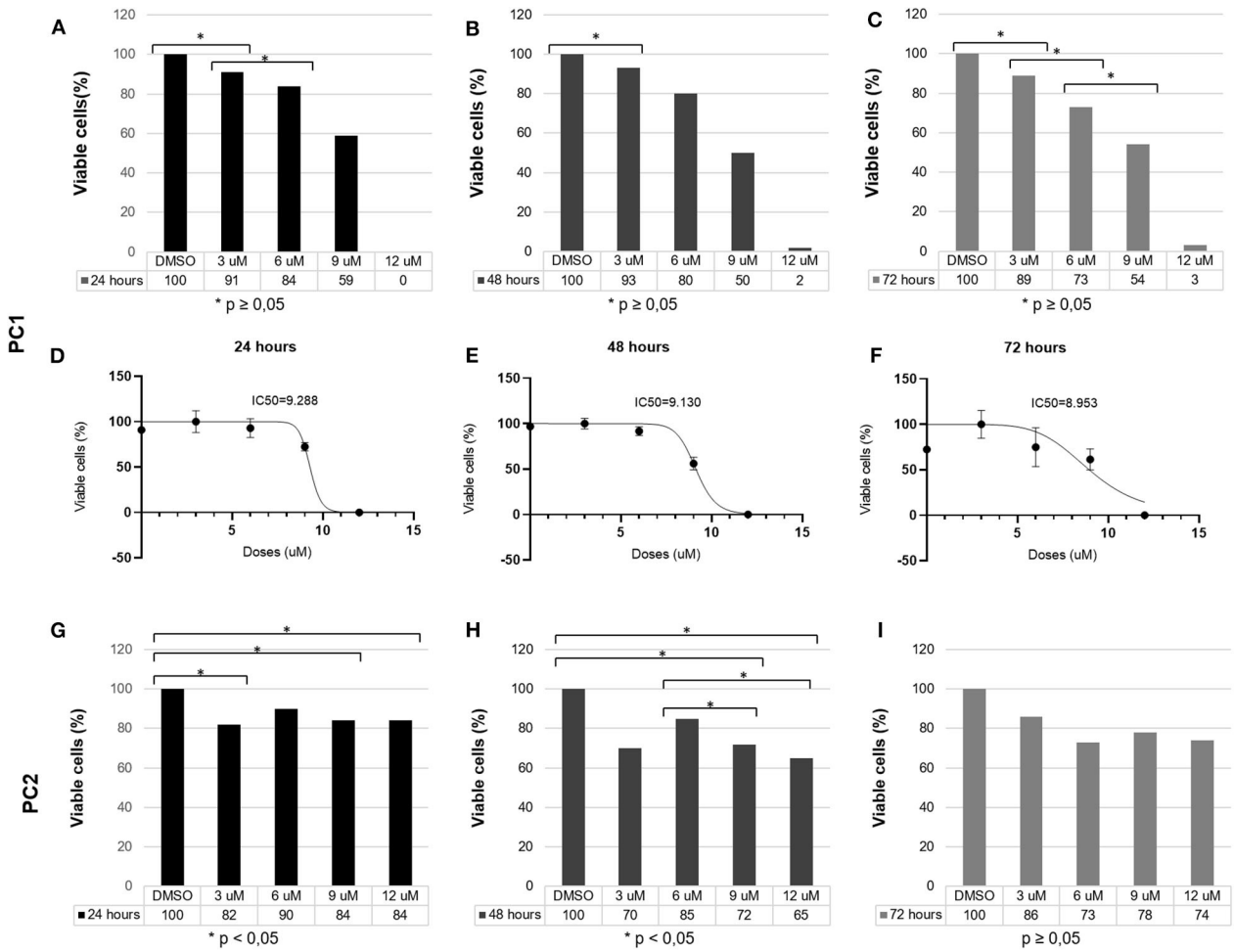

FIGURE 2 | PC1 and PC2 cells were cultured with various concentrations of TP (3, 6, 9, and 12 μM), and cell viability was assessed using an MTT assay. PC1 cells were treated with TP for **(A)** 24, **(B)** 48, and **(C)** 72 h. The IC_{50} values of TP after **(D)** 24, **(E)** 48, and **(F)** 72 h of incubation in PC1 cells. PC2 cells were treated with TP for **(G)** 24, **(H)** 48, and **(I)** 72 h. A value of $p < 0.05$ was considered statistically significant. The symbol * corresponds of *p* value.

Figure 4A, and DEGs were clustered, which can differentiate the treated and untreated PC1 and PC2 cells.

A Venn diagram (**Figure 4B**) was used to compare the DEGs between the PC1 (treated and untreated) and PC2 (treated and untreated) cells. From a total of 525 DEGs, only 17 common DEGs were screened out. Among these genes, *PDGFR-A* was altered in TP-treated PC1 and PC2 cells, but it was upregulated in PC1 cells and downregulated in PC2 cells. Treated PC1 cells also showed upregulation of *PDGFR-β* and *PDGF-D*. There are summarized lists of all the upregulated and downregulated genes in both treated PC1 cells (**Supplementary Tables 1, 2**, respectively) and treated PC2 cells (**Supplementary Tables 3, 4**, respectively).

GO Analysis

We analyzed GO data associated with DEGs in untreated and treated PC1 cells, and we observed 181 enriched genes that were significantly upregulated ($p < 0.05$) and 82 that were significantly downregulated ($p < 0.05$). Redundant GO terms with no statistically significant differences were removed using REVIGO. The analysis revealed that among the 167 biological processes (**Figure 5A**) associated with the upregulated genes in the TP-treated cells, regulation of the PDGFR and PDGFR-β signaling pathway, regulation of the endothelial cell apoptotic process, and negative regulation of vasculature development and morphogenesis of the epithelium were included. A total of 117 biological processes (**Figure 5C**) were associated with the downregulated genes, including regulation of the cell cycle, negative regulation of cell death, sprouting angiogenesis, and DNA synthesis involved in DNA repair. These data suggest an important role for TP in both the PDGF pathway and mechanisms related to angiogenesis and cell growth in PC1 cells. Thirty-one molecular functions were associated with upregulated genes (**Figure 5B**), and 21 were associated with downregulated genes (**Figure 5D**).

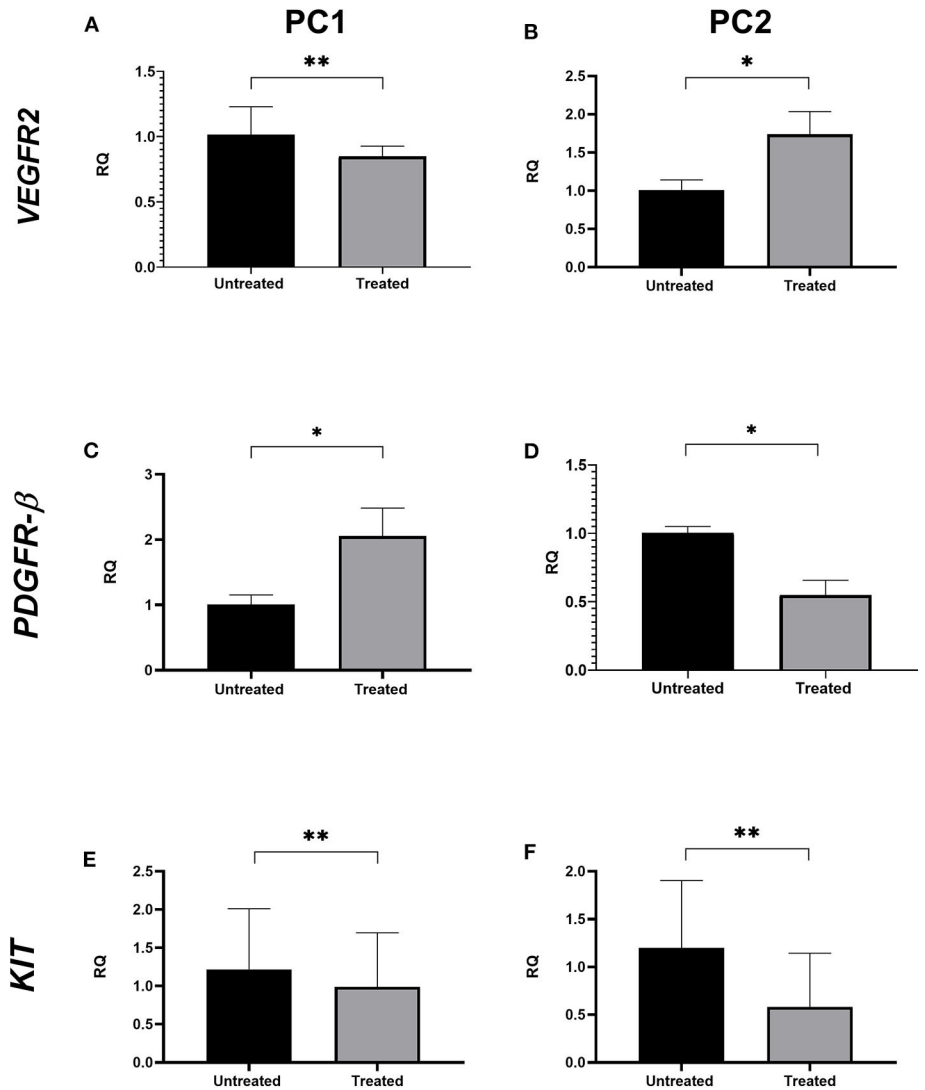

FIGURE 3 | The effect of TP on *VEGFR2*, *PDGFR-β*, and *KIT* transcript levels in PC1 and PC2 cells. Relative quantification levels of *VEGFR2* **(A,B)**, *PDGFR-β* **(C,D)** and *KIT* **(E,F)** were measured by RT-qPCR in PC1 and PC2 cells treated with their respective IC50 value of TP or DMSO. $^{*}p < 0.05$, $^{**}p \geq 0.05$.

When comparing untreated and treated PC2 cells, we observed 18 enriched genes upregulated in treated PC2 cells, from which we identified 139 biological processes (**Figure 6A**) and 19 molecular functions (**Figure 6B**), including phosphatidylinositol 3-kinase (PI3-K) signaling, protein serine/threonine kinase activity, mitotic cell cycle regulation, and cell migration. Among the downregulated genes, 110 biological processes (**Figure 6C**) and 19 molecular functions (**Figure 6D**) were summarized and observed to be related to PDGF/PDGFR binding and signaling and regulation of cell motility and proliferation.

PPI Network

After removing any disconnected nodes in the network, PPI analysis was constructed using upregulated and downregulated genes for each cell line (PC1 and PC2). The analysis comprises a highly interactive PPI network of 170 nodes and 57 interactions in the upregulated genes in PC1 cells (**Figure 7A**), 77 nodes and 51 interactions in the downregulated genes in PC1 cells (**Figure 7B**), 18 nodes and 3 interactions in the upregulated genes in PC2 cells (**Figure 7C**) and 20 nodes and 1 interaction in the downregulated genes in PC2 cells (**Figure 7D**).

Cell Lines Gene Expression Profile Validation in Primary Tumors

We found 1,412 up and 668 downregulated genes shared by the primary tumors from biopsy and needle-aspiration (23). By comparing the gene expression profile of primary tumors with the treated cell lines we observed that PC1 has 59 overlapping genes while PC2 has 11 overlapping genes (**Figure 8A**). Fisher

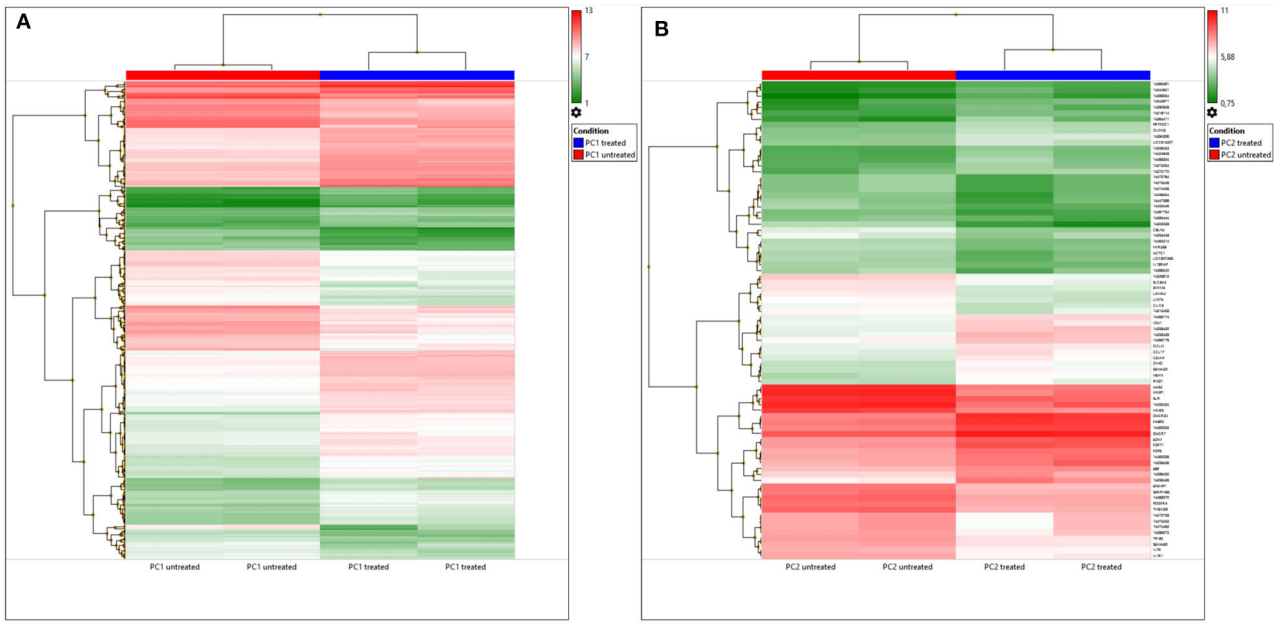

FIGURE 4 | DEGs in untreated and TP-treated PC1 and PC2 cells based on microarray analysis. **(A)** Cluster heatmap: red represents upregulation whereas green indicates downregulation of gene expression relative to that in untreated cells. Each row represents a gene, and each column represents a sample. Each sample was analyzed in duplicate. **(B)** Venn diagram: overlapping sections show common genes deregulated by TP in PC1 (yellow) and PC2 (blue) cells compared to the respective untreated cells.

exact test demonstrated the overlapping of PC1 with the primary tumors the most significant (**Figure 8B**). Two genes were differentially expressed in all conditions (*FABP3* and *SERPINB2*). Interestingly, 25 of 59 genes in PC1 and 6 of 11 in PC2 showed gene expression in opposite direction to that found in tumor tissues (**Supplementary Table 5**).

DISCUSSION

In the present study, we observed VEGFR2 and PDGFR-β protein expression in two primary canine PC cell lines; these receptors could represent suitable targets for therapy with RTK drugs, including TP. Deregulation of RTKs is frequently associated with different cancers and metastases (28–30). Previously, our research group investigated c-KIT expression in canine PC tissue samples and in PC1 and PC2 cells (31). In that study, we identified heterogeneous c-KIT gene and protein expression among PC tissue samples, and no KIT expression was observed in metastatic samples. In addition, we assessed KIT protein expression by western blot, and we did not find KIT expression in either PC1 or PC2 cells, confirming our IF results.

Regarding PDGFR-β and VEGFR2 expression in canine PC, we previously observed increased VEGFR2 protein expression in formalin-fixed paraffin-embedded canine PCs compared to normal prostate cells, suggesting an interesting target for TP (data not published). In humans, VEGFR2 inhibition is associated with reduced osteolysis and growth of prostate carcinoma bone metastasis (32). To the best of our knowledge, no previous study has investigated PDGFR-β in canine PC.

Although we demonstrated the absence of c-KIT protein expression in both canine PC cell lines (PC1 and PC2), we investigated *c-KIT* gene expression after TP treatment, and no significant difference was observed between the transcript levels in treated and untreated cells for either cell line. Although c-KIT protein overexpression may be correlated with more aggressive tumors, higher invasion capacity and tumor recurrence, heterogeneity and/or the absence of c-KIT protein expression has been demonstrated in the epithelial cells of human PC (31, 33–35). Moreover, the stromal microenvironment has an important role in PC biology, and increased c-KIT expression in stromal cells may affect PC development (36). In 2D cell culture, we could not verify the role of TP in the stroma; however, a previous study from our group revealed c-KIT-positive stromal cells in formalin-fixed paraffin-embedded canine PC samples (31).

PC1 and PC2 were positive for PDGFR and VEGFR receptors, but the antitumoral effect of TP was different in the cell lines. After treatment with TP, PC1 cell viability was reduced significantly ($p < 0.05$) in a dose-dependent manner, indicating drug sensitivity. In contrast, the viability of PC2 cells was not significantly altered when subjected to the same TP concentrations. These data reinforce the intertumoral heterogeneity and the importance of PC treatment planning (37). Even at higher TP concentrations (data not shown), the PC2 cell line did not show significant reductions in cell viability. In

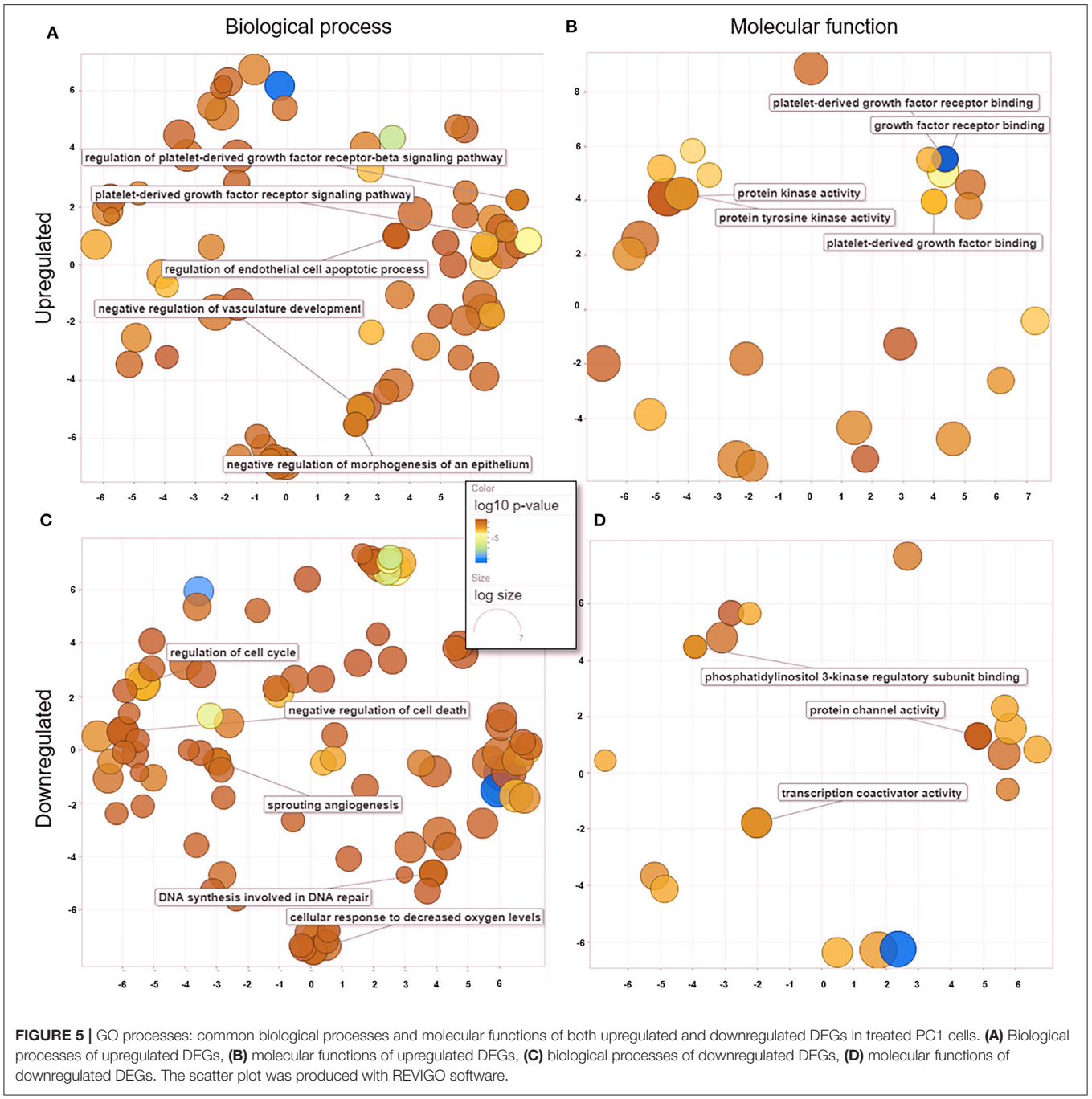

FIGURE 5 | GO processes: common biological processes and molecular functions of both upregulated and downregulated DEGs in treated PC1 cells. **(A)** Biological processes of upregulated DEGs, **(B)** molecular functions of upregulated DEGs, **(C)** biological processes of downregulated DEGs, **(D)** molecular functions of downregulated DEGs. The scatter plot was produced with REVIGO software.

addition, the use of higher concentrations to elicit an effective response would be beyond the maximum safe dosage for *in vivo* use (38). Thus, we considered the PC2 cell line resistant to TP treatment. The global gene expression profile identified DEGs that can help to explain the resistance mechanism. We found only 17 genes in common between PC1 and PC2 cells upon comparison of the respective untreated and treated cells, indicating a difference between them after treatment with TP. Considering the differences in global gene analyses between TP-treated and untreated PC1 and PC2 cell lines, we found that *PDGF-D*, *PDGFR-α*, and *PDGFR-β* genes, which are involved in the PDGFR pathway, were upregulated in PC1 cells. We confirmed the upregulated expression of *PDGFR-β* in PC1 cells after treatment by qPCR. Interestingly, both cell lines presented *PDGFR-β* deregulation after TP treatment; however, the PC2 cell line showed downregulation of *PDGFR-β*. The increases in the release of RTK ligands via autocrine tumor cell production and in RTK expression are potential mechanisms of

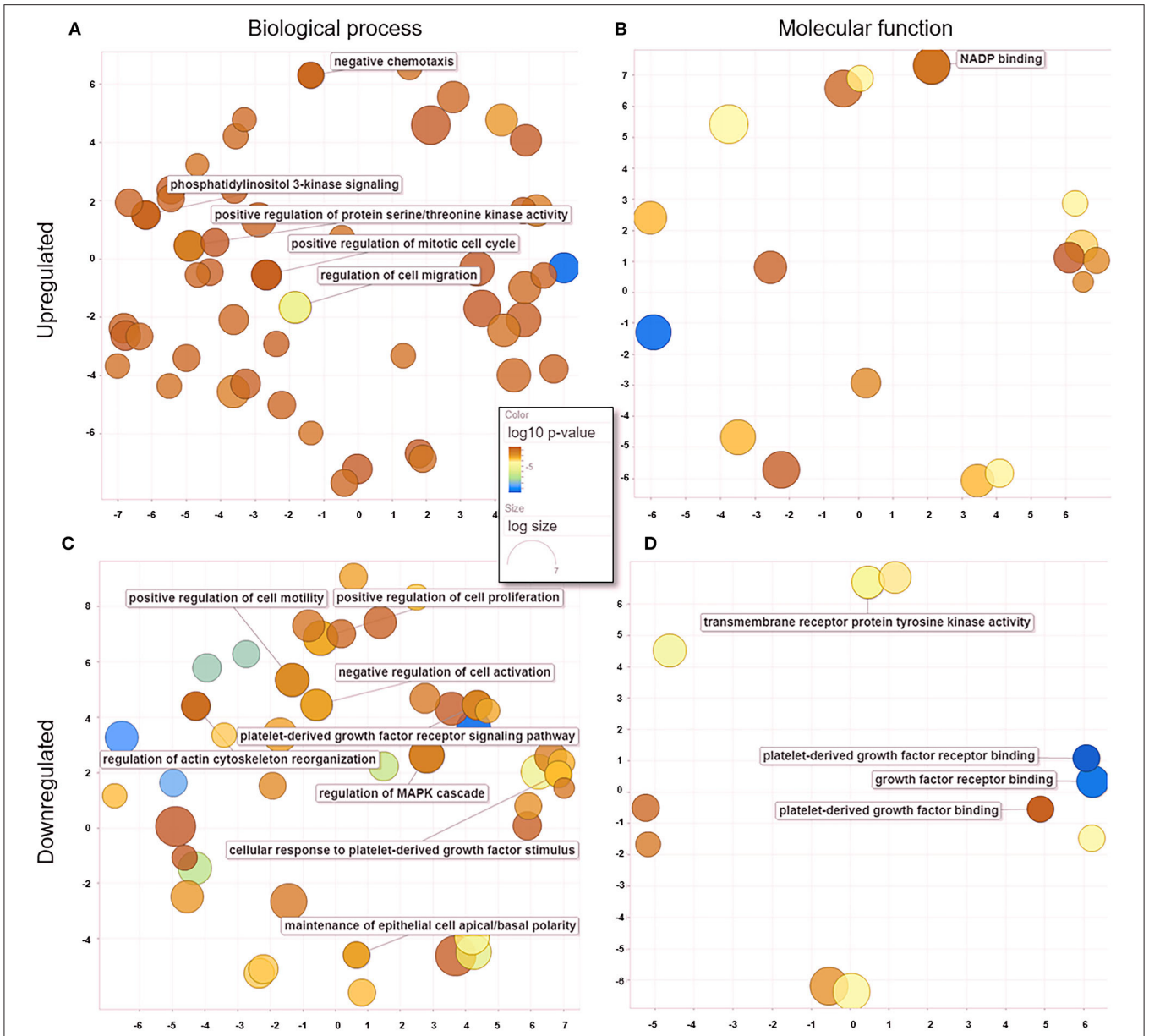

FIGURE 6 | GO processes: common biological processes and molecular functions of both upregulated and downregulated DEGs in treated PC2 cells. **(A)** Biological processes of upregulated DEGs, **(B)** molecular functions of upregulated DEGs, **(C)** biological processes of downregulated DEGs, **(D)** molecular functions of downregulated DEGs. The scatter plot was produced with REVIGO software.

acquired resistance to tyrosine kinase inhibitors (39–41). Thus, the increased *PDGFR-β* expression in PC1 cells could be a response to the direct inhibition of this receptor as an attempt to activate the *PDGFR-β* pathway in the presence of an inhibitor (42). In addition, we observed an increase in *PDGF-D* expression, which seems to activate *PDGFR-β* without the involvement of its classical ligand, *PGDF-B*. Additionally, increased transcription of these genes has been related to tumor aggressiveness and epithelial-mesenchymal transition in multiple cancers, including PC (43–45). On the other hand, PC2 cells showed *PDGFR-β* downregulation, indicating a direct blockage of this pathway. However, associating this molecular feature with the fact that this tumor cell line is resistant to TP, PC2 cells probably did not present PDGFR-*β* as a driving pathway to tumor proliferation.

PC1 and PC2 cell cultures were previously established and characterized by our research group, and both cells were positive for pan-cytokeratin and prostatic specific antigen (PSA) and negative for androgen receptor (20). However, PC1 cells were derived from a nonmetastatic tumor, whereas PC2 cells were derived from a metastatic tumor (20); this expected elevation in

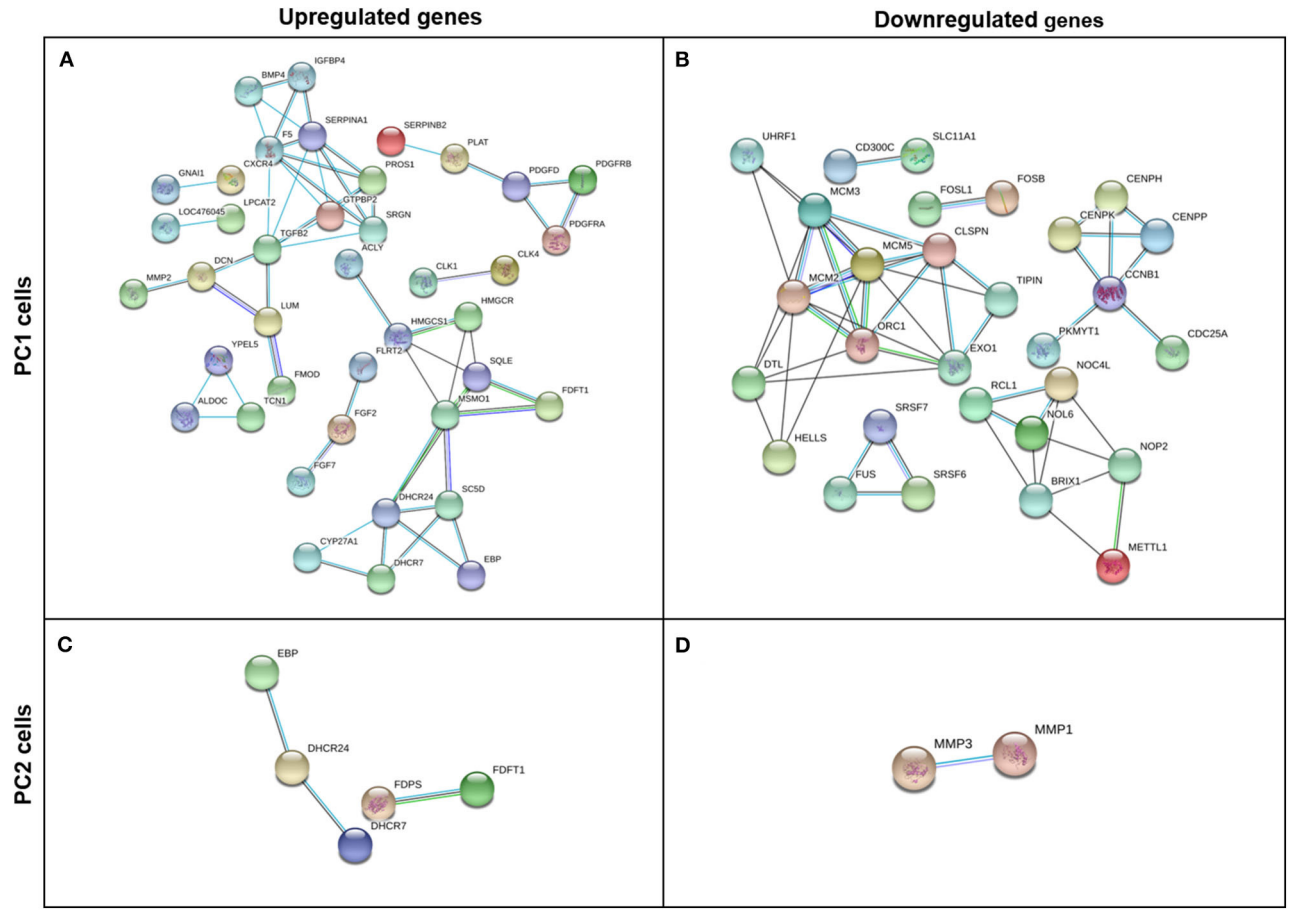

FIGURE 7 | Gene interaction network of upregulated and downregulated DEGs in TP-treated PC1 (**A,B**, respectively) and PC2 cells (**C,D**, respectively) identified by STRING software using a high confidence interaction score (0.700). Circles represent genes, and lines represent protein-protein associations.

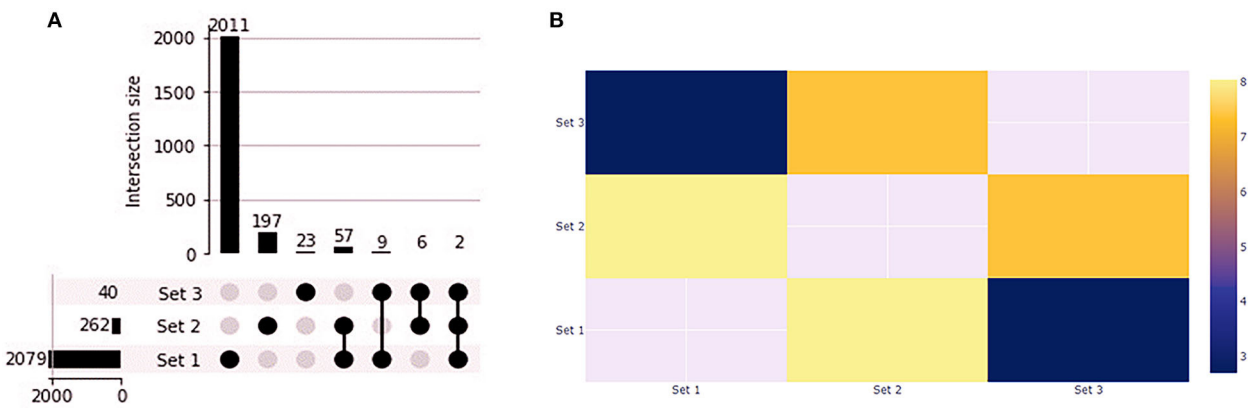

FIGURE 8 | Gene expression profile of PC1 and PC2 compared to canine primary prostate tumors. **(A)** Upset plot displaying the set intersections of differentially expressed genes in the PC1 and PC2 cell lines compared to Thiemeyer et al. (23) RNA-seq dataset of canine primary prostate tumors (GSE122916). **(B)** Heatmap of Fisher's Exact test results. The –log(*p*-values) is shown in the heatmap. Each axis displays which sets are being compared and sets that cannot be compared are given a value of None. Set 1: primary tumors; Set 2: PC1; Set 3: PC2.

number of gene alterations in this cell could have contributed to its resistance to TP.

In addition, we observed an increase in *VEGFR2* expression in TP-treated PC2 cells by qPCR; however, no difference was observed in the microarray analysis. An explanation for that could be the higher sensitivity of qPCR to detect small differences in expression that are undetectable by microarrays due to the flexible number of cycles in qPCR (46).

DEGs involved in the negative regulation of vasculature development and blood vessel endothelial cell proliferation (which are related to a decrease in sprouting angiogenesis) were identified in treated PC1 cells. Moreover, TP significantly affected genes involved in cell growth and activation and DNA repair by different mechanisms, for example, decreases in the mitotic cell cycle and cellular response to DNA damage stimuli. As would be expected of an inhibitor of VEGFR, PDGFR, and c-KIT kinase activity, TP can modify the expression of genes related to antiangiogenic activity and tumor growth inhibition via direct and indirect mechanisms (11, 14).

Another explanation for the resistance of PC2 cells to TP could the upregulation of genes from the PI3K pathway, which positively regulates the mitotic cell cycle, protein serine/threonine kinase activity, and cell migration. Therefore, treated PC2 cells may become resistant by activating parallel signaling pathways and mechanisms.

Dysregulated and/or elevated activation of the PI3K signaling network is one of the most common events in the oncogenesis of different cancers, including PC (47–49). In human PC, inhibition of PI3K signaling has been demonstrated to suppress invasion and induce apoptosis (50). Consistent with the main mechanism of action of TP, our results showed downregulation of genes involved in transmembrane RTK activity and PDGFR binding. The role of negative feedback in growth factor signaling pathways is to generate stability, limiting the duration and extent of signaling and preventing potentially harmful overactivation of signaling (51). However, we observed overexpression of endothelin-1 (*ET-1*), which can be a compensatory cellular mechanism that acts alone or in cooperation with other tyrosine kinase growth factors, such as PDGFR and VEGFR, to activate RTKs, leading to cell proliferation and angiogenesis (52–55). Signaling pathways require both positive and negative feedback loops to adjust their cellular response; these feedback loops act through different genes, mechanisms, and stimuli (56). Therefore, loss of homeostasis of this negative feedback can lead to oncogene activation and uncontrolled growth that can lead to cancer (57).

Migration and invasion are fundamental characteristics of cancer progression and metastasis that consequently reduce both the efficacy of therapeutics and prognosis. This study identified the overexpression of genes involved in these mechanisms, such as Rho family GTPase 1 (*RND1*) and Semaphorin 3D (*SEMA3D*). RND1 protein is concentrated at adherens junctions in both confluent fibroblasts and epithelial cells, and overexpression causes loss of cell-matrix adhesion, leading to cell rounding, which facilitates invasion (58). SEMA3D, a membrane-bound protein, is involved in cell-cell communication and plays an important role in many pathophysiological processes, such as cancer development, and its overexpression has been related to increased cell invasiveness (59, 60).

On the other hand, the expression of other genes involved in metastatic processes, such as matrix metalloproteinase 1 (*MMP1*) and 3 (*MMP3*), was downregulated. MMPs are capable of cleaving extracellular matrix protein substrates and have been identified as key factors involved in carcinogenesis and metastasis (61, 62). Among the members of the MMP family, MMP1 degrades fibrillary collagen and has been associated with invasion and poor prognosis (63). The *MMP3* gene has also been implicated as a contributor to cancer progression and reported to be responsible for inducing epithelial-mesenchymal transition and increasing cell spreading (64, 65). Our PPI network indicated an interaction between MMP1 and MMP3, which suggests a relationship between these two genes and the importance of their role in canine PC.

Interestingly, we observed downregulation of epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*), which encodes a member of the fibulin family of secreted glycoproteins, and its role in carcinogenesis is controversial due to its suppressive and oncogenic activities (66). In the serum and urine of PC patients, decreased EFEMP1 expression was reported, suggesting that this protein participates in the carcinogenesis of human PC (67).

Besides, we validated our results comparing our DEGs found in treated PC cell lines with gene expression profile of primary tumors (23). Interestingly, we found gene expression in opposite direction to that found in tumor tissues and these could be interesting genes to understand the modulation of toceranib phosphate therapy in these canine prostate carcinomas. Therefore, we found *FABP3* and *SERPINB2* as common genes in our treated samples and primary prostate tumor in dogs (**Supplementary Table 5**), with increase of *FABP3* in all our comparative samples, but interestingly *SERPINB2* was downregulated only in PC2, which was resistant to TP. *FABP3* is related to fatty acid transport cell growth and signaling, and gene transcription (68, 69). However, its function in tumor progression still remains controversial and is described as a tumor suppressor gene in breast cancer, and associated with tumor progression in gastric carcinoma and non-small cell lung cancer (69, 70). *SERPINB2* was upregulated in treated PC1 cells as in primary canine prostate tumor, but downregulated in treated PC2 cells. *SERPINB2* overexpression has been linked with inhibition of invasion and cell migration, and prolonged survival in different cancers (71, 72). The differences between PC1 and PC2 cells can explain the differences in therapy response and *SERPINB2* could be studied in the future s as one candidate marker for TP resistance in canine prostate carcinoma.

In summary, we demonstrated that two different primary canine PC cell lines had similar patterns of RTK protein expression but had different responses to TK inhibition upon treatment with TP. Based on a global gene comparative analysis, this study revealed that TP could differentially affect genes involved in the progression of canine PC. These DEGs are important for investigating other mechanisms involved in RTK therapy and are useful targets for treating canine PC. Moreover,

DEGs associated with mechanisms of cancer drug resistance were identified, including PI3K pathway, raising concerns about the development of drug resistance. Further functional studies on PI3K pathways should be carried out, that could provide useful information on possible new candidate multidrug resistance genes in canine prostate carcinoma and discovery of new drug resistance targets. The identification of the common differentially expressed genes among prostate tumor and PC1 and PC2 cells can provide further insight into the discovery of new biomarkers and genes related to resistance in canine prostate carcinoma. These findings support the biological response in some cases of canine PC and the need for more personalized cancer treatments.

DATA AVAILABILITY STATEMENT

The original Microarray dataset presented in the study are publicly available in the ArrayExpress database here: http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9716.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of the São Paulo State University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

PK executed some of the experiments, analyzed the data, prepared the figures and wrote the manuscript. PL, AL-F, and MC performed the experiments and collected data. SC performed the *in silico* comparative analysis from PC cell lines and data from canine PC. FD and RC analyzed and interpreted the data. CF-A conceived the project and helped with data collection and interpretation. RL-A conceived the project and participated in its design and coordination. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.561212/full#supplementary-material

REFERENCES

1. LeRoy BE, Northrup N. Prostate cancer in dogs: comparative and clinical aspects. *Vet J.* (2009) 180:149–62. doi: 10.1016/j.tvjl.2008.07.012
2. Lai C-L, van den Ham R, van Leenders G, van der Lugt J, Mol JA, Teske E. Histopathological and immunohistochemical characterization of canine prostate cancer. *Prostate.* (2008)
3. Smith J. Canine prostatic disease: a review of anatomy, pathology, diagnosis, and treatment. *Theriogenology.* (2008) 70:375–83. doi: 10.1016/j.theriogenology.2008.04.039
4. Cornell KK, Bostwick DG, Cooley DM, Hall G, Harvey HJ, Hendrick MJ, et al. Clinical and pathologic aspects of spontaneous canine prostate carcinoma: a retrospective analysis of 76 cases. *Prostate.* (2000) 45:173–83. doi: 10.1002/1097-0045(20001001)45:2<173::AID-PROS12>3.0.CO;2-R
5. Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S, Contag CH. Animal models of bone metastasis. *Cancer.* (2003) 97:748–57. doi: 10.1002/cncr.11150
6. Bell FW, Klausner JS, Hayden DW, Feeney DA, Johnston SD. Clinical and pathologic features of prostatic adenocarcinoma in sexually intact and castrated dogs: 31 cases (1970-1987). *J Am Vet Med Assoc.* (1991) 199:1623-30.
7. Ravicini S, Baines SJ, Taylor A, Amores-Fuster I, Mason SL, Treggiari E. Outcome and prognostic factors in medically treated canine prostatic carcinomas: A multi-institutional study. *Vet Comp Oncol.* (2018) 16:450–8. doi: 10.1111/vco.12400
8. Johnston S, Kamolpatana K, Root-Kustritz M, Johnston G. Prostatic disorders in the dog. *Anim Reprod Sci.* (2000) 60-61:405–15. doi: 10.1016/S0378-4320(00)00101-9
9. Christensen BW. Canine Prostate Disease. *Vet Clin North Am Small Anim Pract.* (2018) 48:701–19. doi: 10.1016/j.cvsm.2018.02.012
10. Hojjat-Farsangi M. Small-molecule inhibitors of the receptor tyrosine kinases: promising tools for targeted cancer therapies. *Int J Mol Sci.* (2014) 15:13768–801. doi: 10.3390/ijms150813768
11. London C a, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin Cancer Res.* (2003) 9:2755–68. Available online at: https://clincancerres.aacrjournals.org/content/9/7/2755.long
12. Liao AT. Inhibition of constitutively active forms of mutant kit by multitargeted indolinone tyrosine kinase inhibitors. *Blood.* (2002) 100:585–93. doi: 10.1182/blood-2001-12-0350
13. London C. Kinase inhibitors in cancer therapy. *Vet Comp Oncol.* (2004) 2:177–93. doi: 10.1111/j.1476-5810.2004.00059.x
14. Musser ML, Taikowski KL, Johannes CM, Bergman PJ. Retrospective evaluation of toceranib phosphate (Palladia®) use in the treatment of inoperable, metastatic, or recurrent canine pheochromocytomas: 5 dogs (2014-2017). *BMC Vet Res.* (2018) 14:272. doi: 10.1186/s12917-018-1597-7
15. Pellin MA, Wouda RM, Robinson K, Tsimbas K, Kurzman ID, Biller BJ, et al. Safety evaluation of combination doxorubicin and toceranib phosphate (Palladia®) in tumour bearing dogs: a phase I dose-finding study. *Vet Comp Oncol.* (2017) 15:919–31. doi: 10.1111/vco.12232
16. London C a. Tyrosine Kinase Inhibitors in Veterinary Medicine. *Top Companion Anim Med.* (2009) 24:106–12. doi: 10.1053/j.tcam.2009.02.002
17. Pan X, Tsimbas K, Kurzman ID, Vail DM. Safety evaluation of combination CCNU and continuous toceranib phosphate (Palladia®) in tumour-bearing dogs: a phase I dose-finding study. *Vet Comp Oncol.* (2014) 14:202–9. doi: 10.1111/vco.12091
18. London C, Mathie T, Stingle N, Clifford C, Haney S, Klein MK, et al. Preliminary evidence for biologic activity of toceranib phosphate (Palladia®) in solid tumours. *Vet Comp Oncol.* (2012) 10:194–205. doi: 10.1111/j.1476-5829.2011.00275.x

19. Elliott JW, Swinbourne F, Parry A, Baines L. Successful treatment of a metastatic, gastrointestinal stromal tumour in a dog with toceranib phosphate (Palladia). *J Small Anim Pract.* (2017) 58:416–8. doi: 10.1111/jsap.12657
20. Costa CD, Justo AA, Kobayashi PE, Story MM, Palmieri C, Laufer Amorim R, et al. Characterization of OCT3/4, Nestin, NANOG, CD44 and CD24 as stem cell markers in canine prostate cancer. *Int J Biochem Cell Biol.* (2019) 108:21–8. doi: 10.1016/j.biocel.2019.01.002
21. Anwar IB, Santoso A, Saputra E, Ismail R, Jamari J, Van der Heide E. Human bone marrow-derived mesenchymal cell reactions to 316L stainless steel: an *in vitro* study on cell viability and interleukin-6 expression. *Adv Pharm Bull.* (2017) 7:335–8. doi: 10.15171/apb.2017.040
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
23. Thiemeyer H, Taher L, Schille JT, Harder L, Hungerbuehler SO, Mischke R, et al. Suitability of ultrasound-guided fine-needle aspiration biopsy for transcriptome sequencing of the canine prostate. *Sci Rep.* (2019) 9:13216. doi: 10.1038/s41598-019-49271-1
24. Zhou G, Soufan O, Ewald J, Hancock REW, Basu N, Xia J. NetworkAnalyst 3.0: a visual analytics platform for comprehensive gene expression profiling and meta-analysis. *Nucleic Acids Res.* (2019) 47:W234–W241. doi: 10.1093/nar/gkz240
25. Xia J, Gill EE, Hancock REW. NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data. *Nat Protoc.* (2015) 10:823–44. doi: 10.1038/nprot.2015.052
26. Xia J, Lyle NH, Mayer ML, Pena OM, Hancock REW. INVEX-a web-based tool for integrative visualization of expression data. *Bioinformatics.* (2013) 29:3232–4. doi: 10.1093/bioinformatics/btt562
27. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
28. Bellamy WT. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. *Blood.* (2001) 97:1427–34. doi: 10.1182/blood.V97.5.1427
29. Rapisarda A, Melillo G. Role of the VEGF/VEGFR axis in cancer biology and therapy. *Adv Cancer Res.* (2012) 114:237–67. doi: 10.1016/B978-0-12-386503-8.00006-5
30. Ustach CV, Huang W, Conley-LaComb MK, Lin C-Y, Che M, Abrams J, et al. A novel signaling axis of matriptase/PDGF-D/-PDGFR in human prostate cancer. *Cancer Res.* (2010) 70:9631–40. doi: 10.1158/0008-5472.CAN-10-0511
31. Fonseca-Alves CE, Kobayashi PE, Palmieri C, Laufer-Amorim R. Investigation of c-KIT and Ki67 expression in normal, preneoplastic and neoplastic canine prostate. *BMC Vet Res.* (2017) 13:380. doi: 10.1186/s12917-017-1304-0
32. Lee C, Whang YM, Campbell P, Mulcrone PL, Elefteriou F, Cho SW, et al. Dual targeting c-met and VEGFR2 in osteoblasts suppresses growth and osteolysis of prostate cancer bone metastasis. *Cancer Lett.* (2018) 414:205–13. doi: 10.1016/j.canlet.2017.11.016
33. Harris K, Foster B, Shi L, Mobley M, Elliott P, Kerr B. CTC Marker CD117/c-kit represents a prostate cancer stem-like subpopulation driving progression, migration, and TKI resistance. *bioRxiv.* (2018) 1–29. doi: 10.1101/256107
34. Wiesner C, Nabha SM, Bonfil RD, Santos EB Dos, Yamamoto H, Meng H, et al. C-Kit and its ligand stem cell factor: potential contribution to prostate cancer bone metastasis. *Neoplasia.* (2008) 10:996–1003. doi: 10.1593/neo.08618
35. Di Lorenzo G, Autorino R, D'Armiento FP, Mignogna C, De Laurentiis M, De Sio M, et al. Expression of proto-oncogene c-kit in high risk prostate cancer. *Eur J Surg Oncol.* (2004) 30:987–92. doi: 10.1016/j.ejso.2004.07.017
36. Peng Y, Chen Q, Gu M, Chen Y, Zhang M, Zhou J, et al. Human stromal cells in the peripheral zone of the prostate promote tumorigenesis of prostatic cancer stem cells through up-regulation of C-kit expression. *J Cancer.* (2015) 6:776–85. doi: 10.7150/jca.9961
37. Boutros PC, Fraser M, Harding NJ, de Borja R, Trudel D, Lalonde E, et al. Spatial genomic heterogeneity within localized, multifocal prostate cancer. *Nat Genet.* (2015) 47:736–745.
38. Halsey CHC, Gustafson DL, Rose BJ, Wolf-Ringwall A, Burnett RC, Duval DL, et al. Development of an *in vitro* model of acquired resistance to toceranib phosphate (Palladia®) in canine mast cell tumor. *BMC Vet Res.* (2014) 10:105. doi: 10.1186/1746-6148-10-105
39. Minari R, Bordi P, Tiseo M. Third-generation epidermal growth factor receptor-tyrosine kinase inhibitors in T790M-positive non-small cell lung cancer: review on emerged mechanisms of resistance. *Transl Lung Cancer Res.* (2016) 5:695–08. doi: 10.21037/tlcr.2016.12.02
40. D'Amato V, Raimondo L, Formisano L, Giuliano M, De Placido S, Rosa R, et al. Mechanisms of lapatinib resistance in HER2-driven breast cancer. *Cancer Treat Rev.* (2015) 41:877–83. doi: 10.1016/j.ctrv.2015.08.001
41. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature.* (2012) 487:505–9. doi: 10.1038/nature11249
42. Heske CM, Yeung C, Mendoza A, Baumgart JT, Edessa LD, Wan X, et al. The role of PDGFR-β activation in acquired resistance to IGF-1R blockade in preclinical models of rhabdomyosarcoma. *Transl Oncol.* (2016) 9:540–7. doi: 10.1016/j.tranon.2016.09.002
43. Wu Q, Hou X, Xia J, Qian X, Miele L, Sarkar FH, et al. Emerging roles of PDGF-D in EMT progression during tumorigenesis. *Cancer Treat Rev.* (2013) 39:640–6. doi: 10.1016/j.ctrv.2012.11.006
44. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, et al. Emerging roles of PDGF-D signaling pathway in tumor development and progression. *Biochim Biophys Acta Rev Cancer.* (2010) 1806:122–30. doi: 10.1016/j.bbcan.2010.04.003
45. Kong D, Wang Z, Sarkar SH, Li Y, Banerjee S, Saliganan A, et al. Platelet-derived growth factor-D overexpression contributes to epithelial-mesenchymal transition of PC3 prostate cancer cells. *Stem Cells.* (2008) 26:1425–35. doi: 10.1634/stemcells.2007-1076
46. Allanach K, Mengel M, Einecke G, Sis B, Hidalgo LG, Mueller T, et al. Comparing microarray versus RT-PCR assessment of renal allograft biopsies: similar performance despite different dynamic ranges. *Am J Transplant.* (2008) 8:1006–15. doi: 10.1111/j.1600-6143.2008.02199.x
47. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat Rev Cancer.* (2015) 15:7–24. doi: 10.1038/nrc3860
48. Wise HM, Hermida MA, Leslie NR. Prostate cancer, PI3K, PTEN and prognosis. *Clin Sci.* (2017) 131:197–210. doi: 10.1042/CS20160026
49. Shen F, Zong Z, Liu Y, Chen S, Sheng X, Zhao Y. CEMIP promotes ovarian cancer development and progression via the PI3K/AKT signaling pathway. *Biomed Pharmacother.* (2019) 114:108787. doi: 10.1016/j.biopha.2019.108787
50. Shukla S, MacLennan GT, Hartman DJ, Fu P, Resnick MI, Gupta S. Activation of PI3K-Akt signaling pathway promotes prostate cancer cell invasion. *Int J Cancer.* (2007) 121:1424–432. doi: 10.1002/ijc.22862
51. Chandarlapaty S. Negative feedback and adaptive resistance to the targeted therapy of cancer. *Cancer Discov.* (2012) 2:311–9. doi: 10.1158/2159-8290.CD-12-0018
52. Scott Kopetz E, Nelson JB, Carducci MA. Endothelin-1 as a target for therapeutic intervention in prostate cancer. *Invest New Drugs.* (2002) 20:173–82. doi: 10.1023/a:1015630513908
53. Vancauwenberghe E, Lallet Daher H, Derouiche S, Mariot P, Gosset P, Delcourt P, et al. Involvement of ion channels in endothelin-1-induced signalling in human prostate cancer cells. *J Cell Signal.* (2016) 01:126. doi: 10.4172/2576-1471.1000126
54. Rosanò L, Spinella F, Bagnato A. Endothelin 1 in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer.* (2013) 13:637–651. doi: 10.1038/nrc3546
55. Salani D, Di Castro V, Nicotra MR, Rosanò L, Tecce R, Venuti A, et al. Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol.* (2000) 157:1537–47. doi: 10.1016/S0002-9440(10)64791-8
56. Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, et al. A module of negative feedback regulators defines growth factor signaling. *Nat Genet.* (2007) 39:503–12. doi: 10.1038/ng1987
57. Freeman M. Feedback control of intercellular signalling in development. *Nature.* (2000) 408:313–9. doi: 10.1038/35042500
58. Nobes CD, Lauritzen I, Mattei M-G, Paris S, Hall A, Chardin P. A new member of the rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol.* (1998) 141:187–97. doi: 10.1083/jcb.141.1.187

59. Worzfeld T, Offermanns S. Semaphorins and plexins as therapeutic targets. *Nat Rev Drug Discov.* (2014) 13:603–21. doi: 10.1038/nrd4337
60. Foley K, Rucki AA, Xiao Q, Zhou D, Leubner A, Mo G, et al. Semaphorin 3D autocrine signaling mediates the metastatic role of annexin A2 in pancreatic cancer. *Sci Signal.* (2015) 8:ra77. doi: 10.1126/scisignal.aaa5823
61. Radisky ES, Raeeszadeh-Sarmazdeh M, Radisky DC. Therapeutic potential of matrix metalloproteinase inhibition in breast cancer. *J Cell Biochem.* (2017) 118:3531–48. doi: 10.1002/jcb.26185
62. Kessenbrock K, Plaks V, Werb Z. Matrix Metalloproteinases: Regulators of the tumor microenvironment. *Cell.* (2010) 141:52–67. doi: 10.1016/j.cell.2010.03.015
63. Sauter W, Rosenberger A, Beckmann L, Kropp S, Mittelstrass K, Timofeeva M, et al. Matrix metalloproteinase 1 (MMP1) is associated with early-onset lung cancer. *Cancer Epidemiol Biomarkers Prev.* (2008) 17:1127–62. doi: 10.1158/1055-9965.EPI-07-2840
64. Nelson CM, Khauv D, Bissell MJ, Radisky DC. Change in cell shape is required for matrix metalloproteinase-induced epithelial-mesenchymal transition of mammary epithelial cells. *J Cell Biochem.* (2008) 105:25–33. doi: 10.1002/jcb.21821
65. Cichon MA, Nelson CM, Radisky DC. Regulation of epithelial-mesenchymal transition in breast cancer cells by cell contact and adhesion. *Cancer Inform.* (2015) 14s3:CIN.S18965. doi: 10.4137/CIN.S18965
66. Obaya AJ, Rua S, Moncada-Pazos A, Cal S. The dual role of fibulins in tumorigenesis. *Cancer Lett.* (2012) 325:132–8. doi: 10.1016/j.canlet.2012.06.019
67. Shen H, Zhang L, Zhou J, Chen Z, Yang G, Liao Y, et al. Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1) acts as a potential diagnostic biomarker for prostate cancer. *Med Sci Monit.* (2017) 23:216–22. doi: 10.12659/MSM.898809
68. Song GX, Shen YH, Liu YQ, Sun W, Miao LP, Zhou LJ, et al. Overexpression of FABP3 promotes apoptosis through inducing mitochondrial impairment in embryonic cancer cells. *J Cell Biochem.* (2012) 113:3701–8. doi: 10.1002/jcb.24243
69. Tang Z, Shen Q, Xie H, Zhou X, Li J, Feng J, et al. Elevated expression of FABP3 and FABP4 cooperatively correlates with poor prognosis in non-small cell lung cancer (NSCLC). *Oncotarget.* (2016) 7:46253–62. doi: 10.18632/oncotarget.10086
70. Hashimoto T, Kusakabe T, Sugino T, Fukuda T, Watanabe K, Sato Y, et al. Expression of heart-type fatty acid-binding protein in human gastric carcinoma and its association with tumor aggressiveness, metastasis and poor prognosis. *Pathobiology.* (2004) 71:267–73. doi: 10.1159/000080061
71. Zhang X-M, Wang T, Hu P, Li B, Liu H, Cheng Y-F. SERPINB2 overexpression inhibited cell proliferation, invasion and migration, led to G2/M arrest, and increased radiosensitivity in nasopharyngeal carcinoma cells. *J Radiat Res.* (2019) 60:318–27. doi: 10.1093/jrr/rrz003
72. Croucher DR, Saunders DN, Lobov S, Ranson M. Revisiting the biological roles of PAI2 (SERPINB2) in cancer. *Nat Rev Cancer.* (2008) 8:535–45. doi: 10.1038/nrc2400

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Neuroendocrine Carcinomas of the Canine Mammary Gland: Histopathological and Immunohistochemical Characteristics

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Invasive mammary carcinomas with neuroendocrine differentiation are rare in women and were reported only once in female dogs. For the present study, ten cases of solid mammary carcinoma positive for chromogramin A in immunohistochemistry were selected. Histopathological characteristics of these tumors were described and immunohistochemical evaluation was performed with chromogranin A, synaptophysin, CD56, NSE, PGP 9.5, pancitokeratin, Ki67, estrogen receptor (ER), and progesterone receptor (PR). The average animal age was 13.2 years old and the average tumor size was 4.8 cm. In total, 70% of the neoplasms were classified as grade III and 30% as grade II by the Nottingham histological grade system. High mitotic index was observed with a mean of 27.5 mitoses in 10 high magnification fields. Only one case showed typical carcinoid tumor characteristics. In addition, vascular invasion was shown in 3 tumors. All carcinomas were positive for chromogran A, while only two cases were reactive to synaptophysin. For PGP 9.2, NSE and CD56, we observed positivity of 100, 90, and 70%, respectively, in the samples, being that no tumor was positive for all the neuroendocrine markers. All neoplasms showed ER and PR in at least 10% of neoplastic cells, while Ki67 varied from 29 to 95%, with mean mitotic index of 67%. Four of the ten animals died within 1 year of the tumor diagnosis. Neuroendocrine neoplasms occur in the canine mammary gland and are propably underdiagnosed. This is due to their non-specific morphological characteristics and the low use of neuroendocrine immunohistochemistric markers the diagnostic routine. More studies are necessary to determine the prognosis of this new histological type.

Keywords: diagnosis, female dog, histological classification, solid carcinoma, neuroendocrine carcinoma, mammary cancer

INTRODUCTION

Neuroendocrine tumors are a group of biologically and clinically heterogeneous neoplasms that originate most commonly in lungs, gastrointestinal tract and pancreas (1, 2). Although their occurrence is rare, pure neuroendocrine tumors and invasive breast carcinomas with neuroendocrine features have already been reported in women (3–5), while only one case has been reported in a female dog (6).

These tumors were first recognized in women in 1963 by Feyrter and Hartmann (7) based on a "carcinoid" growth pattern seen in two cases of invasive breast carcinoma. Later, in 1977, Cubilla and Woodruff (8) described eight cases of breast cancer showing a growth pattern typical of a "carcinoid tumor," and several recent reviews have since been published (9–12).

Finally, the classification of breast tumors of the World Health Organization (WHO) in 2003 recognized neuroendocrine carcinomas of the breast as a special histological type of invasive carcinoma in which more than 50% of the neoplastic cells express at least one neuroendocrine marker (13). Next, the 2012 classification included a chapter on "Carcinomas with neuroendocrine features," in which the minimum cut-off of tumor cells with positive labeling for neuroendocrine markers was removed (9, 14).

The most recent WHO classification (2019) (15) categorizes breast cancers with neuroendocrine differentiation in three groups: (1) invasive carcinoma with neuroendocrine differentiation; (2) neuroendocrine tumor (NET); (3) neuroendocrine carcinoma (NEC). However, the neuroendocrine differentiation detected by either histochemical or immunohistochemical analysis may be seen in 10–30% of invasive breast carcinomas of no special type (IBC NST). Additionally, special types of breast cancer may also show expression of neuroendocrine markers, particularly solid papillary carcinomas and mucinous carcinomas, and should not be classified as NETs or NECs. When neuroendocrine morphologic characteristics and neuroendocrine marker expression are focal or are not distinct enough to classify a neoplasm as NET or NEC, an IBC NST with neuroendocrine differentiation must be considered. Notably, most breast cancers with neuroendocrine differentiation belong in the first group, as pure neuroendocrine tumors of the breast are exceptionally rare.

Neuroendocrine tumors of the breast correspond to an invasive neoplasm composed of densely cellular solid nests or trabeculae of cells, usually with a low to intermediate grade morphology, separated by delicate fibrovascular stroma. Papillary, insular and alveolar-like patterns may be seen. On the other hand, neuroendocrine carcinomas are invasive carcinomas characterized by the proliferation of small or large, high-grade neoplastic cells (small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma, respectively). Both subtypes present neuroendocrine morphological features, cytoplasmic neurosecretory granules and uniform immunohistochemical positivity for neuroendocrine markers (15).

Data on breast cancers with neuroendocrine differentiation are limited. Also, the true incidence of neuroendocrine neoplasms of the breast is difficult to evaluate, because many of the classic histopathological features of neuroendocrine tumors that occur in other organs are not present in their breast counterpart (13, 16). In addition, neuroendocrine markers are not routinely tested in invasive breast carcinomas, since there is no clinical relevance of neuroendocrine differentiation as an individual characteristic (12, 15).

In the female dog, the classification of mammary neoplasms is mainly based on the histopathological pattern and, to a lesser extent, on the histogenetic classification, due to the difficulty in determining the origin of a specific cell type in certain mammary tumors (17). Among the histological types described, the solid carcinoma is a common pattern of canine mammary tumor that presents some variations in cell characteristics, which makes many researchers believe that there may be several origins for these cells (18–20).

In this study, we aimed to investigate the presence of neuroendocrine differentiation in 10 cases of solid mammary carcinoma of female dogs, in order to promote a greater recognition and appropriate classification of this histological type in this species.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the ethics committee on animal use (CEUA/UFMG) under protocol number 11/2017, on June 5th, 2017.

Animals

Ten cases of canine mammary solid carcinoma, positive for chromogramin A in immunohistochemistry, were selected for this study. The samples were from the Laboratory of Comparative Pathology of Minas Gerais Federal University (UFMG).

Histopathology

Representative samples of tumors removed by incisional or excisional biopsy were obtained and included in paraffin blocks. Consecutive histologic sections were prepared and stained by the hematoxylin and eosin routine method. Neoplasm slides were evaluated and diagnoses were defined according to the "Consensus for the diagnosis, prognosis, and treatment of canine mammary tumors-2013" (20). The Nottingham histologic grade system was applied to determine tumor grade (21).

Immunohistochemistry

Sections of 4 μm thickness from primary tumors were prepared and mounted on common slides for IHC analysis. The antigen was immunodetected by the detection system anti-mouse/anti-rabbit (Novolink Polymer Detection System, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) according to the manufacturer's instructions. The endogenous peroxidase activity was blocked with a 10% hydrogen peroxide (H_2O_2) solution in methyl alcohol. Reagents were manually applied and immunoreactivity was visualized by incubating the slides with diaminobenzidine chromogen (DAB Substrate System, Dako, Carpinteria, CA, USA) for 3 min. Details of the antibodies against Synaptophysin (1, 22, 23), NSE (22, 24), CD56 (24),

PGP 9.5 (25, 26), Chromogranin A (25, 27), Estrogen Receptor - RE (28), Progesterone Receptor - PR (29), Pancitokeratin (22) and Ki67 (30), dilutions, antigen retrieval procedures and incubation times used in the immunostaining process are shown in **Table 1**. Normal canine mammary gland was used as an internal positive control for Estrogen and Progesterone Receptors and Pancitokeratin. For Synaptophysin, NSE, CD56, PGP 9.5 and Chromogranin A, canine adrenal gland was used as positive control. Negative controls were performed using a normal serum (Lab Vision Ultra V Block) in place of the primary antibody.

Immunohistochemical Evaluation

The cell proliferation index (Ki67) was calculated by manually counting the number of positive nuclei in a total of 500 neoplastic cells in areas with the highest levels of positivity (hot spot/hot zones) through image J software (National Institute of Health, Bethesda, Maryland, USA). A 20% score was used as cutoff point to classify cases with high or low proliferation index. Positivity for estrogen receptor (ER) and progesterone receptor (PR) was defined as the presence of nuclear expression in >10% of neoplastic cells (3). The Expression of the neuroendocrine markers chromogranin A, synaptophysin, CD56, NSE and PGP 1.9 was assessed. The evaluation pancytokeratin expression (AE1/AE3) was qualitative and classified as positive positive when there was cytoplasmic staining of neoplastic cells.

RESULTS

The mean age of the ten studied animals was 13.2 years old, ranging from 9 to 16 years old. Mean tumor size was 4.8 cm, excluding one case of incisional biopsy for which the actual size of the neoplasm was not informed. The main pathological parameters are detaleid in **Table 2**.

In general, tumors showed a high mitotic count, with a mean number of 27.5 mitoses in 10 high-power fields (40X). Seventy percent (7/10) of the neoplasms were classified as grade III and 30% (3/10) as grade II. Lymph nodes of only 4 cases were referred for analysis, of which one presented metastasis on histopathological examination.

Similar morphological patterns were observed in the histopathological analysis of all cases, with 100% demonstrating a solid cell arrangement (**Figure 1A**) and at least some proliferation areas *in situ*. Most tumors exhibited an infiltrative growth pattern with invasion into the dermis and adjacent adipose tissue, although a circumscribed pattern was seen

TABLE 1 | Antibodies, dilutions, incubation time and temperature and methods of antigenic recovery for the immunohistochemical reactions.

Antibody	Manufacturer	Clone	Dilution	Incubation time/ temperature	Antigenic recovery method
Synaptophysin	Monosan	SY38	1:100	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
NSE	Dako	BBS/NC/VI-H14	1:1000	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
CD56	Biocare Medical	BC56C04	1:50	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
PGP 9.5	Cell Marque	polyclonal	1:500	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
Chromogranin A	Dako	DAK-A3	1:100	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
Estrogen Receptor (RE)	Dako	1D5	1:50	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
Progesterone receptor (RP)	NeoMarkers	Ab-1 (hPRa2)	1:50	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
Pancitokeratin	Dako	AE1/AE3	1:500	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)
Ki67	Dako	MIB-1	1:50	Overnight 4°C	10 mM citrate (pH 6.0) in the pressure cooker (Pascal^R, Dako)

TABLE 2 | Macroscopic and histopathological characteristics of mammary neoplasms with neuroendocrine differentiation.

Patients	Tumor size measured to its largest extent (cm)	Mitotic figures in 10 HPFs	Histological grade	Presence of lymphatic invasion	Regional lymph node
Patient 1	8.0	43	III	No	Not analyzed
Patient 2	1.5*	46	III	Yes	Not analyzed
Patient 3	8.0	25	III	No	No metastasis
Patient 4	2.0	18	II	Yes	With metastasis
Patient 5	3.0	41	III	No	Not analyzed
Patient 6	5.0	34	III	No	Not analyzed
Patient 7	4.5	22	III	No	Not analyzed
Patient 8	7.0	22	III	No	No metastasis
Patient 9	2.0	11	II	No	No metastasis
Patient 10	4.3	13	II	Yes	Not analyzed

**HPFs, high-power fields.*

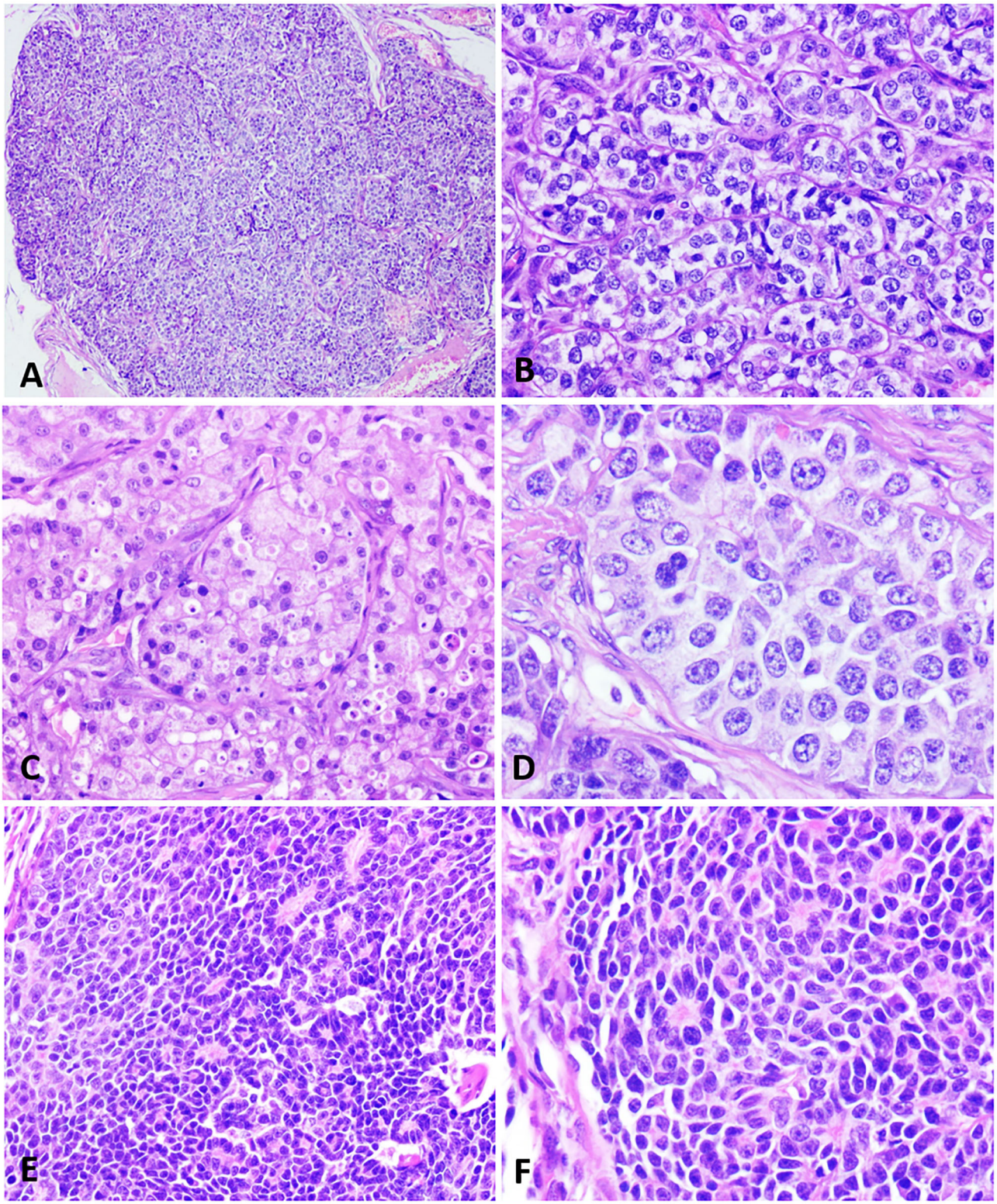

FIGURE 1 | Histopathological characteristics of neuroendocrine carcinomas in the female dog. **(A)** A mammary lump showing a solid arrangement. Hematoxylin and eosin.10x. **(B)** Solid nests of neoplastic cells separated by a delicate fibrovascular stroma. Hematoxylin and eosin.40x. **(C)** Cells with a finely granular eosinophilic cytoplasm, sometimes displaying intracytoplasmic vacuoles. Hematoxylin and eosin.40x. **(D)** Neoplastic cells with round to oval nuclei, finely dotted chromatin, and conspicuous nucleoli. Hematoxylin and eosin.60x. **(E)** Neoplastic cells disposed in a solid arrangement, occasionally in palisades and forming rosettes. Hematoxylin and eosin.40x. **(F)** Cells exhibiting a scarce, slightly eosinophilic cytoplasm, with small and hyperchromatic nuclei. Hematoxylin and eosin.40x.

in some cases. Sixty percent of the cases (6/10) exhibited arrangement in small solid nests of cells delimited by delicate fibrovascular stroma (**Figure 1B**). Neoplastic cells presented a cytoplasm of moderate size, slightly eosnophilic, with varying degrees of fine granulation, or a clear cytoplasm, with vacuolization (**Figure 1C**). The nuclei were large, round to oval, with varying degrees of atypia. In addition, 60, 30, and 10% of the cases showed marked, moderate and mild anisocariosis, respectively. Nuclear chromatin was slightly dotted and dispersed (salt and pepper aspect) in most cases, and sometimes with fine condensation along the nuclear membrane. The nucleoli were either small and multiple or sometimes single and large (**Figure 1D**).

One of the neoplasms (patient 4) showed a distinct, peculiar morphology, with cells arranged in large coalescing solid nests, containing palisade cells, often forming rosettes (**Figure 1E**). The cytoplasm was scarce, slightly eosinophilic, with oval nuclei, small, hyperchromatic and unique nucleoli or occasionally prominent (**Figure 1F**). This morphological pattern was compatible with the histologic characteristics of carcinoid tumors.

Lymphovascular invasion was identified in 3 of the 10 cases (30%). Areas of necrosis, mainly at the center of solid nests, were observed in 70% of the neoplasms.

Immunohistochemistry results are shown in **Table 3**. All cases were positive for chromogranin A (**Figure 2A**) and the cytoplasmic staining was granular with variable intensity. Only two cases were positive for synaptophysin (**Figure 2B**). Seven tumors (7/10) were positive for CD56 (**Figure 2C**), while nine (9/10) were positive for NSE (**Figure 2D**). All cases were positive for PGP 9.5 (**Figure 2E**). No case was positive for all neuroendocrines.

All neoplasms expressed estrogen and progesterone receptors in at least 10% of neoplastic cells and were positive for pancytokeratin (AE1/AE3). Ki67 (**Figure 2F**) was considered high in most carcinomas, with a mean of 67% of positive cells, ranging from 29 to 95%.

Four of the 10 studied animals (patients 1, 2, 6, and 10) died or were euthanized within 1 year of diagnosis due to the neoplasm development. Two animals (patients 3 and 4) died due to other causes unrelated to the tumor and two patients (8 and 9) are still alive with no sings of recurrence and metastasis after 1 year and 6 months and 3 years and 8 months of diagnosis, respectively. The follow-up of patients 5 and 7 after surgery was not feasible. Only one of the eight patients with follow-up (patient 10) received complementary treatment after surgery.

DISCUSSION

Invasive carcinomas with neuroendocrine differentiation of the human breast are under-recognized in the practical routine and represent 0.5–1% of all breast cancers (11, 12, 15). In veterinary medicine, this histological type is not yet well recognized, with a single prior case report in the literature (6), making ours the first retrospective study on neuroendocrine carcinomas in the female dog.

The histogenesis of neuroendocrine tumors of the breast is debatable mainly due to the difficulty in locating neuroendocrine cells in normal mammary glands (16, 31). Viacava et al. (31) did not find histochemical, immunohistochemical and ultrastructural evidence of neuroendocrine differentiation in normal cells of the fetal and adult mammary glands in their study, indicating that this differentiation may happen in the process of tumor progression.

From a clinical point of view, the importance of neuroendocrine differentiation in invasive breast carcinomas is not well stablished. While some studies have stated that there is no prognostic value, others have shown that it is associated to a better or worse prognosis (14, 16, 32). Sapino et al. (33) concluded that the histological grade greatly influenced the clinical evolution of neuroendocrine carcinomas of the breast. Poorly differentiated, grade III neuroendocrine carcinomas with a high proliferative activity behaved aggressively. On the other hand, patients with well-differentiated, grade I tumors with a low proliferative index remained alive after more than 13 years of follow-up. Therefore, the impact of neuroendocrine differentiation on prognosis remains unclear and may be explained by the heterogeneous nature of tumors that fall into this category, as this group includes special breast cancer types

TABLE 3 | Expression of chromogranin A, synaptophysin, NSE, CD56, PGP 9.5, Ki67, ER, PR and pancitokeratin in solid mammary carcinomas with neuroendocrine features.

Patients	Chromogranin A	Synaptophysin	NSE	CD56	PGP 9.5	Ki67	ER	PR	CK AE1/AE3
Patient 1	Positive	Negative	Positive	Negative	Positive	50%	10–25%	>75%	Positive
Patient 2	Positive	Positive	Positive	Negative	Positive	95%	51–75%	>75%	Positive
Patient 3	Positive	Negative	Positive	Positive	Positive	75%	51–75%	>75%	Positive
Patient 4	Positive	Positive	Negative	Positive	Positive	83%	51–75%	>75%	Positive
Patient 5	Positive	Negative	Positive	Positive	Positive	50%	10–25%	>75%	Positive
Patient 6	Positive	Negative	Positive	Positive	Positive	90%	25–50%	>75%	Positive
Patient 7	Positive	Negative	Positive	Positive	Positive	89%	25–50%	>75%	Positive
Patient 8	Positive	Negative	Positive	Positive	Positive	29%	10–25%	>75	Positive
Patient 9	Positive	Negative	Positive	Positive	Positive	56%	10–25%	51–75%	Positive
Patient 10	Positive	Negative	Positive	Negative	Positive	54%	10–25%	51–75%	Positive

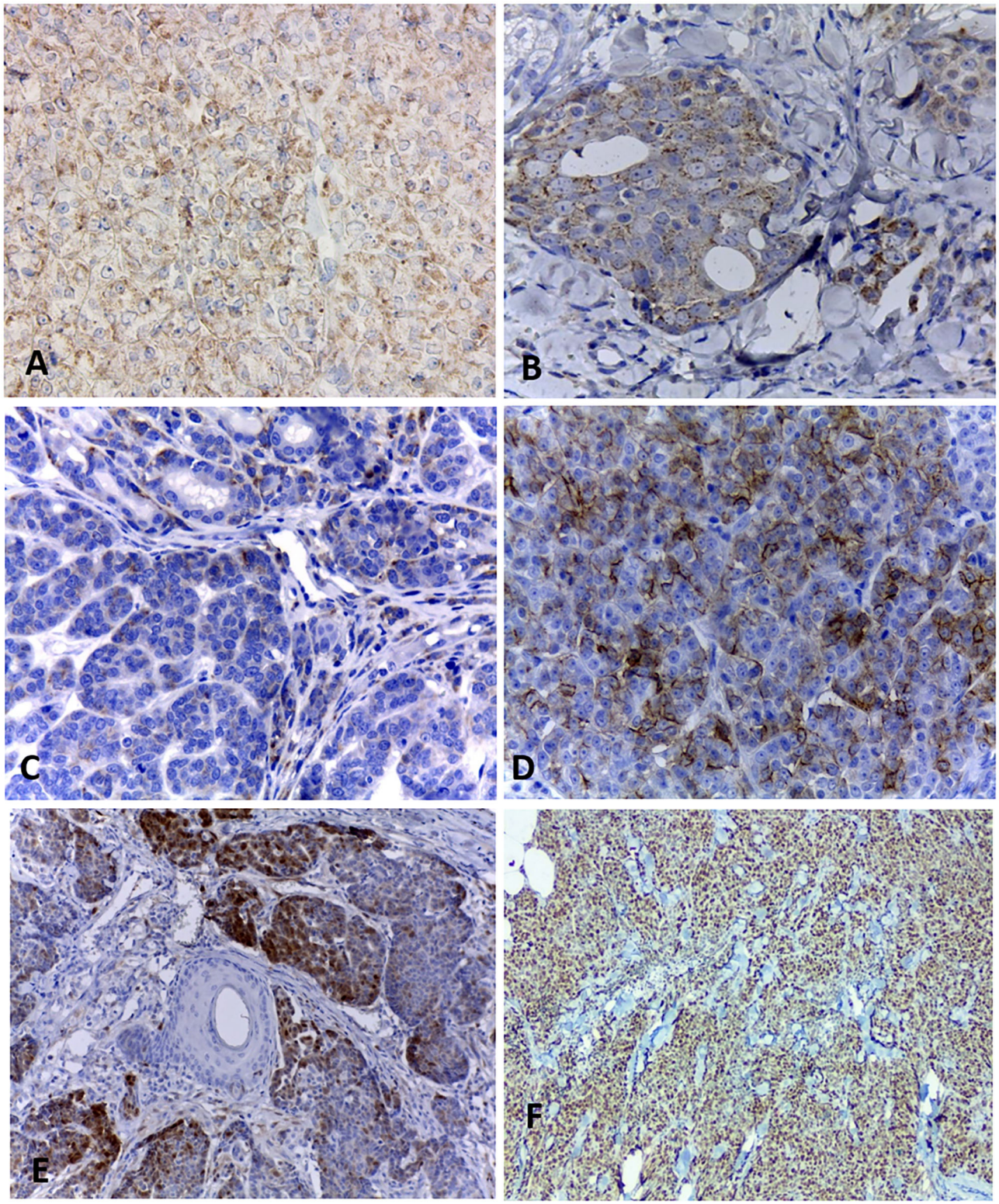

FIGURE 2 | Immunohistochemical staining of canine mammary solid carcinomas. **(A)** Neoplastic cells showing positive cytoplasmic staining for chromogranin A, with a granular pattern. 40x. **(B)** Cytoplasmic expression of synaptophysin in more than 50% of neoplastic cells. 40x. **(C)** Less than 50% of positive neoplastic cells for CD56. 40x. **(D)** Expression of NSE in more than 50% of neoplastic cells. 40x. **(E)** Multifocal staining for PGP 9.5 in 10% of neoplastic epithelial cells. **(F)** Nuclear positivity Ki67 in 95% of neoplastic cells. 10x.

with a low-grade morphology and indolent clinical course, as well as high-grade aggressive carcinomas (32, 34). Of the eight followed-up patients, four died due to the unfavorable neoplasm clinical evolution in <1 year of diagnosis. In this sense, the fact that half of these followed-up patients survived for less than an year after diagnosis may lead to the conclusion that this type of carcinoma presents a guarded to poor prognosis compared to other carcinomas with better prognosis in female dogs, such as carcinoma in mixed tumor, which does not reach the survival median until 2 years of follow-up (35).

The diagnosis of carcinoma with neuroendocrine differentiation based solely on morphologic characterisitics is a challenge and not feasible most of the times, once many of the classic histologic features of neuroendocrine carcinomas that occur in other organs are not present in neuroendocrine carcinomas of the mammary gland (13, 16, 36). Thus, the morphologic features found in this study were similar to those described in other studies that confirmed the presence of neuroendocrine differentiation by immunohistochemistry (11, 14, 32, 37). Only one case exhibited a typical morphology of a carcinoid tumor, with smaller cells, sometimes forming rosettes, hyperchromatic nuclei and scarce cytoplasm, analogous to carcinoid tumors and well-differentiated neuroendocrine carcinomas previously reported in the human breast and other organs (38, 39). According to the WHO classification of breast tumors of 2019, this sole case should be categorized as a neuroendocrine tumor, whereas the other nine tumors should be classified as large cells neuroendocrine carcinomas according to morphologic and immunohistochemical characteristics (15).

Metastases from other neoplasms in women's breast are uncommon and metastases of neuroendocrine carcinoma are even rarer, representing 1–2% of all metastatic tumors of the breast (37). However, the possibility of metastatic neuroendocrine carcinomas should always be excluded with adequate clinical and radiological examinations as well as immunohistochemical studies to attest a breast origin in order to diagnose a primary invasive breast carcinoma with neuroendocrine features (14). The presence of an associated carcinoma *in situ* and positivity for hormonal receptors may also be useful for the diferential diagnosis (14, 16, 37, 40, 41). The animals studied herein had no history of neoplasms in other locations, but imaging data were not obtained. All tumors were positive for ER and PR, and had proliferation areas *in situ*, corroborating the hypothesis that these were primary mammary lesions.

Among neuroendocrine differentiation markers, chromogranin A and synaptophysin are the most frequently used (1, 34). In our study, only 2 of 10 cases showed positivity for both markers and similar results have been reported by Wachter et al. (13). Such findings highlight the importance of a panel including at least two markers in cases suspected for neuroendocrine carcinoma, since these tumors will not always be positive for both antibodies.

Our findings show that neuroendocrine carcinomas occur in the canine mammary gland, as well as in the human breast and may be underdiagnosed when they are included in the group of solid carcinomas. However, a definitive diagnosis based on histopathological examination alone is challenging, stressing the need of using specific markers for neuroendocrine differentiation such as chromogranin and synaptophysin for confirmation. Thus, complementary studies with clinical and therapeutic follow-up are essential to define the prognosis of this new histological type, in addition to establishing implications in target therapy responsiveness.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by CEUA - UFMG. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KN and MN collected patient data and samples from the UFMG Laboratory of Comparative Pathology file. KN, AG, and GC performed histopathological and immunohistochemical analyses. KN, GC, and MD participated in the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Nagahara R, Kimura M, Itahashi M, Sugahara G, Kawashima M, Murayama H, et al. Canine mammary minute oncocytomas with neuroendocrine differentiation associated with multifocal acinar cell oncocytic metaplasia. *J Vet Diagn Invest.* (2016) 28:722–8. doi: 10.1177/1040638716664381
2. Bogina G, Munari E, Brunelli M, Bortesi L, Marconi M, Sommaggio M, et al. Neuroendocrine differentiation in breast carcinoma: clinicopathological features and outcome. *Histopathology.* (2016) 68:422–32. doi: 10.1111/his.12766
3. Wei B, Ding T, Xing Y, Wei W, Tian Z, Tang F, et al. Invasive neuroendocrine carcinoma of the breast: a distinctive subtype of aggressive mammary carcinoma. *BMC Cancer.* (2010) 116:4463–73. doi: 10.1002/cncr.25352
4. Talu CK, Leblebici C, Ozturk TK, Hacihasanoglu E, Koca SB, Gucin Z. Primary breast carcinomas with neuroendocrine features: clinicopathological features and analysis of tumor growth patterns in 36 cases. *Ann Diagn Pathol.* (2018) 34:122–30. doi: 10.1016/j.anndiagpath.2018.03.010
5. Fónyad L, Piros L, Arató G, Kulka J. Primary neuroendocrine tumor of the breast-report of 2 cases. *Diagn Pathol.* (2018) 4:1. doi: 10.17629/www.diagnosticpathology.eu-2018-4:261

6. Nakahira R, Michishita M, Yoshimura H, Hatakeyama H, Takahashi K. Neuroendocrine carcinoma of the mammary gland in a dog. *J Comp Pathol.* (2015) 52:188–91. doi: 10.1016/j.jcpa.2014.12.009
7. Feyrter F, Hartmann G. On the carcinoid growth form of the Mammae carcinoma, especially the carcinoma solidum (Gelatinosum) Mammae. *Frankfurt Z Pathol.* (1963) 73:24–39.
8. Cubilla AL, Woodruff, JM. Primary carcinoid tumor of the breast: a report of eight patients. *Am J Surg Pathol.* (1977) 1:283–92. doi: 10.1097/00000478-197712000-00001
9. Osamura RY, Matsui N, Okubo M, Chen L, Field AS. Histopathology and cytopathology of neuroendocrine tumors and carcinomas of the breast: a review. *Acta Cytol.* (2019) 63:340–6. doi: 10.1159/000500705
10. Wang J, Wei B, Albarracin CT, Hu J, Abraham SC, Wu Y. Invasive neuroendocrine carcinoma of the breast: a population-based study from the surveillance, epidemiology and end results (SEER) database. *BMC Cancer.* (2014) 14:147. doi: 10.1186/1471-2407-14-147
11. Tang F, Wei B, Tian Z, Gilcrease MZ, Huo L, Albarracin CT, et al. Invasive mammary carcinoma with neuroendocrine differentiation: histological features and diagnostic challenges. *Histopathology.* (2011) 59:106–15. doi: 10.1111/j.1365-2559.2011.03880.x
12. Roininen N, Takala S, Haapasaari KM, Jukkola-Vuorinen A, Mattson J, Heikkilä P, et al. Primary neuroendocrine breast carcinomas are associated with poor local control despite favorable biological profile: a retrospective clinical study. *BMC Cancer.* (2017) 17:12. doi: 10.1186/s12885-017-3056-4
13. Wachter DL, Hartmann A, Beckmann MW, Fasching PA, Hein A, Bayer CM, et al. Expression of neuroendocrine markers in different molecular subtypes of breast carcinoma. *Biomed Res Int.* (2014) 2014:408459. doi: 10.1155/2014/408459
14. Kelten Talu C, Savli TC, Huq GE, Leblebici C. Histopathological and clinical differences between primary breast carcinomas with neuroendocrine features and primary breast carcinomas mimicking neuroendocrine features. *Int. J Surg Pathol.* (2019) 27:744–52. doi: 10.1177/1066896919851873
15. Hoon Tan P, Ellis I, Allison K, Brogi E, Fox SB, Lakhani S, et al. The 2019 WHO classification of tumors of the breast. *Histopathology*. (2020). 77:181–5. doi: 10.1111/his.14091
16. Cloyd JM, Yang RL, Allison KH, Norton JÁ, Hernandez-Boussard T, Wapnir IL. Impact of histological subtype on long-term outcomes of neuroendocrine carcinoma of the breast. *Breast Cancer Res Treat*. (2014) 148:637–44. doi: 10.1007/s10549-014-3207-0
17. Gama A, Alves A, Gartner F, Schmitt F. p63: a novel myoepithelial cell marker in canine mammary tissues. *Vet Pathol.* (2003) 40:412–20. doi: 10.1354/vp.40-4-412
18. Moulton JE, Taylor DO, Dorn CR, Andersen, AC. Canine mammary tumors. *Vet Pathol.* (1970) 7:289–320. doi: 10.1177/030098587000700401
19. Goldschmidt M, Peña L, Rasotto R, Zappulli V. Classification and grading of canine mammary tumors. *Vet Pathol.* (2011) 48:117–31. doi: 10.1177/0300985810393258
20. Cassali GD, Lavalle GE, Ferreira E, Estrela-Lima A, De Nardi AB, Ghever, et al. et al. Consensus for the diagnosis, prognosis treatment of canine mammary tumors −2013. *Braz J Vet Pathol.* (2014) 7:38–69.
21. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology.* (1991) 19:403–10. doi: 10.1111/j.1365-2559.1991.tb00229.x
22. Ramírez GA, Rodrígues F, Herráez P, Suárez-Bonnet A, Andrada M, Espinosa-de-los-Monteros A. Morphologic and immunohistochemical freatures of Merkel cells in the dog. *Res Vet Sci.* (2014) 77:475–80. doi: 10.1016/j.rvsc.2014.10.006
23. Ramírez GA, Altimira J, Vilafranca M. Ganglioneuromatosis of the Gallbladder in a dog with cholecystitis and cholestasis. *J Comp Pathol.* (2018) 163:29–32. doi: 10.1016/j.jcpa.2018.07.002
24. Loures FH, Conceição LG, Lauffer-Amorim R, Nóbrega J, Costa EP, Torres Neto R, et al. Histopathology and immunohistochemistry of peripheral neural sheath tumor and perivascular wall tumor in dog. *Arq Bras Med Vet Zootec.* (2019) 71:1100–6. doi: 10.1590/1678-4162-10780
25. Ramos-vara JA, Miller MA. Immunohistochemical detection of protein gene product 9.5 (PGP 9.5) in canine epitheliotropic T-cell lymphoma (mycosis fungoides). *Vet Pathol.* (2007) 44:74–9. doi: 10.1354/vp.44-1-74
26. Arciszewski MB, Barabasz S, Calka J. Immunohistochemical localization of galanin receptors (GAL-R1, GAL-R2, and GAL R3) on myenteric neurons from the sheep and dog stomach. *Ann Anat.* (2008) 190:360–7. doi: 10.1016/j.aanat.2008.04.004
27. Argenta FF, Pereira PR, Bertolini M, Fratini LM, Saccaro RO, Sonne L, et al. Carcinoid of the gallbladder in two dogs. *Cienc Rural.* (2020) 50:2. doi: 10.1590/0103-8478cr20190445
28. Sonremo KU, Durham AC, Radaelli E, Kristiansen V, Pena S, Goldschmidt MH, et al. The estrogen effect; clinical and histopathological evidence of dichotomous influences in dogs with spontaneous mammary carcinomas. *PLoS ONE.* (2019) 14:e0224504. doi: 10.1371/journal.pone.0224504
29. Silveira TL, Campos LM, Dufloth M, Miot HA, Fêo HB, Montoya LM, et al. Cell block sensitivity for immunohistochemical detection of cytokeratin 5, oestrogen and progesterone receptors in canine primary mammary carcinoma. *Austral J Vet Sci.* (2017) 49:99–104. doi: 10.4067/S0719-81322017000200099
30. Kadthurl JC, Rao S, Sonnahallipura M, Thimmanahalli DS, Laxmikanth SM. Prognostic value of Ki67 proliferation antigen in canine malignant mammary gland tumors. *Braz J Vet Pathol.* (2011) 4:36–40.
31. Viacava P, Castagna M, Bevilacqua G. Absence of neuroendocrine cells in fetal and adult mammary glands. Are neuroendocrine breast tumors real neuroendocrine tumors? *Breast.* (1995) 4:143–6. doi: 10.1016/0960-9776(95)90012-8
32. Yussif SM, Soliman N. Assessment of neuroendocrine markers in different molecular subtypes of invasive breast carcinoma and its impact on prognosis. *Merit Res J Med Med Sci.* (2018) 6:204–14.
33. Sapino A, Papotti M, Righi L, Cassoni P, Chiusa L, Bussolati G. Clinical significance of neuroendocrine carcinoma of the breast. *Ann Oncol.* (2001) 12:115–7. doi: 10.1093/annonc/12.suppl_2.S115
34. Tan PH, Schnitt SJ, van de Vijver MJ, Ellis IO, Lakhani SR. Papillary and neuroendocrine breast lesions: the WHO stance. *Histopathology.* (2015) 66:761–70. doi: 10.1111/his.12463
35. Nunes FC, Campos CB, Teixeira SV, Bertagnolli AC, Lavalle GE, Cassali GD. Epidemiological, clinical and pathological evaluation of overall survival in canines with mammary neoplasms. *Arq Bras Med Vet Zootec.* (2018) 70:1714–22. doi: 10.1590/1678-4162-10217
36. Inno A, Bogina G, Turazza M, Bortesi L, Duranti S, Massocco A, et al. Neuroendocrine carcinoma of the breast: current evidence and future perspectives. *Oncologist.* (2016) 21:28–32. doi: 10.1634/theoncologist.2015-0309
37. Mohanty SK, Kim SA, DeLair DF, Bose S, Laury AR, Chopra S, et al. Comparison of metastatic neuroendocrine neoplasms to the breast and primary invasive mammary carcinomas with neuroendocrine differentiation. *Mod Pathol.* (2016) 28:788–98. doi: 10.1038/modpathol.2016.69
38. Bussolati G, Gugliotta P, Sapino A, Eusebi V, Lloyd RV. Chromogranin-reactive endocrine cells in argyrophilic carcinomas ("carcinoids") and normal tissue of the breast. *Am J Pathol.* (1985) 120:186.
39. Visscher DW, Yasir S. Neuroendocrine tumors of the breast. *Endocr Pathol.* (2017) 28:121–7. doi: 10.1007/s12022-017-9477-4
40. Perry KD, Reynolds C, Rosen DG, Edgerton ME, T Albarracin C, Gilcrease MZ, et al. Metastatic neuroendocrine tumor in the breast: a potential mimic of in-situ and invasive mammary carcinoma. *Histopathology.* (2011) 59:619–30. doi: 10.1111/j.1365-2559.2011.03940.x
41. Adams RW, Dyson P, Barthelmes L. Neuroendocrine breast tumors: breast cancer or neuroendocrine cancer presenting in the breast? *Breast.* (2014) 23:120–7. doi: 10.1016/j.breast.2013.11.005

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PDA Indolylmaleimides Induce Anti-Tumor Effects in Prostate Carcinoma Cell Lines Through Mitotic Death

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Castrate resistant prostate cancer in men shares several characteristics with canine prostate cancer (PCa). Due to current insufficient therapies, evaluating novel therapeutic agents for late-stage PCa is of considerable interest for both species. PDA indolylmaleimides showed anticancer effects in several neoplastic cell lines. Herein, a comparative characterization of PDA-66 and PDA-377 mediated effects was performed in human and canine PCa cell lines, which is also the first detailed characterization of these agents on cells derived from solid tumors in general. While PDA-377 showed only weak growth inhibition on human PCa cell lines, PDA-66 inhibited proliferation and induced apoptosis in human and canine cell lines with concentrations in the low micromolar range. Morphological characterization and whole transcriptome sequencing revealed that PDA-66 induces mitotic death through its microtubule-depolymerizing ability. PDA-66 appears to be a worthwhile anti-mitotic agent for further evaluation. The similarities in cellular and molecular response observed in the cell lines of both origins form a solid basis for the use of canine PCa *in vivo* models to gain valuable interchangeable data to the advantage of both species.

Keywords: mitotic death, mitotic slippage, whole transcriptome sequencing, human, dog, prostate cancer

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INTRODUCTION

Prostate cancer (PCa) is the most common malignancy diagnosed among males in almost all western countries (1). PCa emerges in an androgen-dependent or androgen-independent manner with a highly heterogeneous clinical course. Localized PCa has a 5-year survival rate close to 100% due to the availability of a broad range of curative treatment options. However, up to 20% of patients develops incurable and lethal metastatic castrate-resistant PCa within 5 years of follow-up. The development of novel therapeutic options for treating high-risk locally advanced PCa is needed (2–5).

Canine tumors are valuable naturally occurring models helping to reveal mechanisms in cancer development, behavior and treatment (6, 7). Prostatic neoplasia in dogs are reported with a prevalence below 1% (8) and poor prognosis (9–11). However, unlike in men there is no screening for early detection and only late stage cases are diagnosed. Actual numbers are likely to be much higher based on the high frequency of preneoplastic lesions (12). Canine prostate adenocarcinomas share several characteristics with human castrate-resistant PCa, e.g., increased occurrence with age, aggressive tumor progression, similar metastatic spread (lumbar spine and pelvis as well as lymph nodes), and castration-resistance (13, 14). Therefore, identifying and evaluating novel effective agents to treat late-stage, metastatic PCa may be beneficial for both species.

Indolylmaleimides as PDA derivatives PDA-66 and PDA-377 are synthetic molecules characterized by the conjugation of a maleimide compound with a bicyclic indole ring (15, 16). PDA-66 induces mitotic arrest and apoptosis in neuroblastoma, lung cancer, and canine lymphoma cells (15, 17), and has been shown to depolymerize microtubules (15). Known microtubule-destabilizing agents, such as *Vinca* alkaloids, are used in various cancers (18). These antimitotic drugs lead to failures in spindle formation and chromosome segregation in dividing cells, which activates the spindle assembly checkpoint leading to mitotic arrest. Prolonged mitotic arrest eventually triggers mitotic death (MD), an intrinsic form of regulated cell death (19, 20). MD is considered an onco-suppressive mechanism controlling mitotic failures and therefore prevents aneuploidy. Those failures include extensive DNA damage preventing replication, problems with the mitotic machinery (e.g., equal distribution of chromosomes) or failure of mitotic checkpoints leading to premature progress in the cell cycle (19, 21, 22).

A comprehensive characterization of PDA effects in human and canine PCa cells is missing. Before introducing such novel inhibitors into clinics, conducting an evaluation of these agents in model organisms is a prerequisite. Dogs classify as an extraordinary naturally occurring model for human PCa trials. As therapeutic options for dogs are limited and their metabolism is highly comparable to humans, clinical trials in dogs are considered to be of significant value (23). However, before addressing veterinary patients in trials evaluating novel agents, a detailed characterization of its effects *in vitro* is necessary.

Therefore, the aim of this study was to comparatively characterize the influence of PDA-66 and PDA-377 on two human prostate carcinoma cell lines, PC-3 and LNCaP, and on the canine cell line CT1258, which is also the first detailed characterization of these agents on cells derived from solid tumors. Besides cellular analysis, whole transcriptome sequencing was performed. Based on these results, canine PCa is evaluated as a model for clinical trials, accelerating the translation into human patients and providing direct benefit to both species.

Abbreviations: PCa, prostate cancer; MD, mitotic death; DEG, differentially expressed gene; SAC, spindle assembly checkpoint.

MATERIALS AND METHODS

Prostate Carcinoma Cell Lines and Cultivation

Two human and one canine prostate carcinoma cell line were used. The human PC-3 cell line (24) (DSMZ Cat# ACC-465, RRID:CVCL_0035) was cultivated in DMEM/Ham's F-12 medium (Biochrom GmbH, Berlin, Germany). The human LNCaP cell line (25) (DSMZ Cat# ACC-256, RRID:CVCL_0395) was grown in RPMI 1640 medium (Biochrom GmbH). The canine cell line TihoDProAdcarc1258 (26) (CT1258, RRID:CVCL_W737) was established by our group and cultivated in medium 199 (Live Technologies GmbH, Darmstadt, Germany). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS Superior, Biochrom GmbH) and 2% penicillin/streptomycin (Biochrom GmbH). All cells were cultivated at 37°C in a humidified atmosphere of 5% CO_2.

Indolylmaleimides PDA-66 and PDA-377

Synthesis and chemical structures of indolylmaleimides were previously described (15, 16, 27, 28). Both PDA derivatives were dissolved in dimethyl sulfoxide (DMSO, AppliChem GmbH, Darmstadt, Germany), and the stock solutions (10 mM) were stored at −20°C. For experimental use, the PDA dilutions were freshly prepared from the stock solution.

Different PDA concentrations and incubation times were used and compared with DMSO-exposed controls, as the PDA agents themselves were dissolved in DMSO. The final DMSO concentrations of the control samples were equivalent to the highest DMSO doses in the PDA-treated samples to ensure that no possible effects of the solvent were measured.

Live Cell Imaging

PC-3, LNCaP and CT1258 cells were seeded in 96-well plates with a cell density of 1×10^4 cells per well and incubated overnight in 150 µl of culture medium. After 24 h different concentrations of the PDA derivatives (0.25–10 µM) were applied (0.1% DMSO as control). The cells were observed for 72 h in 150 µl incubation medium under a live cell imaging microscope (DMI 6000 B, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) at 37°C with 5% CO_2. An image of each well was captured every 15 min during the incubation period and these single images were combined to create time-lapse movies.

Analysis of Morphology

Morphological changes mediated by PDA were analyzed by May-Grünwald-Giemsa staining.

Microscope slides were placed in 60 cm^2 cell culture dishes and covered with 10 ml of culture medium. Per dish and per cell line, 1×10^6 cells were seeded. On the following day, the microscope slides were transferred to new dishes with incubation medium or 0.15% DMSO for the control cells. The slides were exposed to the PDA agents in two different settings. For the first setting, the slides were incubated with 15 µM PDA-66 or PDA-377 for 24 h, an application equal to the cells used in the transcriptomic analyses. For the second setting, the slides were incubated with 5 µM PDA-66 or PDA-377 for 72 h, a concentration that showed

moderate effects in all cell lines in live cell imaging. Microscope slides were washed with PBS and air dried at room temperature. The slides were stained in May-Grünwald's eosine-methylene blue solution (Merck KGaA, Darmstadt, Germany) (undiluted) for 5 min and rinsed with tap water. Afterwards, they were stained for 15 min in Giemsa's azur eosin methylene blue solution (Merck KGaA) (1:10 dilution) and rinsed thoroughly with tap water again. The slides were left to air dry before analysis.

BrdU Proliferation Assay

Proliferative index of cells in response to PDA-66 and PDA-377 exposure was evaluated using the assay Cell Proliferation ELISA, BrdU (colorimetric) (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). The assay measures the incorporation of the thymidine analog 5-bromo-2-deoxyuridine (BrdU) into newly synthesized DNA of replicating cells using an anti-BrdU monoclonal antibody.

All three cell lines were seeded in 96-well plates with a density of 1×10^4 cells per well and incubated in 150 µl culture medium for 24 h to allow the cells to attach to the surface. Afterwards, the cells were exposed to different concentrations of the PDA derivatives. The human cell lines were treated with 0.25–10 µM of the derivatives and 0.1% (v/v) DMSO as control. CT1258 cells were exposed to 0.25–25 µM of both derivatives (0.25% DMSO as control) as no significant effect was displayed after 24 h with 10 µM PDA-66. The proliferation assay was carried out in accordance with the manufacturer's protocol for adherent cells with the exception that BrdU was added simultaneously with the PDAs. The reaction products were quantified by measuring the absorbance at 370 nm minus the absorbance at 492 nm over a 30 min period using the Multi-Mode Reader Synergy 2 (BioTek Instruments Inc., Winooski, VT, USA). Each single experiment was performed 8-fold.

After this point, no further tests were performed with PDA-377 due to its limited effect on the human cell lines.

Cell Count Analyses

The concentrations used for cell count analysis and apoptosis are based on the 72 h values of the BrdU assay. Concentrations were chosen, that lead to 50% inhibition (2.5 µM PDA-66 in PC-3 and 5 µM PDA-66 in LNCaP). These values were chosen to ensure that there would be some margin in both directions to calculate possible significances, even if the other tests would show higher or lower efficiencies. Based on this criterion, 5 µM PDA-66 should have been used for the CT1258 cell line. However, this concentration showed no effect after 24 h. As it was crucial to be able to detect effects early after exposure for the transcriptomic analysis, 15 µM was used for this cell line, the first concentration to show a significant effect in the BrdU assay after 24 h of exposure.

Cells were seeded in 24-well plates with a cell density of 5×10^4 cells per well in 1 ml culture medium and incubated overnight. Based on the proliferation assay, PC-3 cells were treated with 2.5 µM PDA-66 and 0.025% DMSO, LNCaP with 5 µM PDA-66 (0.05% DMSO), and CT1258 cells with 15 µM PDA-66 (0.15% DMSO). After 24 h, 48 h and 72 h, the cells were detached by TrypLE™ Express (Thermo Fisher Scientific Inc., Waltham, MA, USA) and counted via automatic cell counter (CellometerTM Auto T4, Nexcelom Bioscience LLC, Lawrence, MA, USA). The experiment was carried out in biological replicates three times.

Analyses of Early and Late Stage Apoptosis

Apoptosis rates were determined by staining cells with the fluorescence dyes Annexin V FITC and Propidium Iodide (PI) (Annexin V-FITC Detection Kit, PromoCell GmbH, Heidelberg, Germany) in accordance with the manufacturer's protocol and determined by flow cytometry using a FACSCaliburTM (BD Biosciences GmbH, Heidelberg, Germany). In addition to the cell specific concentrations used in the cell count analysis, two lower concentrations were used to demonstrate that the induction of apoptosis already starts at lower concentrations. The data were evaluated using BD CellQuest software (BD CellQuest Pro, RRID:SCR_014489).

All three cell lines were seeded in 12-well plates with a cell density of 2×10^5 cells per well and incubated in 1.5 ml culture medium overnight and exposed after 24 h to three different PDA-66 concentrations and DMSO based on the BrdU assay and cell count analyses. PC-3 cells were exposed to 0.5, 1.0, and 2.5 µM PDA-66 (0.025% DMSO as reference), LNCaP to 1.0, 2.5, and 5.0 µM PDA-66 (0.05% DMSO), and CT1258 to 7.5, 10 and 15 µM PDA-66 (0.15% DMSO). After the drug exposure period, the medium was saved to collect the non-adherent cell fraction to count all treated cells regardless of their condition. Remaining adherent cells were detached by TrypLE™ Express (Thermo Fisher Scientific Inc, Waltham, MA, USA) and combined with the medium cell fraction. In addition to the double stained samples, for each cell line and each incubation time point, single stained controls were measured. Exposure of the cells to PDA-66 and DMSO as reference was conducted in biological replicates three times.

Transcriptomic Analyses

As PDA-66 is a strong inducer of apoptosis, the transcriptomic analysis was performed on early time points (12 and 24 h). This early time points, before all the apoptosis signaling cascades are fully active and most transcripts would be cell death related, were chosen for the best chance to detect the direct effects of PDA exposure. As 15 µM was the first concentration to show significant effects in CT1258 after 24 h, this concentration was used for all cell lines to keep the transcriptomic data comparable (0.15% DMSO for the control groups). After 12 and 24 h, the cells were harvested and total RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. For each treatment condition (DMSO control and PDA-66 exposed cells), three independent biological replicates were prepared and sequenced.

RNA integrity numbers (RIN) were determined using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). Sequencing libraries were prepared using 1 µg total RNA with RIN > 7. PolyA RNA was enriched and ligated to sequencing adapters using the NEBNext Ultra RNA preparation kit (New England Biolabs Inc., Ipswich, MA,

USA) in accordance with the manufacturer's protocols. Single-read sequencing (75 bp) was conducted on an Illumina NextSeq500 (Illumina Inc., San Diego, CA, USA). Sequences were aligned to the canine genome (Broad CanFam3.1/canFam3, Sep. 2011) using the Burrows-Wheeler Aligner (BWA) (29) (BWA, RRID:SCR_010910). For each of the 24,581 annotated protein-coding canine genes (EMBL gene ID nomenclature), the aligned reads were counted using the R package GAGE (30) (GAGE, RRID:SCR_017067). For the human PC-3 and LNCaP, mapping and read counting were conducted using the RNA Express (version: 1.0.0) workflow within the Illumina basespace environment (BaseSpace, RRID:SCR_011881). In brief, after mapping to the human HG19 reference genome using the STAR (2.3.1s) aligner (STAR, RRID:SCR_015899) read counts for 23,710 annotated RefSeq genes were generated.

Statistical Analyses

Within each experiment, results were described using the mean values of the replicates. The graphs show the mean ± standard deviation. Significant differences between treatment and control were calculated by Dunnett's multiple comparison test for the confidence levels 95, 99, and 99.9% (BrdU proliferation assay and analyses of apoptosis) or student's *t*-test (cell count analyses) using SAS enterprise guide 7.1 (Statistical Analysis System, RRID:SCR_008567). Differences were considered statistically significant for $p < 0.05$. IC50 values were calculated using GraphPad Prism 7.02 (GraphPad Prism, RRID:SCR_002798).

Differential gene expression analyses for canine and human samples were conducted using the BioconductorR package edgeR 3.14.0 (31) (edgeR, RRID:SCR_012802). The average total read counts mapped within annotated genes were 12.2M (STD: 0.7M), 15.8M (STD: 1.1M), 13.5M (STD: 0.9M) for CT1258, PC-3 and LNCaP samples, respectively. Each PDA-66 treatment group (12 or 24 h) was compared to the respective control cells treated with DMSO. After multidimensional scaling and plotting of the data, one PC-3 DMSO treated control sample was identified as outlier and was excluded from further analyses so that the PC-3-24 h-DMSO group consisted of only two independent samples. Genes with a false discovery rate adjusted *p*-value (FDR) of < 0.001 were considered to have significantly different expressions compared to the control samples. These genes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for functional enrichment analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Tool (32, 33) (DAVID, RRID:SCR_001881).

RESULTS

PDA-66 and PDA-377 Cause Morphological Changes

May-Grünwald-Giemsa staining revealed morphological changes in the three tested cell lines (**Figure 1**). Incubation with 5 μM PDA-66 caused an increased amount of large multinucleated cells as well as cells with pyknotic nuclei and karyorrhexis. Moreover, the cells aggregated in clusters and their cytoplasmic borders loosened. In LNCaP and CT1258 cells, 24 h after high-dose (15 μM) PDA-66 application, karyorrhexis was pronounced in most cells.

PDA-377 incubation caused no visual effects after 5 μM treatment for 72 h, and only minor effects on the PC-3 cell line after 15 μM treatment for 24 h. The morphological changes on LNCaP and CT1258 cells were similar to PDA-66 incubation but less pronounced.

Time-lapse imaging movies of the three PCa cell lines showing the cellular response to 5 and 10 μM of either PDA-66 or PDA-377 as well as the control groups are given for demonstration (**Supplementary Movies 1–15**). Moreover, the formation of enlarged multi-nucleated cells as well as apoptosis during or shortly after mitosis was observed at higher PDA concentrations (**Figure 2**).

PDA-66 and PDA-377 Inhibit Proliferation of Prostate Carcinoma Cell Lines

PDA-66 exposure demonstrated an inhibitory effect on both human cell lines and the canine cell line determined by BrdU proliferation assay (**Figure 3**). Application of 2.5 μM PDA-66 resulted in a significant decrease in the proliferative index in PC-3 and LNCaP cells 24 h after treatment. Moreover, PC-3 cells displayed significantly slower proliferation at 1.0 μM PDA-66 exposure after 48 and 72 h. Proliferation was reduced to 50% compared to the DMSO-exposed controls at 2.5 μM PDA-66 for PC-3 and at 5.0 μM for LNCaP cells after 72 h. Compared to the human cell lines, inhibition of proliferation of the canine cell line CT1258 was delayed, but was also more pronounced after 72 h post PDA-66 application, starting with concentrations of 0.5 μM. For all cell lines, the proliferative index did not drop below 25%. IC50 values were 2.07 μM (PC-3), 4.23 μM (LNCaP), and 2.19 μM (canine cell line CT1258) after a 72 h incubation period.

Incubation with PDA-377 also led to a significant decrease in the proliferative index in all tested cell lines. PC-3 and LNCaP cells were inhibited at concentrations of 0.5 μM and 0.25 μM PDA-377 after 48 h. However, proliferation did not drop below 50% compared to the control samples for all tested incubation times and doses in the two human cell lines. For the canine cell line CT1258, the proliferative index dropped below 10% with an IC50 of 3.14 μM after a 72 h incubation period. Compared to PDA-66, PDA-377 treatment displayed a weaker inhibition on the human cell lines, especially within the first 24 h and a slightly stronger effect on the canine PCa cell line.

PDA-66 Induces Apoptosis

Consistent with live cell imaging observations and the proliferation assay, a significant change in the total number of cells was observed 48 h after PDA-66 application in all tested cell lines compared to their tested control cells (**Figure 4**). While the control cells showed a steady increase in cell numbers over time and at least doubled within 72 h, the number of PDA-66 treated cells decreased. The decrease below the amount of seeded cells (5×10^4) indicated the induction of cell death.

PDA-66 significantly induced apoptosis in all three PCa cell lines compared to the DMSO-exposed controls at different concentrations (**Figure 5**, representative dot blots are given

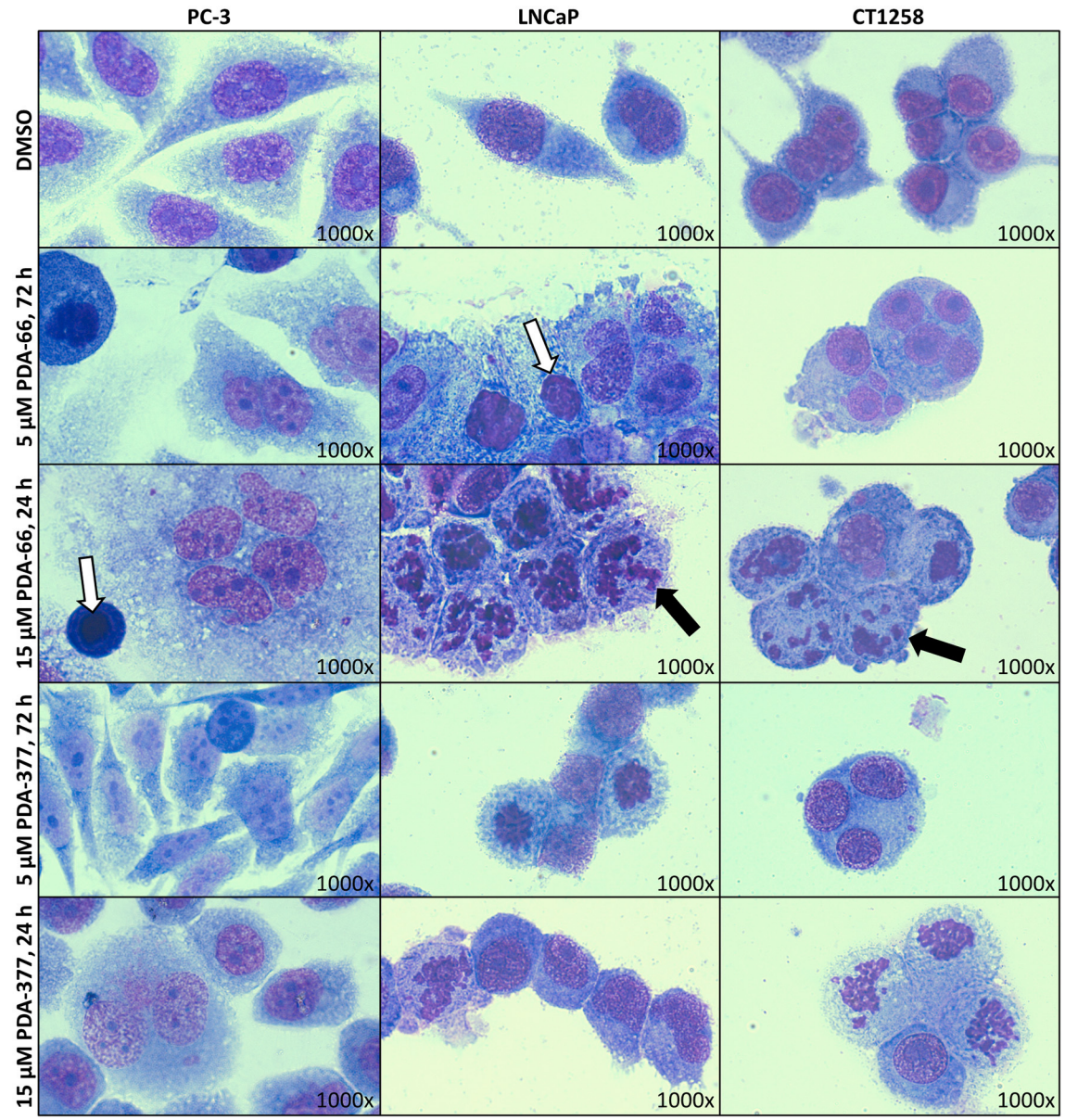

FIGURE 1 | PC-3, LNCaP, and CT1258 cells were grown on microscope slides and incubated with both PDA derivatives for either 72 h with 5 µM PDA or 24 h with 15 µM. Representative pictures are displayed. After incubation with PDA-66, cells tend to aggregate and the cytoplasmic border loosens. Multinucleated cells, pyknosis (white arrows) and karyorrhexis (black arrows) can be determined. Post PDA-377 application, only minor morphological changes can be observed in PC-3 cells. In LNCaP and CT1258 cells, the reactions of cytoplasm and nucleus are similar to incubation with PDA-66.

in **Supplementary Figure 1**). Application of 2.5 µM PDA-66 led to an increase in both early and late apoptotic cells in the two human cell lines after 72 h, reaching up to 35.8%. The rate of late apoptotic/dead cells even increased to 43% in LNCaP cells after incubation with 5 µM PDA-66. In the canine cell line, early apoptotic induction increased over time for all concentrations up to 13.4% (72 h, 15 µM PDA-66), while the rate of late apoptotic/dead cells varied between 20.9 and 52.7%.

Transcriptomic Analyses of PDA-66 Treated Prostate Carcinoma Cells

All cell lines displayed a higher number of differentially expressed genes (DEG; FDR < 0.001) after 12 h compared to the 24 h treatment groups (**Table 1**), correlating with larger distances between clusters of the multidimensional scaling (MDS) plots and the control samples (**Supplementary Figure 2**). PC-3 and CT1258 cells showed more than 4,000 DEG after a 12 h and 24 h incubation period with PDA-66. In the LNCaP cell line, there

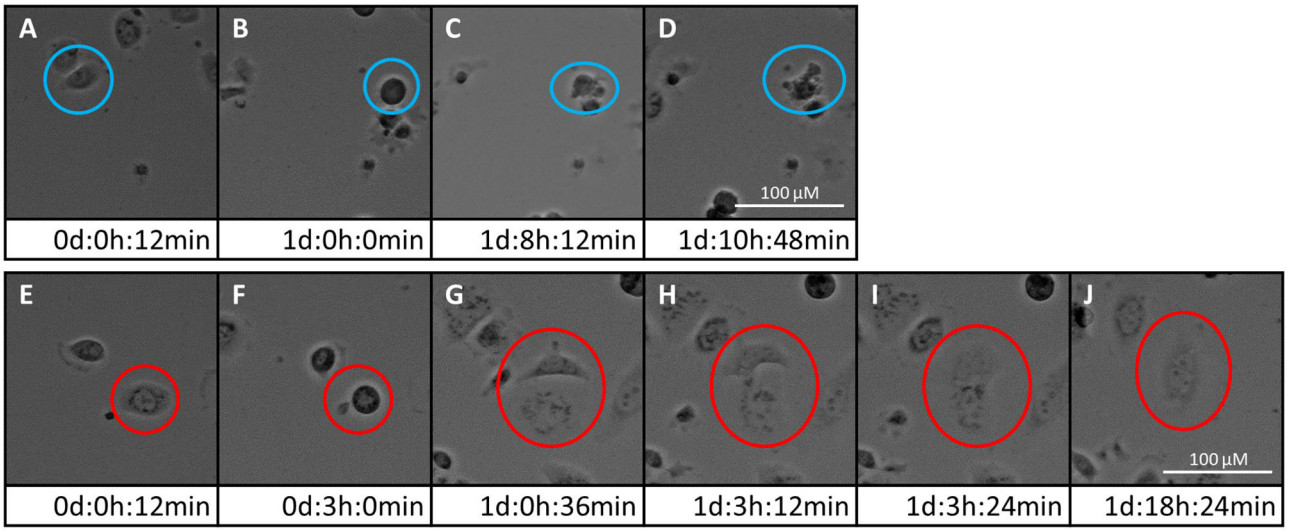

FIGURE 2 | Two fates of PC-3 cells exposed to 5 µM PDA-66. Live cell imaging pictures show the same cell and image section during a 72 h incubation period. **(A–D)** apoptosis of cell in blue circle; **(A)** start of live cell imaging; diploid cell; **(B)** cell becomes round/detached in preparation for cell division; **(C)** formation of apoptotic bodies; **(D)** dead cell; **(E–J)** mitotic slippage of cell in red circle; **(E)** start of live cell imaging; diploid cell; **(F)** cell becomes round/detached in preparation for cell division; **(G)** two daughter cells with unbalanced distribution of chromosomes; **(H,I)** daughter cells merge again; **(J)** survival of a tetraploid/multi-nucleated cell. Please see **Supplementary Material** for the full movie.

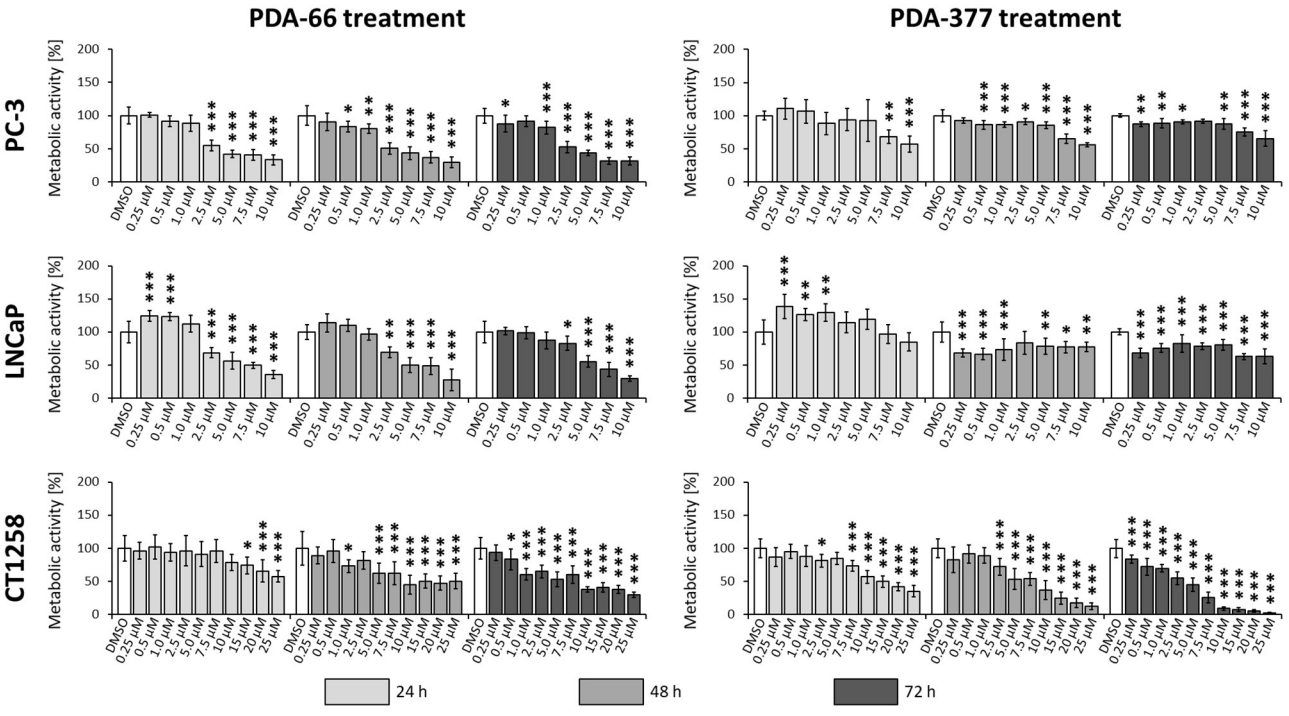

FIGURE 3 | PC-3, LNCaP and CT1258 cells were exposed to increasing concentrations of PDA-66 and PDA-377 ranging from 0.25 to 10 µM and up to 25 µM in the case of CT1258. The cells were incubated for 24, 48, and 72 h, respectively. BrdU proliferation assay was used to determine the proliferative index. The results are expressed as a percentage of the DMSO-exposed control cells. The diagrams show the mean ± standard deviation of eight measurements. Significance of a treatment effect compared to the respective DMSO control (white bar) was determined using Dunnett's Multiple Comparison Test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

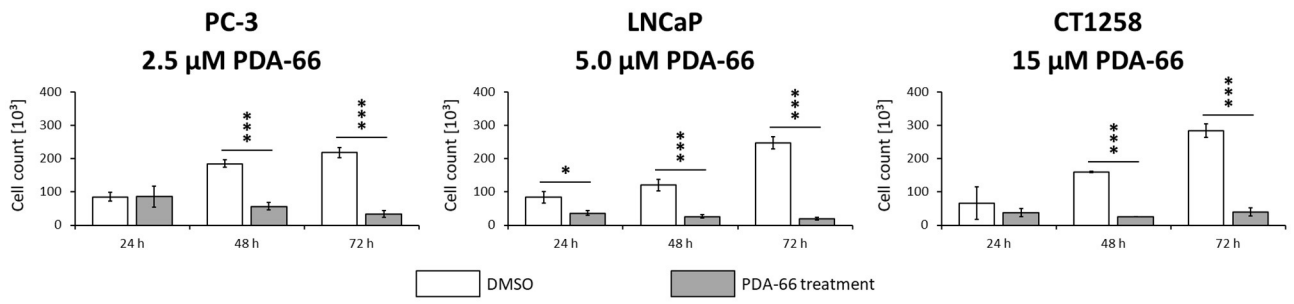

FIGURE 4 | PC-3 cells were incubated with 2.5 µM PDA-66, LNCaP cells with 5 µM PDA-66 and CT1258 cells with 15 µM PDA-66 for 24, 48, and 72 h. After the incubation period, cells were counted via automatic cell counter. The diagrams show the mean ± standard deviation of three independent counting experiments. Significance of a treatment effect compared to the DMSO control was determined using the student's *t*-test. *, $p < 0.05$; ***, $p < 0.001$.

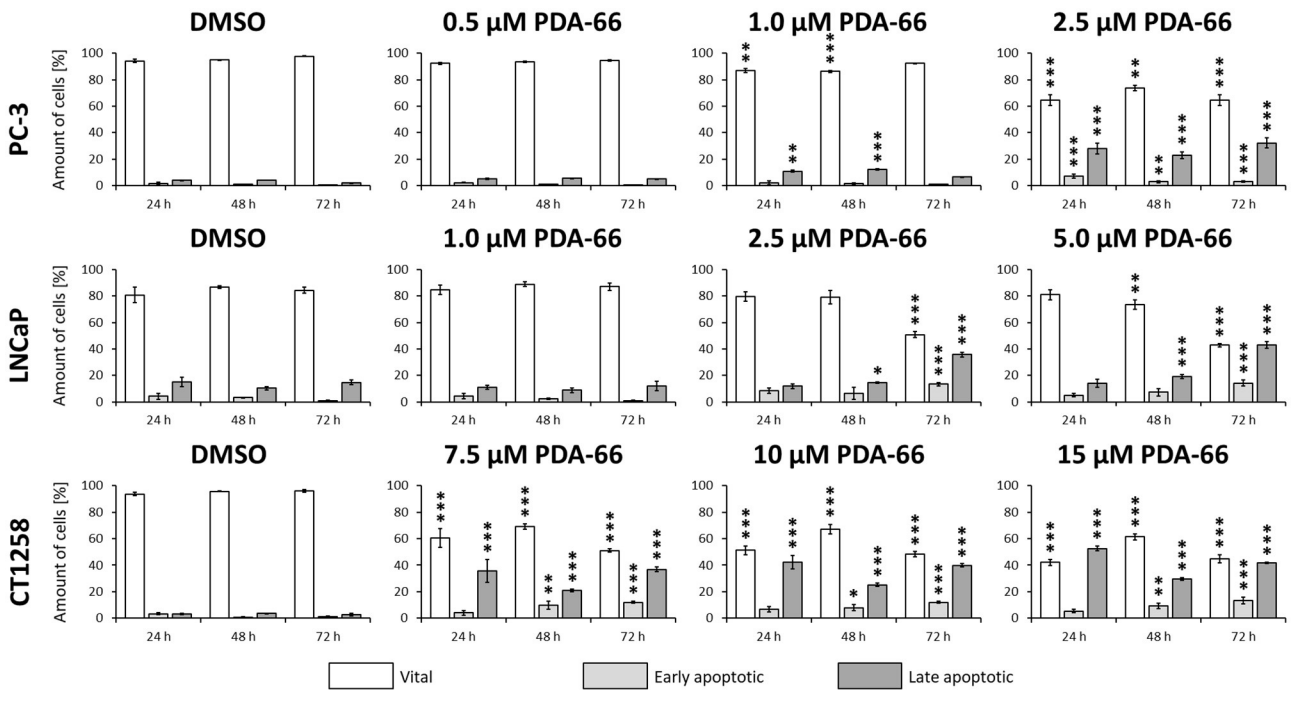

FIGURE 5 | PC-3 cells were exposed to 0.5, 1.0, and 2.5 µM PDA-66, LNCaP cells were exposed to 1.0, 2.5, and 5.0 µM PDA-66, and CT1258 cells were exposed to 7.5, 10, and 15 µM PDA-66 for 24, 48, and 72 h, respectively. As a reference, DMSO-exposed cells were analyzed. Analysis of apoptosis was performed using Annexin V-FITC and propidium iodide (PI) staining with subsequent flow cytometry analysis. Rates of vital (FITC-, PI-) early apoptotic (FITC+, PI-) and late apoptotic (FITC+/-, PI+) cells are displayed as the mean ± standard deviation of three independent measurements. Significances compared to the DMSO control were determined using Dunnett's multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

were only up to 479 DEGs. Therefore, we focused on LNCaP for the KEGG pathway analysis (**Table 2**) with corresponding PC-3 and CT1258 values in comparison. Cell cycle, DNA replication and p53 signaling pathway were the only KEGG pathways significantly enriched (FDR < 0.05) in all three cell lines. Within these three pathways, 38 genes showed at least three significant values compared to the corresponding controls for the six analyzed groups and were further analyzed (**Figure 6**). The fold change pattern was highly consistent between the six groups. Within the relevant 38 genes, only five genes were inconsistent in the direction of their deregulation (upregulated vs. downregulated) across the three cell lines.

DISCUSSION

In the present study, the effects of PDA-66 and PDA-377, two indolylmaleimide derivatives, were comparatively evaluated on three prostate carcinoma cell lines with a diverse genetic

background. The human PC-3 and LNCaP cells are among the most used and best described PCa cell lines (34, 35), both established from metastatic adenocarcinomas. While PC-3 is androgen-independent (36), LNCaP is described as androgen-sensitive (37, 38). Well-characterized canine PCa cell lines are rare. Herein, CT1258 was used, a canine cell line derived by our working group from a metastatic adenocarcinoma, which has been previously established and characterized (26, 39–42).

Both PDA derivatives caused two distinct morphological changes in all tested cell lines. Live cell imaging and May-Grünwald-Giemsa staining revealed clear apoptotic features through observed pyknosis and karyorrhexis in May-Grünwald-Giemsa staining as well as the formation of apoptotic bodies in live cell imaging. These features match the proposed mechanism of induced mitotic arrest and subsequent MD. Secondly, some enlarged multinucleated cells formed. Cells can overcome the mitotic arrest before MD is triggered; a process known as slippage. After DNA replication in the S phase and escaping the mitotic arrest, these cells fail cytokinesis and/or the equal distribution of chromosomes, leading to enlarged multi-nucleated cells similar to those we observed (22, 43, 44).

Proliferation was measured via BrdU assay to quantify growth inhibition seen in live cell imaging. PDA-66 caused significant and dose-dependent anti-proliferative effects in all tested PCa cell lines. The herein demonstrated doses to inhibit proliferation significantly by at least 50% were between 5 and 10 μM PDA-66 within 72 h. The analyzed PCa cell lines were slightly less sensitive compared to other neoplastic cell lines like human neuroblastoma, human acute lymphoblastic leukemia or canine lymphoma (15–17). Consistent with live cell imaging movies, a significant reduction in total cell count and induction of apoptosis were separately quantified after PDA-66 incubation in all cell lines.

PDA-377 also induced anti-proliferative effects in all tested cell lines. However, for both human cell lines, the inhibition of proliferation was limited and most likely dose-independent, as a comparable decrease in proliferation was measured for almost all tested concentrations after 48 h. The proliferative index did not drop below 50%. In the canine cell line, the proliferation inhibition was clearly dose-dependent. After a 72 h incubation period, the proliferative index dropped below 10% with an IC50 of 3.14 μM. PDA-377 may be suitable for the

TABLE 1 | Number of differentially expressed genes (DEGs) in treated prostate carcinoma cell lines compared to control samples.

Cell line	Application	DEGs
PC-3	15 μM PDA-66, 12 h	5,198
LNCaP	15 μM PDA-66, 12 h	479
CT1258	15 μM PDA-66, 12 h	5,316
PC-3	15 μM PDA-66, 24 h	4,013
LNCaP	15 μM PDA-66, 24 h	205
CT1258	15 μM PDA-66, 24 h	4,289

TABLE 2 | KEGG pathways in LNCaP cells with an FDR < 0.05 and the corresponding values of these pathways in PC-3 and CT1258 cells after 12 h **(A)** and 24 h **(B)** of PDA-66 exposure.

(A)	LNCaP		PC-3		CT1258	
KEGG pathways, 12 h PDA-66	**Genes**	**FDR**	**Genes**	**FDR**	**Genes**	**FDR**
Cell cycle	29	4.99E-18	54	8.79E-04	69	2.87E-09
DNA replication	14	3.29E-11	23	1.82E-04	22	1.99E-03
p53 signaling pathway	10	7.24E-04	30	2.98E-02	38	1.49E-05
Fanconi anemia pathway	9	8.06E-04	23	1.60E-01	32	8.51E-06
Retinol metabolism	8	1.88E-02				
Base excision repair	6	1.96E-02	16	1.84E-01	20	8.62E-03
Pyrimidine metabolism	10	2.00E-02	35	7.51E-01	44	3.77E-03
Progesterone-mediated oocyte maturation	9	2.46E-02			45	7.43E-05
Mismatch repair	5	3.46E-02	11	5.62E-01	17	6.13E-04
(B)	**LNCaP**		**PC-3**		**CT1258**	
KEGG pathways, 24 h PDA-66	**Genes**	**FDR**	**Genes**	**FDR**	**Genes**	**FDR**
Cell cycle	14	6.44E-08	46	1.16E-03	54	1.01E-11
Retinol metabolism	7	2.18E-03				
DNA replication	5	1.36E-02	24	5.55E-07		
p53 signaling pathway	6	2.02E-02	21	5.88E-01	27	4.16E-05
Metabolism of xenobiotics by cytochrome P450	6	3.12E-02				
Chemical carcinogenesis	6	4.35E-02				

Missing values showed no enrichment of genes for this pathway.

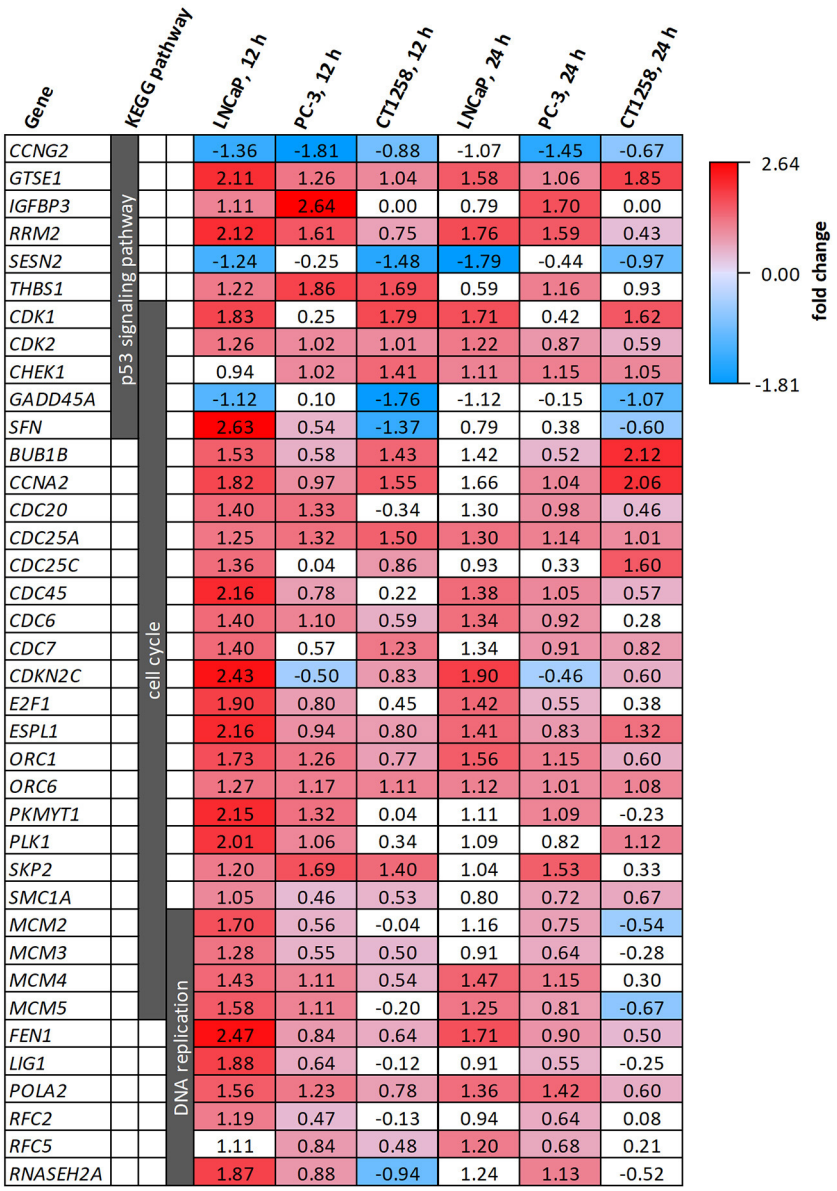

FIGURE 6 | DEGs belonging to the KEGG pathways p53 signaling pathway, cell cycle and DNA replication after PDA-66 exposure. Numbers given for each gene are the fold changes expressed as logarithmized ratios (base 2) in the PCa cell lines after 12 and 24 h. Values highlighted in red or blue indicate significantly higher or lower expression compared to the DMSO control. Non-highlighted values were not significantly different (FDR $\geq$ 0.001).

treatment of prostate cancer in dogs. Nevertheless, no further testing with this substance was conducted, as the limited effect on the human cell lines contradicts the cross-species approach with dogs as a model to generate transferable findings to the benefit of both species.

To confirm whether MD takes place, whole transcriptome sequencing was conducted. The transcriptome analysis revealed the KEGG pathways cell cycle, p53 signaling pathway and DNA replication to be the only pathways with an enrichment of genes across the three PCa cell lines exposed to PDA-66. Other known microtubule-depolymerizing agents, like *Vinca* alkaloids or colchicine-binding site drugs, lead to failures in mitotic spindle formation and therefore chromosome segregation, which triggers the spindle assembly checkpoint (SAC; mitotic checkpoint). SAC activation causes mitotic arrest in the metaphase of the M phase of the cell cycle. Within the enriched KEGG cell cycle pathway, several important genes involved in SAC are upregulated after PDA-66 exposure, e.g., *BUB1B*, *CDC20, CDK1,* and *MAD2.* These DEGs are commonly involved in MD (20, 21, 45, 46).

MD resembles intrinsic apoptosis, and p53 and its pathway play a major role in most cells undergoing MD (19, 21, 46). Little is known about the downstream targets of SAC that trigger MD following a prolonged mitotic arrest (20), but the upregulated cyclin-dependent kinases CDK1 and CDK2, normally known for cell cycle progression, are directly linked with apoptosis after stress events like a mitotic arrest (47, 48).

Interestingly, there was also an upregulation of genes involved in G1/S phase cell cycle progression and DNA replication, especially within the two human cell lines. Given the M phase cell cycle arrest induced by SAC, an enrichment of S phase transcripts is unexpected. Moreover, G1/S phase transcripts were downregulated in canine lymphoma cell lines after PDA-66 exposure (17). However, formation of enlarged multinuclear cells, as observed in the PCa cell lines, has not been described for these lymphoma cells post PDA-66 incubation. These multinuclear cells have three possible fates: Post-slippage cell death (before re-entering mitosis), senescence or further proliferation (43, 49, 50). Senescent polyploid cells, locked in the next interphase after slippage, might be an explanation for the enrichment of G1/S phase transcripts seen in the PCa cell lines after PDA-66 application. For example, tumor suppressor *BRCA1* was significantly upregulated compared to the controls in all PCa cell line samples post PDA-66 exposure (data not shown), a gene that plays an important role in interphase checkpoint activation (51).

The observed morphological changes and the transcriptome analysis match other anti-mitotic drugs causing MD. While most cells die as a result of this intrinsic cell death, some cells remain in a polyploid state after mitotic slippage. These cells are usually senescent and therefore incapable of further proliferation. However, they bear the risk of reinforcing the tumor, as their potential proliferation can lead to aneuploid cells, a hallmark of cancer (43, 49, 50, 52). The selection of aneuploid karyotypes promotes aneuploidy tolerance, a major driving force in cancer evolution and drug resistance (46, 52). Docetaxel, the first-line treatment for castration-resistant prostate cancer in men, is also an anti-mitotic drug causing microtubule perturbations. Similar to PDA-66, treating PC-3 cells with Docetaxel generates multinucleated polyploid cells, which have been linked to clinical relapse and chemoresistance (53). While slippage limits the effectiveness of anti-microtubule drugs, these substances have been proven successful in clinics and are commonly used in a variety of malignancies in addition to PCa (18, 20, 49, 50).

PCa cell lines of human and canine origin showed comparable cellular and molecular reactions, including significant inhibition of proliferation and induced apoptosis after PDA-66 incubation. Furthermore, efficacy of PDA-66 was not influenced by the androgen status of the cell line. As PDA-66 mediates its effects in a hormone-independent manner through microtubule destabilization, the compound is suitable to be used in androgen-dependent as well as -independent PCa subtypes. Based on the findings of this study, PDA-66 appears to be a worthwhile agent for further evaluation and potential treatment of PCa in dogs and, if successfully introduced, also in humans.

The cellular and molecular *in vitro* characterization of PDA-66 effects on PCa cells encourage, as a next step, to initiate an early clinical trial in dogs to evaluate the compound in naturally occurring PCa in the presence of an immune system. First, however, a pharmacokinetic study in dogs needs to be performed. An early study in mice revealed that *i.p.* application of 100 mg/kg PDA-66 once per day was well-tolerated (17). The value for humans will be enhanced by the fact that dogs have several advantages over laboratory models, e.g., mice for comparative pharmacokinetics, as dogs represent an outbred non-immunodeficient model population. As companion animals, dogs are exposed to the same environmental risk factors as humans and the parallel evolution of humans and dogs led to a more similar genomic organization compared to rodents (54–57). Treatment of spontaneously occurring PCa in dogs can provide additional valuable information that can benefit both humans and dogs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162832.

AUTHOR CONTRIBUTIONS

JS carried out partial BrdU assay, May-Grünwald-Giemsa staining, cell treatment and RNA extraction for whole transcriptome analysis, performed data analysis, interpretation and visualization, and drafted the manuscript. IN provided the resources for all cellular analyses, supervised the study, and revised the manuscript. JB performed transcriptomic sequencing and revised the manuscript. DJ carried out partial BrdU assay, live cell imaging, cell count, and flow cytometry analyses. CR helped with primary study design. AP-D synthesized the PDA compounds. AR participated in initial compound generation. LH evaluated the May-Grünwald-Giemsa staining. MB supervised PDA compound design and synthesis. BB and ES provided the resources for the transcriptomic analysis and supervised the transcriptomic work packages. CJ and HM performed the primary study design. In addition, HM supervised the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.558135/full#supplementary-material

Further supplementary videos can be requested from the corresponding author.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2018) 68:394–424. doi: 10.3322/caac.21492
2. Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate cancer population: a systematic review. *Int J Clin Prac.* (2011) 65:1180–92. doi: 10.1111/j.1742-1241.2011.02799.x
3. Attard G, Parker C, Eeles RA, Schroder F, Tomlins SA, Tannock I, et al. Prostate cancer. *Lancet.* (2016) 387:70–82. doi: 10.1016/S0140-6736(14)61947-4
4. Hoang DT, Iczkowski KA, Kilari D, See W, Nevalainen MT. Androgen receptor-dependent and -independent mechanisms driving prostate cancer progression: opportunities for therapeutic targeting from multiple angles. *Oncotarget.* (2017) 8:3724–45. doi: 10.18632/oncotarget.12554
5. Vlachostergios PJ, Puca L, Beltran H. Emerging variants of castration-resistant prostate cancer. *Curr Oncol Rep.* (2017) 19:32. doi: 10.1007/s11912-017-0593-6
6. Rowell JL, McCarthy DO, Alvarez CE. Dog models of naturally occurring cancer. *Trends Mol Med.* (2011) 17:380–8. doi: 10.1016/j.molmed.2011.02.004
7. Pinho SS, Carvalho S, Cabral J, Reis CA, Gartner F. Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl Res.* (2012) 159:165–72. doi: 10.1016/j.trsl.2011.11.005
8. Schrank M, Romagnoli S. Prostatic neoplasia in the intact and castrated dog: how dangerous is castration? *Animals.* (2020) 10:85. doi: 10.3390/ani10010085
9. Cornell KK, Bostwick DG, Cooley DM, Hall G, Harvey HJ, Hendrick MJ, et al. Clinical and pathologic aspects of spontaneous canine prostate carcinoma: a retrospective analysis of 76 cases. *Prostate.* (2000) 45:173–83. doi: 10.1002/1097-0045(20001001)45:2<173::AID-PROS12>3.0.CO;2-R
10. Ravicini S, Baines SJ, Taylor A, Amores-Fuster I, Mason SL, Treggiari E. Outcome and prognostic factors in medically treated canine prostatic carcinomas: a multi-institutional study. *Vet Comp Oncol.* (2018) 16:450–8. doi: 10.1111/vco.12400
11. Bennett TC, Matz BM, Henderson RA, Straw RC, Liptak JM, Selmic LE, et al. Total prostatectomy as a treatment for prostatic carcinoma in 25 dogs. *Vet Surg.* (2018) 47:367–77. doi: 10.1111/vsu.12768
12. Palmieri C, Lean FZ, Akter SH, Romussi S, Grieco V. A retrospective analysis of 111 canine prostatic samples: histopathological findings and classification. *Res Vet Sci.* (2014) 97:568–73. doi: 10.1016/j.rvsc.2014.11.006
13. Leroy BE, Northrup N. Prostate cancer in dogs: comparative and clinical aspects. *Vet J.* (2009) 180:149–62. doi: 10.1016/j.tvjl.2008.07.012
14. Reimann-Berg N, Willenbrock S, Murua Escobar H, Eberle N, Gerhauser I, Mischke R, et al. Two new cases of polysomy 13 in canine prostate cancer. *Cytogenetic Genome Res.* (2011) 132:16–21. doi: 10.1159/000317077
15. Eisenloffel C, Schmole AC, Pews-Davtyan A, Brennfuhrer A, Kuznetsov SA, Hubner R, et al. Interference of a novel indolylmaleimide with microtubules induces mitotic arrest and apoptosis in human progenitor and cancer cells. *Biochem Pharmacol.* (2013) 85:763–71. doi: 10.1016/j.bcp.2012.12.013
16. Kretzschmar C, Roolf C, Langhammer TS, Sekora A, Pews-Davtyan A, Beller M, et al. The novel arylindolylmaleimide PDA-66 displays pronounced antiproliferative effects in acute lymphoblastic leukemia cells. *BMC Cancer.* (2014) 14:71. doi: 10.1186/1471-2407-14-71
17. Liu W, Beck J, Schmidt LC, Roolf C, Pews-Davtyan A, Rutgen BC, et al. Characterization of the novel indolylmaleimides' PDA-66 and PDA-377 effect on canine lymphoma cells. *Oncotarget.* (2016) 7:35379–89. doi: 10.18632/oncotarget.9297
18. Field JJ, Kanakkanthara A, Miller JH. Microtubule-targeting agents are clinically successful due to both mitotic and interphase impairment of microtubule function. *Bioorganic Med Chem.* (2014) 22:5050–9. doi: 10.1016/j.bmc.2014.02.035
19. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. *Cell Death Differ.* (2018) 25:486–541. doi: 10.1038/s41418-018-0102-y
20. Ruan W, Lim HH, Surana U. Mapping mitotic death: functional integration of mitochondria, spindle assembly checkpoint and apoptosis. *Front Cell Dev Biol.* (2018) 6:177. doi: 10.3389/fcell.2018.00177
21. Denisenko TV, Sorokina IV, Gogvadze V, Zhivotovsky B. Mitotic catastrophe and cancer drug resistance: a link that must to be broken. *Drug Resist Updates.* (2016) 24:1–12. doi: 10.1016/j.drup.2015.11.002
22. Mc Gee MM. Targeting the mitotic catastrophe signaling pathway in cancer. *Mediat Inflamm.* (2015) 2015:146282. doi: 10.1155/2015/146282
23. Gardner HL, Fenger JM, London CA. Dogs as a model for cancer. *Ann Rev Anim Biosci.* (2016) 4:199–222. doi: 10.1146/annurev-animal-022114-110911
24. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol.* (1979) 17:16–23.
25. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, et al. LNCaP model of human prostatic carcinoma. *Cancer Res.* (1983) 43:1809–18.
26. Winkler S, Murua Escobar H, Eberle N, Reimann-Berg N, Nolte I, Bullerdiek J. Establishment of a cell line derived from a canine prostate carcinoma with a highly rearranged karyotype. *J Heredity.* (2005) 96:782–5. doi: 10.1093/jhered/esi085
27. Pews-Davtyan A, Tillack A, Ortinau S, Rolfs A, Beller M. Efficient palladium-catalyzed synthesis of 3-aryl-4-indolylmaleimides. *Organ Biomol Chem.* (2008) 6:992–7. doi: 10.1039/b719160j
28. Schmole AC, Brennfuhrer A, Karapetyan G, Jaster R, Pews-Davtyan A, Hubner R, et al. Novel indolylmaleimide acts as GSK-3beta inhibitor in human neural progenitor cells. *Bioorgan Med Chem.* (2010) 18:6785–95. doi: 10.1016/j.bmc.2010.07.045
29. Li H, Durbin R. Fast and accurate short read alignment with burrows-Wheeler transform. *Bioinformatics.* (2009) 25:1754–60. doi: 10.1093/bioinformatics/btp324
30. Luo W, Friedman MS, Shedden K, Hankenson KD, Woolf PJ. GAGE: generally applicable gene set enrichment for pathway analysis. *BMC Bioinform.* (2009) 10:161. doi: 10.1186/1471-2105-10-161
31. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
32. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* (2009) 4:44–57. doi: 10.1038/nprot.2008.211
33. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* (2009) 37:1–13. doi: 10.1093/nar/gkn923
34. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines–part 2. *J Urol.* (2005) 173:360–72. doi: 10.1097/01.ju.0000149989.01263.dc
35. Cunningham D, You Z. *In vitro* and *in vivo* model systems used in prostate cancer research. *J Biol Methods.* (2015) 2:e17. doi: 10.14440/jbm.2015.63
36. Katsuoka Y, Hoshino H, Shiramizu M, Sakabe K, Seiki K. Autoradiographic and cytochemical localization of androgen in human prostatic cancer cell lines. *Urology.* (1986) 28:228–31. doi: 10.1016/0090-4295(86)90048-8
37. Berns EM, de Boer W, Mulder E. Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor containing human prostate tumor cell line LNCaP. *Prostate.* (1986) 9:247–59. doi: 10.1002/pros.2990090305
38. Langeler EG, van Uffelen CJ, Blankenstein MA, van Steenbrugge GJ, Mulder E. Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP. *Prostate.* (1993) 23:213–23. doi: 10.1002/pros.2990230304
39. Fork MA, Murua Escobar H, Soller JT, Sterenczak KA, Willenbrock S, Winkler S, et al. Establishing an *in vivo* model of canine prostate carcinoma using the new cell line CT1258. *BMC Cancer.* (2008) 8:240. doi: 10.1186/1471-2407-8-240
40. Willenbrock S, Wagner S, Reimann-Berg N, Moulay M, Hewicker-Trautwein M, Nolte I, et al. Generation and characterisation of a canine EGFP-HMGA2 prostate cancer *in vitro* model. *PLoS ONE.* (2014) 9:e98788. doi: 10.1371/journal.pone.0098788
41. Liu W, Moulay M, Willenbrock S, Roolf C, Junghanss C, Ngenazahayo A, et al. Comparative characterization of stem cell marker expression, metabolic activity and resistance to doxorubicin in adherent and spheroid cells derived from the canine prostate adenocarcinoma cell line CT1258. *Anticancer Res.* (2015) 35:1917–27.

42. Packeiser EM, Hewicker-Trautwein M, Thiemeyer H, Mohr A, Junginger J, Schille JT, et al. Characterization of six canine prostate adenocarcinoma and three transitional cell carcinoma cell lines derived from primary tumor tissues as well as metastasis. *PLoS ONE.* (2020) 15:e0230272. doi: 10.1371/journal.pone.0230272
43. Vitale I, Galluzzi L, Castedo M, Kroemer G. Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Biol.* (2011) 12:385–92. doi: 10.1038/nrm3115
44. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene.* (2004) 23:2825–37. doi: 10.1038/sj.onc.1207528
45. London N, Biggins S. Signalling dynamics in the spindle checkpoint response. *Nat Rev Mol Cell Biol.* (2014) 15:736–47. doi: 10.1038/nrm3888
46. Haschka M, Karbon G, Fava LL, Villunger A. Perturbing mitosis for anti-cancer therapy: is cell death the only answer? *EMBO Rep.* (2018) 19:e45440. doi: 10.15252/embr.201745440
47. Zhou L, Cai X, Han X, Xu N, Chang DC. CDK1 switches mitotic arrest to apoptosis by phosphorylating Bcl-2/Bax family proteins during treatment with microtubule interfering agents. *Cell Biol Int.* (2014) 38:737–46. doi: 10.1002/cbin.10259
48. Megyesi J, Tarcsafalvi A, Seng N, Hodeify R, Price PM. Cdk2 phosphorylation of Bcl-xL after stress converts it to a pro-apoptotic protein mimicking Bax/Bak. *Cell Death Discov.* (2016) 2:15066. doi: 10.1038/cddiscovery.2015.66
49. Nakayama Y, Inoue T. Antiproliferative fate of the tetraploid formed after mitotic slippage and its promotion; a novel target for cancer therapy based on microtubule poisons. *Molecules.* (2016) 21:663. doi: 10.3390/molecules21050663
50. Cheng B, Crasta K. Consequences of mitotic slippage for antimicrotubule drug therapy. *Endocr Relat Cancer.* (2017) 24:T97–106. doi: 10.1530/ERC-17-0147
51. Goldstein M, Kastan MB. Repair vs. checkpoint functions of BRCA1 are differentially regulated by site of chromatin binding. *Cancer Res.* (2015) 75:2699–707. doi: 10.1158/0008-5472.CAN-15-0400
52. Santaguida S, Amon A. Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nat Rev Mol Cell Biol.* (2015) 16:473–85. doi: 10.1038/nrm4025
53. Mittal K, Donthamsetty S, Kaur R, Yang C, Gupta MV, Reid MD, et al. Multinucleated polyploidy drives resistance to docetaxel chemotherapy in prostate cancer. *Br J Cancer.* (2017) 116:1186–94. doi: 10.1038/bjc.2017.78
54. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest.* (2000) 18:781–92. doi: 10.3109/07357900009012210
55. Axiak-Bechtel SM, Maitz CA, Selting KA, Bryan JN. Preclinical imaging and treatment of cancer: the use of animal models beyond rodents. *Q J Nucl Med Mol Imaging.* (2015) 59:303—16.
56. Park JS, Withers SS, Modiano JF, Kent MS, Chen M, Luna JI, et al. Canine cancer immunotherapy studies: linking mouse and human. *J Immunother Cancer.* (2016) 4:97. doi: 10.1186/s40425-016-0200-7
57. Hansen K, Khanna C. Spontaneous and genetically engineered animal models; use in preclinical cancer drug development. *Eur J Cancer.* (2004) 40:858–80. doi: 10.1016/j.ejca.2003.11.031

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REVIEW
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From Conventional to Precision Therapy in Canine Mammary Cancer: A Comprehensive Review

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Canine mammary tumors (CMTs) are the most common neoplasm in intact female dogs. Canine mammary cancer (CMC) represents 50% of CMTs, and besides surgery, which is the elective treatment, additional targeted and non-targeted therapies could offer benefits in terms of survival to these patients. Also, CMC is considered a good spontaneous intermediate animal model for the research of human breast cancer (HBC), and therefore, the study of new treatments for CMC is a promising field in comparative oncology. Dogs with CMC have a comparable disease, an intact immune system, and a much shorter life span, which allows the achievement of results in a relatively short time. Besides conventional chemotherapy, innovative therapies have a large niche of opportunities. In this article, a comprehensive review of the current research in adjuvant therapies for CMC is conducted to gather available information and evaluate the perspectives. Firstly, updates are provided on the clinical–pathological approach and the use of conventional therapies, to delve later into precision therapies against therapeutic targets such as hormone receptors, tyrosine kinase receptors, *p*53 tumor suppressor gene, cyclooxygenases, the signaling pathways involved in epithelial–mesenchymal transition, and immunotherapy in different approaches. A comparison of the different investigations on targeted therapies in HBC is also carried out. In the last years, the increasing number of basic research studies of new promising therapeutic agents on CMC cell lines and CMC mouse xenografts is outstanding. As the main conclusion of this review, the lack of effort to bring the *in vitro* studies into the field of applied clinical research emerges. There is a great need for well-planned large prospective randomized clinical trials in dogs with CMC to obtain valid results for both species, humans and dogs, on the use of new therapies. Following the One Health concept, human and veterinary oncology will have to join forces to take advantage of both the economic and technological resources that are invested in HBC research, together with the innumerable advantages of dogs with CMC as a spontaneous animal model.

Keywords: canine mammary cancer, targeted therapy, markers, immunotherapy, immunophenotyping, hormonal therapy, tyrosine kinase receptors inhibitors

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INTRODUCTION

Canine mammary tumors (CMTs) are a highly heterogeneous group of neoplasms that represent between 50 and 70% of all tumors in intact female dogs (1–4). The prevalence varies depending on the geographic location, being greater in countries where ovariectomy is not routinely performed (4). In these countries, the prevalence of mammary neoplasms in female dogs is three times higher than the prevalence in women (3). Historically, roughly 50% of CMTs are considered to be malignant (1, 2, 5, 6). However, recent studies have shown an increase in malignant *vs.* benign tumors over the last years, a similar trend that has been detected in human medicine (2). Canine mammary cancer (CMC) and human breast cancer (HBC) share not only the aforesaid trend but also many epidemiological, environmental, biological, clinical, genetic, and pathological features, including a remarkable histological and molecular heterogeneity. Many authors have claimed CMC as a good spontaneous model for the study of HBC, especially the inflammatory mammary cancer, the deadliest type (7–12). In female dogs and in women, mammary cancer is the most frequently diagnosed malignancy (4) and the leading cause of cancer-related death in women worldwide (13). For this reason, over the past two decades, innovative HBC treatments have incredibly evolved to place emphasis on more molecularly directed individual therapies while diminishing radio- and chemotherapy to reduce the adverse effects of treatment (14). Since the publication of the intrinsic molecular classification of Perou and Solie in 2000, which distinguished four subtypes of HBC (luminal A, luminal B, basal-like, and HER-2 enriched), the clinical management shifted to a biology-centered approach, based on the expressions of estrogen and progesterone receptors (luminal subtypes), the human epidermal growth factor receptor 2 (HER-2), and basal markers (positive for high-molecular-weight cytokeratins, as cytokeratins 5/6, 14, and 17) (15). Nowadays, the classification of five molecular subtypes (luminal A, luminal B HER-2–, luminal B HER-2+, HER-2 enriched, and triple negative) is the most widely utilized in human medicine to elect targeted therapy: anti-estrogenic drugs for the luminal subtypes and anti-HER-2 treatment for the HER-2-enriched tumors. Unfortunately, since triple-negative mammary cancer does not currently have a specific targeted therapy, its prognosis is poor (14). Surgery is the treatment of choice in both HBC and CMC, and adjuvant therapies are only given on a routine basis in HBC. On the contrary to HBC treatment, adjuvant chemotherapy has not been proven to have a clear benefit in dogs with CMC yet (16–19). In spite that the application of clinical staging of patients with CMC and the histological grading of the neoplasms have helped to standardize prognosis and treatments, no precision therapies are routinely administered to dogs bearing mammary neoplasms. For all these reasons, the CMC-related mortality is relatively high: over 40% of the patients die within a year after diagnosis (20). There is an urge to developing updated therapeutic protocols and targeted therapies. Therefore, a comprehensive review of the current research in adjuvant therapies for CMC is conducted here to gather available information and evaluate the perspectives.

CONVENTIONAL AND NEW CLINICAL AND HISTOLOGICAL APPROACHES

Staging System

Dogs with CMC are staged according to the modified World Health Organization (WHO) TNM system (T, size of tumor; N, affectation of lymph nodes; M, distant metastasis) (21).

More recently, a pathological staging system inspired in human oncology, in which T is replaced by pathologic tumor size (pT) and N is replaced by the pathologic nodal status (pN), with the addition of lymphovascular invasion (LVI) has been proposed, the stages being 0, I, II, IIIA, and IIIB (22). The use of this system is still limited, the major criticism being its inability to discriminate different stages of malignancy in cases with no LVI or lymph node affectation, which are the vast majority.

The current staging system is not flexible enough to allow prognostic differences between specific tumor types and subtypes and does not consider tumor grades or lymphovascular invasion (21). Therefore, new "bio-score" systems combining the anatomical staging (TNM) with many histological and biological variables have been developed: multivariate scoring (scores from 0 to 40) and refined flexible scoring (scores from 0 to 6.5). Although both systems were accurate in predicting the survival, the refined flexible scoring was superior in differentiating dogs with a high or a low risk of metastasis (23). Greater efforts should be made to implement these bio-score systems in larger prospective studies, allowing for more widespread use and the refinement of these increasingly accurate prognostic systems.

Histopathological Classification and Grading

The new classification of CMTs (24), which substitutes the WHO's 1974 and the last 2011 classification (5), together with the current grading system used worldwide for malignant CMC (9), as an adaptation of the Nottingham method utilized for HBC (25), have given tools to pathologists for accurate diagnosis and prognosis of CMTs since several studies have validated the prognostic significance of histopathological classification and grading (26, 27).

Cell Markers for Diagnosis: Immunophenotyping

Normal mammary gland cells of both humans and dogs have distinct immunoprofiles that can be used for diagnosis and potentially for targeted therapies. Luminal epithelial cells are characterized by the expression of low-molecular-weight (LMW) type I acidic cytokeratins (CKs) 18 and 19 and type II basic CKs 7 and 8. Basal/myoepithelial cells express high-molecular-weight (HMW) type I acidic CKs 14 and 17 and type II basic CKs 5 and 6 (**Figures 1**, **2**). Myoepithelial cells, much more proliferative in CMC than in HBC (5), also express other markers such as p63, vimentin, calponin, smooth muscle actin (SMA), P-cadherin, CD10, epidermal growth factor receptor (EGFR), maspin, and 14-3-3 sigma protein (28–31) (**Figures 3–5**). However, a study performed in 2014 showed that, as happens in human breast

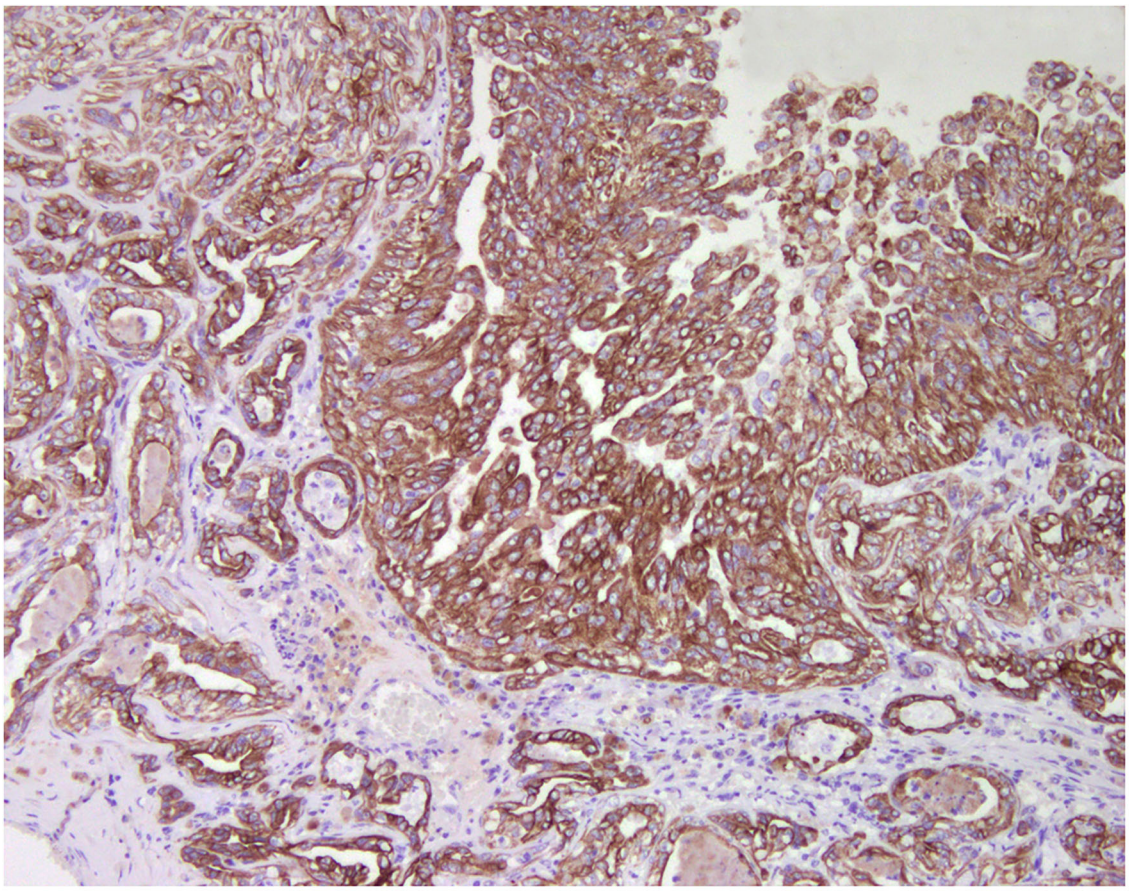

FIGURE 1 | Tubulo-papillary carcinoma, mammary gland, dog. Immunohistochemical cytoplasmic staining of wide-spectrum cytokeratins (*AE1/AE3*). Luminal and basal/myoepithelial cells are positive (*brown*).

(32), the mammary gland subpopulations are more complex than this. Using single and double immunohistochemistry (IHC) on serial sections of normal canine mammary gland, five distinct subpopulations were identified: (1) progenitor cells (CK5+, CK14+, p63+, and VIM+); (2) intermediary myoepithelial cells (CK5+, CK14+, p63+, SMA+, CALP+, and VIM+); (3) terminally differentiated myoepithelial cells (CALP+, SMA+, and VIM+); (4) intermediary luminal glandular cells (CK5+, CK14+, and CK8/CK18+); and (5) terminally differentiated luminal glandular cells (CK8/CK18+). The ducts are considered regenerative niches as they contain progenitor cells and intermediary luminal glandular cells; however, these are located in the basal position (33) and not in the luminal position, as occurs in women (34).

Molecular Classification

In human breast pathology, IHC is routinely used to assist with the prognosis and to determine the specific treatment for patients (35). Over the past years, there has been considerable efforts to characterize and classify HBC at the molecular level to establish effective individual treatments. However, due to time and cost constraints, the surrogate molecular breast cancer classification is still largely based on IHC assessment of biomarkers: estrogen receptor (ER), progesterone receptor (PR), HER-2, and Ki-67, among others (36). Nowadays, HBC is classified on the following molecular subtypes by IHC (**Table 1**): luminal A (ER/PR+, HER-2–, and Ki-67 low), luminal B HER-2 negative (ER+, PR– or low, HER-2–, and Ki-67 high), luminal B HER-2 positive (ER+, PR+/–, HER-2+, and Ki-67 high), HER-2 enriched or overexpressed (ER/PR–, HER-2+, and Ki-67 high), and triple negative (ER–, PR–, HER-2–, and Ki-67 high). Triple-negative breast cancer (TNBC) can be further subdivided, according to genetic signatures, into luminal androgen receptor (LAR), mesenchymal (MES), and two basal-like subtypes (positive for high-molecular-weight cytokeratins): immunosuppressed (basal-like immunosuppressed, BLIS) and immune-activated (basal-like immune-activated, BLIA), depending on the upregulation or downregulation of genes associated with T, B, and natural killer (NK) cells (14, 36, 37).

Despite the relevance of molecular subtyping in HBC, highly variable and even contradictory results have been obtained in CMC (10, 38–43). Regardless of the established guidelines for

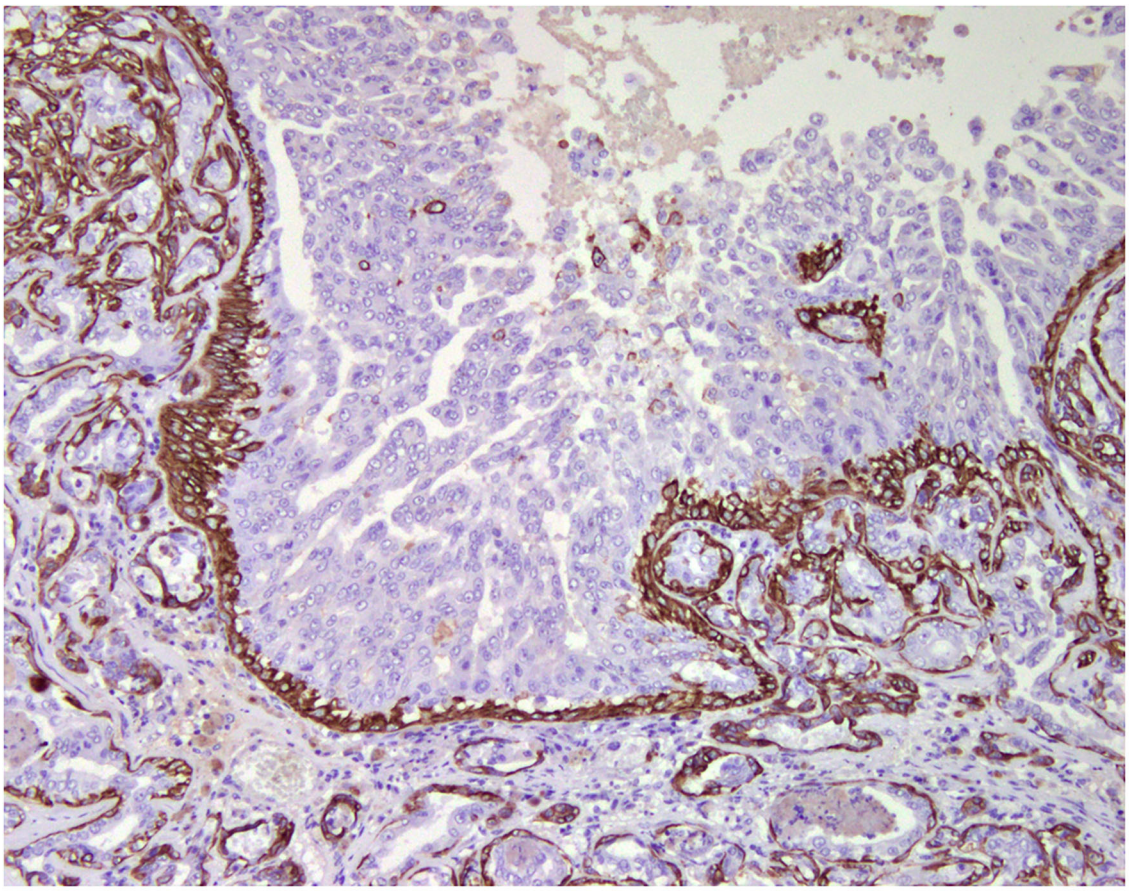

FIGURE 2 | Tubulo-papillary carcinoma, mammary gland, dog. Immunohistochemical cytoplasmic staining of cytokeratin 14. Basal/myoepithelial cells are positive.

immunohistochemical assessment (31), variable application of the criteria has been utilized, and the percentages of the molecular subtypes differ enormously among investigations.

HER-2 immunodetection in CMTs has always remained controversial. In a study performed by Abadie et al. (10), following appropriate standardized intrinsic and extrinsic controls (31), there were no HER-2-enriched tumors, in contrast to other studies in which between 5 and 15% of the mammary neoplasms were classified as HER-2 enriched (38, 42, 43). Previous studies identified HER-2 in normal, hyperplastic, and dysplastic mammary tissues and found no relation with prognostic parameters such as disease-free interval (DFI), overall survival (OS), and lymphovascular invasion, suggesting that HER-2 may play a role in the proliferation of mammary tissue in female dogs, but not conclusively in its malignant transformation (40). Furthermore, in a recent publication (44), HER-2 messenger RNA (mRNA) expression was observed in neoplastic and non-neoplastic mammary tissues using a novel quantitative RNA *in situ* hybridization assay, which correlates with the immunohistochemistry score. Among the non-neoplastic mammary tissues (hyperplasia), all cases showed HER-2: 21.4% were classified as 1+, while 78.6% were positive (2+ and 3+) (**Figure 6**). Moreover, within neoplastic tissues, no significant associations between HER-2 expression and clinical parameters were found.

The specificity of human anti-HER-2 antibody (Dako A0485) for HER-2 immunolabeling in canine tissues is also controversial. While one study showed no evidence of its specificity in canine tissues by Western blotting and subsequent mass spectrometric analysis (45), another work showed the cross-reactivity of the human anti-HER2 antibody in canine tissue (urothelial) by Western blotting (46).

Triple-negative tumors account for approximately half of CMCs (58.6%) (10), and showed significantly shorter disease-free interval (DFI) and overall survival (OS) in comparison to luminal A tumors. Comparable results were obtained in other studies: a triple-negative phenotype was related to a higher histological grade of malignancy, lymphatic invasion, and poorer prognosis. On the other hand, luminal A tumors were frequently complex tumors associated with better prognosis and longer DFI and OS (10, 38, 42, 43). In a study, HER-2-enriched and triple-negative CMCs presented a downregulation of E-cadherin compared to the luminal A and B subtypes, which are related to invasion and metastasis (43).

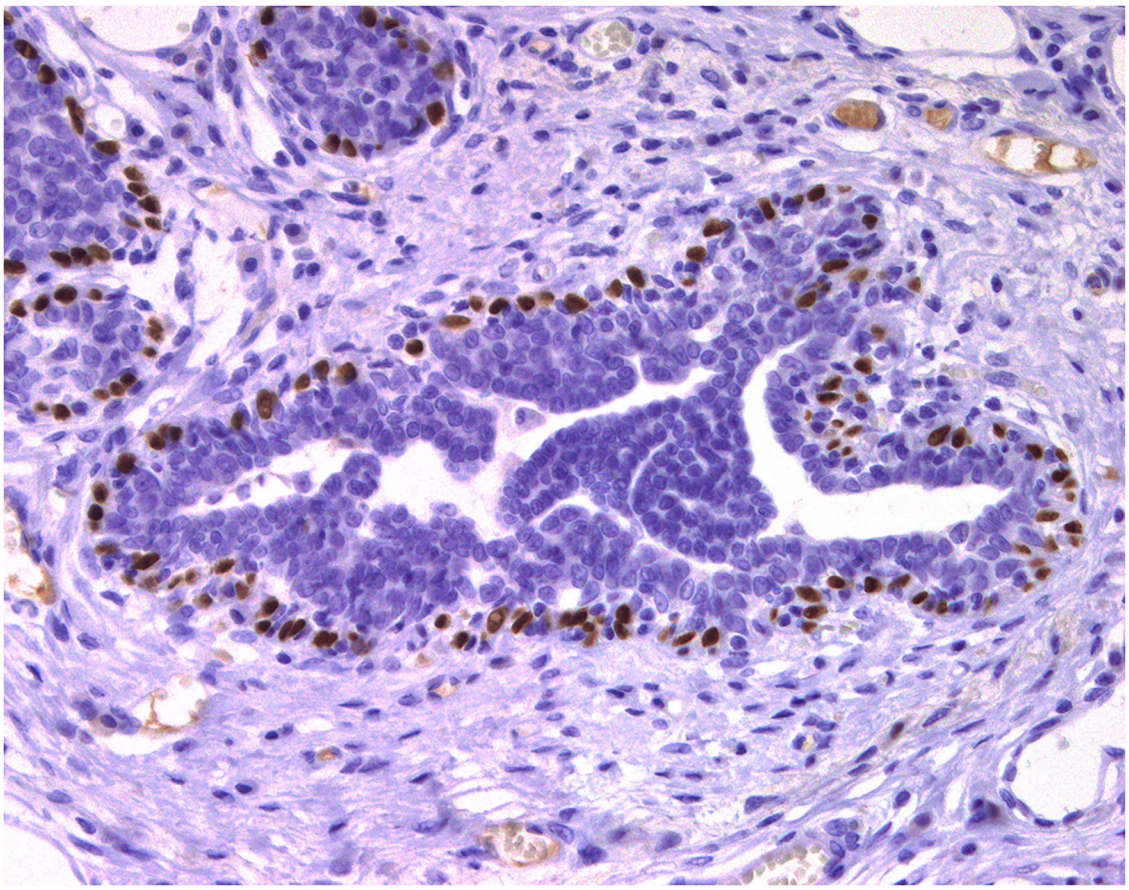

FIGURE 3 | Epitheliosis, mammary gland, dog. Immunohistochemical nuclear staining of *p63*. Myoepithelial cells are positive.

Surgery

Surgery is the primary treatment in the control of CMTs; the goal is to remove the tumor(s) with clean margins and, depending on the case, to prevent the development of new tumors in the remaining glands (4). Clean margins have been found to be predictive of the median survival time (MST) in dogs with stages I–III (19), and very recent publications have elucidated new strategies for the intraoperative assessment of margins using near-infrared light waves to generate real-time, high-resolution images on the microscopic scale, similar to low-power histopathology (47–49).

Despite the elevated frequency of CMTs, there is a lack of prospective clinical trials robust enough to establish the extent of surgical excision: simple lumpectomy, local mastectomy, regional mastectomy, total chain mastectomy, or bilateral total mastectomy (4). Nevertheless, the current literature recommendations are the following: If a single, small (<1 cm) tumor is present, nodulectomy is usually carried out. Simple mastectomy is indicated when the tumor is larger and centrally located within the mammary gland. When multiple tumors are in consecutive glands, or a single tumor is found between two mammary glands, regional mastectomy (excision of adjacent mammary glands, from one to two or from three to five) is performed. Finally, total mastectomy is indicated when multiple tumors are distributed throughout the mammary chain, regardless of the size (4). Those cases in which surgery is not recommended are advanced metastatic (stage V) cancer (17, 50) and inflammatory mammary cancer (IMC) (7, 8, 51).

Additional treatment (adjuvant therapy) can be given after the primary mammary cancer treatment (surgery) to lower the risk of developing further recurrences and metastasis. Adjuvant therapy may include chemotherapy, radiotherapy, and targeted or individualized therapy, this latest based on the specific genetic characteristics of the cancer in a patient (52–55).

Chemotherapy

Approximately 50% of the dogs with CMTs have at least a malignant neoplasm, and these patients would further profit from adjuvant chemotherapy. However, it has not been demonstrated conclusively if adjuvant chemotherapy offers a significant benefit to dogs with CMTs. Although cases have reported measurable tumor responses to doxorubicin (56–58), carboplatin (59, 60), mitoxantrone, and paclitaxel (61, 62), larger studies have not found a significant improvement of the measurable

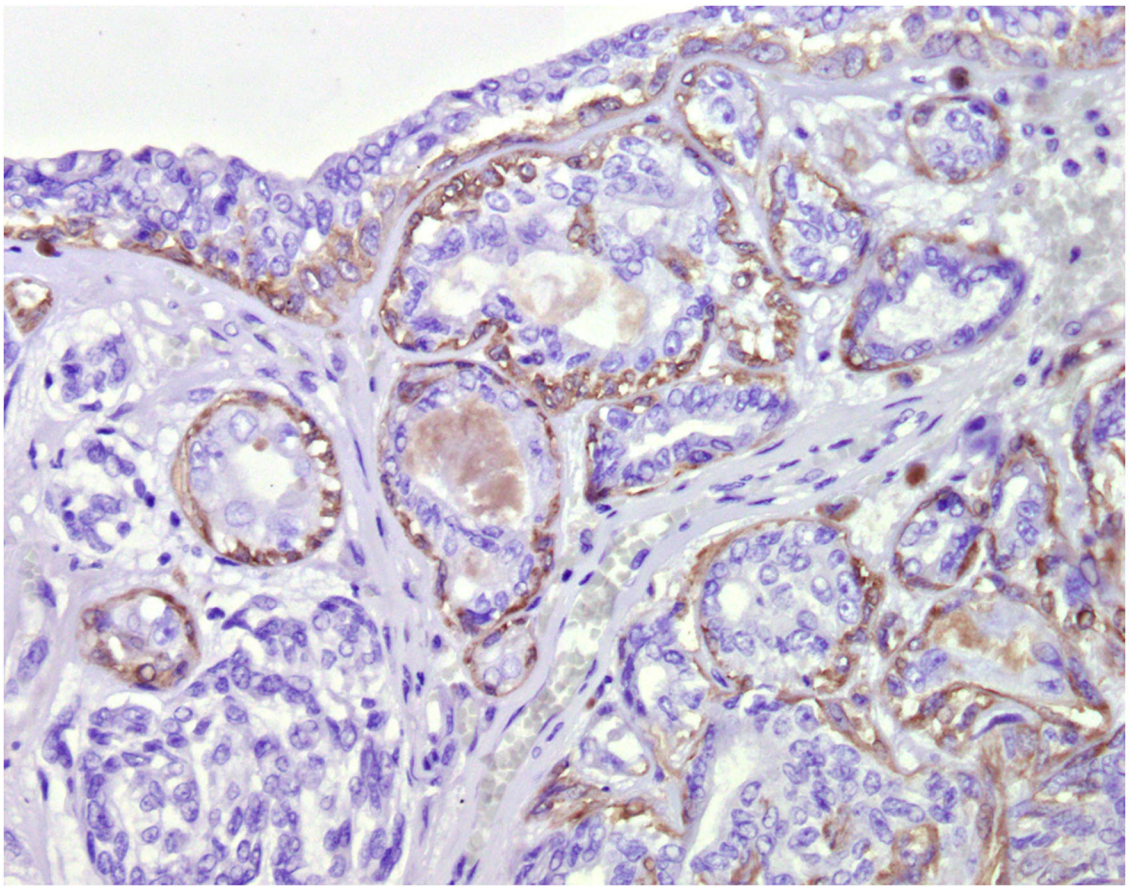

FIGURE 4 | Tubular carcinoma, mammary gland, dog. Immunohistochemical cytoplasmic staining of calponin. Myoepithelial cells are positive.

clinical responses (MST, DFI, or OS) using gemcitabine (17), doxorubicin, docetaxel (16, 19), and mitoxantrone (19). Due to the lack of efficient chemotherapeutics, dogs with malignant CMTs show high rates of recurrence (63) and poor prognosis (64).

Despite this uncertainty, chemotherapy is frequently used in those dogs with tumors considered at high risk of metastasis or recurrence (4).

Another chemotherapeutic approach is oral metronomic chemotherapy, which involves the administration of the lowest biologically effective dose at frequent regular intervals (65). In veterinary medicine, metronomic chemotherapy has been studied since 2007 (66) in tumors that include hemangiosarcoma (67–72), osteosarcoma (73–76), hepatic neuroendocrine carcinoma (77), primary lung carcinoma (78), soft tissue sarcomas (79–82), and transitional cell carcinomas (83), with chemotherapeutics such as cyclophosphamide, lomustine, and chlorambucil. To date, only one study has been published regarding CMC (84), in which longer MSTs were observed in patients treated with surgery and metronomic chemotherapy compared to dogs treated with surgery and conventional chemotherapy.

Radiotherapy

Although radiotherapy is commonly used in HBCs with locoregional treatment (14), only one study has been published in CMC, specifically in dogs bearing IMC. Radiotherapy, in combination with piroxicam, toceranib, and thalidomide, showed significantly longer time to progression than those patients treated with the same regimen, but without radiotherapy (85).

ADJUVANT TARGETED THERAPIES

Targeted therapies involve drugs that block the growth of cancer by interfering with individually expressed specific molecules responsible for tumor cell proliferation, survival, metastasis, or microenvironment (86). Given the poorly efficient available adjuvant therapies in dogs with mammary cancer, several studies have been made *in vitro* in animal models (mice) and few clinical trials, which are shown below.

Hormonal Therapy

Estrogens, progesterone, prolactin (PRL), and growth hormone (GH) are essential for physiological mammary development. The

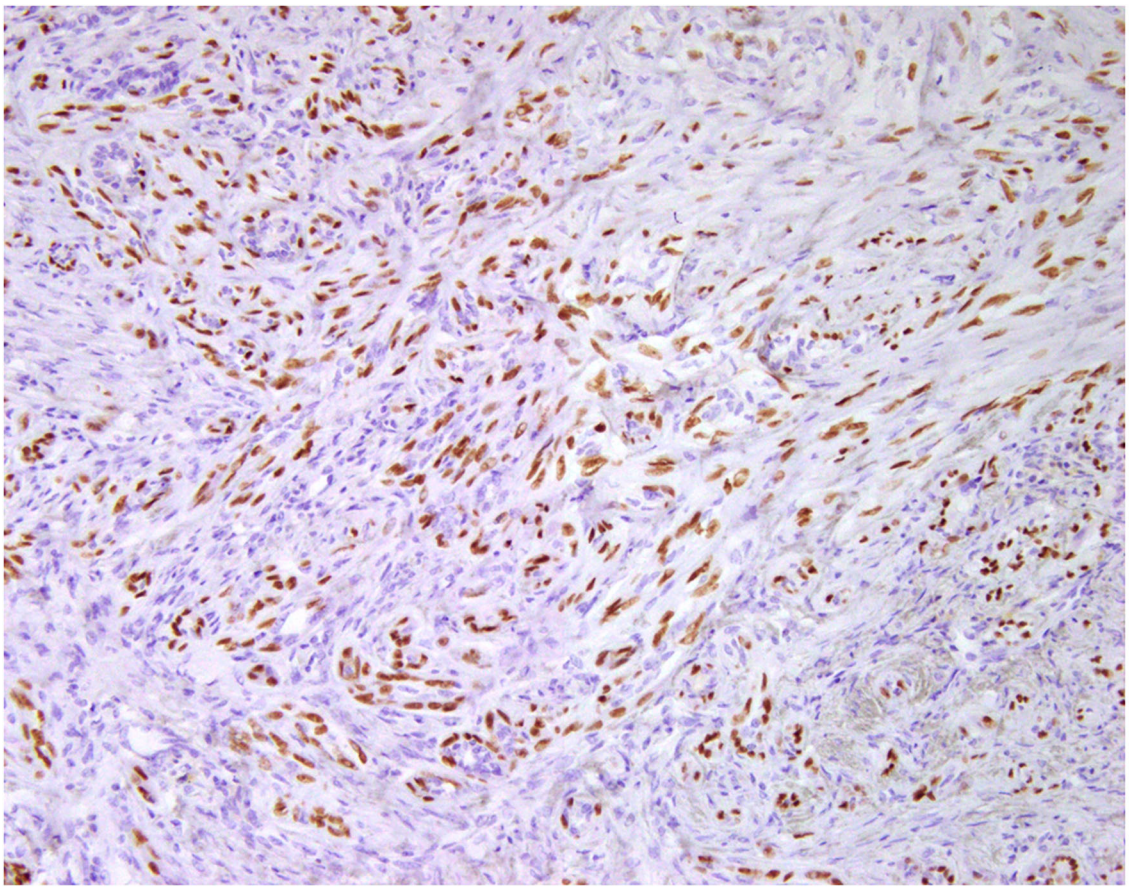

FIGURE 5 | Carcinoma and malignant myoepithelioma, mammary gland, dog. Immunohistochemical nuclear staining of *p63*. Interstitial proliferated myoepithelial cells are positive.

TABLE 1 | Classification of molecular subtypes in human breast cancer.

Molecular subtypes		ER	PR	HER-2	Ki-67	HGM
Luminal A		+++	+++	−	Low	Low
Luminal B HER-2 negative		+	+/−	−	High	Medium/high
Luminal B HER-2 positive		+	+/−	+/++	High	Medium/high
HER-2 enriched		−	−	+++	High	High
Triple-negative		−	−	−	High	High
LAR	AR+, MUC-1+					
MES	EMT genes+, PDGFRα+, c-Kit+					
BLIS	HMWCK+, VTCN1+					
BLIA	HMWCK+, STAT+					

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; HGM, histological grade of malignancy; LAR, luminal androgen receptor; MES, mesenchymal; BLIS, basal-like immunosuppressed; BLIA, basal-like immune-activated; AR, androgen receptor; MUC-1, cell surface-associated mucin-1; EMT, epithelial–mesenchymal transition; PDGFRα, platelet-derived growth factor receptor α; c-Kit, stem cell factor receptor; HMWCK, high-molecular-weight cytokeratins; VTCN1, immunosuppressive V-set domain-containing T cell activation inhibitor 1; STAT, signal transducer and activator of transcription gene family.

effects of these hormones are mediated through binding to their respective receptors within the mammary gland (6) (**Figures 7**, **8**). Estrogens and progesterone have been historically known to have a main role in tumorigenesis in CMTs, as spaying of female dogs before the first or second heat has significant protective effects (87). On the other hand, exposure to exogenous hormones (both estrogens and progestins) increases the risk of developing CMTs in dogs (88). Furthermore, studies have found that dogs with CMC have higher levels of estrogens in the blood, except in IMC, where the serum estrogens levels are lower than

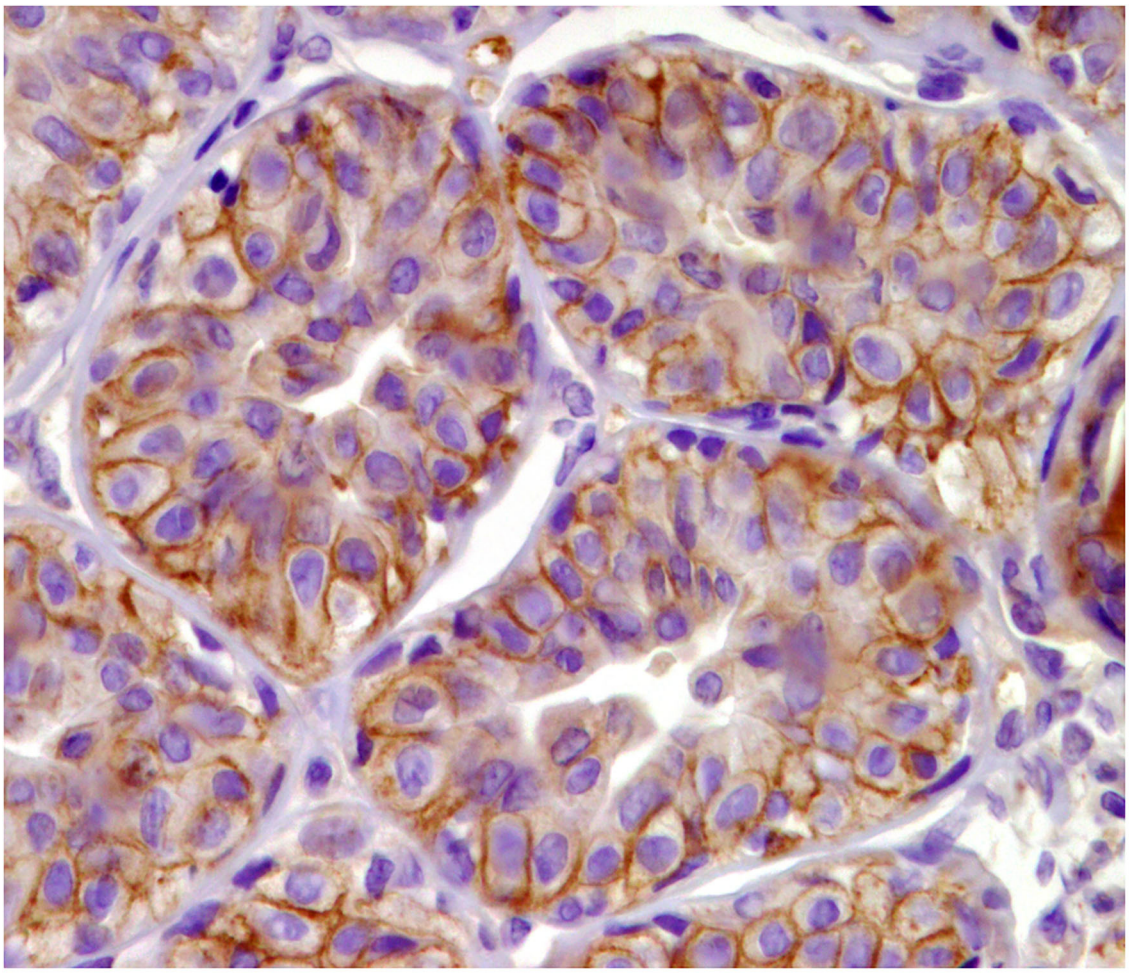

FIGURE 6 | Tubular carcinoma, mammary gland, dog. Immunohistochemical membranous staining of human epidermal growth factor receptor 2 (HER-2). Complete and incomplete membranous staining of neoplastic cells.

in dogs with other malignant mammary tumors (89, 90). On the other hand, ER, PR, PRL, PRL receptor, and GH receptor have been found to be downregulated in malignant mammary tumors compared to normal mammary gland (91, 92) and benign tumors (92–94). Furthermore, lymphatic invasion, high mitotic index, large tumor size, and tumor grade are significantly associated with low ER/PR expressions. Tumors with low ER/PR expressions have poorer prognosis (89, 93, 94).

In human medicine, ER+ breast cancer is the most common subgroup (>70%) (95). The risk of ER+ breast cancer increases with exposure to estrogens during a lifetime, for example due to an earlier menarche or late menopause (14). Moreover, hormone replacement therapy on menopausal women increases the risk of breast cancer (96). In women with HBC, ER+ tumors are susceptible to anti-hormone treatment. This therapy is designed to target mainly ER using antiestrogens, such as tamoxifen or fulvestrant, or by inhibiting the endogenous synthesis of 17β-estradiol using aromatase inhibitors (97).

Tamoxifen is a selective inhibitor of ER that is widely utilized in the treatment of HBC. However, in dogs, severe adverse effects (vulvar edema, vaginal purulent discharge, and pyometra) are repeatedly seen, and that outweighs the possible benefits of this hormone therapy (98, 99).

For this reason, other antiestrogens are being studied for their use in CMC. Indole-3-carbinol is a natural phytochemical found in cruciferous vegetables (i.e., cauliflower, cabbage, and broccoli) that has been proven to suppress cell proliferation and induce apoptosis in breast cancer cell lines by multiple mechanisms such as blocking estrogen receptors (100, 101). In veterinary medicine, a mouse xenograft model of canine IMC was treated with indole-3-carbinol, resulting in decreased tumor proliferation and increased apoptosis, although metastasis and lymphatic embolization were not prevented (102).

When women reach menopause, the ovaries no longer produce estrogen; however, this hormone is produced at other sites (fat, liver, muscle, and mammary tissue) through the

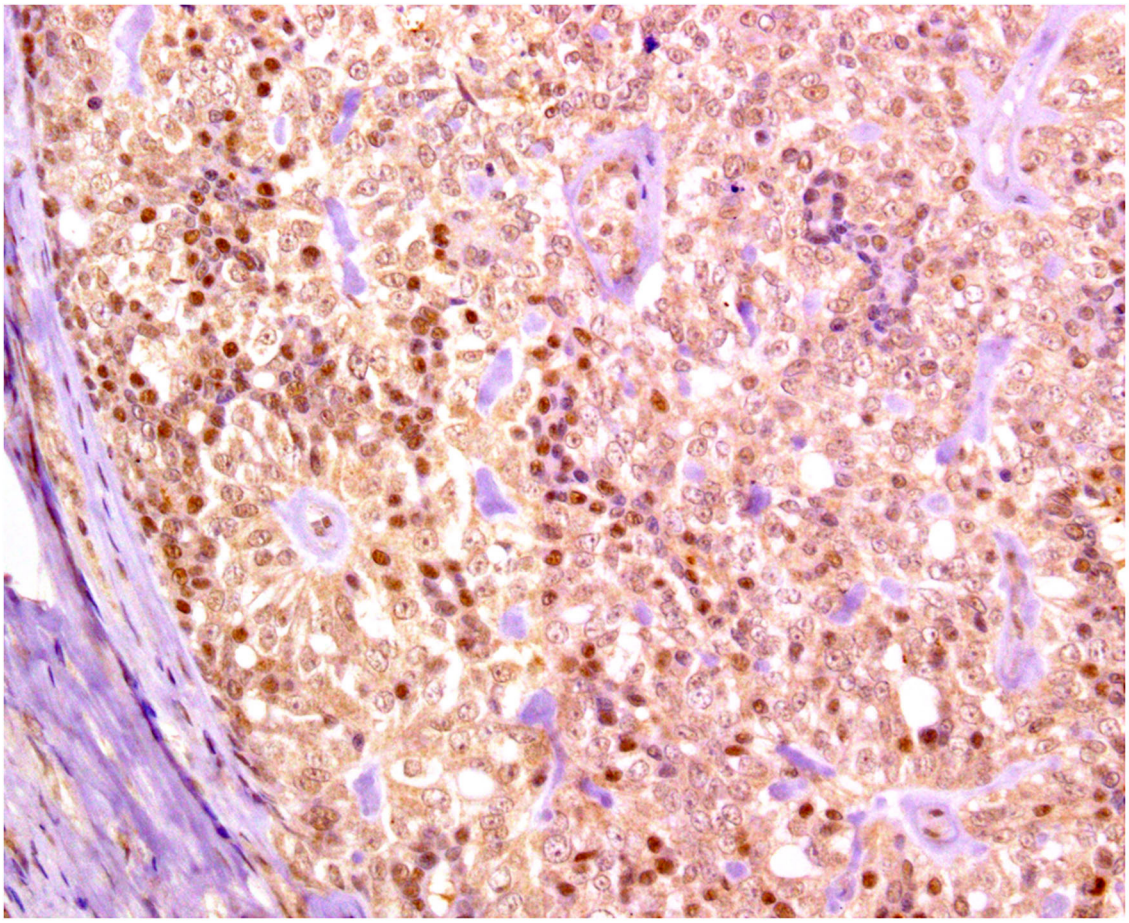

FIGURE 7 | Solid carcinoma, mammary gland, dog. Immunohistochemical nuclear staining of estrogen receptor (ER). Neoplastic cells are positive with different intensities of immunolabeling.

aromatase enzyme (103). Aromatase mRNA levels are higher in cancerous tissues than in normal breast tissues in humans (104). Therefore, while ER inhibitors (e.g., tamoxifen) are preferentially given in premenopausal women with ER+ HBC, aromatase inhibitors are used for the treatment of postmenopausal patients with ER+ breast cancer (103). In dogs, IMC has been shown to express higher levels of aromatase than non-IMC. Moreover, *in vitro* treatment with letrozole, an aromatase inhibitor, significantly reduced cell proliferation in an IMC cell line (105). No clinical trials on the use of aromatase in dogs with CMC have been reported.

Melatonin is a hormone produced by the pineal gland in response to darkness, regulated by photoperiod (106). In breast tissue, melatonin exerts its action through two receptors: melatonin receptors 1 and 2 (MT1 and MT2, respectively) (107). A positive correlation between MT1 and ERα expressions has been demonstrated and recognized as a prognostic marker for OS in HBC (108). Furthermore, melatonin has been shown to suppress the proliferation of HBC cell lines *in vitro* and *in vivo* by disrupting estrogen-dependent signaling as well as inhibiting estrogen production in the gonads and in breast tissues through the aromatase pathway (109–111).

In the last decades, the role of androgens on HBC has started to gain the attention of researchers. Not only can androgens be a source of estrogen through the aromatase pathway but they have also been directly implicated as possible carcinogen factors for breast cancer (112). The androgen receptor (AR) is expressed in ∼70–90% of invasive human breast cancers, a frequency comparable to or higher than those reported for ER (70–80%) and PR (50–70%) (113, 114). On the other hand, AR has been found in 64% of IMC and 40% of non-inflammatory CMC (89) (**Figure 9**). To date, in human medicine, AR-targeted drugs have been approved for the treatment of prostate cancer, and different AR inhibitors are being investigated for the treatment of HBC, specifically for the LAR subtype of triple-negative breast cancer (115). Flutamide is an analog of androgen that blocks the AR (116). It is already used in veterinary medicine to treat canine prostate hyperplasia and cancer (117), but has not been tested in patients with CMC. Flutamide has been shown *in vitro* to decrease the proliferation of an IMC cell line, and reductions in

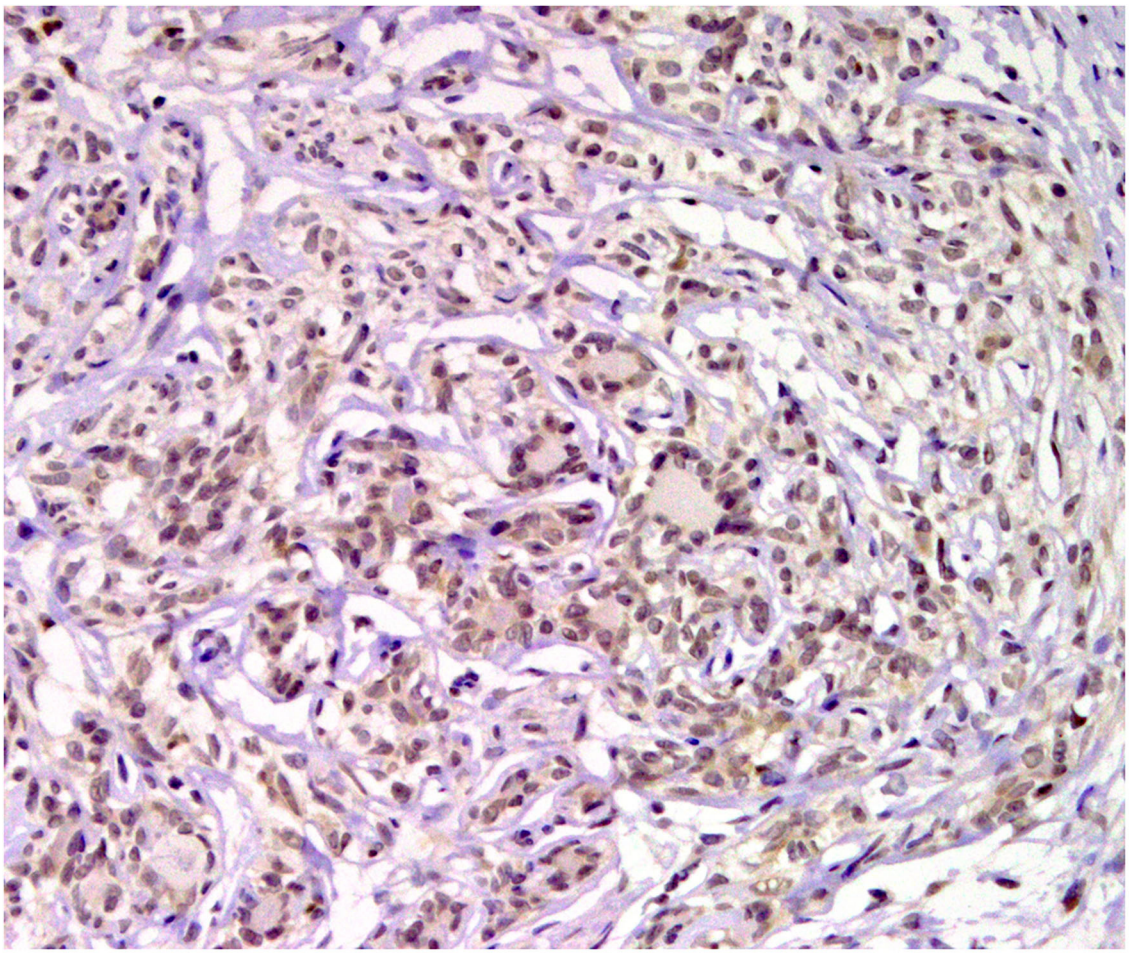

FIGURE 8 | Tubular carcinoma, mammary gland, dog. Immunohistochemical nuclear staining of progesterone receptor (PR). Neoplastic cells are positive.

the tumor size and metastasis rates in IMC xenografted mice were found (118).

Progesterone has also a relevant role in HBC (119) and in CMC (105, 120). Progesterone signals *via* PR, whose expression is stimulated by estrogen (121). Some authors have suggested that progesterone may lead the transition of tumors from luminal to a basal phenotype (122). Upon progesterone exposure, luminal cells secrete growth factors (RANKL and Wnt) that may stimulate the recruitment and differentiation of cancer stem cells (CSCs, characterized by $CD40^{HIGH}$ and $CD24^{LOW}$) (123).

Therapeutic targeting of PRs have been studied in HBC as a treatment of endocrine refractory breast cancer, the most common drugs being megestrol acetate, medroxyprogesterone acetate, mifepristone, and onaprestone (124). The antiprogestin aglepristone is employed in veterinary medicine for abortion, parturition induction, and pyometra treatment in female dogs (125). The study of antiprogestins in oncology has begun relatively recently. Mifepristone and onapristone have been shown to decrease the number of viable tumor cells *in vitro* in a canine mammary carcinoma cell line (126). *In vivo*, aglepristone treatment diminished the expression of PR, reduced the proliferation index in PR+ CMTs (127), and significantly increased DFI and OS in cases with PR+, <3 cm, low and medium grade, low proliferative tumors (128). Although promising for PR+ CMTs, antiprogestins in veterinary oncology need further studies with larger series and longer follow-up periods.

Oxytocin is a peptide hormone mainly synthesized in the hypothalamus that plays a role in uterine contraction and milk ejection, among other functions, and has been linked to the mammary carcinogenic process. Human (129) and canine (130) carcinoma cell lines and HBC xenografted mice (131) have shown reduced proliferation after oxytocin treatment. Recently, it has been shown that the expression of oxytocin receptors in CMTs is associated with ER+, benign tumors, and low-grade malignant tumors compared to high-grade malignant tumors (132).

Desmopressin is a synthetic analogous of vasopressin (antidiuretic hormone) that binds to the V2 membrane receptor (V2R); it has been used for the management of diabetes insipidus

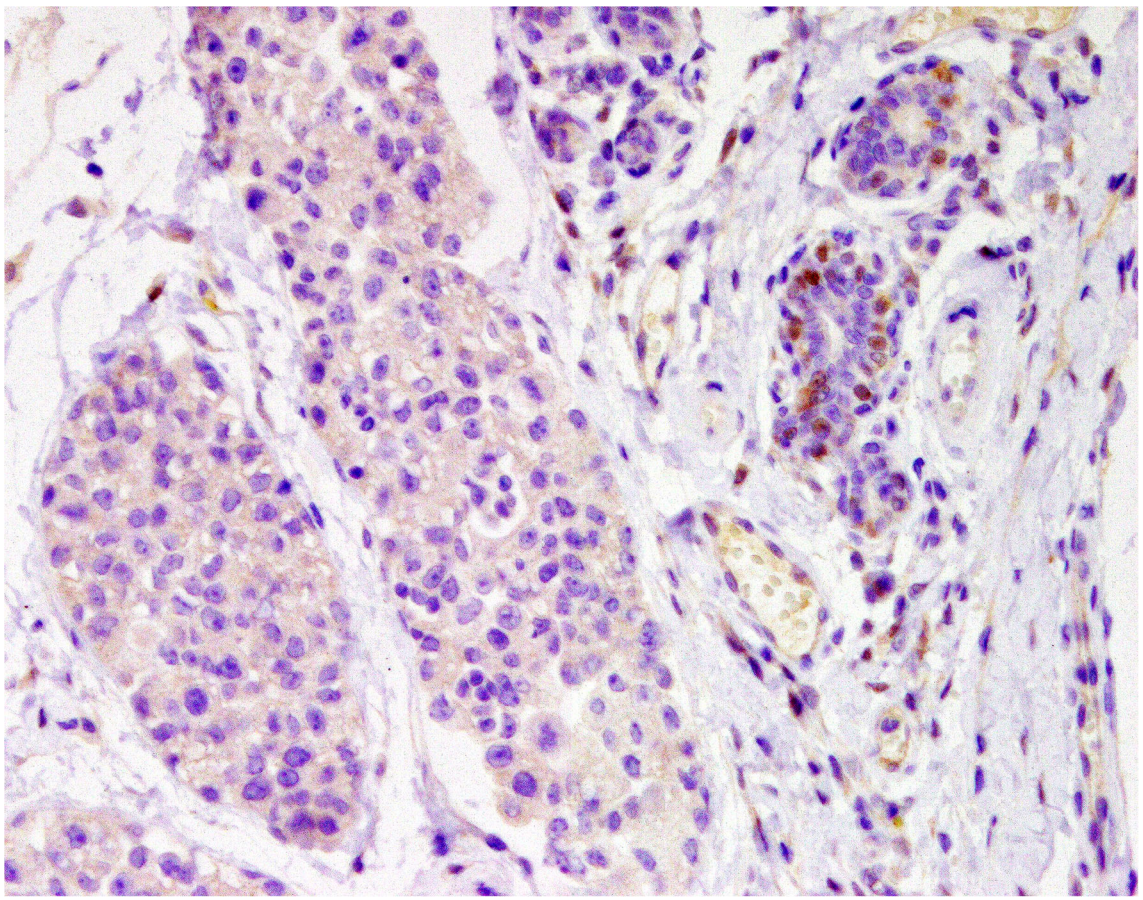

FIGURE 9 | Tubular carcinoma, mammary gland, dog. Immunohistochemical nuclear staining of androgen receptor (AR). Neoplastic cells are negative; adjacent mammary hyperplastic cells are positive.

in humans (133) and dogs (134). Since V2R is also expressed in endothelial cells, desmopressin has been employed in the treatment of different bleeding disorders due to its effects in the hemostatic system (135). In oncology, a number of studies have shown in mouse models of HBC and in different HBC cell lines that this peptide seems to have anti-metastatic and anti-proliferative effects, probably by targeting V2R-expressing cancer cells and raising intracellular cAMP (136, 137). In a study on canine mammary carcinoma cell lines, desmopressin was shown to decrease cell viability at high concentrations (130). Furthermore, a veterinary clinical trial has demonstrated that the perioperative administration of desmopressin increases the DFI and OS in CMC (138). Although it seemed a very promising therapy, there have been no subsequent clinical trials in veterinary or human medicine performed by this group, with the exception of a phase II trial in HBC patients in 2015 (139), where the safety of perioperative administration was established; however, the effect on DFI, OS, or any other clinical parameter has not been reported. Due to this controversy, Sorenmo et al. conducted a prospective randomized trial in dogs with CMC, in which no metastasis-preventing effect of desmopressin was found (140).

Tyrosine Kinase Receptors

Tyrosine kinase receptors (TKRs) catalyze a series of phosphorylation of target proteins that play a significant role in cell proliferation, metabolism, motility, survival, and apoptosis, as well as endothelial cell activation, leading to neovascularization.

Breakthroughs in biotechnology over the past decades have led to the development of new molecules that act on specific targets, among which small-molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs) stand out. Small-molecule (below 900 Da) tyrosine kinase inhibitors rapidly diffuse across cell membranes and target intracellular or extracellular proteins (kinases). To identify TKIs, the suffix "nib" is placed at the end of the generic name (141). On the other hand, monoclonal antibodies cannot cross cell membranes, therefore acting on the extracellular targets. The stem "mab" at the end of the name corresponds to mAbs. When the monoclonal antibody is completely human, the "umab" substem is used (e.g., nivolumab). If the immunoglobulin is chimeric (human constant domain, plus non-human variable domain), the mAb is named with the "ximab" substem; when the antibody is humanized (human framework with grafted murine

complementary determining regions), then the "zumab" substem is utilized (e.g., trastuzumab) (142).

Among TKRs, HER-2, vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), stem cell factor receptor (c-KitR), and colony-stimulating factor 1 (CSF-1) are overexpressed or constitutively activated in human and canine tumors (143, 144). Tyrosine kinase inhibitors act by competitive inhibition of ATP binding, thus avoiding consecutive phosphorylation reactions and blocking signal transduction to the nucleus, inducing the deregulation of cellular proliferation and differentiation (145). Most of the TKIs are given orally, which means a huge benefit for animal welfare, diminishing stressful situations and providing ease of administration by the owner (146). For these reasons, several attempts to block these receptors have been made in veterinary oncology. Some TKIs designed for human oncology can have multiple actions depending on the receptor blocked (i.e., sunitinib inhibits VEGFR, PDGFR, c-KitR, and CSF-2) (147) and can be served for different types of cancer. Below, we review these targets separately.

Anti-Her-2

The family of EGFRs encompasses four tyrosine kinases receptors also named human epidermal receptors (HERs): HER-1, HER-2, HER-3, and HER-4. When activated, these receptors trigger numerous signaling pathways, which regulate cell proliferation and survival, as well as the metastasis of tumor cells (144). Approximately 15–25% of HBC show overexpression of the HER-2 protein and/or amplification of the *HER-2* gene, which is generally associated with a poor prognosis and an aggressive disease course (148).

The targeted therapy with anti-HER-2 agents in HBC is well-established. Several classes of anti-HER-2 agents have been developed, including: (1) monoclonal antibodies that bind to the extracellular domain of HER-2, such as trastuzumab and pertuzumab, which act by direct inhibition of HER-2 and indirect activation of the immune system to evoke antibody-dependent cellular toxicity; (2) small-molecule TKIs, including lapatinib, neratinib, and afatinib, that bind to the intracellular tyrosine kinase domains of HER-2 and other HER family members; and (3) antibody–drug conjugates, e.g., trastuzumab emtansine (T-DM1), composed of a monoclonal antibody directed at the extracellular domain of HER-2 linked to a cytotoxic agent. All of the latter have been approved by the European Medicines Agency (EMA) for clinical use in patients with HER-2+ HBC (149).

The amino acid homology values between canine and human EGFR-1 and HER-2 are reported to be 91 and 92%, respectively (150). *In silico* studies (research conducted by computer modeling or simulation) have shown that cetuximab (monoclonal antibody against EGFR-1) epitopes only differ by four amino acids in canines, while the trastuzumab (monoclonal antibody against HER-2) binding site is identical in humans and canines. *In vitro* studies with canine mammary carcinoma cells have reported a significant growth inhibition and G^0/G^1 phase arrest when treated with either cetuximab or trastuzumab (150). A "caninized" version of cetuximab developed by the same group, fusing the canine constant heavy-chain genes with the variable region murine genes of cetuximab, was able to inhibit the proliferation of canine mammary carcinoma cell lines, enhancing tumor cell killing *via* (151) phagocytosis.

With regard to anti-HER-2 TKIs, only gefitinib has been attested in CMTs. *In vitro* studies showed anti-proliferative effects in a canine mammary carcinoma cell line comparable to those with small interfering RNA (siRNA) targeting EGFR and HER-2 (152).

There are no clinical trials published on the use of HER-2 inhibitors in CMC, and their potential use in veterinary medicine is still far from daily routine.

Anti-angiogenesis

Since 1971, it is well-known that the growth of solid tumors, beyond the size of 1–2 mm^3, is conditioned to a sufficient supply of nutrients and oxygen, for which the new development of blood vessels was hypothesized and called angiogenesis (153). In early tumor development, neoplastic cells are oxygenated through simple diffusion in a phase defined as "avascular state." With time and growth, tumor cells are deprived of oxygen and undergo a phenotypical change into a pro-angiogenic state (angiogenic switch) by inducing specific gene expression to overcome hypoxia through sprouting angiogenesis, vasculogenic mimicry (VM), and/or vascular co-option (VCO) (154). The sprouting angiogenic process occurs in both normal and neoplastic tissues. It is a complex process regulated by pro- and anti-angiogenic factors, among which stands out the VEGF family receptors, especially VEGF-A and its receptor (VEGFR-2), the fibroblast growth factor receptor 2 (FGFR-2), and PDGFR (154). VM and VCO are only found in highly aggressive cancers, as is explained below.

In both, humans (155) and dogs (156), VEGFR-2 and PDGFR are increased in malignant, triple-negative mammary tumors, being higher in those cases with metastatic disease (distant > regional) and positively correlated with tumor grade. Further, in HBC, microvascular density (MVD) is significantly correlated with metastasis, OS, and DFI (157); likewise, MVD is increased in canine primary mammary tumors with distant metastasis (156, 158).

In human medicine, about one third of the molecular therapeutics in clinical development are directed against angiogenesis. Angiogenesis is mediated by two major molecular routes: the VEGF axis-dependent route and the non-VEGF-mediated mechanisms (159). The anti-angiogenic therapies against the VEGF family block either the ligands or the receptors (160). The most widely studied anti-angiogenic therapeutic is bevacizumab (monoclonal antibody against the anti-VEGF-A ligand). In 2008, it became the first Food and Drug Administration (FDA)-approved anti-angiogenic drug for HBC; however, due to the lack of significant clinical improvements in subsequent studies, the approval was revoked in 2011 (161).

Small-molecule TKIs that block the VEGF family receptors have also been developed (pazopanib, sunitinib, and sorafenib, among others), many of them not only acting against angiogenesis but also diminishing other tumor metabolic pathways (162). For instance, pazopanib acts as an anti-angiogenic through the inhibition of VEGFR, PDGFR, and c-Kit

in human renal carcinoma, soft tissue sarcoma, and breast cancer in combination with the anti-HER-2 lapatinib (163). In addition, sunitinib, which inhibits VEGFR, PDGFR, c-KitR, and CSF-1R, has shown promising activity as a single agent for advanced HBC (164). On the other hand, sorafenib, a VEGFR, PDGFR, and rapid accelerated fibrosarcoma-1 (Raf-1) kinase inhibitor, has antitumor effects *in vitro* and inhibits neovascularization in xenograft models of HBC (165). Sorafenib has been assessed in veterinary medicine, and it has shown a promising ability to inhibit VM in CMC cell lines *in vitro* (166). Other TKIs that target VEGFR-2 and that have been proven to significantly diminish the level of active (phosphorylated) VEGFR2, reduce cell proliferation and migration, and increase apoptosis in *in vitro* studies against CMT cell lines are rivoceranib (apatinib) (167) and vandetanib (168).

Despite the logical targeting of angiogenic pathways in cancer treatment and significant efforts in new drug development and HBC clinical trials, no significant clinical benefit has been achieved that outweighs the potential side effects. Some authors have hypothesized that a multi-target approach to angiogenesis is needed to overcome the apparent resistance of tumors to anti-angiogenesis treatment (159).

As stated earlier, angiogenesis is not the exclusive method to nourish tumor cells; two other mechanisms have been discovered in highly aggressive neoplasms—VM (169, 170) and VCO (171)—which have been hypothesized as responsible for the resistance to anti-angiogenic therapy (172–174). VM describes the formation of *de novo* vascular channels lined by genetically deregulated highly malignant cancer cells (175). These cancer cells, also called endothelial-like cells, exhibit cancer stem cell markers and characteristic endothelial morphology under electron microscopy in cell lines of human and canine inflammatory mammary cancer (176). VM has been associated with the spread and metastasis of human (177) and canine mammary tumors (178). VM was found in 33% of canine mammary tumors, and its presence was correlated with histological grade of malignancy and shorter survival times (166).

In VCO, neoplastic cells closely adhere to preexisting blood vessels to obtain nutrients and oxygen and further develop sprouting angiogenesis after the hypoxia switch is turned on (174, 179). In veterinary medicine, VCO has not been recognized yet.

Anti c-Kit and Other Receptors

The stem cell factor receptor c-Kit is an active participant in many vital functions in humans and animals, such as homeostasis, cell maintenance, differentiation, and melanogenesis, in a wide variety of cells (180). However, overexpression or mutation where the receptor is constitutively activated has been detected in a number of tumors in humans (181) and dogs, notably in canine mast cell tumors (143). In canine mast cell tumors, the c-Kit receptor can be labeled by immunohistochemistry in three different staining patterns: pattern I (perimembranous staining), pattern II (focal or stippled cytoplasmic), and pattern III (diffuse cytoplasmic staining), pattern III being the more aggressive (182). In women, c-Kit is expressed in normal breast tissue and is gradually lost during the malignant progression of breast tumors due to a downregulation at the mRNA level (183). On the contrary, c-Kit has been found to be present in 38.5% of CMTs (184) (**Figure 10**) and seems to be overexpressed in malignant mammary tumors (185, 186), in addition to being correlated with the proliferation index (187) and angiogenesis (188).

In veterinary medicine, there are only two approved targeted anti-c-Kit TKIs: toceranib (Palladia) and masitinib. Toceranib, designed and approved for mast cell tumors in dogs, acts mostly through the inhibition of the c-KitR, and it is a sister compound to sunitinib, which was later approved for human therapies (189). Toceranib appears to exert antitumor activity against a variety of dog cancers, including CMC, particularly in cases with pulmonary metastases (143). Toceranib also has anti-angiogenic effects, inhibiting VEGFR and PDGFR, and produces a significant decrease in regulatory T cells, which may increase immune surveillance (189). Toceranib has shown a discrete *in vitro* reduction in cell proliferation on canine mammary carcinoma cell lines (184).

The evaluation of toceranib in clinical trials with dogs with CMC is very limited. In a study with dogs presenting IMC, toceranib was given in combination with piroxicam (anti-COX-2) and thalidomide (an immunomodulatory and anti-angiogenic agent) (190), with or without hypofractionated radiation therapy. The authors found a significant improvement in the clinical benefit rate and overall survival time compared with historical palliative treatment. The response was better when radiotherapy was employed (85).

Masitinib is also a potent and selective inhibitor of the c-Kit receptor that has been recommended for its use in canine mast cell tumors (191). It also inhibits the PDGF receptor and the fibroblast growth factor receptor (FGFR-3). Masitinib has been studied *in vitro* as a "chemosensitizer" by enhancing the anti-proliferative effects of a cytotoxic drug, gemcitabine (192). There are no other studies on the treatment of CMC.

Although TKIs (in particular toceranib) are commonly used for CMC treatment, in the veterinary clinic, the evidence is merely anecdotal, and only the aforementioned studies, one *in vitro* (184) and two *in vivo* (85, 189), have been conducted. There is an urgent need for prospective randomized studies to adequately evaluate the effectiveness of toceranib in patients with CMC in c-Kit-positive or c-Kit-negative tumors.

Other tyrosine kinase inhibitors are under study. Palbociclib is an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, which are key regulators of the cell cycle machinery and, thus, cell proliferation (193). In women, palbociclib improves progression-free survival in ER+, HER-2– breast cancer when combined with an aromatase inhibitor (letrozole) or an ER downregulator (fulvestrant), so it received approval from the FDA and EMA (194). CDK6 has been consistently detected in CMT cells with no association to histotype or grade (64). *In vitro* studies with canine mammary cell lines have shown that palbociclib induces cell cycle arrest, prevents colony formation, and impairs cell migration activity (64). There are no clinical trials on dogs with CMC.

Antitumor Suppressor Gene *p53*

The tumor suppressor gene *p53* plays a central role in tumorigenesis. The p53 protein, after tetramerization, inhibits cell proliferation, functioning as an initiator of cell cycle arrest

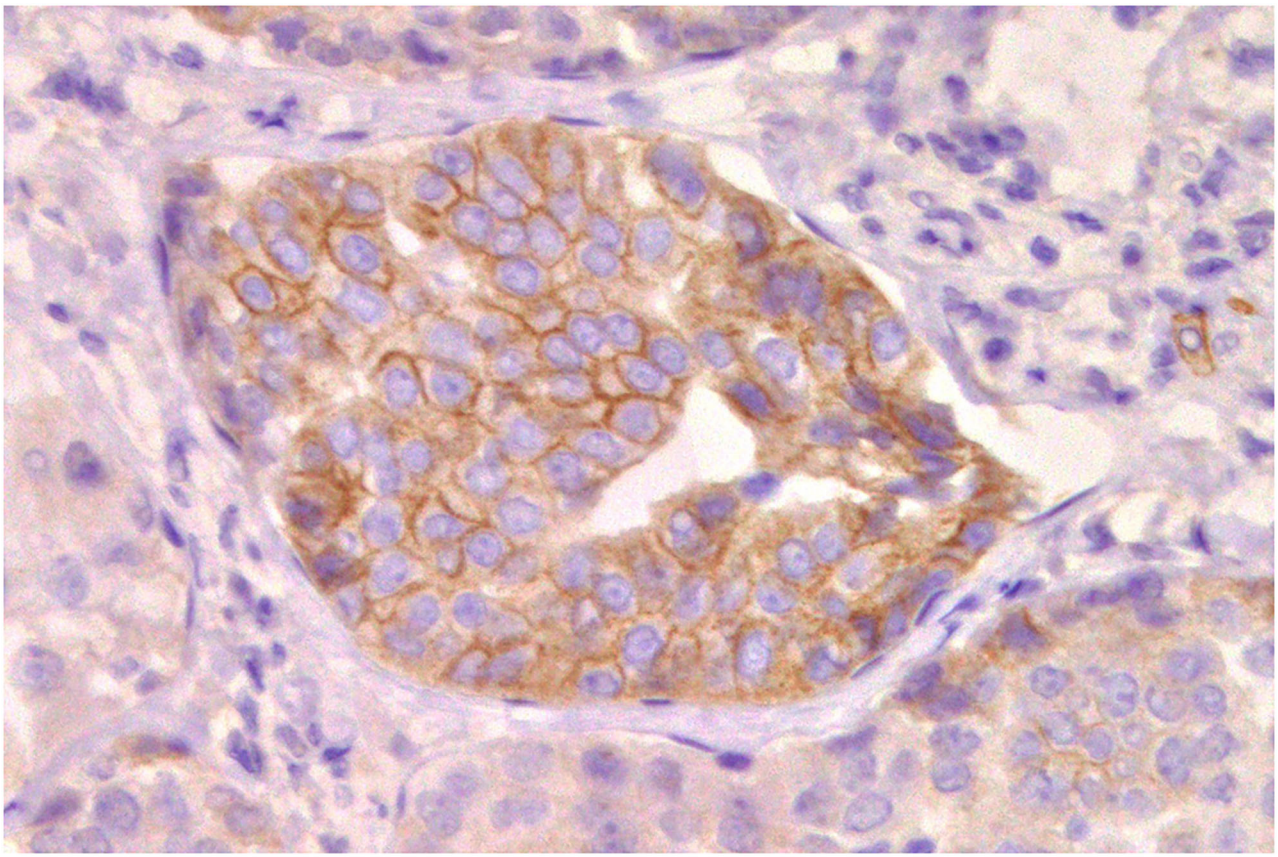

FIGURE 10 | Tubular carcinoma, mammary gland, dog. Immunohistochemical membranous staining of stem cell factor receptor (c-KitR). Neoplastic cells are positive.

and apoptosis. Mutations of *p53* often cause a disruption of its tumor-suppressor function and induce genomic instabilities (195). Mutations in the *p53* gene are associated with more than half of all human cancers and have been described in multiple cancers in dogs (196), including mammary tumors (197, 198). However, its expression and mutation status as prognostic factors in veterinary medicine are controversial: while some studies have found no correlation (199, 200), other authors have associated higher levels of *p*53 with poor overall survival (201, 202).

Mutation of the *p*53 gene can lead to a stable protein that is identifiable by immunohistochemistry (203), although a truncating mutation of the *p*53 gene can lead to an immunohistochemically undetectable protein (204). Therefore, four different patterns of *p*53 immunolabeling have been published: overexpression, complete absence, cytoplasmic staining, and wild-type staining. The first three patterns are related to an underlying *p*53 mutation, while the fourth pattern is primarily associated with no mutation (204, 205). Altered expressions of *p*53 may result from a direct mutation of *p*53 within tumor cells or from an altered localization of *p*53 by increased export of the protein from the nucleus, thereby decreasing its downstream targets and inhibiting apoptosis and cell cycle arrest (195).

Approximately 30% of HBCs have a *p53* mutation, but this frequency is dependent on the molecular subtype, as the luminal subgroup has the lowest mutation rate and TNBC has the highest (up to 88%) (206). Additionally, *p53* overexpression has been correlated with more aggressive HBCs and worst outcomes (207–209).

In CMC, malignant tumors have shown higher levels of *p*53 than benign tumors (210), and the increase of *p*53 is greater in higher-grade tumors with higher proliferation rates (211). Likewise, a significant correlation between increases in *p*53 expression and mutations with shorter OS has been found (212).

Since mutations in the *p*53 gene have an enormous impact on cancer development, great efforts are being made into the mechanisms that can counteract this effect and are brought together in several extensive reviews (213, 214) that compile different experimental approaches to target *p*53 in human cancer: inhibition of mutant *p*53 by promoting its protein degradation, restoration of the wildlife activity of mutant *p*53, and immune stimulation against *p*53 activity.

In contrast, there is only one study on p53 therapy in canine mammary cancer cells. Neoplastic cells can increase the cytoplasmic translocation of nuclear p53 by overexpressing exportin-1, thus preventing its binding to DNA and its

anti-proliferative activities (215). The addition of KPT-185 and KPT-355, engineered molecules that inhibit exportin-1 on canine mammary carcinoma cells, *in vitro* induced cell cycle arrest, apoptosis, and reduced growth (216).

Anti-cyclooxygenases

Cyclooxygenases (COX) are a group of enzymes that catalyzes the conversion of arachidonic acid to prostanoids (prostaglandins, prostacyclins, and thromboxanes). In humans and animals, COX exist in three isoforms: COX-1, constitutively found in most cells, in charge of maintaining homeostasis, protection of the gastric mucosa, and regulation of platelet aggregation and renal blood flow; cyclooxygenase-2 (COX-2), an inducible isoform that is detected in neoplastic and normal cells induced through several stimuli (e.g., mitogens, growth factors, hormones, and pro-inflammatory cytokines); and COX-3, which is expressed mainly in the central nervous system and the aortic wall (217, 218).

Since 1897, with the development of aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) have been developed as analgesic, antipyretic, anti-inflammatory, and anti-rheumatic treatment (219). However, a meta-analysis showed a remarkable preventive effect against colon cancer (220). Other studies found the same effects in different types of cancer, such as breast cancer (221). Nevertheless, the adverse effects of long-term use of NSAIDs were significant, mainly gastrointestinal bleeding, increased uric acid, and coagulation inhibition, among others (222). In an attempt to avoid these adverse effects and target specifically COX-2 for its participation in the neoplastic process, selective COX-2 inhibitors were designed and named as "coxibs." Experimentally, both NSAIDs and coxibs have been shown to inhibit tumorigenesis by inhibiting cancer cell growth and proliferation, modulating apoptotic activity, reducing the metastatic and invasive potential of cells, and by inhibiting angiogenesis (223, 224).

Coxibs had been related in human medicine to thrombotic cardiovascular events, myocardial infarction, and stroke, especially in long-term use (225). Although subsequent meta-analysis revealed that the risk of cardiovascular events was not related to the usage of coxibs (226), the use of coxibs as adjuvants in the treatment of human cancer is an ongoing intense field of research, especially in combination with chemotherapeutic agents (227).

COX-2 is overexpressed in various canine epithelial malignant tumors, including ovarian carcinomas, prostate carcinomas, urothelial and transitional cell carcinomas, colorectal and small intestine tumors, squamous cell carcinomas, osteosarcoma, and melanoma (228).

Since 2003, COX-2 expression has been reported in both benign and malignant canine mammary tumors (229) (**Figure 11**). Cyclooxygenase-2 is overexpressed in 83–95% of the CMC in association with characteristics of aggressiveness, such as high histological and nuclear grades, mitotic index, and lymph node metastasis (230–232). COX-2 has an important role in the angiogenesis of CMC: its presence is correlated with MVD and EGFR and VEGF expressions (233). Interestingly, in canine IMC, which is characterized by exacerbated angiogenesis, lymphangiogenesis, and lymphangiotropism, COX-2 is associated with higher lymphatic proliferation index, VEGF-D (a lymphangiogenic factor), and its receptor VEGFR-3; in contrast, COX-2 is associated with VEGF-A in non-inflammatory mammary cancer (234), indicating a different role of COX-2 in the angiogenesis of IMC.

Moreover, COX-2 also participates in the immunomodulation of the tumor microenvironment by promoting the M2 phenotype of macrophages, inhibiting antigen-presenting cells and reducing $CD8^+$ T cells, all of which impair the antitumoral immune response (235).

The non-selective (anti-COX 1 and 2) NSAIDs meloxicam and piroxicam have been tested *in vitro* against CMC cells, which resulted in apoptosis induction, migration inhibition, and cell cycle arrest (236, 237). Additionally, piroxicam was utilized in a xenograft model of CMC, showing a tumor size reduction (238). Further, a small group of dogs with IMC were treated with piroxicam. The mean survival time was significantly longer in dogs treated with piroxicam compared to those treated with doxorubicin-based chemotherapy (239).

Due to the availability of COX-2 selective drugs in veterinary medicine (firocoxib, deracoxib, cimicoxib, robenacoxib, and mavacoxib), their effectiveness in CMC has been studied in cell lines, mice xenografts, and, in lesser extent, in clinical trials. Celecoxib and mavacoxib have been used in CMC cell lines, showing their cytotoxic activity, proliferation inhibition, apoptosis induction, and migration reduction, even in those cell lines with low COX-2 expression, suggesting their extraordinary therapeutic applicability in COX-2-positive or COX-2-negative CMC (217, 240, 241). Deracoxib has been proven alone or in combination with piroxicam, displaying a significant decrease in cell viability, achieving an arrest in cell cycle, and induction of apoptosis in CMC cell lines (237). Additionally, when combined with doxorubicin, a strong synergistic activity was seen, allowing to reduce the dose of doxorubicin *in vitro* (242). In CMT xenografted mice, deracoxib was given in comparison to piroxicam, although its effect was lower than piroxicam. Nevertheless, the authors did not evaluate the dose of deracoxib, thus concluding that mice could have been underdosed (238).

In a clinical trial, piroxicam showed an increased in OS compared to doxorubicin in 12 IMC-bearing dogs with COX-2-positive tumor cells (239). Likewise, in a case–control prospective study, firocoxib showed higher disease-free survival and overall survival compared to mitoxantrone in dogs with highly malignant CMT-expressing COX-2 (18).

As mentioned above, in the available clinical trials on the use of NSAIDs/coxibs in CMC, patients had COX-2-positive tumors. However, *in vitro* evidence has shown that anti-COX-2 therapy may be therapeutically useful regardless of the COX-2 expression status (217).

As a conclusion, the anti-COX-2 drugs approved for their use in veterinary medicine are being used in veterinary clinics as anti-inflammatory drugs, but also as an adjuvant in neoplastic patients, no matter the expression of the enzyme in the patients' tumors and, in many cases, the lack of proper clinical trials.

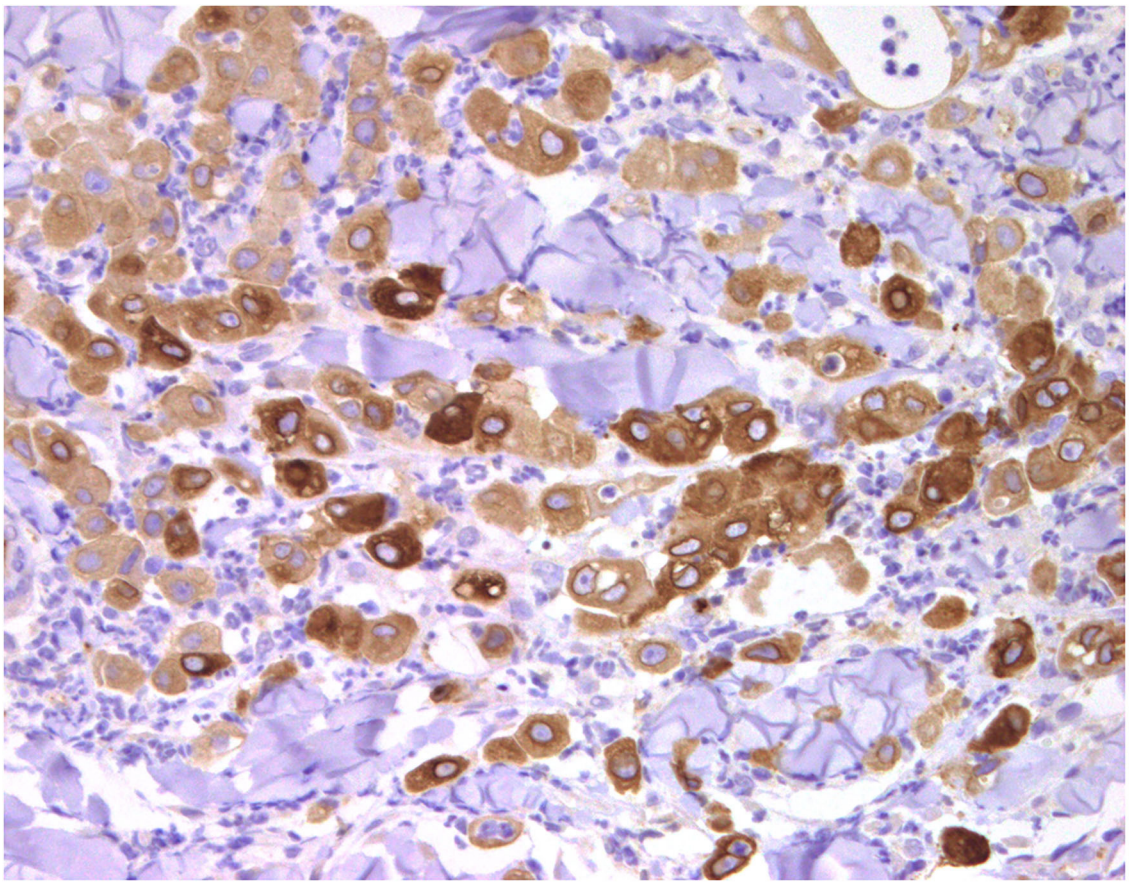

FIGURE 11 | Anaplastic carcinoma, mammary gland, dog, inflammatory carcinoma. Immunohistochemical cytoplasmic staining of cyclooxygenase-2 (COX-2). Neoplastic cells are positive with different intensities of immunolabeling.

Epithelial–Mesenchymal Transition Inhibition: Cancer Stem Cells

Epithelial–mesenchymal transition (EMT) is a complex process in which epithelial cells lose their characteristics and acquire mesenchymal properties. It is essential in different embryonic stages and organ development, wound healing, and neoplastic infiltration and metastasis, cell motility, and invasiveness (243). By this process, epithelial cells undergo detachment and acquire the capacities of motility and invasiveness through the extracellular matrix to finally enter blood and lymphatic vessels and colonize different organs.

EMT is driven by the dysregulation of the adhesion molecules (mainly cadherins). Cadherins are calcium-dependent adhesion molecules responsible for cell-to-cell attachment and for maintenance of the normal structure and polarization of tissues. The most relevant are P-cadherin (placental cadherin), N-cadherin (neural cadherin), and E-cadherin (epithelial cadherin) (244). In normal human mammary tissue, E-cadherin is expressed in luminal epithelial cells, while P-cadherin is found in myoepithelial cells (245). In HBC, E-cadherin is known to be an inhibitor of metastasis, and its downregulation or inactivation leads to aggressive forms of breast cancer, EMT, lymphovascular invasion and metastasis (246, 247), as well as higher histological grade (248). Likewise, in CMC, reduction of E-cadherin expression has been related to large size and ulceration of mammary tumors (249), infiltrative growth, high histological grade, and lymph node metastasis (250–253), as well as shorter overall and disease-free survivals (254). EMT involves a range of transcription factors, including zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2), Snail, signal transducer and activator of transcription 3 (STAT3), and Twist, as well as transcriptional targets such as transforming growth factor-β (TGF-β), all of which suppress the expression of epithelial-associated molecules (E-cadherin and membranous β-catenin) and promote molecules associated with mesenchymal cell phenotypes (N-cadherin, cytoplasmic β-catenin, fibronectin, and vimentin) (255, 256). In CMC, Snail and ZEB2 have been proven to be related to E-cadherin downregulation in invasive micropapillary carcinomas (257, 258).

Not only EMT is required for successful metastatic colonization, but also cells that are capable of initiating tumorigenesis may undergo self-renewal and differentiate into various subsets of cells found in the primary tumor; these cells are called cancer stem cells (CSCs) (259). Molecular pathways

that lead to EMT are markedly overlapping with those of CSC generation, allowing neoplastic epithelial cells to pass through the EMT and generate a population of CSCs (260). Additionally, CSCs are associated with therapeutic resistance (261).

Several reviews are centered on EMT as a source of metastasis, CSC generation, and therapy resistance in HBC (261–263). Likewise, studies on CMC have revealed a positive correlation between cells undergoing EMT and higher tumor grade and metastasis (264) and have identified ZEB1 and ZEB2 as potential therapeutic targets in CMT cells *in vitro*, intended to restore E-cadherin and inhibit EMT, although to date, there are no drugs targeting these molecules (265).

A number of attempts have been made for the therapeutic targeting of EMT and CSCs in HBC and CMC, which are presented below.

As aforementioned, melatonin is capable of disrupting estrogen-dependent cell signaling. In addition, *in vitro* studies on HBC and CMC cell lines have shown that melatonin is also able to reduce EMT through the degradation of β-catenin, an E-cadherin repressor (266), reducing cell migration, invasion, and CSC generation (267, 268). Significantly, the effect of melatonin is higher on ER+ CMC cells overexpressing melatonin receptors (269). In a more recent *in vitro* study, CMC cell lines treated with melatonin plus IL-25 significantly reduced cell viability, increased caspase-3-mediated apoptosis, and reduced pro-angiogenic VEGF-A (268).

Metformin, a commonly used drug in human medicine as oral treatment for type II diabetes, is being experimentally studied due to its anti-carcinogenic properties associated with the inhibition of the EMT process. Metformin has been shown to inhibit EMT in HBC cells by repressing the drivers of TGF-β (270). *In vitro* studies on CMC cell lines proved that metformin is able to induce cell cycle arrest and reduce cell migration and N-cadherin expression while increasing E-cadherin expression. The foreseen effect was ever more prominent when combined with silencing of TGF-β in the CMC cell line (271). In CMC xenografted mice treated with metformin, there was a significant decline in CSCs and reduction of lung metastasis and tumor growth (272–274). When combined with LY294002 (an inhibitor of the PI3K/AKT/mTOR pathway with anti-angiogenic properties), metformin showed a marked reduction in viability and tumor cell growth *in vitro*, while in CMC xenografted mice, both drugs decreased the tumor size and showed an important anti-angiogenic effect (reduction of VEGF-A expression and MVD) (275).

Cancer stem cells are also characterized by the presence of multidrug-resistant (MDR) adenosine triphosphate-binding cassette (ABC) transporter efflux pumps, influencing chemotherapy resistance in cancer due to their capacity to export a wide variety of cell substances (276). It has been seen that the Wnt/β-catenin pathway can upregulate the multidrug resistance protein 1 (MDR-1). Simvastatin (a lipid-lowering drug) has been studied in mammary oncology due to retrospective evidence of an improved OS and recurrence-free survival in HBC-bearing patients (277), and further *in vitro* analyses revealed an induction of apoptosis on HBC cell lines (278). *In vitro* studies using simvastatin on CMC cell lines showed that the expressions of MDR-1 and β-catenin were reduced, contributing to a chemosensitizing effect on CMC cells (279). Furthermore, when combined with doxorubicin, simvastatin exhibited a synergic cytotoxic effect on CMC cells (280). However, despite good *in vitro* results, discouraging large meta-analyses have found no significant benefit from the use of simvastatin in patients suffering from HBC (281–283).

Artemisinin, a derivate from the plant *Artemisia annua*, employed in Chinese traditional medicine, was discovered by Tu Youyou in 1972 for the treatment of malaria. By this discovery, Tu Youyou was co-recipient of the 2015 Nobel Prize in Medicine. Studies on HBC cells showed the *in vitro* suppression of N-cadherin, involved in EMT (284). Additionally, synthetic derivates of artemisinin (e.g., artesunate) are capable of inducing caspase-dependent apoptosis in HBC cells (285). The *in vitro* treatment of CMC cells with an artemisinin derivative (dihydroartemisinin, DHA) showed an inhibition of cell migration and invasiveness by downregulating the expression of EMT-related genes (Slug, ZEB1, ZEB2, and Twist) (286). In human medicine, a few phase I clinical trials have been conducted without significant adverse effects (287, 288), although a patient with HBC on artemisinin treatment showed toxic encephalopathy (289). Knowing the neurotoxicity of artemisinin in experimental animal models (290), a phase I clinical trial should be performed in domestic animals before giving any general recommendation on its use in veterinary patients.

IMMUNOTHERAPY AS A PROGRESSING TARGET

In human oncology, after decades of depleting the immune system of cancer patients with chemotherapy, the tendency is now to protect and increase the action of the immune system against cancer cells (291).

Cancer immunoediting is the process by which the immune system tries to destroy neoplastic cells and is composed of three steps: elimination, equilibrium, and escape (292). In the first step, the host's immune system responds to the newly formed tumor and removes it prior to any clinical evidence. If some resistant clones of the tumor are present, they survive and remain inactive in the second stage, the equilibrium. In the escape or evasion stage, tumor cells improve their ability to evade the immune system, eventually leading to clinical manifestation (293). Some of the evasion mechanisms are as follows: decreased or absent expression of major histocompatibility complex (MHC) molecules, activation of immunoregulatory pathways (immune checkpoints) such as the inhibitory molecule CTLA-4 (cytotoxic T lymphocyte antigen 4), upregulation of PD-L1 (programmed death ligand 1) that binds to the PD-1 receptor on T lymphocytes and represses their function, secretion of immunosuppressive factors such as TGF-β, interleukin-10, and VEGF, as well as induction of regulatory T cells (294). Although many of these mechanisms are already known, the true interplay between the pathways, and the interaction between tumor cells and the immune system and microenvironment, is still largely unknown.

Some mAbs that target specific molecules that intervene in a signaling pathway (e.g., anti-HER-2) have been mentioned in this review. In this section, we will discuss an intriguing class of antibodies designed to modulate the immune response and the use of vaccines, including cellular immunotherapy and DNA vaccines. Cellular immunotherapy, in particular hybrid cell vaccines, are based on cells generated by fusing antigen-presenting cells (i.e., dendritic cells) with the tumor cells of the recipient, intending to present to the immune system the whole tumor-associated antigens and activate an immune response (295). DNA vaccines are based on the introduction of one or more genes (e.g., tumor antigen, cytokines, etc.) into plasmids that are delivered into the patient with the subsequent expression of the introduced gene (296). Finally, the role of oncolytic viruses, which show selective cytotoxicity toward cancer cells and may favor the restoration of the anticancer immune function, will be reviewed (297).

The most successful human immunotherapies to date include mAbs against lymphoma antigens (i.e., CD20—rituximab) as well as mAbs against immune checkpoint molecules such as PD-1 (i.e., pembrolizumab, atezolizumab, and nivolumab) and CTLA-4 (i.e., tremelimumab and ipilimumab), which are able to release the cytotoxic activity of T lymphocytes and activate other immune responses such as antigen presentation and cytokine production (298). Besides targeting immune checkpoint molecules, other immunotherapies are being developed, such as tumor-specific cytotoxic immune cells and cytokines. However, although some cancers, such as melanoma and lymphoma, respond well to immunotherapy, other solid tumors still have weak responses (299).

Cancer cell lines and mouse models, including transgenic mice and patient-derived xenografts, have been extremely useful in the study of human cancer, yielding valuable insights into cancer biology, genetics, and biochemistry (300). However, they have limitations and lack essential features inherent only to spontaneous cancers, like an intact complex immune system (301, 302). Because normal immunocompetent mice reject human tumor grafts, there are no preclinical experimental models to investigate immunotherapy for cancer patient tumors. Canine patients with CMC have been used as an intermediate model in several clinical trials with novel immunotherapy (303).

Several clinical trials have revealed relevant information regarding canine cancer immunotherapy in osteosarcoma, lymphoma, melanoma, meningioma, bladder cancer, soft tissue sarcoma, and hemangiosarcoma using multiple immunotherapeutic approaches (DNA vaccines, cellular immunotherapy, mAbs, bacteria, etc.) (303–310), which are out of the scope of this review.

Antibody Immunotherapy

Even though HBC is not considered a highly immunogenic cancer, immunotherapeutic strategies are being successfully tested especially against TNBC (311). Proof of this is that the FDA approved an immunotherapy for advanced PD-L1+ TNBC, atezolizumab (anti-PD-1 mAB), in combination with paclitaxel (312). Another anti-PD-L1 mAb, pembrolizumab, has also been shown to improve the progression-free survival in PD-1+ TNBC when combined with paclitaxel, gemcitabine, carboplatin, or eribulin (313, 314). Although PD-1 has been detected in CMTs (315, 316), no attempt has been made to use anti-PD-1 or anti-PD-L1 mAbs against CMC, perhaps due to cost restrictions. The only published clinical trial with mAbs against CMC (including IMC) used the mouse monoclonal antibody BR96, which recognizes a specific antigen (Lewis^y-related carbohydrate, Le^y) expressed in several solid tumors and gastrointestinal epithelium, conjugated with a truncated, non-binding derivate of *Pseudomonas* exotoxin A. Stable disease or partial response was achieved in IMC cases, as well as neutralizing antibodies (317). In addition to this, in 2014, a canine anti-EGFR-1 mAb was developed, but it never reached the clinical or preclinical level (151).

Cellular Immunotherapy

Cellular immunotherapy is an emerging field of research in which immune cells are extracted, modified, and reinfused into the patient (318). In HBC, two main types of cellular immunotherapy have been studied: adoptive cell therapy (ACT, based on T lymphocytes) and dendritic cell therapy (319). ACT is based on the isolation of T lymphocytes in the resected tumor, *in vitro* expansion or modification, and subsequent reinfusion (318). Few information on HBC are available to date. Direct T lymphocyte reinfusion in combination with pembrolizumab led to a complete durable regression in a patient with chemotherapy-refractory HBC (320). Chimeric antigen receptor (CAR) T cell therapy is a type of modification where the receptor is engineered to target a specific antigen and combine antigen-binding and T cell-activating functions, hence recognizing antigens in the absence of the presentation by the MHC (321). To date, only preclinical studies have been published using HER-2, mucin 1 cell surface associated (MUC-1), mesothelin (MSLN), epithelial cell adhesion molecule (EPCAM), and carcinoembryonic antigen (CEA) as targets (322). On the other hand, in dendritic cell therapy, the cells are isolated and combined with tumor antigens before reinfusion into the patient (319). Despite clinical settings being currently limited to phase I/II human trials, preclinical studies on HER-2-loaded and cyclin D1-loaded dendritic cell vaccines have been shown to significantly inhibit the HBC xenografted tumor growth in mice (323). Recently, autologous hybrid-cell vaccines were produced for a clinical trial in CMC as an intermediate model for HBC. The therapy was combined with immunostimulatory oligonucleotides and gemcitabine and achieved 3.3 times longer median survival times than the control group, except for the case of IMC, which only resulted in a median of 42 days (324).

DNA Vaccines

DNA, or gene-based, vaccines are intended to deliver functional genes to the target cells for the expression of functional proteins (325). Since naked DNA is readily accessible for endonucleases, the DNA needs an effective and safe delivery method, which can be biological (e.g., viruses and bacteria) or non-biological (e.g., physical methods such as electroporation or chemical methods such as nanoparticles) (326). In HBC, different delivery methods have been tested to transport tumoral antigens, such

as HER-2, *p*53, MUC1, Twist, and mammaglobin-1, as well as immunostimulatory molecules such as IL-6 and IL-12 (326, 327).

Since the intravenous administration of IL-12 has been associated with grave toxicity in humans (328), a clinical trial using nine dogs, one of them with a mammary tumor, used intratumoral IL-12 (plasmid DNA by electroporation) as an immunostimulatory cytokine. Despite transient increases in serum and tumor IL-12 and IFN-ϒ, no clinically relevant outcome benefits were seen (329). Similarly to this, recombinant viral vaccines, based on replication-defective recombinant adenoviruses, have been proven to be safe and to induce strong antibody and cellular antigen-specific immune responses in non-human primates (330). A study evaluated the ability of DNA electroporation and a recombinant adenovirus serotype 6, both expressing telomerase reverse transcriptase (overexpressed in tumor cells, while low to absent in normal cells) and HER-2, to induce immune responses in healthy dogs against these proteins. A detectable and long-standing cellular and humoral immune response was detected in the absence of side effects or autoimmunity (331).

An anticancer DNA vaccine based on p62 (a protein involved in selective macroautophagy that is dispensable for most tissues, but essential for the development and survival of tumors) was utilized in CMC xenografted mice and dogs bearing mammary carcinomas. The intramuscular administration of the *p*62 DNA vaccine achieved a partial response or stable disease in the absence of noteworthy secondary effects. Antitumoral activity was related to lymphocyte infiltration, particularly T lymphocytes, and tumor encapsulation *via* fibrosis (332, 333).

Another clinical trial with dogs presenting CMC utilized nanoparticles carrying DNA plasmids coding canine interferon-β and herpes simplex virus (HSV) thymidine kinase (a suicide gene), which were injected into the tumor bed during mastectomy; afterwards, subcutaneous injections of the nanoparticles associated with a human granulocyte–macrophage colony-stimulating factor and interleukin-2, mixed with allogeneic mammary carcinoma extracts, were periodically administered. The therapy was well-tolerated; only one out of 26 patients had recurrence and none displayed distant metastasis, and overall survival was also improved (334).

Oncolytic Viruses

Oncolytic viruses show selective cytotoxicity toward cancer cells and may favor the restoration of immune anticancer function (297). Clinical trials with oncolytic viruses are currently ongoing in humans, and two viruses have been approved for commercial use. The first, H101 or Oncorine®, is an adenoviral construct with an E1B deletion (in order to avoid replication in normal cells), approved in China in 2005 for the treatment of head-and-neck squamous cell carcinoma (335). The second, T-VEC or Imlygic™, is an engineered herpes simplex virus type I (HSV-1) that expresses the human granulocyte–monocyte colony-stimulating factor as an immune stimulant, approved in 2015 by the United States FDA for the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with recurrent melanoma after initial surgery (336). In veterinary medicine, a number of viruses with natural oncolytic capacity, as well as engineered viruses, are being studied for several neoplasms (337, 337–349). Among them are morbillivirus, poxvirus, and reovirus.

Oncolytic Morbillivirus

In human medicine, the measles virus has demonstrated an oncolytic potential since anecdotical reports describing the regression of hematopoietic neoplasms after natural infection with measles virus (350). In HBC, the measles virus has shown a strong cytolytic effect in cancer cell lines *in vitro* (351–354). Additionally, attenuated measles virus has been proven to overcome chemoresistance in HBC cells. Several studies in mice, however, showed that viral replication also existed in the organs and cells of infected mice and not only in the targeted tumor cells (355–357). Therefore, some modifications have been done to the viral strains. For instance, a recombinant measles virus strain was created by eliminating its ability to bind to the signaling lymphocyte activation molecule (SLAM), a major receptor used by the wild-type virus to infect immune cells in naturally occurring infections. Cancer cells do not express SLAM molecules, but the measles virus is able to use the Nectin-4 receptor to bind and infect the cell (351). Interestingly, Nectin-4 expression has been found in HBC cells (358) and in 45% of CMT tissue samples and in CMC cell lines. Recombinant measles virus strain exerts cytotoxic effects in Nectin-4-expressing CMC cell lines. In Nectin-4-expressing CMC xenografted mice, this oncolytic therapy showed significant suppression of tumor growth without any noticeable adverse effect (340).

Canine distemper virus (CDV) has been shown to induce apoptosis in the cerebellum and lymphoid tissue of naturally infected dogs through the extrinsic pathway, activating caspase-8 and caspase-3 (359, 360). Therefore, this morbillivirus is considered to be a candidate for potential treatment in canine malignancies. An attenuated strain of CDV showed a substantial oncolytic effect in CMC cells both *in vitro* and in xenografted mice, without significant adverse events, by inducing apoptosis through the same pathways as natural infection (extrinsic pathway) with participation of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (346, 361).

Oncolytic Poxvirus

Several studies on the use of multiple strains of vaccinia virus (362–366) have demonstrated the *in vitro* and preclinical effects against HBC, which have led to a randomized phase III clinical trial in patients with metastatic HBC in which the use of a poxviral vaccine in combination with docetaxel resulted in an increase in progression-free survival (367).

Two oncolytic strains of vaccinia virus (strain GLV-1h68 and strain GLV-5b451 expressing GLAF-2, an antibody against VEGF) have been tested against CMC cells *in vitro* or in xenografted mice, resulting in efficient infection and lysis of cells *in vitro* while achieving significant tumor growth inhibition *in vivo* with strong inflammatory and oncolytic-associated effects (368, 369) and a reduction of MVD in the tumors treated with strain GLV-5b451 (337).

An attenuated form of *Myxoma virus* lacking the *serp2* gene (an anti-apoptotic virulence factor) was used to evaluate its

oncolytic activity in canine mammary cancer cells and showed severe cytopathic effects and adequate viral replication (370).

Oncolytic Reovirus

The oncolytic virus pelareorep (REOLYSIN®) is a non-modified serotype 3 reovirus strain that has shown antitumor activity in clinical and preclinical models, especially in pancreatic cancer, and currently is being tested in clinical trials to assess its efficacy as an oncolytic agent against several cancers. In HBC cells, REOLYSIN® infection in the presence of DNA-damaging agents enhances infection and triple-negative breast cancer cell killing by the reovirus (371).

REOLYSIN® was tested in CMC cells *in vitro* and in xenografted mice, demonstrating significant cell death *via* caspase-3-mediated apoptosis (338). When combining this oncolytic therapy with low doses of paclitaxel, carboplatin, gemcitabine, or toceranib, its activity was enhanced with all the therapeutic agents, except toceranib (341). A series of cases of dogs with various malignancies, which included two cases of CMC (one IMC and one non-IMC), were treated with intratumoral or intravenous REOLYSIN®. Less than 50% of the dogs presented grade I or II adverse effects, which included vomiting, diarrhea, and inflammation of the injected tumor. Dogs did not shed virus and had elevated neutralizing antibodies. No specific information about the antitumoral response in CMC patients was provided (341, 345).

OTHER ADJUVANT THERAPIES

Nanotechnology

Nanotechnology has been evolving rapidly in recent years, providing new therapeutic tools for several diseases. Nanoparticles are defined as particles below 100 nm of dimension, although their surface is generally large enough to bind and carry therapeutic compounds (372). Nanoparticles have been proposed as drug carriers in cancer treatment since they can increase drug accumulation in target tissues, optimizing the therapeutic effect (373).

Several nanoparticle-based delivery platforms have been approved by the US FDA, and two nano-based drugs are already in the market for HBC—Doxil® (doxorubicin-loaded nanoparticles) and Abraxane® (albumin-bound paclitaxel-loaded nanoparticles)—whose coating evades the immune system, allowing a precise targeting delivery (374). Additionally, several clinical trials with HBC patients are currently under study using these delivery platforms to carry doxorubicin, paclitaxel, cisplatin, irinotecan, annamycin (synthetic derivate of doxorubicin), and docetaxel (375–379).

In dogs, doxorubicin is a commonly used chemotherapeutic for CMC. However, its toxicity is dose-limiting, reducing treatment efficacy (58). Aldoxorubicin (a prodoxorubicin bound to albumin, which is cleaved from the drug in the acidic tumor microenvironment) (380) was constructed into nanofiber peptides and administered parenterally to xenografted mice bearing HBC, reducing primary tumor burden and lung metastasis as well as improving survival (381). This was also tested in canine mammary tumor cells *in vitro*, showing an excellent anti-proliferative effect at lower doses than free aldoxorubicin or doxorubicin (382).

Gold nanoparticles, associated with other minerals, have been used in human oncology as an aid in diagnostic methods (due to the molecular weight of gold, it captures many x-rays) and as support in thermo- and phototherapy (383). However, direct *in vitro* toxicity has been seen against human colorectal, hepatocellular, and mammary carcinoma cell lines (384). Two metal compounds, Co(III) and Zn(III), that have previously shown remarkable anti-proliferative activity against human colorectal carcinoma cells, hepatocellular carcinoma cells, and breast carcinoma cells were loaded onto 14-nm gold nanoparticles. These metal compounds demonstrated to efficiently lyse CMC cells *in vitro*; moreover, when loaded onto nanoparticles, the effect was even stronger at lower doses (385).

Herbal Medicine

Herbal medicine has long been administered to treat malignancies in Asian countries (386). While 86.4% of HBC patients with Asian background tend to use herbal medicine, the figures in the Western world, although significantly lower, are continually increasing (387). Over the years, numerous herbal compounds with anticancer activities, such as proliferation inhibition, apoptosis induction, anti-angiogenic, and anti-metastatic, have been identified. The most common natural products are curcumin, berberine, artemisinins, ginsenoides, ursolic acid, silibinin, emodin, triptolide, cucurbitacins, tanshinones, ordonin, shikonin, gambogic acid (GA), artesunate, wogonin, β-elemene, and cepharanthine (388).

Few phytochemicals have been studied in veterinary oncology. The following are those related to CMC.

Curcumin is a phytochemical isolated from the rhizome of turmeric (*Curcuma longa*), used in traditional medicine in India and China for a long time (389). *In vitro* studies have demonstrated its anti-proliferative, pro-apoptotic, anti-angiogenic, and chemosensitizing activities against several tumor types, including breast cancer cells (390–396). In veterinary medicine, curcumin and carnosic acid, derived from rosemary leaf extract, were used alone or in combination on mastocytoma, osteosarcoma, and CMC cell lines and resulted in caspase 3 and 7 activation and apoptosis, with a potent synergistic effect when used in combination (397). A major limitation of curcumin is its low absorption (< 1% of orally administered curcumin will be absorbed) (398). Therefore, a liposome-encapsulated curcumin formulation that enables intravenous delivery was developed and tested on canine cancer cells such as CMC, melanoma, and osteosarcoma cell lines, as well as endothelial cells. Cell proliferation was effectively inhibited with the treatment; likewise, the viability, migration, and tube formation of endothelial cells were suppressed. In the same study, a pilot clinical trial was conducted with cancer-bearing dogs (CMC, pulmonary carcinoma, thyroid carcinoma, chest wall sarcoma, osteosarcoma, and malignant melanoma), achieving stable disease in ∼60% of dogs (399). Finally, a combination of curcumin and paclitaxel was loaded into silica nanoparticles and delivered into CMC cell lines, manifesting a clear and persistent cytotoxic effect (400, 401).

The edible wild ginger *Zingiber zerumbet* contains several phytochemicals with healing properties; zerumbone is one of the most important due to its antitumor, anti-inflammatory, antioxidant, antimicrobial, antinociceptive, hepatoprotective, and immunomodulatory activities (402). However, its poor absorption and bioavailability are the main issues for its therapeutic application (403). Therefore, nanostructured lipid carriers loaded with zerumbone have been used as an apoptogenic agent in several neoplastic human and canine cell lines, including CMC. The effect was attributable to increases in caspase-8, caspase-9, caspase-3, and caspase-7 (404).

The last one of the herbal compounds that have been used for canine mammary tumors is berberine, an isoquinolone alkaloid of the plant *Berberis vulgaris* L. that inhibited the proliferation of CMC cells *in vitro* (405).

Old Drugs as New Therapies

Ivermectin is a well-known anti-parasitic agent used to treat a variety of canine parasitic infestations. The mechanism of action of ivermectin in parasites is due to blockade of the parasite chloride channel (406). Currently, ivermectin has been linked to a potential anticancer effect in different tumor types, including breast cancer (407). *In vitro* studies using ivermectin in CMC cell lines, and further in xenografted mice, effectively inhibited cell growth in a dose- and time-dependent manner. The effects were associated with cell cycle arrest *via* the downregulation of CDK4 and cyclin D1 expressions and reduced WNT/β-catenin signaling (408).

Selenium possesses different anti-neoplastic mechanisms: promotion of cell apoptosis, anti-angiogenesis, and immune system regulation. Also, its antioxidant effect may reduce the toxicity of conventional chemotherapeutics if used in combination (409). Different selenium compounds (sodium selenite, methylseleninic acid, and methylselenocysteine) showed *in vitro* anti-proliferative effects on CMC that were even greater when combined with cyclophosphamide. An increase of apoptosis, downregulation of pro-angiogenic VEGFA, angiopoietin-2, and hypoxia-inducible factor-1 alpha, and upregulation of the anti-angiogenic and anti-proliferative phosphatase and tensin homolog (PTEN) were the major features (409). In further CMC xenografted mouse models, the different selenium compounds significantly inhibited tumor growth, generated large necrotic areas, and reduced the MVD compared to the untreated control. This *in vivo* study also found a reduction in pro-angiogenic factors (VEGFA, PDGF, and angiopoietin-2) (410).

Salinomycin is an ionophore antibiotic isolated from *Streptomyces albus*, which is widely used in farm animals as an anticoccidial drug (411). Several studies with HBC cell lines demonstrated that salinomycin inhibits *in vitro* growth by inducing apoptosis and selectively targeting CSC (412–419). Likewise, salinomycin was found to have a profound effect in CMC cell lines by selectively depleting canine mammary CSC and inhibiting the Wnt/β-catenin signaling pathway (preventing cell invasion and migration) (420, 421).

DISCUSSION

A total of 71 studies focused on adjuvant therapies in CMTs, not including those related to surgery or conventional chemotherapy, were analyzed in this review. The majority of those studies were performed *in vitro* (49 studies); 15 used xenografted mice to study CMC (in total, 15 papers of mouse models of CMTs). Only six of those investigations done with CMC cells *in vitro* or in mouse models, have reached the clinical setting (138, 143, 239, 332, 333, 341, 345, 399).

Setting clinical studies of conventional chemotherapy aside, to date, 18 clinical trials have been conducted in dogs with CMC, and a third of them are new immunotherapies, which demonstrate the usefulness of spontaneous CMC as a natural model for the study of HBC in a natural model with a complete immune system. They are also a reflection of the current state of cancer research and the important trend to stimulate the immune system against cancer cells (291). In spite of the relevance of such investigations in dogs, the number of patients recruited in them is very low to obtain conclusive and extrapolative results: five of the 18 clinical studies were done with less than eight dogs with mammary cancer; four of them with less than three dogs. In addition, two more studies were executed in non-tumor-bearing dogs. As a main conclusion, larger prospective randomized studies are needed to provide a strong level of evidence that allows a widespread use of some of these new approaches. Considering that CMTs are the most common malignancy in dogs and the low rate of success of routine adjuvant therapies (i.e., conventional chemotherapy), these clinical trials seem to not be enough. Significantly greater effort must be made to generate knowledge and develop canine-specific targeted therapies. There is a great need for well-planned large prospective randomized clinical trials in dogs with CMC to obtain valid results for both species, humans and dogs, on the use of new therapies.

Following the One Health concept, human and veterinary oncology will have to join forces to take advantage of both the economic and technological resources that are invested in HBC research, together with the innumerable advantages of dogs with CMC as a spontaneous animal model.

AUTHOR CONTRIBUTIONS

LP and GV devised, structured, and wrote the manuscript. GV reviewed the literature. ÁA-D and GV performed the immunohistochemical slides and the photographs. All the authors reviewed and corrected the manuscript.

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REFERENCES

1. Moe L. Population-based incidence of mammary tumours in some dog breeds. *J Reprod Fertil Suppl.* (2001) 57:439–43.
2. Salas Y, Márquez A, Diaz D, Romero L. Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: a growing animal health problem. *PLoS ONE.* (2015) 10:e0127381. doi: 10.1371/journal.pone.0127381
3. Goldschmidt MH, Peña L, Zappulli V. Tumors of the mammary gland. In: Meuten DJ, editor. *Tumors in Domestic Animals.* Fifth ed. Danvers, MA: John Wiley & Sons, Inc (2017). p. 723–65. doi: 10.1002/9781119181200.ch17
4. Sorenmo K, Worley D, Zappulli V. Tumors of the mammary gland. In: Vail D, Thamm D, Liptack J, editors. *Withrow and MacEwen's Small Animal Clinical Oncology.* 6 ed. St. Louis, MO: Elsevier (2020). p. 604–25. doi: 10.1016/B978-0-323-59496-7.00028-1
5. Goldschmidt M, Pena L, Rasotto R, Zappulli V. Classification and grading of canine mammary tumors. *Vet Pathol.* (2011) 48:117–31. doi: 10.1177/0300985810393258
6. Sorenmo KU, Rasotto R, Zappulli V, Goldschmidt MH. Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. *Vet Pathol.* (2011) 48:85–97. doi: 10.1177/0300985810389480
7. Peña L, Perez-Alenza MD, Rodriguez-Bertos A, Nieto A. Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. *Breast Cancer Res Treat.* (2003) 78:141–8. doi: 10.1023/A:1022991802116
8. Clemente M, De Andres PJ, Pena L, Perez-Alenza MD. Survival time of dogs with inflammatory mammary cancer treated with palliative therapy alone or palliative therapy plus chemotherapy. *Vet Rec.* (2009) 165:78–81. doi: 10.1136/vetrec.165.3.78
9. Peña L, De Andres PJ, Clemente M, Cuesta P, Perez-Alenza MD. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. *Vet Pathol.* (2013) 50:94–105. doi: 10.1177/0300985812447830
10. Abadie J, Nguyen F, Loussouarn D, Peña L, Gama A, Rieder N, et al. Canine invasive mammary carcinomas as models of human breast cancer. Part 2: immunophenotypes and prognostic significance. *Breast Cancer Res Treatment.* (2018) 167:459–68. doi: 10.1007/s10549-017-4542-8
11. Lee K-H, Park H-M, Son K-H, Shin T-J, Cho J-Y. Transcriptome signatures of canine mammary gland tumors and its comparison to human breast cancers. *Cancers.* (2018) 10:317. doi: 10.3390/cancers10090317
12. Jeong SJ, Lee KH, Nam AR, Cho JY. Genome-wide methylation profiling in canine mammary tumor reveals miRNA candidates associated with human breast cancer. *Cancers.* (2019) 11:1466. doi: 10.3390/cancers11101466
13. Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and mortality and epidemiology of breast cancer in the world. *Asian Pac J Cancer Prev.* (2016) 17:43–46. doi: 10.7314/APJCP.2016.17.S3.43
14. Harbeck N, Penault-llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. *Nat Rev Dis Primers.* (2019) 5:66. doi: 10.1038/s41572-019-0111-2
15. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature.* (2000) 406:747–52. doi: 10.1038/35021093
16. Simon D, Schoenrock D, Baumgärtner W, Nolte I. Postoperative adjuvant treatment of invasive malignant mammary gland tumors in dogs with doxorubicin and docetaxel. *J Vet Intern Med.* (2006) 20:1184–90. doi: 10.1111/j.1939-1676.2006.tb00720.x
17. Marconato L, Lorenzo RM, Abramo F, Ratto A, Zini E. Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumours in dogs. *Vet Comp Oncol.* (2008) 6:90–101. doi: 10.1111/j.1476-5829.2007.00143.x
18. Arenas C, Peña L, Granados-Soler JL, Pérez-Alenza MD. Adjuvant therapy for highly malignant canine mammary tumours: cox-2 inhibitor versus chemotherapy: a case–control prospective study. *Vet Rec.* (2016) 179:125. doi: 10.1136/vr.103398
19. Tran CM, Moore AS, Frimberger AE. Surgical treatment of mammary carcinomas in dogs with or without postoperative chemotherapy. *Vet Comp Oncol.* (2016) 14:252–62. doi: 10.1111/vco.12092
20. Nguyen F, Pena L, Ibisch C, Loussouarn D, Gama A, Rieder N, et al. Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. *Breast Cancer Res Treat.* (2018) 167:635–48. doi: 10.1007/s10549-017-4548-2
21. Rutteman G, Withrow S, EGM. Tumors of the mammary gland. In: Withrow SJ, MacEwen E, editors. *Small Animal Clinical Oncology.* 3rd ed. Philadelphia, PA: WB Saunders (2001). p. 455–77.
22. Chocteau F, Abadie J, Loussouarn D, Nguyen F. Proposal for a histological staging system of mammary carcinomas in dogs and cats. Part 1: canine mammary carcinomas. *Front Vet Sci.* (2019) 6:388. doi: 10.3389/fvets.2019.00388
23. Sorenmo KU, Durham AC, Kristiansen V, Pena L, Goldschmidt MH, Stefanovski D. Developing and testing prognostic bio-scoring systems for canine mammary gland carcinomas. *Vet Comp Oncol.* (2019) 17:479–88. doi: 10.1111/vco.12509
24. Zappulli V, Peña L, Rasotto R, Goldschmidt M, Gama A, Scruggs J, et al. *Surgical Pathology of Tumors of Domestic Animals*. United States of America: Davis-Thompson DVM Foundation (2019).
25. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology.* (1991) 19:403–10. doi: 10.1111/j.1365-2559.1991.tb00229.x
26. Rasotto R, Berlato D, Goldschmidt MH, Zappulli V. Prognostic significance of canine mammary tumor histologic subtypes: an observational cohort study of 229 cases. *Vet Pathol.* (2017) 54:571–8. doi: 10.1177/0300985817698208
27. Canadas A, França M, Pereira C, Vilaça R, Vilhena H, Tinoco F, et al. Canine mammary tumors: comparison of classification and grading methods in a survival study. *Vet Pathol.* (2019) 56:208–19. doi: 10.1177/0300985818806968
28. Destexhe E, Lespagnard L, Degeyter M, Heymann R, Coignoul F. Immunohistochemical identification of myoepithelial, epithelial, and connective tissue cells in canine mammary tumors. *Vet Pathol.* (1993) 30:146–54. doi: 10.1177/030098589303000207
29. Espinosa Los de Monteros A, Millán MY, Ordás J, Carrasco L, Reymundo C, Martín Las de Mulas J. Immunolocalization of the smooth muscle-specific protein calponin in complex and mixed tumors of the mammary gland of the dog: assessment of the morphogenetic role of the myoepithelium. *Vet Pathol.* (2002) 39:247–56. doi: 10.1354/vp.39-2-247
30. Gama A, Alves A, Gartner F, Schmitt F. p63: a novel myoepithelial cell marker in canine mammary tissues. *Vet Pathol.* (2003) 40:412–20. doi: 10.1354/vp.40-4-412
31. Peña L, Gama A, Goldschmidt MH, Abadie J, Benazzi C, Castagnaro M, et al. Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. *Vet Pathol.* (2014) 51:127–45. doi: 10.1177/0300985813509388
32. Bocker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, et al. Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. *Lab Invest.* (2002) 82:737–46. doi: 10.1097/01.LAB.0000017371.72714.C5
33. Rasotto R, Goldschmidt MH, Castagnaro M, Carnier P, Caliari D, Zappulli V. The dog as a natural animal model for study of the mammary myoepithelial basal cell lineage and its role in mammary carcinogenesis. *J Comp Pathol.* (2014) 151:166–80. doi: 10.1016/j.jcpa.2014.04.013
34. Boecker W, Buerger H. Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept. *Cell Prolif.* (2003) 36:73–84. doi: 10.1046/j.1365-2184.36.s.1.7.x
35. Payne SJ, Bowen RL, Jones JL, Wells CA. Predictive markers in breast cancer–the present. *Histopathology.* (2008) 52:82–90. doi: 10.1111/j.1365-2559.2007.02897.x
36. Lokuhetty D, White V, Watanabe R, Cree I. *WHO Classification of Tumours. Breast Tumors.* 5 ed. Lyon: International Agency for Research on Cancer (2019).
37. Rakha EA, Green AR. Molecular classification of breast cancer: what the pathologist needs to know. *Pathology.* (2017) 49:111–9. doi: 10.1016/j.pathol.2016.10.012

38. Gama A, Alves A, Schmitt F. Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification. *Virchows Archiv.* (2008) 453:123–32. doi: 10.1007/s00428-008-0644-3
39. Sassi F, Benazzi C, Castellani G, Sarli G. Molecular-based tumour subtypes of canine mammary carcinomas assessed by immunohistochemistry. *BMC Vet Res.* (2010) 6:5. doi: 10.1186/1746-6148-6-5
40. Ressel L, Puleio R, Loria GR, Vannozzi I, Millanta F, Caracappa S, et al. HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome. *Res Vet Sci.* (2013) 94:299–305. doi: 10.1016/j.rvsc.2012.09.016
41. Shinoda H, Legare ME, Mason GL, Berkbigler JL, Afzali MF, Flint AF, et al. Significance of ERα, HER2, and CAV1 expression and molecular subtype classification to canine mammary gland tumor. *J Vet Diagn Invest.* (2014) 26:390–403. doi: 10.1177/1040638714527289
42. Im KS, Kim NH, Lim HY, Kim HW, Shin JI, Sur JH. Analysis of a new histological and molecular-based classification of canine mammary neoplasia. *Vet Pathol.* (2014) 51:549–59. doi: 10.1177/0300985813498780
43. Varallo GR, Gelaleti GB, Maschio-Signorini LB, Moschetta MG, Lopes JR, De Nardi AB, et al. Prognostic phenotyping classification for canine mammary tumors. *Oncol Lett.* (2019) 18:6545–53. doi: 10.3892/ol.2019.11052
44. Seung BJ, Cho SH, Kim SH, Lim HY, Sur JH. Quantitative analysis of HER2 mRNA expression by RNA *in situ* hybridization in canine mammary gland tumors: comparison with immunohistochemistry analysis. *PLoS ONE.* (2020) 15:e0229031. doi: 10.1371/journal.pone.0229031
45. Burrai GP, Tanca A, De Miglio MR, Abbondio M, Pisanu S, Polinas M, et al. Investigation of HER2 expression in canine mammary tumors by antibody-based, transcriptomic and mass spectrometry analysis: is the dog a suitable animal model for human breast cancer? *Tumor Biol.* (2015) 36:9083–91. doi: 10.1007/s13277-015-3661-2
46. Tsuboi M, Sakai K, Maeda S, Chambers JK, Yonezawa T, Matsuki N, et al. Assessment of HER2 expression in canine urothelial carcinoma of the urinary bladder. *Vet Pathol.* (2019) 56:369–76. doi: 10.1177/0300985818817024
47. Fabelo C, Selmic LE, Huang PC, Samuelson JP, Reagan JK, Kalamaras A, et al. Evaluating optical coherence tomography for surgical margin assessment of canine mammary tumors. *Vet Comp Oncol.* (2020). doi: 10.1111/vco.12632. [Epub ahead of print].
48. Favril S, Brioschi C, Vanderperren K, Abma E, Stock E, Devriendt N, et al. Preliminary safety and imaging efficacy of the near-infrared fluorescent contrast agent DA364 during fluorescence-guided surgery in dogs with spontaneous superficial tumors. *Oncotarget.* (2020) 11:2310–26. doi: 10.18632/oncotarget.27633
49. Newton A, Predina J, Mison M, Runge J, Bradley C, Stefanovski D, et al. Intraoperative near-infrared imaging can identify canine mammary tumors, a spontaneously occurring, large animal model of human breast cancer. *PLoS ONE.* (2020) 15:e0234791. doi: 10.1371/journal.pone.0234791
50. Chang SC, Chang CC, Chang TJ, Wong ML. Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002). *J Am Vet Med Assoc.* (2005) 227:1625–9. doi: 10.2460/javma.2005.227.1625
51. Marconato L, Romanelli G, Stefanello D, Giacoboni C, Bonfanti U, Bettini G, et al. Prognostic factors for dogs with mammary inflammatory carcinoma: 43 cases (2003-2008). *J Am Vet Med Assoc.* (2009) 235:967–72. doi: 10.2460/javma.235.8.967
52. Haga S, Nakayama M, Tatsumi K, Maeda M, Imai S, Umesako S, et al. Overexpression of the p53 gene product in canine mammary tumors. *Oncol Rep.* (2001) 8:1215–19. doi: 10.3892/or.8.6.1215
53. Enginler SO, Akiş I, Toydemir TS, Oztabak K, Haktanir D, Gündüz MC, et al. Genetic variations of BRCA1 and BRCA2 genes in dogs with mammary tumours. *Vet Res Commun.* (2014) 38:21–7. doi: 10.1007/s11259-013-9577-7
54. Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M, Jurka P. Current biomarkers of canine mammary tumors. *Acta Vet Scand.* (2018) 60:66. doi: 10.1186/s13028-018-0417-1
55. Thumser-Henner P, Nytko KJ, Rohrer Bley C. Mutations of BRCA2 in canine mammary tumors and their targeting potential in clinical therapy. *BMC Vet Res.* (2020) 16:30. doi: 10.1186/s12917-020-2247-4
56. Hahn K, Richardson R, Acvim D, Knapp D. Canine malignant mammary neoplasia: biological behavior, diagnosis, and treatment alternatives. *J Am Anim Hosp Assoc.* (1992) 28:251.
57. Ogilvie GK, Reynolds HA, Richardson RC, Withrow SJ, Norris AM, Henderson RA, et al. Phase II evaluation of doxorubicin for treatment of various canine neoplasms. *J Am Vet Med Assoc.* (1989) 195:1580–3.
58. Zambrano-Estrada X, Landaverde-Quiroz B, Dueñas-Bocanegra AA, De Paz-Campos MA, Hernández-Alberto G, Solorio-Perusquia B, et al. Molecular iodine/doxorubicin neoadjuvant treatment impair invasive capacity and attenuate side effect in canine mammary cancer. *BMC Vet Res.* (2018) 14:87. doi: 10.1186/s12917-018-1411-6
59. Sorenmo K. Canine mammary gland tumors. *Vet Clin North Am Small Anim Pract.* (2003) 33:573–96. doi: 10.1016/S0195-5616(03)00020-2
60. Lavalle GE, De Campos CB, Bertagnolli AC, Cassali GD. Canine malignant mammary gland neoplasms with advanced clinical staging treated with carboplatin and cyclooxygenase inhibitors. *In Vivo.* (2012) 26:375–9.
61. Poirier VJ, Hershey AE, Burgess KE, Phillips B, Turek MM, Forrest LJ, et al. Efficacy and toxicity of paclitaxel (Taxol) for the treatment of canine malignant tumors. *J Vet Intern Med.* (2004) 18:219–22. doi: 10.1111/j.1939-1676.2004.tb00164.x
62. von Euler H, Rivera P, Nyman H, Häggström J, Borgå O. A dose-finding study with a novel water-soluble formulation of paclitaxel for the treatment of malignant high-grade solid tumours in dogs. *Vet Comp Oncol.* (2013) 11:243–55. doi: 10.1111/j.1476-5829.2011.00314.x
63. Stratmann N, Failing K, Richter A, Wehrend A. Mammary tumor recurrence in bitches after regional mastectomy. *Vet Surg.* (2008) 37:82–6. doi: 10.1111/j.1532-950X.2007.00351.x
64. Schoos A, Knab VM, Gabriel C, Tripolt S, Wagner DA, Bauder B, et al. *In vitro* study to assess the efficacy of CDK4/6 inhibitor Palbociclib (PD-0332991) for treating canine mammary tumours. *Vet Comp Oncol.* (2019) 17:507–21. doi: 10.1111/vco.12514
65. Kareva I, Waxman DJ, Lakka Klement G. Metronomic chemotherapy: an attractive alternative to maximum tolerated dose therapy that can activate anti-tumor immunity and minimize therapeutic resistance. *Cancer Lett.* (2015) 358:100–6. doi: 10.1016/j.canlet.2014.12.039
66. Lana S, U'Ren L, Plaza S, Elmslie R, Gustafson D, Morley P, et al. Continuous low-dose oral chemotherapy for adjuvant therapy of splenic hemangiosarcoma in dogs. *J Vet Intern Med.* (2007) 21:764–9. doi: 10.1111/j.1939-1676.2007.tb03019.x
67. Wendelburg KM, Price LL, Burgess KE, Lyons JA, Lew FH, Berg J. Survival time of dogs with splenic hemangiosarcoma treated by splenectomy with or without adjuvant chemotherapy: 208 cases (2001-2012). *J Am Vet Med Assoc.* (2015) 247:393–403. doi: 10.2460/javma.247.4.393
68. Finotello R, Henriques J, Sabattini S, Stefanello D, Felisberto R, Pizzoni S, et al. A retrospective analysis of chemotherapy switch suggests improved outcome in surgically removed, biologically aggressive canine haemangiosarcoma. *Vet Comp Oncol.* (2017) 15:493–503. doi: 10.1111/vco.12193
69. Matsuyama A, Poirier VJ, Mantovani F, Foster RA, Mutsaers AJ. Adjuvant doxorubicin with or without metronomic cyclophosphamide for canine splenic hemangiosarcoma. *J Am Anim Hosp Assoc.* (2017) 53:304–12. doi: 10.5326/JAAHA-MS-6540
70. Alexander CK, Cronin KL, Silver M, Gardner HL, London C. The addition of metronomic chemotherapy does not improve outcome for canine splenic haemangiosarcoma. *J Small Anim Pract.* (2019) 60:32–7. doi: 10.1111/jsap.12926
71. Marconato L, Chalfon C, Finotello R, Polton G, Vasconi ME, Annoni M, et al. Adjuvant anthracycline-based vs metronomic chemotherapy vs no medical treatment for dogs with metastatic splenic hemangiosarcoma: a multi-institutional retrospective study of the Italian society of veterinary oncology. *Vet Comp Oncol.* (2019) 17:537–44. doi: 10.1111/vco.12519
72. Treggiari E, Borrego JF, Gramer I, Valenti P, Harper A, Finotello R, et al. Retrospective comparison of first-line adjuvant anthracycline vs metronomic-based chemotherapy protocols in the treatment of stage I and II canine splenic haemangiosarcoma. *Vet Comp Oncol.* (2020) 18:43–51. doi: 10.1111/vco.12548
73. Bracha S, Walshaw R, Danton T, Holland S, Ruaux C, Obradovich J. Evaluation of toxicities from combined metronomic and maximal-tolerated

dose chemotherapy in dogs with osteosarcoma. *J Small Anim Pract.* (2014) 55:369–74. doi: 10.1111/jsap.12228
74. London CA, Gardner HL, Mathie T, Stingle N, Portela R, Pennell ML, et al. Impact of toceranib/piroxicam/cyclophosphamide maintenance therapy on outcome of dogs with appendicular osteosarcoma following amputation and carboplatin chemotherapy: a multi-institutional study. *PLoS ONE.* (2015) 10:e0124889. doi: 10.1371/journal.pone.0124889
75. Duffy ME, Anderson CL, Choy K, Fidel JL. Metronomic administration of lomustine following palliative radiation therapy for appendicular osteosarcoma in dogs. *Can Vet J.* (2018) 59:136–42.
76. Matsuyama A, Schott CR, Wood GA, Richardson D, Woods JP, Mutsaers AJ. Evaluation of metronomic cyclophosphamide chemotherapy as maintenance treatment for dogs with appendicular osteosarcoma following limb amputation and carboplatin chemotherapy. *J Am Vet Med Assoc.* (2018) 252:1377–83. doi: 10.2460/javma.252.11.1377
77. Morgan E, O'Connell K, Thomson M, Boyd S, Sandy J. Primary hepatic neuroendocrine carcinoma treated with doxorubicin and cyclophosphamide in a dog. *J Am Anim Hosp Assoc.* (2019) 55:e55305. doi: 10.5326/JAAHA-MS-6887
78. Polton G, Finotello R, Sabattini S, Rossi F, Laganga P, Vasconi ME, et al. Survival analysis of dogs with advanced primary lung carcinoma treated by metronomic cyclophosphamide, piroxicam and thalidomide. *Vet Comp Oncol.* (2018) 16:399–408. doi: 10.1111/vco.12393
79. Elmslie RE, Glawe P, Dow SW. Metronomic therapy with cyclophosphamide and piroxicam effectively delays tumor recurrence in dogs with incompletely resected soft tissue sarcomas. *J Vet Intern Med.* (2008) 22:1373–9. doi: 10.1111/j.1939-1676.2008.0179.x
80. Burton JH, Mitchell L, Thamm DH, Dow SW, Biller BJ. Low-dose cyclophosphamide selectively decreases regulatory T cells and inhibits angiogenesis in dogs with soft tissue sarcoma. *J Vet Intern Med.* (2011) 25:920–6. doi: 10.1111/j.1939-1676.2011.0753.x
81. Leach TN, Childress MO, Greene SN, Mohamed AS, Moore GE, Schrempp DR, et al. Prospective trial of metronomic chlorambucil chemotherapy in dogs with naturally occurring cancer. *Vet Comp Oncol.* (2012) 10:102–12. doi: 10.1111/j.1476-5829.2011.00280.x
82. Cancedda S, Marconato L, Meier V, Laganga P, Roos M, Leone VF, et al. Hypofractionated radiotherapy for macroscopic canine soft tissue sarcoma: a retrospective study of 50 cases treated with A 5 × 6 gy protocol with or without metronomic chemotherapy. *Vet Radiol Ultrasound.* (2016) 57:75–83. doi: 10.1111/vru.12308
83. Schrempp DR, Childress MO, Stewart JC, Leach TN, Tan KM, Abbo AH, et al. Metronomic administration of chlorambucil for treatment of dogs with urinary bladder transitional cell carcinoma. *J Am Vet Med Assoc.* (2013) 242:1534–8. doi: 10.2460/javma.242.11.1534
84. Campos CBDE, Lavalle GE, Monteiro LN, Pêgas GRA, Fialho SL, Balabram D. Adjuvant thalidomide and metronomic chemotherapy for the treatment of canine malignant mammary gland neoplasms. *In Vivo.* (2018) 32:1659–66. doi: 10.21873/invivo.11429
85. Rossi F, Sabattini S, Vascellari M, Marconato L. The impact of toceranib, piroxicam and thalidomide with or without hypofractionated radiation therapy on clinical outcome in dogs with inflammatory mammary carcinoma. *Vet Comp Oncol.* (2018) 16:497–504. doi: 10.1111/vco.12407
86. Masoud V, Pagès G. Targeted therapies in breast cancer: new challenges to fight against resistance. *World J Clin Oncol.* (2017) 8:120. doi: 10.5306/wjco.v8.i2.120
87. Schneider R, Dorn CR, Taylor DO. Factors influencing canine mammary cancer development and postsurgical survival. *J Natl Cancer Inst.* (1969) 43:1249–61.
88. Misdorp W. Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins. *Vet Q.* (1988) 10:26–33. doi: 10.1080/01652176.1988.9694142
89. Illera JC, Pérez-Alenza MD, Nieto A, Jiménez MA, Silvan G, Dunner S, et al. Steroids and receptors in canine mammary cancer. *Steroids.* (2006) 71:541–8. doi: 10.1016/j.steroids.2005.11.007
90. Queiroga FL, Pérez-Alenza MD, Silvan G, Peña L, Lopes C, Illera JC. Role of steroid hormones and prolactin in canine mammary cancer. *J Steroid Biochem Mol Biol.* (2005) 94:181–7. doi: 10.1016/j.jsbmb.2004.12.014
91. Spoerri M, Guscetti F, Hartnack S, Boos A, Oei C, Balogh O, et al. Endocrine control of canine mammary neoplasms: serum reproductive hormone levels and tissue expression of steroid hormone, prolactin and growth hormone receptors. *BMC Vet Res.* (2015) 11:235. doi: 10.1186/s12917-015-0546-y
92. Mohr A, Lüder Ripoli F, Hammer SC, Willenbrock S, Hewicker-Trautwein M, Kiełbowicz Z, et al. Hormone receptor expression analyses in neoplastic and non-neoplastic canine mammary tissue by a bead based multiplex branched DNA assay: a gene expression study in fresh frozen and formalin-fixed, paraffin-embedded samples. *PLoS ONE.* (2016) 11:e0163311. doi: 10.1371/journal.pone.0163311
93. Nieto A, Peña L, Pérez-Alenza MD, Sánchez MA, Flores JM, Castaño M. Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. *Vet Pathol.* (2000) 37:239–47. doi: 10.1354/vp.37-3-239
94. Mainenti M, Rasotto R, Carnier P, Zappulli V. Oestrogen and progesterone receptor expression in subtypes of canine mammary tumours in intact and ovariectomised dogs. *Vet J.* (2014) 202:62–8. doi: 10.1016/j.tvjl.2014.06.003
95. Yip CH, Rhodes A. Estrogen and progesterone receptors in breast cancer. *Future Oncol.* (2014) 10:2293–301. doi: 10.2217/fon.14.110
96. Padilla-Rodriguez M, Parker SS, Adams DG, Westerling T, Puleo JI, Watson AW, et al. The actin cytoskeletal architecture of estrogen receptor positive breast cancer cells suppresses invasion. *Nat Commun.* (2018) 9:2980. doi: 10.1038/s41467-018-05367-2
97. Lanari C, Wargon V, Rojas P, Molinolo AA. Antiprogestins in breast cancer treatment: are we ready? *Endocr Relat Cancer.* (2012) 19:R35–50. doi: 10.1530/ERC-11-0378
98. Morris JS, Dobson JM, Bostock DE. Use of tamoxifen in the control of canine mammary neoplasia. *Vet Rec.* (1993) 133:539–42. doi: 10.1136/vr.133.22.539
99. Tavares WL, Lavalle GE, Figueiredo MS, Souza AG, Bertagnolli AC, Viana FA, et al. Evaluation of adverse effects in tamoxifen exposed healthy female dogs. *Acta Vet Scand.* (2010) 52:67. doi: 10.1186/1751-0147-52-67
100. Meng Q, Yuan F, Goldberg ID, Rosen EM, Auborn K, Fan S. Indole-3-carbinol is a negative regulator of estrogen receptor-alpha signaling in human tumor cells. *J Nutr.* (2000) 130:2927–31. doi: 10.1093/jn/130.12.2927
101. Weng J-R, Tsai C-H, Kulp SK, Chen C-S. Indole-3-carbinol as a chemopreventive and anti-cancer agent. *Cancer Lett.* (2008) 262:153–63. doi: 10.1016/j.canlet.2008.01.033
102. Martín-Ruiz A, Peña L, González-Gil A, Díez-Córdova LT, Cáceres S, Illera JC. Effects of indole-3-carbinol on steroid hormone profile and tumor progression in a mice model of canine inflammatory mammarycancer. *BMC Cancer.* (2018) 18:626. doi: 10.1186/s12885-018-4518-z
103. Tremont A, Lu J, Cole JT. Endocrine therapy for early breast cancer: updated review. *Ochsner J.* (2017) 17:405–11.
104. Suzuki T, Moriya T, Ishida T, Ohuchi N, Sasano H. Intracrine mechanism of estrogen synthesis in breast cancer. *Biomed Pharmacother.* (2003) 57:460–2. doi: 10.1016/j.biopha.2003.09.007
105. De Andres PJ, Caceres S, Clemente M, Perez-Alenza MD, Illera JC, Pena L. Profile of steroid receptors and increased aromatase immunoexpression in canine inflammatory mammary cancer as a potential therapeutic target. *Reprod Domest Anim.* (2016) 51:269–75. doi: 10.1111/rda.12676
106. Brainard GC, Hanifin JP, Greeson JM, Byrne B, Glickman G, Gerner E, et al. Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. *J Neurosci.* (2001) 21:6405–12. doi: 10.1523/JNEUROSCI.21-16-06405.2001
107. Brydon L, Roka F, Petit L, de Coppet P, Tissot M, Barrett P, et al. Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. *Mol Endocrinol.* (1999) 13:2025–38. doi: 10.1210/mend.13.12.0390
108. Jablonska K, Pula B, Zemla A, Owczarek T, Wojnar A, Rys J, et al. Expression of melatonin receptor MT1 in cells of human invasive ductal breast carcinoma. *J Pineal Res.* (2013) 54:334–45. doi: 10.1111/jpi.12032
109. Kiefer T, Ram PT, Yuan L, Hill SM. Melatonin inhibits estrogen receptor transactivation and cAMP levels in breast cancer cells. *Breast Cancer Res Treat.* (2002) 71:37–45. doi: 10.1023/A:1013301408464
110. Martínez-Campa C, González A, Mediavilla MD, Alonso-González C, Alvarez-García V, Sánchez-Barceló EJ, et al. Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. *Br J Cancer.* (2009) 101:1613–19. doi: 10.1038/sj.bjc.6605336

111. Blask DE, Hill SM, Dauchy RT, Xiang S, Yuan L, Duplessis T, et al. Circadian regulation of molecular, dietary, and metabolic signaling mechanisms of human breast cancer growth by the nocturnal melatonin signal and the consequences of its disruption by light at night. *J Pineal Res.* (2011) 51:259–69. doi: 10.1111/j.1600-079X.2011.00888.x
112. Dorgan JF, Longcope C, Stephenson HE Jr, Falk RT, Miller R, Franz C, et al. Serum sex hormone levels are related to breast cancer risk in postmenopausal women. *Environ Health Perspect.* (1997) 105(Suppl. 3):583–5. doi: 10.1289/ehp.97105s3583
113. Gonzalez LO, Corte MD, Vazquez J, Junquera S, Sanchez R, Alvarez AC, et al. Androgen receptor expresion in breast cancer: relationship with clinicopathological characteristics of the tumors, prognosis, and expression of metalloproteases and their inhibitors. *BMC Cancer.* (2008) 8:149. doi: 10.1186/1471-2407-8-149
114. Esfahlan RJ, Zarghami N, Esfahlan AJ, Mollazadeh M, Nejati K, Nasiri M. The possible impact of obesity on androgen, progesterone and estrogen receptors (ERα and ERβ) gene expression in breast cancer patients. *Breast Cancer.* (2011) 5:227–37. doi: 10.4137/BCBCR.S7707
115. Kono M, Fujii T, Lim B, Karuturi MS, Tripathy D, Ueno NT. Androgen receptor function and androgen receptor-targeted therapies in breast cancer: a review. *JAMA Oncol.* (2017) 3:1266–73. doi: 10.1001/jamaoncol.2016.4975
116. Ji YK, Lee GS, Choi KC, Jeung EB. Anti-progestogenic effect of flutamide on uterine expression of calbindin-D9k mRNA and protein in immature mice. *Reprod Toxicol.* (2006) 22:694–701. doi: 10.1016/j.reprotox.2006.04.015
117. Frank D, Sharpe N, Scott MC, Mirro E, Hartman B, Halliwell WH. Chronic effects of flutamide in male beagle dogs. *Toxicol Pathol.* (2004) 32:243–9. doi: 10.1080/01926230490274416
118. Caceres S, Monsalve B, Peña L, de Andres PJ, Alonso-Diez A, Illera MJ, et al. *In vitro* and *in vivo* effect of flutamide on steroid hormone secretion in canine and human inflammatory breast cancer cell lines. *Vet Comp Oncol.* (2018) 16:148–8. doi: 10.1111/vco.12324
119. McGuire WL, Horwitz KB. A role for progesterone in breast cancer. *Ann N Y Acad Sci.* (1977) 286:90–100. doi: 10.1111/j.1749-6632.1977.tb29408.x
120. Timmermans-Sprang EPM, Gracanin A, Mol JA. Molecular signaling of progesterone, growth hormone, Wnt, and HER in mammary glands of dogs, rodents, and humans: new treatment target identification. *Front Vet Sci.* (2017) 4:53. doi: 10.3389/fvets.2017.00053
121. Huber JC, Ott J. The dialectic role of progesterone. *Maturitas.* (2009) 62:326–9. doi: 10.1016/j.maturitas.2008.12.009
122. Kariagina A, Aupperlee MD, Haslam SZ. Progesterone receptor isoform functions in normal breast development and breast cancer. *Crit Rev Eukaryotic Gene Exp.* (2008) 18:11–33. doi: 10.1615/CritRevEukarGeneExpr.v18.i1.20
123. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, et al. Progesterone induces adult mammary stem cell expansion. *Nature.* (2010) 465:803–7. doi: 10.1038/nature09091
124. Horwitz KB, Sartorius CA. 90 YEARS OF PROGESTERONE: Progesterone and progesterone receptors in breast cancer: past, present, future. *J Mol Endocrinol.* (2020) 65:T49–63. doi: 10.1530/JME-20-0104
125. Ros L, Holst BS, Hagman R. A retrospective study of bitches with pyometra, medically treated with aglepristone. *Theriogenology.* (2014) 82:1281–6. doi: 10.1016/j.theriogenology.2014.08.011
126. Guil-Luna S, Hellmén E, Sánchez-Céspedes R, Millán Y, Martín de las Mulas J. The antiprogestins mifepristone and onapristone reduce cell proliferation in the canine mammary carcinoma cell line CMT-U27. *Histol Histopathol.* (2014) 29:949–55. doi: 10.14670/HH-29.949
127. Guil-Luna S, Sánchez-Céspedes R, Millán Y, De Andrés FJ, Rollón E, Domingo V, et al. Aglepristone decreases proliferation in progesterone receptor-positive canine mammary carcinomas. *J Vet Intern Med.* (2011) 25:518–23. doi: 10.1111/j.1939-1676.2011.0723.x
128. Guil-Luna S, Millán Y, De Andres J, Rollón E, Domingo V, García-Macías J, et al. Prognostic impact of neoadjuvant aglepristone treatment in clinicopathological parameters of progesterone receptor-positive canine mammary carcinomas. *Vet Comp Oncol.* (2017) 15:391–9. doi: 10.1111/vco.12175
129. Cassoni P, Sapino A, Fortunati N, Munaron L, Chini B, Bussolati G. Oxytocin inhibits the proliferation of MDA-MB231 human breast-cancer cells via cyclic adenosine monophosphate and protein kinase A. *Int J Cancer.* (1997) 72:340–4. doi: 10.1002/(SICI)1097-0215(19970717)72:2<340::AID-IJC23>3.0.CO;2-I
130. Benavente MA, Bianchi CP, Imperiale F, Aba MA. Antiproliferative effects of oxytocin and desmopressin on canine mammary cancer cells. *Front Vet Sci.* (2016) 3:119. doi: 10.3389/fvets.2016.00119
131. Cassoni P, Sapino A, Papotti M, Bussolati G. Oxytocin and oxytocin-analogue F314 inhibit cell proliferation and tumor growth of rat and mouse mammary carcinomas. *Int J Cancer.* (1996) 66:817–20. doi: 10.1002/(SICI)1097-0215(19960611)66:6<817::AID-IJC18>3.0.CO;2-#
132. Benavente MA, Bianchi CP, Aba MA. Expression of Oxytocin receptors in canine mammary tumours. *J Comp Pathol.* (2019) 170:26–33. doi: 10.1016/j.jcpa.2019.05.005
133. Chanson P, Salenave S. Diabetes insipidus and pregnancy. *Ann Endocrinol.* (2016) 77:135–8. doi: 10.1016/j.ando.2016.04.005
134. Vande Walle J, Stockner M, Raes A, Nørgaard JP. Desmopressin 30 years in clinical use: a safety review. *Curr Drug Saf.* (2007) 2:232–8. doi: 10.2174/157488607781668891
135. Kaufmann JE, Vischer UM. Cellular mechanisms of the hemostatic effects of desmopressin (DDAVP). *J Thromb Haemost.* (2003) 1:682–9. doi: 10.1046/j.1538-7836.2003.00190.x
136. Giron S, Tejera AM, Ripoll GV, Gomez DE, Alonso DF. Desmopressin inhibits lung and lymph node metastasis in a mouse mammary carcinoma model of surgical manipulation. *J Surg Oncol.* (2002) 81:38–44. doi: 10.1002/jso.10141
137. Iannucci NB, Ripoll GV, Garona J, Cascone O, Ciccia GN, Gomez DE, et al. Antiproliferative effect of 1-deamino-8-D-arginine vasopressin analogs on human breast cancer cells. *Future Med Chem.* (2011) 3:1987–93. doi: 10.4155/fmc.11.152
138. Hermo GA, Turic E, Angelico D, Scursoni AM, Gomez DE, Gobello C, et al. Effect of adjuvant perioperative desmopressin in locally advanced canine mammary carcinoma and its relation to histologic grade. *J Am Anim Hosp Assoc.* (2011) 47:21–7. doi: 10.5326/JAAHA-MS-5509
139. Weinberg RS, Grecco MO, Ferro GS, Seigelshifer DJ, Perroni NV, Terrier FJ, et al. A phase II dose-escalation trial of perioperative desmopressin (1-desamino-8-d-arginine vasopressin) in breast cancer patients. *Springerplus.* (2015) 4:428. doi: 10.1186/s40064-015-1217-y
140. Sorenmo K, Durham AC, Evans B, Scavello H, Stefanovski D. A prospective randomized trial of desmopressin in canine mammary carcinoma. *Vet Comp Oncol.* (2020) 18:796–803. doi: 10.1111/vco.12619
141. Link W. *Principles of Cancer Treatment and Anticancer Drug Development*. Cham: Springer International Publishing (2019). doi: 10.1007/978-3-030-18722-4
142. Mayrhofer P, Kunert R. Nomenclature of humanized mAbs: early concepts, current challenges and future perspectives. *Hum Antibodies.* (2019) 27:37–51. doi: 10.3233/HAB-180347
143. London C, Mathie T, Stingle N, Clifford C, Haney S, Klein MK, et al. Preliminary evidence for biologic activity of toceranib phosphate (Palladia(®)) in solid tumours. *Vet Comp Oncol.* (2012) 10:194–205. doi: 10.1111/j.1476-5829.2011.00275.x
144. Rimawi MF, Schiff R, Osborne CK. Targeting HER2 for the treatment of breast cancer. *Annu Rev Med.* (2015) 66:111–28. doi: 10.1146/annurev-med-042513-015127
145. London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, et al. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clin Cancer Res.* (2009) 15:3856–65. doi: 10.1158/1078-0432.CCR-08-1860
146. Ranieri G, Pantaleo M, Piccinno M, Roncetti M, Mutinati M, Marech I, et al. Tyrosine kinase inhibitors (TKIs) in human and pet tumours with special reference to breast cancer: a comparative review. *Crit Rev Oncol Hematol.* (2013) 88:293–308. doi: 10.1016/j.critrevonc.2013.05.009
147. Papaetis GS, Syrigos KN. Sunitinib: a multitargeted receptor tyrosine kinase inhibitor in the era of molecular cancer therapies. *BioDrugs.* (2009) 23:377–89. doi: 10.2165/11318860-000000000-00000
148. Mazzotta M, Krasniqi E, Barchiesi G, Pizzuti L, Tomao F, Barba M, et al. Long-term safety and real-world effectiveness of trastuzumab in breast cancer. *J Clin Med.* (2019) 8:254. doi: 10.3390/jcm8020254

149. Goutsouliak K, Veeraraghavan J, Sethunath V, De Angelis C, Osborne CK, Rimawi MF, et al. Towards personalized treatment for early stage HER2-positive breast cancer. *Nat Rev Clin Oncol.* (2020) 17:233–50. doi: 10.1038/s41571-019-0299-9
150. Singer J, Weichselbaumer M, Stockner T, Mechtcheriakova D, Sobanov Y, Bajna E, et al. Comparative oncology: ErbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. *Mol Immunol.* (2012) 50:200–9. doi: 10.1016/j.molimm.2012.01.002
151. Singer J, Fazekas J, Wang W, Weichselbaumer M, Matz M, Mader A, et al. Generation of a canine anti-EGFR (ErbB-1) antibody for passive immunotherapy in dog cancer patients. *Mol Cancer Ther.* (2014) 13:1777–90. doi: 10.1158/1535-7163.MCT-13-0288
152. Gray ME, Lee S, McDowell AL, Erskine M, Loh QTM, Grice O, et al. Dual targeting of EGFR and ERBB2 pathways produces a synergistic effect on cancer cell proliferation and migration *in vitro*. *Vet Comp Oncol.* (2016) 15:890–909. doi: 10.1111/vco.12230
153. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med.* (1971) 285:1182–6. doi: 10.1056/NEJM197111182852108
154. Petrovic N. Targeting angiogenesis in cancer treatments: where do we stand? *J Pharm Pharm Sci.* (2016) 19:226–38. doi: 10.18433/J30033
155. Ghosh S, Sullivan CA, Zerkowski MP, Molinaro AM, Rimm DL, Camp RL, et al. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. *Hum Pathol.* (2008) 39:1835–43. doi: 10.1016/j.humpath.2008.06.004
156. Dos Anjos DS, Vital AF, Lainetti PF, Leis-Filho AF, Dalmolin F, Elias F, et al. Deregulation of VEGFR-2 and PDGFR expression and microvascular density in a triple-negative model of canine malignant mammary tumors with lymph node or lung metastasis. *Vet Sci.* (2019) 6:3. doi: 10.3390/vetsci6010003
157. Ribatti D, Nico B, Ruggieri S, Tamma R, Simone G, Mangia A. Angiogenesis and antiangiogenesis in triple-negative breast cancer. *Transl Oncol.* (2016) 9:453–7. doi: 10.1016/j.tranon.2016.07.002
158. Graham JC, Myers RK. The prognostic significance of angiogenesis in canine mammary tumors. *J Vet Intern Med.* (1999) 13:416–18. doi: 10.1111/j.1939-1676.1999.tb01456.x
159. Clarke JM, Hurwitz HI. Understanding and targeting resistance to anti-angiogenic therapies. *J Gastrointest Oncol.* (2013) 4:253–63.
160. Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D. Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: from the biology to the clinic. *Curr Med Chem.* (2006) 13:1845–57. doi: 10.2174/092986706777585059
161. Li Q, Yan H, Zhao P, Yang Y, Cao B. Efficacy and safety of bevacizumab combined with chemotherapy for managing metastatic breast cancer: a meta-analysis of randomized controlled trials. *Sci Rep.* (2015) 5:15746. doi: 10.1038/srep15746
162. Zhou Z, Yao H, Hu H. Disrupting tumor angiogenesis and "the hunger games" for breast cancer. *Adv Exp Med Biol.* (2017) 1026:171–95. doi: 10.1007/978-981-10-6020-5_8
163. Slamon D, Gomez HL, Kabbinavar FF, Amit O, Richie M, Pandite L, et al. Randomized study of pazopanib + lapatinib vs. lapatinib alone in patients with HER2-positive advanced or metastatic breast cancer. *J Clin Oncol.* (2008) 26:1016. doi: 10.1200/jco.2008.26.15_suppl.1016
164. Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther.* (2005) 4:677–85. doi: 10.1158/1535-7163.MCT-04-0297
165. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res.* (2004) 64:7099–109. doi: 10.1158/0008-5472.CAN-04-1443
166. Mangini Prado MC, Losant Macedo SDA, Gumiero Guiraldelli G, Lainetti Patricia DF, Fernando Leis-Filho A, Emiko Kobayashi P, et al. Investigation of the prognostic significance of vasculogenic mimicry and its inhibition by sorafenib in canine mammary gland tumors. *Front Oncol.* (2019) 9:1445. doi: 10.3389/fonc.2019.01445
167. Lee JH, Li Q, An JH, Chae HK, Choi JW, Kim BJ, et al. Antitumor activity of rivoceranib against canine mammary gland tumor cell lines. *Anticancer Res.* (2019) 39:5483–94. doi: 10.21873/anticanres.13741
168. Kennedy KC, Qurollo BA, Rose BJ, Thamm DH. Epidermal growth factor enhances the malignant phenotype in canine mammary carcinoma cell lines. *Vet Comp Oncol.* (2011) 9:196–206. doi: 10.1111/j.1476-5829.2010.00248.x
169. Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, et al. Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry. *Am J Pathol.* (1999) 155:739–52. doi: 10.1016/S0002-9440(10)65173-5
170. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol.* (2000) 156:361–81. doi: 10.1016/S0002-9440(10)64739-6
171. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* (1999) 284:1994–8. doi: 10.1126/science.284.5422.1994
172. Kuczynski EA, Reynolds AR. Vessel co-option and resistance to anti-angiogenic therapy. *Angiogenesis.* (2020) 23:55–74. doi: 10.1007/s10456-019-09698-6
173. Xu Y, Li Q, Li XY, Yang QY, Xu WW, Liu GL. Short-term anti-vascular endothelial growth factor treatment elicits vasculogenic mimicry formation of tumors to accelerate metastasis. *J Exp Clin Cancer Res.* (2012) 31:16. doi: 10.1186/1756-9966-31-16
174. Pinto MP, Sotomayor P, Carrasco-Avino G, Corvalan AH, Owen GI. Escaping antiangiogenic therapy: strategies employed by cancer cells. *Int J Mol Sci.* (2016) 17:1489. doi: 10.3390/ijms17091489
175. Folberg R, Maniotis AJ. Vasculogenic mimicry. *Apmis.* (2004) 112:508–25. doi: 10.1111/j.1600-0463.2004.apm11207-0810.x
176. Barreno L, Cáceres S, Alonso-Diez Á, Vicente-Montaña A, García ML, Clemente M, et al. Vasculogenic mimicry-associated ultrastructural findings in human and canine inflammatory breast cancer cell lines. *BMC Cancer.* (2019) 19:750. doi: 10.1186/s12885-019-5955-z
177. Zhang J, Qiao L, Liang N, Xie J, Luo H, Deng G. Vasculogenic mimicry and tumor metastasis. *J Buon.* (2016) 21:533–41.
178. Clemente M, Pérez-Alenza MD, Illera JC, Peña L. Histological, immunohistological, and ultrastructural description of vasculogenic mimicry in canine mammary cancer. *Vet Pathol.* (2010) 47:265–74. doi: 10.1177/0300985809353167
179. Kuczynski EA, Vermeulen PB, Pezzella F, Kerbel RS, Reynolds AR. Vessel co-option in cancer. *Nat Rev Clin Oncol.* (2019) 16:469–93. doi: 10.1038/s41571-019-0181-9
180. Ashman LK. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol.* (1999) 31:1037–51. doi: 10.1016/S1357-2725(99)00076-X
181. Abbaspour Babaei M, Kamalidehghan B, Saleem M, Huri HZ, Ahmadipour F. Receptor tyrosine kinase (c-Kit) inhibitors: a potential therapeutic target in cancer cells. *Drug Des Dev Ther.* (2016) 10:2443–59. doi: 10.2147/DDDT.S89114
182. Kiupel M, Webster JD, Kaneene JB, Miller R, Yuzbasiyan-Gurkan V. The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. *Vet Pathol.* (2004) 41:371–7. doi: 10.1354/vp.41-4-371
183. Janostiak R, Vyas M, Cicek AF, Wajapeyee N, Harigopal M. Loss of c-KIT expression in breast cancer correlates with malignant transformation of breast epithelium and is mediated by KIT gene promoter DNA hypermethylation. *Exp Mol Pathol.* (2018) 105:41–9. doi: 10.1016/j.yexmp.2018.05.011
184. Gattino F, Maniscalco L, Iussich S, Biasato I, Martano M, Morello E, et al. PDGFR-α, PDGFR-β, VEGFR-2 and CD117 expression in canine mammary tumours and evaluation of the *in vitro* effects of toceranib phosphate in neoplastic mammary cell lines. *Vet Rec.* (2018) 183:221. doi: 10.1136/vr.104414
185. Kubo K, Matsuyama S, Katayama K, Tsutsumi C, Yonezawa K, Shimada T, et al. Frequent expression of the c-kit proto-oncogene in canine malignant mammary tumor. *J Vet Med Sci.* (1998) 60:1335–40. doi: 10.1292/jvms.60.1335
186. Koltai Z, Szabó B, Jakus J, Vajdovich P. Tyrosine kinase expression analyses in canine mammary gland tumours - A pilot study. *Acta Vet Hung.* (2018) 66:294–308. doi: 10.1556/004.2018.027

187. Brunetti B, Beha G, Benazzi C, Bondin V, De Tolla L, Sarli G. CD117 expression influences proliferation but not survival in canine mammary tumours. *J Comp Pathol.* (2014) 151:202–6. doi: 10.1016/j.jcpa.2014.04.018
188. Carvalho MI, Pires I, Dias M, Prada J, Gregório H, Lobo L, et al. Intratumoral CD3+ T-lymphocytes immunoexpression and its association with c-Kit, angiogenesis, and overall survival in malignant canine mammary tumors. *Anal Cell Pathol.* (2015) 2015:920409. doi: 10.1155/2015/920409
189. London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin Cancer Res.* (2003) 9:2755–68.
190. de Campos CB, Lavalle GE, Fialho Ligório S, Camargo Nunes F, Carneiro RA, Amorim RL, et al. Absence of significant adverse events following thalidomide administration in bitches diagnosed with mammary gland carcinomas. *Vet Rec.* (2016) 179:514. doi: 10.1136/vr.103764
191. Hahn KA, Ogilvie G, Rusk T, Devauchelle P, Leblanc A, Legendre A, et al. Masitinib is safe and effective for the treatment of canine mast cell tumors. *J Vet Intern Med.* (2008) 22:1301–9. doi: 10.1111/j.1939-1676.2008.0190.x
192. Thamm DH, Rose B, Kow K, Humbert M, Mansfield CD, Moussy A, et al. Masitinib as a chemosensitizer of canine tumor cell lines: a proof of concept study. *Vet J.* (2012) 191:131–4. doi: 10.1016/j.tvjl.2011.01.001
193. Clark AS, Karasic TB, DeMichele A, Vaughn DJ, O'Hara M, Perini R, et al. Palbociclib (PD0332991)-a selective and potent cyclin-dependent kinase inhibitor: a review of pharmacodynamics and clinical development. *JAMA Oncol.* (2016) 2:253–60. doi: 10.1001/jamaoncol.2015.4701
194. Turner NC, Ro J, André F, Loi S, Verma S, Iwata H, et al. Palbociclib in hormone-receptor–positive advanced breast cancer. *N Engl J Med.* (2015) 373:209–19. doi: 10.1056/NEJMoa1505270
195. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol.* (2010) 2:a001008. doi: 10.1101/cshperspect.a001008
196. Setoguchi A, Sakai T, Okuda M, Minehata K, Yazawa M, Ishizaka T, et al. Aberrations of the p53 tumor suppressor gene in various tumors in dogs. *Am J Vet Res.* (2001) 62:433–9. doi: 10.2460/ajvr.2001.62.433
197. Ochiai K, Azakami D, Morimatsu M, Hirama H, Kawakami S, Nakagawa T, et al. Endogenous Leu332Gln mutation in p53 disrupts the tetramerization ability in a canine mammary gland tumor cell line. *Oncol Rep.* (2018) 40:488–94. doi: 10.3892/or.2018.6409
198. Abdelmegeed SM, Mohammed S. Canine mammary tumors as a model for human disease. *Oncol Lett.* (2018) 15:8195–205. doi: 10.3892/ol.2018.8411
199. Oliveira TF, Maués T, Ramundo MS, Figueiredo AMS, de Mello MFV, El-Jaick KB, et al. TP53 gene expression levels and tumor aggressiveness in canine mammary carcinomas. *J Vet Diagn Invest.* (2017) 29:865–8. doi: 10.1177/1040638717721730
200. Munday JS, Ariyarathna H, Aberdein D, Thomson NA. Immunostaining for p53 and p16(CDKN2A) protein is not predictive of prognosis for dogs with malignant mammary gland neoplasms. *Vet Sci.* (2019) 6:34. doi: 10.3390/vetsci6010034
201. Dolka I, Czopowicz M, Gruk-Jurka A, Wojtkowska A, Sapierzynski R, Jurka P. Diagnostic efficacy of smear cytology and robinson's cytological grading of canine mammary tumors with respect to histopathology, cytomorphometry, metastases and overall survival. *PLoS ONE.* (2018) 13:e0191595. doi: 10.1371/journal.pone.0191595
202. Klopfleisch R, Gruber AD. Differential expression of cell cycle regulators p21, p27 and p53 in metastasizing canine mammary adenocarcinomas versus normal mammary glands. *Res Vet Sci.* (2009) 87:91–6. doi: 10.1016/j.rvsc.2008.12.010
203. Bartley AN, Ross DW. Validation of p53 immunohistochemistry as a prognostic factor in breast cancer in clinical practice. *Arch Pathol Lab Med.* (2002) 126:456–8. doi: 10.1043/0003-9985(2002)126<0456:VOPIAA>2.0.CO;2
204. Yemelyanova A, Vang R, Kshirsagar M, Lu D, Marks MA, Shih IM, et al. Immunohistochemical staining patterns of p53 can serve as a surrogate marker for TP53 mutations in ovarian carcinoma: an immunohistochemical and nucleotide sequencing analysis. *Modern Pathol.* (2011) 24:1248–53. doi: 10.1038/modpathol.2011.85
205. Köbel M, Ronnett BM, Singh N, Soslow RA, Gilks CB, McCluggage WG. Interpretation of P53 immunohistochemistry in endometrial carcinomas: toward increased reproducibility. *Int J Gynecol Pathol.* (2019) 38(Suppl. 1):S123–31. doi: 10.1097/PGP.0000000000000488
206. Dumay A, Feugeas JP, Wittmer E, Lehmann-Che J, Bertheau P, Espié M, et al. Distinct tumor protein p53 mutants in breast cancer subgroups. *Int J Cancer.* (2013) 132:1227–31. doi: 10.1002/ijc.27767
207. Pan Y, Yuan Y, Liu G, Wei Y. P53 and Ki-67 as prognostic markers in triple-negative breast cancer patients. *PLoS ONE.* (2017) 12:e0172324. doi: 10.1371/journal.pone.0172324
208. Abubakar M, Guo C, Koka H, Sung H, Shao N, Guida J, et al. Clinicopathological and epidemiological significance of breast cancer subtype reclassification based on p53 immunohistochemical expression. *NPJ Breast Cancer.* (2019) 5:20. doi: 10.1038/s41523-019-0117-7
209. Fabi A, Mottolese M, Di Benedetto A, Sperati F, Ercolani C, Buglioni S, et al. p53 and BLC2 immunohistochemical expression across molecular subtypes in 1099 early breast cancer patients with long-term follow-up: an observational study. *Clin Breast Cancer.* (2020) 20:e760–70. doi: 10.1016/j.clbc.2020.05.005
210. Morris JS, Nixon C, King OJ, Morgan IM, Philbey AW. Expression of TopBP1 in canine mammary neoplasia in relation to histological type, Ki67, ERalpha and p53. *Vet J.* (2009) 179:422–9. doi: 10.1016/j.tvjl.2007.10.025
211. Rodo A, Malicka E. Immunohistochemical expression of protein p53 in neoplasms of the mammary gland in bitches. *Pol J Vet Sci.* (2008) 11:89–95.
212. Lee CH, Kim WH, Lim JH, Kang MS, Kim DY, Kweon OK. Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors. *J Vet Sci.* (2004) 5:63–9. doi: 10.4142/jvs.2004.5.1.63
213. Levine AJ. Targeting therapies for the p53 protein in cancer treatments. *Ann Rev Cancer Biol.* (2019) 3:21–34. doi: 10.1146/annurev-cancerbio-030518-055455
214. Zhou X, Hao Q, Lu H. Mutant p53 in cancer therapy—the barrier or the path. *J Mol Cell Biol.* (2019) 11:293–305. doi: 10.1093/jmcb/mjy072
215. Turner JG, Dawson J, Sullivan DM. Nuclear export of proteins and drug resistance in cancer. *Biochem Pharmacol.* (2012) 83:1021–32. doi: 10.1016/j.bcp.2011.12.016
216. Grayton JE, Miller T, Wilson-Robles H. *In vitro* evaluation of selective inhibitors of nuclear export (SINE) drugs KPT-185 and KPT-335 against canine mammary carcinoma and transitional cell carcinoma tumor initiating cells. *Vet Comp Oncol.* (2017) 15:1455–67. doi: 10.1111/vco.12289
217. Tamura D, Saito T, Murata K, Kawashima M, Asano R. Celecoxib exerts antitumor effects in canine mammary tumor cells via COX-2-independent mechanisms. *Int J Oncol.* (2015) 46:1393–404. doi: 10.3892/ijo.2015.2820
218. Szweda M, Rychlik A, Babińska I, Pomianowski A. Significance of cyclooxygenase-2 in oncogenesis. *J Vet Res.* (2019) 63:215–24. doi: 10.2478/jvetres-2019-0030
219. Zhang Z, Chen F, Shang L. Advances in antitumor effects of NSAIDs. *Cancer Manage Res.* (2018) 10:4631–40. doi: 10.2147/CMAR.S175212
220. Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. *Lancet Oncol.* (2009) 10:501–7. doi: 10.1016/S1470-2045(09)70035-X
221. Retsky M, Rogers R, Demicheli R, Hrushesky WJ, Gukas I, Vaidya JS, et al. NSAID analgesic ketorolac used perioperatively may suppress early breast cancer relapse: particular relevance to triple negative subgroup. *Breast Cancer Res Treat.* (2012) 134:881–8. doi: 10.1007/s10549-012-2094-5
222. Derry S, Loke YK. Risk of gastrointestinal haemorrhage with long term use of aspirin: meta-analysis. *BMJ.* (2000) 321:1183–7. doi: 10.1136/bmj.321.7270.1183
223. Arun B, Goss P. The role of COX-2 inhibition in breast cancer treatment and prevention. *Semin Oncol.* (2004) 31:22–9. doi: 10.1053/j.seminoncol.2004.03.042
224. Gurpinar E, Grizzle WE, Piazza GA. NSAIDs inhibit tumorigenesis, but how? *Clin Cancer Res.* (2014) 20:1104–13. doi: 10.1158/1078-0432.CCR-13-1573
225. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA.* (2001) 286:954–9. doi: 10.1001/jama.286.8.954

226. White WB, West CR, Borer JS, Gorelick PB, Lavange L, Pan SX, et al. Risk of cardiovascular events in patients receiving celecoxib: a meta-analysis of randomized clinical trials. *Am J Cardiol.* (2007) 99:91–8. doi: 10.1016/j.amjcard.2006.07.069
227. Li S, Jiang M, Wang L, Yu S. Combined chemotherapy with cyclooxygenase-2 (COX-2) inhibitors in treating human cancers: recent advancement. *Biomed Pharmacother.* (2020) 129:110389. doi: 10.1016/j.biopha.2020.110389
228. Doré M. Cyclooxygenase-2 expression in animal cancers. *Vet Pathol.* (2011) 48:254–65. doi: 10.1177/0300985810379434
229. Doré M, Lanthier I, Sirois J. Cyclooxygenase-2 expression in canine mammary tumors. *Vet Pathol.* (2003) 40:207–12. doi: 10.1354/vp.40-2-207
230. Badowska-Kozakiewicz AM, Malicka E. Expression of cyclooxygenase-2 in neoplasms of the mammary gland in bitches. *Pol J Vet Sci.* (2010) 13:337–42.
231. Guimarães MJ, Carvalho MI, Pires I, Prada J, Gil AG, Lopes C, et al. Concurrent expression of cyclo-oxygenase-2 and epidermal growth factor receptor in canine malignant mammary tumours. *J Comp Pathol.* (2014) 150:27–34. doi: 10.1016/j.jcpa.2013.07.005
232. Millanta F, Asproni P, Canale A, Citi S, Poli A. COX-2, mPGES-1 and EP2 receptor immunohistochemical expression in canine and feline malignant mammary tumours. *Vet Comp Oncol.* (2016) 14:270–80. doi: 10.1111/vco.12096
233. Queiroga FL, Pires I, Parente M, Gregório H, Lopes CS. COX-2 over-expression correlates with VEGF and tumour angiogenesis in canine mammary cancer. *Vet J.* (2011) 189:77–82. doi: 10.1016/j.tvjl.2010.06.022
234. Clemente M, Sanchez-Archidona AR, Sardon D, Diez L, Martin-Ruiz A, Caceres S, et al. Different role of COX-2 and angiogenesis in canine inflammatory and non-inflammatory mammary cancer. *Vet J.* (2013) 197:427–32. doi: 10.1016/j.tvjl.2013.02.009
235. Carvalho MI, Pires I, Prada J, Raposo TP, Gregório H, Lobo L, et al. High COX-2 expression is associated with increased angiogenesis, proliferation and tumoural inflammatory infiltrate in canine malignant mammary tumours: a multivariate survival study. *Vet Comp Oncol.* (2017) 15:619–31. doi: 10.1111/vco.12206
236. Iturriaga MP, Paredes R, Arias JI, Torres CG. Meloxicam decreases the migration and invasion of CF41.Mg canine mammary carcinoma cells. *Oncol Lett.* (2017) 14:2198–206. doi: 10.3892/ol.2017.6400
237. Ustün Alkan F, Ustüner O, Bakirel T, Cinar S, Erten G, Deniz G. The effects of piroxicam and deracoxib on canine mammary tumour cell line. *ScientificWorldJournal.* (2012) 2012:976740. doi: 10.1100/2012/976740
238. Sonzogni-Desautels K, Knapp DW, Sartin E, Doré M. Effect of cyclooxygenase inhibitors in a xenograft model of canine mammary tumours. *Vet Comp Oncol.* (2011) 9:161–71. doi: 10.1111/j.1476-5829.2010.00242.x
239. de M Souza CH, Toledo-Piza E, Amorin R, Barboza A, Tobias KM. Inflammatory mammary carcinoma in 12 dogs: clinical features, cyclooxygenase-2 expression, and response to piroxicam treatment. *Can Vet J.* (2009) 50:506–10.
240. Saito T, Tamura D, Asano R. Usefulness of selective COX-2 inhibitors as therapeutic agents against canine mammary tumors. *Oncol Rep.* (2014) 31:1637–44. doi: 10.3892/or.2014.3010
241. Hurst EA, Pang LY, Argyle DJ. The selective cyclooxygenase-2 inhibitor mavacoxib (Trocoxil) exerts anti-tumour effects *in vitro* independent of cyclooxygenase-2 expression levels. *Vet Comp Oncol.* (2019) 17:194–207. doi: 10.1111/vco.12470
242. Bakirel T, Alkan FÜ, ÜStÜNer O, ÇInar S, Yildirim F, Erten G. Synergistic growth inhibitory effect of deracoxib with doxorubicin against a canine mammary tumor cell line, CMT-U27. *J Vet Med Sci.* (2016) 78:657–68. doi: 10.1292/jvms.15-0387
243. Klymkowsky MW, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. *Am J Pathol.* (2009) 174:1588–93. doi: 10.2353/ajpath.2009.080545
244. Takeichi M. Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol.* (1993) 5:806–11. doi: 10.1016/0955-0674(93)90029-P
245. Knudsen KA, Wheelock MJ. Cadherins and the mammary gland. *J Cell Biochem.* (2005) 95:488–96. doi: 10.1002/jcb.20419
246. Hunt NC, Douglas-Jones AG, Jasani B, Morgan JM, Pignatelli M. Loss of E-cadherin expression associated with lymph node metastases in small breast carcinomas. *Virchows Arch.* (1997) 430:285–9. doi: 10.1007/BF01092751
247. Corso G, Figueiredo J, De Angelis SP, Corso F, Girardi A, Pereira J, et al. E-cadherin deregulation in breast cancer. *J Cell Mol Med.* (2020) 24:5930–6. doi: 10.1111/jcmm.15140
248. Li Z, Yin S, Zhang L, Liu W, Chen B. Prognostic value of reduced E-cadherin expression in breast cancer: a meta-analysis. *Oncotarget.* (2017) 8:16445–55. doi: 10.18632/oncotarget.14860
249. Matos AJ, Lopes C, Carvalheira J, Santos M, Rutteman GR, Gärtner F. E-cadherin expression in canine malignant mammary tumours: relationship to other clinico-pathological variables. *J Comp Pathol.* (2006) 134:182–9. doi: 10.1016/j.jcpa.2005.10.004
250. Brunetti B, Sarli G, Preziosi R, Leprotti S, Benazzi C. E-cadherin expression in canine mammary carcinomas with regional lymph node metastases. *J Vet Med A Physiol Pathol Clin Med.* (2003) 50:496–500. doi: 10.1111/j.1439-0442.2003.00577.x
251. Brunetti B, Sarli G, Preziosi R, Monari I, Benazzi C. E-cadherin and beta-catenin reduction influence invasion but not proliferation and survival in canine malignant mammary tumors. *Vet Pathol.* (2005) 42:781–7. doi: 10.1354/vp.42-6-781
252. De Matos AJ, Lopes CC, Faustino AM, Carvalheira JG, Rutteman GR, Gärtner Mde F. E-cadherin, beta-catenin, invasion and lymph node metastases in canine malignant mammary tumours. *Apmis.* (2007) 115:327–34. doi: 10.1111/j.1600-0463.2007.apm_544.x
253. Asproni P, Ressel L, Millanta F, Vannozzi I, Poli A. Co-localization of PTEN and E-cadherin in canine mammary hyperplasias and benign and malignant mammary tumors. *Res Vet Sci.* (2015) 103:113–18. doi: 10.1016/j.rvsc.2015.09.022
254. Gama A, Paredes J, Gärtner F, Alves A, Schmitt F. Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. *Vet J.* (2008) 177:45–53. doi: 10.1016/j.tvjl.2007.05.024
255. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* (2009) 119:1420–8. doi: 10.1172/JCI39104
256. Wu Y, Zhou BP. Snail: more than EMT. *Cell Adhesion Migr.* (2010) 4:199–203. doi: 10.4161/cam.4.2.10943
257. Gamba CO, Rodrigues MA, Gomes DA, Estrela-Lima A, Ferreira E, Cassali GD. The relationship between E-cadherin and its transcriptional repressors in spontaneously arising canine invasive micropapillary mammary carcinoma. *J Comp Pathol.* (2015) 153:256–65. doi: 10.1016/j.jcpa.2015.08.006
258. Gamba CO, Damasceno KA, Ferreira IC, Rodrigues MA, Gomes DA, Alves MR, et al. The investigation of transcriptional repression mediated by ZEB2 in canine invasive micropapillary carcinoma in mammary gland. *PLoS ONE.* (2019) 14:e0209497. doi: 10.1371/journal.pone.0209497
259. Schatton T, Frank NY, Frank MH. Identification and targeting of cancer stem cells. *Bioessays.* (2009) 31:1038–49. doi: 10.1002/bies.200900058
260. Morel A-P, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS ONE.* (2008) 3:e2888. doi: 10.1371/journal.pone.0002888
261. Gooding AJ, Schiemann WP. Epithelial-mesenchymal transition programs and cancer stem cell phenotypes: mediators of breast cancer therapy resistance. *Mol Cancer Res.* (2020) 18:1257–70. doi: 10.1158/1541-7786.MCR-20-0067
262. Sun X, Wang M, Yao L, Li X, Dong H, Li M, et al. Exploring the metabolic vulnerabilities of epithelial-mesenchymal transition in breast cancer. *Front Cell Dev Biol.* (2020) 8:655. doi: 10.3389/fcell.2020.00655
263. Kong D, Hughes CJ, Ford HL. Cellular plasticity in breast cancer progression and therapy. *Front Mol Biosci.* (2020) 7:72. doi: 10.3389/fmolb.2020.00072
264. Raposo-Ferreira TMM, Brisson BK, Durham AC, Laufer-Amorim R, Kristiansen V, Puré E, et al. Characteristics of the epithelial-mesenchymal transition in primary and paired metastatic canine mammary carcinomas. *Vet Pathol.* (2018) 55:622–33. doi: 10.1177/0300985818776054
265. Xavier PLP, Cordeiro YG, Rochetti AL, Sangalli JR, Zuccari DAPC, Silveira JC, et al. ZEB1 and ZEB2 transcription factors are potential therapeutic targets of canine mammary cancer cells. *Vet Comp Oncol.* (2018) 16:596–605. doi: 10.1111/vco.12427
266. Mao L, Dauchy RT, Blask DE, Slakey LM, Xiang S, Yuan L, et al. Circadian gating of epithelial-to-mesenchymal transition in breast cancer

cells via melatonin-regulation of GSK3β. *Mol Endocrinol.* (2012) 26:1808–20. doi: 10.1210/me.2012-1071
267. Gonçalves Ndo N, Colombo J, Lopes JR, Gelaleti GB, Moschetta MG, Sonehara NM, et al. Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells of canine and human cell lines. *PLoS ONE.* (2016) 11:e0150407. doi: 10.1371/journal.pone.0150407
268. Gelaleti GB, Borin TF, Maschio-Signorini LB, Moschetta MG, Hellmén E, Viloria-Petit AM, et al. Melatonin and IL-25 modulate apoptosis and angiogenesis mediators in metastatic (CF-41) and non-metastatic (CMT-U229) canine mammary tumour cells. *Vet Comp Oncol.* (2017) 15:1572–84. doi: 10.1111/vco.12303
269. Ramos Lopes J, Bazela Maschio L, Victorasso Jardim-Perassi B, Gobbe Moschetta M, Carvalho Ferreira L, Rodrigues Martins G, et al. Evaluation of melatonin treatment in primary culture of canine mammary tumors. *Oncol Rep.* (2015) 33:311–19. doi: 10.3892/or.2014.3596
270. Cufí S, Vazquez-Martin A, Oliveras-Ferraros C, Martin-Castillo B, Joven J, Menendez JA. Metformin against TGFβ-induced epithelial-to-mesenchymal transition (EMT): from cancer stem cells to aging-associated fibrosis. *Cell Cycle.* (2010) 9:4461–8. doi: 10.4161/cc.9.22.14048
271. Custódio PR, Colombo J, Ventura FV, Castro TB, Zuccari D. Melatonin treatment combined with TGF-β silencing inhibits epithelial-mesenchymal transition in CF41 canine mammary cancer cell line. *Anticancer Agents Med Chem.* (2020) 20:989–97. doi: 10.2174/1871520620666200407122635
272. Saeki K, Watanabe M, Tsuboi M, Sugano S, Yoshitake R, Tanaka Y, et al. Anti-tumour effect of metformin in canine mammary gland tumour cells. *Vet J.* (2015) 205:297–304. doi: 10.1016/j.tvjl.2015.04.026
273. Barbieri F, Thellung S, Ratto A, Carra E, Marini V, Fucile C, et al. *In vitro* and *in vivo* antiproliferative activity of metformin on stem-like cells isolated from spontaneous canine mammary carcinomas: translational implications for human tumors. *BMC Cancer.* (2015) 15:228. doi: 10.1186/s12885-015-1235-8
274. Leonel C, Borin TF, de Carvalho Ferreira L, Moschetta MG, Bajgelman MC, Viloria-Petit AM, et al. Inhibition of epithelial-mesenchymal transition and metastasis by combined TGFbeta knockdown and metformin treatment in a canine mammary cancer xenograft model. *J Mammary Gland Biol Neoplasia.* (2017) 22:27–41. doi: 10.1007/s10911-016-9370-7
275. Moschetta MG, Leonel C, Maschio-Signorini LB, Borin TF, Gelaleti GB, Jardim-Perassi BV, et al. Evaluation of angiogenesis process after metformin and LY294002 treatment in mammary tumor. *Anticancer Agents Med Chem.* (2019) 19:655–66. doi: 10.2174/1871520619666181218164050
276. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer.* (2002) 2:48–58. doi: 10.1038/nrc706
277. Manthravadi S, Shrestha A, Madhusudhana S. Impact of statin use on cancer recurrence and mortality in breast cancer: a systematic review and meta-analysis. *Int J Cancer.* (2016) 139:1281–8. doi: 10.1002/ijc.30185
278. Bai F, Yu Z, Gao X, Gong J, Fan L, Liu F. Simvastatin induces breast cancer cell death through oxidative stress up-regulating miR-140-5p. *Aging.* (2019) 11:3198–19. doi: 10.18632/aging.101974
279. Cruz P, Reyes F, Torres CG. Simvastatin modulates β-catenin/MDR1 expression on spheres derived from CF41.Mg canine mammary carcinoma cells. *Pol J Vet Sci.* (2018) 21:95–9.
280. Torres CG, Olivares A, Stoore C. Simvastatin exhibits antiproliferative effects on spheres derived from canine mammary carcinoma cells. *Oncol Rep.* (2015) 33:2235–44. doi: 10.3892/or.2015.3850
281. Bonovas S, Filioussi K, Tsavaris N, Sitaras NM. Use of statins and breast cancer: a meta-analysis of seven randomized clinical trials and nine observational studies. *J Clin Oncol.* (2005) 23:8606–12. doi: 10.1200/JCO.2005.02.7045
282. Cardwell CR, Hicks BM, Hughes C, Murray LJ. Statin use after diagnosis of breast cancer and survival. A population-based cohort study. *Epidemiology.* (2015) 26:68–78. doi: 10.1097/EDE.0000000000000189
283. Ahern TP, Damkier P, Feddersen S, Kjærsgaard A, Lash TL, Hamilton-Dutoit S, et al. Predictive pharmacogenetic biomarkers for breast cancer recurrence prevention by simvastatin. *Acta Oncol.* (2020) 59:1009–15. doi: 10.1080/0284186X.2020.1759820
284. Ko YS, Lee WS, Panchanathan R, Joo YN, Choi YH, Kim GS, et al. Polyphenols from artemisia annua L inhibit adhesion and EMT of highly metastatic breast cancer cells MDA-MB-231. *Phytother Res.* (2016) 30:1180–8. doi: 10.1002/ptr.5626
285. Pirali M, Taheri M, Zarei S, Majidi M, Ghafouri H. Artesunate, as a HSP70 ATPase activity inhibitor, induces apoptosis in breast cancer cells. *Int J Biol Macromol.* (2020) 164:3369–75. doi: 10.1016/j.ijbiomac.2020.08.198
286. Dong J, Yang W, Han J, Cheng R, Guo X, Li L. Effect of dihydroartemisinin on epithelial-to-mesenchymal transition in canine mammary tumour cells. *Res Vet Sci.* (2019) 124:240–7. doi: 10.1016/j.rvsc.2019.03.020
287. von Hagens C, Walter-Sack I, Goeckenjan M, Storch-Hagenlocher B, Sertel S, Elsässer M, et al. Long-term add-on therapy (compassionate use) with oral artesunate in patients with metastatic breast cancer after participating in a phase I study (ARTIC M33/2). *Phytomedicine.* (2019) 54:140–8. doi: 10.1016/j.phymed.2018.09.178
288. von Hagens C, Walter-Sack I, Goeckenjan M, Osburg J, Storch-Hagenlocher B, Sertel S, et al. Prospective open uncontrolled phase I study to define a well-tolerated dose of oral artesunate as add-on therapy in patients with metastatic breast cancer (ARTIC M33/2). *Breast Cancer Res Treat.* (2017) 164:359–69. doi: 10.1007/s10549-017-4261-1
289. Panossian LA, Garga NI, Pelletier D. Toxic brainstem encephalopathy after artemisinin treatment for breast cancer. *Ann Neurol.* (2005) 58:812–13. doi: 10.1002/ana.20620
290. Gordi T, Lepist EI. Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicol Lett.* (2004) 147:99–107. doi: 10.1016/j.toxlet.2003.12.009
291. Lohmueller J, Finn OJ. Current modalities in cancer immunotherapy: immunomodulatory antibodies, CARs and vaccines. *Pharmacol Ther.* (2017) 178:31–47. doi: 10.1016/j.pharmthera.2017.03.008
292. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology.* (2007) 121:1–14. doi: 10.1111/j.1365-2567.2007.02587.x
293. Bates JP, Derakhshandeh R, Jones L, Webb TJ. Mechanisms of immune evasion in breast cancer. *BMC Cancer.* (2018) 18:556. doi: 10.1186/s12885-018-4441-3
294. Spranger S, Gajewski TF. Mechanisms of tumor cell–intrinsic immune evasion. *Ann Rev Cancer Biol.* (2018) 2:213–28. doi: 10.1146/annurev-cancerbio-030617-050606
295. Trefzer U, Walden P. Hybrid-cell vaccines for cancer immune therapy. *Mol Biotechnol.* (2003) 25:63–9. doi: 10.1385/MB:25:1:63
296. Lopes A, Vandermeulen G, Préat V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. *J Exp Clin Cancer Res.* (2019) 38:146. doi: 10.1186/s13046-019-1154-7
297. White CL, Twigger KR, Vidal L, De Bono JS, Coffey M, Heinemann L, et al. Characterization of the adaptive and innate immune response to intravenous oncolytic reovirus (Dearing type 3) during a phase I clinical trial. *Gene Ther.* (2008) 15:911–20. doi: 10.1038/gt.2008.21
298. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* (2012) 12:252–64. doi: 10.1038/nrc3239
299. Wang Q, Wu X. Primary and acquired resistance to PD-1/PD-L1 blockade in cancer treatment. *Int Immunopharmacol.* (2017) 46:210–19. doi: 10.1016/j.intimp.2017.03.015
300. Bock BC, Stein U, Schmitt CA, Augustin HG. Mouse models of human cancer. *Cancer Res.* (2014) 74:4671–5. doi: 10.1158/0008-5472.CAN-14-1424
301. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol.* (2004) 172:2731–8. doi: 10.4049/jimmunol.172.5.2731
302. Kopetz S, Lemos R, Powis G. The promise of patient-derived xenografts: the best laid plans of mice and men. *Clin Cancer Res.* (2012) 18:5160–2. doi: 10.1158/1078-0432.CCR-12-2408
303. Dow S. A role for dogs in advancing cancer immunotherapy research. *Front Immunol.* (2020). 10:2935. doi: 10.3389/fimmu.2019.02935
304. Regan D, Dow S. Manipulation of innate immunity for cancer therapy in dogs. *Vet Sci.* (2015) 2:423–39. doi: 10.3390/vetsci2040423
305. Park JS, Withers SS, Modiano JF, Kent MS, Chen M, Luna JI, et al. Canine cancer immunotherapy studies: linking mouse and human. *J Immunother Cancer.* (2016) 4:97. doi: 10.1186/s40425-016-0200-7
306. Me Finocchiaro L, Glikin G. Recent clinical trials of cancer immunogene therapy in companion animals. *World J Exp Med.* (2017) 7:42–8. doi: 10.5493/wjem.v7.i2.42

307. Addissie S, Klingemann H. Cellular immunotherapy of canine cancer. *Vet Sci.* (2018) 5:100. doi: 10.3390/vetsci5040100
308. Klingemann H. Immunotherapy for dogs: running behind humans. *Front. Immunol.* (2018) 9:133. doi: 10.3389/fimmu.2018.00133
309. Bergman PJ. Cancer immunotherapies. *Vet Clin North Am Small Anim Pract.* (2019) 49:881–902. doi: 10.1016/j.cvsm.2019.04.010
310. Tarone L, Barutello G, Iussich S, Giacobino D, Quaglino E, Buracco P, et al. Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy. *Cancer Immunol Immunother.* (2019) 68:1839–53. doi: 10.1007/s00262-019-02360-6
311. de Melo Gagliato D, Buzaid AC, Perez-Garcia J, Cortes J. Immunotherapy in breast cancer. *Curr Pract Clin Challenges. BioDrugs.* (2020) 34:611–23. doi: 10.1007/s40259-020-00436-9
312. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and Nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med.* (2018) 379:2108–21. doi: 10.1056/NEJMoa1809615
313. Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im S-A, Yusof MM, et al. KEYNOTE-355: Randomized, double-blind, phase III study of pembrolizumab + chemotherapy versus placebo + chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer. *J Clin Oncol.* (2020) 38:1000. doi: 10.1200/JCO.2020.38.15_suppl.1000
314. Tolaney SM, Kalinsky K, Kaklamani VG, D'Adamo DR, Aktan G, Tsai ML, et al. A phase Ib/II study of eribulin (ERI) plus pembrolizumab (PEMBRO) in metastatic triple-negative breast cancer (mTNBC) (ENHANCE 1). *J Clin Oncol.* (2020) 38:1015. doi: 10.1200/JCO.2020.38.15_suppl.1015
315. Maekawa N, Konnai S, Okagawa T, Nishimori A, Ikebuchi R, Izumi Y, et al. Immunohistochemical analysis of PD-L1 expression in canine malignant cancers and PD-1 expression on lymphocytes in canine oral melanoma. *PLoS ONE.* (2016) 11:e0157176. doi: 10.1371/journal.pone.0157176
316. Shosu K, Sakurai M, Inoue K, Nakagawa T, Sakai H, Morimoto M, et al. Programmed cell death ligand 1 expression in canine cancer. *In Vivo.* (2016) 30:195–204.
317. Henry CJ, Buss MS, Hellström I, Hellström KE, Brewer WG, Bryan JN, et al. Clinical evaluation of BR96 sFv-PE40 immunotoxin therapy in canine models of spontaneously occurring invasive carcinoma. *Clin Cancer Res.* (2005) 11:751–5.
318. Cirillo M, Tan P, Sturm M, Cole C. Cellular immunotherapy for hematologic malignancies: beyond bone marrow transplantation. *Biol Blood Marrow Transpl.* (2018) 24:433–42. doi: 10.1016/j.bbmt.2017.10.035
319. Huber A, Dammeijer F, Aerts JGJV, Vroman H. Current state of dendritic cell-based immunotherapy: opportunities for *in vitro* antigen loading of different DC subsets? *Front Immunol.* (2018) 9:2804. doi: 10.3389/fimmu.2018.02804
320. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu Y-C, Zheng Z, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med.* (2018) 24:724–30. doi: 10.1038/s41591-018-0040-8
321. Harris DT, Kranz DM. Adoptive T cell therapies: a comparison of T cell receptors and chimeric antigen receptors. *Trends Pharmacol Sci.* (2016) 37:220–30. doi: 10.1016/j.tips.2015.11.004
322. Venetis K, Invernizzi M, Sajjadi E, Curigliano G, Fusco N. Cellular immunotherapy in breast cancer: the quest for consistent biomarkers. *Cancer Treat Rev.* (2020) 90:102089. doi: 10.1016/j.ctrv.2020.102089
323. Gibb MJ, Kroll M, Su Q, Zurawski S, Zurawski G, Igyarto B. Novel cyclin D1-based DC vaccine inhibits TNBC tumor growth. *J Immunol.* (2018) 200:181. Available online at: https://www.jimmunol.org/content/200/1_Supplement/181.13
324. Bird RC, DeInnocentes P, Church Bird AE, Lutful Kabir FM, Martinez-Romero EG, Smith AN, et al. Autologous hybrid cell fusion vaccine in a spontaneous intermediate model of breast carcinoma. *J Vet Sci.* (2019) 20:e48. doi: 10.4142/jvs.2019.20.e48
325. Santos-Carballal B, Fernández Fernández E, Goycoolea FM. Chitosan in non-viral gene delivery: role of structure, characterization methods, and insights in cancer and rare diseases therapies. *Polymers.* (2018) 10:444. doi: 10.3390/polym10040444
326. Shanmugaraj B, Priya LB, Mahalakshmi B, Subbiah S, Hu RM, Velmurugan BK, et al. Bacterial and viral vectors as vaccine delivery vehicles for breast cancer therapy. *Life Sci.* (2020) 250:117550. doi: 10.1016/j.lfs.2020.117550
327. Allahverdiyev A, Tari G, Bagirova M, Abamor ES. Current approaches in development of immunotherapeutic vaccines for breast cancer. *J Breast Cancer.* (2018) 21:343–53. doi: 10.4048/jbc.2018.21.e47
328. Car BD, Eng VM, Lipman JM, Anderson TD. The toxicology of interleukin-12: a review. *Toxicol Pathol.* (1999) 27:58–63. doi: 10.1177/019262339902700112
329. Cicchelero L, Denies S, Haers H, Vanderperren K, Stock E, Van Brantegem L, et al. Intratumoural interleukin 12 gene therapy stimulates the immune system and decreases angiogenesis in dogs with spontaneous cancer. *Vet Comp Oncol.* (2017) 15:1187–205. doi: 10.1111/vco.12255
330. Aurisicchio L, Mennuni C, Giannetti P, Calvaruso F, Nuzzo M, Cipriani B, et al. Immunogenicity and safety of a DNA prime/adenovirus boost vaccine against rhesus CEA in nonhuman primates. *Int J. Cancer.* (2007) 120:2290–300. doi: 10.1002/ijc.22555
331. Peruzzi D, Mesiti G, Ciliberto G, La Monica N, Aurisicchio L. Telomerase and HER-2/neu as targets of genetic cancer vaccines in dogs. *Vaccine.* (2010). 28:1201–8. doi: 10.1016/j.vaccine.2009.11.031
332. Gabai V, Venanzi FM, Bagashova E, Rud O, Mariotti F, Vullo C, et al. Pilot study of p62 DNA vaccine in dogs with mammary tumors. *Oncotarget.* (2014) 5:12803–10. doi: 10.18632/oncotarget.2516
333. Venanzi FM, Gabai V, Mariotti F, Magi GE, Vullo C, Sufianov AA, et al. p62-DNA-encoding plasmid reverts tumor grade, changes tumor stroma, and enhances anticancer immunity. *Aging.* (2019) 11:10711–22. doi: 10.18632/aging.102486
334. Finocchiaro LME, Spector AIM, Agnetti L, Arbe MF, Glikin GC. Combination of suicide and cytokine gene therapies as surgery adjuvant for canine mammary carcinoma. *Vet Sci.* (2018) 5:70. doi: 10.3390/vetsci5030070
335. Garber K. China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst.* (2006) 98:298–300. doi: 10.1093/jnci/djj111
336. Dolgin E. Oncolytic viruses get a boost with first FDA-approval recommendation. *Nat Rev Drug Discov.* (2015) 14:369–71. doi: 10.1038/nrd4643
337. Adelfinger M, Bessler S, Frentzen A, Cecil A, Langbein-Laugwitz J, Gentschev I, et al. Preclinical testing oncolytic vaccinia virus strain GLV-5b451 expressing an anti-VEGF single-chain antibody for canine cancer therapy. *Viruses.* (2015) 7:4075–92. doi: 10.3390/v7072811
338. Igase M, Hwang CC, Coffey M, Okuda M, Noguchi S, Mizuno T. The oncolytic effects of reovirus in canine solid tumor cell lines. *J Vet Med Sci.* (2015) 77:541–8. doi: 10.1292/jvms.14-0570
339. Gupta SK, Yadav PK, Gandham RK, Sahoo AP, Harish DR, Singh AK, et al. Canine parvovirus NS1 protein exhibits anti-tumor activity in a mouse mammary tumor model. *Virus Res.* (2016) 213:289–98. doi: 10.1016/j.virusres.2015.12.017
340. Shoji K, Yoneda M, Fujiyuki T, Amagai Y, Tanaka A, Matsuda A, et al. Development of new therapy for canine mammary cancer with recombinant measles virus. *Mol Ther Oncolytics.* (2016) 3:15022. doi: 10.1038/mto.2015.22
341. Igase M, Hwang CC, Kambayashi S, Kubo M, Coffey M, Miyama TS, et al. Oncolytic reovirus synergizes with chemotherapeutic agents to promote cell death in canine mammary gland tumor. *Can J Vet Res.* (2016) 80:21–31.
342. Bhat AH, Ganguly B, Tiwari AK, Das AK. Canine parvovirus ns1 gene and chicken anemia vp3 gene induce partial oncolysis of canine transmissible venereal tumor. *Sci Rep.* (2017) 7:15419. doi: 10.1038/s41598-017-15734-6
343. MacNeill AL, Weishaar KM, Séguin B, Powers BE. Safety of an oncolytic myxoma virus in dogs with soft tissue sarcoma. *Viruses.* (2018) 10:398. doi: 10.3390/v10080398
344. Sánchez D, Cesarman-Maus G, Amador-Molina A, Lizano M. Oncolytic viruses for canine cancer treatment. *Cancers.* (2018) 10:404. doi: 10.3390/cancers10110404
345. Hwang CC, Igase M, Sakurai M, Haraguchi T, Tani K, Itamoto K, et al. Oncolytic reovirus therapy: pilot study in dogs with spontaneously occurring tumours. *Vet Comp Oncol.* (2018) 16:229–38. doi: 10.1111/vco.12361
346. Li P, Wang J, Chen G, Zhang X, Lin D, Zhou Y, et al. Oncolytic activity of canine distemper virus in canine mammary tubular adenocarcinoma cells. *Vet Comp Oncol.* (2019) 17:174–83. doi: 10.1111/vco.12466
347. Cecil A, Gentschev I, Adelfinger M, Dandekar T, Szalay AA. Vaccinia virus injected human tumors: oncolytic virus efficiency predicted by antigen

profiling analysis fitted boolean models. *Bioengineered.* (2019) 10:190–6. doi: 10.1080/21655979.2019.1622220
348. Igase M, Shousu K, Fujiki N, Sakurai M, Bonkobara M, Hwang CC, et al. Anti-tumour activity of oncolytic reovirus against canine histiocytic sarcoma cells. *Vet Comp Oncol.* (2019) 17:184–93. doi: 10.1111/vco.12468
349. Hamada K, Takagi S, Kuboshima H, Shimada H, Takagi K, Yasuoka T, et al. Cloning of carrier cells infected with oncolytic adenovirus driven by midkine promoter and biosafety studies. *J Gene Med.* (2019) 21:e3064. doi: 10.1002/jgm.3064
350. Zygiert Z. Hodgkin's disease: remissions after measles. *Lancet.* (1971) 1:593. doi: 10.1016/S0140-6736(71)91186-X
351. Sugiyama T, Yoneda M, Kuraishi T, Hattori S, Inoue Y, Sato H, et al. Measles virus selectively blind to signaling lymphocyte activation molecule as a novel oncolytic virus for breast cancer treatment. *Gene Ther.* (2013) 20:338–47. doi: 10.1038/gt.2012.44
352. Delpeut S, Sisson G, Black KM, Richardson CD. Measles virus enters breast and colon cancer cell lines through a PVRL4-mediated macropinocytosis pathway. *J Virol.* (2017) 91:e02191-16. doi: 10.1128/JVI.02191-16
353. Tai CJ, Liu CH, Pan YC, Wong SH, Richardson CD, Lin LT. Chemovirotherapeutic treatment using camptothecin enhances oncolytic measles virus-mediated killing of breast cancer cells. *Sci Rep.* (2019) 9:6767. doi: 10.1038/s41598-019-43047-3
354. Abdullah SA, Al-Shammari AM, Lateef SA. Attenuated measles vaccine strain have potent oncolytic activity against Iraqi patient derived breast cancer cell line. *Saudi J Biol Sci.* (2020) 27:865–72. doi: 10.1016/j.sjbs.2019.12.015
355. Gentschev I, Adelfinger M, Josupeit R, Rudolph S, Ehrig K, Donat U, et al. Preclinical evaluation of oncolytic vaccinia virus for therapy of canine soft tissue sarcoma. *PLoS ONE.* (2012) 7:e37239. doi: 10.1371/journal.pone.0037239
356. Hwang CC, Umeki S, Kubo M, Hayashi T, Shimoda H, Mochizuki M, et al. Oncolytic reovirus in canine mast cell tumor. *PLoS ONE.* (2013) 8:e73555. doi: 10.1371/journal.pone.0073555
357. Laborda E, Puig-Saus C, Rodriguez-García A, Moreno R, Cascalló M, Pastor J, et al. A pRb-responsive, RGD-modified, and hyaluronidase-armed canine oncolytic adenovirus for application in veterinary oncology. *Mol Ther.* (2014) 22:986–98. doi: 10.1038/mt.2014.7
358. Siddharth S, Goutam K, Das S, Nayak A, Nayak D, Sethy C, et al. Nectin-4 is a breast cancer stem cell marker that induces WNT/β-catenin signaling via Pi3k/Akt axis. *Int J Biochem Cell Biol.* (2017) 89:85–94. doi: 10.1016/j.biocel.2017.06.007
359. Moro L, Martins AS, Alves CM, Santos FG, Del Puerto HL, Vasconcelos AC. Apoptosis in the cerebellum of dogs with distemper. *J Vet Med B Infect Dis Vet Public Health.* (2003) 50:221–5. doi: 10.1046/j.1439-0450.2003.00657.x
360. Del Puerto HL, Martins AS, Moro L, Milsted A, Alves F, Braz GF, et al. Caspase-3/-8/-9, Bax and Bcl-2 expression in the cerebellum, lymph nodes and leukocytes of dogs naturally infected with canine distemper virus. *Genet Mol Res.* (2010) 9:151–61. doi: 10.4238/vol9-1gmr717
361. Garcia JA, Ferreira HL, Vieira FV, Gameiro R, Andrade AL, Eugênio FR, et al. Tumour necrosis factor-alpha-induced protein 8 (TNFAIP8) expression associated with cell survival and death in cancer cell lines infected with canine distemper virus. *Vet Comp Oncol.* (2017) 15:336–44. doi: 10.1111/vco.12168
362. Chon HJ, Lee WS, Yang H, Kong SJ, Lee NK, Moon ES, et al. Tumor microenvironment remodeling by intratumoral oncolytic vaccinia virus enhances the efficacy of immune-checkpoint blockade. *Clin Cancer Res.* (2019) 25:1612–23. doi: 10.1158/1078-0432.CCR-18-1932
363. Choi AH, O'Leary MP, Chaurasiya S, Lu J, Kim SI, Fong Y, et al. Novel chimeric parapoxvirus CF189 as an oncolytic immunotherapy in triple-negative breast cancer. *Surgery.* (2018) 163:336–42. doi: 10.1016/j.surg.2017.09.030
364. Kwilas AR, Ardiani A, Dirmeier U, Wottawah C, Schlom J, Hodge JW. A poxviral-based cancer vaccine the transcription factor twist inhibits primary tumor growth and metastases in a model of metastatic breast cancer and improves survival in a spontaneous prostate cancer model. *Oncotarget.* (2015) 6:28194–210. doi: 10.18632/oncotarget.4442
365. Gholami S, Marano A, Chen NG, Aguilar RJ, Frentzen A, Chen CH, et al. A novel vaccinia virus with dual oncolytic and anti-angiogenic therapeutic effects against triple-negative breast cancer. *Breast Cancer Res Treat.* (2014) 148:489–99. doi: 10.1007/s10549-014-3180-7
366. Chaurasiya S, Yang A, Kang S, Lu J, Kim SI, Park AK, et al. Oncolytic poxvirus CF33-hNIS-ΔF14.5 favorably modulates tumor immune microenvironment and works synergistically with anti-PD-L1 antibody in a triple-negative breast cancer model. *Oncoimmunology.* (2020) 9:1729300. doi: 10.1080/2162402X.2020.1729300
367. Heery CR, Ibrahim NK, Arlen PM, Mohebtash M, Murray JL, Koenig K, et al. Docetaxel alone or in combination with a therapeutic cancer vaccine (PANVAC) in patients with metastatic breast cancer: a randomized clinical trial. *JAMA Oncol.* (2015) 1:1087–95. doi: 10.1001/jamaoncol.2015.2736
368. Gentschev I, Stritzker J, Hofmann E, Weibel S, Yu YA, Chen N, et al. Use of an oncolytic vaccinia virus for the treatment of canine breast cancer in nude mice: preclinical development of a therapeutic agent. *Cancer Gene Ther.* (2009) 16:320–8. doi: 10.1038/cgt.2008.87
369. Gentschev I, Ehrig K, Donat U, Hess M, Rudolph S, Chen N, et al. Significant growth inhibition of canine mammary carcinoma xenografts following treatment with oncolytic vaccinia virus GLV-1h68. *J Oncol.* (2010) 2010:736907. doi: 10.1155/2010/736907
370. Urbasic AS, Hynes S, Somrak A, Contakos S, Rahman MM, Liu J, et al. Oncolysis of canine tumor cells by myxoma virus lacking the serp2 gene. *Am J Vet Res.* (2012) 73:1252–61. doi: 10.2460/ajvr.73.8.1252
371. Rodríguez Stewart RM, Berry JTL, Berger AK, Yoon SB, Hirsch AL, Guberman JA, et al. Enhanced killing of triple-negative breast cancer cells by reassortant reovirus and topoisomerase inhibitors. *J Virol.* (2019) 93:e01411-19. doi: 10.1128/JVI.01411-19
372. De Jong WH, Borm PJA. Drug delivery and nanoparticles:applications and hazards. *Int J Nanomed.* (2008) 3:133–49. doi: 10.2147/IJN.S596
373. Zocchi MR, Tosetti F, Benelli R, Poggi A. Cancer nanomedicine special issue review anticancer drug delivery with nanoparticles: extracellular vesicles or synthetic nanobeads as therapeutic tools for conventional treatment or immunotherapy. *Cancers.* (2020) 12:1886. doi: 10.3390/cancers12071886
374. Mirza Z, Karim S. Nanoparticles-based drug delivery and gene therapy for breast cancer: Recent advancements and future challenges. *Semin Cancer Biol.* (2019). doi: 10.1016/j.semcancer.2019.10.020. [Epub ahead of print].
375. Baselga J, Manikhas A, Cortés J, Llombart A, Roman L, Semiglazov VF, et al. Phase III trial of nonpegylated liposomal doxorubicin in combination with trastuzumab and paclitaxel in HER2-positive metastatic breast cancer. *Ann Oncol.* (2014) 25:592–8. doi: 10.1093/annonc/mdt543
376. Park IH, Sohn JH, Kim SB, Lee KS, Chung JS, Lee SH, et al. An open-label, randomized, parallel, phase III trial evaluating the efficacy and safety of polymeric micelle-formulated paclitaxel compared to conventional cremophor EL-based paclitaxel for recurrent or metastatic HER2-negative breast cancer. *Cancer Res Treat.* (2017) 49:569–77. doi: 10.4143/crt.2016.289
377. Bulbake U, Doppalapudi S, Kommineni N, Khan W. Liposomal formulations in clinical use: an updated review. *Pharmaceutics.* (2017) 9:12. doi: 10.3390/pharmaceutics9020012
378. Li Y, Humphries B, Yang C, Wang Z. Nanoparticle-mediated therapeutic agent delivery for treating metastatic breast cancer-challenges and opportunities. *Nanomaterials.* (2018) 8:361. doi: 10.3390/nano8060361
379. Fujiwara Y, Mukai H, Saeki T, Ro J, Lin YC, Nagai SE, et al. A multi-national, randomised, open-label, parallel, phase III non-inferiority study comparing NK105 and paclitaxel in metastatic or recurrent breast cancer patients. *Br J Cancer.* (2019) 120:475–80. doi: 10.1038/s41416-019-0391-z
380. Kratz F. DOXO-EMCH (INNO-206): the first albumin-binding prodrug of doxorubicin to enter clinical trials. *Expert Opin Invest Drugs.* (2007) 16:855–66. doi: 10.1517/13543784.16.6.855
381. Bellat V, Ting R, Southard TL, Vahdat L, Molina H, Fernandez J, et al. Functional peptide nanofibers with unique tumor targeting and enzyme-induced local retention properties. *Adv Func Mater.* (2018) 28:1803969. doi: 10.1002/adfm.201803969
382. Stokol T, Wan C, Blakely R, Bellat V, Law B. Aldoxorubicin-loaded nanofibers are cytotoxic for canine mammary carcinoma and osteosarcoma cell lines *in vitro*: a short communication. *Res Vet Sci.* (2020) 128:86–9. doi: 10.1016/j.rvsc.2019.11.003
383. Núñez C, Estévez SV, Del Pilar Chantada M. Inorganic nanoparticles in diagnosis and treatment of breast cancer. *J Biol Inorg Chem.* (2018) 23:331–45. doi: 10.1007/s00775-018-1542-z

384. Silva A, Luís D, Santos S, Silva J, Mendo AS, Coito L, et al. Biological characterization of the antiproliferative potential of Co(II) and Sn(IV) coordination compounds in human cancer cell lines: a comparative proteomic approach. *Drug Metabol Drug Interact.* (2013) 28:167–76. doi: 10.1515/dmdi-2013-0015
385. Raposo LR, Roma-Rodrigues C, Jesus J, Martins LMDRS, Pombeiro AJ, Baptista PV, et al. Targeting canine mammary tumours via gold nanoparticles functionalized with promising Co(II) and Zn(II) compounds. *Vet Comp Oncol.* (2017) 15:1537–42. doi: 10.1111/vco.12298
386. Efferth T, Miyachi H, Bartsch H. Pharmacogenomics of a traditional Japanese herbal medicine (Kampo) for cancer therapy. *Cancer Genomics Proteomics.* (2007) 4:81–91.
387. Zhu L, Li L, Li Y, Wang J, Wang Q. Chinese herbal medicine as an adjunctive therapy for breast cancer: a systematic review and meta-analysis. *Evid Based Complement Alternat Med.* (2016) 2016:9469276. doi: 10.1155/2016/1819794
388. Luo H, Vong CT, Chen H, Gao Y, Lyu P, Qiu L, et al. Naturally occurring anti-cancer compounds: shining from Chinese herbal medicine. *Chin Med.* (2019) 14:48. doi: 10.1186/s13020-019-0270-9
389. Devassy JG, Nwachukwu ID, Jones PJ. Curcumin and cancer: barriers to obtaining a health claim. *Nutr Rev.* (2015) 73:155–65. doi: 10.1093/nutrit/nuu064
390. Holy J. Curcumin inhibits cell motility and alters microfilament organization and function in prostate cancer cells. *Cell Motil Cytoskeleton.* (2004) 58:253–68. doi: 10.1002/cm.20012
391. Walters DK, Muff R, Langsam B, Born W, Fuchs B. Cytotoxic effects of curcumin on osteosarcoma cell lines. *Invest New Drugs.* (2008) 26:289–97. doi: 10.1007/s10637-007-9099-7
392. Tian B, Wang Z, Zhao Y, Wang D, Li Y, Ma L, et al. Effects of curcumin on bladder cancer cells and development of urothelial tumors in a rat bladder carcinogenesis model. *Cancer Lett.* (2008) 264:299–308. doi: 10.1016/j.canlet.2008.01.041
393. Liu Q, Loo WT, Sze SC, Tong Y. Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells mediated by down-regulation of NFkappaB, cyclinD and MMP-1 transcription. *Phytomedicine.* (2009) 16:916–22. doi: 10.1016/j.phymed.2009.04.008
394. Watson JL, Hill R, Yaffe PB, Greenshields A, Walsh M, Lee PW, et al. Curcumin causes superoxide anion production and p53-independent apoptosis in human colon cancer cells. *Cancer Lett.* (2010) 297:1–8. doi: 10.1016/j.canlet.2010.04.018
395. Yang CL, Ma YG, Xue YX, Liu YY, Xie H, Qiu GR. Curcumin induces small cell lung cancer NCI-H446 cell apoptosis via the reactive oxygen species-mediated mitochondrial pathway and not the cell death receptor pathway. *DNA Cell Biol.* (2012) 31:139–50. doi: 10.1089/dna.2011.1300
396. Hassan ZK, Daghestani MH. Curcumin effect on MMPs and TIMPs genes in a breast cancer cell line. *Asian Pac J Cancer Prev.* (2012) 13:3259–64. doi: 10.7314/APJCP.2012.13.7.3259
397. Levine CB, Bayle J, Biourge V, Wakshlag JJ. Cellular effects of a turmeric root and rosemary leaf extract on canine neoplastic cell lines. *BMC Vet Res.* (2017) 13:388. doi: 10.1186/s12917-017-1302-2
398. Helson L. Curcumin (diferuloylmethane) delivery methods: a review. *Biofactors.* (2013) 39:21–6. doi: 10.1002/biof.1080
399. Withers SS, York D, Johnson E, Al-Nadaf S, Skorupski KA, Rodriguez Jr CO, et al. *In vitro* and *in vivo* activity of liposome-encapsulated curcumin for naturally occurring canine cancers. *Vet Comp Oncol.* (2018) 16:571–9. doi: 10.1111/vco.12424
400. Jiahao Lin, Qiang Cai, Yinian Tang, Yanjun Xu, Qian Wang, Tingting Li, et al. PEGylated Lipid bilayer coated mesoporous silica nanoparticles for co-delivery of paclitaxel and curcumin: design, characterization and its cytotoxic effect. *Int J Pharm.* (2018) 536:272–82. doi: 10.1016/j.ijpharm.2017.10.043
401. Gao J, Fan K, Jin Y, Zhao L, Wang Q, Tang Y, et al. PEGylated lipid bilayer coated mesoporous silica nanoparticles co-delivery of paclitaxel and curcumin leads to increased tumor site drug accumulation and reduced tumor burden. *Eur J Pharm Sci.* (2019) 140:105070. doi: 10.1016/j.ejps.2019.105070
402. Rahman HS, Rasedee A, Yeap SK, Othman HH, Chartrand MS, Namvar F, et al. Biomedical properties of a natural dietary plant metabolite, zerumbone, in cancer therapy and chemoprevention trials. *BioMed Res Int.* (2014) 2014:920742. doi: 10.1155/2014/920742
403. Rahman HS, Rasedee A, How CW, Abdul AB, Zeenathul NA, Othman HH, et al. Zerumbone-loaded nanostructured lipid carriers: preparation, characterization, and antileukemic effect. *Int J Nanomed.* (2013) 8:2769–81. doi: 10.2147/IJN.S45313
404. Foong JN, Selvarajah GT, Rasedee A, Rahman HS, How CW, Beh CY, et al. Zerumbone-loaded nanostructured lipid carrier induces apoptosis of canine mammary adenocarcinoma cells. *BioMed Res Int.* (2018) 2018:8691569. doi: 10.1155/2018/8691569
405. Sefidabi R, Mortazavi P, Hosseini S. Antiproliferative effect of berberine on canine mammary gland cancer cell culture. *Biomed Rep.* (2017) 6:95–8. doi: 10.3892/br.2016.809
406. Lynagh T, Lynch JW. Molecular mechanisms of Cys-loop ion channel receptor modulation by ivermectin. *Front Mol Neurosci.* (2012) 5:60. doi: 10.3389/fnmol.2012.00060
407. Dou Q, Chen HN, Wang K, Yuan K, Lei Y, Li K, et al. Ivermectin induces cytostatic autophagy by blocking the PAK1/Akt axis in breast cancer. *Cancer Res.* (2016) 76:4457–69. doi: 10.1158/0008-5472.CAN-15-2887
408. Diao H, Cheng N, Zhao Y, Xu H, Dong H, Thamm DH, et al. Ivermectin inhibits canine mammary tumor growth by regulating cell cycle progression and WNT signaling. *BMC Vet Res.* (2019) 15:276. doi: 10.1186/s12917-019-2026-2
409. Liu Y, Li W, Guo M, Li C, Qiu C. Protective role of selenium compounds on the proliferation, apoptosis, and angiogenesis of a canine breast cancer cell line. *Biol Trace Element Res.* (2016) 169:86–93. doi: 10.1007/s12011-015-0387-3
410. Li W, Guo M, Liu Y, Mu W, Deng G, Li C, et al. Selenium induces an anti-tumor effect via inhibiting intratumoral angiogenesis in a mouse model of transplanted canine mammary tumor cells. *Biol Trace Element Res.* (2016) 171:371–9. doi: 10.1007/s12011-015-0554-6
411. Zhou S, Wang F, Wong ET, Fonkem E, Hsieh TC, Wu JM, et al. Salinomycin: a novel anti-cancer agent with known anti-coccidial activities. *Curr Med Chem.* (2013) 20:4095–101. doi: 10.2174/15672050113109990199
412. Mai TT, Hamaï A, Hienzsch A, Cañeque T, Müller S, Wicinski J, et al. Salinomycin kills cancer stem cells by sequestering iron in lysosomes. *Nat Chem.* (2017) 9:1025–33. doi: 10.1038/nchem.2778
413. Kim KY, Park KI, Kim SH, Yu SN, Lee D, Kim YW, et al. Salinomycin induces reactive oxygen species and apoptosis in aggressive breast cancer cells as mediated with regulation of autophagy. *Anticancer Res.* (2017) 37:1747–58. doi: 10.21873/anticanres.11507
414. Li W, Yang H, Li X, Han L, Xu N, Shi A. Signaling pathway inhibitors target breast cancer stem cells in triple-negative breast cancer. *Oncol Rep.* (2019) 41:437–46. doi: 10.3892/or.2018.6805
415. Gao J, Liu J, Xie F, Lu Y, Yin C, Shen X. Co-delivery of docetaxel and salinomycin to target both breast cancer cells and stem cells by PLGA/TPGS nanoparticles. *Int J Nanomed.* (2019) 14:9199–216. doi: 10.2147/IJN.S230376
416. Dewangan J, Srivastava S, Mishra S, Divakar A, Kumar S, Rath SK. Salinomycin inhibits breast cancer progression via targeting HIF-1α/VEGF mediated tumor angiogenesis *in vitro* and *in vivo*. *Biochem Pharmacol.* (2019) 164:326–35. doi: 10.1016/j.bcp.2019.04.026
417. Tyagi M, Patro BS. Salinomycin reduces growth, proliferation and metastasis of cisplatin resistant breast cancer cells via NF-kB deregulation. *Toxicol In Vitro.* (2019) 60:125–33. doi: 10.1016/j.tiv.2019.05.004
418. Hero T, Bühler H, Kouam PN, Priesch-Grzeszowiak B, Lateit T, Adamietz IA. The triple-negative breast cancer cell line MDA-MB 231 is specifically inhibited by the ionophore salinomycin. *Anticancer Res.* (2019) 39:2821–7. doi: 10.21873/anticanres.13410

419. Versini A, Colombeau L, Hienzsch A, Gaillet C, Retailleau P, Debieu S, et al. Salinomycin derivatives kill breast cancer stem cells by lysosomal iron targeting. *Chemistry*. (2020) 26:7416–24. doi: 10.1002/chem.202000335
420. Naujokat C, Steinhart R. Salinomycin as a drug for targeting human cancer stem cells. *J Biomed Biotechnol*. (2012) 2012:950658. doi: 10.1155/2012/950658
421. Du H, Zhou B, Zhang H, Jin Y, Zhang D, Lin D. Salinomycin inhibits canine mammary carcinoma *in vitro* by targeting cancer stem cells. *Oncol Lett*. (2017) 14:427–32. doi: 10.3892/ol.2017.6164

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Quantification of Global DNA Methylation in Canine Mammary Gland Tumors *via* Immunostaining of 5-Methylcytosine: Histopathological and Clinical Correlations

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Mammary tumors are the most prevalent neoplasms in non-neutered female dogs, with genetic and epigenetic alterations contributing to canine mammary carcinogenesis. This study quantified global DNA methylation in 5-methylcytosine (5mC)-immunostained canine mammary tumor samples and established histopathological and clinical correlations. A total of 91 formalin-fixed paraffin-embedded mammary tumor samples from female dogs were retrospectively selected and subjected to immunohistochemistry using an anti-5mC mouse monoclonal antibody. We evaluated 5mC+ stained nuclei of neoplastic epithelial cells in canine mammary glands to obtain semiquantitative histoscores based on staining intensity. Survival rates were estimated based on owners' or veterinary records. Histological samples comprised 28 and 63 benign and malignant canine mammary gland tumors, respectively. Results revealed significant differences between global DNA methylation patterns when mammary samples were categorized as benign or malignant ($p = 0.024$), with hypomethylated patterns more prevalent in malignant tumors and those with higher relapse behavior ($p = 0.011$). Of note, large diameter (>5 cm) tumors revealed a lower methylation pattern ($p = 0.028$). Additionally, we found non-statistically significant differences when tumors were grouped by histopathological characteristics, clinical parameters, or survival. These findings propose global DNA methylation assessment as a promising tool for detecting canine mammary tumors with relapse propensity.

Keywords: mammary adenocarcinoma, methylation, 5mC, epigenetic, epigenome

INTRODUCTION

Mammary tumors are the most prevalent neoplasm in non-neutered female dogs (1) representing ∼50% of tumor diagnoses (2). The risk of developing mammary tumors is associated with hormone levels, breed susceptibility, age, diet, and obesity (3). Although numerous studies investigated genetic alterations in canine mammary cancers (4), including underlying molecular signatures (5),

few studies have investigated epigenetic alterations in these tumors (6, 7). Moreover, to the best of our knowledge, none of the former studies have evaluated the global DNA methylation status.

Cancer development is strongly associated with specific genetic alterations. While epigenetic changes (including DNA methylation, histone modification, and microRNA expression) are independent of hereditary patterns or gene mutations, these changes are also critical to neoplastic initiation and progression. Indeed, several different epigenetic changes have been identified that contribute to the development and maintenance of the neoplastic phenotype (8, 9). In the mammalian genome, methylation occurs at cytosine bases located at the 5′ end of guanine bases, promoting the formation of CpG dinucleotide islands. These CpG dinucleotide islands play a central role in transcriptional repression (when most CpG dinucleotides in an island are methylated). Thus, methylation and demethylation (catalyzed by methyltransferases and demethylases, respectively) are central to transcriptional regulation (9–11). Oncogene activation and inactivation of tumor suppressor genes represent critical steps at every tumorigenesis stage. Furthermore, DNA methylation is central to transcriptional repression once the majority of CpG dinucleotides in the mammalian genome are methylated, except for CG-dense regions located around transcriptional start sites, known as CpG islands. Thus, methylation, which occurs at cytosine bases (C), located 5′ to guanine (G) bases to form CpG dinucleotide islands, can be affected by loss or gain of DNA methyltransferases or demethylases. From an epigenetic standpoint, global DNA hypomethylation can result in overexpression of growth factors, alterations in DNA repair enzymes, and/or loss of genomic stability. Its counterpart, aberrant hypermethylation, can silence tumor suppressor gene promoter regions. These are both well-established mechanisms through which cancer cells may acquire critical features on their pathway to transformation (9–12). In addition, like genetic mutations, epigenetic alterations can be mitotically inherited, resulting in a rapidly growing cancer cell population by conferring advantages to tumor cells, resulting in uncontrolled growth (13, 14).

Global DNA methylation can be studied using several methods, including methylation-sensitive restriction endonucleases followed by analysis of the obtained fragments, and hydrolysis of genomic DNA followed by specific detection and quantification of 5-methylcytosine (5mC) content (15). However, these techniques require DNA extraction and are not compatible with the simple observation of cells and neoplastic tissues. Therefore, an immunochemical approach using monoclonal antibodies that recognize a methyl group on carbon number 5 of cytidine (anti-5mC antibodies) may be more suitable for investigating *in situ* DNA methylation. Moreover, this method allows computer-assisted quantification of global methylation to be performed on interphase nuclei in several cell types and on a cell-by-cell basis using microscopy (16). Piyathilake et al. (17) used a radiolabeled methyl incorporation assay and immunohistochemistry to assess global DNA methylation in squamous cell carcinoma in the human lung and highlighted the advantages of the latter technique, which was superior in demonstrating statistically significant differences between tumor and healthy tissues. More recently, using anti-5mC antibodies to analyze the bone marrow biopsies of patients with myelodysplastic syndrome (in which DNA methylation plays a pathogenic role), researchers have shown this immunohistochemistry assay to be a cost-effective and powerful tool for the prediction of overall survival (18).

As mammary tumors are morphologically complex, with the involvement of numerous patterns and cell types, using an immunohistochemical method associated with microscopic quantification appears more appropriate for evaluating global DNA methylation in these samples. Furthermore, we have previously used this method to evaluate global DNA methylation status in mast cell tumors (19) and lymphomas (20). Therefore, in the present study, we evaluated global DNA methylation in canine mammary gland tumors using immunohistochemistry and correlated our findings with tumor diagnosis and clinical outcomes.

MATERIALS AND METHODS

Case Origin and Data Collection

The study protocol was approved by the Committee on Ethics on the Use of Animals from the School of Veterinary Medicine and Animal Science of the University of São Paulo (No. 1823251013/2014) and by the Human Ethical Committee of Santos Metropolitan University (No. CAAE 10105019.2.0000.5509). Canine mammary tumors were retrieved from the archives of the Veterinary School of the Santos Metropolitan University, UNIMES (Santos, Brazil). The samples

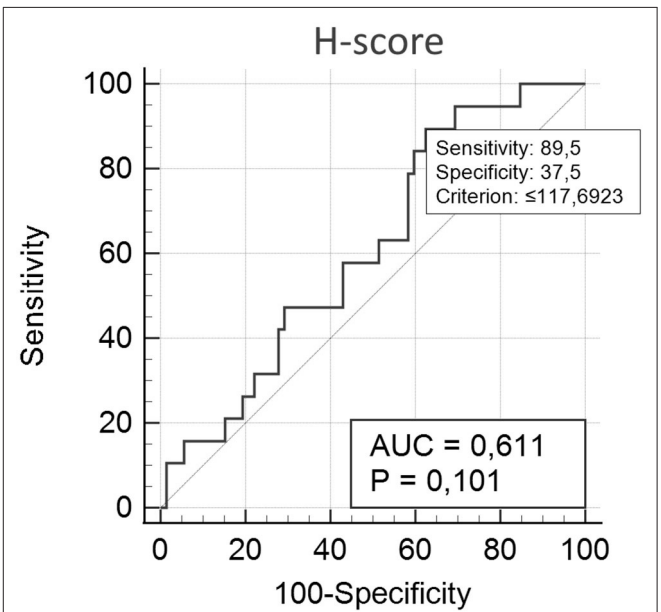

FIGURE 1 | H-score, according to ROC analysis. Variables under analysis were the methylation score and the classification variable (death by mammary gland tumor). Cut-off value: <117.7. Sensitivity and specificity were determined as 89.5 and 37.5, respectively, with an area under the ROC curve of 0.611 ($p = 0.101$; $n = 63$).

were originally obtained from canine females undergoing therapeutic mastectomy over 5 years. A total of 91 formalin-fixed paraffin-embedded (FFPE) mammary gland tumor samples from female dogs were retrospectively included in the study.

Inclusion Criteria

Patients were eligible for this retrospective study when their records included a histologic diagnosis of benign neoplastic mammary lesions or malignant neoplastic mammary lesions. The inclusion criteria were as follows: (1) a minimum follow-up period of 18 months (follow-up intervals of 30, 90, 180, 360, and 540 days after mastectomy, including reexamination and thoracic radiographs); and (2) no previous history of mastectomy and/or history of neoadjuvant chemotherapy. The clinical-stage was obtained according to the World Health Organization TNM classification (21). Distant metastasis was investigated by three-way thoracic radiography and abdominal ultrasound. Lymph node involvement was determined by cytologic and histologic examinations.

TABLE 1 | Neoplastic lesions distribution by histological type and grade.

Histological description	Quantity and frequency %	Grade		
		I	II	III
Benign mixed tumor	11 (10.0)	–	–	–
Adenoma—complex	5 (4.5)	–	–	–
Adenoma—simple tubular	4 (3.6)	–	–	–
Intraductal papillary adenoma	4 (3.6)		–	–
Fibroadenoma	1 (0.9)	–	–	–
Intraductal cystic-papillary adenoma	1 (0.9)	–	–	–
Nipple ductal adenoma	1 (0.9)	–	–	–
Tubulopapillary adenoma	1 (0.9)	–	–	–
Subtotal	**28 (30.8%)**	–	–	–
Complex carcinoma	12 (13.2)	9 (14.3)	3 (4.8)	–
Carcinoma—mixed	9 (9.9)	6 (9.5)	2 (3.2)	1 (1.6)
Carcinoma—simple tubular	11 (12.1)	4 (6.3)	4 (5.9)	3 (4.4)
Intraductal papillary carcinoma	5 (5.5)	3 (4.8)	2 (1.8)	–
Carcinoma in a complex adenoma	4 (4.4)	3 (4.8)	1 (1.5)	–
Carcinoma—simple cribriform	3 (3.3)		3 (4.4)	–
Carcinoma in a benign mixed tumor	3 (3.3)	2 (3.2)	1 (1.6)	–
Carcinoma—anaplastic	1 (1.1)	–	1 (1.5)	1 (1.5)
Carcinoma—solid	2 (2.2)	–	–	2 (3.2)
Comedocarcinoma	2 (2.2)	–	–	2 (3.2)
Inflammatory carcinoma:	2 (2.2)	–	–	–
Carcinosarcoma		–	1 (1.6)	–
Carcinoma—simple tubular		1 (1.6)	–	–
Carcinosarcoma	1 (1.1)	–	–	1 (1.6)
Carcinoma cystic-papillary	1 (1.1)	–	1 (1.6)	–
Carcinoma—micropapillary invasive	1 (1.1)	–		1 (1.6)
Carcinoma—simple tubulopapillary	1 (1.1)	1 (1.6)	–	–
Carcinoma and malignant myoepithelioma	1 (1.1)	1 (1.6)	–	–
Carcinoma in a simple tubular adenoma	1 (1.1)	1 (1.6)	–	–
Carcinoma in a simple tubulopapillary adenoma	1 (1.1)	1 (1.6)	–	–
Carcinoma in a fibroadenomatous dysplasia	1 (1.1)	–	–	–
Carcinoma—*in situ*	1 (1.1)	–	–	–
Ductal carcinoma *in situ*	1 (1.1)	1 (1.6)	–	–
Ductal carcinoma	1 (1.1)	–	–	1 (1.6)
Subtotal	**63 (69.2%)**	**33 (52.4)**	**18 (30.2)**	**12 (17.5)**
Total	**91 (100%)**		**63 (100%)**	

Tissue Processing for Histological Grade Determination and Immunohistochemistry

FFPE mammary tissue fragments were sliced into 5 μm-thick sections, mounted on glass slides, and stained with hematoxylin and eosin for classification according to the histological type. The diagnosis was conducted by three different pathologists (LRB, LBG, and an external private pathology laboratory) based on previously defined classification criteria (21), and histological grading was based on previously described criteria (22, 23).

Immunohistochemistry to detect 5mC involved dewaxing silanized slides containing 5 μm-thick tumor slices using xylene and progressive dehydration in alcohol. Antigen retrieval was performed with citrate buffer (pH 6.0) in a microwave oven at 97°C for 15 min, after which endogenous peroxidases were inactivated with 30% oxygen peroxide in methanol. The slides were then incubated with a primary antibody (ab10805 anti-methylcytosine (5-mC) antibody [33dD3] Abcam, Cambridge, UK) diluted 1:150 and kept at 4°C overnight. After rinsing, the slides were incubated with secondary antibody (LSAB Kit + System-HRP; K0679; DakoCytomation, Glostrup, Denmark) at room temperature (25°C) for 30 min. Samples were stained with a diaminobenzidine chromogen (Dako Corp., Carpinteria, CA, USA) and counterstained with hematoxylin. Between each step, slides were rinsed three times with 1 × phosphate-buffered saline (PBS) or 1 × PBS with Tween 20 (Dako Corp.), according to the manufacturer's instructions. Validated positive tissues (canine mast cell tumor and lymphma) obtained from previous studies performed in our laboratory were used as controls in order to check the quality of the antibody batch in use. Negative controls were obtained by replacing the primary antibody with a control match-isotype antibody (IgG1).

Data Acquisition and Histoscore (H-Score) Quantification

Immunohistochemical sections were evaluated under an optical microscope (NIKON, Tokyo, Japan) over 10 high-power fields, with the images recorded using the Image-Pro Plus system (v.4.5.0.29; Media Cybernetics, Rockville, MD, USA). The goal was to achieve a minimum of 500 cells/tumor, as per previously described methods (16), and tumors with <300 cells were excluded (in summary, 15 samples had more than 300 cells and <400; 10 samples had more than 400 cells and <500 and 66 samples had between 500 and 1,475 cells). Mammary gland epithelial cells were considered 5mC+ if they presented

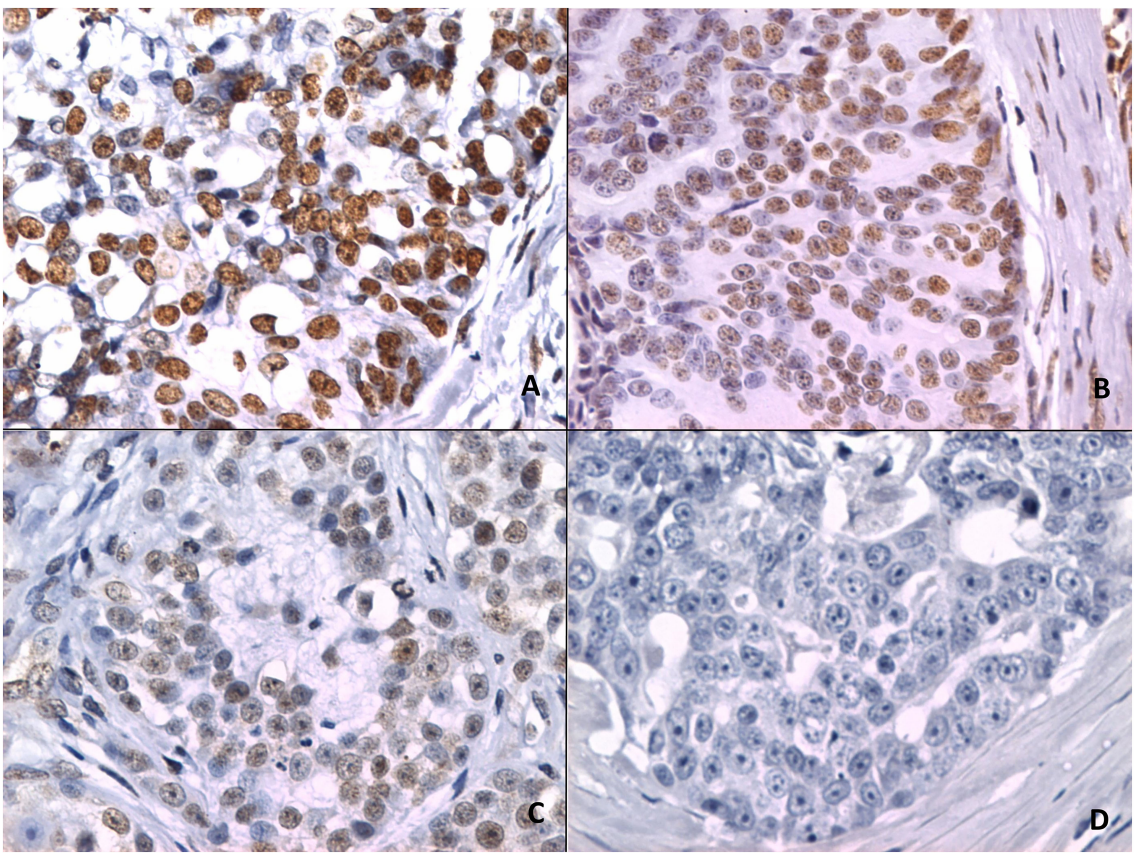

FIGURE 2 | 5mC immunohistochemistry. **(A)** Strong staining is characterized by intense dark brown color in a sample representing nipple ductal adenoma. **(B)** Moderate staining in a sample representing carcinoma in a simple tubular adenoma. **(C)** Weak staining with characteristic vacuolated nuclei in a sample with solid carcinoma. **(D)** A lack of staining in a sample representing comedocarcinoma.

nuclei with dark brown/gold coloration after nuclear staining. Slides were evaluated by two different examiners (LRB and PPCC), and the final score was determined based on the average of the counts. As there is no standardization for 5mC immunoreactivity for canine mammary tumors, quantification of immunohistochemistry results was based on the H-score,

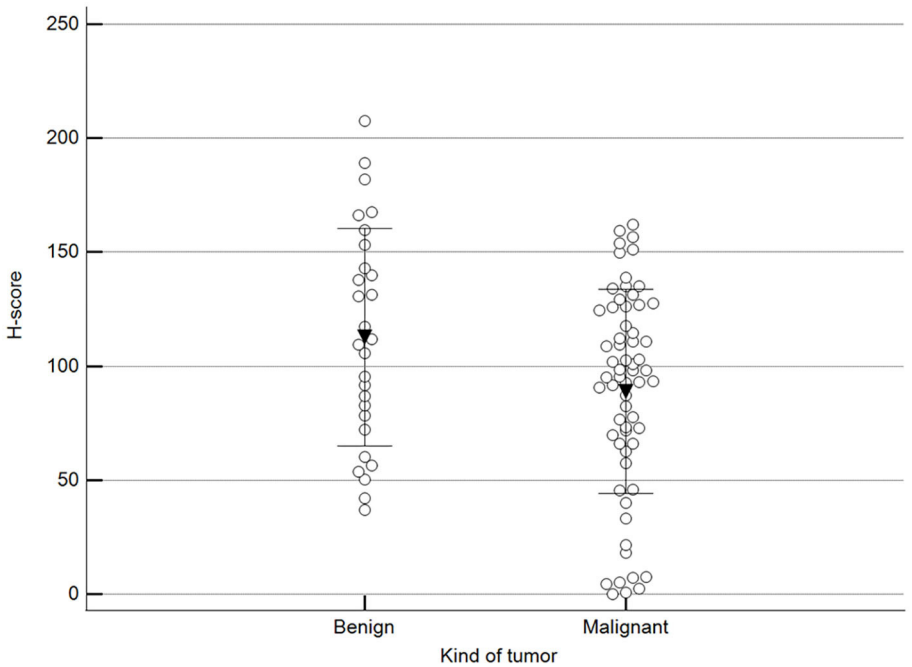

FIGURE 3 | Scoring of methylation patterns according to 5mC immunohistochemistry. Tumors were grouped as benign (mean = 112.8; $n = 28$) or malignant (mean: 88.9; $n = 63$) ($p = 0.024$; Unpaired t-test). Dots represent each case; lines represent 1 standard deviation and the inverted triangle represents the value.

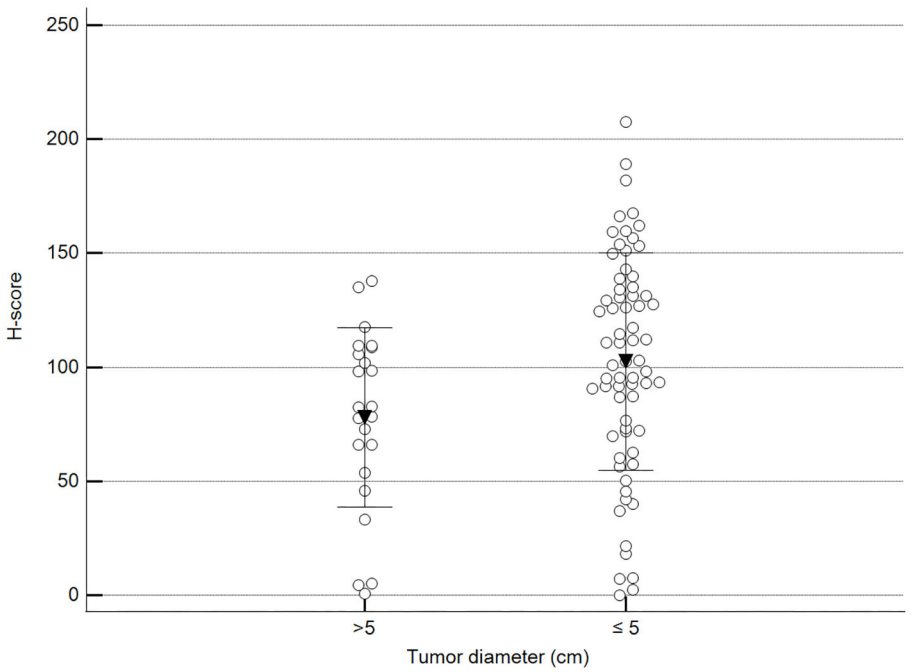

FIGURE 4 | Methylation patterns based on 5mC H-score grouped by the largest tumor diameter. Tumors were grouped according to the largest tumor diameter: >5 cm (mean = 77.9; $n = 23$) and ≤5 cm (mean = 102.5; $n = 68$). Unpaired t-test ($p = 0.028$; $n = 91$). Dots represent each case; lines represent 1 standard deviation and the inverted triangle represents the value.

a semiquantitative method accepted by the American Society of Clinical Oncology (24). Nuclear staining intensity was first classified as 0 (no staining), 1+ (light staining), 2+ (intermediate staining), or 3+ (strong staining), for each cell in a fixed field, and the H-score was then obtained as [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] in a range from 0 to 300 (25). This study considered only epithelial cells for methylation patterns, in line with most recent global DNA methylation studies.

Survival Analyses

For survival and other statistical analyses, scores were converted into two categorical variables (methylated or hypomethylated) either above or below (respectively) a cut-off value of 117.7 (e.g., borderline tumor value: 124.6094 = methylated), as obtained through receiver operating characteristic (ROC) curve analyzes, as per previously described methods (18). The variables under analysis were the methylation score and classification variable (death by mammary gland tumor), as shown in **Figure 1**.

Although prognosis was beyond the scope of this work, information concerning the survival rate was obtained *via* telephone conversation with animal owners or from medical records. The overall survival time was calculated from the date of mastectomy to the date of patient death. The Kaplan–Meier method was used to calculate overall survival time, and the log-rank test was used to identify factors associated with post-mastectomy survival. Live animals, mammary gland tumor-unrelated deaths, or missing animals due to lack of follow-up were removed for statistical purposes.

Other statistical analyzes included a normality test (Kolmogorov-Smirnov), unpaired *t*-test, and one-way analysis of variance (ANOVA). All analyzes were conducted using MedCalc® software (v.19.6.1), and a $p < 0.05$ was considered statistically significant.

RESULTS

Canine mammary gland tissue was obtained from 91 females of different breeds (average age at the time of surgery: 9.2 ± 2.6 years). Of these animals, 16 had been previously neutered, 6 were histopathologically diagnosed with a neoplastic invasion of inguinal or axillary lymph nodes, and 1 received a lung metastasis diagnosis upon radiographic examination during the follow-up.

Histologic diagnosis comprised 28 benign tumors (accounting for 30.8% of all tumors) and 63 malignant tumors (accounting for 69.2% of the tumors in the studied population). Of the malignant tumors, 52.4% (n = 33/63) were classified as grade I, 30.2% (n = 18/63) as grade II, and 17.5% (n = 12/63)

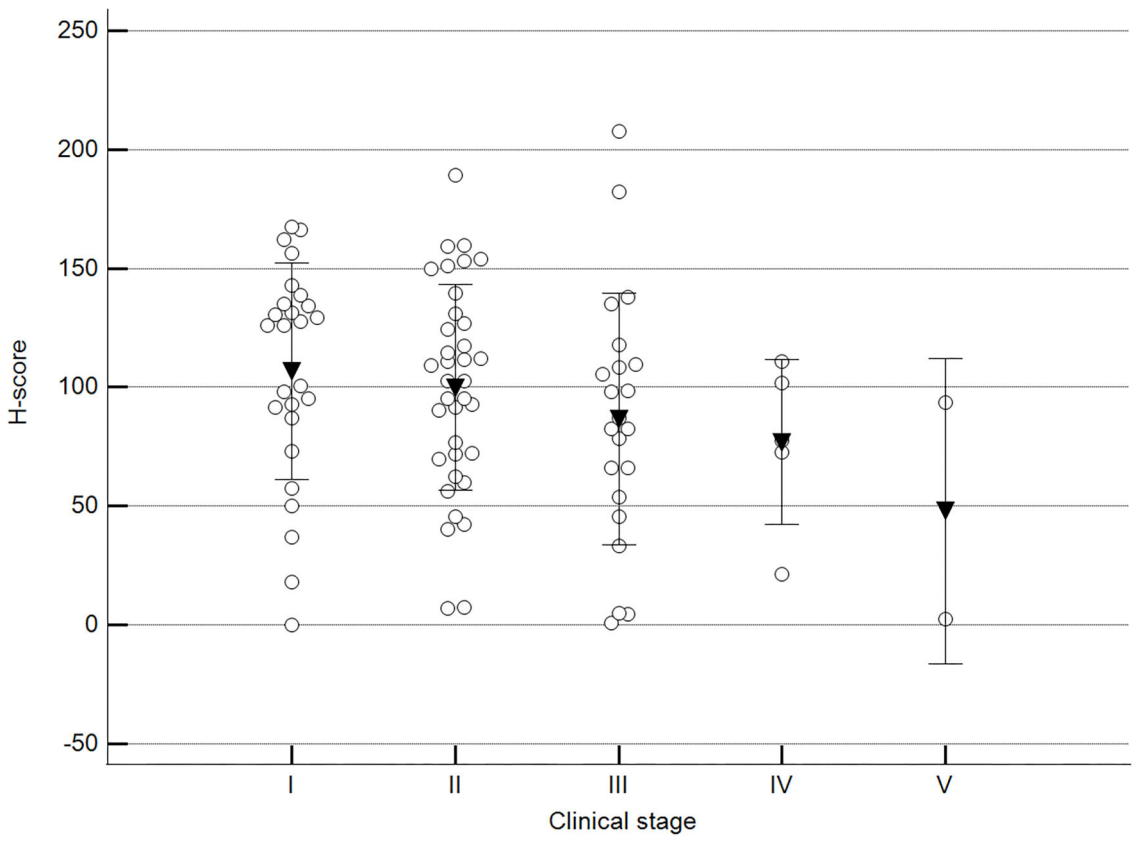

FIGURE 5 | Methylation patterns based on 5mC H-score grouped by stage. Tumors were grouped as clinical stage I to V based on TNM grouping. One-way analysis of variance ($p = 0.247$; $n = 91$). Dots represent each case; lines represent 1 standard deviation, and the inverted triangle represents the value. Post-test not applicable.

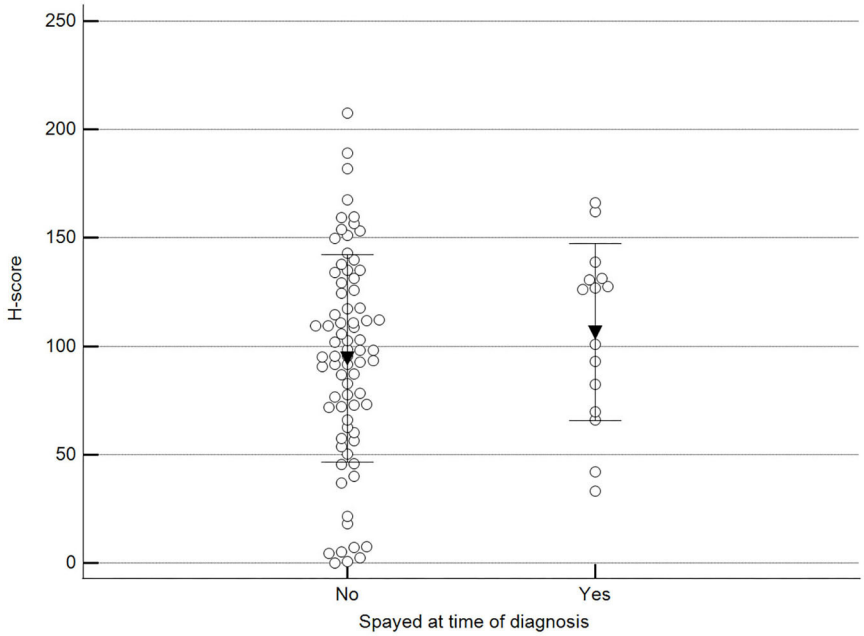

FIGURE 6 | Methylation patterns based on 5mC H-score grouped by females already spayed at the time of diagnosis. Tumors were grouped as previously spayed = yes or no. Unpaired *t*-test ($p = 0.359$; $n = 91$). Dots represent each case; lines represent 1 standard deviation, and the inverted triangle represents the value.

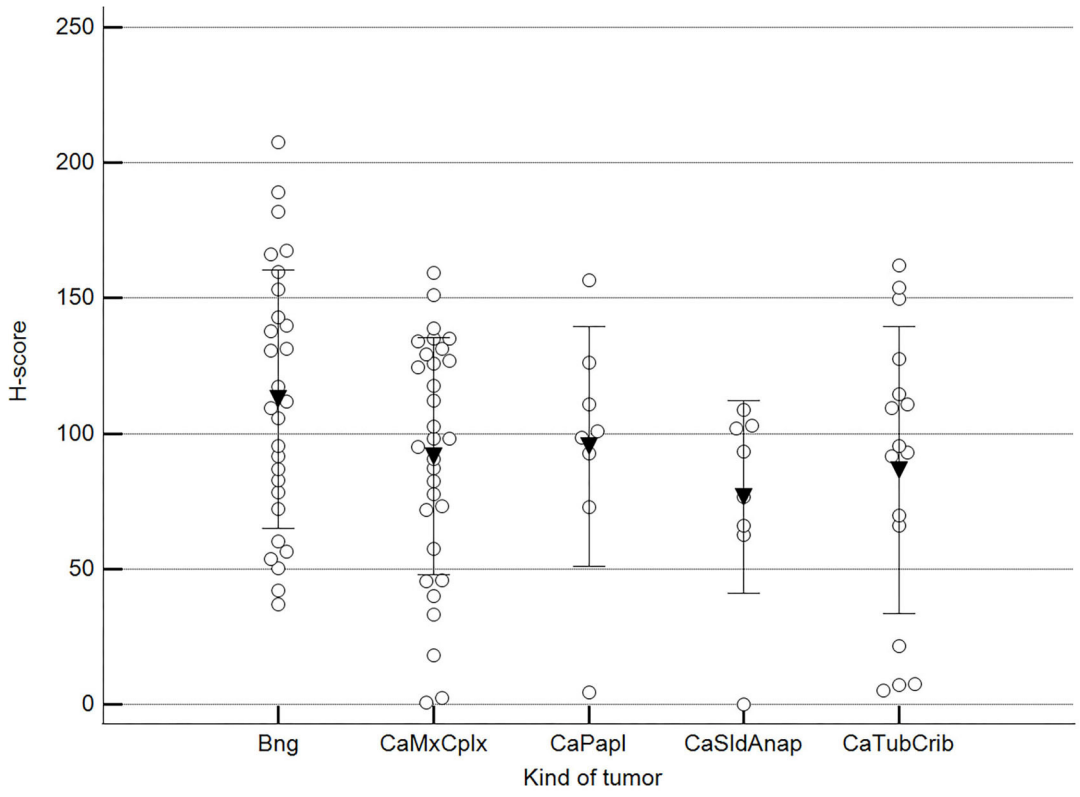

FIGURE 7 | Methylation patterns based on 5mC H-score grouped by tumor histological group. Tumors were grouped as BNG, benign; CaMcCplx, carcinoma mixed and complex; CaPapl, carcinoma papillary; CaSldAnap, carcinoma solid and anaplastic; CaTubCrib, carcinoma simple tubular and cribriform. One-way analysis of variance ($p = 0.206$; $n = 91$). Dots represent each case; lines represent 1 standard deviation, and the inverted triangle represents the value. Post-test not applicable.

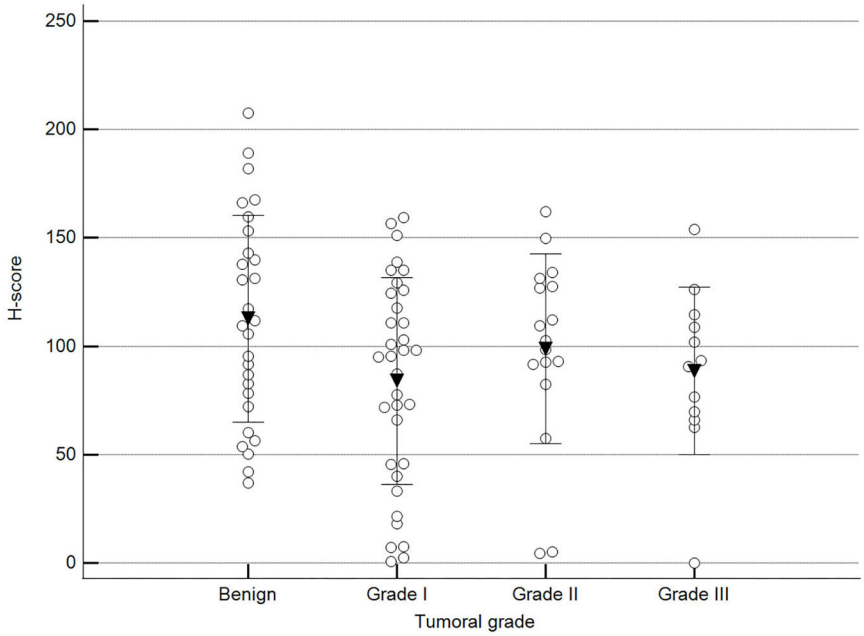

FIGURE 8 | Methylation patterns based on 5mC H-score grouped by tumor histological grade. Tumors were grouped as benign or malignant grade I, II, or III. One-way analysis of variance ($p = 0.100$; $n = 91$). Dots represent each case; lines represent 1 standard deviation, and the inverted triangle represents the value. Post-test not applicable.

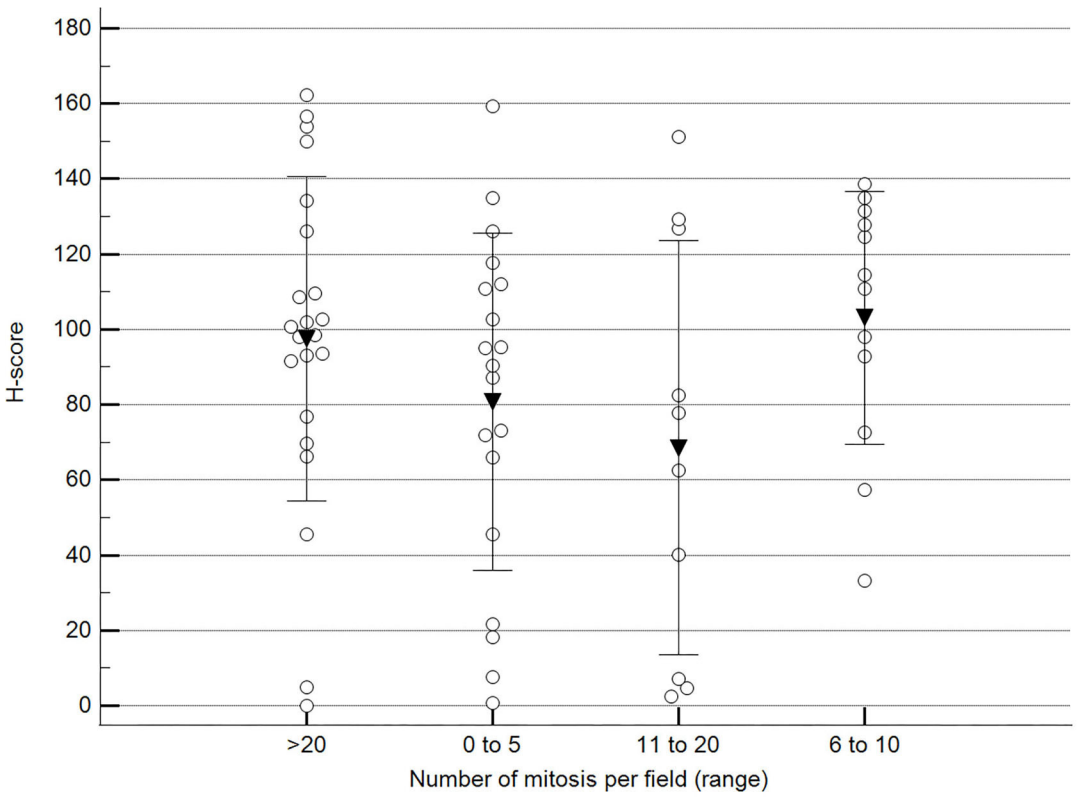

FIGURE 9 | Methylation patterns based on 5mC H-score grouped by the number of mitoses per high-power field. Tumors were grouped according to the number of mitoses per field of 400× magnification. One-way analysis of variance ($p = 0.196$; $n = 63$). Dots represent each case; lines represent 1 standard deviation, and the inverted triangle represents the value. Post-test not applicable. Magnification: 400×.

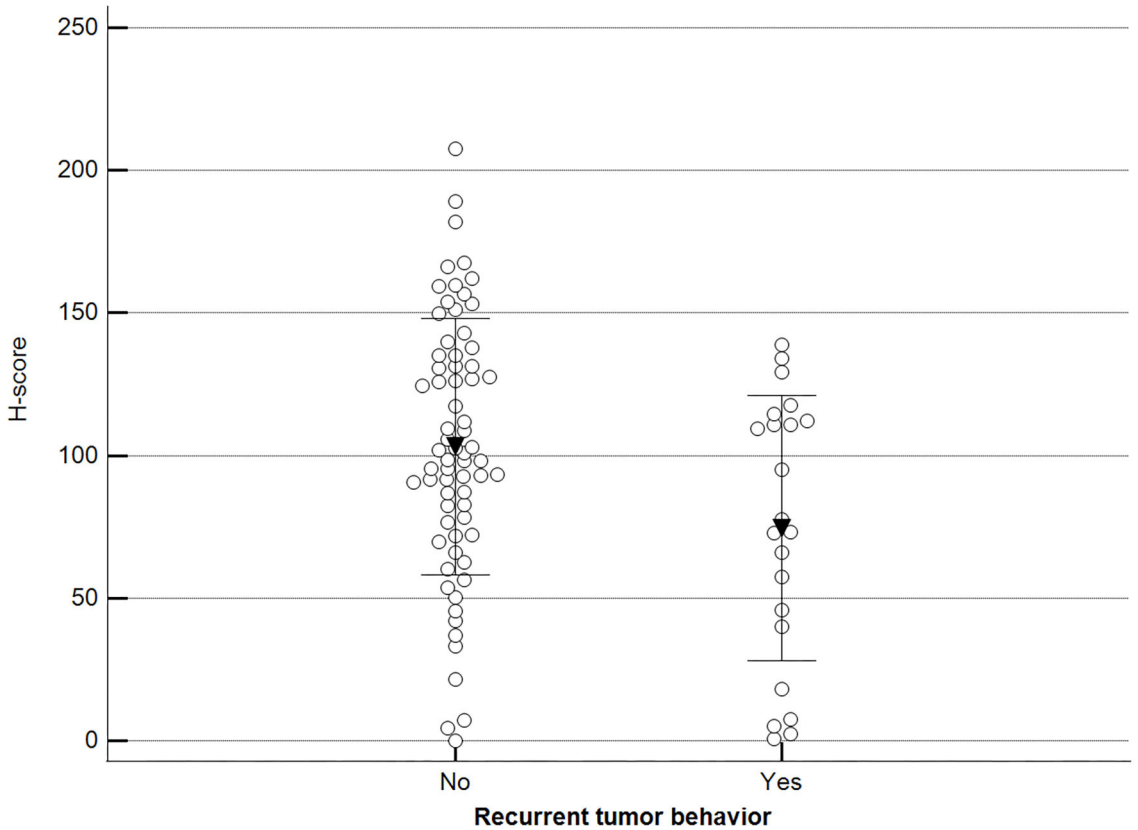

FIGURE 10 | Methylation patterns based on 5mC H-score grouped by tumor relapse. Tumors were grouped as recurrence/new presentation = yes (mean = 74.5; $n = 22$) or no (mean = 103.2; $n = 69$). Unpaired t-test ($p = 0.011$; $n = 91$). Dots represent each case; lines represent 1 standard deviation and the inverted triangle represents the value.

as grade III. Two animals had clinical and histopathologic diagnoses consistent with inflammatory carcinoma with marked vasculogenic skin invasion. **Table 1** summarizes the distribution of tumors according to the histologic diagnosis and grade.

All animals underwent a full or partial mastectomy, and 23 showed disease relapses, including 13 with a new presentation of neoplastic disease in the remaining glands during follow-up, 10 of which had a recurrence in the surgical scar. Overall, 15 animals were alive, 27 animals had missed their follow-up appointments, and 49 animals succumbed. Among the deaths, 17 were attributed to mammary gland tumors, and the remaining were attributed to other causes, such as natural aging, heart or kidney failure, or euthanasia due to other tumor types or conditions.

Figure 2 shows 5mC immunohistochemical staining of neoplastic mammary glands. Evaluation of 5mC immunoreactivity as a continuous variable revealed statistically significant differences that allowed the grouping of tumors as benign or malignant, with the latter being less methylated (**Figure 3**).

We found statistically significant differences upon grouping methylation patterns by tumor size (**Figure 4**). However, we did not find statistically significant differences in methylation patterns when tumors were grouped by clinical parameters such as the stage (**Figure 5**), previous sterilization (**Figure 6**), or other tumoral characteristics, including histological type (**Figure 7**), tumor grade (**Figure 8**), and the number of mitoses in high-magnification fields (**Figure 9**).

Additionally, we observed a significant difference when malignant tumor scores were grouped by the occurrence of relapse (recurrence/new presentation) (**Figure 10**).

5mC H-scores were subsequently transformed into a binary variable (methylated and hypomethylated) according to the ROC curve analysis and subjected to survival analysis. There was no statistically significant difference between the survival of animals with methylated malignant tumors and those with hypomethylated tumors (log-rank Mantel–Cox and chi-squared 1.9799 DF 1, $p = 0.1594$) (**Figure 11**).

DISCUSSION

In the present study, we retrospectively evaluated global DNA methylation status in canine mammary tumors and correlated these results with clinical parameters, survival, and tumor relapse. Few studies have reported the use of immunohistochemical techniques to evaluate global DNA

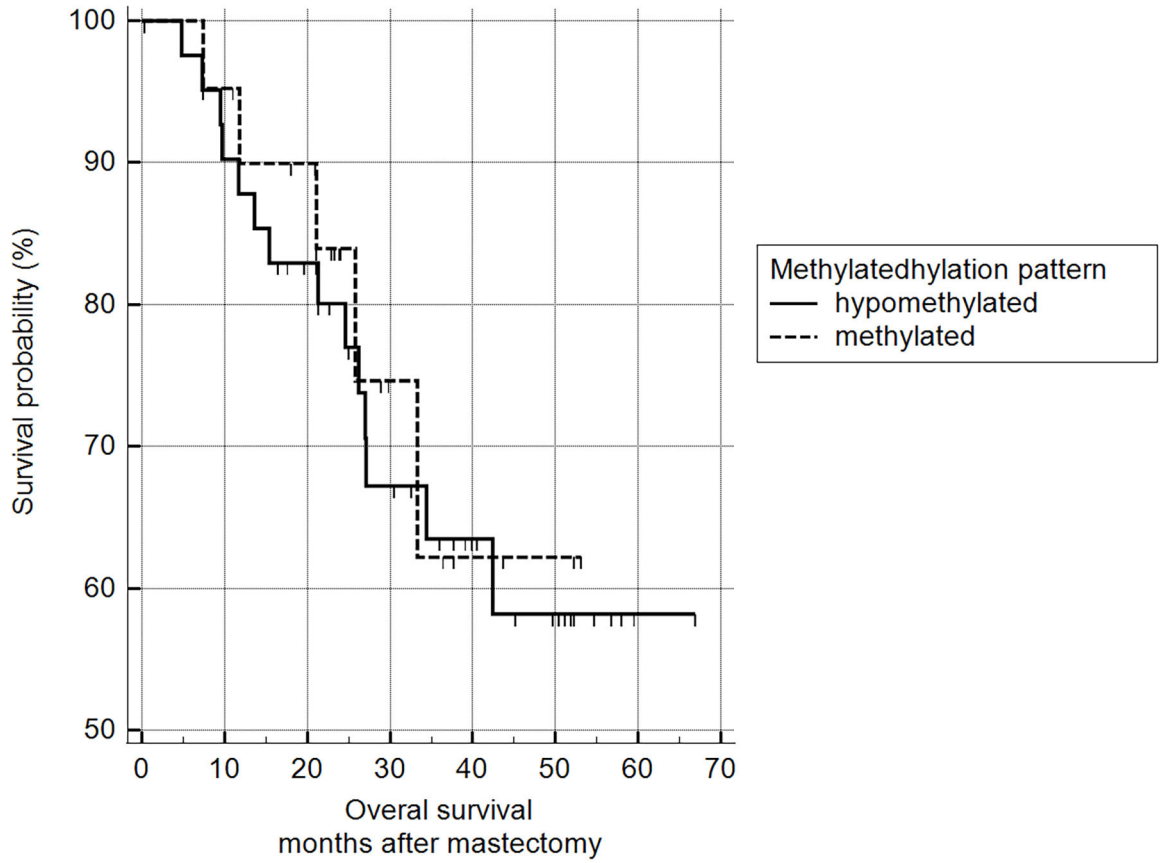

FIGURE 11 | Survival analysis, according to 5mC H-score. Grouping according to hypomethylated or methylated tumor status revealed an H-score cut-off value of 117.7 (ROC) ($n = 63$). Mean survival of hypomethylated = 46.6 months and methylated = 47.0 months. Kaplan-Meier curve and log-rank test ($p = 0.1594$; $n = 63$).

methylation in canine neoplastic disease, and none have focused on global DNA methylation in mammary neoplasms. Moreover, to the best of our knowledge, this is the first study reporting global DNA methylation status in canine mammary tumors. The design of this study was based on the findings of Hernandez et al. (16), who evaluated global DNA methylation patterns in human colon cancer. They validated their immunohistochemistry results using an anti-5mC antibody and with immunoblotting results from genomic DNA samples of *Xanthomonas oryzae* DNA in which almost all the cytosine bases were replaced with 5mC. Furthermore, our results were in line with those reported by Morimoto et al. (19) and Epiphanio et al. (20). Their observations of global methylation patterns in canine mast cell tumors and canine lymphoma were obtained with the same antibody used herein.

We found that malignant mammary tumors demonstrated lower immunohistochemical staining scores compared with t benign tumors, similar to a previous study reporting immunohistochemical evaluation of overall DNA methylation patterns in mouse embryonic tissue and human prostate, breast, and colon tumor tissue (26). In that study, methylated CpG islands were converted to 5-hydroxymethylcytosine (5hC), and they reported high 5hC-staining levels in adult and embryonic tissues, closely related to the degree of cell differentiation and tissue organization. Whereas, low staining levels were observed in prostate carcinomas and breast and colon tumors.

Others studies demonstrated similar findings. One study assessed global DNA methylation profiles in paired normal and neoplastic colon tissue using an anti-5mC antibody (16). They described qualitative and quantitative differences between normal and neoplastic regions of paired colon tissues, with the latter being generally hypomethylated. Another study compared adjacent benign tissue and high and low degree prostate intraepithelial neoplasms (27). Furthermore, immunohistochemistry staining using an anti-5mC antibody and paraffin-embedded tissue in normal uterine cervix, benign lesions (including pre-neoplastic tissue), non-invasive malignant lesions, and samples of invasive squamous cell carcinoma of the uterine cervix, although the authors did not find a significant difference in global DNA methylation between normal, benign, and non-invasive malignant lesions, they reported a significant difference in methylation scores between the normal, benign, and non-invasive malignant lesion groups in comparison with the invasive squamous cell carcinoma group, which demonstrated a hypomethylated pattern (28). Due to the retrospective nature of the present study, there was a lack of adequate normal mammary tissue on the slides that could be used as a paired sample for the corresponding tumor lesion, a limitation of this work.

Interestingly, among the malignant tumors, the hypomethylated pattern was associated with tumors larger than 5 cm in diameter, suggesting that the loss of methylation may be associated with tumor progression, and particularly, with tumor proliferation. This finding is possibly supported by a previous report on the progression of mammary tumors in dogs (29) and breast tumor in women (30). In fact, a study that evaluated the methylation status in human hepatocellular carcinoma, with a sophisticated chip array technique in normal and tumoral liver tissue and tumors in progression, demonstrated that global hypomethylation in hepatocellular carcinoma was associated with chromosomal instability (31).

On the other hand, we also observed a correlation between 5mC immunoreactivity and tumor recurrence, with hypomethylated tumors demonstrating a propensity to relapse. In fact, a previous study reported this phenomenon in prostate tumor in men, and a similar observation was made in post-operative hepatocarcinoma (27, 32). Additionally, Mazzucchelli et al. (33) evaluated global DNA methylation profiles in papillary urothelial neoplasia of low malignant potential using immunohistochemistry and found a statistically significant difference between non-recurring and recurrent tumors, with the latter showing a hypomethylated pattern. As hypomethylation is implicated in genome instability and malignant transformation, we speculate that animals with hypomethylated mammary neoplasms could be at risk of tumor recurrence.

The methylation status and clinical correlations (such as staging, histological grade, and survival) observed here are comparable with previous studies showing no relationship between clinical prognostic factors and overall DNA methylation patterns between normal prostate tissue and prostate cancer samples (27, 34). In contrast with our results, some reports have demonstrated an ambiguous association between methylation status and poor overall survival. One study involving 5mC immunostaining of bone marrow biopsies from patients with myelodysplastic syndrome found significantly worse survival in patients with methylated bone marrow tissues (18). Another study assessed the methylation expression index (MEI) in tissue from women with invasive breast cancer. They found that decreased survival was associated with a low MEI index in 2,500 ER+ patients (35). Good et al. (36) used cell lines from triple-negative breast cancer patients to demonstrate worse overall survival in patients with hypomethylated tumors. These conflicting outcomes may be explained by the methodology adopted for investigating the cause of death. Additionally, as ours was a retrospective study, our data were limited to the quality of the information obtained from medical records or provided by owners, a limitation of our study.

Another study limitation was the inability to validate immunohistochemistry findings using other molecular techniques, as the material available was restricted to FFPE tissues. However, it should be noted that other techniques are biased, as they use complete portions of the triturated tumor tissues, including DNA from both tumor and stromal cells, thereby compromising the results. However, Piyathilake et al. (17) compared the *in vitro* radiolabeled methyl incorporation assay with immunohistochemical staining of the same tissue sections with a monoclonal antibody developed against 5mC. They concluded that immunostaining was a useful technique for evaluating global DNA methylation, especially when cancer-related methylation cannot be normalized to methylation of normal tissues or when the number of samples available for evaluation is small, as in the present study (17).

In summary, this is the first study describing global DNA methylation patterns in canine mammary tumors using 5mC immunostaining as an inexpensive and straightforward technique. Although no correlation between tumor type, grade, or clinical prognostic factors was observed, we effectively demonstrated that hypomethylation was more prevalent in malignant tumors and, particularly, among those with a diameter exceeding 5 cm. Our findings demonstrate the efficacy of this method for global DNA methylation assessment and suggest that DNA hypomethylation is associated with malignant tumors. This tool may help identify recurrent tumors and those with relapse potential, suggesting this marker is potentially useful in the prognostic evaluation of canine mammary neoplasms. However, proper validation of this technique should be performed using a gold standard quantitative technique.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://1drv.ms/u/s!AsfWCRqEd3yHivplXdJRIUScP6GNBQ?e=Eo7Pa6.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee of Ethics on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR'S NOTE

This study is part of the doctoral dissertation project by LB at the Graduate Program on Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

AUTHOR CONTRIBUTIONS

LB and MD: conceptualization and manuscript writing. LB, LG, MT, and MD: methodology. LB: software. MD: grant acquisition and supervision. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Thumser-Henner P, Nytko KJ, Rohrer Bley C. Mutations of BRCA2 in canine mammary tumors and their targeting potential in clinical therapy. *BMC Vet Res*. (2020) 16:30. doi: 10.1186/s12917-020-2247-4
2. Klopfleisch R, von Euler H, Sarli G, Pinho SS, Gärtner F, Gruber AD. Molecular carcinogenesis of canine mammary tumors: news from an old disease. *Vet Pathol*. (2011) 48:98–116. doi: 10.1177/0300985810390826
3. Sorenmo KU, Shofer FS, Goldschmidt MH. Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *J Vet Intern Med*. (2000) 14:266–70. doi: 10.1111/j.1939-1676.2000.tb01165.x
4. Vinothini G, Balachandran C, Nagini S. Evaluation of molecular markers in canine mammary tumors: correlation with histological grading. *Oncol Res*. (2009) 18:193–201.doi: 10.3727/096504009X12596189659042
5. Kim TM, Yang IS, Seung BJ, Lee S, Kim D, Ha YJ, et al. Cross-species oncogenic signatures of breast cancer in canine mammary tumors. *Nat Commun*. (2020) 11:3616. doi: 10.1038/s41467-020-17458-0
6. Brandão YO, Toledo MB, Chequin A, Cristo TG, Sousa RS, Ramos EAS, et al. DNA methylation status of the estrogen receptor α gene in canine mammary tumors. *Vet Pathol*. (2018) 55:510–6. doi: 10.1177/0300985818763711
7. Rybicka A, Mucha J, Majchrzak K, Taciak B, Hellmen E, Motyl T, et al. Analysis of microRNA expression in canine mammary cancer stem-like cells indicates epigenetic regulation of transforming growth factor-beta signaling. *J Physiol Pharmacol*. (2015) 66:29–37.
8. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome–components and functional correlates. *Genes Dev*. (2006) 20:3215–31. doi: 10.1101/gad.1464906
9. Bryan JN, Taylor KH, Henry CJ, Selting KA, Rahmatpanah F, Lewis MR, et al. DNA methylation in cancer: techniques and preliminary evidence of hypermethylation in canine lymphoma. *Cancer Ther*. (2008) 6:137–48.
10. Gargiulo G, Minucci S. Epigenomic profiling of cancer cells. *Int J Biochem Cell Biol*. (2009) 41:127–35. doi: 10.1016/j.biocel.2008.07.021
11. Holliday H, Baker LA, Junankar SR, Clark SJ, Swarbrick A. Epigenomics of mammary gland development. *Breast Cancer Res*. (2018) 20:100. doi: 10.1186/s13058-018-1031-x
12. Qureshi SA, Bashir MU, Yaqinuddin A. Utility of DNA methylation markers for diagnosing cancer. *Int J Surg*. (2010) 8:194–8. doi: 10.1016/j.ijsu.2010.02.001
13. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. (2009) 31:27–36. doi: 10.1093/carcin/bgp220
14. Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE, et al. Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. *Cancer Res*. (2000) 60:4346–8.
15. Rocha MS, Castro R, Rivera I, Kok RM, Smulders YM, Jakobs C, et al. Global DNA methylation: comparison of enzymatic- and non-enzymatic-based methods. *Clin Chem Lab Med*. (2010) 48:1793–8. doi: 10.1515/CCLM.2010.346
16. Hernandez-Blazquez FJ, Habib M, Dumollard JM, Barthelemy C, Benchaib M, de Capoa A, et al. Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis. *Gut*. (2000) 47:689–93. doi: 10.1136/gut.47.5.689
17. Piyathilake CJ, Johanning GL, Frost AR, Whiteside MA, Manne U, Grizzle WE, et al. Immunohistochemical evaluation of global DNA methylation: comparison with *in vitro* radiolabeled methyl incorporation assay. *Biotech Histochem*. (2000) 75:251–8. doi: 10.3109/10520290009085128
18. Chandra D, Tyagi S, Singh J, Deka R, Manivannan P, Mishra P, et al. Utility of 5-methylcytosine immunohistochemical staining to assess global DNA methylation and its prognostic impact in MDS patients. *Asian Pac J Cancer Prev*. (2017) 18:3307–13. doi: 10.22034/APJCP.2017.18.12.3307
19. Morimoto CY, Tedardi MV, Fonseca IIM, Kimura KC, Sanches DS, Epiphanio TF, et al. Evaluation of the global DNA methylation in canine mast cell tumour samples by immunostaining of 5-methyl cytosine. *Vet Comp Oncol*. (2017) 15:1014–8. doi: 10.1111/vco.12241
20. Epiphanio TMF, Fernandes NCCA, de Oliveira TF, Lopes PA, Réssio RA, Gonçalves S, et al. Global DNA methylation of peripheral blood leukocytes from dogs bearing multicentric non-Hodgkin lymphomas and healthy dogs: a comparative study. *PLoS ONE*. (2019) 14:e0211898. doi: 10.1371/journal.pone.0211898
21. Owen LN (editor). *TNM Classification of Tumours in Domestic Animals*. Veterinary Public Health Unit; WHO Collaborating Center for Comparative Oncology. Geneva: World Health Organization (1980).
22. Goldschmidt MH, Peña L, Rasotto R, Zappulli V. Classification and grading of canine mammary tumors. *Vet Pathol*. (2011) 48:117–31. doi: 10.1177/0300985810393258
23. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. (1991) 19:403–10. doi: 10.1111/j.1365-2559.1991.tb00229.x
24. Stenger M. *The ASCO Post. Calculating H-Score. 2015:1*. Available online at: https://ascopost.com/issues/april-10-2015/calculating-\penalty-\@Mh-score/
25. Mazières J, Brugger W, Cappuzzo F, Middel P, Frosch A, Bara I, et al. Evaluation of EGFR protein expression by immunohistochemistry using H-score and the magnification rule: re-analysis of the SATURN study. *Lung Cancer*. (2013) 82:231–7. doi: 10.1016/j.lungcan.2013.07.016
26. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, et al. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget*. (2011) 2:627–37. doi: 10.18632/oncotarget.316
27. Mahapatra S, Klee EW, Young CYF, Sun Z, Jimenez RE, Klee GG. Global methylation profiling for risk prediction of prostate cancer. *Clin Cancer Res*. (2012) 18:2882–95. doi: 10.1158/1078-0432.CCR-11-2090
28. Missaoui N, Hmissa S, Dante R, Frappart L. Global DNA methylation in precancerous and cancerous lesions of the uterine cervix. *Asian Pac J Cancer Prev*. (2010) 11:1741–4.
29. Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, et al. Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. *Vet Comp Oncol*. (2009) 7:162–72. doi: 10.1111/j.1476-5829.2009.00184.x
30. Soares J, Pinto AE, Cunha CV, André S, Barão I, Sousa JM, et al. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. *Cancer*. (1999) 85:112–8. doi: 10.1002/(sici)1097-0142(19990101)85:1<112::aid-cncr16>3.0.co;2-t
31. Nishida N, Nishimura T, Nakai T, Chishina H, Arizumi T, Takita M, et al. Genome-wide profiling of DNA methylation and tumor progression in human hepatocellular carcinoma. *Dig Dis*. (2014) 32:658–63. doi: 10.1159/000367982
32. Itano O, Ueda M., Kikuchi K, Hashimoto O, Hayatsu S. Correlation of post-operative recurrence in hepatocellular carcinoma with demethylation of repetitive sequences. *Oncogene*. (2002) 21:789–97. doi: 10.1038/sj.onc.1205124
33. Mazzucchelli R, Scarpelli M, Lopez-Beltran A, Cheng L, Bartels H, Bartels PH, et al. Global acetylation and methylation changes predict papillary urothelial neoplasia of low malignant potential recurrence: a quantitative analysis. *Int J Immunopathol Pharmacol*. (2011) 24:489–97. doi: 10.1177/039463201102400222
34. Yang B, Sun H, Lin W, Hou W, Li H, Zhang L, et al. Evaluation of global DNA hypomethylation in human prostate cancer and prostatic intraepithelial neoplasm tissues by immunohistochemistry.

Urol Oncol. (2013) 31:628–34. doi: 10.1016/j.urolonc.2011.05.009

35. Figueroa JD, Yang H, Davis S. Integrated analysis of DNA methylation, immunohistochemistry and mRNA expression, data identifies a Methylation Expression Index (MEI) robustly associated with survival of ER-positive breast cancer patients. *Breast Cancer Res*. (2016) 150:457–66. doi: 10.1007/s10549-015-3314-6
36. Good CR, Panjarian S, Kelly AD, Madzo J, Patel B, Jelinek J, et al. TET1-mediated hypomethylation activates oncogenic signaling in triple-negative breast cancer. *Cancer Res*. (2018) 78:4126–37. doi: 10.1158/0008-5472.CAN-17-2082

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REVIEW
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Horizons in Veterinary Precision Oncology: Fundamentals of Cancer Genomics and Applications of Liquid Biopsy for the Detection, Characterization, and Management of Cancer in Dogs

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Cancer is the leading cause of death in dogs, in part because many cases are identified at an advanced stage when clinical signs have developed, and prognosis is poor. Increased understanding of cancer as a disease of the genome has led to the introduction of liquid biopsy testing, allowing for detection of genomic alterations in cell-free DNA fragments in blood to facilitate earlier detection, characterization, and management of cancer through non-invasive means. Recent discoveries in the areas of genomics and oncology have provided a deeper understanding of the molecular origins and evolution of cancer, and of the "one health" similarities between humans and dogs that underlie the field of comparative oncology. These discoveries, combined with technological advances in DNA profiling, are shifting the paradigm for cancer diagnosis toward earlier detection with the goal of improving outcomes. Liquid biopsy testing has already revolutionized the way cancer is managed in human medicine – and it is poised to make a similar impact in veterinary medicine. Multiple clinical use cases for liquid biopsy are emerging, including screening, aid in diagnosis, targeted treatment selection, treatment response monitoring, minimal residual disease detection, and recurrence monitoring. This review article highlights key scientific advances in genomics and their relevance for veterinary oncology, with the goal of providing a foundational introduction to this important topic for veterinarians. As these technologies migrate from human medicine into veterinary medicine, improved awareness and understanding will facilitate their rapid adoption, for the benefit of veterinary patients.

Keywords: dog, cfDNA, cell-free DNA, circulating tumor DNA, cancer, genomic, liquid biopsy, one health

INTRODUCTION

Cancer is frequent in dogs and is by far their most common cause of death (1–5). While dogs and humans have a similar lifetime risk of cancer (between 1:2 and 1:4), dogs have an annual incidence of cancer that is up to 10-fold higher than in humans, as their lifetime risk is compressed into a much-abbreviated lifespan (1, 2). Similar to humans, both genomic and environmental factors drive cancer incidence in dogs: cancer predisposition mutations are concentrated in many breeds as an inadvertent side effect of selective breeding; and dogs share the same environment as humans, including exposure to many carcinogens (6, 7). These considerations help explain why ∼4–6 million dogs are newly diagnosed with cancer per year in the US in a population of under 90 million as compared to 1.8 million cancer diagnoses in humans in a population of ∼330 million (8). Like humans, the burden of cancer in dogs increases with age: up to 50% of dogs over 10 years of age will develop cancer during the remainder of their lives (3, 9, 10).

Canine cancer also carries a significant mortality risk (3, 8, 11), since many canine cancers are diagnosed at advanced stages after there has been microscopic (12, 13) or macroscopic spread (12, 14–16) and a cure is no longer achievable. With rising pet ownership and increased emotional attachment to pets, the substantial burden of canine cancer goes well-beyond the immediate health implications for the dog, with significant emotional and financial impact on dog owners (17–21). Given the high incidence of cancer in dogs, all companion animal practices are exposed to oncology cases on a regular basis, and cancer care is an essential part of pet health care (13).

Over the past decade, genomic medicine has made great strides thanks to technological breakthroughs such as the introduction of next generation sequencing (NGS). In 2005, the National Institutes of Health (NIH) launched The Cancer Genome Atlas (TCGA), a landmark initiative aiming to molecularly characterize the genomic landscape of human cancer (22). By 2013, TCGA concluded enrollment with over 20,000 samples and built a knowledge base across all major human cancer types (22, 23). This effort, together with similar international initiatives such as the International Cancer Genome Consortium (24), enabled rapid cancer biology research and helped facilitate the development of new molecularly targeted therapeutic agents for cancer. As a result, tumor tissue-based molecular testing has become an integral part of the "precision medicine" trend in cancer care for humans (25). More recent innovations in the field have enabled non-invasive testing based on a simple blood draws; typically referred to as "liquid biopsy," this type of testing most commonly relies on analysis of cell-free DNA (cfDNA) fragments released by the tumor cells into the bloodstream and known as circulating tumor DNA (ctDNA) (26–30).

Abbreviations: cfDNA, Cell-Free DNA; CNV, Copy Number Variant; ctDNA, Circulating Tumor DNA; CTC, Circulating Tumor Cell; DNA, Deoxyribonucleic Acid; FNA, Fine Needle Aspiration; MRD, Minimal Residual Disease; NGS, Next Generation Sequencing; SNV, Single Nucleotide Variant; TDT, Tumor Doubling Time; TMB, Tumor Mutational Burden.

The first canine reference genome was published in 2005 (31), not long after the publication of the human reference genome (**Figure 1**) (32–34). However, progress in canine genomics has not been nearly as rapid as in humans, and most advances in genomic medicine have not yet been adopted in veterinary medicine. Certain areas of canine genetics have seen meaningful progress, including breed identification (35, 36), breed-specific disease predisposition (37–39), and genetic determinants of heritable disorders (40). Much of this accumulated knowledge is now available to veterinarians and pet owners through commercial testing options. However, only a small fraction of the scientific progress made in humans has been transferred into the arena of canine oncology. More research and development pertaining to the genetic predispositions underlying canine cancer syndromes, and to the detection, characterization, and management of cancer in dogs, is urgently needed to allow the standard of cancer care in veterinary medicine to catch up with human medicine standards.

With a few notable exceptions—such as *BRAF* testing in urine for detection of canine urinary tract cancer (41), and testing for *c-kit* mutations in mast cell tumors (42–44)—the field of veterinary oncology has yet to utilize the full power of genomics for its precision medicine benefits. However, the rapid adoption of genomics-based testing by the veterinary community could pose risks due to the current lack of regulatory oversight for high complexity molecular testing. Clinical genomic testing for veterinary applications can be currently marketed without any peer-reviewed clinical validation studies, or based on studies in small cohorts that may not be representative of the intended-use population (45). There is currently no established regulatory approval pathway in the United States for veterinary diagnostics, and while a form of accreditation is available through the American Association of Veterinary Laboratory Diagnosticians, this accreditation is limited to publicly funded, full-service laboratories and is not available to privately owned commercial labs (46, 47). In the United States, laboratories conducting high complexity molecular testing in humans must secure certification under CLIA (Clinical Laboratory Improvement Amendments) and may also pursue accreditation through CAP (College of American Pathologists); many laboratory-developed tests (LDTs) intended for oncology applications are also regulated by the US FDA (Food and Drug Administration) (48–50). Because no such standards exist for high complexity molecular testing in veterinary medicine, low-quality tests could easily find their way into clinical use, leading to poor outcomes for patients. The lack of external oversight in veterinary diagnostic testing means that it is critically important for highly complex, novel tests to undergo rigorous analytical and clinical validation, with detailed findings published in peer-reviewed journals for full transparency (51).

To develop reliable genomics-based testing solutions for veterinary applications, significant research and development efforts will be required. This is especially true for blood-based liquid biopsy tests since the proportion of ctDNA in the plasma can be very low and variable, requiring highly sensitive detection with minimal false positive results (52). Analytical validation of any such test must evaluate the entire process – from blood collection to shipping, accessioning, separation of plasma

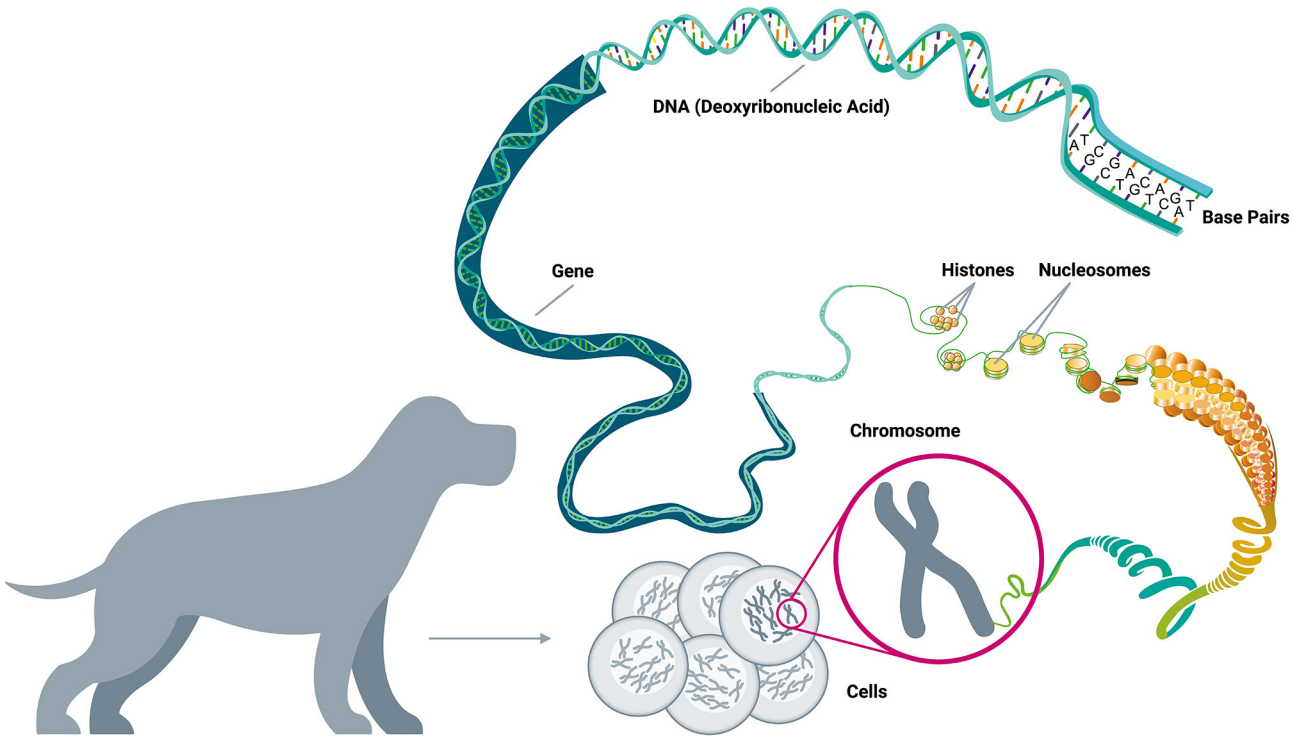

FIGURE 1 | *A brief guide to genomics.* Cancer is a disease of the genome because DNA alterations provide the biological basis of cancer. Each body cell (except for mature red blood cells) contains a full copy of the organism's genome within a set of chromosomes packed in its nucleus. The DNA double-helix is formed by four nucleotides, or bases, assembled in complementary pairs via hydrogen bonds: adenine (A) is always paired with thymine (T), and cytosine (C) is always paired with guanine (G). The gene is the basic unit of heredity and consists of a long sequence of nucleotides that encodes for the synthesis of a protein by transcription to RNA (ribonucleic acid) in the cell's nucleus, followed by translation to a sequence of amino acids in the cytoplasm. The average gene comprises several thousand bases, with wide size variation. The DNA double-helix strand wraps around a set of histone proteins, forming structures known as "nucleosomes" at regular intervals along the length of the strand (Adapted from National Human Genome Research Institute, genome.gov).

and buffy coat (white blood cell - WBC) components, DNA extraction and sequencing library preparation, data generation by NGS, and sophisticated bioinformatics analysis – through adequately designed and powered studies (53). Clinically, the test will need to be validated for each intended use. The unique, non-invasive nature of liquid biopsy allows it to be deployed in multiple clinical use cases across the full spectrum of cancer care in dogs, including: (1) screening for early detection in patients without any signs of cancer; (2) aid in diagnosis in patients with suspected cancer; (3) molecular profiling for targeted treatment selection; (4) detection of minimal residual disease after curative-intent interventions; (5) treatment response monitoring; and (6) recurrence monitoring in patients who achieve complete remission after initial treatment. Each of these use cases will require independent clinical validation in the corresponding intended-use population, with clinical utility ultimately determined by the test's demonstrated ability to inform clinical decision-making or improve clinical outcomes in each use case.

This article will review fundamental principles of cancer genomics for a contemporary understanding of cancer as a disease of the genome; describe key biological and technical considerations for developing and validating a liquid biopsy assay for veterinary cancer applications; and conclude with a review of the six clinical use cases for liquid biopsy described above. Armed with a well-informed appreciation for the validation requirements and the potential of liquid biopsy solutions to significantly improve care for their patients, veterinarians will be well-positioned to evaluate and employ validated liquid biopsy tests as they enter the clinic in the coming years. Once developed and commercialized, liquid biopsy solutions promise to usher in a new era for veterinary medicine, enabling personalized cancer care for pets at the same level of quality and sophistication already available to humans at major cancer centers today.

FUNDAMENTALS OF CANCER GENOMICS

Cancer as a "Disease of the Genome"

Historically, cancer has been defined by its organ or tissue of origin, or by its cellular characteristics, as the ability of clinicians to understand and describe it was limited to gross examination and/or microscopic evaluation. Advances in molecular medicine over the past two decades have revealed that normal cells accumulate random genomic alterations over time as a result of DNA replication errors, as well as exposure to endogenous factors (such as free radicals) and to environmental (exogenous) carcinogens such as various forms of radiation and mutagenic chemicals in food and air (54–57); and that cancer results when

one or more of these alterations confer an uncontrolled growth advantage to a population of cells (58). These random alterations are called *somatic alterations*, as they are acquired "in the body" after birth; in some cases, cancer-predisposing alterations are already present at birth, having been inherited from parents as *germline alterations*.

Most somatic alterations are promptly corrected by intracellular DNA repair mechanisms or (if unrepaired) are severe enough to trigger death of the affected cell, with no ill consequences for the organism; however, when such alterations occur in specific locations in the genome, and are not corrected, a chain of events is set in motion that ultimately leads to the development of cancer. Such alterations confer a growth and/or survival advantage to the affected cells, either by triggering increased cell replication or by inhibiting the processes that keep cell division in check; these are analogous to pressing the gas pedal and cutting the brakes on a car, respectively. Tumor growth can be further accelerated by the accumulation of new somatic alterations with the passing of time; this causes cancer cells to replicate faster, invade surrounding tissues, travel to distant organs by lymphatic and vascular routes, and evade the immune system's surveillance and control mechanisms. When the number of cancer cells reaches around one billion, the malignant mass is ∼1 cm in size and weighs about 1 g (59, 60); at this stage, the mass typically becomes detectable by physical and imaging examinations, and may have already started to cause clinical signs such as bleeding, lameness, weight loss, lethargy, etc. This clinical manifestation is called cancer, and is commonly described by its organ of origin, size, and appearance under the microscope (histological diagnosis and grading). The tumor spread is defined by the TNM (tumor, node, and metastasis) staging system. Fundamentally, however, cancer is a disease of the genome, as it is directly caused by genomic alterations and cannot develop in the absence of such alterations (61).

Genomic Alterations in Cancer

As malignant tumors grow, they develop the ability to invade adjacent areas and metastasize to distant locations in the body through the accumulation of DNA alterations in key genes (58). A primary "gatekeeping" alteration provides an initial growth advantage and allows the affected cell to replicate more quickly than the surrounding cells, becoming a microscopic clone (58); in time, a cell within this clone will randomly acquire a second alteration, typically in another gene, and initiate a subsequent round of clonal expansion with enhanced selective growth advantage for the cells containing both alterations. In this way, the process of novel mutation acquisition followed by clonal expansion continues, leading to the evolution of malignant subclones that can invade surrounding tissues, metastasize to lymph nodes, and spread to distant organs (58).

Genomic alterations that confer a selective growth advantage are termed *driver mutations*. The cumulative effect of this advantage, over many cell divisions, results in a mass of billions of malignant cells growing at an accelerating rate, with multiple subclonal populations emerging through the successive accumulation of additional mutations. In humans, this is a process that begins with a single driver mutation and ends with metastatic disease, and is estimated to take decades (58). On average, a human cancer genome contains 4–5 driver mutations, though there is wide variability across different cancer types (62). Cancer genomes also contain somatic alterations that do not confer a discernible growth advantage to the cell and are referred to as *passenger mutations* (58). Detection of either class (*driver* or *passenger*) can point to the presence of cancer, but only driver mutations can inform the selection of effective targeted therapies (58, 63).

Driver mutations are not randomly distributed across the genome; in fact, of the more than 20,000 human genes, fewer than 1,500 have been implicated in cancer development (58, 64–67). These cancer-related genes are implicated in 12 specific cellular pathways (**Figure 2**), which in turn relate to three main functions: (1) *cell survival* (ability to thrive in nutrient-poor conditions, dysregulation of apoptosis, angiogenic stimulation); (2) *cell fate* (division and differentiation); and (3) *genome maintenance* (ability to survive despite gross chromosomal abnormalities, acceleration of mutation acquisition, and DNA damage control) (58, 68, 69).

As noted previously, cancer-related alterations can be either somatic (acquired after birth, and present in only a subset of cells in the body) or germline (inherited, and present in every cell). Germline alterations resulting in cancer predisposition - for example *BRCA1* and *BRCA2* variants - increase the risk of breast cancer in humans, and alterations in these genes have also been documented in dogs with mammary tumors (70). In humans with cancer-predisposing germline alterations, the diagnosis is often made at a younger age than is typical for that cancer type, and therefore these patients benefit from proactive cancer screening that can detect such cancers at earlier stages (71). As researchers learn more about heritable canine cancer risk, proactive cancer screening in younger dogs, informed by the presence of germline alterations, will likely demonstrate increasing clinical utility and lead to better clinical outcomes.

Somatic driver mutations predominantly occur in two types of genes: oncogenes and tumor-suppressor genes (TSGs) (**Figure 3**) (58). Oncogenes typically acquire *activating* (or gain of function) mutations in very specific locations (known as "hotspots"); these activating mutations increase the rate of cell division, inhibit programmed cell death (apoptosis), or help the cell evade immune surveillance (58). TSGs, on the other hand, typically acquire *inactivating* (or loss of function) mutations, which can occur across the full length of the gene (58). As their name implies, TSGs serve as a built-in control mechanism to suppress the development and growth of tumors; inactivating mutations impair this critical protective function, leaving oncogene-driven cancers to grow unchecked (58).

Successive genomic alterations can accumulate in both oncogenes and TSGs, thereby accelerating the progression of the disease in advanced stages of cancer (72). Early in cancer formation, however, disease progression occurs at a relatively slow pace (72). In humans, many tumors grow over 10 to 30 years before clinical manifestation and remain confined to the organ of origin through most of this period (72). This timeframe represents a considerable window of opportunity for

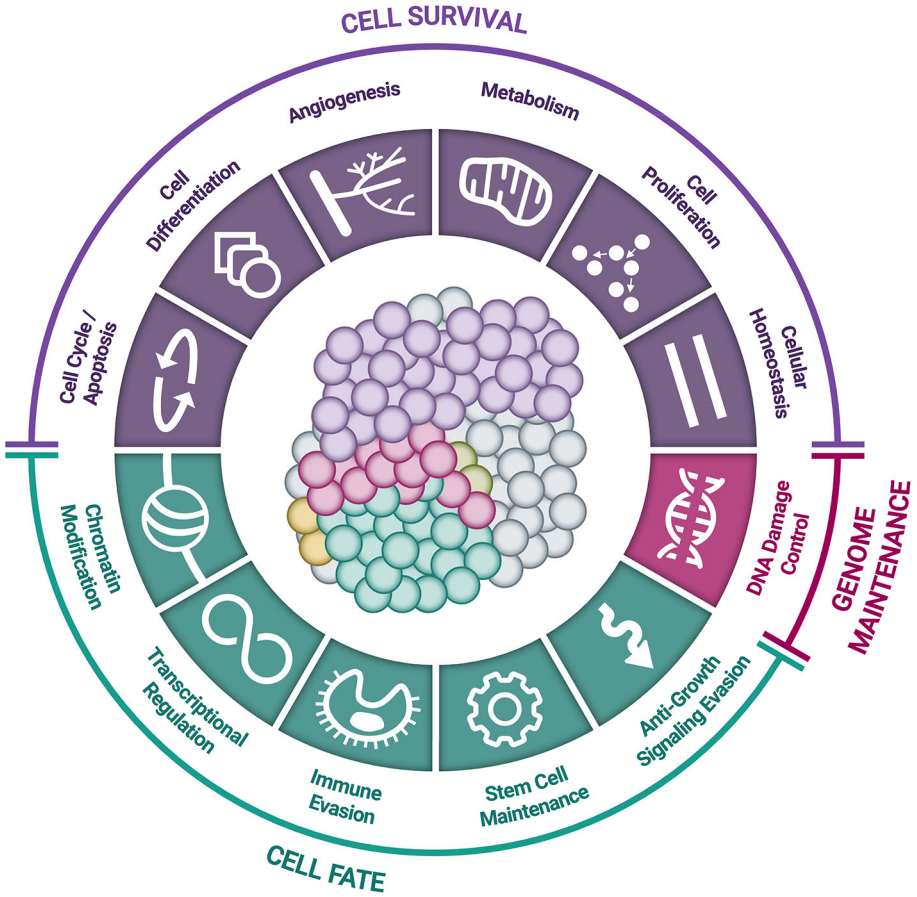

FIGURE 2 | *Cellular pathways and functional processes involved in cancer.* Driver mutations in cancer-related genes are responsible for cancer development. These cancer-related genes are implicated in 12 cellular signaling pathways, which can be grouped into 3 core cellular functions: cell survival, cell fate, and genome maintenance [Inspired by Hanahan & Weinberg (2011) and Vogelstein et al. (2013)] (58, 68).

early detection that can allow for a cure to be achieved by simple surgical removal of the localized mass (72–74). This paradigm holds true in canine cancer as well: in some types of canine cancers, for example mast cell tumors and soft tissue sarcomas, clinical outcomes are often excellent with early detection and proper surgical excision (75, 76).

Each patient's cancer is characterized by a variety of genomic alterations, and even within a particular cancer type (breast, colon, etc.), no two cancers are the same (77). There is no established 1:1 correspondence between a given tumor type and a given genomic alteration. For example, the *BRAF* V600E mutation is most commonly seen in human melanoma but is also seen in other cancers (78); likewise, its canine ortholog V595E is common in transitional cell carcinoma but is also present in different canine cancer types (79). The presence of the same mutation in different cancer types may have different therapeutic implications. For example, in humans, targeting *BRAF* with the agent vemurafenib works more effectively in melanoma than in other cancer types (80). Significant amounts of focused research will be required to understand the efficacy of various targeted agents in specific canine cancers.

Cancer in adult humans typically has dozens to hundreds of mutations per case, while pediatric cancers usually have far fewer mutations per case (58). A commonly employed metric for describing the frequency of mutations in a given cancer case is the tumor mutational burden (TMB), represented by the number of mutations per Mb (megabase, i.e., one million DNA bases) (81). A recent review of over 100,000 human cancer cases across more than 500 cancer types revealed a wide TMB spectrum, ranging from 0 to over 1,000 mutations/Mb, with a median of 3.6 mutations/Mb and increasing with patient age (82). Although less extensively studied, canine cancer genomes have been shown to exhibit similar TMBs in published studies, with a median of 1.98 mutations/Mb in canine osteosarcoma (83), 2.04 mutations/Mb in primary canine lung cancer (84), and a range of 0.1–2.1 mutations/Mb in canine hemangiosarcoma (85, 86). TMB has been shown to be a marker for predicting response to immunotherapy in humans, with high-TMB tumors more likely to respond (87, 88). The ability to non-invasively measure TMB from a blood sample could gain clinical relevance as immunotherapies become increasingly utilized in the management of canine cancers (10).

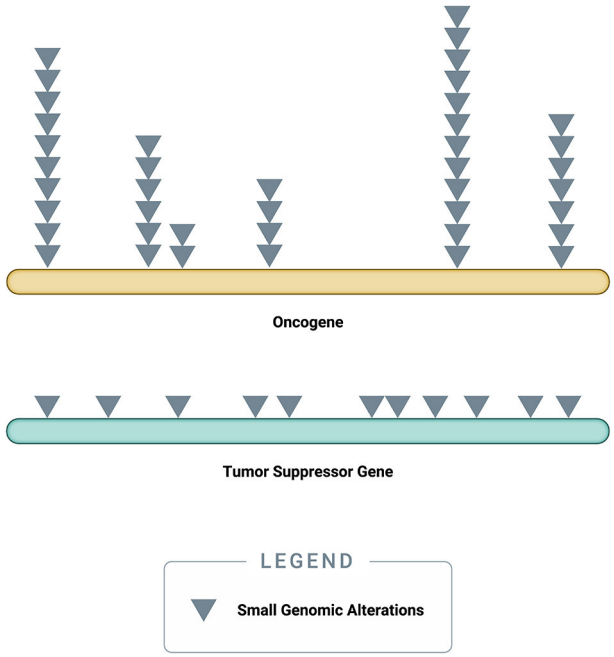

FIGURE 3 | *Accumulation of small genomic alterations in cancer-related genes.* Small genomic alterations in oncogenes tend to be activating mutations, which cluster at very specific locations ("hotspots"), whereas small genomic alterations in tumor suppressor genes (TSGs) tend to be inactivating mutations and may occur across the full length of the gene. The design of a high-quality genomic assay needs to account for these characteristics in order to identify relevant alterations across cancer-related genes in an efficient manner.

The extreme diversity of genomic features across cancer types, coupled with the fundamental understanding of cancer as a "disease of the genome," have opened the door to novel diagnostic approaches that go beyond the notion of a specific test for a specific type of cancer and favor a "pan-cancer" model where a single, highly complex diagnostic assay can be used to detect and characterize a broad range of cancer types (27, 28, 61).

Classes of Genomic Alterations

To understand how genomics-based testing can characterize cancer accurately, it is important to first review the main classes of genomic alterations that drive cancer initiation and progression (**Figure 4**). Though counter-intuitive, many cancers are driven by single nucleotide variant (SNV) "hotspot" alterations that involve a change of just one letter out of several billion letters in the genome (58, 89–91). Another class of small genomic alterations are indels (insertions and deletions), in which one to several nucleotides are inserted into, or removed from, the normal DNA sequence (89–91). Larger genomic events, affecting thousands to millions of nucleotides and known as structural alterations, can also cause significant genomic disruption, leading to cancer (92). Cancer-related structural alterations include: (1) copy number variants (CNVs), in which large segments of DNA (thousands to millions of bases long, up to entire chromosomes) are either completely absent or are abnormally repeated, and (2) translocations, in which DNA strands from unrelated parts of the genome are joined together and result in "fusion genes" in the RNA transcript (92).

Numerous studies have revealed that the disease etiology of a given cancer is typically driven either by focal somatic alterations (SNVs, indels, and/or translocations) or by CNVs, but rarely by both categories (58, 93, 94). This association with specific classes of driver genomic alterations is often cancer type or subtype-specific, with cancers such as sarcomas—which are far more common in dogs than in humans (9)–being mostly CNV-driven while others, such as carcinomas of the lung or gastrointestinal tract, being mostly SNV and indel-driven (93).

Clonality and Tumor Evolution

By the time cancers are diagnosed, they are typically large—measuring centimeters in diameter—and thus comprised of billions of cells (59, 60). As described previously, cancer growth is characterized by the successive accumulation of somatic alterations, meaning that tumors are not static—they constantly evolve to include additional alterations beyond the original clonal (or "truncal") alteration (**Figure 5**) (58). At the time of diagnosis, when the primary tumor is one or more centimeters in size, most patients do not in fact have "cancer"; rather, they have "cancers," as the disease has already evolved to consist of multiple sub-populations of cells (subclones), each sharing the original clonal alteration but further evolved with its own additional unique mutational profile. This phenomenon is known as spatial heterogeneity, which can manifest as intratumor heterogeneity (within a single primary or metastatic tumor mass) and/or intrapatient heterogeneity (between different tumor masses within the same individual) (58, 95–98). Once seeded in a new location, metastatic deposits subsequently accumulate additional alterations, which can be distinct from those present in the primary tumor (58). New alterations, which are unique to a specific subclone within the primary tumor or at a metastatic site, are referred to as *private mutations* (95).

At the time of diagnosis under the current standard of care, a single biopsy of a single tumor will only reveal a set of mutations at one point in time for that one specific physical location in the tumor. However, it is likely that an adjacent area in the primary tumor, or a distant metastatic site, will have a different set of mutations (95, 99). As cancer therapeutics become increasingly guided by the tumor's molecular alterations, a representative and unbiased view of the mutational landscape across all subclones in the body will be essential (100).

Treatment success is currently determined by observing a reduction or apparent disappearance of the tumor mass on imaging or physical examination, but in many cases this is ultimately followed by reemergence of the cancer at the same anatomic site or elsewhere. From a molecular perspective, the treatment may have been successful in eliminating a large subset (perhaps the dominant clone) of cancer cells with a particular genomic signature but left behind other subclones that harbored private resistance mutations to the treatment (**Figure 5**) (101). The treatment-resistant cell populations (subclones) were likely already present in the tumor at the time of initial treatment, albeit in smaller numbers compared to the dominant clone; once

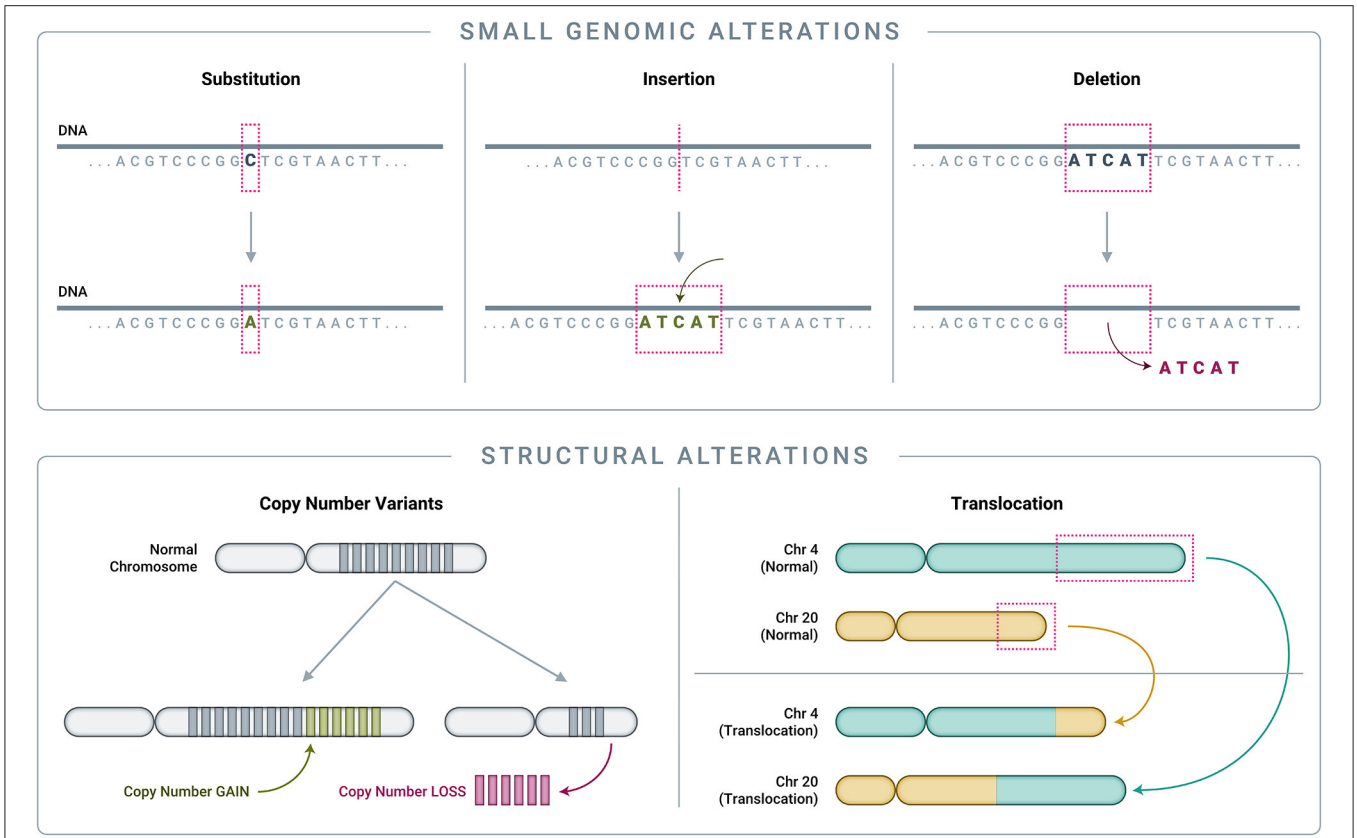

FIGURE 4 | *Classes of genomic alterations.* Small genomic alterations include single nucleotide variants (SNVs) as well as small insertions and deletions (collectively known as "indels"). SNVs arise when one nucleotide is substituted for another, which can result in altered amino acid translation and an altered protein product. Indels involve the insertion or deletion of one or more nucleotides from the normal DNA sequence, resulting in an altered protein product. On a much larger scale, structural alterations typically involve thousands to millions of nucleotides. Copy number variants (CNVs) are a common type of structural alteration, involving gains or losses of large stretches of DNA. Translocations represent another type of structural alteration, whereby two distant, otherwise unrelated genomic regions are joined together, creating "gene fusions" that can drive tumor growth.

the overall disease burden is reduced as a result of treatment pressure on the susceptible clone, these resistant subclones are allowed to prosper, with reduced competition for space and nutrients from the previously dominant clone (58, 102). This highlights an important benefit of detecting cancer earlier, before it accumulates a more diverse clonal composition that may increase its overall resistance to treatments.

In humans, this accumulation of additional somatic alterations is known to progress at relatively predictable rates. By the time a tumor reaches a clinically detectable size (typically 1 g, or 1 cm^3, or 1 billion cells), it has undergone 30 volume doublings (103); the time that the tumor has been present in the body can be roughly estimated by back calculation via the tumor doubling time (TDT), if known. In human breast tumors across multiple subtypes, median tumor volume doubling times of 85–185 days have been reported (104). Assuming constant growth rates, the average breast cancer would need many years to reach a size at which it could be clinically detected. Currently recommended screening intervals in humans take these tumor growth estimates into account. For example, screening for breast cancer with mammography is recommended every 1–2 years beginning at age 45–50 (105, 106), while screening for colorectal cancer is recommended every 3–5 years beginning at age 50 (107). In effect, these recommendations reflect current understanding of the growth rates of specific cancers from early stage to late stage in humans. Routine screening at set intervals also provides the benefit of "cumulative detection"—the combination of detection rates compounded over time, such that after 2+ cycles of screening, the overall detection rate will be higher than if a single screening test were used at just one point in time (108, 109).

The rates of growth of various cancer types in dogs are not as well understood as in humans; however, given the shorter canine lifespan, it can be assumed that the time from a cancer's molecular inception to clinical manifestation is significantly compressed. Though TDTs have been rarely reported in veterinary medicine, those that have been reported support this assessment: for example, the mean TDT for induced canine lung adenocarcinomas was ∼100 days, and for human pulmonary adenocarcinoma was greater than 1 year (110, 111). As in humans, TDT is important for informing the cadence of cancer screening in dogs; given these preliminary estimates, an annual or semiannual screening interval, when such testing becomes available, should allow for the detection

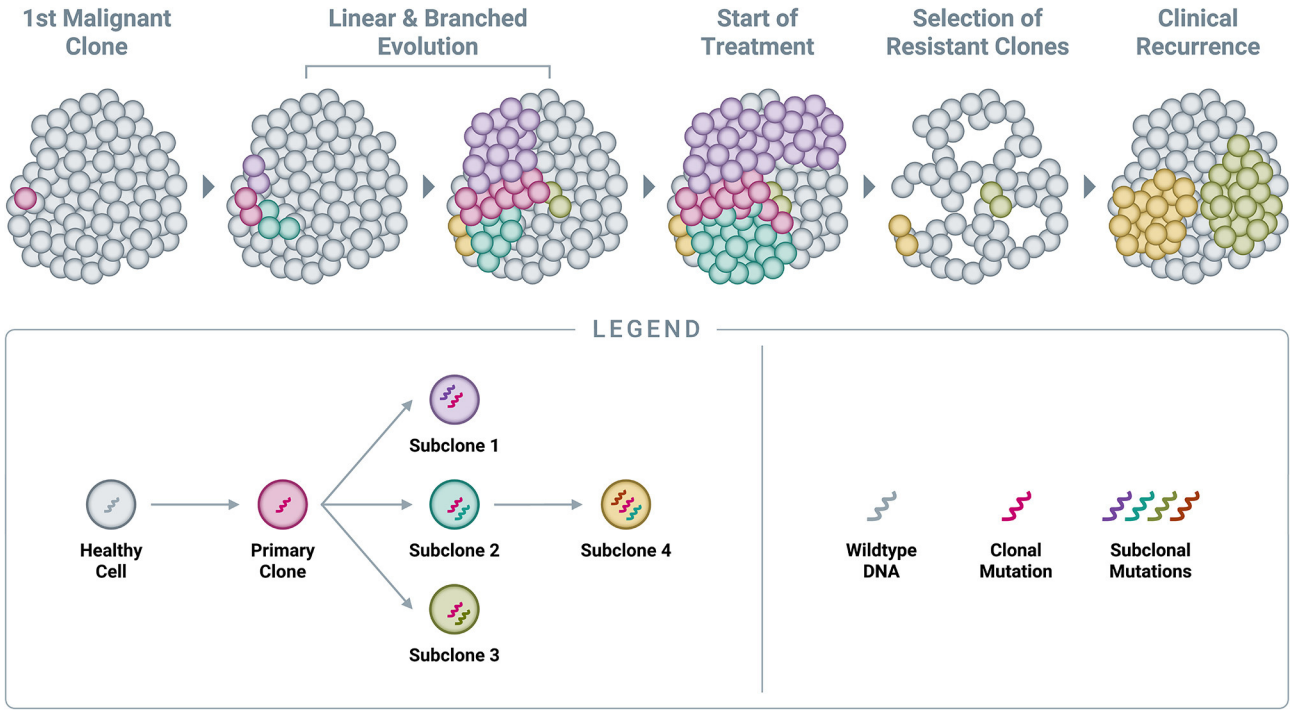

FIGURE 5 | *Accumulation of genomic alterations and emergence of resistance.* Cancer begins with a single genomic alteration in a cancer-related gene, which provides a selective growth advantage that allows the cell with the original "clonal mutation" (also known as the "truncal mutation") to grow and divide more quickly than neighboring healthy cells. Over time, additional genomic alterations accumulate in the DNA of these cancerous cells, leading to both linear and branched evolution from the original clonal population. This leads to a tumor comprised of various subclones, all of which share the original truncal mutation but also feature additional, unique mutations (known as "private mutations"). Administration of an efficacious treatment will typically eliminate many cells in the tumor, resulting in a reduction in tumor burden and clinical remission; however, certain subclones already harboring resistance mutations will often survive treatment at clinically undetectable levels and subsequently expand in the absence of competition. In time, this leads to the clinical observation of recurrence.

of a significant proportion of canine cancers at the localized (resectable) stage.

Comparative Oncology: Dogs and Humans

Comparative oncology is typically described as the study of naturally occurring cancers in veterinary patients to benefit both humans and animals, through the study of cancer biology, pathogenesis, and treatment (112). Canine and human cancers share many histological, molecular, physiological, and even epidemiological features, and this commonality provides the rationale for the field of comparative oncology, wherein a deeper understanding of cancer in one species can drive corresponding insights in the other (7, 113–115). Dogs represent a powerful model system for the study of human cancers and vice versa, as cancers occur spontaneously in both species and are driven by orthologous genomic changes that impact corresponding biological pathways (114, 116, 117).

The human genome is ∼3.1 billion nucleotides in length; the canine genome is ∼20% smaller at ∼2.4 billion nucleotides (31–33, 66, 118). Despite the size difference, the human and canine genomes have a high degree of homology (estimated at around 85%) (31); and among the top 100 human genes most frequently mutated in cancer, the extent of homology in the canine genome is likely even higher. Despite these commonalities, there are important differences between human and canine cancers, and these differences can be intelligently leveraged to drive faster translation of discoveries from one species to the other. For example, while dogs and humans are susceptible to cancers throughout the body, some cancers that are common in dogs are rare in humans (e.g., osteosarcoma, T-cell lymphoma) (83, 119, 120), and vice versa (**Table 1**). It is difficult to perform well-powered studies in rare cancer types; however, research efforts can progress faster in the species where the cancer is more common, and key insights can be translated back to the other species.

LIQUID BIOPSY: THE NEXT FRONTIER IN VETERINARY CANCER CARE

Liquid biopsy broadly refers to the sampling and analysis of analytes from various biological fluids (primarily blood, but in some cases also urine, cerebrospinal fluid, or other secretions) that can be sampled through minimally invasive or non-invasive methods (121). Blood-based liquid biopsy may include analysis of circulating nucleic acids (mainly cfDNA, which includes ctDNA in patients with cancer); circulating tumor cells (CTCs); and proteins (121). The ability to detect cancer-related analytes from

TABLE 1 | Common cancers in humans and dogs (8, 13).

Common cancers in humans	Common cancers in dogs
• Bladder cancer	• Anal sac carcinoma
• Breast cancer	• Lymphoma
• Colorectal cancer	• Mammary gland cancer
• Kidney cancer	• Mast cell tumor
• Lung cancer	• Oral malignant melanoma
• Skin cancer*	• Osteosarcoma
• Non-Hodgkin Lymphoma	• Soft tissue sarcoma
• Prostate cancer	• Splenic hemangiosarcoma

**Including melanomas as well as basal cell and squamous cell skin cancers.*

blood has unique advantages, especially in cancer (or suspected cancer) cases where obtaining a tissue sample for traditional histological analysis might be particularly risky or difficult.

Tumor Tissue Sampling and Analysis as the Current Standard of Care

The conventional path to achieving cancer diagnosis in companion animals varies based on patient characteristics, tumor type, and tumor location (122). Fine needle aspiration (FNA) cytology is less invasive and lower risk compared to biopsy, and FNA is often used to make a preliminary or definitive diagnosis, develop a treatment plan, and predict prognosis (123, 124). However, inconclusive results or misdiagnoses can occur with FNA due to low cellularity, artifact, necrosis, minimal exfoliation of certain cell types, lack of tissue architecture, etc. (124, 125). Also, not all tumors are easily accessible by FNA (such as deep-seated abdominal tumors, many intrathoracic tumors, and tumors of the central nervous system); and some tumors with high vascularization, or those which might seed the body wall (e.g., urinary tract), are not amenable for sampling by FNA.

If FNA cytology is attempted and is non-diagnostic or equivocal, more invasive methods (such as a traditional biopsy or exploratory surgery) are often employed to obtain tissue for analysis prior to making a definitive diagnosis and initiating treatment (126). Compared with FNA, biopsies and surgeries entail higher risks of morbidity and mortality, which are dependent upon the site of the suspected mass and the characteristics of the procedure. Such risks include infection, internal bleeding, fracture after bone biopsy, intestinal perforation with endoscopic biopsy, pancreatitis after pancreatic biopsy, collapse of vertebra at spinal surgery sites, non-diagnostic results, and in the worst cases, death (122, 127–137).

Circulating Biomarkers

The clinical and cost challenges of tissue analysis have stimulated the search for "non-invasive" methods that rely upon analysis of biomarkers found in easily accessible body fluids, such as blood, urine, and secretions. Despite decades of research, few such methods have entered broad clinical use, with the exception of testing for hematological malignancies where blood-based cytology is part of the standard of care. Solid tumors, which make up most malignancies in both humans and dogs, have seen limited benefits to date from methods that employ circulating biomarkers, with cfDNA-based approaches currently showing the greatest promise for the future.

Protein Markers

In humans, blood-based testing has provided the opportunity to profile tumors to aid in the diagnosis of cancers, and to guide treatment decisions; and the earliest such tests have targeted protein markers (138, 139). A number of blood-based protein biomarkers have been used for human cancer screening and monitoring using immunohistochemical methods, including: CEA for colorectal cancer, PSA for prostate cancer, CA-125 for ovarian cancer, and alpha-fetoprotein (AFP) for hepatocellular carcinoma (140–143). Using similar ELISA (Enzyme-Linked ImmunoSorbent Assay) testing methods, recent attempts have been made to measure the concentrations of histone proteins that form the core of nucleosomes in order to detect the presence of cancer (144, 145). The nucleosome is the basic structural unit of DNA packaging, consisting of a segment of DNA wound around eight histone proteins. As cancer cells die, they release histone-bound DNA into circulation, whereupon the histone proteins can be separated and independently assayed (**Figure 1**).

However, biomarker assays based on circulating proteins suffer from high rates of false positives and false negatives, since the same proteins exist in circulation in healthy individuals and can be increased for reasons other than cancer, such as inflammation, sepsis, and trauma (146); also, these markers may not be significantly elevated in a significant proportion of individuals with even advanced stage cancer, reducing the potential for a highly sensitive test (146). Importantly in dogs, nucleosome concentration is also elevated in benign disease and in trauma (147–151), limiting its diagnostic utility for cancer (152). For these reasons, circulating protein markers have not been broadly adopted for cancer detection, and are more commonly used for monitoring cancer in cases where the level of the corresponding protein was shown to be already abnormal at the time of diagnosis.

In veterinary medicine, there has been interest in leveraging protein biomarkers such as thymidine kinase type 1 (TK1), canine C-reactive protein (cCRP), and alpha-fetoprotein receptor (RECAF) for canine cancer detection (153, 154); however, such protein biomarkers are not highly specific for canine cancer and can be elevated due to other reasons including immune-mediated hemolytic anemia, thrombocytopenia, and polyarthropathy (155). In human medicine, protein biomarkers such as RECAF and AFP have demonstrated limited sensitivity and specificity for cancer detection (143, 156).

Circulating Tumor Cells

CTCs are intact tumor cells originating in solid tumors that can sometimes be detected in circulation, a finding that has catalyzed a considerable body of research aimed at using CTCs for cancer detection. However, multiple studies in humans have demonstrated that even in metastatic disease, as many as 20% to over 50% of patients (depending on cancer type) have no detectable CTCs in the typical sample volume collected (157–162). Similar performance has been observed in metastatic canine cancer (163). As a result, CTCs have not seen broad clinical

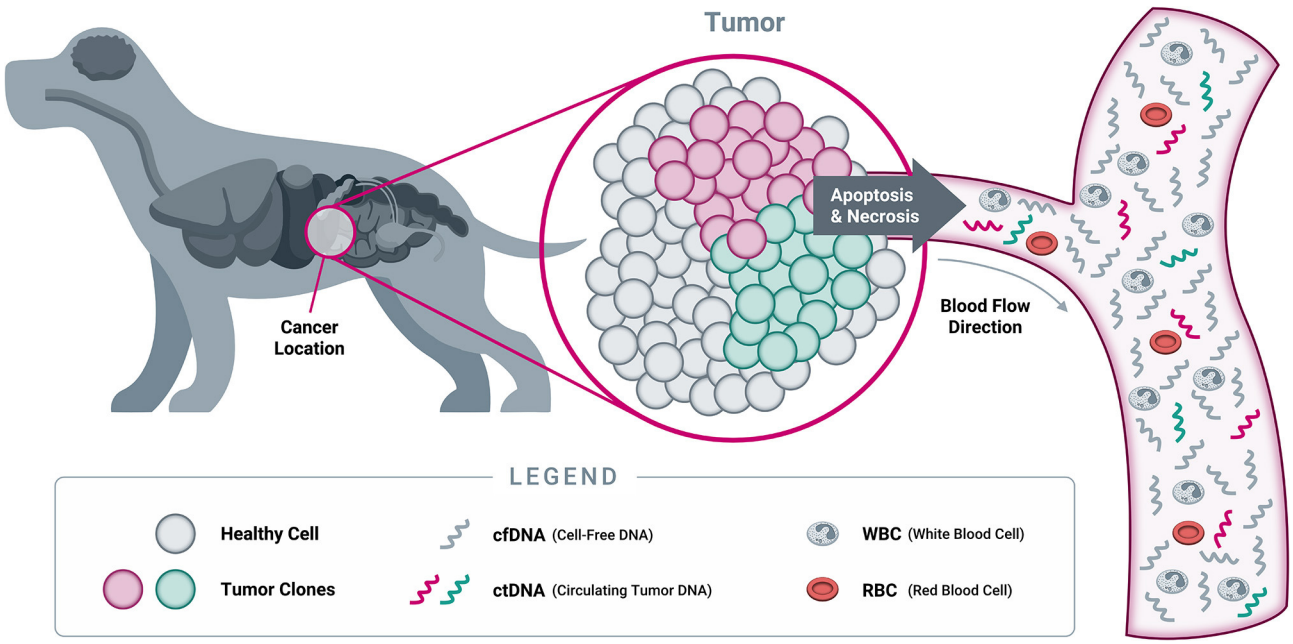

FIGURE 6 | *Origins of cell-free DNA.* When a cell dies through either programmed cell death (apoptosis) or necrosis, its cellular contents (including DNA from the nucleus) are released into the bloodstream. At this point, the DNA becomes "cell-free DNA" and is rapidly degraded into small fragments through the action of circulating enzymes known as "DNAses." As a result, most cfDNA fragments found in circulation are typically short, averaging 167 nucleotides in length in both humans and dogs (166, 167). While both healthy cells and tumor cells contain DNA that becomes cfDNA in circulation, only tumor cells will harbor somatic genomic alterations in cancer-related genes. Detection of such genomic alterations in the cfDNA of a patient is thus indicative of the presence of tumor cells in the body, providing the rationale for "liquid biopsy" testing approaches (Note: cfDNA exists as both single stranded DNA and double stranded DNA; only single stranded DNA is depicted here, for illustrative purposes).

adoption, and remain primarily a research tool in both humans and dogs (164, 165).

Circulating Nucleic Acids

Over the past decade, circulating nucleic acids – in particular cfDNA – have emerged as the most promising class of circulating biomarkers for non-invasive detection and characterization of cancer. cfDNA, which includes ctDNA in cancer subjects, is the focus of the remainder of this review.

cfDNA Origins and Characteristics

As cells undergo programmed cell death (apoptosis) and necrosis, the membranes of cells and nuclei are broken down, and their contents are released into the circulation (**Figure 6**). Among these contents are fragments of DNA, known as "cell-free DNA" once they have left the confines of the cell and its nucleus. These cfDNA fragments are rapidly degraded by normal metabolic processes and have a very short half-life, estimated at 15 min to a few hours in both humans and dogs; as a result, they are usually cleared within a few days (168–170). The constant turnover of cells throughout the body provides a steady supply of cfDNA in the circulation, which is amenable to analysis with sophisticated technologies including NGS.

The presence of cfDNA in humans was first reported in 1948, and while cfDNA was hypothesized to be linked to metastatic cancer in the mid-1960s, it took until 1977 for the first results evaluating cfDNA concentrations in patients with cancer compared to normal controls to be published, and neoplastic characteristics were reported in circulation in 1989 (171–174).

In 1996, two landmark publications reported the detection of cancer-derived alterations in plasma or serum of cancer patients as ctDNA (175, 176). Since then, significant efforts have been devoted to developing molecular tests to detect the presence of cancer-derived alterations in the blood (161, 177), and use the information for cancer detection, characterization, treatment, and monitoring (27, 28, 101, 178–183).

In parallel, fetal-derived cfDNA was discovered in maternal plasma in 1997 (184), leading to the first widely adopted clinical application for cfDNA testing: a screen for common fetal chromosomal abnormalities such as trisomy 21 (Down syndrome) using a sample of the pregnant woman's blood (185). Prior to this revolutionary advance, such fetal genetic information could only be derived from invasive diagnostic tests such as chorionic villus sampling (CVS) or amniocentesis, which carry a risk of miscarriage (186). As a result, the introduction of cfDNA-based non-invasive prenatal testing (NIPT) in 2011 (185) fundamentally changed the way prenatal care is delivered. Tens of millions of pregnancies have been screened with this cfDNA-based technology to date, leading to a marked decrease in the number of invasive diagnostic procedures for detection of fetal chromosomal abnormalities (187).

There are many documented instances of NIPT results incidentally identifying maternal cancer, highlighting plasma as a common repository for both fetal-derived and cancer-derived cfDNA fragments (188, 189), and suggesting the potential of using plasma cfDNA to screen for asymptomatic cancers. Indeed, a population-based study published in 2017 reported the performance of cfDNA-based liquid biopsy to detect nasopharyngeal cancer before symptoms develop (190), which marked the first demonstration of using a cfDNA-based blood test to screen for a specific type of cancer. Multiple commercial providers are currently offering or developing liquid biopsy tests for human cancer applications, and many clinical trials are underway to expand the clinical utility of this technology to additional use cases and/or cancer types.

Published research on canine cfDNA has covered a variety of clinical applications, including trauma, sepsis, thromboembolism, and neoplasia, and has focused primarily on determining the concentration of cfDNA in plasma as correlated to a particular clinical state or as a predictor for certain clinical outcomes (84, 148–151, 167, 169, 191–203). Studies that evaluated cfDNA concentrations in healthy canine subjects have reported median concentrations ranging from less than 1 ng/mL to greater than 500 ng/mL (148, 149, 167, 169, 194–203)—significantly wider than the range documented in healthy humans (typically 0–20 ng/mL) (204). These wide-ranging findings suggest that additional research employing well-controlled, large-scale studies is required to better understand the fundamental characteristics of cfDNA in dogs; they also point to the need for standardized, reproducible methods for blood collection, extraction, and measurement of canine cfDNA. Such standardization will be critical for the successful transfer of cfDNA-based technologies such as liquid biopsy—currently limited to the human space where such methods are well established—to routine clinical use in veterinary medicine.

To provide the highest clinical value, a liquid biopsy test should be able to detect multiple classes of cancer-associated genomic alterations (described above) in cfDNA with high accuracy, even at very low concentrations in the circulation. Furthermore, the biology of cfDNA uniquely facilitates the evaluation of certain genomic features in circulation that can provide additional information about the presence and the origin of cancer.

For example, it is well-known that the attachment of methyl (CH3) groups to the DNA strand at specific locations throughout the genome is associated with cancer; methylation of the promoter regions of tumor suppressor genes can inactivate the expression of these genes, allowing oncogene-driven cancers to proliferate unopposed (205). Furthermore, DNA in cells from specific organs have methylation profiles that are specific to that organ (206). When DNA from cancer cells in a particular organ is released into circulation as ctDNA, its methylation "signature" carries information about the presence of cancer and about the organ of origin of that cancer (27, 207). For this reason, NGS-based analysis of cfDNA methylation profiles has emerged as one of the most promising approaches for detecting cancer and assigning it to a specific organ of origin, which has obvious clinical benefits (27).

Another unique feature of cfDNA is the fact that it is highly fragmented according to specific patterns. In the nucleus of a cell, DNA is organized in chromosomes as an uninterrupted strand ranging in size from tens of millions to over 100 million nucleotides (or bases). However, by the time it enters circulation following cell death and nuclear DNA degradation, cfDNA has been biologically degraded into fragments that are typically less than 1,000 nucleotides in length. In both humans and dogs, much of the cfDNA exists in fragments that are ∼167 bases in length, representing the length of the DNA strand between two nucleosomes plus one full wrap of DNA around the histone proteins that make up the core of the nucleosome (166, 167, 208). Furthermore, it has been shown that in humans with cancer, the fragment length of cfDNA tends to be shorter; one of the key observations that have led to fragment profile analysis becoming an emerging method to improve the sensitivity for cancer detection. In addition, fragmentation features in cfDNA can also encode information about the organ of origin (209–213). As a result, fragmentomics – like methylomics – has the potential to extract unique information from cfDNA that points to both the presence of cancer and its organ of origin (214).

Emerging methylomic and fragmentomic methods leverage features that are unique to circulating tumor DNA and offer additional possibilities for the detection and characterization of cancer in circulation. However, the canine methylome has not been comprehensively characterized, which means that significant research will have to be performed before methylomics-based liquid biopsy solutions can be offered for oncology applications in dogs. Likewise, the canine cfDNA fragmentome is poorly understood at this time, requiring a massive investment in research to fully understand its potential for clinical use.

Currently, the only technology that can simultaneously interrogate all the major classes of genomic alterations in cfDNA, as well as features such as methylation and fragmentation patterns, is next generation sequencing (NGS). Leading liquid biopsy assays currently in use or under development in human medicine use advanced NGS-based techniques to evaluate a broad range of alterations and features across the genome that are known to be associated with cancer. Most of these approaches do not target a particular cancer type; instead, they take a "pan-cancer" approach rooted in the premise that cancer is fundamentally a disease of the genome, and accurate analytical detection of somatic genomic alterations will lead to accurate clinical detection of a wide variety of cancer types. Assays that combine multiple classes of genomic alterations and/or orthogonal genomic features are likely to yield improved clinical performance (such as higher sensitivity and specificity) or provide additional useful information (such as organ of origin prediction and identification of molecular targets for personalized treatment) across a broad range of cancer types. The past few years have also seen the debut of multi-omic liquid biopsy approaches that combine (for example) genomic and proteomic methods, breathing new life into protein analysis as a valuable adjunct to cfDNA analysis (26, 28). Similar combinatorial strategies will likely be required for the successful development of a pan-cancer liquid biopsy test for dogs.

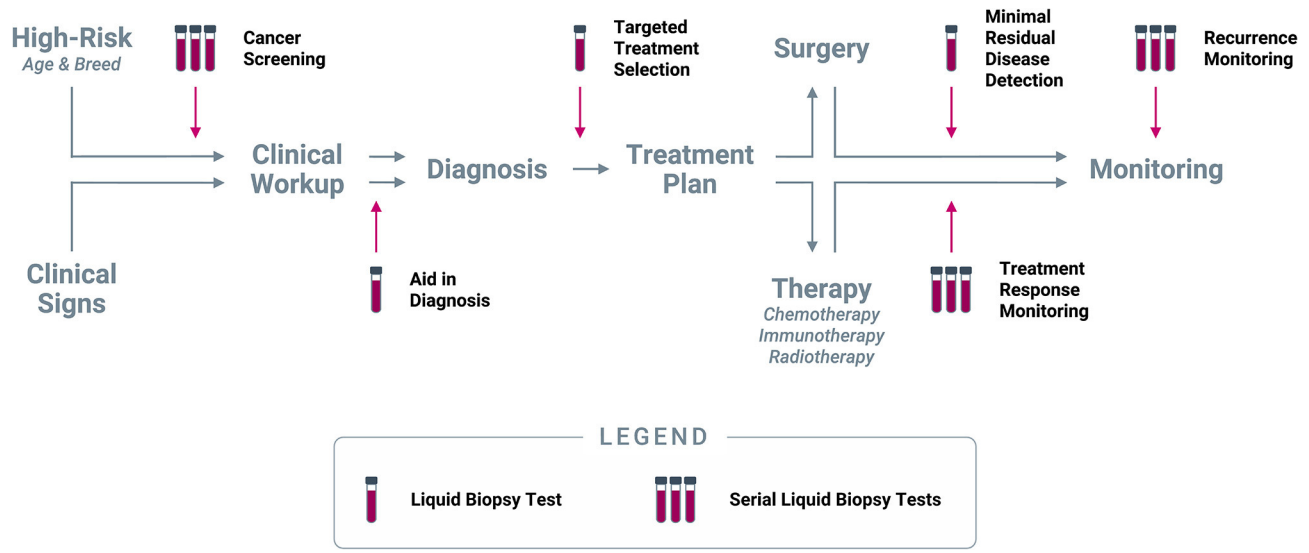

FIGURE 7 | *Clinical use cases for liquid biopsy in cancer.* Liquid biopsy can be used to inform multiple decision points along the entire continuum of cancer care: (1) *Cancer screening* at regular intervals in patients deemed to be at higher risk for cancer based on age and/or breed; (2) *Aid in diagnosis* in patients who present with clinical signs (including incidental findings on imaging or laboratory tests) that are suspicious for cancer; (3) *Targeted treatment selection* based on the unique mutational profile of the tumor in patients diagnosed with cancer; (4) *Minimal residual disease detection* following a curative-intent intervention (such as surgery); (5) *Treatment response monitoring* at regular intervals during extended-duration therapeutic regimens; (6) *Recurrence monitoring* at regular intervals after complete remission or presumed cure.

Clinical Use Cases and Clinical Utility of Liquid Biopsy in Cancer

Liquid biopsy promises the convenience of a blood draw combined with the power of genomic technology. It is unlikely to fully replace the key role that traditional tissue biopsy plays in veterinary cancer diagnosis and management, but the non-invasive nature of liquid biopsy, coupled with its ability to detect tumor signal from any malignant mass in the body, should allow it to provide immediate value in several clinical scenarios once it becomes commercially available. In humans, liquid biopsy has demonstrated feasibility and great clinical potential across multiple use cases, spanning the entire continuum of cancer care; a similar spectrum of applications is in principle available for veterinary uses of the technology (**Figure 7**).

Prior to a cancer diagnosis, liquid biopsy can provide valuable information in (1) presumably cancer-free patients as a *screening* test, and (2) in patients with clinical signs suspicious for cancer as an *aid in diagnosis*. Upon confirmation of a cancer diagnosis, liquid biopsy can be used to (3) identify a personalized treatment path based on the mutational profile of the tumor for *targeted treatment selection*; and (4) if the patient is to undergo a curative-intent intervention (such as a surgical procedure), a liquid biopsy immediately following the intervention can be used to test for *minimal residual disease*. After initiation of a longer-term therapy, such as chemotherapy or radiotherapy, liquid biopsy can be used (5) at regular intervals for *treatment response monitoring*. Finally, once a patient completes their course of treatment and is determined to be cured or in complete remission, liquid biopsy testing at longer intervals can be used for (6) *recurrence monitoring*. Each of these use cases, and their potential applicability in dogs, are described in more detail below.

Screening

Certain dog breeds are known to be more predisposed to cancer than others, presumably due to cancer-predisposing mutations that have become concentrated in the population over time as a result of the breeding process; however, the germline mutations responsible for most of these cancer predispositions are not as well-understood as in humans. It is also well-established that, just as in humans, cancer incidence in dogs increases with age (3). In a large fraction of cases, cancers in dogs are diagnosed at advanced stages after they have spread beyond the organ of origin, when prognosis is poor and the ability to extend life by treatment is limited (12–16). A liquid biopsy-based screening paradigm focused on high-risk populations, such as dogs from predisposed breeds or from geriatric populations, could help identify many of these cancers earlier. Early detection has been shown to drive better clinical outcomes in humans, such as increased life expectancy and higher rates of achieving complete remission following curative-intent interventions (e.g., surgery); historically, this has provided the rationale for well-established screening programs such as colonoscopy, mammograms, Pap smears, PSA screening, and low-dose CT scans (183, 215–217). Liquid biopsy solutions for universal cancer screening in humans are nearing commercialization (216, 218–220), and some of these assays have also shown potential for predicting the organ of origin of the tumor, facilitating the path to a definitive diagnosis (27, 28, 221–223).

State-of-the-art liquid biopsy assays currently in development for pan-cancer screening in humans have demonstrated detection rates (sensitivity) for early-stage cancer ranging from ∼20 to 70% across multiple cancer types, at specificities of 98 to >99% (false positive rates of 2 to <1%) (27, 28, 211). High specificity is particularly important in cancer screening, given potential harms resulting from the diagnostic work-up of false positive screens, and from diagnosis and treatment of cancers that may never have become clinically apparent without screening (overdiagnosed and overtreated cases) (224). Screening results implying the possibility of a cancer diagnosis can also impose a considerable psychological burden on people who receive false positive results (225, 226), and it is reasonable to assume that pet owners would likewise experience distress as a result of false-positive cancer screening results in their companion animal.

A recent health economic modeling study revealed that adding an annual universal cancer screening test to the current standard of care in human medicine would reduce late-stage cancer incidence by 78% in those intercepted by the screening test, and result in an absolute reduction of 26% in all cancer deaths (227). The practice of screening at regular intervals relies on the concept of "cumulative detection" to improve the clinical sensitivity over time at the population level, as sequential testing holds the benefit of detecting cases missed on initial screening (108, 109, 228). Ultimately, this technology may support cancer screening in lower-risk canine populations as well, comparable to how NIPT technology expanded beyond high-risk cases to encompass all pregnancies in humans (229).

Aid in Diagnosis

One of the most common scenarios in which liquid biopsy may add value in the veterinary clinic is as an aid in diagnosis, when cancer is suspected due to clinical signs (including incidental findings on imaging or laboratory tests) or clinical history. Due to the high-risk nature of this patient population, this scenario is likely to provide the initial opportunity for liquid biopsy to be deployed in veterinary medicine. In some cases, clinical signs may be non-specific and not localizing to a certain anatomic site; whereas in other cases an anatomic site may be evident, but the invasive procedures required to obtain tissue for diagnosis may carry a high risk of complications, or the suspected mass is inaccessible by biopsy or surgery. In such cases, liquid biopsy could significantly shorten the time to a definitive diagnosis and help avoid the challenges typically associated with a long diagnostic odyssey. Often, elucidation of such clinical cases requires additional appointments, time, and expense; and diagnosis may be delayed or missed completely. Many pet owners may decline biopsy or exploratory surgery due to the associated risks and cost, missing the opportunity to obtain an adequate diagnosis and select an appropriate treatment. A liquid biopsy can be conveniently performed from a routine blood collection drawn during the initial visit when cancer is first suspected, potentially saving time and money while increasing compliance.

In both the screening and the aid in diagnosis use cases, liquid biopsy can facilitate earlier detection of cancer compared to the current standards of care. In addition to improving outcomes, earlier diagnosis can mitigate the financial burden of treatment, making it a cost-effective paradigm both at the population level and at the level of individual patients. Health economic studies have shown that treatment costs for human cancer patients diagnosed early in the disease course to be 2 to 4 times less than for those diagnosed at later stages (216, 217). Treatment for early-stage cancer typically consists of localized resection, which is often curative and has a short recovery time (28); whereas treatment for late-stage disease involves repeat courses of chemotherapy or radiation therapy aimed at extending life rather than achieving a cure. Availability of an affordable and convenient liquid biopsy testing option for proactive serial screening of dogs at high risk of cancer, or for first-line evaluation of canine patients suspected of cancer, could reshape the clinical and economic landscapes of pre-diagnostic cancer management in veterinary medicine.

Targeted Treatment Selection

In situations where surgical interventions are not feasible, other therapeutic options may be utilized, either with curative intent or as a chronic treatment to extend life and/or improve quality of life. In such cases, selection of a specific therapy may be based on established clinical practice guidelines; however, an emerging area in human medicine, often designated by the terms "precision medicine" or "personalized medicine," aims to utilize the genomic signature of an individual's cancer to select specific targeted therapies (230, 231). For humans, there are over 200 FDA-approved drugs for the treatment of cancer (232) including a subset of more than 50 drugs matched (or "targeted") to specific genomic alterations in a tumor, with many additional targeted-treatment candidates in various phases of development (233–235). For dogs, there are only two drugs that are FDA approved at the time of this writing for the treatment of cancer - toceranib (Palladia™) and tigilanol tiglate (Stelfonta®), with two more drugs - rabacfosadine (Tanovea®-CA1) and verdinexor (Laverdia™-CA1) - available under a conditional FDA approval (236). In the EU, the European Medicines Agency (EMA) has approved toceranib, tigilanol tiglate and mastinib mesylate (Masivet®) (237). Of these approved or conditionally approved drugs, only toceranib (a multi-kinase inhibitor that inhibits c-kit, PDGFR, and VEGFR2), and mastinib (a c-kit inhibitor) can be used as a targeted drugs linked to specific genomic features of a tumor, as improvements in tumor response (43, 238) and outcome (239) have been demonstrated for tumors with an activating kit mutation; however, many targeted drugs used to treat human disease are currently used off-label in dogs (236, 240). Many compounds developed (and FDA-approved) for use in humans underwent preclinical safety testing in dogs; significant safety and dosing data are thus available to help inform the treatment of canine cancer patients with these agents (241).

State of the art liquid biopsy approaches have the potential to comprehensively evaluate the genomic signature of a patient's cancer directly from blood – the final common pathway for ctDNA derived from all tumor subclones in the patient's body; this unique capability makes therapy selection based on liquid biopsy results less susceptible to treatment selection bias resulting

from tumor heterogeneity, a bias that is unavoidable when a tumor is only sampled by a single tissue biopsy. Liquid biopsy results could be used for targeted treatment selection, especially for treatments where the genomic alteration targeted in humans has a direct ortholog in the canine genome. This could lead to more rapid and widespread utilization in canine cancer patients of targeted therapies currently approved for human use. The availability of liquid biopsy assays, as subject selection tools during the drug development process and as companion diagnostics following regulatory approval, can also accelerate the development of canine-specific targeted therapeutics; ultimately this will likely be the preferred path to bringing targeted treatments into veterinary oncology, as human-oriented targeted treatments might not have the same efficacy in canine cancer even if the targeted genomic alteration is perfectly homologous across the two species (242).

Minimal Residual Disease (MRD) Detection

After curative intent treatment (such as surgery) has been performed to remove the tumor, adjuvant therapy is often considered because of the risk of malignant deposits remaining in the body and resulting in relapse (or recurrence) in the future (243). MRD is defined as occult malignant disease that exists immediately after surgery and is undetectable by conventional methods; however, it can often be detected by the presence of ctDNA in the circulation (244, 245). The short half-life of cfDNA (minutes to hours in both humans and dogs) makes it an ideal analyte for MRD testing, as detection of any amount of ctDNA starting within a few days after surgery would point to the persistent presence of malignant disease in the body (168, 169). Many cancer types in humans have been studied in the context of MRD detection, including breast, pancreatic, lung, nasopharyngeal, and colorectal, as well as hematological malignancies (246, 247). In colorectal cancer for example, MRD detection has strong prognostic value, as patients with undetectable ctDNA post-operatively have significantly improved recurrence-free survival compared to those with detectable ctDNA in plasma (178, 244). In fact, detectable ctDNA post-operatively has a stronger prognostic association than many of the other traditional high-risk pathological and clinical features typically used by oncologists when considering adjuvant chemotherapy for patients with stage II colon cancer (246, 248). Similarly, the adoption of liquid biopsy-based MRD testing for canine patients could be used to inform the clinician about the relative risk of recurrence following curative-intent interventions, and thereby guide decisions regarding initiation of adjuvant treatment as soon as the patient has recovered from surgery.

Treatment Response Monitoring

Traditionally, treatment response monitoring has been performed by clinical observation and by imaging (mainly ultrasound and radiography, in the veterinary setting). Formalized procedures for documenting treatment response in dogs, such as the Canine Response Evaluation Criteria for Solid Tumors (cRECIST v1.0), have been published based on these methods (249, 250). However, reliance upon imaging alone for ascertaining treatment response has significant shortcomings. There are well-documented high inter-observer variabilities with imaging approaches in both dogs and humans, which can complicate the interpretation of imaging studies read by different radiologists (251–256).

In addition, hyperprogression (faster-than-expected tumor growth while under treatment) (257) and pseudoprogression (an initial apparent increase in tumor size or appearance of a new lesion on imaging during treatment, followed by tumor regression) (258, 259) can confound the interpretation of imaging for evaluation of treatment response. Lesion growth observed on imaging after treatment initiation may be due to advancing disease (secondary to ineffective treatment), an inflammatory response (resulting from tumor destruction by the treatment or from a direct side effect of the treatment), or simply ongoing tumor growth in the setting of a delayed treatment effect (257). Due to these complexities, real-time monitoring of tumor dynamics via serial liquid biopsy testing may help the clinician differentiate among these challenging scenarios and obtain more frequent updates on the patient's response to treatment than might be feasible with imaging alone.

The concentration of ctDNA in plasma can serve as a surrogate for the overall tumor burden (161), and patients with undetectable ctDNA after treatment are more likely to have had a complete response (178, 248, 260). Furthermore, the precise genomic variants in an individual's cancer can be used to follow the efficacy of the treatment in real time. This monitoring for treatment response may be useful regardless of the treatment modality (e.g., IV vs. oral chemotherapy, radiation, etc.). Since many chemotherapeutics are costly and typically require multiple clinic visits (20, 261), a ctDNA-based treatment response monitoring approach can offer significant value by detecting treatment response or treatment failure sooner than imaging or clinical observation would. This earlier detection may allow for early discontinuation of non-efficacious therapies in favor of alternate therapies that might have a better efficacy profile; or it may reassure the pet owner to continue a course of successful treatment even if clinical improvement is not readily apparent, or when a mixed clinical picture raises the question of disease progression vs. side effects of an otherwise efficacious treatment.

Monitoring for treatment response will also likely yield insights into the genomic evolution of tumor clones under the selective pressures of treatment—for example, the emergence of resistance mutations, or the emergence of new genomic variants potentially targetable by a different drug (262–264). Such molecular insights into tumor evolution are currently possible with standard tumor biopsy; however, even if molecular profiling of tumor tissue were widely available, longitudinal monitoring through repeat tissue biopsies would not be feasible in actual practice due to clinical, ethical, and financial considerations. Compared to current methods for monitoring treatment response, liquid biopsy would represent a complementary tool to better understand the evolution of the tumor, and its non-invasive nature could pave the way for liquid biopsy to become a routine monitoring test during cancer treatment in dogs.

Recurrence Monitoring

Even in patients who are thought to have achieved complete remission or a cure following successful treatment, the possibility of disease recurrence remains an ever-present concern. Sequential cfDNA testing during the post-treatment period aims to detect residual disease at a pre-clinical stage and flag a "molecular relapse" well before clinical relapse becomes otherwise evident (246). Many recent studies have described the use of liquid biopsy to identify human patients with molecular relapse many months before clinical or radiological relapse (246). Early identification of cancer relapse may help guide treatment and management decisions in canine patients as well, with the goal of improving clinical outcomes through earlier adjuvant therapeutic intervention.

DISCUSSION AND A LOOK TO THE FUTURE

Development of high-quality liquid biopsy tests for dogs comparable to those currently available for human testing has the potential to revolutionize the detection, characterization, and management of cancer in pets. However, the challenges involved in such development are significant. To observe cancer-related genomic variants at low concentrations in blood, the assay must interrogate a large number of cfDNA fragments, the majority of which will not be tumor-derived. This drives the need to focus on genomic regions of known clinical relevance for cancer. Pending results from large-scale discovery efforts across all major canine cancer types, these clinically-relevant genomic regions can only be identified from the—limited—available literature describing genomic alterations in canine cancers, or by homology mapping from the much more substantial human knowledge base. Identifying high-confidence orthologous regions in dogs for the top cancer-related regions in humans is non-trivial and will require significant effort and expertise.

After defining the genomic regions and features of interest, the process of developing a robust assay to detect low ctDNA signal presents a number of challenges, including: (1) optimizing best practices for the collection and isolation of cfDNA from canine plasma; (2) optimizing enrichment of targeted genomic regions; (3) maximizing the signal-to-background ratio of tumor-derived ctDNA vs. non-cancer cfDNA during data analysis; and (4) establishing a normal reference baseline, so that a signal indicative of cancer can be confidently segregated from random signals in patients without cancer who may have other clinical conditions that also could present with cancer-like signatures. For example, a well-documented challenge in the human liquid biopsy field is posed by the presence of clonal hematopoiesis of indeterminate potential (CHIP), also known as age related clonal hematopoiesis (ARCH) and defined as the accumulation of somatic mutations in hematopoietic stem cells that are clonally propagated to their progeny, a process that is associated with aging (265, 266). This phenomenon has not yet been documented in dogs, but it is reasonable to expect that it could also be a confounder in canine liquid biopsy, requiring sophisticated approaches to mitigate the impact on the false positive rate of such tests.

An analytically robust and clinically accurate liquid biopsy assay for use in canine patients will be highly complex, potentially generating billions of data points (base reads) for each test from NGS data; and will require extensive analytical and clinical validation to demonstrate reliability and clinical performance. Although the veterinary diagnostics space is not subject to the extensive regulations that apply to human diagnostics, it is imperative that any candidate liquid biopsy solution undergo validation at a level similar to that expected for human use, to maximize benefit for veterinary patients and clinicians. Clinical validation should be performed in adequately sized cohorts of canine subjects with a variety of cancers as well as presumably cancer-free canine subjects, to demonstrate both high sensitivity (few missed cases of cancer) and high specificity (few false positives). The results of such studies should be published in peer-reviewed journals so that the veterinary community is able to review the full corpus of supporting data before starting to use liquid biopsy tests in routine practice.

As liquid biopsy solutions become available in veterinary medicine, the clinical paradigm can be expected to shift in order to accommodate the inclusion of additional information afforded by the new modality; over time, veterinarians will develop an informed appreciation for the clinical utility of liquid biopsy in each care setting and incorporate this new tool judiciously into their clinical algorithms. Specifically, screening and aid in diagnosis will likely show the most immediate clinical utility for liquid biopsy by shifting diagnosis to an earlier timepoint when clinical outcomes are superior. In addition, the use of liquid biopsy for detection of minimal residual disease and for recurrence monitoring promises to provide an earlier opportunity to determine if a curative-intent intervention (i.e., surgery) was successful – and to inform the timely use of adjuvant treatments if the disease has not been eradicated. Finally, as more treatment options become available in veterinary medicine in the form of targeted therapies aimed at specific genomic alterations, the standard of care may evolve to include liquid biopsy as a routine pre-treatment selection step, and as a complement to imaging for evaluating response to treatment.

Liquid biopsy solutions based on cfDNA analysis are well-positioned to revolutionize certain aspects of cancer care in veterinary medicine by enabling safe, non-invasive testing at frequent intervals as dictated by the needs of each clinical case. However, liquid biopsy is not a panacea for all the challenges facing veterinary cancer management, and limitations exist. Certain tumors may not shed sufficient ctDNA into circulation to allow for confident detection and characterization of the disease by liquid biopsy; this can happen with smaller sized tumors in early disease, or with certain malignancies that tend to release lower levels of ctDNA into the bloodstream (such as tumors of the central nervous system) (161, 267). Also, the novelty of liquid biopsy means that extensive education will be required before its use can become widespread in the veterinary community, presenting a practical limitation to the speed and extent of adoption. Finally, the economics of a liquid biopsy-based approach to veterinary cancer diagnostics are yet unknown, which can present challenges – especially in the early years. In some use cases, such as aid in diagnosis when cancer is already suspected on clinical grounds, liquid biopsy may offer

obvious cost advantages over invasive diagnostic procedures; in other cases, the economic value of liquid biopsy may be less apparent, such as with annual screening of dogs who will never go on to develop cancer, or with testing for targeted treatment selection when the only available options are off-label human therapeutics that have not been directly shown to be efficacious in canine cancer. Pricing considerations will certainly play an important role in the overall economics of the emerging liquid biopsy paradigm; ongoing decreases in the cost of sequencing, rapid improvements in assay design and automation, volume-driven economies of scale, and competition among providers should all contribute to favorable developments in pricing, making liquid biopsy an increasingly affordable testing option for pet owners.

Tumor tissue analysis is likely to remain a core component of the standard of care, especially for cases where malignant masses can be easily sampled by biopsy or surgery. Traditional tissue histopathology can provide unique and highly valuable information, such as: establishing a definitive diagnosis of cancer; determining aggressiveness and prognosis; and selecting a treatment – this being especially useful in cases where genomic analysis of the tumor does not provide any obvious targeted treatment options. As experience with liquid biopsy builds within the veterinary community, this new testing method may prove to be a replacement for older methods in some cases but is more likely to establish itself as a complementary or backup method alongside existing approaches, expanding the overall ability of the clinician to provide the most personalized care to each patient.

The genomic revolution has already had a marked impact on cancer care for human patients and is poised to revolutionize veterinary medicine in a similar manner in the coming years. As genomics becomes a routine part of veterinary care, expansion into *multi-omic* liquid biopsy approaches is likely to follow, including epigenomics (methylation and histone-modification analyses), transcriptomics (gene expression, micro RNAs), proteomics (tumor markers, other peptides), metabolomics, fragmentomics, etc. (121, 214). When combined, these orthogonal datasets will enable a multidimensional view of the cancer in real-time, enabling delivery of the highest quality of care. The introduction of high-quality, clinically validated pan-cancer liquid biopsy tests into the realm of veterinary medicine has the potential to substantially impact every step along the clinical journey of a canine cancer patient, from early detection to recurrence monitoring.

Long known as "man's best friends," dogs live much shorter lives than humans, yet they form exceptionally close bonds with their human companions as well as with other pet dogs in the family; the loss of a pet dog often has a devastating emotional impact on the surviving family members, whether humans or other pet dogs (268–270). Cancer is by far the single most common cause of death in dogs, and having a pet companion that is fighting a losing battle with late-stage cancer is particularly difficult for families because of the financial strain of managing the disease in its final stages, and because the process is often drawn out over weeks or months and may involve considerable physical pain for the patient (271–273). The decision to euthanize a pet family member is one of the most difficult decisions a family will make. As veterinary medicine stands on the threshold of the new era of genomic medicine, novel tools – convenient, affordable, non-invasive, and widely available – will enable veterinarians to routinely screen for cancer and detect it early, when it can be cured; pursue rapid diagnosis of cancer as soon as the disease is suspected; and select targeted treatments and monitor for response and recurrence after a diagnosis has been made. These new tools will allow countless families to spend more time with their beloved pet family members and will further empower veterinarians to honor their professional oath to use their "scientific knowledge and skills for the benefit of society through the protection of animal health and welfare... [and] the prevention and relief of animal suffering" (274).

Humans have benefited extensively from medical advances that were first trialed in our canine sidekicks. By implementing lessons learned from recent genomic advances in cancer care for humans, we can now raise the level of cancer care for our canine companions as well. It is fitting to consider that widespread adoption of liquid biopsies in veterinary medicine may represent an upcoming historic opportunity to repay our "best friends" for their many prior contributions to our well-being.

AUTHOR CONTRIBUTIONS

JC and DG contributed to conception and design of the manuscript and co-wrote the first draft. All authors contributed to the manuscript and provided critical revisions, read, and approved the submitted version.

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REFERENCES

1. Pang LY, Argyle DJ. Using naturally occurring tumours in dogs and cats to study telomerase and cancer stem cell biology. *Biochim Biophys Acta.* (2009) 1792:380–91. doi: 10.1016/j.bbadis.2009.02.010
2. Pang LY, Argyle DJ. Veterinary oncology: biology, big data and precision medicine. *Vet J.* (2016) 213:38–45. doi: 10.1016/j.tvjl.2016.03.009
3. Fleming JM, Creevy KE, Promislow DEL. Mortality in North American dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med.* (2011) 25:187–98. doi: 10.1111/j.1939-1676.2011.0695.x
4. LeBlanc AK, Mazcko CN. Improving human cancer therapy through the evaluation of pet dogs. *Nat Rev Cancer.* (2020) 20:727–42. doi: 10.1038/s41568-020-0297-3

5. Baioni E, Scanziani E, Vincenti MC, Leschiera M, Bozzetta E, Pezzolato M, et al. Estimating canine cancer incidence: findings from a population-based tumour registry in northwestern Italy. *BMC Vet Res.* (2017) 13:203. doi: 10.1186/s12917-017-1126-0
6. Dobson JM. Breed-predispositions to cancer in pedigree dogs. *Isrn Vet Sci.* (2013) 2013:1–23. doi: 10.1155/2013/941275
7. Pinello KC, Niza-Ribeiro J, Fonseca L, Matos AJ. Incidence, characteristics and geographical distributions of canine and human non-Hodgkin's lymphoma in the Porto region (North West Portugal). *Vet J.* (2019) 245:70–6. doi: 10.1016/j.tvjl.2019.01.003
8. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* (2020) 70:7–30. doi: 10.3322/caac.21590
9. Schiffman JD, Breen M. Comparative oncology: what dogs and other species can teach us about humans with cancer. *Philos Trans R Soc B Biol Sci.* (2015) 370:20140231. doi: 10.1098/rstb.2014.0231
10. Klingemann H. Immunotherapy for dogs: running behind humans. *Front Immunol.* (2018) 9:133. doi: 10.3389/fimmu.2018.00133
11. Cancer Statistics. *National Cancer Institute*. NCI (2020) Available online at: https://www.cancer.gov/about-cancer/understanding/statistics (accessed October 5, 2020)
12. Williams LE, Packer RA. Association between lymph node size and metastasis in dogs with oral malignant melanoma: 100 cases (1987-2001). *J Am Vet Med Assoc.* (2003) 222:1234–6. doi: 10.2460/javma.2003.222.1234
13. Biller B, Berg J, Garrett L, Ruslander D, Wearing R, Abbott B, et al. 2016 AAHA oncology guidelines for dogs and cats*. *J Am Anim Hosp Assoc.* (2016) 52:181–204. doi: 10.5326/jaaha-ms-6570
14. Polton GA, Brearley MJ. Clinical stage, therapy, and prognosis in canine anal sac gland carcinoma. *J Vet Intern Med.* (2007) 21:274–80. doi: 10.1111/j.1939-1676.2007.tb02960.x
15. Flory AB, Rassnick KM, Stokol T, Scrivani PV, Erb HN. Stage migration in dogs with lymphoma. *J Vet Intern Med.* (2007) 21:1041–7. doi: 10.1111/j.1939-1676.2007.tb03062.x
16. Wendelburg KM, Price LL, Burgess KE, Lyons JA, Lew FH, Berg J. Survival time of dogs with splenic hemangiosarcoma treated by splenectomy with or without adjuvant chemotherapy: 208 cases (2001-2012). *J Am Vet Med Assoc.* (2015) 247:393–403. doi: 10.2460/javma.247.4.393
17. Nakano Y, Matsushima M, Nakamori A, Hiroma J, Matsuo E, Wakabayashi H, et al. Depression and anxiety in pet owners after a diagnosis of cancer in their pets: a cross-sectional study in Japan. *BMJ Open.* (2019) 9:e024512. doi: 10.1136/bmjopen-2018-024512
18. Spitznagel MB, Jacobson DM, Cox MD, Carlson MD. Caregiver burden in owners of a sick companion animal: a cross-sectional observational study. *Vet Rec.* (2017) 181:321. doi: 10.1136/vr.104295
19. Bowen J, García E, Darder P, Argüelles J, Fatjó J. The effects of the Spanish COVID-19 lockdown on people, their pets and the human-animal bond. *J Vet Behav.* (2020) 40:75–91. doi: 10.1016/j.jveb.2020.05.013
20. Rebhun RB, Kent MS, Borrofka SAEB, Frazier S, Skorupski K, Rodriguez CO. CHOP chemotherapy for the treatment of canine multicentric T-cell lymphoma. *Vet Comp Oncol.* (2011) 9:38–44. doi: 10.1111/j.1476-5829.2010.00230.x
21. Shaevitz MH, Tullius JA, Callahan RT, Fulkerson CM, Spitznagel MB. Early caregiver burden in owners of pets with suspected cancer: owner psychosocial outcomes, communication behavior, and treatment factors. *J Vet Intern Med.* (2020) 34:2636–44. doi: 10.1111/jvim.15905
22. The Cancer Genome Atlas (TCGA) Timeline & Milestones. *National Cancer Institute*. NCI. Available online at: https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga/history/timeline (accessed October 5, 2020)
23. National Institutes of Health, National Cancer Institute. *TCGA Research Network Publications*. NCI (2020) Available online at: https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga/publications (accessed October 5, 2020)
24. International Cancer Genome Consortium (ICGC). ICGC (2020). Available online at: https://icgc.org/ (accessed November 20, 2020)
25. National Institutes of Health (NIH). *The Promise of Precision Medicine*. NIH (2020). Available online at: https://www.nih.gov/about-nih/what-we-do/nih-turning-discovery-into-health/promise-precision-medicine (accessed November 20, 2020)
26. Lennon AM, Buchanan AH, Kinde I, Warren A, Honushefsky A, Cohain AT, et al. Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. *Science.* (2020) 369:eabb9601. doi: 10.1126/science.abb9601
27. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Consortium C, et al. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann Oncol.* (2020) 31:745–59. doi: 10.1016/j.annonc.2020.02.011
28. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science.* (2018) 359:eaar3247. doi: 10.1126/science.aar3247
29. Plagnol V, Woodhouse S, Howarth K, Lensing S, Smith M, Epstein M, et al. Analytical validation of a next generation sequencing liquid biopsy assay for high sensitivity broad molecular profiling. *PLoS ONE.* (2018) 13:e0193802. doi: 10.1371/journal.pone.0193802
30. Gale D, Lawson ARJ, Howarth K, Madi M, Durham B, Smalley S, et al. Development of a highly sensitive liquid biopsy platform to detect clinically-relevant cancer mutations at low allele fractions in cell-free DNA. *PLoS ONE.* (2018) 13:e0194630. doi: 10.1371/journal.pone.0194630
31. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature.* (2005) 438:803–19. doi: 10.1038/nature04338
32. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature.* (2001) 409:860. doi: 10.1038/35057062
33. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science.* (2001) 291:1304–51. doi: 10.1126/science.1058040
34. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. *Nature.* (2004) 431:931. doi: 10.1038/nature03001
35. Koskinen MT. Individual assignment using microsatellite DNA reveals unambiguous breed identification in the domestic dog. *Anim Genet.* (2003) 34:297–301. doi: 10.1046/j.1365-2052.2003.01005.x
36. Parker HG, Kim LV, Sutter NB, Carlson S, Lorentzen TD, Malek TB, et al. Genetic structure of the purebred domestic dog. *Science.* (2004) 304:1160–4. doi: 10.1126/science.1097406
37. Grall A, Guaguère E, Planchais S, Grond S, Bourrat E, Hausser I, et al. PNPLA1 mutations cause autosomal recessive congenital ichthyosis in golden retriever dogs and humans. *Nat Genet.* (2012) 44:140–7. doi: 10.1038/ng.1056
38. Plassais J, Guaguère E, Lagoutte L, Guillory A-S, Citres CD de, Degorce-Rubiales F, et al. A spontaneous KRT16 mutation in a dog breed: a model for human focal non-epidermolytic palmoplantar keratoderma (FNEPPK). *J Invest Dermatol.* (2015) 135:1187–90. doi: 10.1038/jid.2014.526
39. Donner J, Anderson H, Davison S, Hughes AM, Bouirmane J, Lindqvist J, et al. Frequency and distribution of 152 genetic disease variants in over 100,000 mixed breed and purebred dogs. *PLoS Genet.* (2018) 14:e1007361. doi: 10.1371/journal.pgen.1007361
40. Nicholas FW. Online Mendelian Inheritance in Animals (OMIA): a comparative knowledgebase of genetic disorders and other familial traits in non-laboratory animals. *Nucleic Acids Res.* (2003) 31:275–7. doi: 10.1093/nar/gkg074
41. Mochizuki H, Kennedy K, Shapiro SG, Breen M. BRAF mutations in canine cancers. *PLoS ONE.* (2015) 10:e0129534. doi: 10.1371/journal.pone.0129534
42. London CA, Galli SJ, Yuuki T, Hu Z-Q, Helfand SC, Geissler EN. Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. *Exp Hematol.* (1999) 27:689–97. doi: 10.1016/s0301-472x(98)00075-7
43. London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, et al. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clin Cancer Res.* (2009) 15:3856–65. doi: 10.1158/1078-0432.ccr-08-1860
44. Kurita S, Miyamoto R, Tani H, Kobayashi M, Sasaki T, Tamura K, et al. Genetic alterations of KIT during clonal expansion and subsequent

acquisition of resistance to toceranib in a canine mast cell tumor cell line. *J Vet Pharmacol Ther.* (2019) 42:673–81. doi: 10.1111/jvp.12816
45. Moses L, Niemi S, Karlsson E. Pet genomics medicine runs wild. *Nature.* (2018) 559:470–2. doi: 10.1038/d41586-018-05771-0
46. American Association of Veterinary Laboratory Diagnosticians (AAVLD). *AAVLD Accreditation Program*. AAVLD (2017). Available online at: https://www.aavld.org/accreditation-explained (accessed January 29, 2021)
47. American Association of Veterinary Laboratory Diagnosticians (AAVLD). *Accreditation Requirements*. AAVLD (2018). Available online at: https://www.aavld.org/accreditation-requirements-page (accessed October 5, 2020)
48. College of American Pathologists (CAP). *Accreditation*. CAP. Available online at: https://www.cap.org/laboratory-improvement/accreditation (accessed October 5, 2020)
49. Centers for Medicare & Medicaid Services (CMS). *Clinical Laboratory Improvement Amendments (CLIA)*. CMS (2020). Available online at: https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index (accessed October 5, 2020)
50. US Food & Drug Administration (FDA). *List of Cleared or Approved Companion Diagnostic Devices (In Vitro and Imaging Tools)*. FDA (2020). Available online at: https://www.fda.gov/medical-devices/vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-vitro-and-imaging-tools (accessed January 29, 2021)
51. Cawley J, Khanna C. The challenge of cognitive dissonance in the delivery of precision medicine in veterinary oncology. *Arch Cancer Biol Ther.* (2020) 1:37–41. doi: 10.33696/cancerbiology.1.008
52. Stetson D, Ahmed A, Xu X, Nuttall BRB, Lubinski TJ, Johnson JH, et al. Orthogonal comparison of four plasma NGS tests with tumor suggests technical factors are a major source of assay discordance. *Jco Precis Oncol.* (2019) 3:1–9. doi: 10.1200/po.18.00191
53. Tsui DWY, Blumenthal GM, Philip R, Barrett JC, Montagut C, Bramlett K, et al. Development, validation, and regulatory considerations for a liquid biopsy test. *Clin Chem.* (2020) 66:408–14. doi: 10.1093/clinchem/hvaa010
54. Morley AA, Turner DR. The contribution of exogenous and endogenous mutagens to *in vivo* mutations. *Mutat Res Fundam Mol Mech Mutagen.* (1999) 428:11–5. doi: 10.1016/s1383-5742(99)00026-5
55. Tomasetti C, Vogelstein B. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science.* (2015) 347:78–81. doi: 10.1126/science.1260825
56. Wu S, Powers S, Zhu W, Hannun YA. Substantial contribution of extrinsic risk factors to cancer development. *Nature.* (2016) 529:43. doi: 10.1038/nature16166
57. Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science.* (2017) 355:1330–4. doi: 10.1126/science.aaf9011
58. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. *Science.* (2013) 339:1546–58. doi: 10.1126/science.1235122
59. Fiala C, Diamandis EP. Utility of circulating tumor DNA in cancer diagnostics with emphasis on early detection. *BMC Med.* (2018) 16:166. doi: 10.1186/s12916-018-1157-9
60. DelMonte U. Does the cell number 10 9 still really fit one gram of tumor tissue? *Cell Cycle.* (2009) 8:505–6. doi: 10.4161/cc.8.3.7608
61. The Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, et al. The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet.* (2013) 45:1113. doi: 10.1038/ng.2764
62. Campbell PJ, Getz G, Korbel JO, Stuart JM, Jennings JL, Stein LD, et al. Pan-cancer analysis of whole genomes. *Nature.* (2020) 578:82–93. doi: 10.1038/s41586-020-1969-6
63. Bailey MH, Tokheim C, Porta-Pardo E, Sengupta S, Bertrand D, Weerasinghe A, et al. Comprehensive characterization of cancer driver genes and mutations. *Cell.* (2018) 173:371–85.e18. doi: 10.1016/j.cell.2018.02.060
64. Yates AD, Achuthan P, Akanni W, Allen J, Allen J, Alvarez-Jarreta J, et al. Ensembl 2020. *Nucleic Acids Res.* (2019) 48:D682–8. doi: 10.1093/nar/gkz966
65. Sondka Z, Bamford S, Cole CG, Ward SA, Dunham I, Forbes SA. The COSMIC Cancer Gene Census: describing genetic dysfunction across all human cancers. *Nat Rev Cancer.* (2018) 18:696–705. doi: 10.1038/s41568-018-0060-1
66. Ensembl. *Human (GRCh38.p13)*. Ensembl (2020). Available online at: https://uswest.ensembl.org/Homo_sapiens/Info/Annotation (accessed October 5, 2020)
67. Catalogue of Somatic Mutations in Cancer (COSMIC). *COSMIC Census.* COSMIC. Available online at: https://cancer.sanger.ac.uk/census (accessed October 5, 2020)
68. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
69. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* (2000) 100:57–70. doi: 10.1016/s0092-8674(00)81683-9
70. Rivera P, Melin M, Biagi T, Fall T, Häggström J, Lindblad-Toh K, et al. Mammary tumor development in dogs is associated with BRCA1 and BRCA2. *Cancer Res.* (2009) 69:8770–4. doi: 10.1158/0008-5472.can-09-1725
71. Forbes C, Fayter D, de Kock S, Quek RG. A systematic review of international guidelines and recommendations for the genetic screening, diagnosis, genetic counseling, and treatment of BRCA-mutated breast cancer. *Cancer Manage Res.* (2019) 11:2321–37. doi: 10.2147/cmar.s189627
72. Jones S, Chen W, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, et al. Comparative lesion sequencing provides insights into tumor evolution. *Proc Natl Acad Sci USA.* (2008) 105:4283–8. doi: 10.1073/pnas.0712345105
73. Chenam A, Lau C. Genitourinary cancers. *Canc Treat.* (2018) 175:105–26. doi: 10.1007/978-3-319-93339-9_5
74. Zigras T, Lennox G, Willows K, Covens A. Early cervical cancer: current dilemmas of staging and surgery. *Curr Oncol Rep.* (2017) 19:51. doi: 10.1007/s11912-017-0614-5
75. Horta RS, Lavalle GE, Monteiro LN, Souza MCC, Cassali GD, Araújo RB. Assessment of canine mast cell tumor mortality risk based on clinical, histologic, immunohistochemical, and molecular features. *Vet Pathol.* (2018) 55:212–23. doi: 10.1177/0300985817747325
76. Milovancev M, Tuohy JL, Townsend KL, Irvin VL. Influence of surgical margin completeness on risk of local tumour recurrence in canine cutaneous and subcutaneous soft tissue sarcoma: a systematic review and meta-analysis. *Vet Comp Oncol.* (2019) 17:354–64. doi: 10.1111/vco.12479
77. Stephens PJ, Tarpey PS, Davies H, Loo PV, Greenman C, Wedge DC, et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature.* (2012) 486:400–4. doi: 10.1038/nature11017
78. Luu L-J, Price TJ. BRAF mutation and its importance in colorectal cancer. *Advances in the Molecular Understanding of Colorectal Cancer*. Intech Open. (2019) doi: 10.5772/intechopen.82571
79. Mochizuki H, Breen M. Comparative aspects of BRAF mutations in canine cancers. *Vet Sci.* (2015) 2:231–45. doi: 10.3390/vetsci2030231
80. Sclafani F, Gullo G, Sheahan K, Crown J. BRAF mutations in melanoma and colorectal cancer: a single oncogenic mutation with different tumour phenotypes and clinical implications. *Crit Rev Oncol Hemat.* (2013) 87:55–68. doi: 10.1016/j.critrevonc.2012.11.003
81. Shao C, Li G, Huang L, Pruitt S, Castellanos E, Frampton G, et al. Prevalence of high tumor mutational burden and association with survival in patients with less common solid tumors. *JAMA Netw Open.* (2020) 3:e2025109. doi: 10.1001/jamanetworkopen.2020.25109
82. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med.* (2017) 9:34. doi: 10.1186/s13073-017-0424-2
83. Gardner HL, Sivaprakasam K, Briones N, Zismann V, Perdigones N, Drenner K, et al. Canine osteosarcoma genome sequencing identifies recurrent mutations in DMD and the histone methyltransferase gene SETD2. *Commun Biol.* (2019) 2:266. doi: 10.1038/s42003-019-0487-2
84. Lorch G, Sivaprakasam K, Zismann V, Perdigones N, Contente-Cuomo T, Nazareno A, et al. Identification of recurrent activating HER2 mutations in primary canine pulmonary adenocarcinoma. *Clin Cancer Res.* (2019) 25:5866–77. doi: 10.1158/1078-0432.ccr-19-1145
85. Wang G, Wu M, Maloneyhuss MA, Wojcik J, Durham AC, Mason NJ, et al. Actionable mutations in canine hemangiosarcoma. *PLoS ONE.* (2017) 12:e0188667. doi: 10.1371/journal.pone.0188667
86. Megquier K, Turner-Maier J, Swofford R, Kim J-H, Sarver AL, Wang C, et al. Comparative genomics reveals shared mutational landscape in canine hemangiosarcoma and human angiosarcoma. *Mol Cancer Res.* (2019) 17:2410–21. doi: 10.1158/1541-7786.mcr-19-0221

87. Büttner R, Longshore JW, López-Ríos F, Merkelbach-Bruse S, Normanno N, Rouleau E, et al. Implementing TMB measurement in clinical practice: considerations on assay requirements. *Esmo Open.* (2019) 4:e000442. doi: 10.1136/esmoopen-2018-000442
88. Klempner SJ, Fabrizio D, Bane S, Reinhart M, Peoples T, Ali SM, et al. Tumor mutational burden as a predictive biomarker for response to immune checkpoint inhibitors: a review of current evidence. *Oncology.* (2020) 25:e147–59. doi: 10.1634/theoncologist.2019-0244
89. Zook JM, Chapman B, Wang J, Mittelman D, Hofmann O, Hide W, et al. Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls. *Nat Biotechnol.* (2014) 32:246–51. doi: 10.1038/nbt.2835
90. Dunnen JT, Dalgleish R, Maglott DR, Hart RK, Greenblatt MS, McGowan-Jordan J, et al. HGVS recommendations for the description of sequence variants: 2016 update. *Hum Mutat.* (2016) 37:564–9. doi: 10.1002/humu.22981
91. Wagner J, Olson ND, Harris L, Khan Z, Farek J, Mahmoud M, et al. Benchmarking challenging small variants with linked and long reads. *BioRxiv.* (2020)2020.07.24.212712. doi: 10.1101/2020.07.24.212712
92. Li Y, Roberts ND, Wala JA, Shapira O, Schumacher SE, Kumar K, et al. Patterns of somatic structural variation in human cancer genomes. *Nature.* (2020) 578:112–21. doi: 10.1038/s41586-019-1913-9
93. Ciriello G, Miller ML, Aksoy BA, Senbabaoglu Y, Schultz N, Sander C. Emerging landscape of oncogenic signatures across human cancers. *Nat Genet.* (2013) 45:1127–33. doi: 10.1038/ng.2762
94. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature.* (2013) 500:415. doi: 10.1038/nature12477
95. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* (2012) 366:883–92. doi: 10.1056/nejmoa1113205
96. McQuerry JA, Chang JT, Bowtell DDL, Cohen A, Bild AH. Mechanisms and clinical implications of tumor heterogeneity and convergence on recurrent phenotypes. *J Mol Med.* (2017) 95:1167–78. doi: 10.1007/s00109-017-1587-4
97. McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell.* (2017) 168:613–28. doi: 10.1016/j.cell.2017.01.018
98. Dzobo K, Senthebane DA, Thomford NE, Rowe A, Dandara C, Parker MI. Not everyone fits the mold: intratumor and intertumor heterogeneity and innovative cancer drug design and development. *Omics J Integr Biol.* (2018) 22:17–34. doi: 10.1089/omi.2017.0174
99. Crowley E, Nicolantonio FD, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol.* (2013) 10:472. doi: 10.1038/nrclinonc.2013.110
100. Murtaza M, Dawson S-J, Pogrebniak K, Rueda OM, Provenzano E, Grant J, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun.* (2015) 6:8760. doi: 10.1038/ncomms9760
101. Diaz LA, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature.* (2012) 486:537. doi: 10.1038/nature11219
102. Waclaw B, Bozic I, Pittman ME, Hruban RH, Vogelstein B, Nowak MA. A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. *Nature.* (2015) 525:261–4. doi: 10.1038/nature14971
103. Tubiana M. Tumor cell proliferation kinetics and tumor growth rate. *Acta Oncol.* (2009) 28:113–121. doi: 10.3109/02841868909111193
104. Nakashima K, Uematsu T, Takahashi K, Nishimura S, Tadokoro Y, Hayashi T, et al. Does breast cancer growth rate really depend on tumor subtype? Measurement of tumor doubling time using serial ultrasonography between diagnosis and surgery. *Breast Cancer.* (2019) 26:206–14. doi: 10.1007/s12282-018-0914-0
105. Siu AL, Force USPST. Screening for breast cancer: U.S. preventive services task force recommendation statement. *Ann Intern Med.* (2016) 164:279. doi: 10.7326/m15-2886
106. American Cancer Society (ACS). *American Cancer Society Recommendations for the Early Detection of Breast Cancer*. ACS (2020). Available online at: https://www.cancer.org/cancer/breast-cancer/screening-tests-and-early-detection/american-cancer-society-recommendations-for-the-early-detection-of-breast-cancer.html (accessed November 20, 2020)
107. US Preventive Services Task Force, Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW, et al. Screening for colorectal cancer: US preventive services task force recommendation statement. *JAMA.* (2016) 315:2564. doi: 10.1001/jama.2016.5989
108. Melnikow J, Henderson JT, Burda BU, Senger CA, Durbin S, Weyrich MS. Screening for cervical cancer with high-risk human papillomavirus testing: updated evidence report and systematic review for the US preventive services task force. *JAMA.* (2018) 320:687–705. doi: 10.1001/jama.2018.10400
109. Zorzi M, Hassan C, Capodaglio G, Fedato C, Montaguti A, Turrin A, et al. Long-term performance of colorectal cancer screening programmes based on the faecal immunochemical test. *Gut.* (2018) 67:2124. doi: 10.1136/gutjnl-2017-314753
110. Perry RE, Weller RE, Buschbom RL, Dagle GE, Park JF. Radiographically determined growth dynamics of primary lung tumors induced in dogs by inhalation of plutonium. *Am J Vet Res.* (1992) 53:1740–3.
111. Aoki T, Nakata H, Watanabe H, Nakamura K, Kasai T, Hashimoto H, et al. Evolution of peripheral lung adenocarcinomas: CT findings correlated with histology and tumor doubling time. *Am J Roentgenol.* (2000) 174:763–68. doi: 10.2214/ajr.174.3.1740763
112. Paoloni MC, Khanna C. Comparative oncology today. *Vet Clin North Am Small Anim Pract.* (2007) 37:1023–32. doi: 10.1016/j.cvsm.2007.08.003
113. Rowell JL, McCarthy DO, Alvarez CE. Dog models of naturally occurring cancer. *Trends Mol Med.* (2011) 17:380–8. doi: 10.1016/j.molmed.2011.02.004
114. LeBlanc AK, Mazcko CN, Khanna C. Defining the value of a comparative approach to cancer drug development. *Am Assoc Cancer Res.* (2016) 22:2133–8. doi: 10.1158/1078-0432.ccr-15-2347
115. Sakthikumar S. *Characterizing the Spectrum of Somatic Alterations in Canine and Human Cancers.* Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1624. Uppsala: Acta Universitatis Upsaliensis (2020). p. 64.
116. Alsaihati BA, Ho K-L, Watson J, Feng Y, Wang T, Zhao S. Canine tumor mutation rate is positively correlated with TP53 mutation across cancer types and breeds. *Biorxiv.* (2020)2020.07.15.205286. doi: 10.1101/2020.07.15.205286
117. LeBlanc AK, Breen M, Choyke P, Dewhirst M, Fan TM, Gustafson DL, et al. Perspectives from man's best friend: National Academy of Medicine's Workshop on Comparative Oncology. *Sci Transl Med.* (2016) 8:324ps5. doi: 10.1126/scitranslmed.aaf0746
118. Ensembl. *Dog Assembly and Gene Annotation*. Ensembl (2020). Available online at: https://uswest.ensembl.org/Canis_lupus_familiaris/Info/Annotation (accessed October 5, 2020)
119. McDonald JT, Kritharis A, Beheshti A, Pilichowska M, Burgess K, Ricks-Santi L, et al. Comparative oncology DNA sequencing of canine T cell lymphoma via human hotspot panel. *Oncotarget.* (2018) 9:22693–702. doi: 10.18632/oncotarget.25209
120. Villarnovo D, McCleary-Wheeler AL, Richards KL. Barking up the right tree. *Curr Opin Hematol.* (2017) 24:359–66. doi: 10.1097/moh.0000000000000357
121. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet.* (2019) 20:71–88. doi: 10.1038/s41576-018-0071-5
122. Ehrhart N, Culp W. Chapter 1: Principles of surgical oncology. In: Kudnig S, S9guin B, editors. *Veterinary Surgical Oncology*. Wiley Blackwell (2012). Retrieved from: www.mazon.com
123. Friedrichs K, Young K. Diagnostic cytopathology in clinical oncology. In: Vail D, Thamm D, Liptak J, editors. *Withrow & MacEwen's Small Animal Clinical Oncology*. 6th ed. St. Louis, MO: Elsevier (2020). p. 126–45.
124. Sharkey LC, Dial SM, Matz ME. Maximizing the diagnostic value of cytology in small animal practice. *Vet Clin North Am Small Animal Pract.* (2007) 37:351–72. doi: 10.1016/j.cvsm.2006.11.004
125. DeBerry JD, Norris CR, Samii VF, Griffey SM, Almy FS. Correlation between fine-needle aspiration cytopathology and histopathology of the lung in dogs and cats. *J Am Anim Hosp Assoc.* (2014) 38:327–36. doi: 10.5326/0380327
126. Ehrhart N. Biopsy and sentinel lymph node mapping principles. In: Vail D, Thamm D, Liptak J, editors. *Withrow & MacEwen's Small Animal Clinical Oncology*. 6th ed. St. Louis, MO: Elsevier (2020). p. 164–74.

127. Simon MA. Biopsy of musculoskeletal tumors. *J Bone Jt Surg.* (1982) 64:1253–57. doi: 10.2106/00004623-198264080-00020
128. Guilford W. Gastrointestinal endoscopy. In: Guilford W, Center S, Strombeck D, editors. *Strombeck's Small Animal Gastroenterology*. 3rd ed. Philadelphia, PA: Saunders. (1995). p. 114–29.
129. Willard M, Schuz K, Hayashi K. Chapter 14: Principles of minimally invasive surgery and imaging of the surgical patient. In: Fossum T, Duprey LP, editors. *Small Animal Surgery*. 5th ed. Elsevier. Retrieved from: www.Amazon.com
130. Pratschke KM, Ryan J, McAlinden A, McLauchlan G. Pancreatic surgical biopsy in 24 dogs and 19 cats: postoperative complications and clinical relevance of histological findings. *J Small Anim Pract.* (2015) 56:60–6. doi: 10.1111/jsap.12262
131. de Rycke LMJH, Bree HJJ van, Simoens PJM. Ultrasound-guided tissue-core biopsy of liver, spleen and kidney in normal dogs. *Vet Radiol Ultrasound.* (1999) 40:294–9. doi: 10.1111/j.1740-8261.1999.tb00364.x
132. Bagley RS. Spinal neoplasms in small animals. *Vet Clin North Am Small Animal Pract.* (2010) 40:915–27. doi: 10.1016/j.cvsm.2010.05.010
133. Shales CJ, Warren J, Anderson DM, Baines SJ, White RAS. Complications following full-thickness small intestinal biopsy in 66 dogs: a retrospective study. *J Small Anim Pract.* (2005) 46:317–21. doi: 10.1111/j.1748-5827.2005.tb00326.x
134. Weisse C, Soares N, Beal MW, Steffey MA, Drobatz KJ, Henry CJ. Survival times in dogs with right atrial hemangiosarcoma treated by means of surgical resection with or without adjuvant chemotherapy: 23 cases (1986-2000). *J Am Vet Med Assoc.* (2005) 226:575–9. doi: 10.2460/javma.2005.226.575
135. Mamelak AN, Owen TJ, Bruyette D. Transsphenoidal surgery using a high definition video telescope for pituitary adenomas in dogs with pituitary dependent hypercortisolism: methods and results. *Vet Surg.* (2014) 43:369–79. doi: 10.1111/j.1532-950x.2014.12146.x
136. Rijn SJ van, Galac S, Tryfonidou MA, Hesselink JW, Penning LC, Kooistra HS, et al. The influence of pituitary size on outcome after transsphenoidal hypophysectomy in a large cohort of dogs with pituitary-dependent hypercortisolism. *J Vet Intern Med.* (2016) 30:989–95. doi: 10.1111/jvim.14367
137. Harris BJ, Lourenço BN, Dobson JM, Herrtage ME. Diagnostic accuracy of three biopsy techniques in 117 dogs with intra-nasal neoplasia. *J Small Anim Pract.* (2014) 55:219–24. doi: 10.1111/jsap.12187
138. Diaz LA, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol.* (2014) 32:579–86. doi: 10.1200/jco.2012.45.2011
139. Amirouchene-Angelozzi N, Swanton C, Bardelli A. Tumor evolution as a therapeutic target. *Cancer Discov.* (2017) 7:805–17. doi: 10.1158/2159-8290.cd-17-0343
140. Nicholson BD, Shinkins B, Pathiraja I, Roberts NW, James TJ, Mallett S, et al. Blood CEA levels for detecting recurrent colorectal cancer. *Cochrane Database Syst Rev.* (2015) 12:CD011134. doi: 10.1002/14651858.cd011134.pub2
141. Fenton JJ, Weyrich MS, Durbin S, Liu Y, Bang H, Melnikow J. Prostate-specific antigen–based screening for prostate cancer: evidence report and systematic review for the US preventive services task force. *JAMA.* (2018) 319:1914–31. doi: 10.1001/jama.2018.3712
142. Henderson J, Webber E, Sawaya G. *Evidence Synthesis Number 157 Screening for Ovarian Cancer: An Updated Evidence Review for the U.S. Preventive Services Task Force*. USPSTF (2018).
143. Zhang J, Chen G, Zhang P, Zhang J, Li X, Gan D, et al. The threshold of alpha-fetoprotein (AFP) for the diagnosis of hepatocellular carcinoma: a systematic review and meta-analysis. *PLoS ONE.* (2020) 15:e0228857. doi: 10.1371/journal.pone.0228857
144. Bauden M, Pamart D, Ansari D, Herzog M, Eccleston M, Micallef J, et al. Circulating nucleosomes as epigenetic biomarkers in pancreatic cancer. *Clin Epigenetics.* (2015) 7:106. doi: 10.1186/s13148-015-0139-4
145. Rahier J-F, Druez A, Faugeras L, Martinet J-P, Géhénot M, Josseaux E, et al. Circulating nucleosomes as new blood-based biomarkers for detection of colorectal cancer. *Clin Epigenetics.* (2017) 9:53. doi: 10.1186/s13148-017-0351-5
146. Fiala C, Kulasingam V, Diamandis EP. Circulating tumor DNA for early cancer detection. *J Appl Lab Med.* (2018) 3:300–13. doi: 10.1373/jalm.2018.026393
147. Lawson C, Smith SA, O'Brien M, McMichael M. Neutrophil extracellular traps in plasma from dogs with immune-mediated hemolytic anemia. *J Vet Intern Med.* (2018) 32:128–34. doi: 10.1111/jvim.14881
148. Letendre J-A, Goggs R. Concentrations of plasma nucleosomes but not cell-free DNA are prognostic in dogs following trauma. *Front Vet Sci.* (2018) 5:180. doi: 10.3389/fvets.2018.00180
149. Letendre J-A, Goggs R. Determining prognosis in canine sepsis by bedside measurement of cell-free DNA and nucleosomes: cell-free DNA and nucleosomes in canine sepsis. *J Vet Emerg Crit Care.* (2018) 28:503–11. doi: 10.1111/vec.12773
150. Goggs R. Effect of sample type on plasma concentrations of cell-free DNA and nucleosomes in dogs. *Vet Rec Open.* (2019) 6:e000357. doi: 10.1136/vetreco-2019-000357
151. Martiny P, Goggs R. Biomarker guided diagnosis of septic peritonitis in dogs. *Front Vet Sci.* (2019) 6:208. doi: 10.3389/fvets.2019.00208
152. McAnena P, Brown J, Kerin M. Circulating nucleosomes and nucleosome modifications as biomarkers in cancer. *Cancers.* (2017) 9:5. doi: 10.3390/cancers9010005
153. Lechowski R, Jagielski D, Hoffmann-Jagielska M, Zmudzka M, Winnicka A. Alpha-fetoprotein in Canine Multicentric Lymphoma. *Vet Res Commun.* (2002) 26:285–96. doi: 10.1023/a:1016086508286
154. Selting KA, Ringold R, Husbands B, Pithua PO. Thymidine kinase type 1 and C-reactive protein concentrations in dogs with spontaneously occurring cancer. *J Vet Intern Med.* (2016) 30:1159–66. doi: 10.1111/jvim.13954
155. Grobman M, Outi H, Rindt H, Reinero C. Serum thymidine kinase 1, canine-C-reactive protein, haptoglobin, and vitamin D concentrations in dogs with immune-mediated hemolytic anemia, thrombocytopenia, and polyarthropathy. *J Vet Intern Med.* (2017) 31:1430–40. doi: 10.1111/jvim.14787
156. Smrkolj T, Gubina B, Bizjak J, Kumer K, Fabjan T, Osredkar J. Tumor marker α-fetoprotein receptor does not discriminate between benign prostatic disease and prostate cancer. *Adv Clin Exp Med.* (2017) 26:1085–90. doi: 10.17219/acem/65432
157. Bidard F-C, Proudhon C, Pierga J-Y. Circulating tumor cells in breast cancer. *Mol Oncol.* (2016) 10:418–30. doi: 10.1016/j.molonc.2016.01.001
158. Punnoose EA, Atwal S, Liu W, Raja R, Fine BM, Hughes BGM, et al. Evaluation of circulating tumor cells and circulating tumor DNA in non–small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. *Clin Cancer Res.* (2012) 18:2391–401. doi: 10.1158/1078-0432.ccr-11-3148
159. Beinse G, Berger F, Cottu P, Dujaric M -E., Kriegel I, Guilhaume M -N., et al. Circulating tumor cell count and thrombosis in metastatic breast cancer. *J Thromb Haemost.* (2017) 15:1981–8. doi: 10.1111/jth.13792
160. Politaki E, Agelaki S, Apostolaki S, Hatzidaki D, Strati A, Koinis F, et al. A comparison of three methods for the detection of circulating tumor cells in patients with early and metastatic breast cancer. *Cell Physiol Biochem.* (2017) 44:594–606. doi: 10.1159/000485115
161. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med.* (2014) 6:224ra24. doi: 10.1126/scitranslmed.3007094
162. Menarini Silicon Biosystems Inc. *CellSearch Circulating Tumor Cell Kit (Epithelial) [Package Insert]*. Available online at: https://documents.cellsearchctc.com/pdf/e631600006/e631600006_EN.pdf (accessed January 2, 2021)
163. Marconato L, Facchinetti A, Zanardello C, Rossi E, Vidotto R, Capello K, et al. Detection and prognostic relevance of circulating and disseminated tumour cell in dogs with metastatic mammary carcinoma: a pilot study. *Cancers.* (2019) 11:163. doi: 10.3390/cancers11020163
164. Costa A da, Oliveira JT, Gärtner F, Kohn B, Gruber AD, Klopfleisch R. Potential markers for detection of circulating canine mammary tumor cells in the peripheral blood. *Vet J.* (2011) 190:165–8. doi: 10.1016/j.tvjl.2010.09.027
165. Wright T, Brisson BA, Wood GA, Oblak M, Mutsaers AJ, Sabine V, et al. Flow cytometric detection of circulating osteosarcoma cells in dogs. *Cytom Part A.* (2019) 95:997–1007. doi: 10.1002/cyto.a.23847
166. Sanchez C, Snyder MW, Tanos R, Shendure J, Thierry AR. New insights into structural features and optimal detection of circulating tumor DNA

determined by single-strand DNA analysis. *Npj Genome Med.* (2018) 3:31. doi: 10.1038/s41525-018-0069-0
167. Akter S, Nakao R, Imasato Y, Alam MZ, Katakura K. Potential of cell-free DNA as a screening marker for parasite infections in dog. *Genomics.* (2018) 111:906–12. doi: 10.1016/j.ygeno.2018.05.020
168. Kustanovich A, Schwartz R, Peretz T, Grinshpun A. Life and death of circulating cell-free DNA. *Cancer Biol Ther.* (2019) 20:1057–67. doi: 10.1080/15384047.2019.1598759
169. Wilson IJ, Burchell RK, Worth AJ, Burton SE, Gedye KR, Clark KJ, et al. Kinetics of plasma cell-free DNA and creatine kinase in a canine model of tissue injury. *J Vet Intern Med.* (2018) 32:157–64. doi: 10.1111/jvim.14901
170. Chen K, Zhao H, Shi Y, Yang F, Wang LT, Kang G, et al. Perioperative dynamic changes in circulating tumor DNA in patients with lung cancer (DYNAMIC). *Clin Cancer Res.* (2019) 25:7058–67. doi: 10.1158/1078-0432.ccr-19-1213
171. Mandel P, Metais P. [Nuclear acids in human blood plasma]. *Comptes Rendus Séances Soc Biol Fil.* (1948) 142:241–3.
172. Bendich A, Wilczok T, Borenfreund E. Circulating DNA as a possible factor in oncogenesis. *Science.* (1965) 148:374–6. doi: 10.1126/science.148.3668.374
173. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* (1977) 37:646–50.
174. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology.* (1989) 46:318–22. doi: 10.1159/000226740
175. Chen XQ, Stroun M, Magnenat J-L, Nicod LP, Kurt A-M, Lyautey J, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med.* (1996) 2:1033–5. doi: 10.1038/nm0996-1033
176. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med.* (1996) 2:1035–7. doi: 10.1038/nm0996-1035
177. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci USA.* (2005) 102:16368–73. doi: 10.1073/pnas.0507904102
178. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med.* (2007) 14:985–90. doi: 10.1038/nm.1789
179. Yung TKF, Chan KCA, Mok TSK, Tong J, To K-F, Lo YMD. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non–small cell lung cancer patients. *Clin Cancer Res.* (2009) 15:2076–84. doi: 10.1158/1078-0432.ccr-08-2622
180. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature.* (2012) 486:532–6. doi: 10.1038/nature11156
181. Wan N, Weinberg D, Liu T-Y, Niehaus K, Ariazi EA, Delubac D, et al. Machine learning enables detection of early-stage colorectal cancer by whole-genome sequencing of plasma cell-free DNA. *BMC Cancer.* (2019) 19:832. doi: 10.1186/s12885-019-6003-8
182. Ulz P, Perakis S, Zhou Q, Moser T, Belic J, Lazzeri I, et al. Inference of transcription factor binding from cell-free DNA enables tumor subtype prediction and early detection. *Nat Commun.* (2019) 10:4666. doi: 10.1038/s41467-019-12714-4
183. Aravanis AM, Lee M, Klausner RD. Next-generation sequencing of circulating tumor DNA for early cancer detection. *Cell.* (2017) 168:571–4. doi: 10.1016/j.cell.2017.01.030
184. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet.* (1997) 350:485–7. doi: 10.1016/s0140-6736(97)02174-0
185. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med.* (2011) 13:913–20. doi: 10.1097/gim.0b013e3182368a0e
186. Akolekar R, Beta J, Picciarelli G, Ogilvie C, D'Antonio F. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. *Ultrasound Obst Gyn.* (2015) 45:16–26. doi: 10.1002/uog.14636
187. Samura O. Update on noninvasive prenatal testing: a review based on current worldwide research. *J Obstet Gynaecol Res.* (2020) 46:1246–54. doi: 10.1111/jog.14268
188. Bianchi DW, Chudova D, Sehnert AJ, Bhatt S, Murray K, Prosen TL, et al. Noninvasive prenatal testing and incidental detection of occult maternal malignancies. *JAMA.* (2015) 314:162–9. doi: 10.1001/jama.2015.7120
189. Dharajiya NG, Grosu DS, Farkas DH, McCullough RM, Almasri E, Sun Y, et al. Incidental detection of maternal neoplasia in noninvasive prenatal testing. *Clin Chem.* (2018) 64:329–35. doi: 10.1373/clinchem.2017.277517
190. Chan KCA, Woo JKS, King A, Zee BCY, Lam WKJ, Chan SL, et al. Analysis of plasma epstein–barr virus DNA to screen for nasopharyngeal cancer. *N Engl J Med.* (2017) 377:513–22. doi: 10.1056/nejmoa1701717
191. Beck J, Hennecke S, Bornemann-Kolatzki K, Urnovitz HB, Neumann S, Ströbel P, et al. Genome aberrations in canine mammary carcinomas and their detection in cell-free plasma DNA. *PLoS ONE.* (2013) 8:e75485. doi: 10.1371/journal.pone.0075485
192. Devall VC, Goggs R, Hansen C, Frye CW, Letendre J, Wakshlag JJ. Serum myoglobin, creatine kinase, and cell-free DNA in endurance sled dogs and sled dogs with clinical rhabdomyolysis. *J Vet Emerg Crit Care.* (2018) 28:310–6. doi: 10.1111/vec.12731
193. Tagawa M, Tambo N, Maezawa M, Tomihari M, Watanabe K-I, Inokuma H, et al. Quantitative analysis of the BRAF V595E mutation in plasma cell-free DNA from dogs with urothelial carcinoma. *PLoS ONE.* (2020) 15:e0232365. doi: 10.1371/journal.pone.0232365
194. Schaefer DMW, Forman MA, Kisseberth WC, Lehman AM, Kelbick NT, Harper P, et al. Quantification of plasma DNA as a prognostic indicator in canine lymphoid neoplasia. *Vet Comp Oncol.* (2007) 5:145–55. doi: 10.1111/j.1476-5829.2007.00122.x
195. Uzuelli JA, Dias-Junior CAC, Izidoro-Toledo TC, Gerlach RF, Tanus-Santos JE. Circulating cell-free DNA levels in plasma increase with severity in experimental acute pulmonary thromboembolism. *Clin Chim Acta.* (2009) 409:112–6. doi: 10.1016/j.cca.2009.09.011
196. Burnett DL, Cave NJ, Gedye KR, Bridges JP. Investigation of cell-free DNA in canine plasma and its relation to disease. *Vet Q.* (2016) 36:1–8. doi: 10.1080/01652176.2016.1182230
197. Jeffery U, Ruterbories L, Hanel R, LeVine DN. Cell-free DNA and DNase activity in dogs with immune-mediated hemolytic anemia. *J Vet Intern Med.* (2017) 31:1441–50. doi: 10.1111/jvim.14808
198. Beffagna G, Sammarco A, Bedin C, Romualdi C, Mainenti M, Mollo A, et al. Circulating cell-free DNA in dogs with mammary tumors: short and long fragments and integrity index. *PLoS ONE.* (2017) 12:e0169454. doi: 10.1371/journal.pone.0169454
199. Letendre J, Goggs R. Measurement of plasma cell-free DNA concentrations in dogs with sepsis, trauma, and neoplasia. *J Vet Emerg Crit Care.* (2017) 27:307–14. doi: 10.1111/vec.12592
200. Lee K-H, Shin T-J, Kim W-H, Lee S-Y, Cho J-Y. Methylation of LINE-1 in cell-free DNA serves as a liquid biopsy biomarker for human breast cancers and dog mammary tumors. *Sci Rep.* (2019) 9:175. doi: 10.1038/s41598-018-36470-5
201. Tagawa M, Shimbo G, Inokuma H, Miyahara K. Quantification of plasma cell-free DNA levels in dogs with various tumors. *J Vet Diag Invest.* (2019) 31:836–43. doi: 10.1177/1040638719880245
202. Favaro PF, Stewart SD, McDonald BR, Cawley J, Contente-Cuomo T, Wong S, et al. Feasibility and promise of circulating tumor DNA analysis in dogs with naturally-occurring sarcoma. *BioRxiv.* (2020) 2020.08.20.260349. doi: 10.1101/2020.08.20.260349
203. Prouteau A, Denis JA, Fornel PD, Cadieu E, Derrien T, Kergal C, et al. Circulating tumor DNA is detectable in canine histiocytic sarcoma, oral malignant melanoma, and multicentric lymphoma. *Sci Rep.* (2021) 11:877. doi: 10.1038/s41598-020-80332-y
204. Chang H-W, Lee SM, Goodman SN, Singer G, Cho SKR, Sokoll LJ, et al. Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst.* (2002) 94:1697–703. doi: 10.1093/jnci/94.22.1697
205. Baylin SB, Jones PA. A decade of exploring the cancer epigenome — biological and translational implications. *Nat Rev Cancer.* (2011) 11:726–34. doi: 10.1038/nrc3130

206. Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H, et al. A DNA methylation fingerprint of 1628 human samples. *Genome Res.* (2012) 22:407–19. doi: 10.1101/gr.119867.110
207. Sun K, Jiang P, Chan KCA, Wong J, Cheng YKY, Liang RHS, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci USA.* (2015) 112:E5503–12. doi: 10.1073/pnas.1508736112
208. Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FMF, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med.* (2010) 2:61ra91. doi: 10.1126/scitranslmed.3001720
209. Jiang P, Chan CWM, Chan KCA, Cheng SH, Wong J, Wong VW-S, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. *Proc Natl Acad Sci USA.* (2015) 112:E1317–25. doi: 10.1073/pnas.1500076112
210. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an *in vivo* nucleosome footprint that informs its tissues-of-origin. *Cell.* (2016) 164:57–68. doi: 10.1016/j.cell.2015.11.050
211. Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature.* (2019) 570:385–9. doi: 10.1038/s41586-019-1272-6
212. Jiang P, Sun K, Peng W, Cheng SH, Ni M, Yeung PC, et al. Plasma DNA end-motif profiling as a fragmentomic marker in cancer, pregnancy, and transplantation. *Cancer Discov.* (2020) 10:664–73. doi: 10.1158/2159-8290.cd-19-0622
213. Sun K, Jiang P, Cheng SH, Cheng THT, Wong J, Wong VWS, et al. Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Res.* (2019) 29:418–27. doi: 10.1101/gr.242719.118
214. Chiu RWK, Heitzer E, Lo YMD, Mouliere F, Tsui DWY. Cell-free DNA fragmentomics: the new "omics" on the block. *Clin Chem.* (2020) 66:1480–4. doi: 10.1093/clinchem/hvaa258
215. Cho H, Mariotto AB, Schwartz LM, Luo J, Woloshin S. When do changes in cancer survival mean progress? The insight from population incidence and mortality. *Jnci Monogr.* (2014) 2014:187–97. doi: 10.1093/jncimonographs/lgu014
216. Beer TM. Novel blood-based early cancer detection: diagnostics in development. *Am J Manage Care.* (2020) 26:S292–9. doi: 10.37765/ajmc.2020.88533
217. Brill JV. Screening for cancer: the economic, medical, and psychosocial issues. *Am J Manage Care.* (2020) 26:S300–6. doi: 10.37765/ajmc.2020.88534
218. Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic — implementation issues and future challenges. *Nat Rev Clin Oncol.* (2021). doi: 10.1038/s41571-020-00457-x. [Epub ahead of print].
219. Oncology TL. Cancer detection: the quest for a single liquid biopsy for all. *Lancet Oncol.* (2020) 21:733. doi: 10.1016/s1470-2045(20)30033-4
220. Bradley SH, Barclay ME. "Liquid biopsy" for cancer screening. *Bmj.* (2021) 372:m4933. doi: 10.1136/bmj.m4933
221. Kalinich M, Haber DA. Cancer detection: seeking signals in blood. *Science.* (2018) 359:866–7. doi: 10.1126/science.aas9102
222. Penson A, Camacho N, Zheng Y, Varghese AM, Al-Ahmadie H, Razavi P, et al. Development of genome-derived tumor type prediction to inform clinical cancer care. *JAMA Oncol.* (2020) 6:84–91. doi: 10.1001/jamaoncol.2019.3985
223. Liu ET, Mockus SM. Tumor origins through genomic profiles. *JAMA Oncol.* (2020) 6:33–4. doi: 10.1001/jamaoncol.2019.3981
224. Pinsky PF. Principles of cancer screening. *Surg Clin N Am.* (2015) 95:953–66. doi: 10.1016/j.suc.2015.05.009
225. Wu GX, Raz DJ, Brown L, Sun V. Psychological burden associated with lung cancer screening: a systematic review. *Clin Lung Cancer.* (2016) 17:315–24. doi: 10.1016/j.cllc.2016.03.007
226. Vermeer NCA, Valk MJM, Snijders HS, Vasen HFA, Hoop AG van der, Guicherit OR, et al. Psychological distress and quality of life following positive fecal occult blood testing in colorectal cancer screening. *Psycho Oncol.* (2020) 29:1084–91. doi: 10.1002/pon.5381
227. Hubbell E, Clarke CA, Aravanis AM, Berg CD. Modeled reductions in late-stage cancer with a multi-cancer early detection test. *Cancer Epidemiol Prev Biomarkers.* (2020). doi: 10.1158/1055-9965.epi-20-1134. [Epub ahead of print].
228. Kooyker AI, Toes-Zoutendijk E, Winden AWJO, Spaander MCW, Buskermolen M, Vuuren HJ, et al. The second round of the Dutch colorectal cancer screening program: impact of an increased fecal immunochemical test cut-off level on yield of screening. *Int J Cancer.* (2020) 147:1098–106. doi: 10.1002/ijc.32839
229. Rose NC, Kaimal AJ, Dugoff L, Norton ME, Bulletins—Obstetrics AC of O and GC on P, Genetics C on, et al. Screening for fetal chromosomal abnormalities: ACOG practice bulletin summary, number 226. *Obstetr Gynecol.* (2020) 136:859–67. doi: 10.1097/aog.0000000000004107
230. Hyman DM, Taylor BS, Baselga J. Implementing genome-driven oncology. *Cell.* (2017) 168:584–99. doi: 10.1016/j.cell.2016.12.015
231. Wise HC, Solit DB. Precision oncology: three small steps forward. *Cancer Cell.* (2019) 35:825–6. doi: 10.1016/j.ccell.2019.05.009
232. American Association for Cancer Research (AACR). *AACR Cancer Progress Report 2020.* (2020). Available online at: http://www.cancerprogressreportr.org (accessed October 5, 2020)
233. National Cancer Institute (NCI). *Targeted Cancer Therapies.* NCI (2020). Available online at: https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet (acessed October 5, 2020)
234. Chakravarty D, Gao J, Phillips S, Kundra R, Zhang H, Wang J, et al. OncoKB: a precision oncology knowledge base. *Jco Precis Oncol.* (2017) 2017:1–16. doi: 10.1200/po.17.00011
235. OncoKB. *OncoKB Level 1 FDA-Approved Drugs*. OncoKB (2020). Available online at: https://www.oncokb.org/actionableGenes#levels=1 (accessed October 5, 2020)
236. US Food & Drug Administration (FDA). *Animal Drugs at FDA*. FDA. Available online at: https://animaldrugsatfda.fda.gov/ (accessed October 5, 2020)
237. European Medicines Agency (EMA). *European Medicines Agency (EMA)*. EMA (2021). Available online at: https://www.ema.europa.eu/en/medicines (accessed January 29, 2021)
238. London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin Cancer Res.* (2003) 9:2755–68.
239. Thamm DH, Weishaar KM, Charles JB, Ehrhart EJ. Phosphorylated KIT as a predictor of outcome in canine mast cell tumours treated with toceranib phosphate or vinblastine. *Vet Comp Oncol.* (2020) 18:169–175. doi: 10.1111/vco.12525
240. US Food & Drug Administration (FDA). *The Ins and Outs of Extra-Label Drug Use in Animals: A Resource for Veterinarians*. FDA (2020). Available online at: https://www.fda.gov/animal-veterinary/resources-you/ins-and-outs-extra-label-drug-use-animals-resource-veterinarians (accessed October 5, 2020)
241. Londhe P, Gutwillig M, London C. Targeted therapies in veterinary oncology. *Vet Clin North Am Small Anim Pract.* (2019) 49:917–31. doi: 10.1016/j.cvsm.2019.04.005
242. Cronise KE, Hernandez BG, Gustafson DL, Duval DL. Identifying the ErbB/MAPK signaling cascade as a therapeutic target in canine bladder cancer. *Mol Pharmacol.* (2019) 96:36–46. doi: 10.1124/mol.119.115808
243. Murray NP. Biomarkers detecting minimal residual disease in solid tumors: what do they mean in the clinical management of patients? *Biomark Med.* (2019) 13:1535–8. doi: 10.2217/bmm-2019-0401
244. Bork U, Grutzmann R, Rahbari NN, Scholch S, Distler M, Reissfelder C, et al. Prognostic relevance of minimal residual disease in colorectal cancer. *World J Gastroenterol.* (2014) 20:10296–304. doi: 10.3748/wjg.v20.i30.10296
245. Pantel K, Alix-Panabières C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. *Nat Rev Clin Oncol.* (2019) 16:409–24. doi: 10.1038/s41571-019-0187-3
246. Coakley M, Garcia-Murillas I, Turner NC. Molecular residual disease and adjuvant trial design in solid tumors. *Clin Cancer Res.* (2019) 25:6026–34. doi: 10.1158/1078-0432.ccr-19-0152
247. Sánchez R, Ayala R, Martínez-López J. Minimal residual disease monitoring with next-generation sequencing methodologies in hematological malignancies. *Int J Mol Sci.* (2019) 20:2832. doi: 10.3390/ijms20112832
248. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence

in patients with stage II colon cancer. *Sci Transl Med.* (2016) 8:346ra92. doi: 10.1126/scitranslmed.aaf6219
249. Nguyen SM, Thamm DH, Vail DM, London CA. Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consensus document. *Vet Comp Oncol.* (2015) 13:176–83. doi: 10.1111/vco.12032
250. Vail DM, Michels GM, Khanna C, Selting KA, London CA, Group VCO. Response evaluation criteria for peripheral nodal lymphoma in dogs (v1.0)–a veterinary cooperative oncology group (VCOG) consensus document. *Vet Comp Oncol.* (2010) 8:28–37. doi: 10.1111/j.1476-5829.2009.00200.x
251. Erasmus JJ, Gladish GW, Broemeling L, Sabloff BS, Truong MT, Herbst RS, et al. Interobserver and intraobserver variability in measurement of non–small-cell carcinoma lung lesions: implications for assessment of tumor response. *J Clin Oncol.* (2003) 21:2574–82. doi: 10.1200/jco.2003.01.144
252. Alexander K, Joly H, Blond L, D'Anjou M, Nadeau M, Olive J, et al. A comparison of computed tomography, computed radiography, and film-screen radiography for the detection of canine pulmonary nodules. *Vet Radiol Ultrasound.* (2012) 53:258–65. doi: 10.1111/j.1740-8261.2012.01924.x
253. McErlean A, Panicek DM, Zabor EC, Moskowitz CS, Bitar R, Motzer RJ, et al. Intra- and interobserver variability in CT measurements in oncology. *Radiology.* (2013) 269:451–9. doi: 10.1148/radiol.13122665
254. Guglielmini C, Toaldo MB, Quinci M, Romito G, Luciani A, Cipone M, et al. Sensitivity, specificity, and interobserver variability of survey thoracic radiography for the detection of heart base masses in dogs. *J Am Vet Med Assoc.* (2016) 248:1391–8. doi: 10.2460/javma.248.12.1391
255. Leffler AJ, Hostnik ET, Warry EE, Habing GG, Auld DM, Green EM, et al. Canine urinary bladder transitional cell carcinoma tumor volume is dependent on imaging modality and measurement technique. *Vet Radiol Ultrasound.* (2018) 59:767–76. doi: 10.1111/vru.12652
256. Woo M, Lowe SL, Devane AM, Gimbel RW. Intervention to reduce inter-observer variability in CT measurement of cancer lesions among experienced radiologists. *Curr Probl Diagn Radiol.* (2020). doi: 10.1067/j.cpradiol.2020.01.008. [Epub ahead of print].
257. Zhou L, Zhang M, Li R, Xue J, Lu Y. Pseudoprogression and hyperprogression in lung cancer: a comprehensive review of literature. *J Cancer Res Clin.* (2020) 146:3269–79. doi: 10.1007/s00432-020-03360-1.
258. Yekula A, Muralidharan K, Rosh ZS, Youngkin AE, Kang KM, Balaj L, et al. Liquid biopsy strategies to distinguish progression from pseudoprogression and radiation necrosis in glioblastomas. *Adv Biosyst.* (2020) 4:2000029. doi: 10.1002/adbi.202000029
259. Ma Y, Wang Q, Dong Q, Zhan L, Zhang J. How to differentiate pseudoprogression from true progression in cancer patients treated with immunotherapy. *Am J Cancer Res.* (2019) 9:1546–53.
260. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Sci Transl Med.* (2015) 7:302ra133. doi: 10.1126/scitranslmed.aab0021
261. Hosoya K, Kisseberth WC, Lord LK, Alvarez FJ, Lara-Garcia A, Kosarek CE, et al. Comparison of COAP and UW-19 protocols for dogs with multicentric lymphoma. *J Vet Intern Med.* (2007) 21:1355–63. doi: 10.1111/j.1939-1676.2007.tb01959.x
262. Quigley D, Alumkal JJ, Wyatt AW, Kothari V, Foye A, Lloyd P, et al. Analysis of circulating cell-free DNA identifies multiclonal heterogeneity of BRCA2 reversion mutations associated with resistance to PARP inhibitors. *Cancer Discov.* (2017) 7:999–1005. doi: 10.1158/2159-8290.cd-17-0146
263. Lin JJ, Riely GJ, Shaw AT. Targeting ALK: precision medicine takes on drug resistance. *Cancer Discov.* (2017) 7:137–55. doi: 10.1158/2159-8290.cd-16-1123
264. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol.* (2018) 15:81–94. doi: 10.1038/nrclinonc.2017.166
265. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood.* (2015) 126:9–16. doi: 10.1182/blood-2015-03-631747
266. Razavi P, Li BT, Brown DN, Jung B, Hubbell E, Shen R, et al. High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. *Nat Med.* (2019) 25:1928–37. doi: 10.1038/s41591-019-0652-7
267. Wang Y, Springer S, Zhang M, McMahon KW, Kinde I, Dobbyn L, et al. Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. *Proc Natl Acad Sci USA.* (2015) 112:9704–9. doi: 10.1073/pnas.1511694112
268. Field NP, Orsini L, Gavish R, Packman W. Role of attachment in response to pet loss. *Death Stud.* (2009) 33:334–55. doi: 10.1080/07481180802705783
269. Uccheddu S, Cataldo LD, Albertini M, Coren S, Pereira GDG, Haverbeke A, et al. Pet humanisation and related grief: development and validation of a structured questionnaire instrument to evaluate grief in people who have lost a companion dog. *Animals.* (2019) 9:933. doi: 10.3390/ani9110933
270. Winch G. Why We Need to Take Pet Loss Seriously. *Scientific American.* (2018). Available online at: https://www.scientificamerican.com/article/why-we-need-to-take-pet-loss-seriously/ (accessed January 29, 2021)
271. Bishop G, Cooney K, Cox S, Downing R, Mitchener K, Shanan A, et al. 2016 AAHA/IAAHPC End-of-Life Care Guidelines*. *J Am Anim Hosp Assoc.* (2016) 52:341–56. doi: 10.5326/jaaha-ms-6637
272. Hetts S, Lagoni L. The owner of the pet with cancer. *Vet Clin North Am Small Animal Pract.* (1990) 20:879–96. doi: 10.1016/s0195-5616(90)50076-5
273. Butler CL, Lagoni L, Dickinson KL, Withrow SJ. Animal illness and human emotion. *Probl Vet Med.* (1991) 3:21–37.
274. American Veterinary Medical Association (AVMA). Veterinarian's Oath. *AVMA Policies*. (2021) Available online at: https://www.avma.org/resources-tools/avma-policies/veterinarians-oath (accessed January 29, 2021)

Conflict of Interest: JC, AF, KK, IC, JT, KL, LH, DT, and DG are employed by or affiliated with PetDx. JC, AF, KK, NL, AN, ND, DB, TJ, JF, MS, IC, JT, KL, LH, MM, LD, DT, and DG hold vested or unvested equity in PetDx. TJ is employed by Laboratory Corporation of America. JF is Managing Partner at Friedman Bioventure, Inc. MS is Managing Director at RS Technology Ventures LLC. KK is an inventor on multiple patent applications related to bioinformatics methods for cancer diagnostics and holds equity in Illumina. MM is an inventor on multiple patent applications covering technologies for canine and human cancer diagnostics, and has licensing or consulting relationships with PetDx, Exact Sciences, AstraZeneca, Bristol Myers Squibb, and TGen. LD is a member of the board of directors of Personal Genome Diagnostics (PGDx) and Jounce Therapeutics. LD is a compensated consultant to PGDx, 4Paws (PetDx), Innovatus CP, Se'er, Kinnate and Neophore. LD is an uncompensated consultant for Merck but has received research support for clinical trials from Merck. LD is an inventor of multiple licensed patents related to technology for circulating tumor DNA analyses and mismatch repair deficiency for diagnosis and therapy from Johns Hopkins University. Some of these licenses and relationships are associated with equity or royalty payments directly to Johns Hopkins and LD. LD holds equity in PGDx, Jounce Therapeutics, Thrive Earlier Detection, Se'er, Kinnate and Neophore. LD's spouse holds equity in Amgen. The terms of all these arrangements for LD are being managed by Johns Hopkins and Memorial Sloan Kettering in accordance with their conflict of interest policies.

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Effects of the Latex of *Synadenium grantii* Hook F. (*Euphorbiaceae*) on a Preclinical Model of Canine Prostate Cancer

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Prostatic cancer (PC) stands out in terms of its occurrence, pathophysiology, and unfavorable prognostics in humans and dogs. Natural drugs bear an integrative potential for conventional antineoplastic treatments. In this context, the bioproducts of *Synadenium grantii* have been empirically used in different parts of Brazil for the integrative treatment of prostate cancer in humans. However, there is no availability of scientific evidence of the antitumor effects of *S. grantii*. Therefore, this study aimed to investigate the bioactive compounds in the latex of *S. grantii* using the high-resolution mass spectrophotometry (HRMS) and to evaluate its cytotoxic effects on primary canine PC cell cultures. Four fragments of phorbol ester were identified as potential bioactive compounds using the HRMS. With the help of an MTT ([3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide]) assay, two canine prostatic carcinoma cell lines (PC 1 and PC2) showed a decrease in the tumor cell count, with an Inhibitory concentration 50 (IC_{50})of 0.8469 and 0.6068 mg/ml, respectively, for PC1 and PC2. In conclusion, the latex of *S. grantii* contains phorbol esters in its composition, and its aqueous solution has a cytotoxic effect on canine metastatic PC cells *in vitro*.

Keywords: dog, cell culture, prostate cancer model, janauba, neoplasia, prostate

INTRODUCTION

Prostate cancer (PC) is one of the most important problems worldwide. Most recent statistical report in the USA demonstrated that PC is the most common cancer among men excluding skin cancer. The 2020 cancer statistics also highlighted that one of five men may develop PC cancer (1). Recent release of the Global Cancer Observatory (2) pointed out that PC is one of the main leading causes of malign neoplasia-related cancer deaths in humans next to lung cancer and female breast cancer. It is the second most frequently occurring cancer among males and its occurrence has grown vertiginously throughout the globe (2). Due to its importance, several models, including PC spontaneous models, have been proposed (3). Dogs and humans are the only species in which PC occurs spontaneously, thus spontaneous canine PC has been used in comparative studies (3, 4)

Canine PC has a low incidence but is characterized by its aggressive behavior, metastatic at diagnosis and poor outcome (5, 6). In addition, it is usually unresponsive to the androgen stimulation due to a lack of androgen receptor (AR) (3). Thus, dogs can be considered as an important model for the human androgen-independent PC (4, 7). Although dogs can be considered as a model, the veterinary literature is focused on comparative morphological characters, and the information on the molecular pattern of canine PC is limited (3, 4). Moreover, a very limited number of studies have investigated the antitumor effect of different compounds on the preclinical models of canine PC (8, 9). Thus, the evaluation of new potential drugs for preclinical models can benefit both dogs and humans.

Usually, anticancer drugs used in the conventional chemotherapy are originated from natural compounds, such as vinca alkaloids (10). Currently, 60% of the drugs for the anticancer therapy are sourced naturally, including from plants, microorganisms, and marine organisms (11). Selective and effective treatments, as well as new mechanisms to limit the illness progression, have been the goal of researchers for the development of anticancer agents (12). Thus, the plant-derived products are the sources of substances used in chemotherapy and other drug therapies (11, 13). A large number of natural active agents are present in cancer therapies (14, 15) because of their structural models and unique action mechanisms. The drugs obtained from natural products can be used as the drugs of primary chemotherapy (16), chemopreventives (17), or chemosensitizers, with the effects synergistic to the conventional chemotherapy (18). However, before using these compounds in clinical practice, the antitumor response and toxicity should be evaluated by *in vitro* models.

Brazil has a great natural plant biodiversity with a great potential to identify natural products with therapeutic properties (19). Most of the used plants are based on popular medicine, where people describe plant potentials to heal diseases without any scientific evidence (11). Among those plants, *Synadenium grantii* Hook F. (Euphorbiaceae) has been proposed to have an antitumor effect on human PC. The latex of *S. grantii* has been empirically used to treat allergic and gastric disorders and cancer (20). It is commonly administered orally in a solution diluted in water to treat malignant neoplasia. The latex of *S. grantii* is rich in non-polar substances (21, 22) and its activity against tumor cell lines has already been demonstrated (23–25). However, there is no finding of clinical evidence on the effect of the latex of *S. grantii* on patients with cancer. Based on this scenario, new studies are required for investigating such an effect on cancer cells. As dogs are considered as a model for human PC, the evaluation of the latex of *S. grantii* in canine cancer cell lines can be considered as a preclinical model, allowing future clinical studies in dogs. Unfortunately, no standard treatment has been tested on PC-affected dogs. Instead, chemotherapy with doxorubicin or carboplatin associated with non-steroidal anti-inflammatory drugs are commonly used (26). Thus, investigating new compounds for preclinical models can provide a new perspective. Therefore, this study aimed to investigate the bioactive compounds of the latex of *S. grantii* and evaluate its effects on canine PC cells as a preclinical model of canine PC.

METHODS

Ethics Statement

This research was approved by the Research Ethics Committee of the Federal University of Goiás (CEP/UFG, protocol no. 2.269.572) and by the Ethics Commission for Animal Use of the São Paulo State University Júlio de Mesquita Filho (CEUA/UNESP, protocol no. 0004/2017).

Plant Material

A fresh sample of the latex of *S. grantii* was collected in July 2018, in Nerópolis, Goiás, Brazil (16°24′31.8″ S, 49°13′11.3″ W, 812-m altitude) after cleaning and transverse cutting of the plant stem. The sample was acclimatized in a 50-ml Falcon tube and kept at 4°C. An exsiccate was prepared and deposited at the Herbarium of the UFG, under number 65903. Portion of the sample was used in the physicochemical characterization test and in the identification of active compounds. For *in vitro* and *in vivo* studies, 1 g of latex was dissolved in distilled water into a 10 ml final stock solution according to Luz et al. (27). Then, a decrease in concentrations was made, and new stock solutions with concentrations of 0.75, 1, 1.5, 3, and 6 mg/ml were originated.

Physicochemical Characterization of the Latex of *S. grantii* Latex

Relative density of the latex was determined by a pycnometer method, weighing 10 ml pure latex in an AG200 analytical scale (Gehaka®, AG 200). The method was performed according to AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA (28). Briefly, the relative density was the ratio of pure latex sample and distilled water weights. The same 10-ml sample was used in the other assays, including pH and humidity measurements, as well as the screening of phenolic and alkaloid compounds.

The pH was obtained by using a pH microprocessor (Marconi®, MA 522). Humidity was determined in the three 1-g latex samples by a desiccation loss analysis, using a moisture analyzer (Shimadzu®, MOC63u). For the detection of phenolic compounds, 1 ml of latex diluted in 10 ml distilled water was used, with the solution being heated for 5 min. Then, four drops of 4.5% ferric chloride were added, with hydroxyls determined qualitatively by evaluating dark precipitate in the solution.

Alkaloids were detected by diluting 2 g of the latex in 20 ml of 5% sulfuric acid. The solution was heated for 3 min, and drops of iodized and complex polyacids reagents were added to independent samples. Then, Mayer's reactive (potassium tetra-iodine mercurate), Dragendorf reactive (potassium iodine-bismuthate), Bertrand reactive (1% silico-tungstic acid), Hager reactive (2% picric acid), and 1% tannic acid were used.

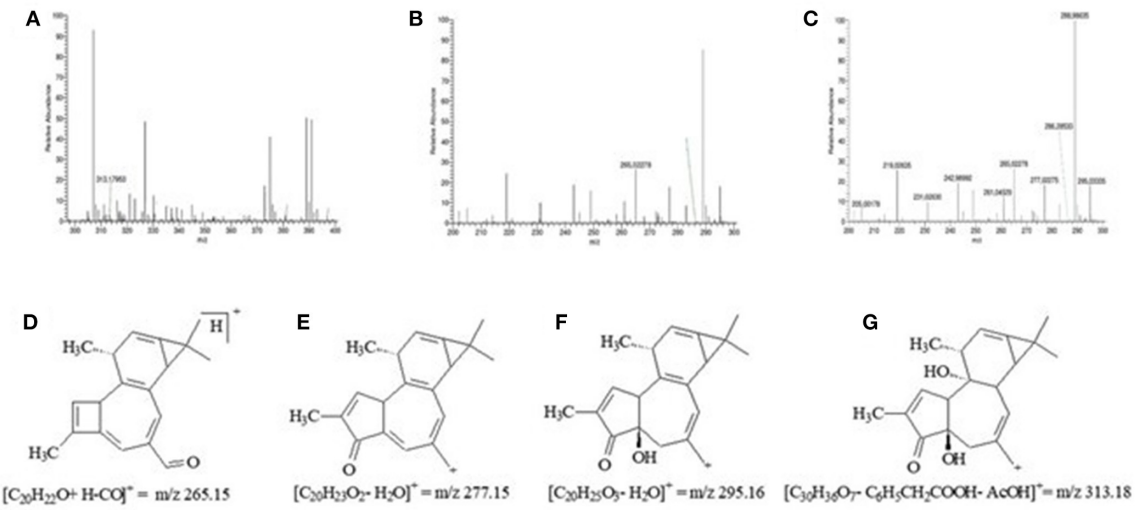

FIGURE 1 | Peak generated in the mass spectrum (HRMS) of the latex of *Synadenium grantii* by magnifying the general chromatogram and its respective structures, molecular forms, and fragment masses. Chromatograms: **(A)** m/z 313.17; **(B)** m/z 265.02; **(C)** m/z 277.02 and 295.03. Structure, molecular form, and fragment mass: **(D)** m/z 265.02; **(E)** m/z 277.02; **(F)** m/z 295.03; and **(G)** m/z 313.17.

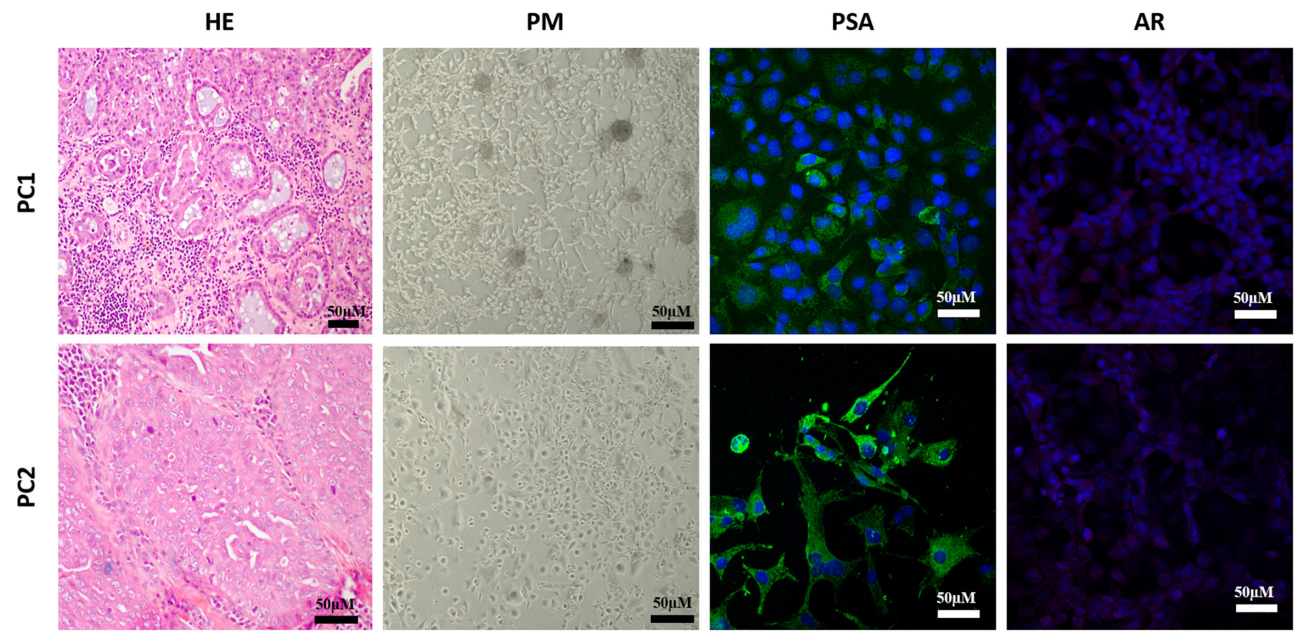

FIGURE 2 | Morphological evaluation of two canine prostatic cell lines (PC1 and PC2). Both originated from Gleason 10 PC, with cribriform to solid morphology. In culture conditions, PC1 grew in groups, forming colonies, and tubular-like structures while PC2 grew isolated. The prostatic origin was confirmed by a prostatic-specific antigen (PSA) positive expression, and both were negative for androgen receptor (AR) expression. HE, hematoxylin and eosin; PC, phase contrast.

S. grantii Latex Phytochemical Research by High-Resolution Mass Spectrometry

A latex sample was subjected to the HRMS to identify 3,4,12,13-tetraacetylphorbol-20-phenylacetate, a compound previously isolated from *S. grantii* plants (29). Another sample was used to check whether the latex contain 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA). Lyophilized standards of both compounds were used for a comparison with the latex samples.

High-resolution mass spectrometry of latex samples and 3,4,12,13-tetraacetylphorbol-20-phenylacetate and dPPA standards were acquired by direct infusions, using the Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer, with the H-ESI (Thermo Fisher Scientific,

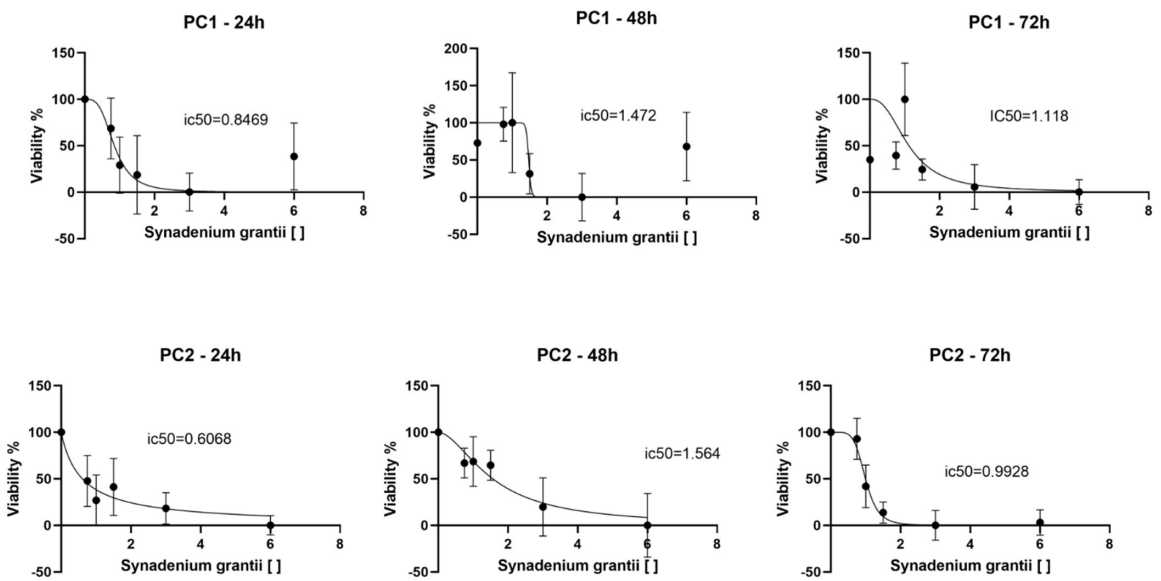

FIGURE 3 | Determination of IC_{50} of PC1 and PC2 cell lines at 24, 48, and 72 h. The cell lines presented low IC_{50} at 24 h but high at 48 h. Then, at 72 h, IC_{50} decreased compared to the previous moment. The graphic representation also shows a dose-dependent response trend over time.

Waltham, MA, USA) as a source. The equipment was operated in a positive mode under the following conditions: full scan (m/z 200–800), spray voltage of 3.5 kV, resolution of 70,000, 10 µl/min flow, sheath gas at 15 (arbitrary units), auxiliary gas at 2 (arbitrary units), capillary temperature of 32°C, auxiliary gas temperature of 37°C, and tube lens at 50 (arbitrary units).

Latex and standard samples were prepared in different ways. About 500 µl methanol and water (1:1) were added to a 0.5-ml *S. grantii* latex sample, and the solution was then subjected to an ultrasound for 3 min, filtrated, and acidified with formic acid.The standard sample of phorbol esters was prepared with methanol solvent and was prepared at a concentration of 10 ppm without acidification. The results were analyzed by using the Therm Platform, which allowed viewing of the total ion chromatogram at a range of 200–800 m/z. The compounds were evaluated by using a latex sample and phorbol ester standard fragments subjected to an impact analysis by electrospray mass spectrometry. Used solvent, mass number, carbons and unsaturation, Na+ and H+ gains, and H+ loss were all considered. A "blank" was performed to confirm whether processed ions were obtained from the sample or the solvent.

Canine Prostate Cell Culture

Two canine prostate cancer cells lines (PC1 and PC2) were used in this study. PC1 was established from a 10-year-old, intact, mixed breed dog with non-metastatic PC (Gleason 10) and PC2 from an 11-year-old, intact, poodle dog with metastatic PC (Gleason 10). Cell morphology and phenotype were previously determined with both cell lines being negative for the nuclear AR expression, positive for prostatic-specific antigen (PSA) and pan-cytokeratin (AE1/AE3), and negative for uroplakin III, vimentin, and P63 (30).

PC1 and PC2 were used at passage 15 (P15) and cultured in a PrEBM medium (Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), with 1% penicillin-streptomycin solution (Sigma, Portland, OR, USA), in a humid atmosphere at 37°C and CO_2 at 5%. To confirm the cell phenotype, PSA and AR immunofluorescence analyses were performed according to Costa et al. (30). Briefly, sterile circular coverslips (15 mm, Knitell) were placed at the center of each plate well with 12 sterile wells. A total of 1×10^5 cells were sown in 250 µl of PrEBM medium (Lonza, Basel, Switzerland), supplemented with 10% FBS and incubated in a moist chamber at 5% CO_2 and 37°C. When adherent cells reached 50% confluence, the medium was removed, and the cells were washed thrice with PBS and fixated with chilled methanol (4°C) for 15 min, followed by a permeabilization with the anionic surfactant solution Triton-X 0.25% (Sigma, Portland, OR, USA). The cells were incubated for 45 min with FBS at 3% in PBS and then used as a blocking solution. Afterward, they were incubated with rabbit polyclonal anti-PSA (Biorbyt, Cambridge, UK) and anti-rabbit polyclonal AR (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, the cells were incubated by using Alexa Fluor 484 (Invitrogen, Carlsbad, CA, USA) diluted at 2 µg/ml. The slides were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma, Portland, OR, USA). The coverslips were analyzed by using a confocal laser scanning microscope TCS SP 5 (Leica), and the images were captured by using the Leica Application Suite Advanced Fluorescence (LAS AF) software.

Treatment and Cell Viability of the Latex of *S. grantii*

PC1 and PC2 cells were cultured for 24 h in 96-well plates, at 1×10^4 cells/well, in a humidified incubator at 37°C and 5% CO_2. Treatments of the latex of *S. grantii* were

performed by using the samples of 0.75, 1, 1.5, 3, and 6 mg/ml, which were evaluated at 24 (G24), 48 (G48), and 72 h (G72). Negative control and blank groups were preformed according to Chaves et al. (31). After treatments, the media were discarded, and 10-µl MTT [3-[4,5-dimethylthiazol-2-l]-2,5-diphenyltetrazolium bromide, Sigma Aldrich, Portland, OR, USA] solution was used while maintained for 3 h in an incubator according to the manufacturer's recommendation. Thereafter, 100-µl solubilizing solution was added to each well. The plate was homogenized for 10 min, and optical density readings were performed in a microplate reader (Expert Plus, 595 nm). We determined the concentration at which 50% of the cell viability is inhibited [Inhibitory concentration 50 (IC_{50})] (32). Three independent experiments were performed thrice.

Statistical Analysis

Descriptive statistics was performed, which describe qualitative outcomes as a positive and negative expression for each marker. For the IC_{50} determination, a dose-response curve was performed by using the GraphPad Prism v.8.1.0 (GraphPad Software Inc., La Jolla, CA, USA), at 5% significance level ($p < 0.05$).

RESULTS

Characterization of *S. grantii*

The physicochemical characterization showed that the latex had a density of 1.02 g/ml and pH of 5.72.The percentage of total solids was 29.19% with 70.8% humidity. No precipitation occurred or change in coloration was noted in the sample used for a qualitative detection of phenolic hydroxyls. All reagents showed a discreet precipitation in the detection of alkaloid, which confirmed their presence in *S. grantii* latex samples. The standards used in the HRMS were standardized according to a technique wherein the molecular ion corresponds to its respective molecular mass. No specific molecular ion fragments characterizing the 12-deoxifolia-13-phenylacetate-20-acetate (dPPA), with m/z = 531.23 [M+Na]+, or the 3,4,12,13-tetraacetylforbol-20-phenylacetyl, with m/z 675. 27[M+Na]+z were found in the *S. grantii* latex.

The compounds undergoing a test were not observed in the chromatogram of the latex of *S. grantii*. However, the HRMS analysis showed precisely the molecular weight of fragments of other compounds in the tested latex, including [$C_{20}H_{22}O$+H-CO]+ m/z 265.15, [$C_{20}H_{23}O_2$-H_2O]+ m/z 277.15, [$C_{20}H_{25}O_3$-H_2O]+ m/z 295.16, and [$C_{30}H_{36}O_7$-$C_6H_5CH_2$-COOH-AcOH]+ m/z 313.18 (**Figure 1**), which are all from the phorbol family.

Cell Phenotype and Viability After the Treatment of Latex of *S. grantii*

PC1 cells showed a polygonal morphology at P15 and grew in small clusters, forming cell colonies. PC2 cells also showed a polygonal morphology but grew isolated in the culture flask. In P15, cells are positive and negative, respectively, for PSA for RA (**Figure 2**).

We identified different IC_{50} concentrations as a function of exposure time. Overall, after 24 h exposure, PC1 had an IC_{50} of 0.8469 mg/ml and PC2 0.6068 mg/ml (**Figure 3**). Both cell lines had high IC_{50} at 48 h but low at 72 h (**Figure 3**). All tested *S. grantii* latex concentrations had a dose-dependent effect on PC1 cells. When comparing 3 and 6 mg/ml with the untreated group, considering PC1 and PC2 within 24 h, 6 mg/ml had the best effects (0.19 and 20 abs), followed by 3 mg/ml in PC2 (0.27 abs). But within 72 h, cell viability had no differences in either PC1 or PC2 for both latex concentrations (**Table 1**).

TABLE 1 | Mean cell viability for canine PC1 and PC2 cell lineages per concentration and exposure time to the latex of *S. grantii* by a tetrazolium reduction method.

	PC1		PC2	
	Exposure time			
Concentrations	**24 h**	**72 h**	**24 h**	**72 h**
0	$0.24 \pm 0.02^{a^*,A^{**}}$	$0.48 \pm 0.05^{a,B}$	$0.56 \pm 0.11^{a,A}$	$0.48 \pm 0.05^{a,B}$
3 mg/ml	$0.26 \pm 0.02^{c,A}$	$0.21 \pm 0.02^{c,A}$	$0.27 \pm 0.02^{b,A}$	$0.21 \pm 0.02^{b,A}$
6 mg/ml	$0.19 \pm 0.02^{d,A}$	$0.21 \pm 0.02^{c,A}$	$0.20 \pm 0.02^{c,A}$	$0.21 \pm 0.02^{b,A}$

*Scott-Knott test ($p < 0.05\%$). *lowercase letters in the same column are the comparing concentrations within the same time and **uppercase letters in the same row are the comparing concentration over different treatment times.*

DISCUSSION

Brazil has a great biodiversity with a potential to identify several compounds with antitumor activity based on native plants. Using technological databases, botanical taxonomists have classified plants into several kinds. In addition, advances in research have led to a finding of medicinal plants with a similar phenotype but differences in microscopic or molecular scales. Synonymy is quite common and Brazil counts with a species list, the "Lista de Espécies da Flora do Brasil" (33), which is an online list of accepted names of native species and their geographic distribution.

S. grantii is a species of the Euphorbiaceae family, also known as "milagrosa" or "janauba," has been a scientific subject for the anticancer drug treatment since different compounds can be found in its plant material. Scientific evidence has found the anticancer activity of steroids, terpenes (diterpenes, sesquiterpenes, and triterpenes), phenolic compounds, and proteins from *S. grantii* (25, 27, 34, 35). Taxon evidence shows that *Euphorbia umbellata* (Pax) Bruyns and *Synadenium umbellatum* are scientific synonyms, as well as their role in anticancer drug development (27).

The use of the latex of *S. grantii* use as an antiproliferative agent has been experimentally demonstrated in several cancer cell culture studies. Other *Euphorbia* genera have been used in melanoma cell culture (25) and leukemic cell culture (36) for antiproliferative cancer research (37). In popular beliefs, the latex of *S. grantii* has a high antitumor effect when taking orally and is often advised for men with prostate cancer. However, no previous studies have demonstrated such an antitumor effect. Different *in vitro* and *in vivo* models have been used in a comparative oncology for preclinical research. For example, since prostate

carcinoma affects both humans and dogs, the canine model has been widely accepted as a preclinical model for prostate cancer (38, 39). In addition, in recent years, several cultures of canine prostate cancer have been published, which allow testing of new drugs still in the preclinical phase (40).

Given the recent studies still on the latex of *S. grantii*, the first stage of our research was to characterize this latex. Previously, the density of this latex was reported to be of 1 g/ml when collected from the plant stem (41), which is close to water density and similar to what we observed. Total dissolved solids after the evaporation were not found for the latex in the literature. This parameter is useful in measuring working solutions since the water concentration in the latex must be accounted.

Plant primary and secondary metabolites are useful in determining the bioactive compounds for cancer research (42). Phenolic compounds are a group of substances with different levels of chemical complexity and are able to neutralize reactive oxygen species (ROS) (43). However, no phenolic compounds were detected in the samples of latex of *S. grantii* by using a qualitative analysis.

Cell morphology was analyzed to verify cell stability and confirm their phenotypes in cultures. Both cell cultures were previously characterized (30). However, we opted to confirm the AR status of cells and the cells being primary prostatic (based on PSA expression). In our study, more than 90% of the cells in both cell lineages of the canine PC-expressed PSA, which confirmed the prostatic origin of neoplastic cells. The role of androgens and the impact of early or late neutering are still controversial even though a few studies have not reported androgen effects on the canine prostate adenocarcinoma initiation or progression (6, 44, 45). In our study, AR immunoexpression was negative demonstrating androgen hormone independence for both cell lines.

We determined the IC_{50} for both cell lines and observed a dose-dependent response; therefore, the latex of *S. grantii* has antitumor activity in canine PC cells. The cytotoxic effect of plants of the *Synadenium* genus has been demonstrated mainly by the evaluation of plant shoot extracts (46, 47), with a few reports about it in the latex. This, in turn, has been used empirically in therapies and may be potentially dangerous due to its high toxicity, cytotoxic effects, and mutagenic potential (dose-dependent) besides its low lethal dosage to mice (110–168 mg/kg) (41, 48).

Such scientific information allied to the IC_{50} value of PC 1 and 2 suggests a potential effective and safe dose to be applied in future analysis. Thus, this safe dose can be tested in *in vivo* evaluation by using mice as a scientific model. The latex possesses a lethal action on cells at the proliferative stage (S-G2/M), which had been observed when using 1.7–7 *ug* latex per well in a melanoma cell culture by flow cytometry (25). Interestingly, PC2 come from a metastatic canine PC and presented an IC_{50} lower than PC1. In general, our results suggested an antitumor effect of the latex of *S. grantii in vitro*, which is important for future clinical trials involving this plant material in dogs with PC. The CAM assay showed significant results by comparing IC_{50} in PC1 and PC2 cells whereas metastatic cell lineage had a lower IC_{50}.

CONCLUSION

The latex of *S. grantii* plants contains phorbol esters in its composition. Its aqueous solution has an *in vitro* cytotoxic effect on metastatic canine PC cells. Still, the latex of *S. grantii* should be further verified for other biological effects, such as bioactive compound identification and respective action mechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are include in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by this research was approved by Research Ethics Committee of the Federal University of Goiás (CEP/UFG, protocol no. 2.269.572) and by the Ethics Commission for the Use of Animals of the São Paulo State University Júlio de Mesquita Filho (CEUA/UNESP, protocol no. 0004/2017).

AUTHOR CONTRIBUTIONS

EB, LP, and LA were responsible for all experimental stages and for the writing of the first draft of the manuscript. EA was involved in statistical analysis. JP and LR were responsible for physicochemical and phytochemical characterization of the *S. grantii*. CF-A, MM, and VdM were responsible for the hypotheses, supervision of the *in vitro* and *in vivo* assays, as well as the writing and reviewing of the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *Cancer J Clin.* (2020) 70:7–30. doi: 10.3322/caac.21590
2. Bray F, Ferlay J, Soerjomataram I, Siegel RLS, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2018) 68:394–424. doi: 10.3322/caac.21492
3. Palmieri C, Foster RA, Grieco V, Fonseca-Alves CE, Wood GA, Culp WTN, et al. Histopathological terminology standards for the reporting of prostatic epithelial lesions in dogs. *J Comp Pathol.* (2019) 171:30–7. doi: 10.1016/j.jcpa.2019.07.005
4. Laufer-Amorim R, Fonseca-Alves CE, Villacis RAR, Linde SAD, Carvalho M, Larsen SJ, et al. Comprehensive genomic profiling of androgen-receptor-negative canine prostate cancer. *Int J Mol Sci.* (2019) 20:1555. doi: 10.3390/ijms20071555
5. Teske E, Naan EC, Van Dijk EM, Van Garderen E, Schalken JA. Canine prostate carcinoma: epidemiological evidence of an increased risk in castrated dogs. *Mol Cell Endocrinol.* (2002) 197:251–5. doi: 10.1016/S0303-7207(02)00261-7
6. Bryan JN, Keeler MR, Henry CJ, Bryan ME, Hahn AW, Caldwell CW. A population study of neutering status as a risk factor for canine prostate cancer. *Prostate.* (2007) 67:1174–81. doi: 10.1002/pros.20590
7. Rivera-Calderón LG, Fonseca-Alves CE, Kobayashi PE, Carvalho M, Drigo SA, Vasconcelos RO, et al. Alterations in PTEN, MDM2, TP53 and AR protein and gene expression are associated with canine prostate carcinogenesis. *Res Vet Sci.* (2016) 106:56–61. doi: 10.1016/j.rvsc.2016.03.008
8. Harting T, Stubbendorff M, Willenbrock S, Wagner S, Schadzek P, Ngezahayo A, et al. The effect of dichloroacetate in canine prostate adenocarcinomas and transitional cell carcinomas in vitro. *Int J Oncol.* (2016) 49:2341–50. doi: 10.3892/ijo.2016.3720
9. Schille JT, Nolte I, Packeiser EM, Wiesner L, Hein JI, Weiner F, et al. Isoquinolinamine FX-9 exhibits anti-mitotic activity in human and canine prostate carcinoma cell lines. *Int J Mol Sci.* (2019) 20:5567. doi: 10.3390/ijms20225567
10. Bhanot A, Sharma R, Noolvi MN. Natural sources as potential anti-cancer agents: a review. *Int J Phytomed.* (2011) 3:9–26.
11. Cragg GM, Newman DJ. Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta.* (2013) 1830:3670–95. doi: 10.1016/j.bbagen.2013.02.008
12. Brandao HN, DavidII JP, CoutoII RD, NascimentoII JAP, David JM. Química e farmacologia de quimioterápicos antineoplásicos derivados de plantas. *Quím Nova.* (2010) 33:1359–69. doi: 10.1590/S0100-40422010000600026
13. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Products.* (2012) 75:311–35. doi: 10.1021/np200906s
14. Shukla SI, Mehta A. Anticancer potential of medicinal plants and their phytochemicals: a review. *Braz J Bot.* (2015) 38:199–210. doi: 10.1007/s40415-015-0135-0
15. Ruiz-Torres V, Encinar JA, Herranz-López M, Pérez-Sánchez A, Galiano V, Barrajón-Catalán E, et al. An updated review on marine anticancer compounds: the use of virtual screening for the discovery of small-molecule cancer drugs. *Molecules.* (2017) 22:1037. doi: 10.3390/molecules22071037
16. Calcabrini C, Catanzaro E, Bishayee A, Turrini E, Fimognari C. Marine sponge natural products with anticancer potential: an updated review. *Mar Drugs.* (2017) 15:310. doi: 10.3390/md15100310
17. Kotecha R, Takami A, Espinoza JL. Dietary phytochemicals and cancer chemoprevention: a review of the clinical evidence. *Oncotarget.* (2016) 7:52517–29. doi: 10.18632/oncotarget.9593
18. Vinod BS, Maliekal TT, Anto RJ. Phytochemicals as chemosensitizers: from molecular mechanism to clinical significance. *Antioxid Redox Signal.* (2013) 18:1307–48. doi: 10.1089/ars.2012.4573
19. Valli M, Russo HM, Bolzani VS. The potential contribution of the natural products from Brazilian biodiversity to bioeconomy. *An Acad Bras Ciênc.* (2018) 90:763–78. doi: 10.1590/0001-3765201820170653
20. Ortêncio WB. *Medicina popular do centro-oeste*. Thesaurus Editora (1997). p. 459.
21. Costa LLG, David VC, Pinto RMC, Minozzo BR, Kozlowski VA Jr, Campos LA, et al. Anti-ulcer activity of *Synadenium grantii* latex. *Rev Bras Farmacogn.* (2012) 22:1070–8. doi: 10.1590/S0102-695X2012005000050
22. Hassan EM, Mohammed M, Mohamed SM. Two new phorbol-type diterpene esters from *Synadenium grantii* Hook F. *Leaves Rec Nat Prod.* (2012) 6:255–62. Available online at: https://acgpubs.org/doc/2018080619493836-RNP-1102-510.pdf
23. Nogueira IAL, Leão ABB, Vieira MS, Benfica PL, Cunha LC, Valadares MC. Antitumoral and antiangiogenic activity of *Synadenium umbellatum* Pax. *J Ethnopharmacol.* (2008) 120:474–8. doi: 10.1016/j.jep.2008.08.026
24. Sadeghi-Aliabadi H, Sajjadi SE, Khodamoradi M. Cytotoxicity of Euphorbia macroclada on MDA-MB-468 breast cancer cell line. *Iranian J Pharm Sci.* (2009) 5:103–8. Available online at: http://www.ijps.ir/article_2083_bcd36e8f68b2d1817e5e634928ba6e8d.pdf
25. Oliveira TL, Munhoz ACM, Lemes BM, Minozzo BR, Nepel A, Barison A, et al. Antitumoural effect of *Synadenium grantii* Hook f. (*Euphorbiaceae*) latex. *J Ethnopharmacol.* (2013) 150:263–9. doi: 10.1016/j.jep.2013.08.033
26. Ravicini S, Baines SJ, Taylor A, Amores-Fuster I, Mason SL, Treggiari E. Outcome and prognostic factors in medically treated canine prostatic carcinomas: a multi-institutional study. *Vet Comp Oncol.* (2018) 16:450–8. doi: 10.1111/vco.12400
27. Luz LEC, Paludo KS, Santos VLP, Franco CRC, Klein T, Silva RZ, et al. Cytotoxicity of latex and pharmacobotanical study of leaves and stem of *Euphorbia umbellata* (Janaúba). *Rev Bras Farmacogn.* (2015) 25:344–52. doi: 10.1016/j.bjp.2015.07.005
28. AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA. *Farmacopeia Brasileira, Vol. 1*. 6ª Ed. Brasília (2019). Available online at: https://www.gov.br/anvisa/pt-br/assuntos/farmacopeia/farmacopeia-brasileira/arquivos/7985json-file-1 (accessed March 18, 2021).
29. Campos A, Vendramini-Costa DB, Longato GB, Zermiani T, Ruiz ALTG, Carvalho JE, et al. Antiproliferative effect of *Synadenium grantii* Hook f. stems (*Euphorbiaceae*) and a rare phorbol diterpene ester. *Int J Toxicol.* (2016) 35:666–71. doi: 10.1177/1091581816659660
30. Costa CD, Justo AA, Kobayashi PE, Story MM, Palmieri C, Laufer-Amorim R, et al. Characterization of OCT3/4, Nestin, NANOG, CD44 and CD24 as stem cell markers in canine prostate cancer. *Int J Biochem Cell Biol.* (2019) 108:21–8. doi: 10.1016/j.biocel.2019.01.002
31. Chaves DA, Lemes SR, Araujo LA, Sousa MAM, Freitas GB, Lino RS Jr, et al. Avaliação da atividade angiogênica da solução aquosa do barbatimão (*Stryphnodendron adstringens*). *Rev Bras Plantas Med.* (2016) 18:524–30. doi: 10.1590/1983-084X/15_093
32. Carneiro CC, Santos SC, Lino RS Jr, Bara MTF, Chaibub BA, Melo-Reis PR, et al. Chemopreventive effect and angiogenic activity of punicalagin isolated from leaves of *Lafoensia pacari* A. St-Hil. *Toxicol Appl Pharmacol.* (2016) 310:1–8. doi: 10.1016/j.taap.2016.08.015
33. Flora do Brasil (2020). *Jardim Botânico do Rio de Janeiro*. Available online at: http://floradobrasil.jbrj.gov.br/ (accessed March 18, 2021).
34. Premaratna A, Shdaksharaswamy M, Nanjappa S. Some biological properties of *Synadenium grantil* lectin. *Indian J Pathol Microbiol.* (1984) 27:91–7.
35. Dawidar AAM, Keshk EM, Saad HH, Mogib MA. GC/MS analysis of sesquiterpenes in *Synadenium grantii*. *Man J Chem.* (2011) 38:107–19.
36. Kanunfre CC, Leffers T, Cruz LS, Luz LEC, Crisma AR, Wang M, et al. *Euphorbia umbellata* bark extracts–an in vitro cytotoxic study. *Rev Bras Farmacogn.* (2017) 27:206–13. doi: 10.1016/j.bjp.2016.11.002
37. Luz LEC, Kanunfre CC, Paludo KS, Justo AS, Petry VK, Lemes BM, et al. Cytotoxic biomonitored study of *Euphorbia umbellata* (Pax) Bruyns. *J Ethnopharmacol.* (2016) 183:29–37. doi: 10.1016/j.jep.2016.02.025
38. He J, Li CL, Wilson BC, Fisher CJ, Ghai S, Weersink RA. A clinical prototype transrectal diffuse optical tomography (TRDOT) system for *in vivo* monitoring of photothermal therapy (PTT) of focal prostate cancer. *IEEE Trans Biomed Eng.* (2020) 67:2119–29. doi: 10.1109/TBME.2019.2955354

39. Wang-Leandro A, Willmitzer F, Karol A, Porcellini B, Kronen P, Hiltbrand EM, et al. MRI-guided, transrectal, intraprostatic steam application as potential focal therapeutic modality for prostatic diseases in a large animal translational model: a feasibility follow-up study. *PLoS ONE.* (2019) 14:e0226764. doi: 10.1371/journal.pone.0226764
40. Liu W, Sender S, Kong W, Beck J, Sekora A, Bornemann-Kolatzki K, et al. Establishment and characterization of stable red, far-red (fR) and near infra-red (NIR) transfected canine prostate cancer cell lines. *Cancer Cell Int.* (2020) 20:1–11. doi: 10.1186/s12935-020-01211-0
41. Melo-Reis PR, Andrade LS, Silva CB, Araújo LMM, Pereira MS, Mrue F, et al. Angiogenic activity of *Synadenium umbellatum* Pax latex. *Braz J Biol.* (2010) 70:189–94. doi: 10.1590/S1519-69842010000100026
42. Tariq M, Rossiter JT, Wright DJ, Staley JT. Drought alters interactions between root and foliar herbivores. *Oecologia*. (2013) 172:1095-104. doi: 10.1007/s00442-012-2572-9
43. Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L. Plant polyphenols: chemical properties, biological activities, and synthesis. *Angew Chem Int Ed Engl.* (2011) 50:586–621. doi: 10.1002/anie.201000044
44. Leav I, Schelling KH, Adams JY, Merk FB, Alroy J. Role of canine basal cells in prostatic post natal development, induction of hyperplasia, sex hormone-stimulated growth; and the ductal origin of carcinoma. *Prostate.* (2001) 48:210–24. doi: 10.1002/pros.1100
45. LeRoy BE, Nadella MVP, Toribio RE, Leav I, Rosol TJ. Canine prostate carcinomas express markers of urothelial and prostatic differentiation. *Vet Pathol.* (2004) 41:131–40. doi: 10.1354/vp.41-2-131
46. Cunha LC, Azeredo SF, Mendonça ACV, Vieira MS, Pucci LL, Valadares MC, et al. Avaliação da toxicidade aguda e subaguda, em ratos, do extrato etanólico das folhas e do látex de *Synadenium umbellatum* Pax. *Rev Bras Farmacogn.* (2019) 19:403–11. doi: 10.1590/S0102-695X2009000300012
47. Da Mota MF, Benfica PL, Batista AC, Martins FS, Paula JR, Valadares MC. Investigation of Ehrlich ascites tumor cell death mechanisms induced by *Synadenium umbellatum* Pax. *J Ethnopharmacol.* (2012) 139:319–29. doi: 10.1016/j.jep.2011.04.055
48. Oliveira RB, Cunha LC, Valadares MC, Filho MJP, Araújo DM, Paula JR, et al. Toxicidade aguda de látex e extrato etanólico de folhas de *Synadenium umbellatum* em camundongos. *Rev Eletrôn Farm.* (2005) 2:143–5. Available online at: https://revistas.ufg.br/REF/article/view/1998/1966

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Blood-Based Liquid Biopsy for Comprehensive Cancer Genomic Profiling Using Next-Generation Sequencing: An Emerging Paradigm for Non-invasive Cancer Detection and Management in Dogs

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This proof-of-concept study demonstrates that blood-based liquid biopsy using next generation sequencing of cell-free DNA can non-invasively detect multiple classes of genomic alterations in dogs with cancer, including alterations that originate from spatially separated tumor sites. Eleven dogs with a variety of confirmed cancer diagnoses (including localized and disseminated disease) who were scheduled for surgical resection, and five presumably cancer-free dogs, were enrolled. Blood was collected from each subject, and multiple spatially separated tumor tissue samples were collected during surgery from 9 of the cancer subjects. All samples were analyzed using an advanced prototype of a novel liquid biopsy test designed to non-invasively interrogate multiple classes of genomic alterations for the detection, characterization, and management of cancer in dogs. In five of the nine cancer patients with matched tumor and plasma samples, pre-surgical liquid biopsy testing identified genomic alterations, including single nucleotide variants and copy number variants, that matched alterations independently detected in corresponding tumor tissue samples. Importantly, the pre-surgical liquid biopsy test detected alterations observed in spatially separated tissue samples from the same subject, demonstrating the potential of blood-based testing for comprehensive genomic profiling of heterogeneous tumors. Among the three patients with post-surgical blood samples, genomic alterations remained detectable in one patient with incomplete tumor resection, suggesting utility for non-invasive detection of minimal residual disease following curative-intent treatment. Liquid biopsy allows for non-invasive profiling of cancer-associated genomic alterations with a simple blood draw and has potential to overcome the limitations of tissue-based testing posed by tissue-level genomic heterogeneity.

Keywords: cell-free DNA, dog, cancer, tumor, genomic, liquid biopsy, circulating tumor DNA, mutation

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INTRODUCTION

Non-invasive blood-based genomic profiling, often called liquid biopsy, is increasingly employed in human medicine to support cancer diagnosis and management (1, 2). It refers to the measurement of circulating biomarkers present in blood that can be used to study the genomic profiles of underlying conditions (e.g., cancer). The most commonly used form of liquid biopsy is based on analysis of cell-free DNA (cfDNA) in plasma; cfDNA is comprised of DNA fragments released by cells into the circulation through secretion, apoptosis, or necrosis (3), including cfDNA derived from tumor cells (which is known as circulating tumor DNA or ctDNA). It has been demonstrated in studies of human cancer that plasma cfDNA analysis can recapitulate the genomic profile of the underlying tumor tissue (4, 5). Additionally, it offers distinct advantages through its ability to capture the diversity of mutations derived from spatially separated sites within a primary tumor or across metastatic deposits (a fundamental biological feature of cancer, referred to as "tumor heterogeneity") that may be missed by testing a single tissue sample from a single tumor site (6–8). Liquid biopsy enables genomic profiling of cancer cases that are difficult to biopsy, and its non-invasive nature allows it to be performed safely and repeatedly for disease monitoring. Liquid biopsy has also shown considerable potential as a multi-cancer early detection test that can be used at regular intervals (e.g., annually) for cancer screening in high-risk populations, where detection of the disease at earlier stages can result in improved clinical outcomes (9–12).

A number of studies have demonstrated the presence of ctDNA in canine plasma across multiple cancer types (13–16). The general strategy for detecting tumor-specific cfDNA relies on identifying genomic alterations specific to cancer cells and absent in healthy cells (17). Different cancer types in humans are driven by different classes of genomic alterations (18), a phenomenon that is encountered in canine cancers as well: for instance, single nucleotide variants (e.g., in the *KIT* gene) are frequently found in mast cell tumors (19) whereas copy number variants (gains and losses) are often encountered in canine sarcomas (20).

Here, we report results generated using an advanced prototype of a novel blood-based liquid biopsy assay (hereinafter "the test") specifically developed for the non-invasive detection, characterization, and management of cancer in dogs. This is the first report of a next-generation sequencing (NGS) based multi-cancer early detection test designed to simultaneously interrogate multiple classes of cancer-associated genomic alterations, including single nucleotide variants (SNVs), insertions and deletions (indels), translocations, and copy number variants (CNVs), in cfDNA extracted from canine blood samples. This proof-of-concept study aims to demonstrate the potential of liquid biopsy to broadly profile tumor-derived genomic alterations, and to capture the genomic heterogeneity of multiple, spatially separated tumor clones (within the same primary site or across different metastatic sites) that would be missed by traditional single-site tissue biopsy, suggesting broad utility as a diagnostic tool in veterinary oncology.

METHODS

Eleven client-owned dogs with a variety of confirmed cancer diagnoses (including localized/regional as well as disseminated/metastatic disease) that were scheduled for surgical resection, and five dogs presumed to be cancer-free (no history of cancer and no clinical signs indicative of cancer at the time of enrollment), were enrolled across multiple care settings. All cancer subjects were enrolled under a dedicated protocol that was independently approved by the institutional animal care and use committee (IACUC) or internal review board at two veterinary centers, and all cancer-free subjects were enrolled under a separate dedicated protocol that was independently approved by the institutional clinical review board at one veterinary center; the protocols were also approved by institutional clinical review boards at the other participating sites, based on each site's requirements. All subjects were client-owned, and written informed consent was obtained from all owners.

From each patient, a baseline blood sample was collected at the time of enrollment. In 9 of the 11 patients with cancer, samples of resected tumor specimens from one or more sites within each primary tumor, and from one or more metastatic sites if available, were collected during the subsequent surgery. In three cancer patients, a post-surgical blood sample was collected 10, 15, and 189 days after surgery, respectively. Clinical and demographic data, plasma volumes, and cfDNA yields for all enrolled subjects are summarized in **Table 1**.

Blood samples were processed with a double-centrifugation protocol to separate plasma from white blood cells (WBCs) (21–23). CfDNA was extracted from plasma using a proprietary bead-based chemistry protocol optimized to maximize cfDNA yield in canine subjects. DNA from WBC and tumor tissue samples was extracted using QIAamp DNA Mini Blood Kit (Qiagen). Sequencing libraries were generated from DNA extracted from matched tumor (if available), plasma, and WBC samples for each subject. Libraries were prepared by incorporating universal adapters and barcodes into sample DNA via ligation and universal PCR amplification. Amplified libraries were subjected to genome-wide sequencing for CNV analysis. In parallel, the libraries underwent enrichment with a proprietary method targeting selected regions of the canine genome for SNV analysis. All libraries were sequenced using an Illumina NovaSeq 6000.

All sequencing reads were aligned to the CanFam3.1 reference genome (24), and somatic variant calling was performed with a custom bioinformatics pipeline. For subjects who had only a baseline plasma sample available for testing, a proprietary algorithm to detect ctDNA based on fragment length profiles was also applied.

RESULTS

In the nine cancer patients that had tumor tissues analyzed, all nine had somatic alterations detected in tissue, based on aggregate variant calls across all available tissue samples (**Supplementary Table 1**). In 5 of these subjects, a subset of these variants was also independently detected in the matched pre-operative plasma sample. In two cancer patients without

TABLE 1 | Patient demographics and clinical history.

Subject ID	Age (years)	Sex	Weight (kg)	Reported breed	Plasma volume (ml)	Plasma cfDNA yield (ng)	Primary diagnosis	Cancer status	Lymph node involvement (yes/no)	Tumor size (cm)
Cancer patients										
PT01	12	MI	9.1	West Highland White Terrier	0.6	3.1	Cystic renal carcinoma	Localized/ Regional	No	>5
PT02	15	FS	7.3	American Eskimo Dog	8.0	6.5	Cholangiocellular carcinoma	Localized/ Regional	No	>5
PT03	14	FS	18.6	Mixed	4.9	6.8	Metastatic pancreatic carcinoma; hepatocellular carcinoma; metastatic splenic hemangiosarcoma	Disseminated/ Metastatic	No	>5
PT04	11	FS	23.2	Border Collie	8.0	4.1	Anal sac adenocarcinoma	Localized/ Regional	Yes	>5
PT05	12	MN	27.3	Mixed	8.0	5.7	Bilateral anal sac adenocarcinoma	Localized/ Regional	Yes	<2
PT06	10	MN	40.0	Mixed	6.6	4.2	Multifocal mast cell tumor	Localized/ Regional	No	>5
PT07	11	MN	36.4	Labrador	8.0	1.7	Anal sac adenocarcinoma	Localized/ Regional	Yes	<2
PT08	9	FS	29.1	Mixed	8.0	2.9	Soft tissue sarcoma	Localized/ Regional	No	2-5
PT09	13	FS	23.2	Mixed	5.6	3.3	Multifocal soft tissue sarcoma	Localized/ Regional	No	>5
PT10	8	MN	27.7	Mixed	9.5	1.4	Osteosarcoma	Localized/ Regional	No	2-5
PT11	10	FS	24.1	Goldendoodle	6.0	2.8	Hemangiosarcoma (renal, splenic)	Disseminated/ Metastatic	No	>5
Presumably cancer-free subjects*										
PT12	10	MN	18.6	Cardigan Welsh Corgi	0.5	0.4		NA		
PT13	10	MN	7.3	Miniature Poodle	5.5	2.2		NA		
PT14	13	MN	19.5	Mixed	7.5	2.1		NA		
PT15	10	MI	53.6	Mixed	5.0	2.9		NA		
PT16	13	FS	16.8	Basenji	6.3	2.5		NA		

*NA, not applicable; FI, female, intact; FS, female, spayed; MI, male, intact; MN, male, neutered. "Localized/Regional" for solid tumors designates cancer that is limited to the organ of origin or to nearby lymph nodes, tissues, or organs; "Localized/Regional" for lymphomas designates cancer that is limited to a single lymph node (Stage I) or multiple lymph nodes on one side of the diaphragm (Stage II). "Disseminated/Metastatic" for solid tumors designates cancer that has spread to areas of the body distant from the primary tumor; "Disseminated/Metastatic" for lymphomas designates cancer that involves two or more lymph nodes on both sides of the diaphragm and/or one or more extra-nodal sites (Stages III, IV, and V); "Disseminated/Metastatic" also includes all non-lymphoma hematological malignancies. *Subjects presumed to be cancer-free due to no history of cancer and no clinical signs consistent with cancer at the time of blood collection.*

a tumor tissue sample available for analysis, both had somatic alterations detected in their respective pre-operative plasma sample, and both were also classified as cancer-positive by fragment length profiling. Among the seven plasma-positive cancer subjects, two had disseminated/metastatic disease and five had localized/regional disease (one with adjacent lymph node involvement and four without lymph node involvement). All four remaining cancer subjects who had no variants detected in pre-operative plasma had localized disease (two with lymph node involvement and two without lymph node involvement). No SNV or CNV alterations were detected in plasma samples from any of the five presumably cancer-free dogs, and all were also classified as cancer-negative by fragment length profiling. Full subject-level results are summarized in **Supplementary Table 1**.

The test detected genomic alterations in genes such as *TP53, KRAS, EGFR,* and *PIK3CA* that are frequently associated with cancer in both humans and dogs (25, 26). Homologs of some of these alterations are clinically actionable in humans; for example, both the plasma and the tissue of a cholangiocarcinoma patient (PT02) featured an *EGFR* p.L805R mutation, which is homologous to the human *EGFR* p.L858R mutation, a common target for EGFR tyrosine kinase inhibitors (27). We also detected mutations in genes that are more specifically associated with canine cancer, such as *PTPN11, PDGFRA,* and *PDGFRB;* in particular, the PDGFR protein is a target for the multikinase

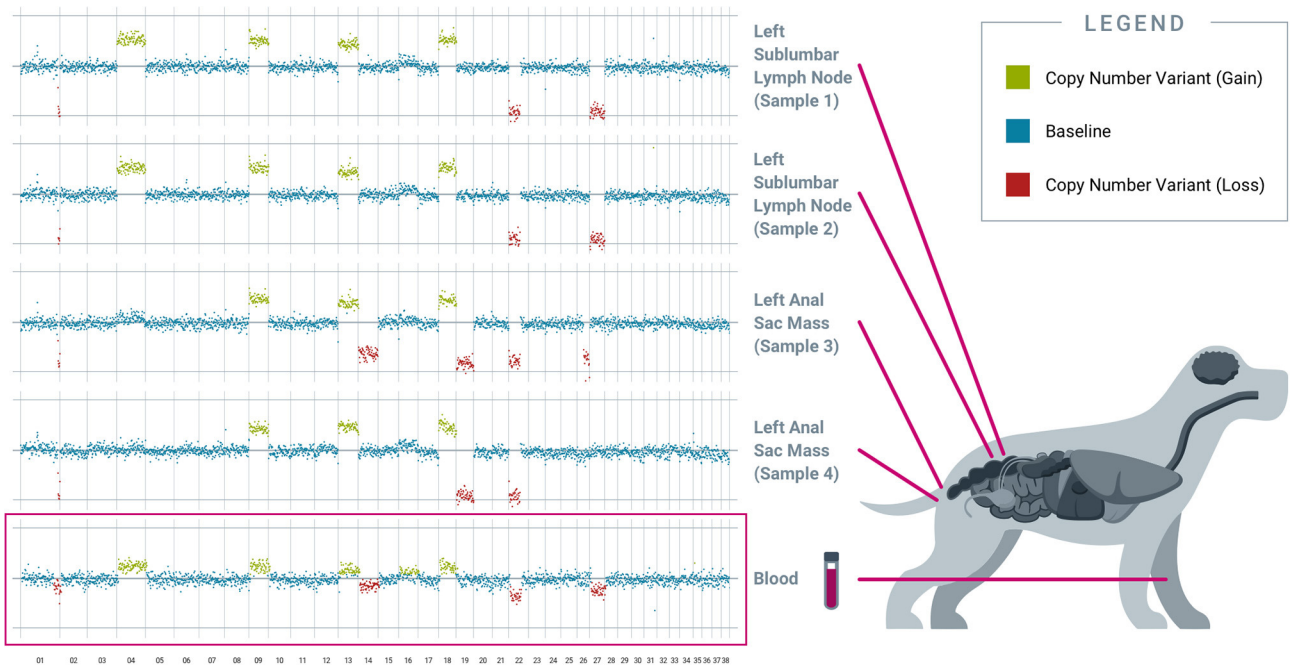

FIGURE 1 | *Individual tissue samples reveal distinct somatic copy number alterations, collectively captured in the blood-derived pre-operative plasma.* Results from PT04, an 11-year-old female spayed Border Collie diagnosed with apocrine gland anal sac adenocarcinoma of the left anal sac with metastasis to a left sublumbar lymph node, are shown. Four different tumor samples were collected from spatially separated locations at two sites: the left sublumbar lymph node (two samples) and the left anal sac mass (two samples). Copy number variation analysis revealed both inter-site and intra-site tumor heterogeneity. Plasma cfDNA captured shared and private alterations from both tumor sites. All chromosomes shown were interrogated for cancer-associated genomic alterations by the test.

inhibitor toceranib phosphate (Palladia™) (28, 29), which is commonly used in the treatment of canine cancers.

Genomic profiling of spatially separated tumor tissue samples from the same subject demonstrated genomic heterogeneity across sites within the primary mass as well as across distant sites, in keeping with the polyclonal nature of cancer (17); and these different genomic alterations were collectively captured in plasma. For example, in PT04 (diagnosed with anal sac adenocarcinoma with lymph node metastases), tissue samples were collected from two different locations within the left anal sac primary tumor, and two different locations within the left sublumbar lymph node. Four CNVs (on chromosomes 9, 13, 18, 22) are shared across all four samples, while other CNVs are sample-specific: e.g., the CNVs on chromosomes 4 and 27 are specific to the two lymph node samples, while the CNV on chromosome 19 is specific to the two primary tumor samples. The corresponding pre-operative plasma captured most of this heterogeneity, with both shared and private (site-specific) CNV alterations from the various tumor samples reflected in cfDNA (**Figure 1**).

Apart from capturing tumor heterogeneity, the test was also able to identify residual disease in plasma after incomplete surgical resection. PT03 was simultaneously diagnosed with multiple primary cancers, including metastatic pancreatic carcinoma, hemangiosarcoma of the liver and spleen, and hepatocellular carcinoma; at surgery, five tissue samples representing primary and metastatic tumor deposits were obtained for testing. Four somatic alterations, including one SNV in *NRAS*, one SNV in *KRAS*, and two distinct SNVs in *TP53*, were identified across four of the five tissue samples (**Figure 2**), and multiple CNV alterations were also identified in most of the tissue samples. Three of the four SNVs were also detected in the pre-operative plasma sample, along with the majority of the CNV alterations. Complete histologic resection of the hepatic hemangiosarcoma, splenic hemangiosarcoma, and hepatocellular carcinoma were achieved; however, the metastatic pancreatic carcinoma was incompletely excised. This was consistent with the observation of persistent CNV and SNV alterations in the post-operative plasma sample, confirming the presence of residual disease (**Figure 3A**). For comparison, curative-intent resection of a cystic renal carcinoma in PT01 was complete, and the post-operative plasma sample showed no evidence of any of the somatic alterations that had been present in the pre-operative plasma or the resected tumor (**Figure 3B**).

DISCUSSION

This proof-of-concept study is the first to demonstrate the feasibility of using blood-based liquid biopsy to non-invasively detect multiple classes of genomic alterations in dogs with cancer, in both localized and metastatic settings. An advanced prototype of a novel liquid biopsy test, designed to interrogate multiple classes of cancer-associated genomic alterations across the canine genome, revealed alterations in plasma that were shared between different tumor sites as well as alterations that were private

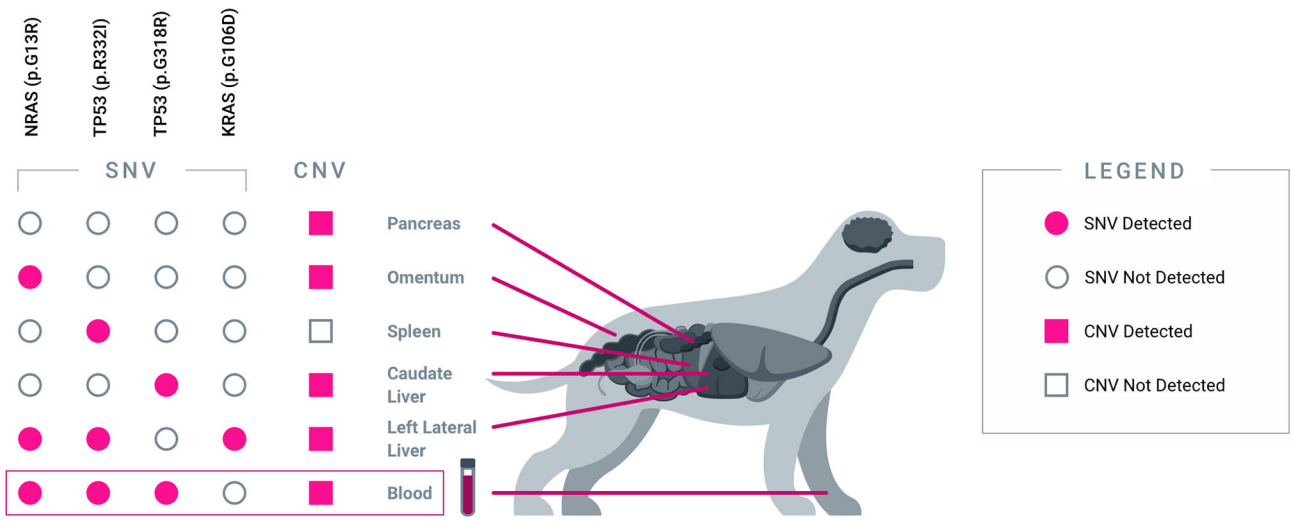

FIGURE 2 | *Blood-derived plasma cfDNA captures heterogeneous alterations from five spatially separated sites across multiple organs.* Results from PT03, a 14-year-old female spayed mixed breed dog diagnosed with multiple cancers are shown. Five tissue samples were collected from five distinct tumor sites: pancreas (pancreatic carcinoma), omentum (metastatic pancreatic carcinoma), spleen (hemangiosarcoma), caudate liver (metastatic hemangiosarcoma), and left lateral liver (hepatocellular carcinoma). Each tumor sample revealed a unique combination of genomic alterations (SNVs and/or CNVs), some of which were shared across multiple sites. The majority of SNV and CNV alterations were collectively captured in pre-operative plasma cfDNA.

FIGURE 3 | *Analysis of post-operative blood-derived plasma cfDNA reveals residual genomic alterations after incomplete resection of tumor.* Genomic alterations were detected in post-op blood samples from tumors with incomplete tumor resection but not in those with complete resection. **(A)** depicts pre- and post-operative plasma CNV traces from PT03, a 14-year-old female spayed mixed breed dog that had incompletely excised metastatic pancreatic carcinoma. **(B)** depicts pre- and post-operative plasma CNV traces from PT01, a 12-year-old male intact West Highland White Terrier that had completely excised cystic renal carcinoma. All chromosomes shown were interrogated for cancer-associated genomic alterations by the test.

to individual tumor sites within a subject; these observations highlight the potential of plasma cfDNA analysis by NGS to capture the genomic heterogeneity of spatially separated tumors within individual canine cancer patients.

Importantly, among the seven patients for whom cancer was detected from blood, three had only one class of genomic alteration (either SNV or CNV) detected, suggesting that profiling multiple classes of genomic alteration improves overall

detection of tumor-derived cfDNA compared to relying on just one class. Among patients for whom cancer was not detected in blood, all had localized disease; these results are consistent with the observation in humans that the proportion of patients with detectable tumor-derived cfDNA in plasma is higher in metastatic disease compared to localized disease (30). Larger studies are needed to confirm these observations in dogs.

Furthermore, our results suggest utility for liquid biopsy in detecting minimal residual disease (MRD) following curative-intent surgery. After a complete resection, tumor-specific genomic alterations should no longer be present in plasma cfDNA, whereas such alterations would persist—potentially at lower levels—following an incomplete resection.

The ability of liquid biopsy to detect genomic alterations originating from one or multiple tumor sites within a patient offers several advantages compared to traditional tissue-based testing. First, it is non-invasive and thus poses a lower risk to patients compared to biopsy or surgery. Second, it allows for a more comprehensive characterization of the full genomic landscape of the patient's malignancy, across multiple sites within the primary tumor and across metastatic deposits. This tissue-level heterogeneity, which reflects the polyclonal nature of most clinical cancers, would be otherwise difficult to evaluate in dogs due to the morbidity and expense associated with obtaining multiple tissue samples from the primary as well as metastatic sites. Furthermore, obtaining multiple tissue samples is generally not performed in dogs with gross metastatic disease, as aggressive therapy is not typically pursued in these individuals, and the invasiveness of collecting samples from metastatic lesions would cause an unacceptable level of morbidity in a patient with advanced cancer (31). A blood test to characterize and monitor malignant disease, regardless of its location(s) within the body, is less burdensome and facilitates serial testing for longitudinal monitoring of the disease.

Liquid biopsy testing can support multiple applications across the continuum of canine cancer care, including: screening (for early detection) in high-risk populations, aid in diagnosis, detection of MRD, selection of targeted therapies, and monitoring for cancer recurrence or for treatment response by serial testing; and promises to bring the power of precision oncology to veterinary practice through a simple blood draw that does not require changes to the clinical routine (17). Finally, a liquid biopsy test covering cancer-associated regions of the genome that have high homology between dogs and humans can enable identification of somatic alterations in dogs that have clinically actionable human homologs. Such insights gained from liquid biopsy based genomic profiling could be used to speed the adoption of targeted human cancer therapeutics for the treatment of canine cancer.

Widespread access to multi-cancer early detection testing would be highly beneficial for canine patients, given that cancer is by far the leading cause of death in dogs (32). The current results demonstrate the value of interrogating multiple classes of genomic alterations simultaneously in order to improve sensitivity for detection of cancer. Furthermore, to our knowledge these results are the first to suggest that fragment length profiling may provide utility for cancer detection in dogs. It is important to note that an advanced prototype version of the test was employed in the current study, and further validation in larger cohorts of cancer-diagnosed and cancer-free subjects will be required to fully characterize the analytical performance as well as the clinical sensitivity and specificity of the test.

CONCLUSION

Blood-based liquid biopsy testing—first used for non-invasive prenatal testing (33) and more recently for cancer detection and management—is becoming increasingly common in human medicine. Historically, successful advances in human medicine have been quickly adopted for veterinary use after demonstration of utility in companion animals. This study represents the first-ever application of liquid biopsy to simultaneously profile multiple classes of genomic alterations using next-generation sequencing for the non-invasive detection and characterization of cancer in dogs, an important first step toward demonstrating such utility. Additionally, we demonstrate that canine cancers, like human cancers, exhibit significant intra-patient genomic heterogeneity at the tissue level. The sampling challenges imposed by this heterogeneity can be significantly mitigated by a non-invasive liquid biopsy testing approach, which provides a comprehensive, systemic view of the genomic landscape of malignant lesions throughout the body, and which can be easily incorporated into the current clinical routine. Finally, we demonstrate the potential utility of the test for detecting MRD by showing that residual somatic genomic alterations in plasma post-surgery correlate with incomplete surgical resection. Taken together, the findings of this study demonstrate that non-invasive liquid biopsy testing has the potential to support multiple use cases across the continuum of cancer care, for the benefit of canine patients.

DATA AVAILABILITY STATEMENT

Restrictions apply to the datasets: The datasets presented in this article are not readily available because they contain proprietary information. Requests to access the datasets should be directed to science@petdx.com.

ETHICS STATEMENT

All cancer-diagnosed subjects were enrolled under a dedicated protocol, which was independently reviewed and approved by the institutional animal care and use committees (IACUCs) at the University of Guelph Ontario Veterinary College and the University of Minnesota Veterinary Medical Center, as well as by institutional clinical review boards at additional sites based on each study site's requirements; all cancer-free subjects were enrolled under a separate dedicated protocol, which was independently approved by the Veterinary Centers for America (VCA) Clinical Studies Institutional Review Board, as well as by institutional clinical

review boards at additional sites based on each study site's requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KK, JC, DG, and AF contributed to the conception and design of the study. LM, RM-P, LH, and AF managed the logistics of patient recruitment and sample collections. KK, PN, GH, DF, JT, IC, and DT generated the data and performed data analysis. KK and DT wrote the first draft of the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.704835/full#supplementary-material

REFERENCES

1. Husain H, Velculescu VE. Cancer DNA in the circulation: the liquid biopsy. *JAMA*. (2017) 318:1272–4. doi: 10.1001/jama.2017.12131
2. Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic — implementation issues and future challenges. *Nat Rev Clin Oncol.* (2021) 18:297–312. doi: 10.1038/s41571-020-00457-x
3. Diaz LA, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol.* (2014) 32:579–86. doi: 10.1200/JCO.2012.45.2011
4. Jahangiri L, Hurst T. Assessing the concordance of genomic alterations between circulating-free DNA and tumour tissue in cancer patients. *Cancers.* (2019) 11:1938. doi: 10.3390/cancers11121938
5. Chae YK, Davis AA, Carneiro BA, Chandra S, Mohindra N, Kalyan A, et al. Concordance between genomic alterations assessed by next-generation sequencing in tumor tissue or circulating cell-free DNA. *Oncotarget.* (2016) 7:65364–73. doi: 10.18632/oncotarget.11692
6. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* (2012) 366:883–92. doi: 10.1056/NEJMoa1113205
7. Russano M, Napolitano A, Ribelli G, Iuliani M, Simonetti S, Citarella F, et al. Liquid biopsy and tumor heterogeneity in metastatic solid tumors: the potentiality of blood samples. *J Exp Clin Canc Res.* (2020) 39:95. doi: 10.1186/s13046-020-01601-2
8. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol.* (2018) 15:81–94. doi: 10.1038/nrclinonc.2017.166
9. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Consortium C, et al. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann Oncol.* (2020) 31:745–59. doi: 10.1016/j.annonc.2020.04.013
10. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science.* (2018) 359:926-930. doi: 10.1126/science.aar3247
11. Hubbell E, Clarke CA, Aravanis AM, Berg CD. Modeled reductions in late-stage cancer with a multi-cancer early detection test. *Cancer Epidemiol Prev Biomarkers.* (2021) 30:460–8. doi: 10.1158/1055-9965.EPI-20-1134
12. Ofman J, Raza A. Taking early cancer detection to the next level. *Sci Am.* (2020) 18:297–312. Available online at: https://www.scientificamerican.com/article/taking-early-cancer-detection-to-the-next-level/
13. Prouteau A, Denis JA, Fornel PD, Cadieu E, Derrien T, Kergal C, et al. Circulating tumor DNA is detectable in canine histiocytic sarcoma, oral malignant melanoma, and multicentric lymphoma. *Sci Rep.* (2021) 11:877. doi: 10.1038/s41598-020-80332-y
14. Tagawa M, Shimbo G, Inokuma H, Miyahara K. Quantification of plasma cell-free DNA levels in dogs with various tumors. *J Vet Diag Invest.* (2019) 31:836-43. doi: 10.1177/1040638719880245
15. Beck J, Hennecke S, Bornemann-Kolatzki K, Urnovitz HB, Neumann S, Ströbel P, et al. Genome aberrations in canine mammary carcinomas and their detection in cell-free plasma DNA. *PLoS ONE.* (2013) 8:e75485. doi: 10.1371/journal.pone.0075485
16. Lorch G, Sivaprakasam K, Zismann V, Perdigones N, Contente-Cuomo T, Nazareno A, et al. Identification of recurrent activating HER2 mutations in primary canine pulmonary adenocarcinoma. *Clin Cancer Res.* (2019) 25:5866–77. doi: 10.1158/1078-0432.CCR-19-1145
17. Chibuk J, Flory A, Kruglyak KM, Leibman N, Nahama A, Dharajiya N, et al. Horizons in veterinary precision oncology: fundamentals of cancer genomics and applications of liquid biopsy for the detection, characterization, and management of cancer in dogs. *Frontiers Vet Sci.* (2021) 8:664718. doi: 10.3389/fvets.2021.664718
18. Ciriello G, Miller ML, Aksoy BA, Senbabaoglu Y, Schultz N, Sander C. Emerging landscape of oncogenic signatures across human cancers. *Nat Genet.* (2013) 45:1127–33. doi: 10.1038/ng.2762
19. Webster JD, Yuzbasiyan-Gurkan V, Kaneene JB, Miller R, Resau JH, Kiupel M. The role of c-KIT in tumorigenesis: evaluation in canine cutaneous mast cell tumors. *Neoplasia.* (2006) 8:104–11. doi: 10.1593/neo.05622
20. Gardner HL, Sivaprakasam K, Briones N, Zismann V, Perdigones N, Drenner K, et al. Canine osteosarcoma genome sequencing identifies recurrent mutations in DMD and the histone methyltransferase gene SETD2. *Commun Biol.* (2019) 2:266. doi: 10.1038/s42003-019-0487-2
21. Kumar M, Choudhury Y, Ghosh SK, Mondal R. Application and optimization of minimally invasive cell-free DNA techniques in oncogenomics. *Tumor Biol.* (2018) 40:1010428318760342. doi: 10.1177/1010428318760342
22. Pérez-Barrios C, Nieto-Alcolado I, Torrente M, Jiménez-Sánchez C, Calvo V, Gutierrez-Sanz L, et al. Comparison of methods for circulating cell-free DNA isolation using blood from cancer patients: impact on biomarker testing. *Transl Lung Cancer Res.* (2016) 5:665–72. doi: 10.21037/tlcr.2016.12.03
23. Sorber L, Zwaenepoel K, Jacobs J, Winne KD, Goethals S, Reclusa P, et al. Circulating cell-free DNA and RNA analysis as liquid biopsy: optimal centrifugation protocol. *Cancers.* (2019) 11:458. doi: 10.3390/cancers11040458

24. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature.* (2005) 438:803–19. doi: 10.1038/nature04338
25. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. *Science.* (2013) 339:1546–58. doi: 10.1126/science.1235122
26. Alsaihati BA, Ho K-L, Watson J, Feng Y, Wang T, Zhao S. Canine tumor mutation rate is positively correlated with TP53 mutation across cancer types and breeds. *Biorxiv [Preprint].* (2020). doi: 10.1101/2020.07.15.205286
27. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to Gefitinib. *N Engl J Med.* (2004) 350:2129–39. doi: 10.1056/NEJMoa040938
28. Berger EP, Johannes CM, Jergens AE, Allenspach K, Powers BE, Du Y, et al. Retrospective evaluation of toceranib phosphate (Palladia®) use in the treatment of gastrointestinal stromal tumors of dogs. *J Vet Intern Med.* (2018) 32:2045–53. doi: 10.1111/jvim.15335
29. Yamazaki H, Miura N, Lai Y-C, Takahashi M, Goto-Koshino Y, Yasuyuki M, et al. Effects of toceranib phosphate (palladia) monotherapy on multidrug resistant lymphoma in dogs. *J Vet Med Sci.* (2017) 79:16–0457. doi: 10.1292/jvms.16-0457
30. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med.* (2014) 6:224ra24. doi: 10.1126/scitranslmed.3007094
31. Withers SS, York D, Choi JW, Woolard KD, Laufer-Amorim R, Sparger EE, et al. Metastatic immune infiltrates correlate with those of the primary tumour in canine osteosarcoma. *Vet Comp Oncol.* (2019) 17:242–52. doi: 10.1111/vco.12459
32. Fleming JM, Creevy KE, Promislow DEL. Mortality in North American dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med.* (2011) 25:187–98. doi: 10.1111/j.1939-1676.2011.0695.x
33. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al. DNA sequencing of maternal plasma to detect down syndrome: an international clinical validation study. *Genet Med.* (2011) 13:913–20. doi: 10.1097/GIM.0b013e3182368a0e

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