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## Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy

### **This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1729218> since 2020-02-20T14:00:56Z

*Published version:*

DOI:10.1007/s00262-019-02360-6

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## 1 Natural occurring cancer in pet dogs as pre-clinical models for cancer immunotherapy

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35 **18 Keywords:** Comparative Oncology; CSPG4; DNA vaccination; melanoma; osteosarcoma; PIVAC 18

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38 **19 Précis:**

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40 Interest in exploiting naturally occurring cancers in dogs as an important predictive tool for human oncology

41

42 **is on the rise. Herein,** we discuss the relevance of these comparative oncology studies and our experience in

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44 the field.

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**1 Abbreviations:**

1			
2	2	BTK	Bruton's tyrosine kinase
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4	3	CAR-T	Genetically engineered T cells with chimeric antigen receptors
5			
6	4	CIs	Checkpoint inhibitors
7	5	COTC	Comparative Oncology Trials Consortium
8			
9	6	CSC	Cancer stem cells
10			
11	7	CSPG4	Chondroitin sulphate proteoglycan 4
12	8	CTLA-4	Cytotoxic T lymphocyte antigen-4
13			
14	9	ECM	Extracellular matrix
15			
16	10	FAK	Focal adhesion kinase
17	11	FDA	Food and Drug Administration
18			
19	12	L-MTP-PE	Liposomal muramyl tripeptide phosphatidyl ethanolamine
20			
21	13	MM	Malignant melanoma
22	14	NCI	National Cancer Institute
23			
24	15	OSA	Osteosarcoma
25			
26	16	PAC-1	Procaspase-activating compound-1
27	17	PD-1	Programmed cell death receptor-1
28			
29	18	PDX	Patients-derived xenograft
30			
31	19	TWT	Triple wild type
32	20	USDA	United States Department of Agriculture
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34	21	WT	Wild-type
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## 1 Abstract

2 Despite the significant progress in tumor prevention, early detection, diagnosis and treatment made over recent  
3 decades, cancer is still an enormous public health challenge all around the world, with the number of people  
4 affected increasing every year. A great deal of effort is therefore being devoted to the search for novel safe,  
5 effective and economically sustainable treatments for the growing population of neoplastic patients. One main  
6 obstacle to this process is the extremely low percentage of therapeutic approaches that, after successfully  
7 passing pre-clinical testing, actually demonstrate activity when finally tested in humans. This disappointing  
8 and expensive failure rate is partly due to the pre-clinical murine models used for *in-vivo* testing, which cannot  
9 faithfully recapitulate the multifaceted nature and evolution of human malignancies. These features are better  
10 mirrored in natural disease models, i.e., companion animals affected by cancers. Herein, we discuss the  
11 relevance of spontaneous canine tumors for the evaluation of the safety and anti-tumor activity of novel  
12 therapeutic strategies before in-human trials, and present our experience in the development of a vaccine that  
13 targets chondroitin sulphate proteoglycan (CSPG)4 as an example of these comparative oncology studies.

## 24 Introduction

25 Since the concept of translational oncology officially emerged from the National Cancer Institute (NCI) of the  
26 United States in 1992, an increasing number of comprehensive mouse models have been developed and used  
27 to test new therapies before their clinical application, strongly consolidating the bridge between basic research  
28 and clinical practice (1). This has greatly contributed to our knowledge of cancer biology and to the improved  
29 clinical outcomes observed for many types of cancer over recent decades. Nevertheless, the survival benefits  
30 achieved are relatively modest, often measurable in months, and the short- and long-term toxicities of therapies  
31 are quite significant and not predicted by pre-clinical testing in mice. Even though phylogenetic and  
32 physiological similarities between mice and humans do exist, experimental therapies tested in murine models  
33 have, all too often, elicited responses that only poorly predict the outcomes of that therapy being translated to  
34 a human setting (2). Indeed, transplantable models, genetically engineered mice and patient-derived xenograft  
35 models have been shown to not accurately mimic the complexity of human cancer, limiting their reliability for  
36 subsequent translational applications (2-4). One of the main criticisms raised is the limited life-span of mice,  
37 which does not allow several fundamental features of the nature of human cancers, i.e., growth over long  
38 periods of time, genomic instability and tumor heterogeneity, to be reproduced (4, 5). Furthermore, the  
39 microenvironment of the tumors that are modeled in mice is quite different from that which characterizes  
40 human neoplastic lesions, resulting in a favorable predictive response to chemo- and radio-therapy (6).  
41 Importantly, from the safety point of view, murine bone marrow is generally less sensitive to the toxicity  
42 induced by chemotherapy than human bone marrow, suggesting that mice are not suitable for use in the  
43 evaluation of the adverse effects of novel chemotherapies or combinatorial approaches with chemotherapeutic  
44 agents (7). Similar considerations can also be made for the response to immunotherapy, which has now become  
45 the fourth pillar of cancer treatment. The discrepancies between the immune systems of mice and humans, in  
46 terms of both innate and adaptive immunity, highlight the concerns raised as to the use of mouse models for

1 the rigorous evaluation of immunotherapeutic strategies (3, 8). Overall, many of these limitations may be  
2 overcome by evaluating novel treatments in companion animals – particularly dogs – that are affected by  
3 naturally occurring malignancies, in accordance with another important concept promoted by the NCI, that of  
4 comparative oncology.

5 The rationale for evaluating therapeutics in domestic tumor-bearing dogs before carrying out in-human studies  
6 will be discussed in the following sections. In particular, the unique opportunity found in assessing, with high  
7 translational value, both the safety and anti-tumor activity of novel immunotherapies in canine patients will be  
8 uncovered, with a specific focus on the comparative oncology studies that we have performed in recent years.

### 9 Why dogs are humans' best friends, even in disease

10 Tumor-bearing dogs capture the “essence” of the problem of cancer in a way that is not achievable with other  
11 animal models (4, 5). This awareness comes from decades of investigations into canine oncology. In 1929, the  
12 Nobel laureate August Krogh was the first to propose the study of diseases that naturally occur in animals and  
13 not just those induced experimentally in laboratory animals (9). However, it took more than 30 years for the  
14 first anti-cancer therapy to be evaluated in dogs (5). From that moment on, the concept of comparative  
15 oncology has spread all over the scientific world.

16 Several different factors have contributed to the solid rationale for the use of naturally occurring cancer in pet  
17 dogs as a translational model for human malignancies. In fact, new dimensions in the comparative oncology  
18 field opened up with the decoding of the canine genome in 2005 (10). Dog-genome sequencing revealed that  
19 all 19,000 identified genes are orthologous, or at least similar, to human genes (11). In particular, comparative  
20 gene expression studies in canine and human tumors have revealed that there is close correspondence in terms  
21 of genetics and molecular markers (4, 12), thus supporting the overlap between canine and human cancer  
22 biology.

23 Cancer incidence in the pet animal population has increased in recent years, due to pets' increased life  
24 expectancy (5). It is estimated that 1 out of 3 people develop cancer; almost the same incidence is estimated in  
25 dogs. For certain tumor types, the incidence is higher in dogs than in humans, and this may be important for  
26 those low occurrence-rate human cancers whose treatment is still an unmet need. In this case, studying the  
27 same tumor in dogs could provide a larger patient population for the evaluation of new strategies, with rapid  
28 enrolment and faster study completion (13). Moreover, canine cancers have shown some breed predispositions,  
29 providing us with an opportunity to understand the genetic links to different types of cancer (13).

30 Tumor initiation and progression processes in both human and dogs are influenced by the same factors,  
31 including age, nutrition, sex and environment (5). Living in close proximity and sharing the same environment  
32 with their owners, dogs show the same pattern of cancer development, and could, therefore, be considered  
33 epidemiologic or etiologic sentinels of the disease (4, 13).

34 Pet tumors grow slowly in an intact immune system, allowing immune and cancer cells to interact for a long  
35 period of time, shaping one each other as well as showing the intratumor heterogeneity and genetic instability  
36 that is typical of human lesions (5). Moreover, cancer development in companion animals resembles the natural  
37 step-wise evolution of human tumors, giving rise to spontaneous recurrences and metastasis. Overall, dog

1 tumors reflect, better than any other animal model, the complex genetic, environmental, and physiological  
2 aspects present in human malignancies (2, 4, 5, 14). An additional and fundamental point for translational  
3 research is the evidence that canine cancer patients often show the same clinical response to conventional  
4 treatments as those observed in human patients. Indeed, it has been demonstrated that several therapeutic  
5 protocols used in human clinics have a similar spectrum of activity in veterinary application (5). Furthermore,  
6 drugs that have failed to give rise to significant effects in humans are also ineffective in dogs (5).

7 All these considerations mean that it is now widely accepted that cancer in canine patients faithfully reproduces  
8 fundamental aspects of the corresponding human malignancies. In fact, on one hand, we have growing  
9 scientific interest in exploiting naturally occurring cancers in dogs as an important predictive tool for human  
10 oncology, and, on the other, there are the owners who are increasingly willing to secure innovative  
11 experimental therapies for their pets (5). The combination of these two considerations contributes to the “one  
12 medicine” concept, opening up possibilities to quite easily investigate innovative therapeutic approaches, with  
13 high translational power for human patients, in client-owned dogs. In this panorama, performing clinical trials  
14 in tumor-bearing companion animals could provide such considerable advantages over conventional pre-  
15 clinical mouse testing that a Comparative Oncology Trials Consortium (COTC) was established at the NCI to  
16 provide the infrastructure and resources needed to integrate veterinary oncology studies into the development  
17 pathways of new therapies for human cancers. More recently, not only veterinary teaching hospitals but also  
18 several private veterinary hospitals are contributing to the “one medicine” practice by providing cutting-edge  
19 options and clinical trials for pet cancer patients.

20 While the patients entering human clinical trials generally have already been treated with standard-of-care  
21 therapies or have a disease in its advanced stages, in-dog trials, also newly diagnosed patients not yet been  
22 exposed to other treatment modalities can be enrolled, especially for those tumors for which standard-of-care  
23 is still inadequate.

24 As a result, clinical trials for pet patients can enhance and accelerate drug-development efforts by providing  
25 unique information that cannot be obtained from traditional pre-clinical models or trials performed directly on  
26 human patients. However, this does not mean that some limitations cannot be envisaged. Using pet as a model  
27 for studying human tumors and the potential of immunotherapeutic approaches entails possible high cost and  
28 long time to get the proper number of canine patients needed for a single veterinary study. Moreover, non-  
29 homogenous results can be obtained due to the influence of the owners when applying post-operative  
30 treatments and following up the study (15). Moreover, a critical point could be related to the difficulties in the  
31 readout of results coming from veterinary immunotherapy trials, since the availability of tools for immune-  
32 monitoring is reduced as compared to those used in traditional inbred mouse model experiments (16).

### 33 **The importance of veterinary clinical trials for translation to human patients**

34 Despite the unquestionable role that murine models have had and still hold for human cancer research, attrition  
35 rates for oncological therapies that move from the pre-clinical stage to human clinics are significantly higher  
36 than those in other therapeutic areas. Indeed, approximately 60% of anti-neoplastic drugs entering Phase III  
37 clinical trials fail, and only around 10% of anti-cancer treatments that proved successful in mice have been

1 approved in human oncology (17). This is even more dramatic if we consider that the development of a new  
2 cancer therapy from discovery to the marketplace is extremely time consuming and expensive. These  
3 disappointing results place the emphasis on the need of a “bridge” between murine models and human clinical  
4 trials, which could increase this success rate and improve our ability to select the safest and most  
5 promising therapeutics to be tested in humans. Because of this and all the previously mentioned  
6 considerations, we and  
7 others support the translational value of oncological canine patients (2, 4). Interestingly, after the NCI  
8 established the Comparative Oncology Program and a European initiative launched the LUPA project to foster  
9 the use of naturally occurring cancer in dogs as a model for human tumors, several companies also  
10 introduced clinical trials in pet patients into their overall work flow, as was highlighted by the National Academies of  
11 Sciences back in 2015 .

12 The use of canine models to evaluate innovative therapies has a long-standing history in other branches of  
13 medicine, with the first successful blood transfusion performed in dogs by Richard Lower in 1666; this  
14 technique was perfected much later, in the early 1900s, again in dogs (18). The 1950s was the turn of surgical  
15 techniques for kidney transplantation and for the reduction of rejection risk, which were refined in dogs before  
16 becoming routine in humans (19). Again, in the 1970s, one of the first clinical trials involving dogs assisted in  
17 the development of a regimen for bone marrow transplantation and then for the treatment of lymphoma canine  
18 patients with chemotherapy and myeloablative radiation (20, 21), leading to clinical protocols that were  
19 then  
20 used in human medical centers. These early examples of studies in pet dogs paved the way for important  
21 achievements in human clinics, and were a foretaste of how veterinary trials could strongly benefit both  
22 species.

23 Soon after, a number of studies performed in canine cancer patients collected proofs of clinical efficacy, dose  
24 definitions and toxicity assessments of anti-cancer drugs in a way that would be impossible to achieve in  
25 murine models. For example, two similar molecules, sunitinib and toceranib, which have been approved for  
26 the treatment of gastrointestinal tumors, renal cell carcinoma and pancreatic neuroendocrine tumors in human  
27 patients, and of mastocytoma in canine patients, were demonstrated to have similar toxicities in the two species,  
28 leading to lethargy, weakness and vomiting that could not be observed so easily in mice (22, 23). Clinical trials  
29 on pet patients can therefore also allow graded and standardized toxicity assessments to be performed.

30 Other interesting examples include recent Phase I/II veterinary trials using Ibrutinib (24), exportin-1, protein  
31 inhibitor KPT-335 (23) and the GS-9219 drug (25); these trials were all helpful in demonstrating not only the  
32 anti-tumor activity of the drugs, but also in giving important clues regarding the toxicity profile and the re-  
33 definition of the dosing schedule prior to human clinical trials.

34 A particularly interesting case is that of Ibrutinib, a Bruton’s tyrosine kinase (BTK) inhibitor. This drug was  
35 proved to be effective in the treatment of lymphoma *in vitro* (8). However, no appropriate *in vivo* murine  
36 models of lymphoma were available to confirm the efficacy of this inhibitor. The availability of pet dogs  
37 bearing naturally occurring lymphomas with sustained B cell receptor signaling was fundamental to the ability  
38 to demonstrate the drug’s clinical efficacy and to identify a useful biomarker for use as an endpoint in human  
39 clinical trials. Moreover, the regimen of Ibrutinib administration in human patients was re-defined thanks to

1 the data, obtained in dogs, on the minimum tolerated and biologically effective dose (8). Another remarkable  
2 story is that of GS-9291, an anti-proliferative nucleotide analog prodrug, which was found to be ineffective in  
3 murine models, while subsequent studies showed that the drug did have effects on canine lymphocytes (26).  
4 When tested in canine patients with hematological malignancies, GS-9291 proved its clinical safety and  
5 efficacy (25), providing the basis for its evaluation in human patients. This molecule was entered into the  
6 process of regulatory approval for veterinary commercialization for the treatment of canine B-cell lymphomas  
7 (27).

8 Another exciting pillar in the comparative oncology field is procaspase-activating compound-1 (PAC-1), a  
9 synthesized chemical product (28), which has now been granted Orphan Drug Designation by the Food and  
10 Drug Administration (FDA) for the treatment of advanced human cancers. PAC-1 is an outstanding paradigm  
11 because of the unique development path that has brought it to the human clinic, since it was first evaluated in  
12 pet dogs with spontaneous cancer to identify the best application for human clinical trials. Indeed, the safety,  
13 tolerability and anti-tumor potential of PAC-1, whether used as a single agent, or in combination with  
14 conventional drugs, was first demonstrated in canine patients (29), leading quite promptly to the approval of a  
15 first human trial (NCT02355535) for the treatment of advanced malignancies, such as breast cancer,  
16 lymphomas, melanomas and other solid tumors. Thanks to the veterinary trials, PAC-1 was also shown to be  
17 able to penetrate the blood-brain barrier, suggesting that this drug may be promising for the treatment of  
18 cancers of the central nervous system (30). All these results drove the approval of an additional clinical trial  
19 for the combination of PAC-1 with temozolomide for the treatment of glioblastoma (NCT03332355).

20 Other fundamental achievements are found in the immune-oncology field. Indeed, as explained above, canine  
21 patients are of extraordinary relevance for the evaluation of immunotherapeutic strategies since tumors  
22 spontaneously develop in an immune-competent environment, and long-lasting and mutual relationships  
23 develop between host immune system and cancer cells.

24 In 2003, Bergman and collaborators started veterinary trials in dogs affected by advanced malignant melanoma  
25 (MM) to exploit the safety, immunogenicity and the anti-tumor potential of a xenogeneic DNA vaccine coding  
26 for the human tyrosinase (31-33). The positive results obtained by these studies, led, in 2010, to the approval,  
27 by the United States Department of Agriculture (USDA), of the first anti-human tyrosinase DNA vaccine  
28 (ONCEPT, Merial) for the treatment of MM-bearing dogs and to a rapid translation of the proposed therapeutic  
29 approach to human clinical trials (34, 35). Even though with the coming out of the most recent results from  
30 multiple veterinary and human trials, the therapeutic efficacy of ONCEPT has been questioned in both species  
31 (36-38), in-human trials demonstrated the safety and immunogenicity profile of the vaccine previously found  
32 in dogs. This is currently the only licensed anti-cancer DNA vaccine in any species and has driven several  
33 groups, including our own (see below), to investigate the translational efficacy of the immune-targeting of  
34 other antigens that are relevant for human and canine cancer tumors (39, 40).

35 The study of another immunomodulatory agent, the liposomal muramyl tripeptide phosphatidyl ethanolamine  
36 (L-MTP-PE), corroborated the valuable potential of canine tumor models for the advancement of human  
37 treatments. L-MTP-PE has been studied because of its ability to activate macrophages and monocytes, which



1 in turn can release proinflammatory cytokines with tumoricidal effects. The first evidence of L-MTP-PE's  
2 potential efficacy in the treatment of osteosarcoma (OSA) came from veterinary studies in OSA-bearing dogs  
3 who showed higher survival when treated with this agent than controls that were treated with the placebo (41).  
4 Considering the strong similarities between canine and human OSA (see below), the results of these veterinary  
5 assessments laid the foundation for L-MTP-PE's evaluation in human clinical studies (NCT00631631,  
6 NCT02441309, NCT03643133) (42-44) and to its approval in Europe for the adjuvant treatment of patients  
7 with non-metastatic, resectable OSA (45). Interestingly, strong anti-metastatic potential was shown when L-  
8 MTP-PE was tested in a mouse model of OSA. However, no increase in survival was observed, unlike findings  
9 that had previously been described in dogs, and this was most likely because OSA progression in mice was  
10 too rapid (46). This confirms the idea that investigating immunotherapy in models that display the slow and  
11 stepwise progression of spontaneous metastatic disease may be of paramount importance for the identification  
12 of a survival benefit, which may be masked when using fast-progressing tumors in mice.  
13 A vaccine named ADXS31-164, which is based on recombinant *Listeria monocytogenes* that express a  
14 chimeric human HER2/neu, has more recently been successfully investigated in canine OSA patients, resulting  
15 in a significant reduction in metastatic disease and increased overall survival (47). Soon afterwards, this  
16 became the first *Listeria*-based vaccine to gain conditional approval for its clinical use in veterinary clinics,  
17 and a Phase I/II trial in human patients (NCT02386501) is ongoing. Many other immunotherapies and  
18 immunotherapeutic combination approaches are now under investigation in well-designed clinical trials using  
19 dogs with cancers, and thus provide increasing amounts of evidence to support the value of comparative  
20 oncology approaches to advance both canine and human oncological patient management (see below).

### 21 **Melanoma and osteosarcoma on the comparative stage**

22 As discussed above, canine oncological patients that spontaneously develop tumors in the same anatomic sites  
23 as humans are an interesting avatar for pre-clinical therapeutic studies endowed with a high translational value  
24 (4). This is particularly true for MM and OSA, which are the two most challenging tumors “under the  
25 microscope” of comparative oncology nowadays.

26 MM is the most aggressive form of skin cancer in humans. It represents the sixth most common cancer  
27 worldwide and its incidence is increasingly rising (2, 48). Several advantages for MM clinical outcome have  
28 undoubtedly been achieved (2) with the introduction of checkpoint inhibitors (CIs), i.e., monoclonal antibodies  
29 directed against the Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) and the Programmed Cell Death Receptor-  
30 1 (PD-1) or its ligands. However, CIs have been proven to work well in an, as yet, unsatisfactory percentage  
31 of patients, the vast majority of whom displayed a pre-existing T-cell mediated immune response against the  
32 tumor (49). A high proportion of MM patients, however, exhibit innate or acquired resistance to CIs and suffer  
33 from disease progression despite the treatment, and most display severe toxicity issues. Improvements and  
34 new therapies are therefore needed to increase the survival of patients. Although pre-clinical mouse models  
35 have contributed to our understanding of the molecular mechanisms of melanoma carcinogenesis, they are  
36 inadequate for the study of novel (immune) therapeutic approaches (2). As a consequence, we, and others,  
37 have looked at spontaneous MM-bearing dogs as models because the canine malignancy shares many

1 characteristics with human MM, including overlapping cytological, histopathological and architectural  
2 features (50). Clinical behavior is another important aspect. Indeed, canine MM comes in a very aggressive  
3 form, as in humans, with a strong resistance to treatment (2, 4). Furthermore, conventional therapies are quite  
4 effective in the early stages of the disease both in canine and human MM patients, but not very  
5 successful in  
6 the advanced stages, with one third of patients experiencing recurrence and metastasis (4). Moreover, once the  
7 tumor has metastasized, the survival rate of canine MM patients after 1-year is only 30%, resembling the  
8 human-patient 5-year survival rate, which is only 15–20% (4, 48). From the genetic point of view, several  
9 alterations and signaling-pathway abnormalities have been found in canine MM, including phosphorylated  
10 forms of AKT and ERK1/2, alterations in KIT and PTEN, which overlap with some of those widely described  
11 in specific human MM subtypes.

12 However, it must be noted that the well-known BRAF<sup>V600E</sup> mutation, which has been widely identified in  
13 almost 60% of human MM, is absent in canine MM, which are universally BRAF wild-type (WT). Moreover,  
14 although MM in dogs can affect a range of anatomical sites, such as the lips, skin and digit/footpad, the oral  
15 MM subtype is the most prevalent clinically significant form affecting dogs. Therefore, canine MM can serve,  
16 in particular, to model human mucosal MM, an aggressive histological subtype that is predominantly BRAF,  
17 RAS and NF1 WT (Triple Wild Type or TWT), with markedly poor survival. The possibility of deeply  
18 investigating this subtype in humans is limited by its very low prevalence, increasing the value of canine MM,  
19 which instead accounts for up to 100,000 diagnoses/year in the United States alone (51). These characteristics  
20 mean that canine oral MM has been proposed as an invaluable pre-clinical model of mucosal, TWT MM and  
21 UV-independent melanomagenesis (52). The consequent identification of novel effective therapies may be  
22 successful for both veterinary and human oncology fields.

23 Another urgent medical need is found in OSA, an aggressive malignancy with poor prognosis and that still has  
24 few therapeutic options (53). OSA is one of the most common malignant bone tumors in both humans and  
25 dogs. Several investigations have brought to light the considerable similarities that exist in OSA biological  
26 behavior in human and canine patients, including an identical site of onset, histology and proclivity for  
27 metastasis (54). Moreover, it has been demonstrated that genomic alterations that have been linked to OSA  
28 pathogenesis and progression are highly conserved in human and canine tumors (13). Moreover, a similar  
29 pattern of response to traditional treatments has been observed in both species. A combination of surgery and  
30 radio- or chemotherapy is the first line treatment and has been shown to enhance the survival time for both  
31 human and dog OSA patients (55). However, for those patients with the metastatic form of the disease, which  
32 is indeed the vast majority, the prognosis remains dismally poor, with a 1-year survival for canine patients (55)  
33 and a 5-year survival for human patients (53) of only about 20%. Therefore, the identification of novel and  
34 effective approaches to improve patient survival is urgently needed. In particular, the use of canine OSA as a  
35 surrogate for pediatric OSA could be of paramount impact. Indeed, there is still a lack of knowledge regarding  
36 the etiology of this tumor and a paucity of therapeutic targets involved in OSA initiation, progression and  
37 development. One of the major challenges to overcome when developing OSA clinical trials in the human  
38 setting is the young age and the low percentage of affected patients. In this condition, the high number of

1 canine OSA patients diagnosed each year offers a tremendous opportunity **that can** accelerate advancements  
2 in the identification of **the** key initiating events **that are** involved in the etiopathogenesis and progression of  
3 OSA, **thus improving** the management of the disease for both humans and dogs.

4 Overall, spontaneously occurring canine MM and OSA **are, in our opinion**, attractive models for the  
5 identification and development of novel therapeutic strategies.

#### 8 **CSPG4: “all for one and one for all”**

10 The power of comparative oncology studies obviously relies on the identification of shared tumor antigens,  
11 **that are significantly** relevant for both human and canine cancers. This would allow unique therapeutic  
12 strategies, which can benefit both species, **to be developed**.

13 **Of the numerous** tumor antigens **that have been** identified so far, our attention **has been focused** on chondroitin  
14 sulphate proteoglycan (CSPG)4. **CSPG4 is restrictedly present in normal healthy tissues, as it was widely**  
15 **stated (56-61) and recently supported by Rivera and colleagues (62) which performed an IHC analysis of an**  
16 **FDA Standard Frozen Tissue Array, including 30 different organs, demonstrating that no CSPG4 expression**  
17 **was found in healthy tissues. Indeed, in adults CSPG4 expression is mainly limited to stem-cells and adult**  
18 **progenitor cells, while it is post-translationally down-regulated at terminal differentiation (63).**

19 **It is becoming increasingly clear from the literature that CSPG4 is implicated in several of the most aggressive**  
20 **and treatment-resistant forms of cancer, including MM, basal-like breast cancers, leukaemia, mesothelioma,**  
21 **glioblastoma, soft-tissue sarcomas, pancreatic carcinoma and squamous-cell carcinoma of the head and neck,**  
22 **where it plays a key, and indispensable, oncogenic role (56, 64). CSPG4 therefore** meets all the requirements  
23 of the definition of “oncoantigen” (40, 65, 66), i.e., **it is** an ideal target for anti-tumor (immuno)therapy.

24 CSPG4 is endowed with multivalent functions, which make it a sort of master regulator of several cancer cell-  
25 associated pathways. A **great deal** of data **have demonstrated** that CSPG4 can be involved in the sustenance of  
26 tumor cell proliferation through its ability to sequester growth factors and concomitantly to associate with the  
27 corresponding receptors to form ternary complexes (56). This has been demonstrated for platelet derived  
28 growth factor AA and several fibroblast growth factors (67). CSPG4 perceive and capture these mitogens,  
29 while promoting ligand-binding and dimerization of the corresponding receptors. In this way, CSPG4 **can**  
30 potentiate the activation of the MAPK pathway, resulting in the selective growth of CSPG4-positive tumor  
31 cells **and providing a survival advantage**. Moreover, its extended extracellular arm **means that** CSPG4 **can** link  
32 different components of the extracellular matrix (ECM), such as tenascin-C, laminin, perlecan and collagens  
33 (types II, V and VI) (68). **Its** strong interplay with ECM molecules suggests **that** CSPG4 **is involved** in optimal  
34 cancer-cell adhesion and migration. **Furthermore,** CSPG4 has been demonstrated to interact with several  
35 integrins, **and thus to cooperate** in the activation of integrin-dependent cellular phenomena, such as cell  
36 proliferation, motility and survival. Filopodial CSPG4 can also sequester plasminogen and **has** consequently  
37 **been** implicated in the control of matrix degradation (69, 70). All these data suggest that binding through the  
38 extracellular portion of CSPG4 **to** a huge variety of molecules in the extracellular space **means that** this unique  
39 proteoglycan **may** be involved in **numerous** steps **in** cancer progression, from sustained proliferation to  
40 migration and invasion. Indeed, the CSPG4 cytoplasmic tail is directly linked to a multitude of different

1 signaling cascades, with the two major involved pathways being PI3K–AKT-1 and focal adhesion kinase  
2 (FAK) (71).

3 As mentioned above, the oncogenic role of CSPG4 in a number of tumor histotypes has recently been revealed.  
4 Nevertheless, the best-established implication is with MM, because of its widespread expression in the  
5 majority of human MM patients (72). In this regard, we have evaluated two publicly available comprehensive  
6 microarray datasets that include gene expression data from 214 samples of primary MM (73) and 44 samples  
7 from MM metastatic lesions (74). Interestingly, we observed, by querying the R2 Kaplan Meier scanner  
8 (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) for prognostic studies, that CSPG4 over-expression in MM  
9 tumors showed a significant correlation with shorter overall survival (Fig. 1a). Furthermore, CSPG4 over-  
10 expression was associated with significantly reduced overall survival in a selected metastatic setting (Fig. 1b).  
11 These data corroborate the link between high CSPG4 expression and poor prognosis, supporting the idea of  
12 the potential direct implication of CSPG4 in melanoma progression (64, 72).

13 These considerations and evidence that the amino-acid sequence of CSPG4 is highly evolutionarily conserved,  
14 showing over 82% homology with its canine counterpart, led us to evaluate the potential relevance of CSPG4  
15 for comparative oncology in MM. We were the first to investigate, by means of immunohistochemical analysis,  
16 CSPG4 expression in canine MM. After evaluating a cohort of 65 canine MM samples, collected between  
17 2000 and 2010 at the Diagnostic Laboratory of the Department of Animal Pathology at the University of Turin  
18 (Italy), we demonstrated the over-expression of CSPG4 antigen in almost 60% of canine MM, in which the  
19 staining was mostly restricted to the tumor cell membrane (75). Moreover, positive staining was more frequent,  
20 albeit not significantly so, in amelanotic rather than in melanotic tumors, and this correlation with a more  
21 aggressive phenotype was also suggested by the Kaplan-Meier curve, which indicate lower survival in cases  
22 of higher CSPG4 expression levels (40). In addition to the well-known role of CSPG4 in human MM, this  
23 molecule therefore also constitutes a potential IHC marker and a promising targetable antigen in canine MM.  
24 These results laid the foundation for the evaluation of CSPG4 as a prototype oncoantigen for translational  
25 immunotherapy studies against MM (66).

26 What makes anti-CSPG4 directed therapies an even more attractive approach is the recently recognized  
27 widespread expression of this oncoantigen in a huge variety of other aggressive tumors (66). We have recently  
28 expanded our focus of research to another challenging malignancy with very poor prognosis and few treatments  
29 available; OSA. We demonstrated that CSPG4 is over-expressed in both human and canine OSA biopsies and  
30 that an evident correlation exists between CSPG4 over-expression and a shorter survival for both OSA-affected  
31 humans and dogs (Riccardo *et al.*, *under revision*). This study indicates that CSPG4 may possibly be clinically  
32 implicated in OSA progression, highlighting that CSPG4 is also an interesting therapeutic target in the  
33 comparative oncology field of OSA.

34 Finally, it has been emerging in recent decades that a minority of cells inside a tumor, named cancer stem cells  
35 (CSC), are endowed with more resistant behavior to conventional therapies, i.e., chemo- and radio-therapy,  
36 than more differentiated cancer cells (76, 77). This implies that CSC are the cells that are principally  
37 responsible for treatment failure and local or distant recurrences/metastases. Considering that conventional

1 anti-cancer therapies are predominantly directed against the bulk of differentiated tumor cells, the CSC model  
2 has important clinical implications, and suggests that there is a need for innovative approaches that can also  
3 impact upon the CSC compartment. Against this background, the potential of immunotherapies against CSC  
4 has recently become an appealing field of research that may yet succeed where conventional therapies have  
5 failed.

6 Considering its significant oncogenic role, it is not surprising that CSPG4 over-expression has been identified  
7 in CSC subsets in several tumor histotypes (56, 78). We have also confirmed the overexpression of CSPG4 in  
8 human (Fig. 2a) and canine (Fig. 2b) MM- and OSA-derived CSC (Riccardo et al., *under revision*), thanks to  
9 the generation of “melanospheres” and “osteospheres” (79). These findings make CSPG4 an even more  
10 interesting target for the design of approaches to target both differentiated cells and CSC.

11 In conclusion, the development of effective anti-CSPG4 therapies may represent a “crosswise bullet” that can  
12 simultaneously strike a wide range of tumors, and impair a number of oncogenic features in tumor cells. , We  
13 consider the possibility of investigating anti-CSPG4 targeting in spontaneous canine tumors that express  
14 CSPG4 to be a priceless opportunity for the development of advancements in the veterinary field that can be  
15 successfully and rapidly translated into treatment in human clinics.

#### 16 **Testing anti- CSPG4 DNA vaccines**

17 Once an appealing tumor antigen has been identified, as in the case of CSPG4, the rational design of  
18 immunotherapeutic strategies becomes a precious opportunity in the fight against cancer.

19 The potential of targeting CSPG4 by means of passive and active immunotherapeutic strategies has been well  
20 documented in recent decades. For this reason, monoclonal antibody (mAb)-based anti-tumor approaches (78,  
21 80), and genetically engineered T cells with chimeric antigen receptors (CAR-T) (81) that are reactive against  
22 CSPG4 have been developed. These techniques have demonstrated the efficacy of anti-CSPG4 immune-  
23 targeting in impairing cancer cell proliferation, migration and invasion in a number of cancer types and in  
24 various experimental settings. Furthermore, active immunization approaches, such as anti-idiotypic antibodies  
25 or mimotopes (82, 83) have been investigated. These approaches never reached clinics because of the  
26 difficulties in the standardization and the induction of a frequent and efficient immune response. Nevertheless,  
27 they have shown evidence of immunogenicity and clinical effectiveness, without collateral effects. This has  
28 provided a strong rationale for the development of innovative and more effective strategies of immunization  
29 against CSPG4. DNA vaccination may well represent an easy and versatile strategy with which to achieve this  
30 aim (84). DNA vaccination offers many advantages over other immunotherapies, as DNA plasmids are  
31 relatively simple and inexpensive to design and produce on large scales, as well as being well tolerated and  
32 safe (40). Indeed, it has been demonstrated in preclinical models and by many clinical trials that the risk for  
33 plasmid genomic integration is very low, and no evidence of anti-DNA immune response following  
34 vaccination have been reported so far, which allows multiple administrations to be carried out. We therefore  
35 investigated the immunogenic potential of two plasmids, one carrying the human (Hu; Gene ID\_1464) and one  
36 the dog (Do; Gene ID\_487658) sequence of CSPG4, initially in a murine model, where both Hu- and Do-  
37 CSPG4 are xenogeneic antigens. Specifically, we vaccinated C57BL/6 mice twice, at 2-week intervals, with

1 either the Hu- or Do-CSPG4 plasmids. DNA vaccination was performed by plasmid intramuscular injection  
2 followed by electroporation, one of the most effective methods for securing safe and efficient DNA  
3 immunization (85). Sera of vaccinated mice were collected two weeks after the last vaccination and tested, by  
4 flow cytometry, for their ability to stain B16 murine melanoma cells that had been stably transfected with  
5 either the Hu- or Do-CSPG4. No staining was found on the B16 WT cells with all the tested sera. However,  
6 as shown in Fig. 3, sera from Hu-CSPG4 DNA vaccinated mice were effective in binding the B16-Hu-CSPG4  
7 (Fig. 3a, left panel) and to a lesser extent the B16-Do-CSPG4 (Fig. 3a, right panel) cell lines, indicating the  
8 presence of anti-CSPG4 antibodies. Similarly, Do-CSPG4 DNA vaccination was effective in inducing a  
9 significant antibody response that could bind B16-Do-CSPG4 cells (Fig. 3a, right panel), but the induced  
10 antibodies had a very low ability to bind B16-Hu-CSPG4 cells (Fig. 3a, left panel). The empty plasmid did not  
11 induce antibodies that were able to bind any of the two cell lines tested. Overall, these results demonstrate that  
12 both Hu-CSPG4 and Do-CSPG4 DNA vaccines can be immunogenic in a xenogeneic host. However, one of  
13 the major limitations in anti-cancer vaccination is host immune tolerance to the self-target antigen. Indeed, the  
14 homologous sequence used as an immunogen frequently fails to induce an effective immune response. To  
15 overcome this issue, we decided to test the Hu-CSPG4 vaccine in dogs in order to circumvent immune  
16 tolerance and induce a proper immunogenic response.

17 To this aim, we conducted a non-randomized prospective veterinary clinical trial of adjuvant vaccination with  
18 the xenogeneic Hu-CSPG4 DNA plasmid in client-owned dogs with *en bloc* surgically resected CSPG4-  
19 positive oral MM (39, 40). This trial included, after written informed consent signed by the owners, dogs  
20 without concurrent life-threatening diseases and with histologically confirmed oral stage II and III surgically  
21 resected MM and a minimum follow-up of 6-months. Basically, after primary MM resection, canine patients  
22 included in the vaccination group were injected intramuscularly with the Hu-CSPG4 DNA plasmid, and then  
23 *in-vivo* electroporation was performed (40). The purpose of this adjuvant vaccination was to eliminate the  
24 tumor cells that may remain after surgery, hampering the development of recurrences and metastasis, which  
25 are actually the main causes of MM-related death.

26 The trial demonstrated the safety, immunogenicity and clinical efficacy of the vaccine. No evidence of acute  
27 or late (up to 3-years for some of the vaccines), local or systemic side effects were observed. Moreover, the  
28 vaccine was able to induce, in the sera of all the vaccinated dogs, an IgG antibody response that was able to  
29 bind not only the Hu- CSPG4, but also the Do-CSPG4 antigen (39, 40). In addition, recent preliminary data  
30 suggest that the vaccine also induces IgA antibodies (Fig. 3b). This could be of paramount relevance for  
31 mucosal protection and consequently for blocking recurrences in the oral cavity. A deeper analysis of the  
32 immunoglobulin repertoire, not only in the sera, but also in the saliva of vaccinated dogs, may provide  
33 interesting insights to better explain the clinical-protection mechanisms observed in the vaccines. Indeed, the  
34 most important result from this veterinary trial is the significant prolongation of the overall and the disease-  
35 free survival of vaccinated dogs compared to dogs treated with conventional therapies alone (39, 40) and Fig.  
36 4).

1 The polyvalent role that CSPG4 plays in regulating numerous pathways of the “life” of cancer cells means that  
2 there may be many different mechanisms of action by which anti-CSPG4 antibodies exert their therapeutic  
3 effects. We demonstrated that sera derived from both vaccinated mice and dogs were able to interfere with  
4 MM cell proliferation (40), by inducing CSPG4 down-regulation (39) and CSPG4 internalization (data not  
5 shown). Moreover, after demonstrating the over-expression and clinical relevance of CSPG4 in human and  
6 canine OSA (see above), we explored whether CSPG4 immune-targeting by monoclonal antibodies, or sera  
7 derived from vaccinated dogs, were able to inhibit both human and canine OSA cell proliferation and  
8 osteosphere viability (Riccardo et al., *under revision*). The results show that this is indeed the case, suggesting  
9 that anti-CSPG4 DNA vaccination also exerts a potential therapeutic effect in the treatment of OSA. It is highly  
10 likely that other mechanisms, such as the ability of vaccine-induced antibodies to interfere with cancer cell  
11 migration and/or adhesion, may impact upon the clinical efficacy of Hu-CSPG4 DNA vaccination, and these  
12 are currently under investigation.

13 Overall, the results reported in these studies ((39, 40); Riccardo et al., *under revision*) have endorsed DNA  
14 vaccination against CSPG4 as a valid adjuvant option for the treatment of strongly aggressive diseases, such  
15 as MM and OSA, while also indicating that it has the potential to be extended to the treatment of a wide range  
16 of CSPG4-expressing tumors. To this end, we are now testing a second-generation anti-CSPG4 DNA vaccine  
17 that codes for a chimeric human/dog protein. The chimeric CSPG4 protein provides xenogeneic epitopes to  
18 both human and dog patients, granting a tolerance brake in both species.

## 19 Conclusions

20 As discussed, companion animals naturally develop tumors in a chronologically relevant time and in an  
21 immunocompetent environment, realistically reproducing most of the fundamental processes involved in  
22 human tumor development, which are major clinical hurdles in the treatment of human patients. For these  
23 reasons, tumors arising in companion dogs are becoming an increasingly recognized tool with which to study  
24 the therapeutic potential of anti-cancer treatments. This is particularly true for some types of tumors, for which  
25 physiological, anatomical, biological and clinical features are shared by the canine and human diseases, as has  
26 been clearly demonstrated.

27 In recent years, several groups have performed veterinary studies in order to test their innovative strategies in  
28 a high translational setting, against a wide range of comparative tumors, such as lymphomas (86-89),  
29 melanoma (90-92), osteosarcomas (47) and many others ([http://vetcancersociety.org/pet-owners/clinical-](http://vetcancersociety.org/pet-owners/clinical-trials/)  
30 [trials/](https://ebusiness.avma.org/aahsd/study_search.aspx); [https://ebusiness.avma.org/aahsd/study\\_search.aspx](https://ebusiness.avma.org/aahsd/study_search.aspx)).

31 We focused our research on CSPG4, demonstrating that it is expressed by both human and canine MM and  
32 OSA, and that its targeting with antibodies can reduce tumor proliferation *in vitro*. Moreover, our DNA vaccine  
33 coding for Hu-CSPG4 was safe and immunogenic in dogs with surgically resected MM and significantly  
34 increased their survival (39, 40). Interestingly, our results also demonstrate that CSPG4 is over-expressed by  
35 human and canine melano- and osteospheres, suggesting that the use of immunotherapeutic strategies against  
36 CSPG4 might not only be effective against the tumor bulk population, but also against CSC (Riccardo *et al.*,  
37 *under revision*). This could be of paramount importance for the ability to target cells with more aggressive and

1 stem features, in order to more efficiently counteract the onset of recurrences and metastatic lesions. In  
2 conclusion, our observations i) support the idea that comparative oncology **may have a significant** impact **on**  
3 the development of effective new anti-cancer therapies; and ii) underline the relevance of anti-CSPG4  
4 vaccination for the treatment of the wide range of CSPG4-expressing tumors, starting from MM and OSA.  
5 As a final, more general consideration, we believe that, on one hand, new therapies **that are** developed in dogs  
6 **can be quickly** translated for the management of human patients. **On** the other hand, it **may** be also true that  
7 human therapies **that have already been approved** (e.g., CIs) could be used to treat canine tumors, **making the**  
8 investigation of combinatorial approaches that can be added to clinical protocols **easier**. This mutual benefit  
9 **for** the veterinary and the human clinical worlds is **also** starting to capture the attention of industry and financial  
10 markets, **leading to the hope** that **there will be a** time reduction **in the** jump from pre-clinic to in-human clinical  
11 trials and a consequent acceleration **in** the drug-development process.

## 12 **Author contributions**

13 Lidia Tarone, Giuseppina Barutello, Selina Iussich, Davide Giacobino and Federica Riccardo produced the  
14 results discussed in this review. Federica Riccardo, Lidia Tarone and Giuseppina Barutello performed mouse  
15 experiments and flow cytometry analysis, supervised by Federica Cavallo. Selina Iussich and Davide  
16 Giacobino, under the supervision of Paolo Buracco, collaborated to produce the results in canine patients.  
17 Federica Cavallo, Federica Riccardo and Lidia Tarone provided major contributions in writing and discussing  
18 the manuscript. Federica Cavallo and Elena Quaglino critically revised the manuscript. All authors read and  
19 approved the final version of the manuscript.

## 20 **Acknowledgements**

21 **Monoclonal antibodies directed towards different epitopes of the CSPG4 antigen (225.2, TP32, TP49 and**  
22 **VF20-VT87.41) used to perform flow cytometry analysis were kindly provided by Prof. Soldano Ferrone**  
23 **(Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA). We**  
24 **thank Dr. Dale Lawson for his revision and editing of the manuscript.**

## 25 **Funding**

26 This work was supported by grants from Fondazione Ricerca Molinette Onlus, the University of Turin (ex  
27 60% 2018, intramural funds) and the Italian Ministry of Health (Progetti ordinari di Ricerca Finalizzata, RF-  
28 2013-02359216).

## 29 **Compliance with ethical standards**

### 30 *Conflict of interest*

31 The authors declare that no potential conflicts of interest exist

### 32 *Ethical approval and ethical standards*



1 All the *in-vivo* experiments were approved by the Italian Ministry of Health, authorization numbers 0006939-  
2 P-18/03/2015 (164/2015-PR) and 0004230-20/02/2018-DGSAF-MDS-P.

### 3 *Animal source*

4 Mice used for the vaccination experiments reported in this paper were purchased from Charles River  
5 Laboratories or bred at the Molecular Biotechnology Center, University of Turin, where all mice were  
6 maintained and treated in accordance with University Ethical Committee and European Union guidelines under  
7 Directive 2010/63. The canine patients that were enrolled in veterinary trials were client-owned dogs, whose  
8 institutes of reference were the Veterinary Teaching Hospital of the University of Turin and the Veterinary  
9 clinics of South Rome, Italy. Dogs were treated according to the Good Clinical Practice guidelines for animal  
10 clinical studies, and rules imposed by the Ethical Committee of the University of Turin (Italy).

### 11 **Figure legends**

12 *Figure 1. CSPG4 clinical impact on melanoma patient survival.* The mRNA expression levels of CSPG4 in  
13 human MM samples were determined by querying the R2 Kaplan Meier scanner ([https://hgserver1.amc.nl/cgi-  
14 bin/r2/main.cgi](https://hgserver1.amc.nl/cgi-bin/r2/main.cgi)) using previously deposited gene expression analysis datasets from a) (73) (GSE65904,  
15 including 214 melanoma tumor samples) and b) (74) (GSE19234, including 44 metastatic melanoma biopsies).  
16 For prognostic studies, R2 analysis software was used and patients were stratified according to CSPG4  
17 expression.

18 Kaplan-Meier curves depict overall survival probability, in years, for melanoma patients stratified by high  
19 (blue) or low (red) mRNA CSPG4 expression. In order to define the cutoff between high and low gene  
20 expression, all percentiles between the lower and upper quartiles were computed; the best performing threshold  
21 was used as a cutoff. Overall survival data were tested for significance using the log-rank test.

22 *Figure 2. CSPG4 expression in melanospheres.* a, b) Representative images of human SK-Mel28- (a) and  
23 canine CMM-12- (b) derived melanospheres. Both human and canine melanospheres were generated  
24 according to the protocol described in (79). c, d) Flow cytometry analysis of CSPG4 expression on Ep and P1-  
25 derived human SK-Mel28 (c) and canine CMM-12 (d) cells. Flow cytometry was performed using a FACS  
26 Verse (BD Biosciences) and the results were analyzed using BDFacs Suite software. Results are expressed as  
27 percentage (%) of CSPG4 positive cells (left panels) and as P1/Ep fold-change of CSPG4 mean fluorescence  
28 intensity (MFI, right panels).

29 *Figure 3. Anti CSPG4 vaccine-induced antibody response.* a) Anesthetized C57BL/6 mice were vaccinated as  
30 previously described in (93) and sera collected 2 weeks after vaccination were tested for their ability to stain  
31 murine B16 melanoma cells stably transfected with either the human (left panel) or canine (right panel) CSPG4  
32 antigen. Results are expressed as percentage (%) of CSPG4 positive cells. Student's t-test \*\*\* P < 0.0006;  
33 \*\*\*\* P < 0.0001. b) Canine MM patients were vaccinated with the Hu-CSPG4 DNA plasmid, as previously  
34 described in (39, 40), and sera collected before the first immunization (Pre-Vax) and after the fourth  
35

1 vaccination (Post-Vax) were selected for further analysis. Sera were tested for their ability to stain the canine  
2 CSPG4 antigen on the canine CSPG4<sup>+</sup> MM cell line (CMM-12). The IgA specific binding was revealed using  
3 a goat anti-dog IgA secondary antibody. Results are expressed as percentage (%) of CSPG4 positive cells.  
4  
5 Flow cytometry was performed using a FACS Verse (BD Biosciences) and the results were analyzed using  
6 BDFacs Suite software.  
7

8  
9  
10 Figure 4. Clinical efficacy of the Hu-CSPG4 vaccine in canine MM patients. Swimmer plot depicting the  
11 overall survival of canine MM patients enrolled in the veterinary trials (39, 40). Briefly, the survival (in days)  
12 of dogs with surgically resected CSPG4-positive MM, either vaccinated (Vax) or non-vaccinated (Ctrl), is  
13 reported. Arrows indicate that the patients were still alive at the time of publication (39). The purple dots  
14 indicate, for each patient, the day of recurrence or metastasis (Rec/Mets) detection, if any. Black dots indicate  
15 patients who died because of unrelated reasons, while red dots indicate patients who died because of MM.  
16  
17 Percentage of canine patients, vaccinated or treated with conventional therapies alone, which are still alive at  
18 1-year after the diagnosis, is indicated in the plot.  
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## 23 Bibliography

- 24 1. Dragani TA, Castells A, Kulasingam V, Diamandis EP, Earl H, Iams WT, Lovly CM, Sedelaar JP, Schalken  
25 JA (2016) Major milestones in translational oncology. BMC Med. 14: 110. doi: 10.1186/s12916-016-0654-y
- 26 2. Barutello G, Rolih V, Arigoni M, Tarone L, Conti L, Quaglino E, Buracco P, Cavallo F, Riccardo F (2018)  
27 Strengths and Weaknesses of Pre-Clinical Models for Human Melanoma Treatment: Dawn of Dogs'  
28 Revolution for Immunotherapy. International journal of molecular sciences. 19. doi: 10.3390/ijms19030799
- 29 3. Mak IW, Evaniew N, Ghert M (2014) Lost in translation: animal models and clinical trials in cancer  
30 treatment. Am J Transl Res. 6: 114-8.
- 31 4. Riccardo F, Aurisicchio L, Impellizzeri JA, Cavallo F (2015) The importance of comparative oncology in  
32 translational medicine. Cancer immunology, immunotherapy : CII. 64: 137-48. doi: 10.1007/s00262-014-  
33 1645-5
- 34 5. Paoloni M, Khanna C (2008) Translation of new cancer treatments from pet dogs to humans. Nature  
35 reviews. Cancer. 8: 147-56. doi: 10.1038/nrc2273
- 36 6. Talmadge JE, Singh RK, Fidler IJ, Raz A (2007) Murine models to evaluate novel and conventional  
37 therapeutic strategies for cancer. The American journal of pathology. 170: 793-804. doi:  
38 10.2353/ajpath.2007.060929
- 39 7. Gordon MY, N. B (1976) The Sensitivities of Human and Murine Hemopoietic Cells Exposed to  
40 Cytotoxic Drugs in an in Vivo Culture. Cancer Res. . 36: 2822-6.
- 41 8. Honigberg LA, Smith AM, Sirisawad M et al. (2010) The Bruton tyrosine kinase inhibitor PCI-32765  
42 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc Natl  
43 Acad Sci U S A. 107: 13075-80. doi: 10.1073/pnas.1004594107
- 44 9. Krogh A (1929) The Progress of Physiology. Science. 70: 200-4. doi: 10.1126/science.70.1809.200
- 45 10. Lindblad-Toh K, Wade CM, Mikkelsen TS et al. (2005) Genome sequence, comparative analysis and  
46 haplotype structure of the domestic dog. Nature. 438: 803-19. doi: 10.1038/nature04338
- 47 11. Olson PN (2007) Using the canine genome to cure cancer and other diseases. Theriogenology. 68:  
48 378-81. doi: 10.1016/j.theriogenology.2007.04.016
- 49 12. Mueller F, Fuchs B, Kaser-Hotz B (2007) Comparative biology of human and canine osteosarcoma.  
50 Anticancer research. 27: 155-64.
- 51 13. Gardner HL, Fenger JM, London CA (2016) Dogs as a Model for Cancer. Annu Rev Anim Biosci. 4: 199-  
52 222. doi: 10.1146/annurev-animal-022114-110911

- 1 14. Medicine Io, National Academies of Sciences E, Medicine (2015) The Role of Clinical Studies for Pets  
1 2 with Naturally Occurring Tumors in Translational Cancer Research: Workshop Summary. The National  
2 3 Academies Press, Washington, DC
- 3 4 15. Abdelmegeed SM, Mohammed S (2018) Canine mammary tumors as a model for human disease.  
4 5 Oncology letters. 15: 8195-205. doi: 10.3892/ol.2018.8411
- 5 6 16. Fan TM, Selting KA (2018) Exploring the Potential Utility of Pet Dogs With Cancer for Studying  
6 7 Radiation-Induced Immunogenic Cell Death Strategies. *Frontiers in oncology*. 8: 680. doi:  
8 8 10.3389/fonc.2018.00680
- 9 9 17. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J (2014) Clinical development success  
10 10 rates for investigational drugs. *Nat Biotechnol*. 32: 40-51. doi: 10.1038/nbt.2786
- 11 11 18. Barsoum N, Kleeman C (2002) Now and then, the history of parenteral fluid administration. *Am J*  
12 12 *Nephrol*. 22: 284-9. doi: 10.1159/000063775
- 13 13 19. Murray JE, Sheil AG, Moseley R, Knight P, McGavic JD, Dammin GJ (1964) Analysis of Mechanism of  
14 14 Immunosuppressive Drugs in Renal Homotransplantation. *Ann Surg*. 160: 449-73.
- 15 15 20. Weiden PL, Storb R, Lerner KG, Kao GF, Graham TC, Thomas ED (1975) Treatment of canine  
16 16 malignancies by 1200 R total body irradiation and autologous marrow grafts. *Exp Hematol*. 3: 124-34.
- 17 17 21. Storb R, Tsoi MS, Weiden PL, Graham TC, Thomas ED (1976) Studies on the mechanism of stable  
18 18 graft-host tolerance in canine and human radiation chimeras. *Transplant Proc*. 8: 561-4.
- 19 19 22. Faivre S, Delbaldo C, Vera K et al. (2006) Safety, pharmacokinetic, and antitumor activity of SU11248,  
20 20 a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. *J Clin Oncol*. 24: 25-35. doi:  
21 21 10.1200/JCO.2005.02.2194
- 22 22 23. London CA, Bernabe LF, Barnard S et al. (2014) Preclinical evaluation of the novel, orally bioavailable  
23 23 Selective Inhibitor of Nuclear Export (SINE) KPT-335 in spontaneous canine cancer: results of a phase I study.  
24 24 *PLoS One*. 9: e87585. doi: 10.1371/journal.pone.0087585
- 25 25 24. Burger JA, Keating MJ, Wierda WG et al. (2014) Safety and activity of ibrutinib plus rituximab for  
26 26 patients with high-risk chronic lymphocytic leukaemia: a single-arm, phase 2 study. *The Lancet Oncology*. 15:  
27 27 1090-9. doi: 10.1016/s1470-2045(14)70335-3
- 28 28 25. Vail DM, Thamm DH, Reiser H et al. (2009) Assessment of GS-9219 in a pet dog model of non-  
29 29 Hodgkin's lymphoma. *Clin Cancer Res*. 15: 3503-10. doi: 10.1158/1078-0432.CCR-08-3113
- 30 30 26. Reiser H, Wang J, Chong L et al. (2008) GS-9219--a novel acyclic nucleotide analogue with potent  
31 31 antineoplastic activity in dogs with spontaneous non-Hodgkin's lymphoma. *Clin Cancer Res*. 14: 2824-32. doi:  
32 32 10.1158/1078-0432.CCR-07-2061
- 33 33 27. De Clercq E (2018) Tanovea(R) for the treatment of lymphoma in dogs. *Biochem Pharmacol*. 154:  
34 34 265-9. doi: 10.1016/j.bcp.2018.05.010
- 35 35 28. West DC, Qin Y, Peterson QP et al. (2012) Differential effects of procaspase-3 activating compounds  
36 36 in the induction of cancer cell death. *Mol Pharm*. 9: 1425-34. doi: 10.1021/mp200673n
- 37 37 29. Peterson QP, Hsu DC, Novotny CJ, West DC, Kim D, Schmit JM, Dirikolu L, Hergenrother PJ, Fan TM  
38 38 (2010) Discovery and canine preclinical assessment of a nontoxic procaspase-3-activating compound. *Cancer*  
39 39 *Res*. 70: 7232-41. doi: 10.1158/0008-5472.CAN-10-0766
- 40 40 30. Joshi AD, Botham RC, Schlein LJ et al. (2017) Synergistic and targeted therapy with a procaspase-3  
41 41 activator and temozolomide extends survival in glioma rodent models and is feasible for the treatment of  
42 42 canine malignant glioma patients. *Oncotarget* 8: . doi: doi: 10.18632/oncotarget.19085
- 43 43 31. Bergman PJ, Camps-Palau MA, McKnight JA et al. (2006) Development of a xenogeneic DNA vaccine  
44 44 program for canine malignant melanoma at the Animal Medical Center. *Vaccine*. 24: 4582-5. doi:  
45 45 10.1016/j.vaccine.2005.08.027
- 46 46 32. Liao JC, Gregor P, Wolchok JD, Orlandi F, Craft D, Leung C, Houghton AN, PJ. B (2006 ) Vaccination  
47 47 with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. *Cancer Immun*  
48 48 21.
- 49 49 33. Grosenbaugh DA, Leard AT, Bergman PJ et al. (2011) Safety and efficacy of a xenogeneic DNA vaccine  
50 50 encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following  
51 51 surgical excision of the primary tumor. *Am J Vet Res*. 72. doi: doi: 10.2460/ajvr.72.12.1631.

1 34. Wolchok JD, Yuan J, Houghton AN et al. (2007) Safety and immunogenicity of tyrosinase DNA vaccines  
 1 2 in patients with melanoma. *Molecular therapy : the journal of the American Society of Gene Therapy*. 15:  
 2 3 2044-50. doi: 10.1038/sj.mt.6300290

3 4 35. Yuan J, Ku GY, Adamow M et al. (2013) Immunologic responses to xenogeneic tyrosinase DNA vaccine  
 4 5 administered by electroporation in patients with malignant melanoma. *J Immunother Cancer*. 18. doi: doi:  
 5 6 10.1186/2051-1426-1-20

7 7 36. Ottnod JM, Smedley RC, Walshaw R, Hauptman JG, Kiupel M, Obradovich JE (2013) A retrospective  
 8 8 analysis of the efficacy of Oncept vaccine for the adjunct treatment of canine oral malignant melanoma.  
 9 9 *Veterinary and comparative oncology*. 11: 219-29. doi: 10.1111/vco.12057

10 10 37. Treggiari E, Grant JP, North SM (2016) A retrospective review of outcome and survival following  
 11 11 surgery and adjuvant xenogeneic DNA vaccination in 32 dogs with oral malignant melanoma. *J Vet Med Sci*.  
 12 12 78: 845-50. doi: 10.1292/jvms.15-0510

13 13 38. Verganti S, Berlatto D, Blackwood L, Amores-Fuster I, Polton GA, Elders R, Doyle R, Taylor A, Murphy  
 14 14 S (2017) Use of Oncept melanoma vaccine in 69 canine oral malignant melanomas in the UK. *J Small Anim  
 15 15 Pract*. 58: 10-6. doi: 10.1111/jsap.12613

16 16 39. Piras LA, Riccardo F, Iussich S et al. (2017) Prolongation of survival of dogs with oral malignant  
 17 17 melanoma treated by en bloc surgical resection and adjuvant CSPG4-antigen electrovaccination. *Vet Comp  
 18 18 Oncol*. 15: 996-1013. doi: 10.1111/vco.12239

19 19 40. Riccardo F, Iussich S, Maniscalco L et al. (2014) CSPG4-specific immunity and survival prolongation in  
 20 20 dogs with oral malignant melanoma immunized with human CSPG4 DNA. *Clinical cancer research : an official  
 21 21 journal of the American Association for Cancer Research*. 20: 3753-62. doi: 10.1158/1078-0432.CCR-13-3042

22 22 41. Kurzman ID, MacEwen EG, Rosenthal RC et al. (1995) Adjuvant therapy for osteosarcoma in dogs-  
 23 23 results of randomized clinical trials using combined liposome-encapsulated muramyl tripeptide and cisplatin.  
 24 24 *Clin Cancer Res* 1.

25 25 42. Kleinerman ES, Gano JB, Johnston DA, Benjamin RS, N. J (1995) Efficacy of liposomal muramyl  
 26 26 tripeptide (CGP 19835A) in the treatment of relapsed osteosarcoma. *Am J Clin Oncol* 18

27 27 43. Kleinerman ES, Jia SF, Griffin J, Seibel NL, Benjamin RS, N. J (1992) Phase II study of liposomal muramyl  
 28 28 tripeptide in osteosarcoma: the cytokine cascade and monocyte activation following administration. *J Clin  
 29 29 Oncol* 10.

30 30 44. Creaven PJ, Cowens JW, Brenner DE et al. (1990) Initial clinical trial of the macrophage activator  
 31 31 MTP-PE encapsulated in liposomes in patients with advanced cancer. *J Biol Resp Modifier* 9.

32 32 45. PA M (2009) Muramyl tripeptide (mifamurtide) for the treatment of osteosarcoma. *Expert Rev  
 33 33 Anticancer Ther* 9 doi: doi: 10.1586/era.09.69.

34 34 46. Biteau K, Guiho R, Chatelais M, Taurelle J, Chesneau J, Corradini N, Heymann D, F. R (2016) L-MTP-PE  
 35 35 and zoledronic acid combination in osteosarcoma- preclinical evidence of positive therapeutic combination  
 36 36 for clinical transfer. *Am J Cancer Res* 6

37 37 47. Mason NJ, Gnanandarajah JS, Engiles JB, Gray F, Laughlin D, Gaurnier-Hausser A, Wallecha A,  
 38 38 Huebner M, Paterson Y (2016) Immunotherapy with a HER2-Targeting *Listeria* Induces HER2-Specific  
 39 39 Immunity and Demonstrates Potential Therapeutic Effects in a Phase I Trial in Canine Osteosarcoma. *Clinical  
 40 40 cancer research : an official journal of the American Association for Cancer Research*. 22: 4380-90. doi:  
 41 41 10.1158/1078-0432.CCR-16-0088

42 42 48. Siegel RL, Miller KD, Jemal A (2018) Cancer statistics, 2018. *CA Cancer J Clin*. 68: 7-30. doi:  
 43 43 10.3322/caac.21442

44 44 49. Jenkins RW, Barbie DA, Flaherty KT (2018) Mechanisms of resistance to immune checkpoint  
 45 45 inhibitors. *Br J Cancer*. 118: 9-16. doi: 10.1038/bjc.2017.434

46 46 50. Nishiya AT, Massoco CO, Felizzola CR et al. (2016) Comparative Aspects of Canine Melanoma. *Vet Sci*.  
 47 47 3. doi: 10.3390/vetsci3010007

48 48 51. Bosenberg M, Arnheiter H, Kelsh R (2014) Melanoma in mankind's best friend. *Pigment Cell  
 49 49 Melanoma Res*. 27: 1. doi: 10.1111/pcmr.12196

50 50 52. Mochizuki H, Kennedy K, Shapiro SG, Breen M (2015) BRAF Mutations in Canine Cancers. *PLoS One*.  
 51 51 10: e0129534. doi: 10.1371/journal.pone.0129534

52 52 53. Taran SJ, Taran R, Malipatil NB (2017) Pediatric Osteosarcoma: An Updated Review. *Indian J Med  
 53 53 Paediatr Oncol*. 38: 33-43. doi: 10.4103/0971-5851.203513

1 54. Varshney J, Scott MC, Largaespada DA, Subramanian S (2016) Understanding the Osteosarcoma  
 1 2 Pathobiology: A Comparative Oncology Approach. *Vet Sci*. 3. doi: 10.3390/vetsci3010003

2 3 55. Fenger JM, London CA, Kisseberth WC (2014) Canine osteosarcoma: a naturally occurring disease to  
 3 4 inform pediatric oncology. *ILAR J*. 55: 69-85. doi: 10.1093/ilar/ilu009

4 5 56. Nicolosi PA, Dallatomasina A, Perris R (2015) Theranostic impact of NG2/CSPG4 proteoglycan in  
 5 6 cancer. *Theranostics*. 5: 530-44. doi: 10.7150/thno.10824

7 7 57. Benassi MS, Pazzaglia L, Chiechi A, Alberghini M, Conti A, Cattaruzza S, Wassermann B, Picci P, Perris  
 8 8 R (2009) NG2 expression predicts the metastasis formation in soft-tissue sarcoma patients. *Journal of*  
 9 9 *orthopaedic research : official publication of the Orthopaedic Research Society*. 27: 135-40. doi:  
 10 10 10.1002/jor.20694

11 11 58. Wang X, Katayama A, Wang Y et al. (2011) Functional characterization of an scFv-Fc antibody that  
 12 12 immunotherapeutically targets the common cancer cell surface proteoglycan CSPG4. *Cancer research*. 71:  
 13 13 7410-22. doi: 10.1158/0008-5472.CAN-10-1134

14 14 59. Garusi E, Rossi S, Perris R (2012) Antithetic roles of proteoglycans in cancer. *Cellular and molecular*  
 15 15 *life sciences : CMLS*. 69: 553-79. doi: 10.1007/s00018-011-0816-1

16 16 60. Beard RE, Abate-Daga D, Rosati SF, Zheng Z, Wunderlich JR, Rosenberg SA, Morgan RA (2013) Gene  
 17 17 expression profiling using nanostring digital RNA counting to identify potential target antigens for melanoma  
 18 18 immunotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*.  
 19 19 19: 4941-50. doi: 10.1158/1078-0432.CCR-13-1253

20 20 61. Ziai MR, Imberti L, Nicotra MR, Badaracco G, Segatto O, Natali PG, Ferrone S (1987) Analysis with  
 21 21 monoclonal antibodies of the molecular and cellular heterogeneity of human high molecular weight  
 22 22 melanoma associated antigen. *Cancer research*. 47: 2474-80.

23 23 62. Rivera Z, Ferrone S, Wang X, Jube S, Yang H, Pass HI, Kanodia S, Gaudino G, Carbone M (2012) CSPG4  
 24 24 as a target of antibody-based immunotherapy for malignant mesothelioma. *Clinical cancer research : an*  
 25 25 *official journal of the American Association for Cancer Research*. 18: 5352-63. doi: 10.1158/1078-0432.CCR-  
 26 26 12-0628

27 27 63. Kozanoglu I, Boga C, Ozdogu H, Sozer O, Maytalan E, Yazici AC, Sahin FI (2009) Human bone marrow  
 28 28 mesenchymal cells express NG2: possible increase in discriminative ability of flow cytometry during  
 29 29 mesenchymal stromal cell identification. *Cytotherapy*. 11: 527-33. doi: 10.1080/14653240902923153

30 30 64. Campoli M, Ferrone S, Wang X (2010) Functional and clinical relevance of chondroitin sulfate  
 31 31 proteoglycan 4. *Advances in cancer research*. 109: 73-121. doi: 10.1016/B978-0-12-380890-5.00003-X

32 32 65. Cavallo F, De Giovanni C, Nanni P, Forni G, Lollini PL (2011) 2011: the immune hallmarks of cancer.  
 33 33 *Cancer Immunol Immunother*. 60: 319-26. doi: 10.1007/s00262-010-0968-0

34 34 66. Rolih V, Barutello G, Iussich S, De Maria R, Quagliano E, Buracco P, Cavallo F, Riccardo F (2017) CSPG4:  
 35 35 a prototype oncoantigen for translational immunotherapy studies. *J Transl Med*. 15: 151. doi:  
 36 36 10.1186/s12967-017-1250-4

37 37 67. Cattaruzza S, Ozerdem U, Denzel M et al. (2013) Multivalent proteoglycan modulation of FGF  
 38 38 mitogenic responses in perivascular cells. *Angiogenesis*. 16: 309-27. doi: 10.1007/s10456-012-9316-7

39 39 68. Price MA, Colvin Wanshura LE, Yang J et al. (2011) CSPG4, a potential therapeutic target, facilitates  
 40 40 malignant progression of melanoma. *Pigment cell & melanoma research*. 24: 1148-57. doi: 10.1111/j.1755-  
 41 41 148X.2011.00929.x

42 42 69. Goretzki L, Lombardo CR, Stallcup WB (2000) Binding of the NG2 proteoglycan to kringle domains  
 43 43 modulates the functional properties of angiostatin and plasmin(ogen). *The Journal of biological chemistry*.  
 44 44 275: 28625-33. doi: 10.1074/jbc.M002290200

45 45 70. Tamburini E, Dallatomasina A, Quartararo J, Cortelazzi B, Mangieri D, Lazzaretti M, Perris R (2019)  
 46 46 Structural deciphering of the NG2/CSPG4 proteoglycan multifunctionality. *FASEB journal : official publication*  
 47 47 *of the Federation of American Societies for Experimental Biology*. 33: 3112-28. doi: 10.1096/fj.201801670R

48 48 71. Tamburini E, Dallatomasina A, Quartararo J, Cortelazzi B, Mangieri D, Lazzaretti M, Perris R (2018)  
 49 49 Structural deciphering of the NG2/CSPG4 proteoglycan multifunctionality. *FASEB J*. fj201801670R. doi:  
 50 50 10.1096/fj.201801670R

51 51 72. Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, Ferrone S (2004) Human high molecular  
 52 52 weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate  
 53 53 proteoglycan (MSCP) with biological and clinical significance. *Critical reviews in immunology*. 24: 267-96.

1 73. Cirenajwis H, Ekedahl H, Lauss M et al. (2015) Molecular stratification of metastatic melanoma using  
 1 2 gene expression profiling: Prediction of survival outcome and benefit from molecular targeted therapy.  
 2 3 *Oncotarget*. 6: 12297-309. doi: 10.18632/oncotarget.3655

3 4 74. Bogunovic D, O'Neill DW, Belitskaya-Levy I et al. (2009) Immune profile and mitotic index of  
 4 5 metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci U S A*.  
 5 6 106: 20429-34. doi: 10.1073/pnas.0905139106

7 7 75. Mayayo SL, Prestigio S, Maniscalco L et al. (2011) Chondroitin sulfate proteoglycan-4: a biomarker  
 8 8 and a potential immunotherapeutic target for canine malignant melanoma. *Vet J*. 190: e26-30. doi:  
 9 9 10.1016/j.tvjl.2011.02.020

10 10 76. Ruiu R, Rolih V, Bolli E et al. (2019) Fighting breast cancer stem cells through the immune-targeting  
 11 11 of the xCT cystine-glutamate antiporter. *Cancer immunology, immunotherapy* : CII. 68: 131-41. doi:  
 12 12 10.1007/s00262-018-2185-1

13 13 77. Koren A, Rijavec M, Kern I, Sodja E, Korosec P, Cufer T (2016) BMI1, ALDH1A1, and CD133 Transcripts  
 14 14 Connect Epithelial-Mesenchymal Transition to Cancer Stem Cells in Lung Carcinoma. *Stem Cells Int*. 2016:  
 15 15 9714315. doi: 10.1155/2016/9714315

16 16 78. Rivera Z, Ferrone S, Wang X, Jube S, Yang H, Pass HI, Kanodia S, Gaudino G, Carbone M (2012) CSPG4  
 17 17 as a Target of Antibody-Based Immunotherapy for Malignant Mesothelioma. *Clinical Cancer Research*. 18:  
 18 18 5352-63. doi: 10.1158/1078-0432.Ccr-12-0628

19 19 79. Conti L, Lanzardo S, Arigoni M, Antonazzo R, Radaelli E, Cantarella D, Calogero RA, Cavallo F (2013)  
 20 20 The noninflammatory role of high mobility group box 1/Toll-like receptor 2 axis in the self-renewal of  
 21 21 mammary cancer stem cells. *FASEB journal : official publication of the Federation of American Societies for  
 22 22 Experimental Biology*. 27: 4731-44. doi: 10.1096/fj.13-230201

23 23 80. Wang X, Osada T, Wang Y et al. (2010) CSPG4 protein as a new target for the antibody-based  
 24 24 immunotherapy of triple-negative breast cancer. *Journal of the National Cancer Institute*. 102: 1496-512. doi:  
 25 25 10.1093/jnci/djq343

26 26 81. Wang Y, Geldres C, Ferrone S, Dotti G (2015) Chondroitin sulfate proteoglycan 4 as a target for  
 27 27 chimeric antigen receptor-based T-cell immunotherapy of solid tumors. *Expert Opin Ther Targets*. 19: 1339-  
 28 28 50. doi: 10.1517/14728222.2015.1068759

29 29 82. Mittelman A, Chen GZ, Wong GY, Liu C, Hirai S, Ferrone S (1995) Human high molecular weight-  
 30 30 melanoma associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: modulation of  
 31 31 the immunogenicity in patients with malignant melanoma. *Clinical cancer research : an official journal of the  
 32 32 American Association for Cancer Research*. 1: 705-13.

33 33 83. Wang X, Ko EC, Peng L, Gillies SD, Ferrone S (2005) Human high molecular weight melanoma-  
 34 34 associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: enhancement of  
 35 35 immunogenicity of anti-idiotypic monoclonal antibody MK2-23 by fusion with interleukin 2. *Cancer Res*. 65:  
 36 36 6976-83. doi: 10.1158/0008-5472.CAN-04-2328

37 37 84. Quaglino E, Riccardo F, Macagno M, Bandini S, Cojoca R, Ercole E, Amici A, Cavallo F (2011) Chimeric  
 38 38 DNA Vaccines against ErbB2+ Carcinomas: From Mice to Humans. *Cancers*. 3: 3225-41. doi:  
 39 39 10.3390/cancers3033225

40 40 85. Aurisicchio L, Mancini R, Ciliberto G (2013) Cancer vaccination by electro-gene-transfer. *Expert Rev  
 41 41 Vaccines*. 12: 1127-37. doi: 10.1586/14760584.2013.836903

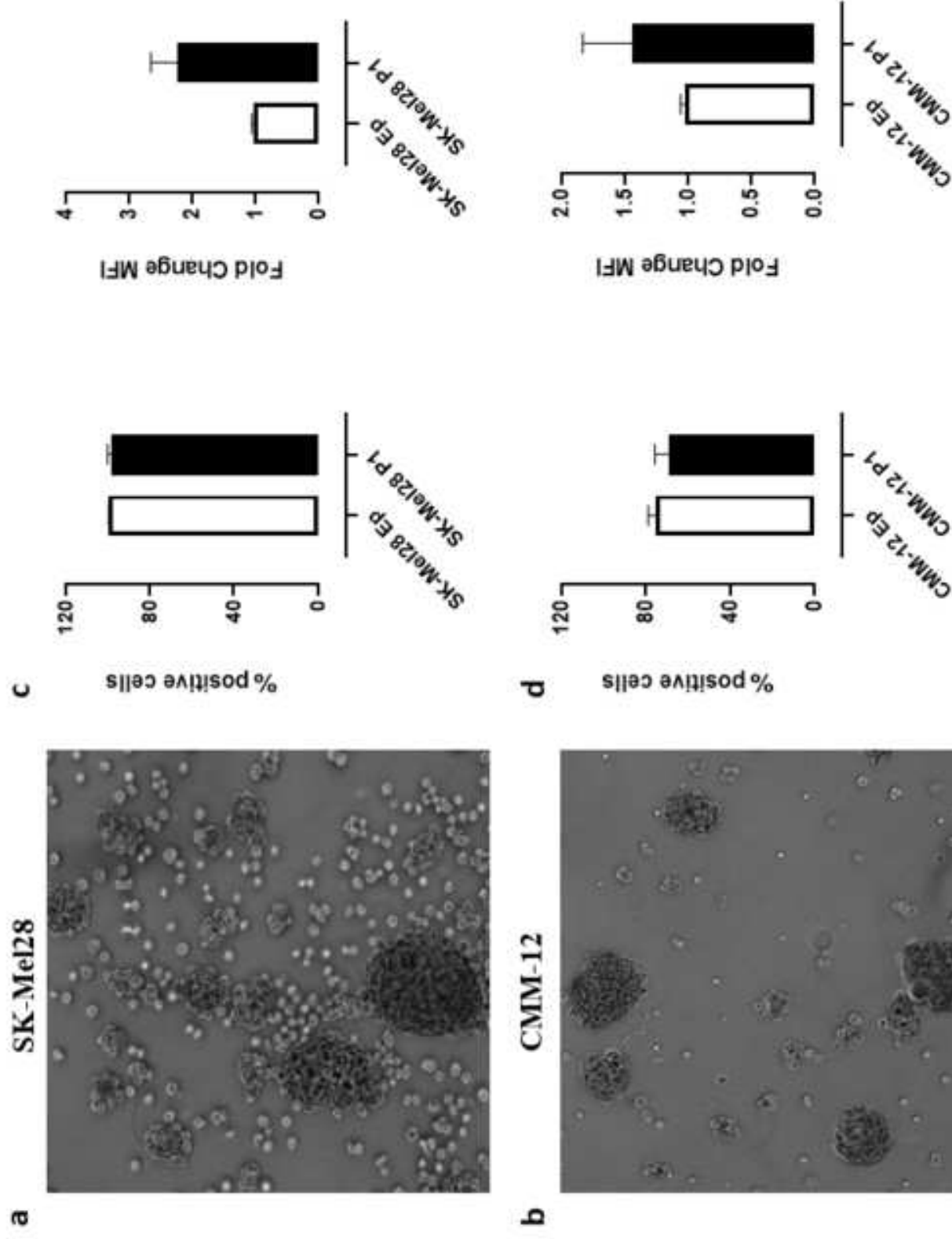
42 42 86. Impellizeri JA, Gavazza A, Greissworth E, Crispo A, Montella M, Ciliberto G, Lubas G, Aurisicchio L  
 43 43 (2018) Tel-eVax: a genetic vaccine targeting telomerase for treatment of canine lymphoma. *Journal of  
 44 44 translational medicine*. 16: 349. doi: 10.1186/s12967-018-1738-6

45 45 87. Gavazza A, Lubas G, Fridman A et al. (2013) Safety and efficacy of a genetic vaccine targeting  
 46 46 telomerase plus chemotherapy for the therapy of canine B-cell lymphoma. *Human gene therapy*. 24: 728-38.  
 47 47 doi: 10.1089/hum.2013.112

48 48 88. Peruzzi D, Gavazza A, Mesiti G et al. (2010) A vaccine targeting telomerase enhances survival of dogs  
 49 49 affected by B-cell lymphoma. *Molecular therapy : the journal of the American Society of Gene Therapy*. 18:  
 50 50 1559-67. doi: 10.1038/mt.2010.104

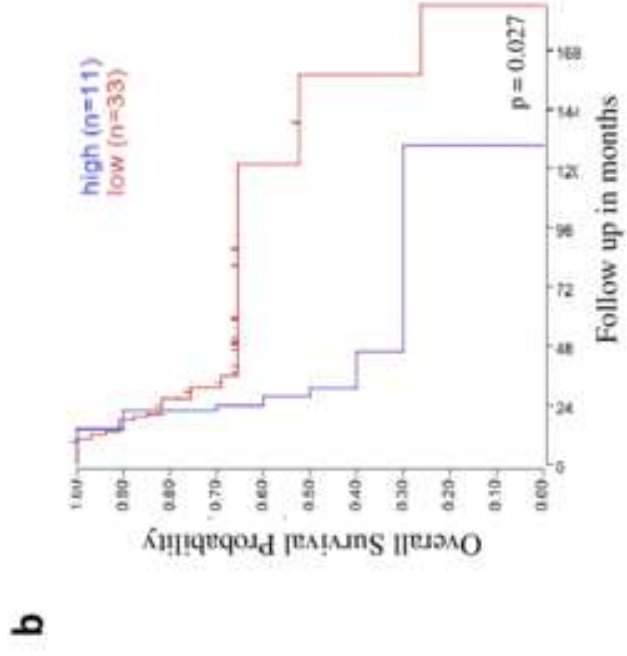
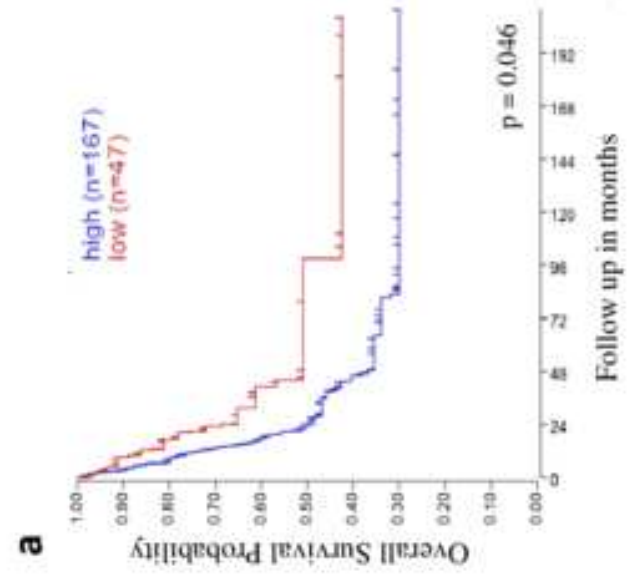
51 51 89. Marconato L, Stefanello D, Sabattini S et al. (2015) Enhanced therapeutic effect of APAVAC  
 52 52 immunotherapy in combination with dose-intensive chemotherapy in dogs with advanced indolent B-cell  
 53 53 lymphoma. *Vaccine*. 33: 5080-6. doi: 10.1016/j.vaccine.2015.08.017

1 90. Milevoj N, Tratar UL, Nemeč A, Brožič A, Znidar K, Sersa G, Cemazar M, Tozon N (2019) A combination  
2 of electrochemotherapy, gene electrotransfer of plasmid encoding canine IL-12 and cytoreductive surgery in  
3 the treatment of canine oral malignant melanoma. *Research in veterinary science*. 122: 40-9. doi:  
4 10.1016/j.rvsc.2018.11.001  
5 91. Kurupati RK, Zhou X, Xiang Z, Keller LH, Ertl HCJ (2018) Safety and immunogenicity of a potential  
6 checkpoint blockade vaccine for canine melanoma. *Cancer immunology, immunotherapy* : CII. 67: 1533-44.  
7 doi: 10.1007/s00262-018-2201-5  
8 92. Finocchiaro LM, Fondello C, Gil-Cardesa ML, Rossi UA, Villaverde MS, Riveros MD, Glikin GC (2015)  
9 Cytokine-Enhanced Vaccine and Interferon-beta plus Suicide Gene Therapy as Surgery Adjuvant Treatments  
10 for Spontaneous Canine Melanoma. *Human gene therapy*. 26: 367-76. doi: 10.1089/hum.2014.130  
11 93. Riccardo F, Bolli E, Macagno M, Arigoni M, Cavallo F, Quaglino E (2017) Chimeric DNA Vaccines: An  
12 Effective Way to Overcome Immune Tolerance. *Curr Top Microbiol Immunol*. 405: 99-122. doi:  
13 10.1007/82\_2014\_426  
14

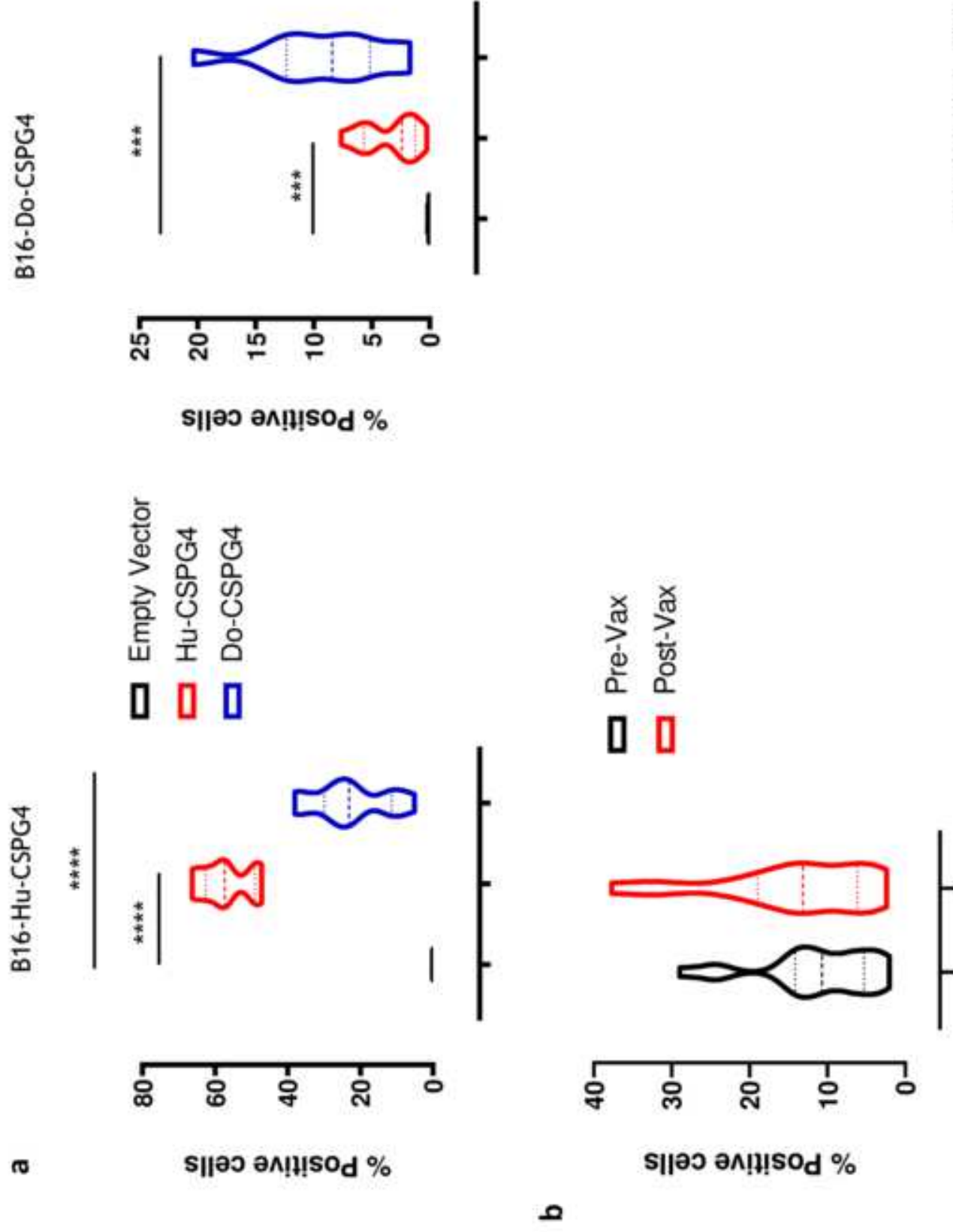


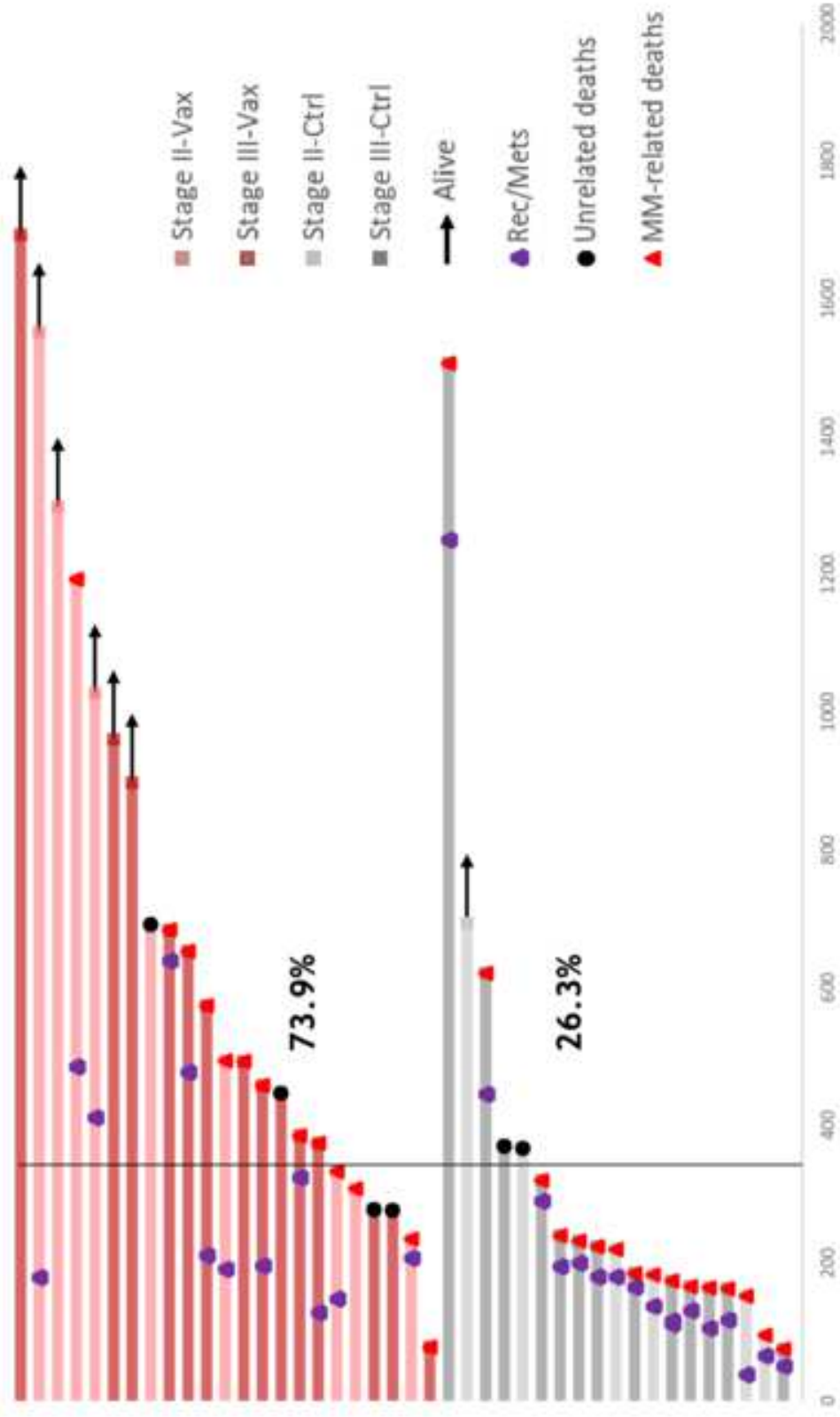
Tarone et al., Figure 2





Tarone et al., Figure 1





Tarone et al., Figure 4

This paper is a Focussed Research Review based on a presentation given at the *Eighteenth International Conference on Progress in Vaccination against Cancer (PIVAC 18)*, held in Oslo, Norway, 3<sup>rd</sup> – 5th October, 2018. It is part of a *Cancer Immunology, Immunotherapy* series of *PIVAC 18* papers.