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Evaluation of the efficacy of a chimeric CSPG4 DNA vaccine for the treatment of malignant melanoma and osteosarcoma

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To my family

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Abstract

Melanoma (MM) and osteosarcoma (OSA) are aggressive tumors, for which standard therapies are poorly effective in counteracting recurrences and metastases. The survival of patients with advanced MM and OSA is still dismally poor; hence, the finding of alternative anti-cancer treatments is urgently needed. The progression of research aimed at developing and testing novel therapeutic options is often hampered by pre-clinical testing in mice that do not faithfully reproduce the tumor complexity and response to therapies. As a step forward, availing of pet dogs that naturally develop tumors as avatars of human diseases, represents an invaluable opportunity to advance research for anti-tumor purposes, endowed with a high translational value.

Immunization using DNA plasmids encoding hybrid molecules to break tolerance against self-antigens represents an innovative strategy to treat cancer. An ideal tumor antigen to be targeted through DNA vaccination should be susceptible to both cellular and humoral immunity. By fulfilling these requirements, the Chondroitin Sulfate Proteoglycan (CSPG)4 is prominent in the immune-oncology panorama, and has emerged as a good immunotherapeutic target. CSPG4 is highly expressed in tumors - including MM and OSA - where it plays a key role in sustaining the malignant behavior of tumor cells, while it is barely expressed in healthy normal tissues.

Thanks to the high similarities between human and canine MM and OSA, and the high homology between the corresponding CSPG4 sequences, we used canine models to evaluate the clinical efficacy of a chimeric DNA vaccine targeting CSPG4. We generated a DNA plasmid coding for a hybrid CSPG4 protein, composed partly by the human (Hu-) and partly by the dog (Do)-CSPG4 (HuDo-CSPG4) sequences. We evaluated HuDo-CSPG4 immunogenicity and anti-tumor potential in tumor-bearing dogs in an adjuvant setting, in association with electroporation. We found that HuDo-CSPG4 DNA vaccination elicited both CSPG4 specific humoral and cellular immune responses in canine MM and OSA patients. The vaccine-induced immunity was safe and effective, since we observed a correlation with an increased overall survival in vaccinated dogs. Additionally, HuDo-CSPG4 vaccination delayed tumor growth and metastasis development in immunodeficient human OSA-bearing mice, and induced a cytotoxic response in a human surrogate setting. Finally, preliminary data suggest a possible role of CSPG4 in sustaining OSA-genesis.

Overall, based on these findings and considering the high predictive value of spontaneous canine tumors, these results might open up the potential translation of this novel immunotherapeutic approach into a human setting, eventually extending this strategy to other CSPG4-positive tumor types.

Introduction

The field of cancer research is constantly growing, with the testing and development of more and more anti-tumor therapeutic options aimed at raising the tail of the survival curve of oncological patients. However, despite significant efforts, cancer is still a leading cause of death worldwide, accounting for nearly 610,000 predicted deaths in the United States in 2023 (1) and more than twice in the European Union (2). Among all tumors, melanoma and osteosarcoma represent the focus of my interest because they are critical challenges in the human oncology panorama.

Melanoma is the fifth most common cancer among women and men, and the deadliest form of skin cancer worldwide (3). It can be stated that the history of cancer immunology is inextricably linked to melanoma research, being this tumor considered one of the most immunogenic human cancers. Therefore, in the past decades, failures and triumphs in the immune-oncology field have often been interconnected with the study of melanoma. Ultimately, the positive impact of novel immune-based treatments on melanoma patients' prognosis led to the evaluation of immunotherapy as the fifth pillar in the armamentarium to fight against this tumor type, and more generally against cancer (4). Despite the great initial enthusiasm raised by the success of checkpoint inhibitors (5), there is still a high percentage of patients dying because of progressive disease. Awareness campaigns as well as screening tests are increasingly aiming to sensitize the population to the evidence of the high aggressiveness and lethality of this disease, emphasizing that there is still a great need to develop more effective therapies for a higher percentage of patients. From this perspective, melanoma is the best stage for developing and testing proof of concept studies of novel immunotherapies.

On the opposite side, osteosarcoma, a malignant bone cancer, is considered a "cold" tumor, in which immunotherapy is clinically quite unexplored. Osteosarcoma is the third most common cancer in the first decade of life, with only lymphomas and brain tumors occurring more frequently (6). As a cancer patient, a child with osteosarcoma needs ongoing aggressive care, and given the very young age, managing treatment could be devastating for both patients and parents who assist in the path of cures. That is why there is an urgent need of identifying and developing novel safer and more effective therapeutic strategies.

Hence, the achievements reported in this thesis could be important for advancing knowledge and finding novel therapeutic opportunities for both melanoma and osteosarcoma patients management.

1. Malignant melanoma and Osteosarcoma: two medical issues in the oncology panorama

1.1 Malignant melanoma (MM)

Malignant melanoma (MM) is the fifth most common cancer worldwide affecting people of any age, and its incidence and mortality have steadily increased over the past two decades (3, 7). MM originates from the malignant transformation of melanocytes; as such, due to the wide distribution of these cells throughout the body, it can occur in any anatomical location or organ and tissue types (8). Based on the site of onset, major subtypes have been defined such as *cutaneous melanoma*, the most common subtype that arises in non-glabrous skin; *acral melanoma,* which originates in the glabrous skin of the palms, soles and nail beds; *mucosal melanoma*, which arises from melanocytes in the mucosa; and *uveal melanoma*, which develops from melanocytes in the uveal tract of the eye and represents the rarest subtype (9). In general, the etiology of MM is still unknown; however, some of the tumor subtypes, and particularly the cutaneous one, are strongly influenced by environmental factors. Indeed, exposure to sunlight and ultra-violet (UV)B radiation are associated with a high risk of developing MM, with fair-haired individuals with freckles and nevi having a major risk (10). A genetic classification of MM has also been established based on the driver genetic alteration(s), which categorizes the tumor into four different genomic subtypes: BRAF-mutant, RAS (N/H/K)-mutant, NF1-loss, and triple wild-type (TWT) subtype (9). The latter is characterized by the lack of BRAF, RAS, and NF1 alterations, and includes mostly non-cutaneous tumors, such as the mucosal and uveal ones, and a small subgroup of cutaneous and acral MM, mainly non-UV-induced (11-13). It has been observed that different mutations are present in respect to the site of onset. For example, cutaneous MM harboring the BRAF mutations is the most common, with 50% of cases presenting the V600E/K abnormality in the BRAF gene ($BRAF^{V600E/K}$). Mutations in N/H/K-*RAS* (20%), and/or NF1 (25%) genes are found to a lesser extent in the cutaneous subtype. Contrarily, mutations in BRAF and NF1 occur at a lower rate in the acral subtype (<35% and 5%, respectively), while N-RAS is mutated to a similar extent as the cutaneous one (28%) (14) .

From a clinical perspective, the surgical resection of the primary tumor is the first-line treatment for early stage, localized MM, resulting in a 5-year survival rate of 98% (15). However, in most cases, patients already have metastatic disease at the time of diagnosis or develop metastasis at a later stage, that requires the combination of surgery with adjuvant chemotherapy/radiotherapy (3). Both single-agent and combination chemotherapies have been evaluated in MM, but they

frequently do not achieve complete response, with a median overall survival (OS) that significantly drops to no more than 9 months (16-18). Indeed, MM is considered to be one of the most chemotherapy-resistant malignancies. The possibility of testing and developing targeted therapies against the most common genetic alterations occurring in melanoma cells has pursued a dramatic evolution of possible therapeutic approaches. This led to approval in 2011 of the first targeted therapy - *Vemurafenib* - against the $BRAF^{V600E/K}$ mutation for the treatment of advanced - metastatic or unresectable - melanoma. *Vemurafenib* is a tyrosine kinase inhibitor that selectively recognizes the ATP-binding domain of the oncogenic $BRAF^{V600E}$ mutation. It has shown a favorable impact on the survival of patients with advanced MM patients, prolonging the median OS to 15.9 months (19, 20). Nonetheless, most of MM patients harboring the $BRAF^{V600E/K}$ mutation present intrinsic or acquired resistance to monotherapy with BRAF inhibitors. The combination with MEK inhibitors that block the downstream activation of the mitogen-activated protein kinase (MAPK) enzymes (MEK1/2), to inhibit tumor growth and induce cell death in BRAF-mutant MM, has therefore been introduced (21). This combinatorial approach has improved patients' prognosis, even potentially attenuating adverse events observed with monotherapy (8, 22, 23). However, the clinical benefit of this treatment is still limited; moreover, in the case of the TWT subtype, targeted therapies cannot be applied (24).

The approval of the immune checkpoint inhibitors (ICIs; discussed in the following sections) *Ipilimumab*, targeting the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), in 2011 and *Nivolumab* and *Pembrolizumab*, targeting the Programmed death-1 (PD-1) molecule in 2014 (25) has drastically changed the management of advanced melanoma patients. Compared to 10 years ago indeed, the 5-year survival rate of metastatic patients has increased considerable from <5% to around 30% (8). However, not all MM patients could benefit from these innovative treatment options, with most of them having innate or acquired resistance to ICIs or developing mild-to-severe side effects (26). Overall, despite advancements in MM management, there is still room for improvement, and the finding of more effective therapies is still an ongoing critical issue.

1.2 Osteosarcoma (OSA)

Osteosarcoma (OSA) is the most common cancer of bones affecting patients with a bimodal age distribution. The first peak is observed in children and young adolescents, whereas the second peak is observed in the elderly. A middle-lower plateau is observed in people aged 25-59 years (27) . OSA is usually defined, however, as a rare pediatric cancer, accounting for 60% of bone tumors in the childhood population (28), and has a high mortality rate (29).

OSA most commonly occurs in the metaphysis of long bones, while it rarely arises in axial skeleton and other sites (30). Epidemiological studies have provided etiological clues to OSA onset, such as associations with puberty, height, and disorders of bone growth and remodeling; thus far, definitive environmental risk factors have not been identified (31).

From a clinical point of view, the resection of the primary tumor through limb-sparing surgery alongside neoadjuvant/adjuvant multi-agent chemotherapy using several cytotoxic agents, such as doxorubicin, cisplatin, high doses of methotrexate, and eventually ifosfamide, is curative in up to 80% of cases with localized OSA (32, 33). Radiotherapy is rarely used because of OSA's intrinsic resistance (34).

Nonetheless, OSA shows a very aggressive behavior, with lung micrometastasis often already present at the time of diagnosis (33). For both these patients and for those who expire recurrence/metastasis within 2-3 years after the end of the primary treatment, survival is dismal. Metastasectomy and radiofrequency ablation could be implemented to restrain metastatic dissemination (35), but conventional treatments remain inefficient against the rapid metastatic spread (33). Only <30% of metastatic OSA patients survive 5-years (27).

Till now, the lack of a comprehensive understanding of the molecular mechanisms of OSA genesis has limited the development of targeted therapies. OSA is a genetically and epigenetically heterogeneous tumor, as described by Poos et al. (36), who identified 911 proteins and 81 miRNAs potentially associated with OSA development. Nevertheless, at present, no activated driver oncogenes have been sufficiently characterized to develop a target-based effective strategy; on the contrary, several tumor suppressor genes have been identified in inherited familial syndromes with a predisposition to OSA such as Li-Fraumeni, hereditary retinoblastoma, Rothmund-Thomson, Bloom or Werner syndromes. Genes such as p53, Rb, Cyclin-Dependent Kinase Inhibitor (CDKN) 2A and 2B, c-myc, RECQL4, BLM, and WRN have been shown to play a critical role in the development of OSA (34, 37). Driver mutations in p53 have been detected in 65-90% of pediatric OSA (34). Synergistic drivers, such as Rb, Twist, PTEN, and Jun, may accelerate tumor initiation and growth. In particular, 56% OSA patients have both p53- and Rb-inactivating mutations (37). However, while these mutations are not directly targetable in the clinic, the possibility of p53 reactivation has been explored (38). As well, novel alterations in the ErbB, PI3K-, AKT-mTOR, VEGF/PDGFR and MAPK signaling pathways have been identified as new candidates for developing targeting strategies, but they are not yet available in the clinic (37, 39).

Also the immunotherapeutic approaches have not provided significant clinical results in terms of increased survival of OSA patients (40). This is at least in part due to the immunosuppressive nature of the tumor microenvironment and low T-cell activity in OSA. The genomic complexity

and significant heterogeneity of the immune landscape of OSA primarily promotes an immunosuppressive phenotype. Most of OSA are characterized by a "cold" immune microenvironment lacking most types of immune cells and associated with genes with copy number alterations as well as decreased HLA expression that contribute to immune suppression (41). Indeed, ICIs used both as single agents and in combination with other drugs, have demonstrated limited clinical efficacy (41-44).

Overall, OSA management and survival rates have remained frozen since 1970. Certainly, the disease rarity, the high genetic variability, and the very young age of patients, together with the lack of predictive pre-clinical models, have contributed to halting the progress of OSA therapeutic management. The identification of more reliable pre-clinical models for testing novel anti-cancer therapies, could help to advance OSA research towards novel effective therapeutic options.

2. Pre-clinical modeling for cancer research

For many years, the study of the molecular mechanisms of cancer has been performed exploiting two-dimensional (2D) *in vitro* platforms. Cancer cell lines are in fact easy to handle and grow; thus, they have been widely used as a first step in the pre-clinical study of novel anti-tumor drugs, allowing fast testing and comparison of experimental results (45, 46).

Nevertheless, a major concern in the use of *in vitro* cell cultures is the reliability of the information that they can provide about processes and pathological outcomes in humans (47). 2D cultures fail to mimic tissue organization *in vivo*, particularly the tumor microenvironment (TME) composition, that includes the strict interaction between tumor cells, immune cells, and stromal and non-cellular components, that may vary between tumor types and patients (48). As such, the use of *in vitro* cell models per se does not reproduce intra- and inter-patient tumor heterogeneity, generating poorly accurate results (47).

These considerations have brought to the shift toward three-dimensional (3D) *in vitro* cultures, in the form of spheroids or organoids, with the aim achieving a structural and functional complexity that can better recapitulate the architecture and the mechanical and biochemical signals naturally observed in human tumors. Spheroids are generated by self-assembling tumor cells in an anchorage-independent manner, into a serum-free medium enriched in specific growth factors; while organoids are self-organized organotypic cultures that arise from tissue-specific adult stem cells (ASC), embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Among these 3D platforms, ASC-derived organoids are considered the best models for mimicking physiopathological features of tumors (48).

Depending on their complexity, 3D models can be derived from cells of the same type (homotypic), or from two or more cell types (heterotypic). More cell types are included in 3D models, more closely they recapitulate the interactions of cancer cells with their microenvironment, including the extracellular matrix (ECM). In addition, reproduction of the challenging conditions of the TME, such as hypoxia, may further increase the reliability of 3D models (45, 46, 48-50). Nevertheless, some concerns remain regarding such models, mainly regarding their failure to faithfully predict the response to therapy.

Based on the technical and ethical guidelines articulated for human experimentation, the validation of new discoveries needs to face out the critical step of pre-clinical animal testing (51). For decades, laboratory animals, particularly murine models, have represented an invaluable tool not only for explicating mechanisms of tumor initiation and progression, but also for evaluating the *in vivo* efficacy of novel anti-cancer treatments (52, 53). Due to the several homologies in genetic, physiological, and pathological features (54), it is widely recognized that mice are a good model for studying human cancers. Mice and humans share a high homology in their inner organ systems, including the immune system; for this reason, these models have always been used to address questions in cancer research. Indeed, when the first transgenic mouse models of cancer were generated in the 1980s (55), several aspects of cancer development as well as novel therapeutic targets have been identified and validated. Since that time, many in-mice investigations of novel anti-cancer treatments have been performed with the aim of translating them into human trials.

Vorious murine models have been generated and exploited. Among them, genetically engineered mouse models (GEMMs) harboring one or more known genetic drivers of carcinogenesis and spontaneously developing specific cancers, have greatly enhanced the understanding of the genetics underlying tumor formation and progression (56). Studies employing immunodeficient murine models injected or implanted with human cells or tissues, such as cell line-derived xenograft (CDX) or patient-derived xenograft (PDX) models, have now become an integral part of the pre-clinical screening of new anti-cancer agents (57, 58). As well, third generation murine models aimed at circumventing the natural histocompatibility mismatch between human and murine tumor cells have also been developed, to establish humanized microenvironments within the mouse host (59).

2.1 Mouse models of MM

Understanding the genetic alterations that trigger melanoma development has been and still is an indispensable step in the discovery of novel therapeutics (60).

In 1991, one of the first GEMM of melanoma was generated via melanocyte-specific expression of simian virus 40 (SV40) T-antigen (61), which was analogous to the phenotype obtained with the loss of the CDKN2A locus. These mice predominantly developed melanomas in the eyes, while skin melanomas were infrequent and mostly benign. When in 1994 Klein-Szanto et al. exposed this model to UV radiations, a higher incidence of cutaneous MM was observed (62). These are the first examples of how mice could be helpful in elucidating the relationship between causative mutations and disease initiation and progression.

Owing to the advancement in the characterization of the molecular profiles of different MM subtypes, several mouse models have been genetically engineered to harbor all major genetic drivers of the disease (i.e., BRAF, N-RAS, NF1), to better elucidate the functions of these key genes in melanoma progression and their potential as therapeutic targets (24). GEMMs have indeed widely proven the oncogenic role of the somatic point $BRAF^{V600E}$ mutation in melanomagenesis (7, 63). In 2014, it was demonstrated that the $BRAF^{V600E}$ mutation evokes accelerated melanomagenesis upon UV radiation exposure, resembling the UV-induced genetic signature prevalent in human MM, thus supporting the influence of such environmental factor in MM development (64). However, the $BRAF^{V600E}$ mutation alone is not sufficient to initiate melanoma, and additional alterations are required (60). In fact, it was demonstrated that $BRAF^{V600E}$ activation paired with the homozygous loss of the tumor suppressor gene PTEN in cutaneous melanocytes in Tyr:Cre-ERT2 BrafCA/+; $PTEN^{lox/lox}$ GEMMs, showed a rapid initiation and progression of melanoma with high penetrance and metastasis to the lungs and distant organs (65). Similarly, the $BRAF^{V600E}$ activating mutation combined with the conditional inactivating mutation in the NF1 gene in GEMMs (tyrosinase (Tyr):CreERT2; BrafCA/+; $Nf1^{flox/flox}$ mice), demonstrated the induction of melanocyte hyperproliferation and tumor onset in most animals (66). Recently, Sun and colleagues (67) employed a *c-Kit-CreER*-driven model to show that *c-Kit* expression in follicular melanocyte stem cells combined with $BRAF^{V600E}$ mutation and PTEN loss gives rise to epidermal melanoma.

Since the upregulation of the RAS/RAF/MEK/ERK pathway has emerged as an obligate event in the etiology of a large majority of melanomas, several mouse models have also been developed to exploit the causal role of RAS oncogene mutations in melanomagenesis. Ackerman and colleagues (68) generated a mouse model carrying the $N\text{-}RAS^{Q61K}$ mutation under the Tyr promoter that developed tumors with low penetrance and high latency. However, when crossed with the Ink4a/Arf-null background, melanomagenesis and lung metastasis onset were significantly accelerated.

Mouse models of MM have also been generated via induction with chemical and physical agents. Because of the strict correlation between UV exposure and MM risk and onset (69), the most important model used is the UV-induced one. The hepatocyte growth factor or scatter factor (HGF/SF) transgenic model is the one that better resembles the human melanoma etiology, and is therefore the most used one. The overexpression of HGF/SF under the control of the metallothionein gene promoter acts through the direct binding of the MET receptor and activates the MAPK and PI3K pathways (60). Upon UV ray exposure, this model generates lesions resembling melanoma and its invasive behavior (60). In addition, mouse models have contributed to reveal that also epigenetic factors may be affected by UV exposure. For example, methylation of CpG islands along the DNA transcript of PTEN and other MM-associated genes, as well as histone acetylation and deacetylation, strongly contribute to speeding up melanoma development (70-72). As well, topical treatment with chemical carcinogens such as 1,3-dimethylbutylamine (DMBA), an immune-suppressing polycyclic aromatic hydrocarbon, and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)(60) coupled with UV irradiation, can induce skin irritation and black lesions that finally generate melanoma (73).

2.2 Mouse models of OSA

OSA occurs spontaneously in <1% of mice of different strains (74). Therefore, GEMMs harboring genetic mutations have been widely used to model human OSA, to better understand the molecular genetics behind its onset, and to study tumor metastatic features (37).

Consistent with human OSA genetic profile, the consequences of the alteration of two of the most common and relevant genes involved in osteosarcomagenesis have been widely investigated. Mutations in Rb alone have been demonstrated to not, or poorly induce OSA formation in mice; while mice in which the TP53 gene was deleted develop OSA with a low incidence (75).

The c-fo*s* overexpression, alone or paired with c-Jun (76), has also been appointed as a critical gene for the genesis of OSA in mouse models. Indeed, elevated expression of the Fos protein in osteoblasts promotes the formation of OSA with short latency and 100% penetrance (77). As well, OSA cells isolated from c-Fos transgenic mice and orthotopically injected in immunocompromised mice showed the formation of lung nodules in two weeks after challenge (78).

In recent decades, genetic studies in mouse models of OSA have also provided evidence of somatic driver mutations in genes that are already well described in other common forms of human cancers. Among these, somatic mutations in components of the Notch signaling pathway were discovered

by Tao and colleagues in 2010. The expression of an activating truncated form of the Notch1 receptor in osteoblasts, was sufficient to drive OSA development with an increased manifestation of bone remodeling before tumor establishment (79). Notch1 activation is now considered a first potential driver for OSA initiation, since it can drive OSA formation in mice with complete penetrance. Moreover, the synergistic pairing of p53 loss/Notch1 activation has revealed a more rapid development of murine OSA (80). Other genes, such as Wif1 and Brca2, can instead induce OSA tumorigenesis in only a small percentage of mice (37, 81). Myc amplification has also been associated with the pathogenesis and chemoresistance of OSA, and its inactivation in mouse-derived bone marrow stromal cells (BMSC) induces proliferative arrest and promotes differentiation toward an OSA phenotype (82). Alterations in factors involved in osteoprogenitor cells differentiation have also been explored. A prominent role in OSA-genesis has been attributed to Runx2, a pre-osteoblast transcription factor that acts as a tumor suppressor gene that interacts with p53/Rb. It has been demonstrated that Rb loss could disrupt the feed-forward cycle between Runx2 and p27, resulting in the impairment of terminal differentiation of osteoprogenitor cells (83, 84).

The use of chemical carcinogens for inducing OSA in mice was also exploited in the 90s as well. Most models have been generated through the direct orthotopic injection of carcinogens, with a very low incidence of tumor onset and high latency (85). However, this approach was useful for determining the low impact of environmental exposure on OSA genesis. As well, OSA has been induced in mice by exposure to radioactive heavy metals. Ions produced from such metals naturally home to the bone matrix in the metaphysis of long bones and emit radiations that have proved to induce osteosarcoma (84).

Human and mouse OSA cells, genetically engineered to carry the most common genetic mutations found in human tumors, have also been widely used to challenge immunodeficient and immunocompetent recipient mice, respectively. In these settings, the site of tumor cell injection is relevant to better recapitulate the human situation. Indeed, both heterotopic (i.e., subcutaneously) or orthotopic (i.e., para-tibial or intraosseous) implantation of cells have been tested, being the former the easiest model, but the latter the better to recapitulate the tumor microenvironment and the metastatic cascade observed in the human clinical disease (74, 86)

In the search or reliable pre-clinical models for OSA research, PDX models have also come to prominence, demonstrating to faithfully recapitulate the histologic and radiographic features of primary OSA tumors, maintaining the genomic profile of the original ones, the same heterogeneity and drug sensitivity properties of the original tumor when injected in the xenogeneic host. Studies in PDX mice have provided an opportunity to identify recurrent genetic alterations that occur in

human OSA, to investigate treatment resistance mechanisms, and to test novel agents (87, 88). However, despite the orthotopic tumor implantation has demonstrated to improve the development of spontaneous metastasis as compared to the subcutaneous one, most of the times PDX models fail to reproduce the high metastatic phenotype encountered in human patients (87).

2.2.2 Modeling OSA-genesis in mice

As still little is known about the cell of origin of OSA, GEMMs represent a suitable tool for studying this aspect to eventually advance OSA research. OSA is assumed to originate at some stage in the differentiation process of MSCs into pre-osteoblasts/mature osteoblasts (OSB), and the moment at which any alteration occurs can affect the final tumor phenotype (86). Recently, OSA is generally classified as a tumor of mesenchymal derivation, and some studies appoint OSB precursors as the cell of origin when they are unable to proceed to terminal differentiation (86). Little is known, however, about the events that occur prior to the clinical manifestation of the disease. Therefore, the characterization of such genetic modifications could be crucial for understanding OSA pathogenesis and improving patients' outcomes.

Unlike other sarcomas, OSA is characterized by high and complex genetic heterogeneity (89). Nevertheless, as for a broad range of tumor types, alterations in p53 and Rb genes have shown to play a key role even in OSA pathogenesis. As such, murine MSCs and/or OSB and mouse models have been genetically modeled by introducing relevant mutations in p53, alone or in combination with Rb inactivation, as previously reported in this thesis. Inactivation of both p53 and Rb in murine MSCs has been demonstrated to generate sarcomas with a low incidence (20-30%). Contrarily, once committed to the OSB lineage, inactivation of these genes has been shown to induce OSA with higher penetrance and endowed with strong metastatic potential (90, 91). Berman and coworkers generated a double knock out (KO) mouse model by concomitantly inactivating both p53 and Rb, using an Osx1-Cre transgene, to selectively induce the inactivation of these tumor suppressors in murine OSB precursors. Cell lines isolated from primary tumors developed in this mouse model showed mesenchymal properties, being able to differentiate *in vitro*, displaying higher proliferative ability as compared to normal OSB and MSCs, and demonstrated a retained expression of the stem marker Sca-1. These results supported the hypothesis that OSA cell-of-origin could be MSCs cells already undergoing osteoblast commitment, rather than immature MSCs (90).

As well, the role of genes involved into OSB development and differentiation have also been explored. Runx2, one of the main sustainers of OSB formation from osteo-chondroprogenitor

cells, has been shown to drive OSA-genesis through its interaction and consequent deregulation by Rb and Myc (92, 93). Similarly, BMP/TGFβ family members, as well as signaling pathways involved in OSA metastatization, such as the ERK1/2 pathway, have shown to promote OSA pathogenesis at different stages of malignant transformation from OSB (94).

Other genes with a well-defined role as a selectable oncogenic drivers of tumor proliferation, such as MET (95), have also been investigated as possible driver genes of OSA-genesis.; and the contribution of MET receptor overexpression in the initiation of transformation of human OSB has been studied. Even though MET is not reported to be involved in the differentiation of OSB, Patanè and coworkers investigated whether its overexpression in primary human OSB could induce oncogenic transformation, given the high expression and the involvement of MET in human OSA. MET overexpression in patient-derived OSB promoted their transformation into OSA cells *in vitro,* being its overexpression essential for the persistence of the transformed phenotype. These transformed cells also displayed tumorigenicity *in vivo* in immunocompromised mice, even though at low incidence. Nonetheless, all established tumors showed the distinguishing features of highly aggressive human OSA (96, 97).

Finally, Wnt/β-catenin signaling has also been implicated in sarcomas development, as β-catenin is precisely regulated at different stages of MSCs commitment toward the osteoblastic phenotype, raising the possibility that its deregulation could lead to OSA pathogenesis (98-100). In this context, Sato and coworkers have reported that in mouse models the concomitant deregulation of β-catenin, together with p53 inactivation in chondroitin sulfate proteoglycan (CSPG)4 (*described in other sections of this thesis*) expressing pericytes, could drive the development of sarcomas, including OSA. In this study, OSA occurred with a 66-76% incidence and 20% lung metastasis development (101).

2.3 From mice to men: translation of therapeutic strategies for MM and OSA

Several pre-clinical studies in the melanoma setting have been successfully translated from mice to men, providing new options to treat this complex disease. One of the most relevant translations of pre-clinical findings for MM management in human clinic is represented by the selective inhibitor of mutated-$BRAF^{V600E}$, PLX4720, better known today as *Vemurafenib* (102). Following striking demonstrations of the anti-tumor efficacy of *Vemurafenib* in pre-clinical xenograft models (103-105), Phase III randomized clinical trials in humans were performed (106), leading to its approval by the Food and Drug Administration (FDA) in 2011, and EMA in 2012, to treat $BRAF^{V600E}$-mutated, advanced MM patients. *Vemurafenib* administration showed clinically

meaningful results, achieving prolonged progression-free and overall survival in treated patients as compared to chemotherapy treated controls (107).

Contrarily, NRAS has often been considered an undruggable molecule to date, with no targeted therapies approved in recent decades. Developing an effective compound for targeting NRAS mutations remains a hurdle to overcome. Even though pre-clinical studies in melanoma xenografts have demonstrated the efficacy of its targeting by means of small-molecule inhibitors alone or in combination with receptor tyrosine kinase (RTK) inhibitors (108, 109), such results were not confirmed in the clinical setting, where treatments were not effective in hampering NRAS-driven MM (110). As a step forward, *in vitro* studies in cells containing both the activating $NRAS^{Q61R}$ and $BRAF^{V600E}$ mutations, suggested the sensitivity of MM cells to MEK inhibitors (MEKi, (111)). Hence, some studies have successfully explored the applicability of targeting RAS-MAPK downstream effectors by testing the efficacy of MEKi in NRAS-mutated melanomas in pre-clinical xenograft models (112). When these inhibitors were then translated to human melanoma patients, a longer progression-free survival was observed compared to those treated with chemotherapy alone (110, 113). These are some examples of the positive relationship that might exist between pre-clinical studies in mice and the clinical panorama, emphasizing the importance of this segment of pre-clinical research for advancing clinical application. Indeed, other than these, different new combination therapies, including targeted and immune-based therapies, have been tested and approved in the last 10 years in the United States, some of which are now part of the standard-of-care armamentarium, strongly improving patients' survival and quality of life (114). Among them, the approval in 2018 of the combination of *Enconafenib*, the newest BRAF-inhibitor, with the MEKi *Binimetinib* (115, 116), as well as the more recent report of the successful combination of *Atezolizumab*, an anti-Programmed Death Ligand (PD-L)1 monoclonal antibody (mAb), with *Vemurafenib* and the MEKi *Cobimetinib* (117) approved for the treatment of unresectable metastatic MM, for which the first evidence of efficacy was achieved in murine models.

Concerning OSA, till now, very few successful studies conducted in syngeneic mouse models or GEMMs have seen their translation in a human setting. To overcome this brake, in 2013, the Pediatric Preclinical Testing Program (PPTP) established that PDX are the most efficient pre-clinical models for testing novel therapeutic agents for OSA treatment (118).

Previously known targeted therapies have been tested in pre-clinical OSA PDX models, based on the mutational profile found in human patients. As an example, the effects of anti-VEGFR/PDGFR therapies, including antibodies and small molecule tyrosine kinase inhibitors, such as *Sunitinib*

and *Regorafenib*, have been explored in these models. When administered in a human intratibial xenograft OSA model, *Sunitinib* reduced tumor burden and suppressed pulmonary metastasis (119). Similarly, PPTP promoted a study in which *Regorafenib* was tested in more than one hundred PDX, demonstrating a slowing in tumor progression in most cases (120). *Sorafenib*, a multi-tyrosine kinase inhibitor approved for the treatment of hepatocellular carcinoma, renal cell carcinoma, and thyroid carcinoma, has also been tested in OSA PDX models demonstrating its efficacy in inhibiting the growth, angiogenesis and metastatic ability of OSA cells interfering with the ERK1/2, MCL-1 and ERM pathways (121). Recently, the combination of mTOR (*Rapamycin*) and VEGF/VEGFR inhibitors has also demonstrated impressive results in overcoming chemotherapy resistance in PDX models based on an OSA-derived lung metastasis cell line (122). Nowadays, OSA research is turning on using PDX models as "avatars behind the scenes", so patient-derived tumor-implanted mice are supposed to receive in parallel the same therapeutics as the donor patient enrolled in the clinical trial, with the aim of predicting the clinical response (87). The main advantage of such an approach relies on the possibility of either testing the therapy chosen for the donor patient or testing novel treatment combinations and evaluating side effects as well, elevating the translatability of novel precision therapies in humans (87, 123). However, to date, no specific therapies have been clinically approved using this approach.

2.4 Limitations of murine models

Despite their well-documented relevance in cancer research, murine models have limitations that should be considered. Among the criticisms regarding the use of murine models, there is a limited lifespan, that does not allow monitoring tumor growth over long periods of time; the low degree of heterogeneity in mouse tumors as compared to human ones, that might be initially traduced in higher therapeutic responses in mice, ultimately leading to an overestimation of the effectiveness of the treatments. When analyzed in depth, mouse models of cancer often do not faithfully recapitulate human tumor biology, drug responses, and therapy resistance. All these barriers limit the predictive value of pre-clinical results and create challenges for their clinical translation in human clinical trials (55). Moreover, in spite the advantages provided by using mice carrying mutations relevant to their human counterparts and developing spontaneous cancers, it must be noted that some transgenic mouse models could develop multiple tumor histotypes during their lifespan, due to overlapping genetic mutations that could drive different cancer types. For example, most mice carrying the deletion of TP53 have demonstrated the development of different tumors, including lymphomas and hemangiosarcomas (124). Therefore, the high incidence of multiple

tumor types may preclude the use of certain mouse models, particularly if the tumor of interest is not the most commonly developed (74). In mouse melanoma models, the difference in melanocyte localization in the skin between mice and humans is an important concern. In fact, melanoma development in mice often occurs with distinct histological properties, which are not representative of human disease, which limits the reliability of the findings (60). Also in the case of OSA, GEMMs have demonstrated to not fully summarize all events that occur in human tumor progression and the typical molecular heterogeneity that characterize the human tumor (78). In general, evidence of such limitations is that 90% of drugs fail to be effective in human trials, even though they succeed in pre-clinical studies (125).

The use of PDX models has also been likewise criticized, and the question of how accurately xenografts resemble the human situation remains open. Indeed, while on one hand PDX models have demonstrated to be valid tools to recapitulate the drug-sensitivity patterns seen in the patients from who they derive (126), on the other hand, they have resulted non-representative of the clonal dynamics of some tumors, because of initial selective pressure during culture or after cell transplantation, in which more stable and aggressive clones tend to prevail (127). Some studies have also shown that clones surviving upon injection in PDX models often lose their ability to recapitulate organ-specific metastatic spread and therapy response. In addition, engrafting human cells in immunocompromised mice precludes the evaluation of immunomodulatory or stroma-directed therapeutic approaches (127). Finally, PDX models are expensive, and not all laboratories may have direct access to patient material in real time (87).

One of the major challenges in pre-clinical cancer research in mice is the modeling of metastasis. Time to metastasization metastasis is difficult to predict in human patients, since metastasis can either rapidly occur and be detected early, or it can remain dormant and take decades to manifest (114). Hence, recapitulating the metastatic process that occurs in humans in mice remains a critical point today. Indeed, an important limitation of GEMMs is, for example, the low incidence of spontaneous metastatic spread, which often does not reflect the organ tropism seen in human disease (128, 129). Moreover, very often the primary tumors grow rapidly, and their resection is required to allow the development of metastases. Surgery is nonetheless not always feasible in such cases. As well, intravenous injection of tumor cells, which is the most common method for inducing experimental metastasis, has the limitation of not recapitulating the first steps of the metastatic cascade (130).

Albeit mice will undoubtedly remain a critical first step in pre-clinical research, key questions in cancer biology and therapy cannot be sufficiently asked or answered with the exclusive use of such models. The need for additional animal models that could integrate and overcome some of

the limitations of murine models is being increasingly recognized, with dogs with naturally occurring tumors being among the more reliable ones (131, 132).

Actually, in the oncology panorama, the study of spontaneous tumors in dogs has recently emerged as an invaluable opportunity for advancing the understanding, diagnosis, and management of cancer, with a high translational perspective (133), as *"tumor-bearing dogs capture the essence of the problem of cancer in a way that is not achievable with other animal models"* (134). By flanking pre-clinical traditional rodent models, the study of cancers in dogs may drive mutual clinical benefits for both human and canine patients, representing the basis of the *comparative oncology* concept.

3. *One Health*: The importance of comparative oncology for cancer research

As in humans, cancer represents a major health problem in veterinary settings, being the leading cause of disease-associated death in pet animals. However, advancements in veterinary medicine are still running behind humans, and the development of novel therapies for exclusive veterinary purposes is still limited, with cancer management of pets having reached a therapeutic plateau. The origin of the *One Health* concept dates to the 20th century, to promote a complex initiative that aims to stimulate a multidisciplinary approach towards the well-being of all living species (135). Starting with these ideas, a specific focus has been placed on cancer, promoting the collaboration between medical and veterinary disciplines through the study of naturally occurring spontaneous diseases that are comparable to each other. A first revolution in this direction came in 2003, when the canine genome was decoded and consequently publicly released (136), revealing that dogs share 80% genetic similarity with humans, compared to 67% of mice. Nowadays, the canine genome is the third most detailed mammalian genome assembled, in addition to the human and mouse ones (131). This finding is becoming increasingly important in the era of personalized medicine,, facilitating comparative genomic, transcriptomic, and proteomic investigations in dogs, with the final aim of developing novel targeted therapies for both canine and human species (132). Roughly in parallel, the National Cancer Institute (NCI) initiated the *Comparative Oncology Program* in 2003, to foster studies in canines that could inform the design of trials in humans (137). Right after, a European project also joined this concept, launching a similar purpose through the LUPA project (138).

Among all companion animals, the dog is the species in which the discipline of comparative oncology has grown the most (131). Dogs spontaneously develop tumors in the context of the same environment as their owners; thus, they are influenced by the same risk factors and are

susceptible to various disease states that may influence carcinogenesis (134). A breed predilection for the development of certain cancers has been identified, providing additional advantages in determining genetic susceptibility towards the development of these neoplastic diseases (139) . Importantly, dogs have similar responses to treatments as humans, such as chemotherapy, and develop resistance mechanisms and toxicities as well (132). The relatively shorter lifespan and generally faster progression of cancer in this species, compared to humans, also allows for a more timely assessment of clinical outcomes. Moreover, dogs develop tumors in the presence of an intact immune system, nearly like the human one, and therefore they are suitable models for studying tumor cells-immune cells interactions and the responses to immune-based therapies. This is of paramount importance for studying novel immunotherapies in the setting of a permissive tumor microenvironment and the potential side effects associated with profound immune activation (131). Finally, the tumor heterogeneity of canine cancers closely models the situation encountered in human tumors (140, 141). Of note, dogs spontaneously develop with a higher frequency some tumor histotypes that are rare in humans, offering the opportunity to perform statistically relevant studies that could more rapidly inform about the efficacy of novel therapies. All of these considerations place the dog in a unique position to well reflect cancer development, progression, and response to treatments, as occurs in humans. It is hence not surprising that pre-clinical testing in dogs has been introduced as a necessary step to accelerate the translation and approval of novel drugs for human use (142).
Among solid tumors of high comparative interest, MM and OSA have emerged because of the strong similarity with their human counterparts (132, 134).

4. Spontaneously occurring MM and OSA in dogs

4.1 Canine oral melanoma

Melanocytic tumors are widespread in dogs, with 70% of cases displaying a malignant phenotype (van der Weyden et al., 2020 (143)). MM is indeed one of the most common cancers in dogs (13). The anatomical locations of MM onset are oral (62%), cutaneous (27%), digit (6%), ungual (4%), and ocular (1%;(143)). The anatomical location influences tumor behavior, with metastasis in up to 97% of dogs with oral melanomas, 100% with subungual tumors, and 84% with digital tumors, whereas cutaneous and ocular melanomas are predominantly benign (144).
However, the most diffuse and fatal subtype, affecting 20.3 cases per 100,000 dogs per year (13) is oral MM (OMM), which accounts for 30–40% of all canine oral malignancies (145). OMM

affects several breeds including Scottish Terriers, Golden and Labrador Retrievers, Poodles, Dachshunds, Cocker Spaniel, Miniature Poodle, Chow Chow, Gordon Setter, and Anatolian Sheepdog, with a higher prevalence in elderly dogs, without any gender predilection (145, 146). The first-line treatment is the surgical removal of the primary mass with better outcomes achieved when correct surgical excision is performed for local control of the tumor (147). Surgery is usually implemented with radiotherapy and/or chemotherapy (148). Both neoadjuvant and adjuvant radiotherapies have been demonstrated to be good options for local control of the tumor, with complete remission in up to 70% of treated cases (149). However, OMM is a highly malignant disease, characterized by local invasiveness and a high metastatic propensity to the lungs and lymph nodes (145, 147). When OMM-bearing dogs develop distant metastasis, they also rapidly acquire resistance to chemotherapeutic drugs (13). Metastases are the leading cause of death (143, 150). The survival time of metastatic OMM-affected dogs is very short being, of approximately 200 days after diagnosis (151). Thanks to the release of the canine genome, the characterization of the molecular landscape of canine MM has revealed genetic alterations in BRAF, NF1 and KIT genes that parallel the human ones (132), although with a lower frequency. BRAF gene mutations are in fact observed in a very small percentage of canine MM cases (6%; (152)). Contrarily, MAPK and PI3K/AKT/mTOR pathways have been found to be activated in canine OMM, highlighting the convergence between humans and dogs (132). Although genetic alterations in OMM have not been totally described yet, the mutational profile of OMM resembles an UV-independent molecular etiology, which is typical of human non-UV-induced cutaneous, mucosal, uveal, and TWT subtypes (153). Hence, OMM would represents a highly translational model for human tumors lacking the most common alterations for which targeted therapies have already been developed and tested.

Overall, due to the limited response to conventional treatments, the survival times of OMM-affected dogs have not improved in the last few decades (154). Hence, the pursuit of more efficacious and tolerable treatments is spurring the finding of novel anti-cancer approaches to improve the long-term prognosis of canine patients, with a high translational value for human MM patients too.

4.2 Canine osteosarcoma

OSA occurs in 10,000 dogs/year in the USA (155-157), with a wider peak of incidence observed in larger and giant breed dogs, such as the Saint Bernard, Great Dane, Irish setter, Doberman Pinscher, Rottweilers, German Shepherds, and Golden Retriever (158). Predisposing factors such

as increased weight and height appear to be related to OSA onset (158, 159). OSA most commonly occurs in the appendicular skeleton, usually affecting the metaphysis of long bones, such as distal radius, proximal humerus, distal femur, proximal and distal tibia, and ulna (159).

Several similarities with the human counterpart have been identified regarding OSA genomic drivers; among them copy-number aberrations of several key genes, such as RB, MET Proto-Oncogene, c-FOS, insulin-like growth factor receptor-1 (IGF-1R), MYC Proto-Oncogene, RUNX Family Transcription Factor 2 (RUNX2), CDKN2A, CDKN2B, Human epidermal growth factor receptor 2 (HER2) and others; being TP53 the most mutated gene (160-162). Recently, several studies have revealed additional similarities between human and canine OSA, such as the low overall mutational burden (~2 mutations per Mb in humans younger than 18 years of age, ~1.98 mutations per Mb in dogs) and dysregulation of pathways such as the ERK and PI3K–mTOR ones (132). Additionally, studies on cell populations infiltrating canine OSA have revealed levels of T cells and macrophages that resemble those found in human OSA (163). Furthermore, myeloid derived suppressor cells (MDSC) represent the predominant population observed in circulation and within the tumor microenvironment in canine OSA (163-165), while high T regulatory (Treg) cell levels have been correlated with worse prognosis (166, 167), both contributing to mechanisms of immune evasion (165).

Standard-of-care treatment includes limb-amputation or limb-sparing surgery, followed by systemic adjuvant platinum-based or doxorubicin chemotherapy, alone or in combination. Such treatments are effective in modestly prolonging the survival of patients, with a 1-year survival rate of less than 45% (168). However, in most cases metastases occur rapidly and spread to secondary organs, including distant bones and lungs. In this case, the survival of OSA-bearing dogs dramatically drops to a few months (158, 168).

The high biological and clinical similarities, including cancer management and response to treatments, between canine and human OSA strongly support the ongoing characterization of the dog as a potential model of pediatric OSA, representing an invaluable opportunity to advance knowledge for this rare pediatric malignancy.

Table 1. Features of MM and OSA on a comparative lens

	Malignant Melanoma		***Osteosarcoma***	
	Humans	**Dogs**	**Humans**	**Dogs**
Incidence	Estimated 97,610 diagnoses/year in the USA in 2023	Up to 60.000 diagnoses/year in the USA	1000 cases/year in the USA (https://www.cancer.org/cancer/oste	Up to 10.000 diagnoses/year in the USA

			osarcoma/about/key-statistics.html)	
Breed	/	Scottish Terriers, Golden Retrievers, Poodles, Dachshunds, Cocker Spaniel, Miniature Poodle, Chow Chow, Gordon Setter, Anatolian Sheepdog, and mixed-breed dogs	/	Saint Bernard, Great Dane, Irish setter, Doberman pinscher, Rottweilers, German Shepherds, Golden Retriever
Age of patients	Affects people of any age, major peak of incidence Over 50 years of age	Median age 10–11 years	Median age: -first peak of incidence10-30 years -second peak of incidence 25-59 years	Median age 7 years
Causes and risk factors	Etiology generally unknown Major risk factors for cutaneous subtype: UVB radiation exposure Major genetic mutations: – $BRAF^{V600E/K}$ – N/H/K-RAS – NF-1	The major risk factors include: – Consanguinity, pigmentation characteristics – Environmental carcinogens (mucosal melanomas) – Sun exposure (cutaneous melanomas) Major genetic mutations: – NRAS, KRAS, PTEN, KIT, TP53 – $BRAF^{V600E}$ mutation not detected	Etiology unknown Possible risk factors include: – Genetic alterations – Mutagenic effects of ionizing radiation – Multiple minor trauma – Hormonal influence Major genetic mutations: TP53, RB1, PTEN, MYC, CDKN2A, CDKN2B RECQL4, BLM, and WRN	Etiology generally unknown Possible risk factors include: – Genetic alterations – Mutagenic effects of ionizing radiation – Multiple minor trauma Major genetic mutations: TP53, RB1, PTEN, MYC, HER2, CDKN2A, CDKN2B, RUNX2
Anatomic sites	- *Cutaneous melanoma*, non-glabrous skin; - *Acral melanoma,* glabrous skin of the palms, soles and nail beds; - *Mucosal melanoma*, from melanocytes in the mucosa; -*Uveal melanoma*, from melanocytes in the uveal tract of the eye	Any portion of the oral cavity Gingival mucosa, mandibular labial mucosa, tongue, skin, digit/footpad Metastasis: lymph nodes, lungs, tonsils, other distant organs	Mostly metaphysis of long bones Femur, tibia, humerus Other potential sites include the skull or jaw (8%) and the pelvis (8%) Metastasis: Lungs, bones, lymph nodes	Mostly metaphysis of long bones Forelimbs, hind limbs, distal radius, proximal humerus, distal femur, proximal tibia, distal tibia, diaphyseal ulna Metastasis: Lungs, bones, soft tissues, regional lymph nodes

	Metastasis: Skin and subcutaneous, lungs, liver, bones and brain			
Conventional treatments	– Surgery and/or radiation therapy – Chemotherapy (decarbazine, temozolomide) – Targeted therapies (BRAF inhibitors, MEK inhibitors) – Immunotherapy (Checkpoint inhibitors; IL-2; TVEC)	– Surgery and/or radiation therapy – Chemotherapy (carboplatin)	– Surgery – Neoadjuvant/ adjuvant chemotherapy (doxorubicin, cisplatin, high doses of methrotrexate, and eventually ifosfamide – Metastectomy and/or radiofrequency ablation in case of advanced disease	– Surgery – Postoperative chemotherapy (doxorubicin, carboplatin and cisplatin)
Survival	5-year survival rate: – Localized disease 98% – Metastatic disease 30%	1-year survival rate: – Localized disease 70% – Metastatic disease 30%	5-year survival rate: – Localized disease 80% – Metastatic disease at diagnosis <30%	1-year survival rate: <45%

Adapted from Tarone et al., 2021 (146)

5. Cancer Immunotherapy

Increasing evidence supports a key role of the immune system in the treatment of cancer. By flanking surgery, radiotherapy, chemotherapy and targeted therapy, immunotherapy is emerging in the anticancer arsenal, as well as in the field of veterinary oncology. Immunology and oncology have a long-time relationship that has become even more consolidated in recent decades. Cancer immunology investigates the complex mechanisms and interactions between developing tumors and immune cells, with the aim of identifying novel immunotherapy-based approaches (169).

The insight of the tumor-immune cell interactions dates back several decades, finding its greatest expression in Paul Ehlrich's concept of *immunological surveillance,* which supported the idea that the natural immunity of the host can protect against growing malignant cells. However, at that time the lack of immunological tools and the poor knowledge of the immune system did not let to experimentally prove this hypothesis (170).

Subsequent advancements in the immunology field have allowed for a deeper investigation of both immune cell populations and their functions. As a step forward, in the 50s' Burnet and Thomas reintroduced the *immunesurveillance theory,* proposing that the immune system continuously recognized and eliminated nascent transformed cells (171, 172). However, their hypothesis was rejected by the scientific community. Approximately 35 years ago, pre-clinical and clinical data from human patients provided surprising evidence that immune cells are essential to prevent and counteract cancer (170, 173).

Nonetheless, the presence of an intact immune system does not always imply the prompt elimination of malignant cells. Indeed, tumor-associated immune cells can play a dual role by both participating in tumor eradication on one side and shaping the immunogenicity of tumors on the other side, thus promoting the progression of more aggressive tumor clones (174, 175). Therefore, the concept of cancer immunosurveillance was refined and extended to the concept of the now well-known *cancer immunoediting theory (170)*.

Cancer immunoediting comprises three distinct phases: (i) *elimination*, (ii) *equilibrium,* and (iii) *escape*. The *elimination* phase is likely related to the original concept of cancer immunosurveillance, in which activated immune cells can kill cancer cells before the clinical appearance of the tumor. Here, a strict cooperation of both innate and adaptive immunity is required. When cancer cells manage to prevail and avoid killing by immune cells, they can enter a phase of *equilibrium*. In this phase, cancer cells can remain dormant and reside in patients for decades. Their interaction with immune cells may eventually sculpt the phenotype of the developing tumor, resulting in the selection of less immunogenic clones that could more easily hide from the immune system. Consequently, in the phase of *escape,* these surviving cancer cells are no longer susceptible to attack by immune cells, and can grow uncontrollably. In this perspective, the immune escape of cancer cells is the result of the selective pressure exerted by the immune system on malignant cells. Indeed, when the immune response against harboring tumors is triggered, it can also prompt the onset of tumor variants that can no longer be recognized by effector immune cells (176, 177).

On their side, tumor cells put in place different mechanisms to evade $CD8^+$ cytotoxic T cell (CTL) recognition and killing, for example through the downregulation of the Major Histocompatibility Complex (MHC) class I molecules or the engagement of the PD-1/PD-L1 axis. Indeed, tumor cells can upregulate PD-L1 expression and its interaction with PD-1 on T cells causes their chronic inhibition and anergy. Among the other mechanisms of tumor escape, the secretion of immunosuppressive cytokines, such as TGF-β or IL-10, or the recruitment of MDSC and Treg

cells in the tumor environment could have a high impact (178). This third phase of escape is now recognized to be one of the *Hallmarks of Cancer* (170, 175, 179).

Thus, how the immune escape of cancer cells can be overcome remains an open question, paving the way for a growing field of research towards strategies aimed at potentiating patients' immune system against cancer cells (180). Hence, cancer immunotherapy now stands out as the fifth pillar of cancer care (181). Immune-based treatments have strikingly changed the management of patients with cancer, with a significant prolongation of their survival. Immunotherapies can be broadly categorized into two main types of interventions: *passive* and *active* therapies. This classification is based on their mechanism of action, as well as on their way to engage the host immune system to counteract cancer.

5.1 Passive immunotherapy

Passive immunotherapy involves the transfer of *ex vivo*-generated immune elements into patients, including the systemic administration of tumor-specific antibodies, pre-activated immune cells endowed with anti-neoplastic activity, and oncolytic viruses (182). Once these passive immunotherapeutics are inside the body, they are supposed to compensate for the missing or deficient immune functions of patients.

Tumor-targeting mAbs are the most characterized and employed form of passive immunotherapy in human clinical practice. The most commonly used are of the IgG class, due to their long half-life and stability in the serum (182). Anti-cancer mAbs are supposed to selectively recognize cancer cells based on the selection of the target, ideally a tumor-associated antigen (TAA), overexpressed on tumors but not in non-transformed cells (183).

Anti-cancer mAbs can act by directly impairing the signaling mediated by molecules expressed on the surface of malignant cells, inhibiting the tumorigenic functions, or by indirect mechanisms, inducing antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and/or antibody-dependent cellular phagocytosis (ADCP). mAbs can also be conjugated to radioisotopes, cytokines, and toxins to deposit active agents in the proximity of tumor cells to avoid the spread of adverse effects (182).

As another passive immunotherapy strategy, the adoptive cell transfer (ACT) involves the isolation of autologous immune cells, such as tumor infiltrating lymphocytes (TIL), cytokine-induced killer (CIK), or cascade-primed (CAPRI) cells, their selection and enrichment in potentially tumor-reactive immune effectors *ex vivo*, and their re-administration back to the patients. Patients' T cells can be engineered *ex vivo* to express T cell receptors (TCRs) with high avidity for specific tumor

antigens. Moreover, patient-derived T cells can be also modified to express Chimeric Antigen Receptors (CAR). A typical CAR construct comprises four main components: (i) an extracellular target antigen recognition domain, (ii) a hinge region, (iii) a transmembrane region, and (iv) one or more intracellular signal activation domains (184). The antigen-binding domain is generally an antibody single-chain variable fragment (scFv) that confers antigen specificity by directly targeting the membrane-bound surface receptors of cancer cells. Their unique structure makes the use of CAR advantageous, since it makes T cells capable of recognizing, and hence potentially killing cancer cells expressing the selected TAA, in an MHC-independent manner.

Oncolytic viruses (OVs) are another promising approach that is being increasingly used in the treatment of cancer. Although OVs are a quite novel approach to treat cancer, they have already well proven anti-tumor efficacy in various clinical trials (185), and have been recently introduced as agents that induce both local and abscopal immunity (186, 187). An example of the successful application of oncolytic virotherapy is the T-VEC (Imlygic™) a herpes simplex type-1 virus (HSV-1) encoding the human granulocyte macrophage colony-stimulating factor (GM-CSF) gene. Following relevant achievements in Phase III clinical trial for the treatment of unresectable melanoma patients (188-190) it received FDA approval in 2015-2016. Several advantages can be attributed to OVs-therapy. OVs are tumor-selective, providing higher cancer specificity and better safety margins, can induce immunogenic cell death of both cancer and stromal cells, and provide the loaded tumor antigen in conjunction with danger signals like damage-associated molecular pattern (DAMP) and OV-derived pathogen-associated molecular pattern (PAMP) molecules, and inflammatory cytokines that culminate in the induction of strong anti-tumor immunity (191). For their properties, OVs are considered at the edge between passive and active immunotherapy.

One of the main limitations of passive immunotherapy is the onset of mechanisms of resistance and/or adverse effects and, even though they have a quick time to action, they fail to induce long-lasting immune memory.

5.2 Active immunotherapy

The goal of active immunotherapy is to safely stimulate the patient's own immune system to induce a long-lasting activation of both humoral and cellular responses (192). The most important active immunotherapy approaches comprise recombinant cytokines, mAbs targeting molecules involved in T cell activation such as ICIs, and cancer vaccines.

5.2.1 Immunostimulatory cytokines

The administration of cytokines is the oldest immunotherapeutic intervention for treating cancer. Cytokines can be administered either locally or systemically, and have demonstrated positive results in the early stages of testing and in larger clinical trials. However, the administration of most immunostimulatory cytokines to cancer patients as standalone therapeutic interventions is generally associated with little clinical activity; therefore, they are usually administered as adjuvants in combination with other immunotherapeutics such as immunomodulatory mAbs or anti-cancer vaccines, rather than as monotherapy (183).

Exceptions are represented by interleukin (IL)-2 (*Aldesleukin and Proleukin®*), and interferon (IFN)-α2b (*Intron A®),* which were approved by the FDA in 1992 and 2011, respectively, for the treatment of different cancers, including melanoma (193, 194). GM-CSF (also known as *Molgramostim, Sargramostim, Leukomax®, Mielogen® or Leukine®*), was approved by the US FDA and EMA human use, and when combined with other immunotherapies such as peptide-based vaccines or immunomodulatory mAbs, has shown a synergistic clinical activity (183). Anyway, their use has never achieved wide clinical application due to high toxicities and even due to the advent of targeted therapies and novel immunotherapies with superior safety and efficacy profiles (195).

In recent years, other several immunostimulatory cytokines, including IL-10 (196), IL-15 (197) and IL-12 (198), have been explored in Phase I trials combined with other immunotherapies, demonstrating promising clinical results.

5.2.2 Immune checkpoint inhibitors

It is well-known that tumor infiltrating lymphocytes (TIL) specific for tumor antigens exist in several types of cancer. Despite the presence of T cells infiltrating the tumor, inhibitory mechanisms in the tumor microenvironment suppress their functions. Indeed, cancer cells try to escape the immune response by upregulating molecules that can inhibit the activation of T cells (199). In the context of a growing tumor, during T-cell priming, a key co-stimulatory signal is provided by the interaction between B7–1 and B7–2 molecules expressed on antigen-presenting cells and the CD28 receptor on T cells. Soon after T cells are activated, when they are still in secondary lymphoid organ, they upregulate the expression of CTLA-4 as part of a compensatory mechanism necessary to attenuate and regulate T cell function. The competitive binding of CTLA-4 to B7 molecules results in the inhibition of T cell activation. Consequently, anti-CTLA-4 mAb

may restore T cell stimulation, mainly in secondary lymphoid organs. Also, following long-term stimulation, the PD-1 receptor is upregulated by T cells, while its ligands PD-L1 and PD-L2 can be highly expressed on cancer cells and cancer infiltrating myeloid cells, leading to T-cell inhibition following receptor–ligand interaction. Therefore, mAbs targeting the PD-1/PD-L1 or PD-L2 axis may enhance the functional properties of effector T cells inside the tumor (146, 200). The approval of immunomodulatory mAbs developed for blocking immune checkpoint molecules such as CTLA-4, PD-1 and its ligands, has raised great excitement in the immuno-oncology panorama. Since 2011, when the FDA approved the anti-CTLA-4 mAb *Ipilimumab* to treat patients with advanced melanoma (201), several mAbs have received approval for the clinical use (i.e., anti-PD1 *Pambrolizumab, Nivolumab, Cemiplimab, Dostarlimab* and anti-PD-L1 *Atezolizumab, Avelumab and Durvalumab; anti-LAG3 Relatlimab*), demonstrating significant results in the progression free and overall survival of treated patients, either as monotherapy or as combination therapy (202). As a step forward, several "next generation immune checkpoints" have been identified, including the B7 homolog 3 protein (B7-H3), lymphocyte activation gene-3 (LAG- 3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), and CD200. These molecules exert a co-inhibitory function, and strictly act by co-operating with the CTLA-4 and PD-1/PD-L1/L2 axes to modulate the anti-cancer immune response (203). A treatment combining *Relatlimab* and *Nivolumab* has been recently approved for the treatment of unresectable or metastatic melanoma (204); while the combination of a novel anti-CTLA-4 mAb (*Tremelimumab*) with *Durvalumab* has been indicated for the treatment of advanced hepatocellular carcinoma in the USA (205). Currently, mAbs targeting B7-H3, TIGIT and TIM-3 are being investigated in human Phase I/II clinical trials.
Nonetheless, despite the impressive clinicalresults for some types of tumors, not all tumor patients can benefit from such treatments, and in addition, the development of several adverse effects and toxicity following ICIs administration has been reported (206-208)).

5.2.3 Cancer vaccines

Among the active immunotherapeutic approaches designed to fight cancer, a special regard should be dedicated to anti-tumor vaccination that represents a very attractive tool thanks to its potential safety, specificity, and induction of long-lasting responses (209, 210).
The concept behind antitumor vaccination is to properly stimulate the patient's own immune system to induce both a specific T-cell and an antibody response against cancer cells to directly control tumor growth, and eventually develop an immune memory that can ensure a long-term

protection from recurrences and metastasis and to eradicate minimal residual disease (146, 186). On one hand, vaccines against carcinogenic viruses have provided a novel insight for primary cancer prevention. Such *prophylactic* vaccines are designed to prevent the development of specific virus-associated cancers by generating neutralizing antibodies that prevent the oncogenic virus from infecting target cells (211-213). The licensing of two prophylactic vaccines by the FDA to prevent cancer-promoting viral infections such as hepatitis B (HBV) and human papillomavirus (HPV;(214, 215) in 1986 and 2014, respectively, paved the way to the development and approval of more vaccines in the 2000s (216). The use of such vaccines has led to a consistent decrease in the incidence of hepatocellular carcinoma and cervical cancer cases worldwide (217, 218).

These positive results lay the groundwork for another growing field of research, aimed at developing cancer vaccines against virus-unrelated cancers, with a therapeutic window of application.

Therapeutic vaccines are supposed to elicit an immune response to an existing tumor by directly targeting a specific TAA. Basically, therapeutic vaccination enables the delivery of large amounts of antigen to dendritic cells (DC), with consequent optimal DC activation and induction of effective T helper and cytotoxic responses, ensuring the maintenance of anti-cancer immunity (186). Several therapeutic cancer vaccines have been successfully tested in pre-clinical mouse models, as well as in Phase I and II clinical trials, alone or in combination. However, very few have effectively reached Phase III trials, demonstrating less impressive achievements (146, 219-221). The reasons for vaccine failure are not fully understood; however, speculations related to the (advanced) stage of the disease, an inherent difficulty in mounting a strong cellular immune response, the choice of the right targetable antigens, and the suppressive nature of the tumor microenvironment would more likely impact on patients' responses to the vaccine (219).

Many different vaccination strategies have been investigated, including DC-, viral-, peptide-based, and genetic vaccines (222-224).

DC-based vaccines occupy a prominent position among active immunotherapy approaches. DC are major antigen presenting cells (APC) that process and present antigens via MHC class I and class II molecules. They are often referred to as "nature's adjuvant" (225), and are capable of activating both naive and memory immune responses. They play a key role in the interface between innate and adaptive immunity. As a proof of principle study, Dhodapkar et al. demonstrated that a single injection of an antigen-pulsed DC vaccine was sufficient to induce antigen-specific immune responses *in vivo* in healthy subjects as compared to unpulsed DC or soluble antigens alone, which did not display any immunogenicity (226). Several studies have exploited the anti-tumor efficacy of DC-based immunotherapies in different tumors, including leukemia, melanoma, lung cancer

and brain tumors (227), leading to the first FDA-approved cell-based therapy, *Sipuleucel-T* (Provenge). It consists of autologous DC expanded and activated *ex vivo* with a recombinant fusion protein containing prostatic acid phosphatase (PAP) fused with GM-CSF, and then infused back in patients with hormone-refractory prostate cancer (228-231). Despite promising results, some technical aspects remain to be optimized, such as the number of cells to be injected, route of injection, and best vaccination schedule. Moreover, the high costs of such therapy and its modest therapeutic benefit in terms of overall survival seriously limit its application (232, 233).

Peptide-based vaccines consist of immunogenic epitopes, ranging from 8 to 30 amino acids, identified within a TAA sequence. However, even though this kind of vaccination has demonstrated promising achievements in pre-clinical settings (234), no peptide-based vaccines against cancers have achieved FDA approval until now (235). Limitations in fact outweigh the advantages. Indeed, peptide immunization has shown a very limited ability to overcome antigen heterogeneity or loss of antigen expression within the tumor, ultimately failing to trigger effective cancer cell elimination. The mechanisms underlying the priming of anticancer immune responses by peptide-based vaccines, and hence their efficacy, depend (at least in part) on their size. Short peptides (8-12 amino acids) have been demonstrated to induce lower immune responses than longer peptides (25-30 amino acids), since they are processed by non-professional APC that finally lead to a tolerogenic signal and T cell dysfunction, only eliciting $CD8^+$ T cell responses. In addition, short peptides tend to be MHC-restricted, thus limiting the application of the same vaccine to a smaller portion of patients. As short peptides do not bind MHC II and fail to activate $CD4^+$ helper T cells, they do not stimulate the full activation of cytotoxic $CD8^+$ T lymphocytes (236). Contrarily, longer peptides efficacy is especially higher when they include epitopes recognized by both cytotoxic and helper T cells (237). However, the weak immunogenic nature of peptide-based vaccination requires the implementation of these vaccine platforms with carrier molecules or adjuvants to induce of a robust immune response (236).

Genetic vaccines, including either RNA or DNA vaccines, are used for direct delivery of antigen-coding sequences *in vivo* (186). RNA vaccines are produced by the *in vitro* transcription of template DNA using RNA polymerase. RNA molecules from tumor samples can be amplified by PCR, yielding a large amount of complementary DNA encoding patient-specific tumor antigens (238). The major advantage of RNA vaccines is that they do not integrate into the host cell genome, thereby avoiding potential safety problems (239). Contrarily, a concern regarding the development and the approval of RNA vaccines is their very high susceptibility to degradation. Several

formulations have been explored to overcome this issue, with most RNA vaccines administered through lipid nanoparticulate formulations as carriers and via different routes of delivery (240). The clinical efficacy and immunogenicity of these vaccines have been evaluated across different cancers. Nonetheless, a few trials have reported objective responses in patients, in which a significant T cell response and improvement in the survival have been observed (241, 242). However, no phase III clinical trials are ongoing to date, and even though very promising, no anti-cancer therapeutic mRNA vaccines have yet been approved by the FDA. Progress in using mRNA vaccines in pre-clinical and clinical trials has resulted from improvements in mRNA vaccine technology for cancer treatment, culminating in the translation of knowledge that has led to the rapid development and success of such vaccines during the COVID-19 pandemic (243).

DNA-based genetic vaccines use either viral or plasmid DNA vectors to partially or entirely deliver the coding region of the target antigen (244). Viruses used as vectors for DNA delivery, are generally structured with three components (i) the protein capsid and/or envelope that encapsidates the genetic payload and defines the vector's tissue or cell tropism and antigen recognition; (ii) the transgene of interest; and (iii) the "regulatory cassette," that controls stable or transient somatic expression of the transgene (245, 246). Expression cassettes carrying the cDNA encoding the antigen of interest, are inserted into the viral genome in place of one or more essential viral genes. Vaccinia virus and adenovirus are the most widely used viral vectors, due to their ability to induce a particularly robust cytotoxic immune response against the expressed foreign antigens (247). Concerns regarding the use of viral vectors include the possible intrinsic immunogenicity due to the presence of pre-existing immunity against the vector and the possibility of integration mechanisms into the host genome (247).

To overcome these issues, a new area of cancer-targeted therapy is to exploit the advantages of OVs as vectors for gene delivery (248). The efficacy of this approach has been widely demonstrated in human clinical trials employing OVs armed with chemokines, cytokines, or tumor antigens (249) with positive results. The T-VEC is a successful example of this approach. Among the different OVs, the bovine herpesvirus 4 (BoHV4) is widely investigated as a potential candidate for gene transfer in vaccination and cancer therapy. BoHV4 can replicate and cause cytopathic effects in several cancer cell lines and primary tumor cultures (250). Moreover, vector-neutralizing antibodies isolated from bovine species, are not present in humans, making it a good candidate for the human clinical use. The effects of BoHV4-based vaccination have already been documented *in vitro* and in pre-clinical *in vivo* studies (250-252).

As a virus-based therapeutic approach, virus-like particle (VLP)–based technology has also been explored for cancer treatment. VLP are self-assembling nanoparticles derived from viral proteins that are structurally indistinguishable from their corresponding viruses, resulting in a highly effective vaccine against these pathogens. For example, upon assembly into a VLP, the structural protein of HPV elicits antibodies that protect against viral infection and prevent cervical cancer (253). The advantages of VLP-based approaches include the evidence that they harness immunogenicity without compromising patients' safety, as VLP cannot infect or replicate into the host due to the absence of the viral genome. VLP could even be used to vehicle genetic cargo such as RNA (254) or DNA encoding for a TAA (255). The efficacy of VLP has been demonstrated against both primary tumors (256, 257) and metastasis development (258) in pre-clinical models of solid cancers, with significant impact on survival. VLP-based vaccines have also been explored in human clinical setting (Mohsen et al., 2019).

Vaccination by means of DNA plasmid coding for a protein antigen is the simplest and cheapest gene-based approach among anti-cancer active immunotherapy strategies (244).

DNA-plasmid immunization has received great attention in the immuno-oncology field over the last decades. Given the long-lasting experience of my laboratory with DNA vaccines for anti-tumor purposes, I have focused on this type of active immunotherapy strategy.

6. DNA vaccines

DNA vectors consist of bacterial plasmid double-strain DNA containing multiple cloning sites, in which the antigen-coding sequence and possibly sequences coding for immune-modulating molecules can be easily inserted. The presence of a bacterial replication origin and an antibiotic selection gene, which confers antibiotic resistance, allows efficient plasmid replication and selection in bacterial cells (146, 209, 259). High expression of the TAA-coding sequence is facilitated by the presence of a ubiquitarian enhancer promoter and a transcription termination site.

6.1 Mechanism of action

Once delivered *in vivo*, plasmid DNA is internalized and expressed by local cells, such as myocytes, and resident APC. The antigen-coding sequence is transcribed and then translated into proteins or peptide strings within the cytoplasm. These sequences are then processed by the cells and presented by MHC-I molecules. APC attracted to the injection site can be directly transfected

with the plasmid DNA or can uptake secreted proteins or engulf apoptotic cells or debris containing the antigen, resulting in the subsequent cross-presentation of the antigens through MHC-I and MHC-II complexes. Antigen-loaded APC migrate to lymph nodes, where they interact and activate antigen-specific T cells. Therefore, one of the main advantages of DNA-based vaccines is the potential stimulation of both arms (cellular and humoral) of the immune system (**Figure 1**; (260, 261)).

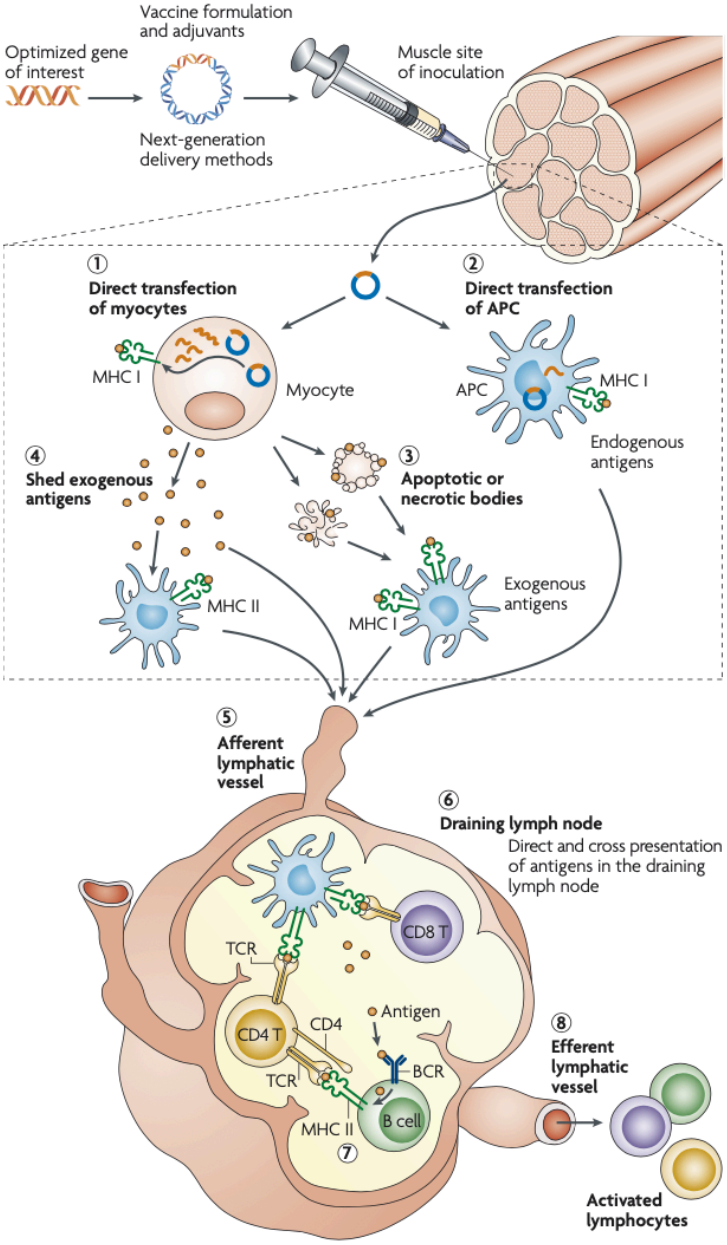

Figure 1: *Mechanism of action of DNA vaccine*. DNA plasmid injected into muscles, is internalized by (1) myocytes and (2) resident APC. (3) APC uptake of apoptotic or necrotic bodies and cross-present the antigen. (4) APC mediate the display of peptides on MHC II molecules after protein antigens that have been shed from transfected cells are captured and processed within the endocytic pathway. (5) Antigen-

loaded APCs travel to the draining lymph node via the afferent lymphatic vessels. (6) APC present peptide antigens to naive T cells via MHC and the T cell receptor (TCR) in combination with co-stimulatory molecules, initiating an immune response and expansion of T cells. (7) Activated CD4 T helper cells secrete cytokines during cell-to-cell interaction with B cells and bind to co-stimulatory molecules that are required for B cell activation. Specific immunity against plasmid-encoded antigen is induced by activating both T and B cells, which, can travel through the efferent lymphatic vessels (8) and provide a surveillance system (Adapted from (260)).

The bacterial origin of the DNA plasmid makes this type of vaccines also capable of evoking the innate immune system by acting as adjuvants by themselves. It is indeed recognized that exogenous DNA containing hypometilated CpG dinucleotide motifs can induce the production of type I IFN and other cytokines by stimulating cytoplasmic DNA sensors such as Toll-like receptor (TLR)9 (262). Nevertheless, some studies have reported that TLR9-induced innate immune responses are not required for the immunogenicity of DNA vaccines *in vivo* (262-265). Stimulators of interferon genes (STING) and the downstream TANK-Binding Kinase (TBK)-1 have been suggested to cooperate to convey signals that trigger the production of type I IFN. This axis facilitates to shape the adaptive immune response, increasing DNA vaccine-induced immunogenicity (264, 266-268).

Compared to other vaccine platforms, DNA vaccines have the advantages of being of simple and relatively inexpensive production (210). They are temperature-stable and can be amplified in large quantities, conferring advantages for both transport and storage. In addition, plasmids employed for DNA vaccines are safe, being non-infectious, non-self-replicating in mammals, without any evidence of genomic integration after plasmid injection (269). Of note, DNA plasmids can ensure antigen expression over long periods of time *in vivo* (261). Their major limitation is their relatively low degree of immunogenicity, that limits the potential efficacy of the vaccine in human clinical trials (270).

Naked DNA plasmids alone can be administered via different routes, either intramuscularly, intratumorally, or intradermally, but the standard needle injections have often failed to show strong immunogenicity (271). This is particularly evident when DNA vaccines are tested and translated from pre-clinical mouse models to larger animals, such as primates and dogs. Different variables can influence DNA plasmid immunogenicity, for example its inefficient uptake by targeted cells that could eventually explain the lack of efficacy in clinical trials (272). Also, the volume of the DNA solution administered could have a major role in the immunization success, as small volumes are unable to produce a hydrodynamic pressure strong enough to allow high plasmid transfection efficiency and trigger a proper immune response (273). Moreover, it has been postulated that the

extracellular matrix composition of different target cells at the site of injection could influence DNA vaccine efficacy (146, 274).

6.2 *In vivo* electroporation for DNA vaccine delivery

DNA vaccination combined with *in vivo* electroporation (electrovaccination) has been used since 1970 and represents a useful tool for improving the efficacy of anti-tumor immunization (146, 272). Electroporation consists in the delivery, at the injection site, of brief electric pulses through electrodes immediately after plasmid injection. By causing the formation of transient pores in the cell membrane, it facilitates plasmid entry into target cells. In addition, electroporation can increase DNA vaccine efficacy by acting as an adjuvant itself, by creating an injured environment in the injection site. Indeed, electric pulses can also induce the release of necrotic debris or apoptotic bodies from transfected cells, and an important recruitment of inflammatory cells, including APC. These events facilitate the uptake and presentation of the plasmid-encoded antigen and the activation of immune cells (**Figure 2**; (209)). Therefore, electroporation demonstrates to positively affect DNA plasmid efficacy even when low doses of DNA vaccine are used. It significantly increases antigen delivery of 100- to 1000-folds, and its immunogenicity as compared to naked DNA delivery alone, and in general to the other delivery systems exploited till now (275, 276).

Therefore, electrovaccination represents a safe and easy method of efficient DNA delivery currently employed for several medical applications (277). In the last years, several human (198, 278-280) and veterinary clinical trials (281-285) have demonstrated the efficacy of electrovaccination in inducing a strong and long-lasting immune response in patients.

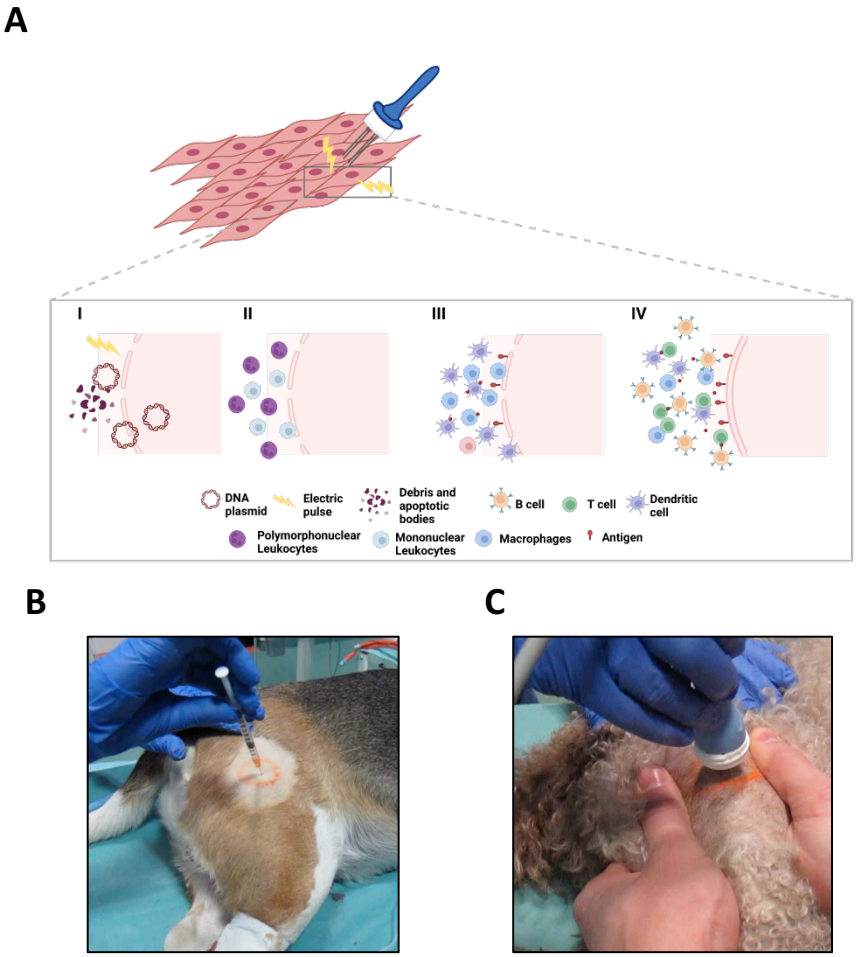

Figure 2: (A) *Cellular events following DNA plasmid intramuscular electroporation*. *I*) Insertion of the electroporator needles into the quadriceps muscle and delivery of two low voltage pulses. *II)* Damaged myofibers and cell debris (dotted red) are surrounded by polymorphonuclear and mononuclear leukocytes. *III*) Mature and differentiated tissue macrophages and DC progressively become prominent among inflammatory cells infiltrating the numerous necrotic myofibers. *IV*) On the third-fourth day from electroporation, intact and regenerating muscular fibers are overexpressing the protein encoded by the plasmid (red dots), while the area is being infiltrated by B, T-cells and DC. Adapted from (209). **(B, C)** In vivo electroporation procedure through electrodes by using the Cliniporaotor (IGEA, Carpi, Italy) in dogs. Adapted from (146).

7. Tumor antigens as vaccination targets

In the design of a novel anti-cancer vaccine, the choice of the right tumor antigen to target is of outstanding importance. Ideally, the right antigen should retain its almost exclusive expression in cancer cells, in which it must play a key and not disposable role in sustaining their malignant behavior (209, 210, 286). On the contrary, the wide expression of the antigen also in healthy cells,

could lead to autoimmune reactions (287). Another key feature for broad application of the vaccine is the high expression of the antigen in a wide percentage of cancer patients.
Tumor antigens employed in cancer vaccines can be broadly divided into two main categories: tumor-specific antigens (TSA) and TAA.

7.1 Tumor specific antigen

TSA are proteins uniquely expressed in tumors; they arise from non-synonymous mutations, genetic alterations, or virally introduced genetic information in cancer cells, and are generally patient and/or tumor-lesion-specific (288). In contrast, they are not expressed in normal tissues (289). Neoantigens are included in this category. Since normal cells do not express such mutated antigens, they are recognized as non-self and are thus susceptible to recognition by high-affinity T cells (233). A higher number of mutations within the cells composing the tumor is generally associated with an increased number of potentially targetable neoantigens. This would likely increase the chance of identifying a patient's relevant TSA against which personalized immunotherapies could be directed (290). However, the generation of cancer vaccines against patients' TSA/neoantigens requires a personalized approach, as most of them are unique to each patient, with a pattern of expression that varies between different cancer entities. The identification of commonly mutated antigens shared by multiple patients could advance the development of anti-cancer vaccines with broader applications.

7.2 Tumor associated antigens

TAA, instead, are non-mutated self-antigens abnormally expressed by tumor cells as compared to normal ones. Three main TAA groups can be identified: (i) cancer-testis (CT) antigens, which are normally expressed only in tumors and in immune-privileged germline cells such as the testis and placenta (i.e., MAGE-A1, MAGE-A3, NY-ESO-1, and PRAME); (ii) cell differentiation antigens which are not normally expressed in adult tissues (i.e., tyrosinase, gp100, MART-1, prostate-specific antigen (PSA); prostatic acid phosphatase (PAP); (iii) overexpressed antigens, that are expressed in some normal tissues, but are highly expressed in cancer cells (i.e., RAGE-1, hTERT, HER2, mesothelin, and MUC-1;(287, 291)).
The development of effective vaccines against TAA is challenging. As TAA are self-antigens, self-reacting T cells that recognize these antigens may be removed from the immune repertoire or subjected to clonal inactivation or anergy by central and peripheral tolerance mechanisms. In

addition, low-affinity TCRs cannot mediate effective responses. When a normal protein is overexpressed, as occurs in tumors, it may exceed the threshold for T cell recognition, and potentially overcome the tolerogenicity barrier (287, 292). Cancer vaccines targeting TAA must, therefore, be potent enough to break the host's tolerance mechanisms by stimulating the low affinity or TAA-reactive T cells that remain while maintaining immune tolerance to self-antigens (210, 293). One way to overcome this limitation and increase immunogenicity of a DNA vaccine, is to use xenogeneic proteins or peptides from a different species, which is significantly homologous with the self-ortholog, as immunogens (259).

8. Xenogeneic vaccination to overcome immune tolerance

As a way for the design of highly immunogenic DNA vaccines, it has been suggested the use of modified forms of TAA that could act as "*non-self*" or "*altered self*" proteins to circumvent host tolerance. A great opportunity in this direction is provided by highly evolutionarily conserved proteins acting as TAA. Indeed, exploiting xenogeneic sequences significantly similar to the self-orthologous one is an effective way of overcoming the immunological tolerance and likely induce an immune response against the self-proteins.

When vaccinating with a plasmid coding for a xenogeneic antigen, the differences in the sequences between the xenogeneic and the native antigens allows the recognition of the coded TAA by the immune system as a non-self antigen. Aminoacidic changes in the coded xenogeneic antigen sequence could be sufficient to overcome immune tolerance and induce T and/or B cell responses against the foreign protein, likely able to cross-react with the self-homologous TAA (259, 293, 294).

Immunization with DNA vaccines coding for xenogeneic antigens that share a significant homology with the self-antigen have shown to trigger a better immune response as compared to self-homologous ones. The first demonstration of the impact of using an altered-self form of a tumor antigen to induce protection from tumor progression, was derived from studies by Naftzger and colleagues who immunized mice with a human melanoma cell lysate expressing the gp75 antigen. Following immunization, mice developed antibodies that could recognize the murine antigen, as compared to those that were immunized with murine cells, resulting in hampered tumor growth and decreased lung metastatization (295). The efficacy of this strategy has been extensively demonstrated in several other murine models of cancer using prophylactic and therapeutic studies. This xenovaccination approach has been tested with success also in veterinary cancer patients

(281, 282, 284, 296-300). Nevertheless, xenovaccination induces relatively low-intensity and low-affinity immune responses against homologous self-antigens (294).

9. Chimeric vaccines

The induction of low-affinity immune responses against self-TAAs by xenogenic immunization, could be overcome using hybrid DNA plasmids, coding chimeric TAA derived in part from the self-antigen (homologous domain) and in part from a xenogeneic ortholog (heterologous domain) (259, 293, 294). The innovation and potential efficacy of these plasmids rely on the hybrid nature of the TAA sequence, encompassing both a homologous portion, which ensures the specificity of the immune response, and a xenogeneic portion, which is instrumental in circumventing immune tolerance.

As for DNA vaccines, in general, also the chimeric protein encoded by the hybrid plasmid and produced by transfected cells can be taken up by DC and recognized and internalized by B cells. In this way, peptides from both the xenogeneic and homologous domains of the internalized protein are presented by DCs and B cells through MHC I and MHC II. Upon presentation of xenogeneic peptides by DC, effective priming and expansion of both CD8+ and CD4+ T cells specific for these xenogeneic moieties occurs. T cells are also potentially cross-reactive with the homologous moiety of the chimeric proteins.

Simultaneously, the interaction between the expanded CD4+ T cells and B cells, which recognize the xenogeneic domain of the chimeric protein, leads to the differentiation of plasma cells which produce antibodies against the xenogeneic domain of the vaccine. In contrast, the interaction of expanded CD4+ T cells with B cells which recognize the homologous domain leads to the production of antibodies recognizing the self-tolerated homologous domain of the protein (293).

The slight differences in the amino acid sequence and tertiary structure of the chimeric protein encoded by the hybrid plasmid, may result in the exposure of subdominant and/or new conformational epitopes, triggering an even more efficient humoral and cellular immune response than that induced by the fully xenogeneic or homologous vaccines (**Figure 3**; (259)).

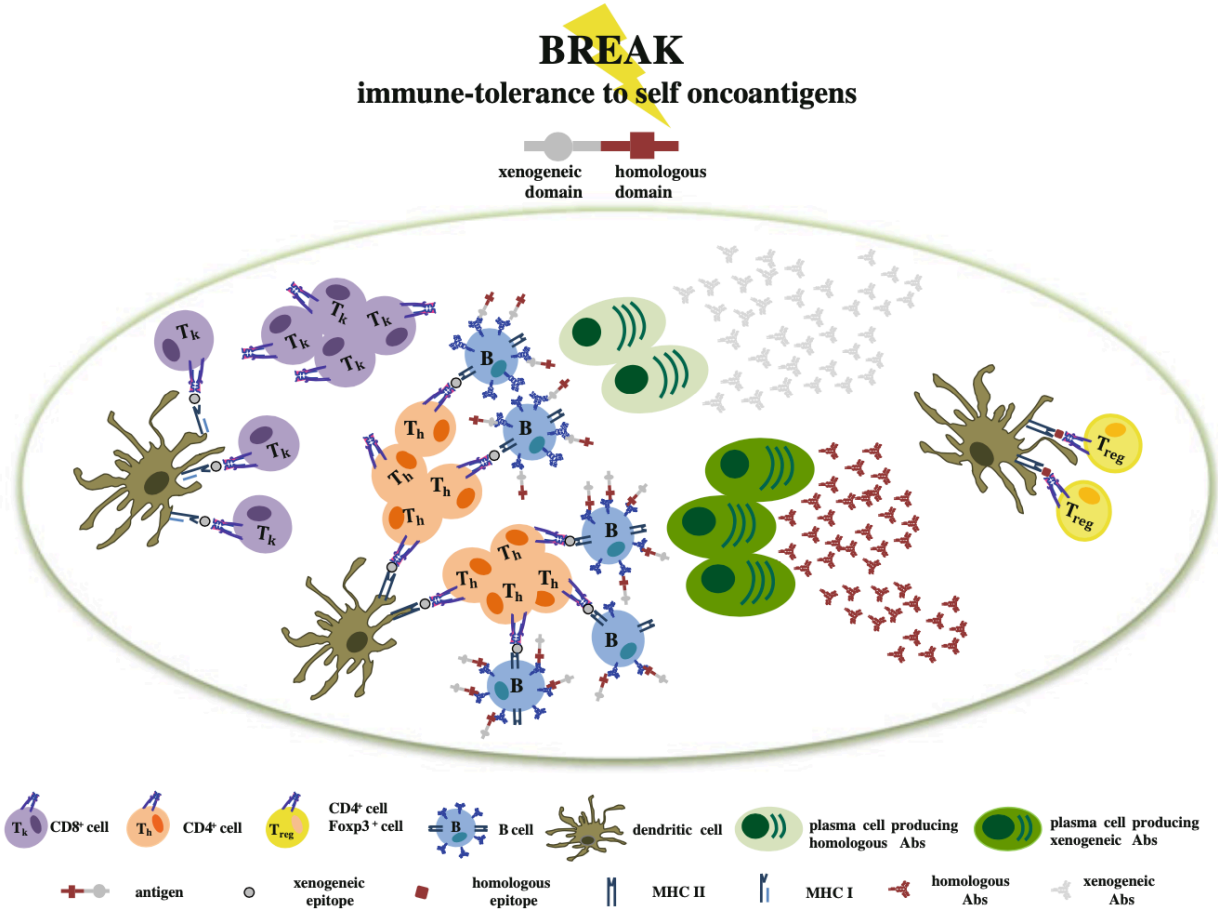

Figure 3: Schematic representation of the mechanisms leading to the induction of potent cross- reactive T cell and antibody responses using chimeric vaccines **(259).**

The first proof of the effectiveness of this approach was investigated in murine models of ErbB2+ tumors (259, 301, 302). When Quaglino et al. immunized cancer prone mice transgenic for the rat or human ErbB2 protein with xenogeneic plasmids encoding for either the fully human (HuHuT) or the fully rat (RRT) ErbB-2 proteins, they observed the induction of a specific antibody titer to either the human or the rat antigens, respectively. Contrarily, the vaccine-induced immune response was poorly cross-reactive with the ortholog ErbB2 protein expressed by transgenic mice and their tumors. To overcome this hindrance, the high amino acid homology (84.5 %) between rat and human extracellular and transmembrane domains of the ErbB2 protein was exploited for the generation of hybrid plasmids coding for ErbB-2 proteins composed in part by rat and in part by human sequences (RHuT and HuRT plasmids), that were then tested in human or rat ErbB2 transgenic mice. Vaccination with such chimeric plasmids demonstrated induced a stronger and more cross-reactive antibody and cellular immune responses, reflected by better protection against tumors overexpressing either rat or human ErbB2 than the fully human or fully rat plasmids (209, 294, 301, 302). Taken together, these data suggest that chimeric DNA-plasmids ensure the

specificity of the immune response through the homologous moiety, while leading to a break of immune tolerance through the xenogeneic portion, finally triggering a powerful anti-tumor immune response.
This strategy can be theoretically applied to any TAA that shares a high level of sequence similarity (294).

10. Testing novel immunotherapies in tumor-bearing canine patients: advancing knowledge for both human and veterinary purposes

In the wake of human discoveries underlying the positive impact of immunotherapy for cancer management, also veterinary oncology is recently focusing on its use as a potentially effective option for treating tumor-bearing dogs that cannot benefit from first-line therapies (146, 162). Nonetheless, advancing anti-cancer treatment for exclusive veterinary purposes is still moving slowly, also due to the high costs required for such therapies and for which not all owners are willing to spend. In view of the recognized similarities between human and canine tumors, on one side veterinary oncology could avail of in-human studies to establish novel hypotheses and test innovative therapeutic options; on the other side, the results obtained in clinical trials enrolling dogs with cancer, could speed up the translation of therapeutic successes in human clinical management, with a final mutual benefit for both veterinary and human patients.

10.1 Anti-cancer immunotherapies for canine MM

Several immunotherapy-based strategies have been developed and tested for canine MM-affected patients, ranging from those that nonspecifically boost the immune system to those that aid the immune system to specifically target cancer cells. The most relevant immunotherapeutic strategies for the treatment of MM-bearing dogs are summarized below.
Therapeutic approaches combining local treatment with a suicide gene (SG)/pro-drug non-viral system plus in situ cytokine release, have been investigated in dogs with MM and other solid tumors. In a first study, lipoplex-mediated herpes simplex thymidine kinase (HSVtk) SG therapy was able to generate objective responses, resulting in the control of metastatic spreading in most of treated dogs. However, in some patients, the treatment was not fully effective in counteracting metastatic disease (303). The strategy was optimized, and in subsequent studies it was tested in the adjuvant setting with surgery, alone or combined with canine interferon (IFN)-β (303, 304). However, the lack of a complete local tumor control eventually results in relapse. As a step

forward, a novel controlled trial was performed to test the effects of a combination of local and systemic gene therapy. The previous treatment strategy was flanked by periodic subcutaneous injection of allogeneic formolized tumor cell extracts and lipoplexes carrying human (h)IL-2, and hGM-CSF genes. In case of clinical manifestation of local recurrence or disease progression, the patients were subjected to a second treatment schedule. This approach significantly enhanced the previous results, improving the median survival times of treated MM-bearing dogs and increasing the disease-free interval in most cases by preventing recurrence and metastasis (305).

Considering the clinical relevance of checkpoint molecules in human oncology, their expression has also been investigated in canine tumors (203, 306, 307). High PD-L1 expression has been found in canine tumors of different histology, including MM; while PD-1 upregulation has been shown on tumor-infiltrating lymphocytes (306, 308-310). These findings provided the first evidence of the possible therapeutic benefit of immune checkpoint blockade in canine cancer patients (309) with ongoing veterinary studies exploiting PD-1/PD-L1 axis blockade, which is considered the most promising. A chimeric rat-dog anti-PD-L1 mAb has been generated and tested in a pilot study in OMM and sarcoma-bearing dogs (309, 311). Additionally, a fully canine anti-PD-1 mAb (ca-4F12-E6) has been developed and tested in stage IV OMM-bearing dogs enrolled in a pilot clinical study (312). Similarly, CTLA-4 expression is barely detected in PBMC from healthy canine donors, while its upregulation has been detected in canine T cells following activation. Recently, a full-length canine anti-CTLA4 mAb has been developed, and its pharmacokinetics and safety profile have been analyzed in pre-clinical mouse models, paving the way for its testing in dogs with immune-responsive cancers (313). The use of ICIs developed for human purposes could be considered even for treating veterinary patients, as the evidence of the cross-reactivity of antibodies targeting human checkpoint molecules such as Atezolizumab (anti-PD-L1 mAb; (314)) as well as mAb targeting the less explored B7-H3 (203) with canine ones, has provided the rationale for deepening investigations and consequent clinical testing in veterinary oncology.

Among all the immunotherapy approaches, vaccines also come to prominence in veterinary oncology. Several target antigens that have been discovered in the context of human melanoma, have opened the possibility of exploiting their targeting through anti-cancer vaccination also in dogs (203) with veterinary clinical testing starting in the early 2000s. Following in-human studies demonstrating that melanoma cells express immunogenic TAA that could be exploited for mediating tumor cell destruction (315, 316), the use of xenogeneic antigens have been one of the strategies of election to induce both antibody and cellular responses in the host against otherwise poorly immunogenic self-proteins. Based on pre-clinical in-mouse studies (317-319), the gp100

antigen was suggested as an appealing comparative target and, as such, it has been explored for the development of a xenogeneic human gp100-vaccination to be tested in canine patients with MM of different subtypes. As an example, autologous canine DC transfected *ex vivo* with an adenovirus expressing the human melanoma antigen gp100, were administered to MM-bearing dogs in combination with surgery and fractionation radiation therapy, reporting a modest ability to stimulate immunologic activity, but resulting therapeutically effective only in stage I MM (320). Important clinical responses were instead achieved through the administration of an allogeneic whole-cell vaccine, consisting of a canine melanoma cell line transfected with the human gp100 and killed via irradiation. This vaccine was tested in a Phase II veterinary clinical trial in stage II-IV MM-affected dogs, with some cases displaying tumor control, associated with delayed-type hypersensitivity occurrence and the induction of both cytotoxic and antibody responses (321).

A relevant achievement in canine cancer immunotherapy field came in 2010 with the approval by the United States Department of Agriculture (USDA) of the xenogeneic DNA vaccine ONCEPT® for the treatment of OMM-bearing dogs (322). ONCEPT® is a DNA-plasmid vaccine encoding human tyrosinase, which is 91% similar to the canine protein (296). It was licensed as an adjuvant treatment for stage II and III OMM-bearing dogs and was reported to induce both cellular and antibody responses. The antibody response was quite unexpected, given the intracellular nature of the tyrosinase glycoprotein, which is normally inaccessible to antibodies. The authors postulated that canine tyrosinase could be expressed at low levels on the cell surface, thus representing a target for the vaccine-induced antibodies, that were documented to persist for three to nine months in the circulation of immunized dogs (297). Of note, a correlation between the antibody titer and clinical response was observed. Tyrosinase targeting by means of xenogeneic DNA vaccination has been also proposed for the treatment of canine digit MM, where its efficacy in improving the survival of vaccinated dogs as compared to controls treated with surgery alone was reported (300). Recently, Zuleger and colleagues have also explored the microseeding of DNA encoding human tyrosinase into the skin of MM-bearing dogs as a potentially novel method for vaccine delivery (323).

Despite the good results achieved, the reliability of ONCEPT® has been questioned, with several studies reporting its efficacy with contradictory results (324, 325). The study by Ottnod et al. retrospectively evaluated 45 dogs with stage I and III OMM, treated with the locoregional control of the primary tumor, combined with radiation therapy, and reported no significant differences between the survival of dogs treated either with standard therapies or plus the adjuvant ONCEPT® vaccine; however, a better disease-free survival in dogs that did not receive the vaccination was observed (326). Moreover, some discrepancies in the setting of the study that led to USDA

approval have been highlighted, including the inclusion criteria, the low number of dogs enrolled, and the fact that 50% of the dogs were censored from the analysis (146, 324).
Nevertheless, to date, ONCEPT® is the first and only approved anticancer DNA-based treatment for MM-bearing dogs and has laid the foundation for its testing in human clinics, resulting safe and immunogenic as well (327, 328). In general, the success and the approval of ONCEPT® raised up a renewed enthusiasm for the development of DNA vaccines against different TAA, and on this wave, the search of novel melanoma associated antigens to target by means of DNA vaccination is advancing.

10.2 Anti-cancer immunotherapies for canine OSA

The recognized high similarities between human and canine OSA (132, 168) have addressed the use of pet dogs with spontaneously occurring OSA to evaluate the effectiveness of novel immune-based therapies to finally inform human clinical trials (146, 162). In the last years, different strategies have been exploited in the veterinary field, attempting to potentiate OSA patient's immune cells to attack cancer. Among them, several studies have investigated the antitumor potential of recombinant cytokines, such as IFN-γ, interleukin (IL)-2, IL-12, IL-15, and others, administered as soluble factors, encapsulated in liposomes, or delivered using viral and non-viral gene vectors (146). Overall, either as local intra-tumor or systemic treatments, these approaches promise to be safe and effective for large animals with various spontaneous tumor types, including OSA.
In a preliminary combinatorial proof-of-concept study, Magee *et al.,* have tested the efficacy of an immune-radiotherapy strategy in which *in-situ* radiation therapy and intra-tumor injection of the cytokine fusion protein hu14.18-IL2 (human recombinant IL-2 fused to the humanized anti-disialoganglioside mAb) has been investigated. This strategy has been tested in companion dogs with advanced metastatic tumors, including OSA. However, because of this treatment, non-irradiated metastatic lesions may become a niche for immunosuppressive cells, leading to systemic immune tolerance that can limit the benefits of this *in-situ* application. To overcome this problem, the authors added systemic targeted radionuclide therapy to modulate the TME and abrogate immunosuppression in secondary lesions with promising results (329).
Again, in the context of cytokine therapy, a first-in-dog phase I clinical trial has recently reported an emerging interest in the development of strategies to activate immune cell subsets, such as natural killer (NK) cells, which might play a relevant role in cancer elimination. Rebhun *et al.*, have tested the safety profile and the clinical activity of an inhaled recombinant human (rh)IL-15

in metastatic canine OSA patients, demonstrating the induction of a cytotoxic immune response that correlated with a clinical benefit (330). In this study, the treatment via inhalation of OSA-derived lung metastasis overcame the problem of toxicity induced by systemic delivery of ILs, that has usually hampered their use in human clinical trials (331, 332).
Evidence of the success of immune modulation in OSA is found in the use of the liposome-encapsulated lipophilic derivative of muramyl dipeptide (L-MTP-PE). This immunomodulatory agent can stimulate a systemic anti-cancer immune response through the activation of macrophages and monocytes. The activation of these immune mediators may lead to tumor cell elimination via both direct lysis and the release of tumoricidal pro-inflammatory cytokines. The adjuvant administration of L-MTP-PE has proven to be a highly effective treatment in a randomized double-blind clinical trial in surgically resected OSA-bearing dogs (333, 334). When combined with chemotherapy, L-MTP-PE was found to be more effective in counteracting metastatic spread and improving dog survival than placebo-treated controls, enhancing both monocyte activation and cytotoxic activity of macrophages against OSA cells (159, 333, 335, 336). The positive results obtained in veterinary medicine have promoted the development of L-MTP-PE in human clinical trials, with FDA approval for the adjuvant treatment of non-metastatic human OSA patients (337-339).

Moving on from successful pre-clinical murine studies (340), Regan and coworkers have tested the effects of the small-molecule anti-hypertensive drug *Losartan* as a novel monocyte-targeting immunotherapy for the treatment of advanced OSA in dogs. They have demonstrated that the administration of Losartan and the anti-angiogenic drug *Toracenib phosphate* (Palladia) impaired the CCR2/CCL2 axis, which is involved in macrophage-mediated metastatic colonization, thus preventing monocyte recruitment and stimulating an anti-metastatic effect in lungs. In this trial, the combinatorial approach was found to be safe and with a clinical benefit rate of 50% (341).

As a step forward, an attempt to combine some of the aforementioned immune-based approaches has been tested in a pilot veterinary study using a *vaccine-enhanced* adoptive T-cell treatment (ACT) with a cytokine boost in dogs with OSA treated with amputation alone. Canine patients were vaccinated with irradiated autologous cancer cells, and ex vivo-activated T-cells combined with low-dose IL-2 were administered after surgery to dogs that did not show overt gross metastatic disease. The treatment resulted in a modest therapeutic benefit. The novelty of this study was the complete absence of chemotherapy cycles; the modest improvement in survival is

therefore entirely attributable to the novel treatment (342). One limitation of this study was the lack of a control group and the lack of data regarding the induction of immunity in treated patients. Recently, an adjuvant peptide-based anticancer vaccine, composed of non-conventional endoplasmic reticulum-stress-response-derived immunogenic peptides (ERstrePs) that are released following the infection of OSA cells with Salmonella, has been tested in a veterinary trial in OSA-bearing dogs with no evidence of metastasis at the time of diagnosis. The dermal administration of this peptide-based vaccine was well tolerated, and antitumor efficacy was reported, with the time-to-metastasis and tumor-specific survival being longer in vaccinated dogs than in historical controls. The induction of anti-tumor humoral and cellular immune responses was also observed (343, 344).

Among the TAA explored for OSA therapy, HER2 has gained attention. HER2 is overexpressed in approximately 40% of human and canine primary OSA cases, and its overexpression correlates with higher metastatic rates and chemoresistance (345, 346). Anti-HER2 therapy has been explored using different immune-based approaches, including passive CAR-T cell therapy (347) and peptide-based vaccination (348). Nonetheless, the most successful treatment is represented by a bacterial-based immunization strategy consisting of a recombinant Listeria monocytogenes (Lm)-based vaccine expressing the human HER2/neu protein (ADXS31–164). The construct consists of fragments of two extra- and one intracellular domain of the human HER2/neu protein, and the most of the known HLA-A2-restricted epitopes of the human protein, fused to a truncated listeriolysin O fragment (LLO) derived from the highly attenuated Listeria vector Lmdda; (349)). This vaccine was tested in a phase I clinical veterinary trial, with the aim of preventing metastatic spreading in OSA-bearing dogs. The vaccine hampered the development of lung metastasis and prolonged the overall survival of treated patients. Based on these positive results, a lyophilized formulation of the live Listeria vector vaccine (the canine OSA vaccine-live Listeria vector; COV-LLV) received a conditional license from the USDA in 2017 for the adjuvant treatment of dogs with OSA (346, 350). However, the safety of this vaccine has been recently contested in a large veterinary study. The occurrence of important side events, such as Listeria abscesses and severe infections following administration, was reported in some treated dogs. In addition, considering the potential hazard of zoonotic spread of the disease, not only for canine patients receiving the vaccine, but also for the healthcare workers and family caring for the pet, the vaccine license has been terminated by the company (351, 352).

Overall, despite successful achievements, the application of some of these approaches is still limited due to several logistical challenges. For example, the potential dangers associated with live virus or bacteria use or the presence of pre-existing neutralizing anti-vector immune responses in

the patients are among the limitations in the use of viral and bacterial vaccines (353). However, the results of these veterinary clinical trials could support the evaluation of such approaches as novel therapeutic strategies for the treatment of both localized and high-risk metastatic OSA in the human setting, opening the possibility of finding novel effective therapies for OSA management.

11.The CSPG4 antigen

In the plethora of TAA exploited as suitable targets for immunotherapy studies, the chondroitin sulfate proteoglycan (CSPG)4 has attracted great attention.

CSPG4, also known as High Molecular Weight-Melanoma Associated Antigen (HMW-MAA), melanoma chondroitin sulfate proteoglycan (MCSP) or Neuro-glia antigen 2 (NG2), was first discovered in 1981 by Wilson and colleagues to be overexpressed on melanoma cells (354). Recently, CSPG4 overexpression and its role have been characterized in other cancer histotypes besides MM, including both hematological and solid tumors (355-360), among which OSA (361, 362).

CSPG4 is a single-pass type I transmembrane protein consisting of an N-linked glycoprotein of ~250 kDa and a proteoglycan component of ~450 kDa (363). The extracellular region is largely decorated with chondroitin sulfate (CS) chains that influence CSPG4 distribution on the cell membrane and has a large core protein composed of three structural domains (D1-D2-D3). Each of these domains has a specific function that together confer to CSPG4 unique structural features among the members of its family. The D1 domain comprises the N-terminal portion and contains two laminin G-type regions and disulfide linkages that are important for ligand binding, integrin interactions and cell-matrix or cell-cell connections. The middle D2 domain consists of acceptor sites for CS chains and 15 “CSPG repeat” motifs necessary for direct binding with ECM components, such as collagens V and VI. D3 is the membrane proximal domain, which contains carbohydrate modifications that might bind to galectin 3, other lectins (i.e., p-selectin), and integrins. This portion also contains putative proteolytic cleavage sites. The transmembrane region of CSPG4 is involved in membrane localization, whereas the cytoplasmic portion contains tyrosine phosphoacceptor sites for PKCa and ERK 1,2. The inner C-terminus contains a 4 residue PDZ domain-binding motif (PDZ), that is responsible for interactions with various PDZ domain-containing binding partners. The cytoplasmic part of CSPG4 plays the functional role of the proteoglycan (**Figure 4;** 364).

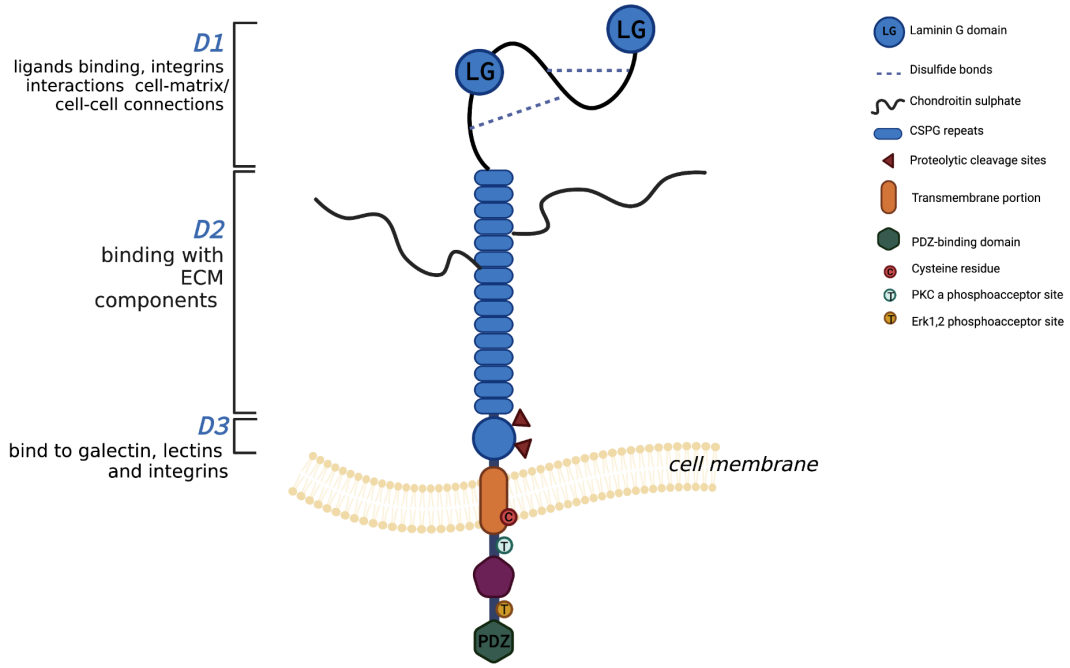

Figure 4: Chondroitin Sulfate Proteoglycan (CSPG)4 structure. Adapted from (364).

Even though CSPG4 does not have intrinsic catalytic activity, it participates in signal transduction by acting as a co-receptor for receptors with intrinsic tyrosine kinase activity. In this way, it actively sustains the activation of downstream signaling pathways such as FAK and MAPK pathways, conferring a malignant behavior to tumor cells, i.e. enhanced cell proliferation and motility. Moreover, by directly binding to ECM components, such as fibronectin and collagen, it is involved in enhanced cell adhesion and invasiveness (365). Finally, CSPG4 mediates the activation of the α3β1 integrin/PI3K signaling and its downstream targets, promoting cell chemoresistance and survival (**Figure 5;** (366)).

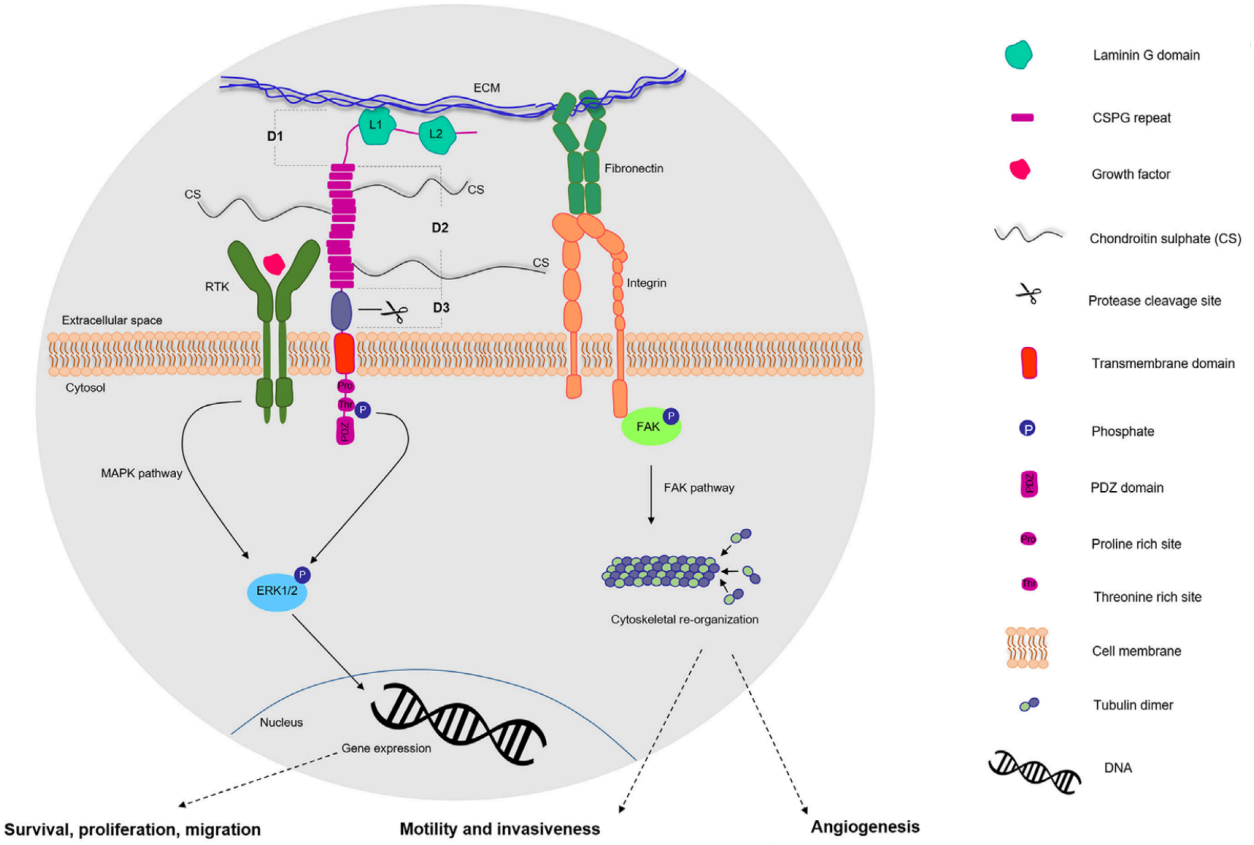

Figure 5: CSPG4 activates two major overlapping but distinct signaling pathway cascades: MAPK pathway signaling (left) and integrin/focal adhesion kinase (FAK) signaling (right). In this manner, it promotes several pro-tumoral functions. Adapted from (360).

CSPG4 generally has a very low expression in normal tissues; nonetheless, it has been found to be expressed in certain benign cell types in the developing central nervous system (CNS), being appointed as a marker of oligodendrocyte precursor cells together with platelet-derived growth factor receptor (PDGFR)-α (367), and vessel-surrounding pericytes (368). It has also been identified in immature cell types outside the CNS, such as mesenchymal stem cells (MSC), chondroblasts, osteoblasts, immature keratinocytes, muscle progenitors, brain perivascular and microvascular cells. However, its expression decreases with terminal differentiation (360, 369).

CSPG4 expression is finely regulated at different levels, depending on specific cell functions and tissues. Several conditions, such as enhanced inflammation and hypoxia, and regulatory genetic and epigenetic factors such as methyltransferases, transcription factors and miRNAs contribute to the dysregulation of its expression, triggering the alteration of cell physiological conditions that ultimately drive diseases such as cancer (369).

Overall, the features of CSPG4 and the evidence that its overexpression has been appointed as a negative prognostic factor for unfavorable clinical outcomes in human patients (356, 361, 370, 371), make CSPG4 an ideal antigen for investigating novel immunotherapies to treat CSPG4-expressing tumors. As a surface tumor antigen, CSPG4 could be susceptible to the attack of both antibodies and T cells (203, 209) and the effects of its immune targeting have been explored in a variety of tumor histotypes (358, 372-374).

11.1 CSPG4 and immunity

Proteoglycans may influence the immune response (375). Although the definitive role of CSPG4 in influencing the immune system in the setting of an established cancer still must be elucidated, it has been observed that either CS chains and/or the products of their degradation appear to influence the activation, maturation, proliferation, and migration of different immune cell subsets (360).

In general, the CS chains of proteoglycans, have been reported to influence murine B cell proliferation *in vitro*, and stimulate monocytes to secrete IL-1β (376, 377). Early in vivo studies on the murine NG2 ortholog instead, have reported its role in the regulation of the expression of pro-inflammatory cytokines such as IL-1β and TNF-α and of nitric oxide synthase, suggesting its role in the inflammatory reaction and chemotaxis in the CNS under pathological conditions (378). It has also been suggested that CS play a role in supporting the maturation of DC, accelerating the differentiation from monocytes as compared to GM-CSF and IL-4 (379). Another study has shown that the addition of CS to *ex vivo* culture of splenocytes from ovalbumin (OVA)-immunized mice, stimulated the secretion of Th1-type cytokines, including IFN-γ, IL-2, and IL-12, while suppressing the secretion of Th2-type cytokines (IL-5 and IL-10;(380)).

In addition, CSPG4 is supposed to be expressed in certain immune cell subsets such as NK cells and macrophages. In fact, metabolic products of macrophages have shown secreted CSPG4 following LPS stimulation (381); while the treatment of NK cells with chondroitinase, an enzyme that cuts the CS chains that bind proteoglycans, has shown a substantial decrease in IFN-γ secretion (382). Finally, CSPG4-reactive CD4+ T cells have been found in both healthy individuals and melanoma patients, but with no significant differences with respect to clinical condition (383).

Overall, further studies are required to clarify whether CSPG4 interaction with different immune cell subsets could impact on patients' response to immunotherapy and, particularly, to anti-CSPG4 immune-based therapies.

11.2 The long path toward the development of an effective immunotherapy against CSPG4 for malignant melanoma

Since its discovery, CSPG4 has been comprehensively characterized in melanoma. In humans, CSPG4 is expressed in 90% of melanomas (384, 385), being its overexpression negatively correlated with patients' survival (386).

Because of its properties as an ideal TAA, CSPG4 has become the subject of intensive studies aimed at defining the implications of its targeting for treating this tumor type and has stimulated interest in the development and application of different immunotherapy approaches for MM, most of which have been tested in pre-clinical mouse models (387). For example, de Bruyn and coworkers have demonstrated, both *in vitro* and *in vivo,* the anti-tumor effects of an anti-CSPG4:TRAIL, resulting from the genetic fusion of TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand) to an anti-CSPG4 scFv antibody fragment derived from the mAb 9.2.27 (388). In another study, CSPG4-specific CARs have been generated using mAbs reactive against CSPG4, and pre-clinically tested in human xenografts, demonstrating striking effects in controlling melanoma growth (389). Anti-CSPG4 specific mAbs have been poorly tested *in vivo*; however, early investigations regarding the potential of using mAbs for therapeutic purposes, have been widely exploited in melanoma models *in vitro*. The combination of an anti-CSPG4 mAb with PLX4032, a BRAF inhibitor, has demonstrated efficacy in impairing cancer cell tumorigenic functions, suggesting the possible effects that could be mediated upon this combinatorial administration *in vivo* (390).

Self-TAA overexpressed on tumor cells, such as CSPG4, are generally well tolerated by the host immune system. CSPG4 targeting has been widely explored through the *antigen mimicry* concept as a possible way to overcome self-unresponsiveness. Molecular mimicry occurs when similarities between foreign and self-peptides favor the activation of autoreactive T or B cells by a foreign-derived antigen in a susceptible individual. First attempts of developing an effective immunization strategy against CSPG4 foresaw the use of such antigen mimics in human MM patients. The mouse anti-idiotypic mAb MK2–23, mimicking the determinant defined by a mouse anti-CSPG4 mAb (763.74), was tested in a phase I clinical trial in metastatic MM patients. Following immunization, the development of an anti-CSPG4 antibody response, which occurred in 60% of patients, was positively correlated with a significant prolongation of survival and metastatic spreading impairment, as compared to those that did not develop anti-CSPG4 antibodies (391). To achieve better results, anti-idiotypic antibodies were exploited and administered in conjunction with adjuvants (i.e. keyhole limpet hemocyanin (KLH) or Bacillus Calmette-Guerin (BCG)). However,

because of the occurrence of side effects associated with BCG administration and the difficulties in stabilizing the KLH-mAb conjugates, along with the lack of a vaccine-induced T cell response, the clinical use of this combination was hampered (392). The optimization of the MK2-23 anti-idiotypic antibody led to the development and the subsequent pre-clinical testing of the IL-2-MK2-23 fusion mAb. This novel antibody demonstrated an enhanced immunogenicity; nonetheless, this strategy has never reached the clinical testing (393).

One evidence of cellular immunity induced following anti-idiotypic antibodies administration came in 2004 when Murray et al., reported that epitopes present in the anti-idiotypic mAb formulation, named MELIMMUNE, consisting in murine anti-idotypic mAbs MEL-2 and MF11–30, could activate *ex vivo* CTL from patients previously immunized against CSPG4. This combination induced CTL able to lyse melanoma cells expressing CSPG4 in an MHC-II -restricted fashion (394). Concurrently, peptide mimics was explored as an alternative and easiest way of developing immunity as compared to anti-idiotypic mAb. Immunization with peptides mimicking CSPG4 determinants resulted in the induction of antibodies capable of mediating cell-lysis through ADCC and inhibiting both the migration and invasion of CSPG4-positive MM cells *in vitro* (395). However, despite decades of testing and some successful results, the anti-idiotypic mAb/peptide mimotope approach was eventually abandoned. One of the main limitations of such immunotherapies was the difficulty in being strongly immunogenic and the failure to induce an effective cellular immune response, which is considered of outstanding importance for counteracting established tumors.

11.3 A xenogeneic human CSPG4 DNA vaccine for the treatment of canine OMM

One of the principles of comparative oncology, is the identification of shared tumor antigens that are significantly relevant to both human and canine cancers. In this way, testing novel therapeutic strategies would allow the development of unique treatment options that could eventually benefit both species. Because CSPG4 is a highly evolutionarily conserved molecule, showing over 82% homology and 88% similarity between the human and canine counterparts, Mayayo and colleagues determined for the first time whether canine MM expressed CSPG4 and the possibility of appointing it as a novel tumor biomarker for canine MM. The evaluation of a cohort of 65 canine MM samples, revealed CSPG4 overexpression in 57% of the MM biopsies analyzed (396). The percentage of CSPG4 expression in canine OMM in that study was probably underrated, as recent, provisional results of the same research group indicate a percentage of expression of more than 80% (Prof. Selina Iussich, personal communication).

Considering the high frequency of expression, similar to that of other canine MM antigens used as diagnostic markers, such as Melan A (397) and PNL2 (398), CSPG4 was appointed as a potential immunohistochemical marker and a promising targetable antigen for canine MM, with a strong translational potential.

To this end, the safety and therapeutic efficacy of an adjuvant DNA-based vaccine against CSPG4 were evaluated (282), and the immunogenicity of the vaccine, in terms of both antibody and cellular-induced immunity, was investigated. Owing to the high similarities in the amino acid sequences of the canine and human CSPG4 molecules, a DNA plasmid coding for the xenogeneic Human (Hu)-CSPG4 was used, to break the tolerance in dogs against the otherwise well tolerated self-dog (Do)-CSPG4 protein. A non-randomized prospective clinical veterinary trial was conducted in stage II-III CSPG4+ OMM-affected dogs, that were electrovaccinated with the Hu-CSPG4 DNA vaccine. From a clinical point of view, Hu-CSPG4 vaccination conferred a significant survival advantage to treated dogs, compared to controls treated with surgery alone, both in terms of median survival time (MST) and disease-free interval (DFI). The 1-year survival of dogs was significantly longer in patients that received the adjuvant Hu-CSPG4 vaccination as compared to conventionally treated controls. Moreover, some canine patients who received the vaccination displayed delayed metastasis onset, as compared to non-vaccinated dogs, which rapidly exhibited metastatic spreading, suggesting the efficacy of the anti-CSPG4 vaccination to confer long-term immune protection against tumor progression. The improvement in the overall survival and the delayed metastasis onset in immunized canine patients were mainly associated with the development of antibodies against the human CSPG4 antigen coded by the vaccine, which can also bind the Do-CSPG4 antigen in most cases. Nevertheless, only two vaccinated dogs showed a specific T cell response against the canine CSPG4 protein, suggesting that the xenogeneic DNA vaccine induced a low frequency of dog-CSPG4 circulating reactive T cells (281, 282).

Thus, these data provided the first evidence that a xenogeneic DNA vaccination against CSPG4 in dogs can break immune tolerance against the self-antigen and be effective in treating canine OMM.

Aims of the thesis

In this thesis, I investigated the efficacy of a second-generation DNA-based vaccine targeting CSPG4 for the treatment of both MM and OSA. In our studies, my collaborators and I have availed of dogs spontaneously developing tumors, considered relevant pre-clinical models for testing novel immune-based approaches, endowed with high translational value.

In the results discussed in ***Chapter I***, I have contributed to the evaluation of the safety, immunogenicity, and potential clinical benefit of an innovative hybrid DNA plasmid, encoding for a chimeric human/dog (HuDo)-CSPG4 protein. We tested our HuDo-CSPG4 vaccine in client-owned dogs affected by naturally occurring CSPG4-positive MM, resulting in a high safety profile, strong immunogenicity, and promising anti-tumor potential. Our results represent not only an important revolution in the veterinary clinic, proposing a valid adjuvant therapy for canine melanoma affected patients that do not respond to standard-of-care, but, thanks to the hybrid structure of this vaccine, the important results obtained in dogs could be translated with a similar foreseen success in human clinic.

Based on these results demonstrating the relevance of CSPG4 immunotherapy for the treatment of a CSPG4-positive tumor such as MM, in ***Chapter II***, I have evaluated the possibility of appointing CSPG4 as a novel, targetable OSA associated antigen, in both human and canine settings. Because of the very poor prognosis of OSA patients undergoing standard treatments, there is an unmet need of finding and developing novel therapeutic options. We confirmed the role of CSPG4 in sustaining tumorigenic properties in both human and canine OSA cells, and this evidence spurred us to investigate the potentiality of applying our HuDo-CSPG4 DNA vaccine in relevant pre-clinical models of OSA. Our vaccine was able to induce anti-tumor immunity not only in pre-clinical mouse models of human OSA but also in naturally occurring OSA-bearing dogs, resulting in an overall clinical benefit of treated patients.

Finally, in ***Chapter III*** I report very preliminary investigations regarding the involvement of CSPG4 in driving OSA-genesis. Although CSPG4 function in MSCs has still not been fully clarified, it seems to play an important role in their differentiation toward the osteoblastic lineage. We think that uncovering which is the genetic event triggering OSA-genesis, and which is CSPG4 involvement in this process, could open the possibility of developing novel therapeutic interventions for the management of OSA in the pediatric population.

Overall, the results reported in this thesis confirm the important role that CSPG4 exerts when it is overexpressed in two clinically relevant solid tumors, such as MM and OSA. Keeping investigating the impact of anti-CSPG4 immunotherapy in valuable pre-clinical models, as in this thesis, could eventually bridge the translation from basic and pre-clinical research to human clinic, finally benefiting both melanoma and osteosarcoma patients for who standard therapies are not effective.

Chapter I

A Human/Dog-CSPG4 DNA vaccination for advancing canine and human melanoma therapy

(For detailed results and materials & methods, please refer to Paper I at the end of the paragraph**)**

After the initial demonstration of the immunogenicity of the xenogeneic Hu-CSPG4 vaccine in MM-affected dogs by my coworkers (281, 282), I have contributed to reinforcing the first evidence of the safety profile of this immunization approach by collecting and analyzing follow-up data (386).

Hu-CSPG4 treated dogs didn't develop side effects following the electrovaccination, up to 3 years (for some of the vaccinees) following the first vaccine administration. Nevertheless, Hu-CSPG4 xenovaccination induced relatively low intensity and low-affinity immune responses against the Do-CSPG4 in treated dogs ((209, 399) and *discussed below*), that could explain why most dogs enrolled in this first veterinary clinical trial eventually died because of progressive disease. The clinical efficacy of the vaccine was indeed lower when the primary tumor expressed low levels of CSPG4 antigen (386). This could be probably linked to the presence of escaping clones with low or no CSPG4 expression within the tumor lesion, which could be responsible for the progression of the disease, thus limiting the efficacy of the vaccine (146, 259, 294). Moreover, from the translational point of view, the Hu-CSPG4 vaccine cannot be successfully applied in a human setting. For this reason, a second-generation vaccine that carries a hybrid CSPG4 sequence has been developed (400), and the first application of this chimeric concept in veterinary oncology has been recently tested (399).

To this aim, we have taken advantage of the high degree of conservation of CSPG4 between human and dogs to develop an antigen mimicry DNA-based vaccination strategy to be studied in canine models. Specifically, a hybrid plasmid encoding a chimeric CSPG4 protein, partially derived from the human (Hu) and partially from the dog (Do) CSPG4 sequences (HuDo-CSPG4) was generated (**Figure 6**; (400)) and I contributed to its testing in MM-bearing dogs.

We carried out a Phase I, non-randomized, clinical veterinary trial enrolling client-owned dogs with spontaneous, locally controlled, stage II-IV, CSPG4-positive OMM, whose first objective was to evaluate the safety and the immunogenicity of the adjuvant HuDo-CSPG4 electrovaccination.

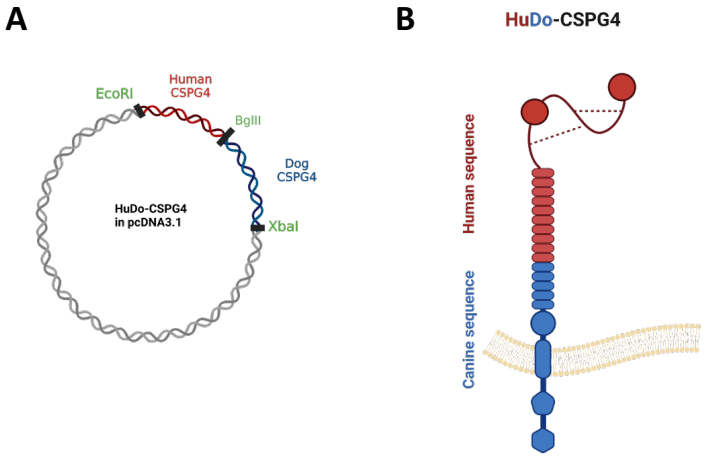

Figure 6: (A) Schematic drawing of the HuDo-CSPG4 DNA plasmid containing part of the Hu- (red) and part of the Do- (blue) CSPG4 cDNA. Restriction enzymes sites used for HuDo-CSPG4 cloning are indicated in green (399). **(B)** Drawing of HuDo-CSPG4 encoded chimeric protein.

To evaluate the efficacy of our vaccine in inducing protective immunity against CSPG4-positive OMM, we first analyzed the vaccine-induced antibody response in the sera of immunized canine patients. HuDo-CSPG4 vaccination was effective in inducing an antibody response, progressively increasing after repeated boosts. Vaccine-induced antibodies could bind the canine CSPG4 molecule, demonstrating the ability of the chimeric plasmid to overcome the immunological tolerance to the self-autologous antigen and triggering a cross-reactive humoral response. Vaccine-induced antibodies could hamper the progression of the disease by means of different mechanisms of action, including the elimination of melanoma cells by ADCC, CSPG4 downregulation, and inhibition of proliferation and migratory ability of melanoma cells *in vitro*, suggesting possible functional effects that could be also exhibited *in vivo*. In addition, we detected anti-CSPG4 IgA in the serum of a high percentage of the vaccinated dogs, allowing us to speculate that the induction of mucosal immunity may be relevant for counteracting the local recurrence in the oral cavity.

Importantly, this study demonstrated the ability of HuDo-CSPG4 vaccination in inducing a higher affinity antibody response, as compared to the one induced by the fully xenogeneic Hu-CSPG4 vaccine used in the previous clinical veterinary trial (281, 282). As a demonstration, only HuDo-CSPG4 vaccine-induced antibodies in the sera collected after the fourth vaccination, were able to significantly inhibit the proliferation of a canine MM cell line that expresses very low levels of the CSPG4 antigen.

In the analysis of the vaccine-elicited cellular immunity we observed an increased percentage of B and CD4+ T cells in the PBMC of vaccinated dogs, and a reduction in circulating MDSC in the majority of analyzed samples. In more than half immunized dogs analyzed, we also detected a CD8+ T cell-mediated cytotoxic activity.

Clinically, HuDo-CSPG4 immunized canine patients showed significantly prolonged overall survival (OS) as compared to conventionally treated controls. We observed a positive correlation between the induction of both vaccine-induced humoral and cellular responses and OS and disease-free interval (DFI) in immunized dogs. HuDo-CSPG4 vaccination demonstrated to be equally effective for the treatment of OMM with high or low CSPG4 expression as compared to Hu-CSPG4 vaccine that instead was more effective for the treatment of melanomas with a high CSPG4 score. Furthermore, dogs which eventually developed recurrence, showed a reduction of CSPG4 expression in the tumor lesion as compared to the primary mass. Overall, with this first clinical veterinary trial we confirmed the safety of the HuDo-CSPG4 vaccination, since immunized dogs didn't display any signs of toxicity or side effects during the whole observational period. In addition, we demonstrated its immunogenicity and potential clinical benefit in CSPG4-positive OMM canine patients.

Ultimately, thanks to the highly recognized predictive power of comparative veterinary studies and to the unique structure of the vaccine, these findings suggested the potential of its translation for the treatment of human CSPG4-positive melanomas.

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Antigen mimicry as an effective strategy to induce CSPG4-targeted immunity in dogs with oral melanoma: a veterinary trial

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ABSTRACT

Background Melanoma is the most lethal form of skin cancer in humans. Conventional therapies have limited efficacy, and overall response is still unsatisfactory considering that immune checkpoint inhibitors induce lasting clinical responses only in a low percentage of patients. This has prompted us to develop a vaccination strategy employing the tumor antigen chondroitin sulfate proteoglycan (CSPG)4 as a target.

Methods To overcome the host's unresponsiveness to the self-antigen CSPG4, we have taken advantage of the conservation of CSPG4 sequence through phylogenetic evolution, so we have used a vaccine, based on a chimeric DNA molecule encompassing both human (Hu) and dog (Do) portions of CSPG4 (HuDo-CSPG4). We have tested its safety and immunogenicity (primary objectives), along with its therapeutic efficacy (secondary outcome), in a prospective, non-randomized, veterinary clinical trial enrolling 80 client-owned dogs with surgically resected, CSPG4-positive, stage II–IV oral melanoma.

Results Vaccinated dogs developed anti-Do-CSPG4 and Hu-CSPG4 immune response. Interestingly, the antibody titer in vaccinated dogs was significantly associated with the overall survival. Our data suggest that there may be a contribution of the HuDo-CSPG4 vaccination to the improvement of survival of vaccinated dogs as compared with controls treated with conventional therapies alone.

Conclusions HuDo-CSPG4 adjuvant vaccination was safe and immunogenic in dogs with oral melanoma, with potential beneficial effects on the course of the disease. Thanks to the power of naturally occurring canine tumors as predictive models for cancer immunotherapy response, these data may represent a basis for the translation of this approach to the treatment of human patients with CSPG4-positive melanoma subtypes.

BACKGROUND

Melanoma in humans is the sixth most common cancer in the world, and its incidence has increased over the past 50 years.[1] It can affect multiple anatomical sites, defining four major subtypes, each one with distinct clinical characteristics: cutaneous melanoma, arising in non-glabrous skin; acral melanoma, that originates in glabrous skin of the palms, soles and nail beds; mucosal melanoma, which arises from melanocytes in the mucosa; and uveal melanoma, which develops from melanocytes in the uveal tract of the eye.[2] Major progress has been recently made mainly in the treatment of cutaneous melanoma thanks to the introduction of BRAF/MEK-targeted[3] and immune checkpoint inhibitor (ICIs)-based therapies.[4 5] These treatments have induced impressive clinical responses in 20%–50% of patients with melanoma, nevertheless, a still high proportion of patients does not benefit clinically from these therapies.[4 5] The other melanoma subtypes, including non-ultraviolet (UV)-induced cutaneous, mucosal and uveal melanomas, are rare and less characterized clinical entities with few therapeutic options and a very poor prognosis.[6–8]

Tumor antigen (TA)-based vaccination strategies, able to stimulate a long-lasting antitumor immune response, could represent an effective therapeutic option for patients with melanoma. In our study, the TA used as a target is the membrane bound chondroitin sulfate proteoglycan (CSPG)4, which is a member of the CSPG family. Members of this family are key bioactive molecules that play a major role in tumor growth, migration, and neoangiogenesis.[9–12] CSPG4 is an attractive target for antitumor vaccination, since it is highly expressed on melanoma cells in a high percentage of patients with limited inter-lesion and intra-lesion heterogeneity, independently of tumor stage and subtypes, with a restricted expression in normal tissues.[11–14] However, CSPG4 is a non-mutated self-antigen, and as such it is poorly immunogenic.[11 15]

In previous studies, anti-idiotypic monoclonal antibodies (mAbs) which mimic CSPG4 have been shown to be able to overcome a host's unresponsiveness to this self TA in patients with melanoma and to induce CSPG4-specific antibodies. This humoral immunity appears to have clinical relevance, since it was associated with patients' survival prolongation. In spite of these encouraging results, the anti-idiotypic mAb approach was abandoned, since the assumed lack of a cellular immune response was thought to be a major deficiency of this type of immunotherapy.[16]

Guided by these results, we have taken advantage of the high degree of conservation through phylogenetic evolution of CSPG4 sequence to develop an antigen mimicry DNA-based vaccination strategy. Specifically, we have generated and tested a hybrid plasmid encoding a chimeric CSPG4 protein, partially derived from the human (Hu) and partially from the dog (Do) CSPG4 sequence (HuDo-CSPG4). We have previously demonstrated that plasmids coding for chimeric proteins that include both xenogeneic and autologous domains of the target antigen delivered by in vivo electroporation can elicit a humoral and a cellular immune response.[17–19]

To test the validity of our strategy we have treated dogs affected by spontaneous oral melanoma, since they represent a clinically relevant model of human non-UV-induced and 'triple wild-type' cutaneous, mucosal and uveal melanomas.[20–22] Canine oral melanoma shares the same aggressive behavior as its human counterpart, with a high propensity to metastasize to lymph nodes and lungs, and has comparable treatment options and clinical responses.[22–24] Moreover, dogs affected by oral melanoma have been widely used in immunotherapy trials[25] and led to the US Department of Agriculture (USDA)-approval of the DNA vaccine ONCEPT (Merial), carrying the sequence of the human tyrosinase, for the treatment of this canine tumor.[26] Importantly, a high percentage of oral canine melanomas express the CSPG4 antigen.[27] Therefore, our goals were the evaluation of the safety, immunogenicity, and antitumor potential of HuDo-CSPG4 vaccination in the adjuvant setting, in a prospective, multicentric, phase I, non-randomized, veterinary clinical trial enrolling 80 client-owned dogs affected by spontaneous, CSPG4-positive, stage II–IV, oral melanoma, after the surgical removal of the tumor. HuDo-CSPG4 vaccination, used in association with in vivo electroporation in 52 out of 80 dogs, was found to be well tolerated and immunogenic. The improvement in the overall survival of vaccinated dogs as compared with controls suggest a potential clinical benefit of adjuvant HuDo-CSPG4 vaccination for the treatment of patients affected by malignant melanoma.

METHODS

Cell lines and reagents

The canine CMM-12 and OLGA cells were derived from a primary oral melanoma[28] and from a metastatic lymph node,[11] respectively; the human skin melanoma SK-MEL-28 cells were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 20% fetal bovine serum (FBS, Sigma-Aldrich) and penicillin/streptomycin (both from Sigma-Aldrich) and maintained at 37°C in a 5% CO_2 atmosphere. Cell lines were routinely checked for contamination by mycoplasma using the Mycoalert Detection Kit (Lonza). CSPG4 expression by cell lines was assessed as described[29–31] utilizing western blotting, flow cytometric analysis and immunofluorescence of cells stained with a pool of the mAbs TP32, TP49 and VF20-VT87.41, which recognize distinct and spatially distant CSPG4 epitopes.

Generation of the hybrid human/dog (HuDo)-CSPG4 plasmid

The hybrid HuDo-CSPG4 plasmid (pCDNA3.1 backbone) was generated as described.[30] Briefly, the first 3737 bp of the Hu-CSPG4 sequence (Gene ID: 1464)[29] were ligated to the last 3187 bp of the Do-CSPG4 sequence (Gene ID: 487658). The hybrid HuDo-CSPG4 complementary DNA was then cloned into the pCDNA3.1 plasmid and verified by sequencing (BMR Genomics). The large-scale preparation of the plasmids was carried out with EndoFree Plasmid Giga kits (Qiagen) according to *Good Laboratory Practice*. The hybrid HuDo-CSPG4 plasmid encodes for a chimeric protein which includes at the N-terminal portion the domain 1 and part of the domain 2 (amino acid, aa 1–1245) of the Hu-CSPG4 protein and part of domain 2 and the full domain 3 (aa 1246–2307) of the Do-CSPG4 protein at the C-terminal.

Dog enrollment and vaccination

Eighty client-owned dogs were enrolled following owners' informed consent during the period October 1, 2016 to June 30, 2021. The study protocol was approved by the Italian Ministry of Health (0015537-28/06/2017-DGS AF-MDS-P) and conducted at the Veterinary Teaching Hospital, University of Turin, Grugliasco (Turin), Italy, and the Tyrus Veterinary Clinic, Terni, Italy. Dogs without concurrent life-threatening diseases and with stage II (2–4 cm diameter, negative lymph nodes (LN)), III (>4 cm diameter and negative LN or any tumor size with ipsilateral-positive LN) and IV (any tumor size, with bilateral positive LN without distant metastasis), surgically resected CSPG4-positive oral melanomas, were included in the study. Preoperatively, full tumor staging, defined according to the tumor, node, metastases staging system by Owen,[32] included a skull and three-view chest radiography and abdominal ultrasound examination and/or a total body CT. Tumor samples were immunohistochemically tested for CSPG4 expression as previously described.[29 33] Briefly, a total score ranging from 0 to 8 was assigned to each melanoma sample considering the value assigned to the proportion of CSPG4 positively stained tumor cells (score from 0 to 5) and the average staining intensity of CSPG4-positive tumor cells (score from 0 to

3). Only dogs bearing an oral melanoma with a CSPG4 score ≥3 were enrolled in the study.

Dogs included in the vaccination arm were adjuvantly immunized with the HuDo-CSPG4 plasmid as previously described,[29] starting 2 weeks after surgery. Briefly, 500 µg of HuDo-CSPG4 plasmid per each dog, diluted in 200 µL of 0.03% NaCl solution, were injected into the muscle of the caudal thigh. Two minutes after plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 µs, frequency 3 HZ; 1 s pause; eight low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were applied to the injection site using the CLINIPORATOR (Igea). Immunization was repeated after 2 weeks and then monthly, for a minimum of 4 and a maximum of 24 cycles. Clinical examinations, three-view chest radiographs and/or CT were performed before each vaccination, as well as sera and peripheral blood mononuclear cells (PBMC) were collected, whenever possible.[29 33] The Veterinary Co-operative Oncology Group-Common Terminology Criteria for Adverse Events V.1.1, (VCOG-CTCAE)[34] was used to classify the adverse events.

Antibody binding assays

ELISA was performed as previously described.[35] Briefly, 96-well plates (Costar, Sigma-Aldrich) were coated overnight at 4°C with the recombinant D2 (Do–D2) and D3 (Do–D3) domains of the Do-CSPG4 protein (obtained from Genscript), and of the commercially available D3 (Hu-D3) domain of the Hu-CSPG4 protein (R&D Systems) (50 ng/well). Plates were then sequentially incubated with diluted canine sera (1:100) for 2 hours at 37°C and horseradish peroxidase-conjugated anti-dog IgG or IgA xenoantibodies (1:10000; both from R&D system). Plates were washed and chromogenic 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) substrate was added. The reaction was stopped by the addition of 2N hydrochloric acid and absorbance was measured at 450 nm on a 680XR microplate reader (Bio-Rad). Vaccinated dogs were considered responders when the fold change between the O.D. at 450 nm of the post-vaccination/pre-vaccination sera was >1.1. Avidity of anti-CSPG4 antibodies produced by vaccinated dogs was tested in a chaotropic ELISA as previously described.[35]

For flow cytometric analysis, CMM-12 and SK-MEL-28 melanoma cells were incubated for 30 min at 4°C with canine sera diluted 1:40 in phosphate-buffered saline (PBS; Sigma-Aldrich). Total IgG and IgA binding was evaluated as previously described.[23] Samples were acquired using a BD FACSVerse (BD BioScience) and analyzed with FlowJO V.10.5.3.

Immunofluorescence staining was performed as described[29] using 1×10^5 CMM-12 and SK-MEL-28 cells seeded onto glass coverslips. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature (RT) and blocked with PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 0.1% Tween-20 (Sigma-Aldrich). For internalization analysis, CMM-12 cells were seeded for 3 hours at RT onto glass coverslips that had been previously coated with fibronectin (5 µg/mL) (Sigma-Aldrich). Cells were then incubated with canine sera (1:10) for 1 hour at 37°C, permeabilized with 0.5% saponin (Fluka), blocked for 1 hour at RT and incubated overnight at 4°C with a pool of the CSPG4-specific mAbs TP32, TP49, VF20-VT87.41 (1:40). Specific antibody binding was revealed with a goat anti-mouse IgG antibody (Alexa Fluor 568; Thermo Fisher Scientific). Nuclei were stained with 4',6-diamidino-2-phenylindole, coverslips were air-dried and mounted. Immunofluorescence images were acquired with an Eclipse 80i-ViCO system (Nikon), using a 60×/1.4NA oil immersion objective and analyzed using a Fiji Software (Rasband, W.S., ImageJ, US National Institutes of Health).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

CMM-12 (8×10^3)[28] and OLGA (5×10^3)[11] cells were starved for 4 hours in 96-well plates. Pooled sera (1:50) from vaccinated dogs were then added and incubation was continued for 48 and 72 hours for CMM-12 and OLGA cells, respectively. Cell viability was then evaluated as previously described.[31]

Cytofluorimetric analysis of circulating leukocytes

Thawed PBMC were incubated with human IgG to block the Fc receptor and then stained with the following mAbs: rat anti-dog CD5-fluorescein isothiocyanate (FITC), rat anti-dog CD4-RPE-cy7, rat anti-dog CD8-Pacific Blue, mouse anti-dog B cells-Alexa Fluor 647, purified mouse anti-dog CD11b, rat anti-dog major histocompatibility complex (MHC) II-FITC and mouse anti-human CD14-Alexa fluor 647 (all from AbD Serotec). To reveal CD11b positivity a secondary PE-conjugated anti-mouse IgG (DakoCytomation) was used. Samples were acquired using a BD FACSVerse (BD BioScience) and analyzed with FlowJO V.10.5.3.

Cytotoxicity assay

CMM-12 target cells (1×10^4) were labeled with 2 µM of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) and then cultured with thawed PBMC at the effector:target ratio (E:T) of 50:1 for 48 hours at 37°C in a 5% CO_2 atmosphere. PBMC were from 19 dogs of the vaccination arm, selected based on sample accessibility. Antibody-dependent cell-mediated cytotoxicity (ADCC) was performed by incubating PBMC with CMM-12 cells at the E:T ratio of 50:1 overnight at 37°C in a 5% CO_2 atmosphere in the presence of a 1:50 dilution of canine sera collected before and after the vaccination cycles. After staining with 1 µg/mL 7-Amino-ActinomycinD (7-AAD, BD BioSciences), cells were acquired using a BD FACSVerse and analyzed using FlowJO V.10.5.3. Percentage of killing was obtained by back-gating on the $CFSE^+$ targets and measuring the percentage of $7\text{-}AAD^+$ dead cells, as previously described.[36] Briefly, percentage of specific lysis was calculated with the formula ((dead targets in sample (%) − spontaneously dead targets (%)/

(dead target maximum (%) − spontaneously dead targets (%)) × 100. Spontaneous death was obtained by culturing target cells without PBMC, whereas maximal death was obtained after treatment with 1% saponin.

Western blotting

Western blotting for CSPG4 detection was performed as previously described.[31 33] β-Actin (Santa Cruz Biotechnology) and Vinculin (Cell Signaling) were used as protein-loading controls.

Cell migration assay

CMM-12 (2×10^4) cells were incubated with pooled sera (1:10 dilution) from vaccinated dogs for 1 hour at 37°C in a 5% CO_2 atmosphere and then seeded into the top chamber of a 24-transwell plate (8 µm pore size; Corning). Migration assay was performed as previously described.[31]

Statistical analysis

Shapiro-Wilk or Kolmogorov Smirnoff test were used to evaluate normal distribution. The non-parametric Mann-Whitney test was used when the distribution was not normal. Two-tailed unpaired and paired Student's t-tests were used to perform the statistical analyses for normally distributed data. The Kaplan-Meier method was used to estimate overall survival and disease-free interval (DFI) of dogs enrolled in the study. Differences in survival times were analyzed using the log-rank test. Pearson's correlation method was used to estimate the correlation between the antibody response determined by ELISA and survival. Statistical significance was evaluated using GraphPad V.9 software (GraphPad) and values of $p<0.05$ were considered significant.

RESULTS

Molecular and antigenic profile of the HuDo-CSPG4 plasmid used as a vaccine

The hybrid HuDo-CSPG4 plasmid includes the N-terminal portion of the Hu-CSPG4 and the C-terminal portion of the Do-CSPG4 sequences[30] (online supplemental figure S1A). The predicted chimeric HuDo-CSPG4 aa sequence (online supplemental figure S1B) has 89.0% identity with the full Hu-CSPG4 sequence and 93.0% with the full Do-CSPG4 sequence.[37] National Institute of Health (NIH)/3T3 fibroblasts transfected with the HuDo-CSPG4 plasmid demonstrated the presence of the two CSPG4 components with the molecular weight of 250 kDa and >450 kDa (online supplemental figure S1C) as revealed by western blotting using a pool of three mAbs (the TP32, TP49 and VF20-VT87.41), recognizing distinct Hu-CSPG4 epitopes.[38–40] Moreover, using this mAb pool, a specific binding on NIH/3T3 fibroblasts transfected with HuDo-CSPG4 was also revealed by flow cytometry analysis (online supplemental figure S1D) and immunofluorescence (online supplemental figure S1E). Overall, these results confirm that the chimeric protein is correctly coded by the hybrid construct and expressed on the cell membrane.

In addition, sera from C57BL/6 mice vaccinated with the HuDo-CSPG4 plasmid stained murine B16 melanoma cells stably overexpressing either the Hu-CSPG4 (B16-Hu-CSPG4; online supplemental figure S2A) or the Do-CSPG4 (B16-Do-CSPG4; online supplemental figure S2B) proteins. Lastly, a significant delay of the tumor incidence was observed in HuDo-CSPG4 vaccinated mice challenged subcutaneously with either B16-Hu-CSPG4 (online supplemental figure S2C) or B16-Do-CSPG4 (online supplemental figure S2D) cells. These results are compatible with the preservation of the antigenic and immunogenic properties of both the Hu-CSPG4 and the Do-CSPG4 domains encoded by the hybrid construct.

Phase I veterinary clinical trial: eligibility criteria and patient enrollment

Eighty client-owned dogs with oral melanoma were prospectively enrolled in the study. Their principal characteristics are summarized in (online supplemental table S1).

All dogs were treated with an *en-bloc* resection of the primary tumor, with the inclusion, if feasible, of at least 2 cm of macroscopically normal tissue around the tumor, and regional lymphadenectomy. In some cases (10% of vaccinated dogs and 3.6% of controls), adjuvant radiotherapy was given in addition to surgery. Tumor samples were immunohistochemically tested for CSPG4 expression (n=80) (online supplemental table S1), Ki67 expression (n=78), mitotic index (n=80) and nuclear atypia (n=70) (online supplemental table S2). Dogs were then assigned to the adjuvant vaccination treatment group or the control one according to the owner's decision. The clinical stage distribution and the CSPG4 expression score[27] were similar in the two arms (online supplemental table S1,S3,S4).

HuDo-CSPG4 vaccination is safe and with potential beneficial effects on the overall survival of canine melanoma patients

HuDo-CSPG4 vaccination was started 2 weeks after surgery, repeated 14 days later and then monthly in 52 out of the 80 dogs enrolled in the study (vaccination arm) (figure 1A and online supplemental figure S3). The remaining 28 dogs that did not receive the adjuvant HuDo-CSPG4 vaccination were included in the control arm (online supplemental figure S3).

No significant changes in blood counts, body weight and temperature were detected, as well as no allergic/immunologic events were recorded throughout the entire course of the study. Sixteen out of the 22 dogs with a body weight below 15 kg exhibited transient hind/limb limping after electroporation lasting from some hours to days (grade 1 toxicity, according to the VCOG-CTCAE[34]). No hospitalization was required for any dog.

Adjuvantly vaccinated dogs exhibited significantly longer overall survival than the control population treated with conventional therapies alone (log-rank test,

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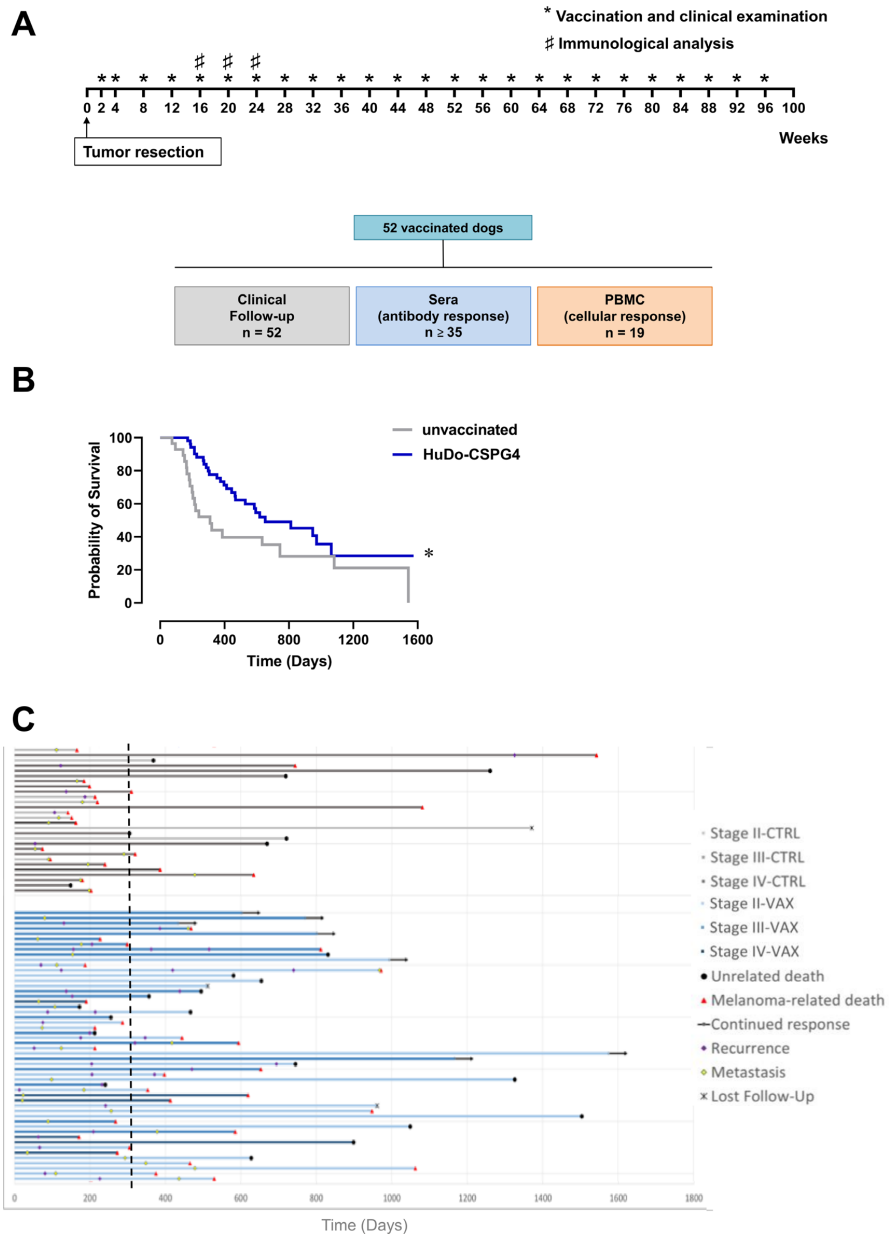

Figure 1 Adjuvant chimeric HuDo-CSPG4 vaccination improves the survival of canine patients affected by CSPG4-positive oral melanomas. (A) Immunization protocol (upper panel) and study design (lower panel). (B) Kaplan-Meier curves comparing the overall survival (in days) of HuDo-CSPG4 vaccinated (blue line) and unvaccinated (gray line) dogs, after the local control of CSPG4-positive oral melanoma, updated to December 2021. Log-rank test, *p=0.0320. (C) Swimmer plot depicting the overall survival of canine melanoma patients enrolled in the study. Briefly, the survival (in days) of dogs with surgically resected CSPG4-positive melanoma, either vaccinated (Vax) or non-vaccinated (Ctrl), is reported, considering the day 0 as the moment of the surgery for each dog. Arrows indicate that the patients were still alive at the time of publication (continued response). For each patient, the moment of recurrence or metastasis detection, if any has been indicated. Black dots indicate patients who died because of unrelated reasons, while red triangles indicate patients who died because of melanoma. Dogs lost in the follow-up (n=3) were also indicated. The median survival time (310 days) for the control group has been indicated by a dotted vertical line. CSPG4, chondroitin sulfate proteoglycan 4; PBMC, peripheral blood mononuclear cells.

Table 1 Survival times for canine melanoma patients calculated up to December 31, 2021

Groups	MST (days)	Survival (months)			
		6	12	18	24
HuDo-CSPG4 (n = 52)	653	96.15%	69.23%	50.00%	37.25%
Control (n = 28)	310	71.43%	39.29%	32.14%	21.43%

CSPG4, chondroitin sulfate proteoglycan 4 ; Do, dog; Hu, human; MST, median survival time.

*p=0.0320) with a median survival time (MST) of 653 and 310 days in the vaccinated and the control group, respectively (figure 1B and C and table 1).

At the end of the observation period (1575 days), 7 out of the 52 (13%) vaccinated dogs were still alive, of which 5 (71%) without evidence of recurrence nor metastasis developed during the study, while in the remaining 2 (29%) dogs we observed the regression of metastasis, and both are currently in remission. Forty-three out of the 52 (83%) vaccinated dogs died during the study and 2 dogs (4%) were lost to follow-up on day 512 and 962, respectively; the latter dog developed both a local recurrence and metastasis on day 214 and 276, respectively. Of the 43 dead dogs, 27 (63%) died because of melanoma and the remaining 16 (37%) succumbed to cancer-unrelated events. In the study period, 40 out of 52 (77%) vaccinated dogs experienced progressive disease; 15 of them (37%) developed local recurrence, 13 (33%) distant metastasis and 12 (30%) both.

In the control arm, 27 out of 28 dogs (96%) were dead at the end of the study and 1 (4%) was lost to follow-up on day 1371, with no recurrence and metastasis developed during the observation period. Out of the 27 deceased dogs, 20 (74%) died because of melanoma and 7 (26%) because of cancer-unrelated events. During the study period 22 out of the 28 (79%) dogs experienced progressive disease; 6 of them (27%) developed local recurrence, 13 (59%) distant metastasis and 3 (14%) both.

HuDo-CSPG4 is immunogenic in canine melanoma patients

Antibody response

Sera collected from HuDo-CSPG4 vaccinated dogs after the fourth immunization were tested in an ELISA to investigate the ability of the HuDo-CSPG4 vaccine to induce an antibody response against the Do-CSPG4 protein. An increased IgG binding to the recombinant Do–D2 and the Do–D3 domains in the post-vaccination as compared with pre-immunization sera was observed in 44% (figure 2A-C) and 46% (figure 2F-H) of dogs, respectively. Interestingly, in the responder dogs, a correlation between an increased IgG binding and the overall survival was observed, with statistical significance for Do–D2 responders (figure 2D,E) and a reliable trend for Do–D3 responders (figure 2I,J). Only a trend was observed between an increased IgG antibody level in responder dogs and the DFI (online supplemental figure S4A, B); although, it must be noted the reduction of the CSPG4 antigen in recurrences for most dogs (online supplemental figure S4C).

When samples were available, the anti-Do–D2 and anti-Do–D3 antibody response was measured also after the fifth and the sixth immunizations (online supplemental figure S5A, C). The percentage of responder dogs to the Do–D2 and the Do–D3 increase from 44% after the fourth vaccination (figure 2B) to 51% (online supplemental figure S5B) and from 46% (figure 2G) to 55% (online supplemental figure S5D), respectively. A significant progressive increase in the antibody levels to the Do–D2 was observed after repeated vaccinations (figure 2C).

Noteworthy, when sera from non-responder dogs were tested against the Do–D2 domain using a chaotropic ELISA, a higher percentage of IgG remained bound to the plate in 70% of the post-vaccination as compared with pre-vaccination sera analyzed (online supplemental figure S6A, B). This increased IgG avidity in the post-vaccination sera suggests that HuDo-CSPG4 vaccination can improve the spontaneous anti-CSPG4 antibody response detectable before vaccination in some of the dogs affected by CSPG4-positive melanoma. However, it must be noted that the spontaneous antibody response to both the Do–D2 and Do–D3 is not predictive of a better patient's survival (online supplemental figure S6C-H), either considering the entire canine population (online supplemental figure S6D, G) or only dogs who were responders (figure 2) to HuDo-CSPG4 vaccination (online supplemental figure S6E, H).

In view of the clinical risk of recurrence in the oral mucosa following local tumor control, we evaluated whether HuDo-CSPG4 vaccination could also induce anti-CSPG4 IgA antibodies. About 36% and 46% of vaccinated dogs developed an IgA response against the Do–D2 (online supplemental figure S7A, B) and Do–D3 (online supplemental figure S7E, F), respectively. In 71% of responder dogs, we observed the development of a local recurrence as compared with 76% in the non-responder group to the Do–D2; while 66% of dogs who respond to the Do–D3 developed a local recurrence as compared with 81% of non-responder dogs. Moreover, a positive trend between an increased antibody level and a prolonged DFI, considering the local recurrences, was found for the responder dogs to Do–D2 (online supplemental figure S7C, D), but not for those to Do–D3 (online supplemental figure S7G, H).

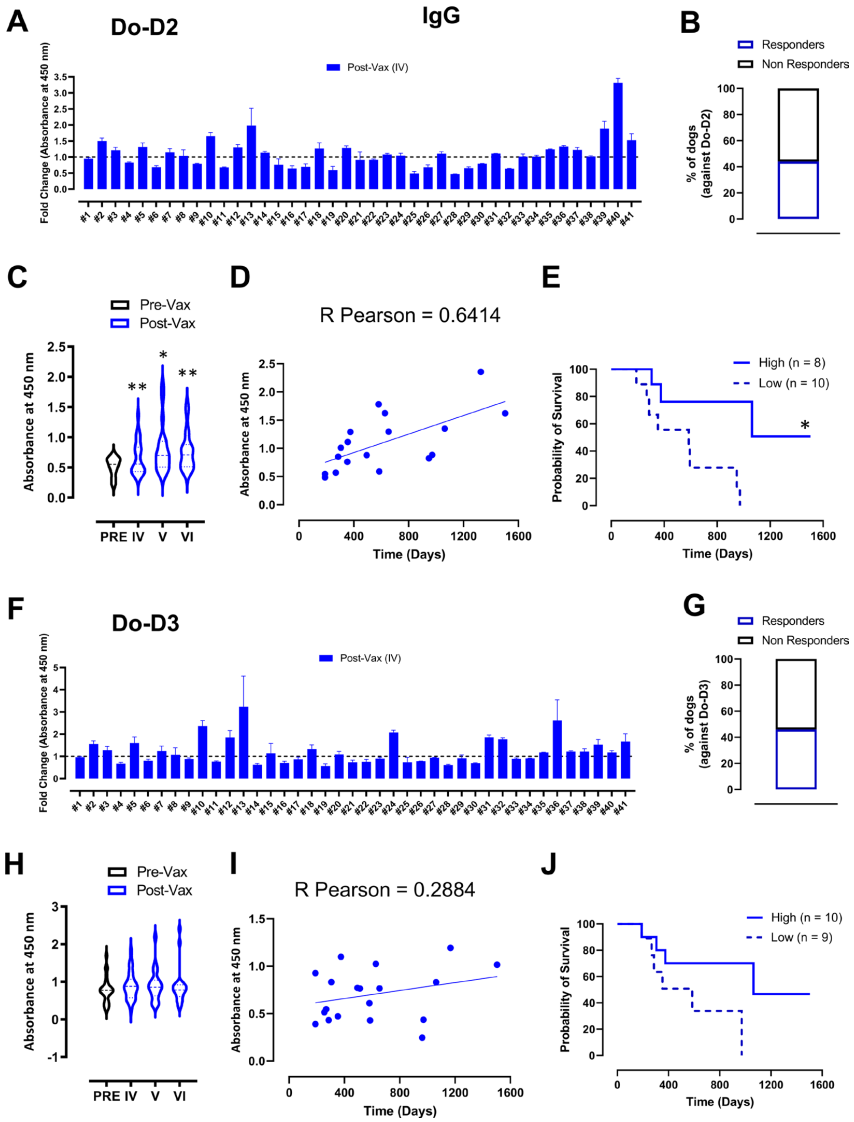

Figure 2 HuDo-CSPG4 vaccination is effective in inducing a specific anti-canine CSPG4 antibody response in dogs. (A) and (F) Analysis of the presence of IgG antibodies against the Do–D2 (A) and Do–D3 (F) domains of the canine CSPG4 protein in the sera of dogs before the first immunization (Pre-Vax, dotted black line) and after the fourth HuDo-CSPG4 vaccination (Post-Vax, blue bars), measured by ELISA. Results are expressed as the ratio (fold change) between the absorbance measured at 450 nm of the Post-Vax and the Pre-Vax sera. Each bar represents a canine patient. (B) and (G) Histograms representing the percentage of responders (blue) and non-responders (black) calculated by enumerating dogs in which sera collected after the fourth immunization displayed an increased ability to bind the Do–D2 (B) and the Do–D3 (G) domains as compared with sera collected before the first immunization. (C) and (H) Violin graphs representing the absorbance measured at 450 nm by ELISA against the Do–D2 (C) and Do–D3 (H) of pre-vaccination and post-vaccination sera from dogs who respond after the fourth, the fifth or the sixth immunizations. Student's t-test, p*=0–0108, p**<0.0085. (D) and (I) Correlation between the absorbance values measured at 450 nm by ELISA of the post-vaccination IgG of responder dogs against the Do–D2 (D) and Do–D3 (I) domains and the overall survival. Each dot represents a responder dog. Pearson correlation coefficients (r) are shown. (E) and (J) Kaplan-Meier curves correlating the overall survival of vaccinated dogs who develop (responders) a specific IgG response against the Do–D2 (E) and Do–D3 (J) domains with a high (continuous blue lines) or low (dotted blue lines) antibody level measured in their post-vaccination sera by ELISA, considering as cut-off the mean of the absorbance at 450 nm. Log-rank test, *p=0.0120. CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human.

Interestingly, sera collected from HuDo-CSPG4 vaccinated dogs after the fourth immunization exhibited an increased ability to stain canine CMM-12 cells, expressing the Do-CSPG4 protein in its natural conformation (online supplemental figure S8A, B), as compared with sera collected before the first vaccination (figure 3A). In 62% of the vaccinated dogs, post-vaccination sera showed a higher binding to CMM-12 cells as compared with the corresponding pre-vaccination sera (figure 3B). The increased binding of post-vaccination sera was validated by testing them with canine CMM-12 cells in immunofluorescence (figure 3C). An increased binding of vaccine-induced IgA to the canine CMM-12 cells was also found in post-vaccination sera (figure 3D).

Moreover, in 33% (online supplemental figure S9A-D) and 51% (online supplemental figure S9E-G) of HuDo-CSPG4 immunized dogs, vaccine-induced IgG and IgA, respectively, bind also the recombinant human CSPG4 D3 domain (Hu-D3), and the detection of this antibody response is associated with an improved overall and disease-free survival (online supplemental figure S9C, D, G). An increased binding of post-vaccination as compared with pre-vaccination sera was observed also using the human SK-MEL-28 melanoma cells as targets (figure 3E), naturally overexpressing the Hu-CSPG4 antigen (online supplemental figure S8A, B). Specifically, in 82% of HuDo-CSPG4 vaccinated dogs, post-vaccination sera displayed a higher ability to bind SK-MEL-28 cells as compared with those from the same patients before the vaccination cycle (figure 3E,F). The binding of the sera from HuDo-CSPG4 vaccinated dogs was also confirmed by testing them with SK-MEL-28 cells in immunofluorescence (figure 3G).

Cellular response

An increase in the percentage of both B and $CD4^+$ T cells was observed in the peripheral blood of 63% and 53%, respectively, of the 19 analyzed dogs following the fourth HuDo-CSPG4 vaccination, as compared with that in the peripheral blood collected before the first immunization (figure 4A). Moreover, in 53% of the analyzed dogs, PBMC collected after the fourth vaccination displayed an increased percentage of $CD8^+$ T cells (figure 4A), and when co-cultured with CMM-12 cells, they were significantly effective in killing CSPG4-positive tumor cells, as compared with those collected before vaccination (figure 4B). Vaccinated dogs that developed an increased cytotoxicity against CSPG4-positive canine melanoma cells (responders) displayed a longer, despite not significant, overall survival as compared with those that did not (figure 4C), with a MST of 972 days for responders as compared with 594 days for non-responder dogs. A slight increase of the DFI in responder dogs was also observed (figure 4D). Lastly, a decrease (fold change Post-Vax/Pre-Vax <1.0) in the percentage of myeloid derived suppressor cells (MDSC) was observed between the pre-vaccination and corresponding post-vaccination samples in 68% of the vaccinated dogs analyzed (figure 4A).

Mechanisms underlying the role of HuDo-CSPG4-vaccine-induced antibodies

The vaccine-induced IgG antibodies recognizing Do-CSPG4 may mediate melanoma cell elimination through an ADCC mechanism. Indeed, in 36% of the sera tested, vaccination-induced antibodies effectively mediated the killing of canine CMM-12 cells (figure 5A). In addition, post-vaccination sera induced Do-CSPG4 internalization (figure 5B) and downregulation (figure 5C), and significantly reduced the proliferative (figure 5D) and migratory (figure 5E) ability of CMM-12 cells. Post-vaccination sera were also able to inhibit the proliferation (figure 6A, left panel) of the canine melanoma cell line OLGA, naturally expressing low levels of CSPG4 (online supplemental figure S8A, B). Interestingly, no inhibition (figure 6A, right panel) was detected when OLGA cells were incubated with dog sera collected after the fourth vaccination with a fully xenogeneic Hu-CSPG4 vaccine, used in previous trials.[29 33] This difference may reflect the induction of a higher avidity antibody response by HuDo-CSPG4 as compared with Hu-CSPG4 vaccination, as demonstrated by a chaotropic ELISA against the Do–D2 (figure 6B).

These data are supported by the clinical observation that HuDo-CSPG4 vaccination is equally effective for the treatment of melanomas with a low (<5) or high (>5) CSPG4 score (figure 6C), while the antitumor efficacy of Hu-CSPG4 vaccine, was higher for the treatment of melanomas with a CSPG4 score ≥5, weakening its efficacy against oral melanoma with lower CSPG4 positivity.[33] These results emphasize the benefit of using the chimeric HuDo-CSPG4 vaccine over the fully Hu-CSPG4.

DISCUSSION

The CSPG4 antigen is an appealing comparative immunotherapeutic target, highly expressed on melanoma cells in primary and metastatic lesions in both humans and dogs, with a pivotal role for cancer cell malignancy.[11] However, being a self, non-mutated tumor associated antigen (TAA), CSPG4 is poorly immunogenic in both species. To overcome this limitation, we revisited the antigen mimicry concept.

Unlike other vaccines based on the use of xenogeneic TAs as a strategy to break immune-tolerance against a self-antigen,[19] including ONCEPT[41–43] and our Hu-CSPG4 DNA vaccine,[29 33] we developed a chimeric CSPG4 DNA vaccine, HuDo-CSPG4, resulting from the fusion of the cell membrane proximal portion of the dog CSPG4 with the cell membrane distal portion of the human CSPG4, taking advantage of the high homology between human and canine CSPG4 sequences. We evaluated, as primary objectives, the safety and the immunogenicity and, as a secondary outcome, the antitumor potential of HuDo-CSPG4 vaccination in a prospective, non-randomized, veterinary clinical trial, enrolling 80 client-owned dogs affected by locally controlled oral, CSPG4-positive, stage II–IV melanoma. Dogs affected by a CSPG4-negative

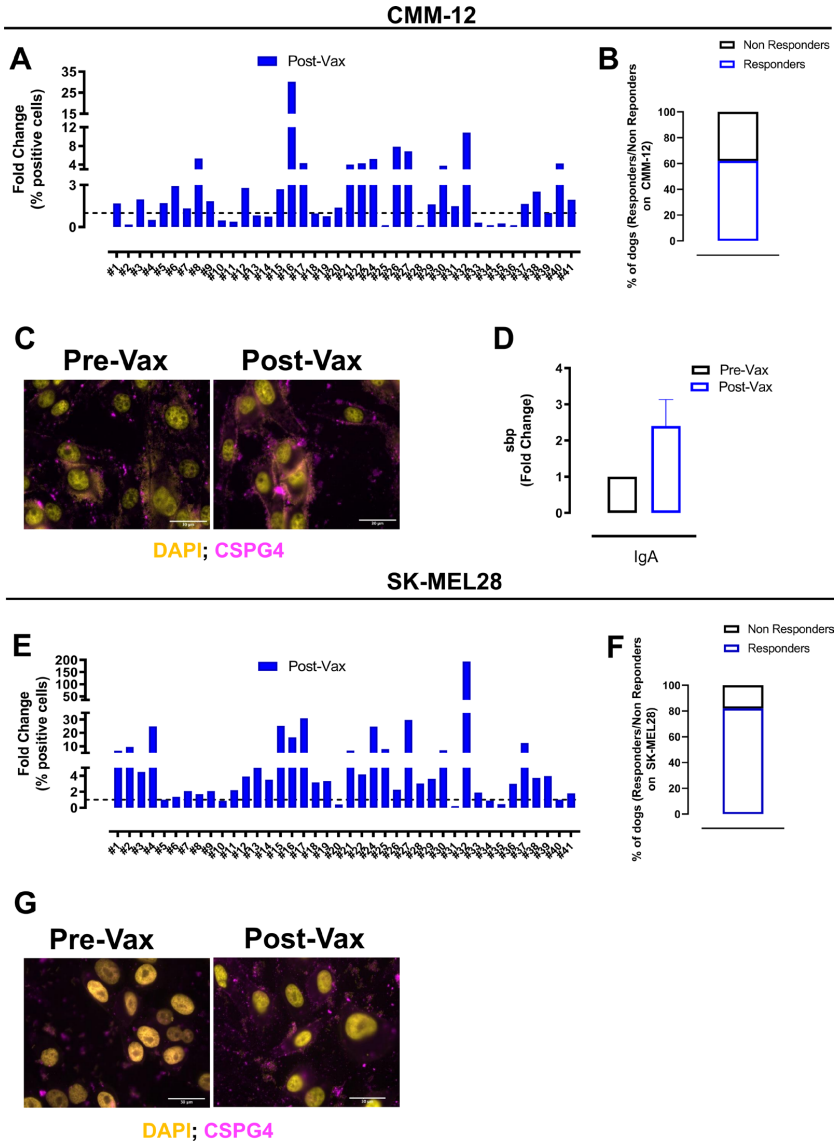

Figure 3 HuDo-CSPG4 vaccine-induced antibodies bind CSPG4-overexpressing canine and human melanoma cells. (A) and (E) Flow cytometry analysis of naturally CSPG4-expressing canine CMM-12 (A) and human SK-MEL-28 (E) melanoma cells incubated with sera from canine patients before the first immunization (Pre-Vax, dotted black line) and after the fourth HuDo-CSPG4 vaccination (Post-Vax, blue bars). Total IgG binding was evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as the ratio (fold change) between the percentages (%) of stained cells incubated with the Post-Vax and the Pre-Vax sera. Each bar represents a canine patient. (B) and (F) Histograms representing the percentage of responders (blue) and non-responders (black) calculated by enumerating dogs in which sera collected after the fourth immunization displayed an increased ability (ratio >1.1) to stain the CMM-12 (B) and the SK-MEL-28 (F) melanoma cells as compared with sera collected before the first immunization. (C) and (G) Representative immunofluorescence images (one out of three independent experiments) of canine CMM-12 (C) and human SK-MEL-28 (G) cells stained with Pre-Vax and Post-Vax sera from HuDo-CSPG4 vaccinated dogs. Bound antibodies were revealed using a FITC rabbit anti-dog IgG secondary antibody and nuclei were stained with DAPI. (D) IgA specific binding of Pre-Vax and Post-Vax sera collected from HuDo-CSPG4 vaccinated dogs on canine CMM-12 cells. Results are expressed as the ratio (fold change) between the serum binding potential (sbp) of the Post-Vax and the Pre-Vax sera. CSPG4, chondroitin sulfate proteoglycan 4; DAPI, diamidino-2-phenylindole; Do, dog; Hu, human; FITC, fluorescein isothiocyanate.

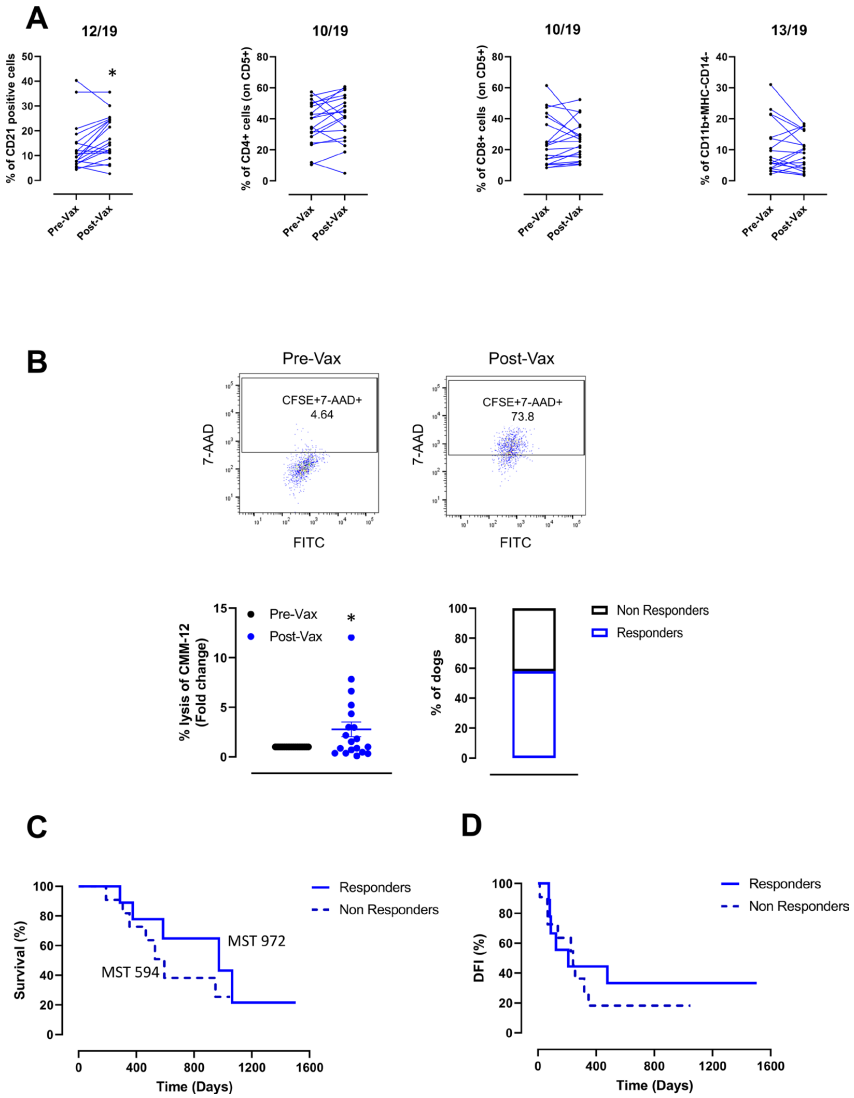

Figure 4 HuDo-CSPG4 vaccination is effective in inducing an anti-CSPG4 cellular immune response in dogs. (A) Flow cytometry analysis of the frequency of circulating B cells, $CD4^+$ and $CD8^+$ T cells, and MDSC collected from canine melanoma patients before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Graphs show the percentage of $CD21^+$ B cells (gated on live cells), of $CD4^+$ and $CD8^+$ T cells (gated on $CD5^+$ cells) and of MHC-II^-CD14^- (gated on $CD11b^+$ cells) cells. The numbers of dogs in which a difference in the frequency (fold change >1.1 or fold change <1.1) of a cell population was observed comparing Pre-Vax and Post-Vax PBMC are indicated above in each graph. Student's t-test, *p=0.0151. (B) Cytotoxic assays to quantify the ability of Pre-Vax and Post-Vax PBMC to kill CSPG4-positive CMM-12 cells. Representative dot plots of one dog analyzed, showing the percentage of 7-AAD^+ dead cells among $CFSE^+$ cells (upper panels) are shown. Results are shown as the fold change between the percentage of CMM-12 cells lysed after incubation with Post-Vax and Pre-Vax PBMC for each dog analyzed (lower, left panel, Student's t-test, *p=0.0260), and as the percentage of dogs of which PBMC induced an increased CMM-12 cell lysis (responders) or not (non responders) (lower, right panel). (C) and (D) Kaplan-Meier curves comparing the overall survival (C) and the disease-free-interval (DFI, (D), in days, of vaccinated dogs who develop (responders, continuous blue line) or not (non responders, dotted blue line) a cytotoxic response against the canine CMM-12 cell line. The median survival times (MST) in days for each group has been reported in the overall survival graph. Log-rank test, p=0.2819. 7-AAD, 7-Amino-ActinomycinD; CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human; MDSC, myeloid derived suppressor cells; PBMC, peripheral blood mononuclear cells; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; CFSE, carboxyfluorescein succinimidyl ester.

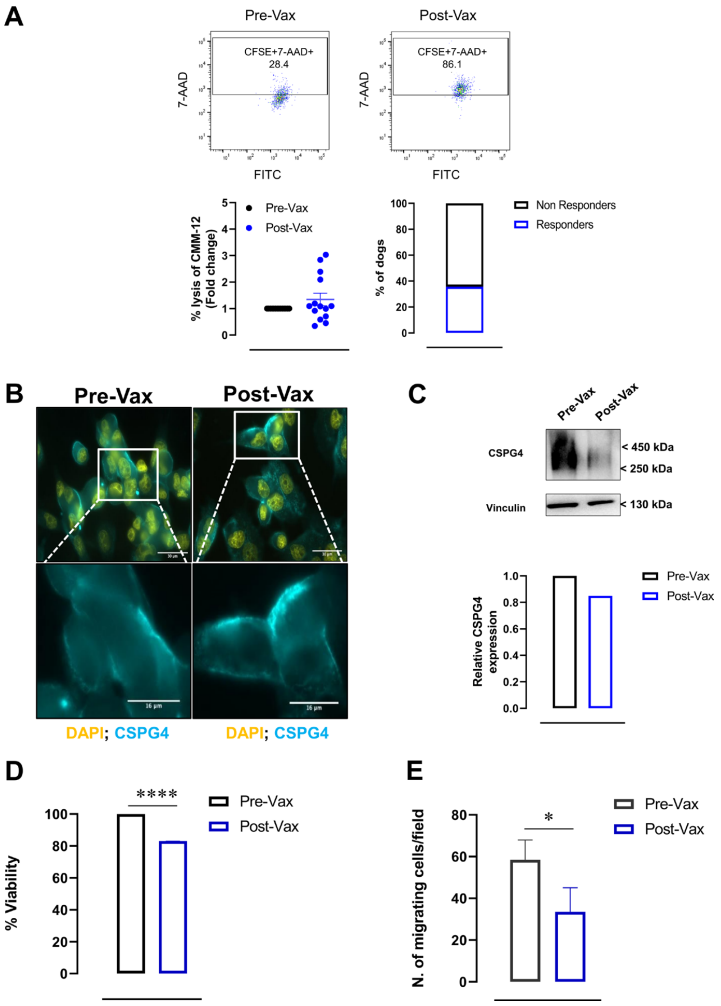

Figure 5 Potential mechanisms of action of HuDo-CSPG4 vaccine-induced antibodies. (A) Cytotoxic assay to quantify the ability of Pre-Vax and Post-Vax sera to induce the killing (ADCC) of CSPG4-positive CMM-12 cells. Representative dot plots of one dog analyzed, showing the percentage of 7-AAD$^+$ dead cells among CFSE$^+$ cells (upper panels) are shown. Results are reported as the fold change between the percentage of CMM-12 cells lysed after incubation with Post-Vax and Pre-Vax sera for each dog analyzed (lower, left panel), and the percentage of dogs whose sera induced an increased CMM-12 cell lysis (responders) or not (non responders) (lower right panel). (B) Representative immunofluorescence images (one out of three independent experiments) of canine CMM-12 cells incubated at 37°C with pooled sera, collected before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Anti-CSPG4 IgG binding and localization was detected using a Texas red-conjugated anti-mouse IgG and nuclei were stained with DAPI. (C) Representative Western blot analyses (upper panel) of CSPG4 expression in the lysates of CMM-12 melanoma cells incubated at 37°C for 48 hours with pooled sera collected before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Relative protein loading was shown using an anti-vinculin antibody. Immunoreactive band density quantification is shown (lower panel); results are reported as relative CSPG4 protein expression, considering Pre-Vax condition as 1. (D) MTT proliferation assay performed on CSPG4-positive canine CMM-12 cells after 48 hours of incubation at 37°C with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 vaccination (Post-Vax). Results are expressed as the percentage (%) of cell viability, considering Pre-Vax conditions as 100%. Student's t-test, ****p<0.0001. (E) Migratory ability of canine CMM-12 melanoma cells incubated with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 vaccination (Post-Vax). Results show the number of migrated cells in four randomly selected fields per well. Student's t-test, *p=0.0155. 7-AAD, 7-Amino-ActinomycinD; ADCC, antibody-dependent cell-mediated cytotoxicity; CSPG4, chondroitin sulfate proteoglycan 4; DAPI, diamidino-2-phenylindole; Do, dog; Hu, human; FITC, fluorescein isothiocyanate; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; CFSE, carboxyfluorescein succinimidyl ester.

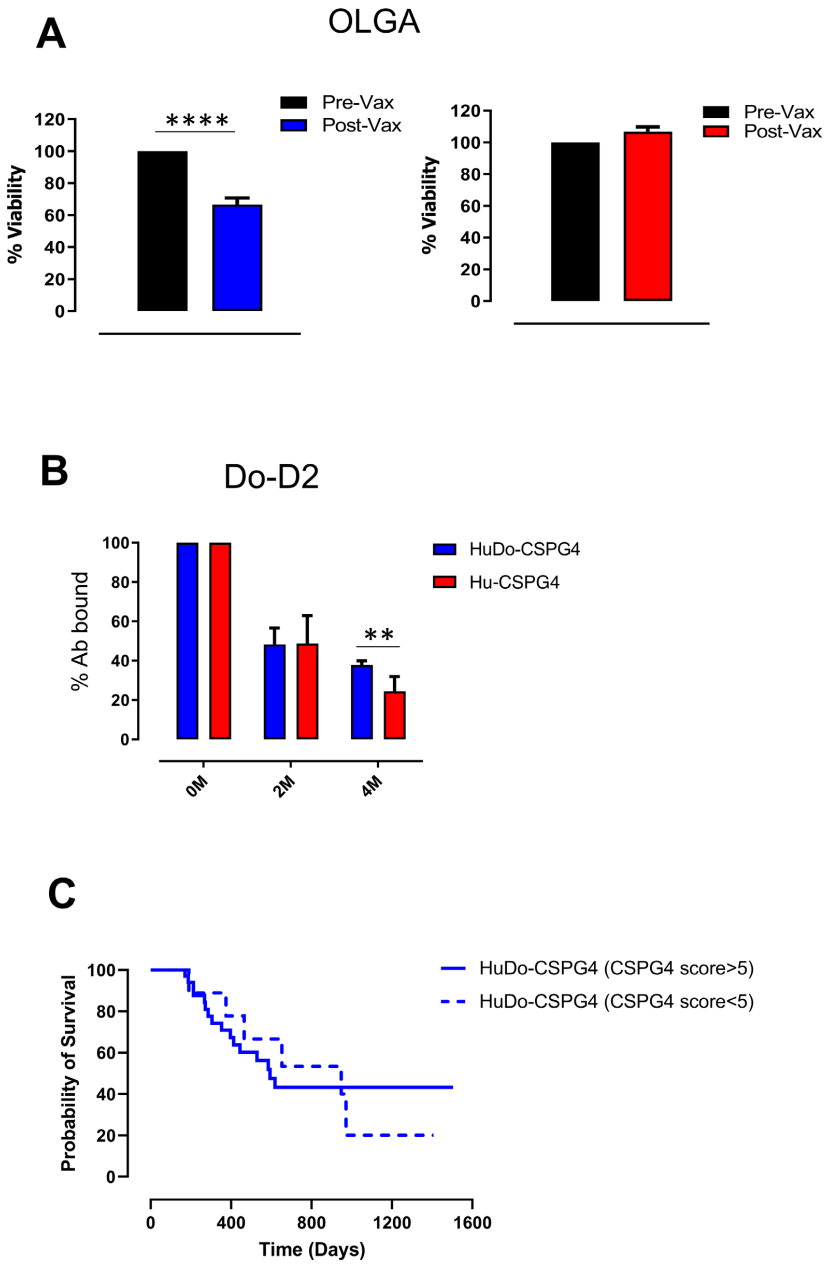

Figure 6 HuDo-CSPG4 vaccination induces a high avidity antibody response. (A) MTT proliferation assay performed on a low CSPG4-expressing canine melanoma cell line (OLGA) incubated for 72 hours at 37°C with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 (blue bars) or Hu-CSPG4 (red bars) vaccination. Results are expressed as percentage (%) of viability, considering Pre-Vax conditions as 100%. Student's t-test, ****p=0.0002. (B) Avidity of anti-Do–D2 vaccine-induced antibodies in the sera of dogs immunized with either the HuDo-CSPG4 (blue bars) or the (Hu)-CSPG4 (red bars) plasmids, evaluated by a chaotropic ELISA. Results are expressed as percentage (%) of antibodies (Ab) that remain bound after the treatment with the chaotropic agent, as compared with the medium alone considered as 100%. Student's t-test, **p=0.0050. (C) Kaplan-Meier curves comparing overall survival of HuDo-CSPG4 vaccinated dogs bearing a melanoma with CSPG4-positivity score <5 (dotted blue line) and ≥5 (continuous blue line). CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide).

melanoma were not enrolled, since they could not benefit from CSPG4-immune-targeting and display a better prognosis as compared with CSPG4-positive melanoma-affected dogs.[29]

HuDo-CSPG4 vaccination was effective in inducing an antibody response against the canine CSPG4 protein. After four immunizations, an increased IgG antibody response against both the recombinant D2 and D3 domains of Do-CSPG4 and the native Do-CSPG4 protein was detected in the post-vaccination as compared with pre-vaccination sera in a high percentage of dogs. It must be noted that a spontaneous low affinity anti-CSPG4 antibody response in melanoma-bearing dogs after the local control of the tumor and before vaccination was detected, however this is not predictive for the outcome. This would have resulted in an underestimation of the percentage of responder dogs in our trial. Indeed, spontaneous anticancer autoantibodies are present in patients with a variety of malignancies, including melanoma.[12 44] However, in dogs with this spontaneous antibody response we observed the production of antibodies with a higher avidity for CSPG4 after immunization, highlighting the benefit of HuDo-CSPG4 vaccination in improving the quality of the antibody response over the quantity. In addition, for most of the dogs, a further increase in vaccine-induced antibodies was detected following subsequent vaccinations (after the fifth and the sixth immunization cycles). Therefore, we cannot exclude the possibility that, for some dogs, other time points than those that we selected[29 33] would have been more informative as to the development of anti-CSPG4 antibodies, also considering the differences among dogs, including size and age.

However, the finding of a vaccine-induced anti-Do-CSPG4 antibody response is likely to reflect the ability of the antigen mimicry strategy to overcome host's unresponsiveness to the self-CSPG4, thanks to 86% homology in the aa sequence of the human moiety inside the chimeric HuDo-CSPG4 sequence with its dog counterpart. Under our experimental conditions, the immune clones triggered by the Hu-CSPG4 moiety which cross react with the self-antigen may be amplified by the Do-CSPG4 moiety encoded in the chimeric HuDo-CSPG4. This mechanism may account for the herein observed higher avidity and more marked functional effects of the anti-Do-CSPG4 antibodies elicited by the chimeric HuDo-CSPG4 as compared with those elicited in dogs by immunization with the fully Hu-CSPG4 vaccine, used in a previous pilot veterinary trial.[29 33 45]

The anti-CSPG4 immune response elicited appears to be clinically relevant in the immunized population, since a significant correlation was observed between the vaccine-induced IgG level of responder dogs and the overall survival. In addition, the induction of a mucosal immunity, potentially relevant for the treatment of oral malignancies, was suggested by the detection of anti-CSPG4 IgA in the serum of a high percentage of the vaccinated dogs. Ultimately, these results allow to speculate that the induction of anti-CSPG4 IgA may be partially protective against local recurrences. Flow cytometry and immunofluorescence analysis demonstrated that vaccine-induced antibodies can bind the Do-CSPG4 antigen over-expressed in its natural conformation on canine CMM-12 melanoma cells, and this is important to assume their effectiveness in mediating antitumor activities in vivo. As suggested by our in vitro results, anti-CSPG4 antibodies that we have detected in the immunized dogs are likely to mediate multiple mechanisms. They include the elimination of melanoma cells by an ADCC mechanism, CSPG4 downregulation, and inhibition of its role in the biology and functional properties of melanoma cells. Such effective anti-CSPG4 antibody response may overcome the ability of melanoma cells to downregulate MHC-I molecules and escape from T cells.[46]

Finally, an increased percentage of B and $CD4^+$ T cells in the PBMC of vaccinated dogs was observed. A cytotoxic activity of PMBC against canine CMM-12 melanoma cells was found in 11 out of 19 vaccinated dogs analyzed. This response in the immunized population is associated with a better overall survival. Moreover, the reduction in circulating MDSC in the majority of analyzed vaccinees, suggest that, following HuDo-CSPG4 vaccination, the immunosuppression that persists after the local control of the tumor can be, at least partially, reduced.

Based on the evidences of a detectable and effective vaccine-induced immune response, the potential clinical consequences of HuDo-CSPG4 vaccination have been evaluated, considering the overall survival as the most objective measure, for both arms, based on the design of the trial.[47]

The DFI instead was not considered as a proper clinical endpoint for this study, since it might be affected by the timing and, potentially, by the different imaging diagnostic procedures adopted. Indeed, both X-rays and CT scan were used for diagnosis, but the different methods depended on the improvement of diagnostic techniques over time, and on the owners' financial resources. While no differences regarding the staging system at baseline were observed between the two arms (ie, vaccinated vs control dogs), in accordance with owners' decision, unvaccinated dogs underwent only a 3–6 monthly check-up, thus potentially limiting the early detection of local recurrence and metastasis as compared with dogs of the vaccinated arm. This makes the evaluation of the overall survival, rather than the DFI, the sole reasonable endpoint for analysis and comparison with the vaccinated dogs in this study.

The adjuvant HuDo-CSPG4 vaccination envisages a potential benefit on the overall survival of immunized dogs as compared with unvaccinated controls, treated with conventional therapies alone (surgery with or without radiotherapy), prompting its more extensive evaluation in a randomized trial.

Beside these promising results, one evidence which is noteworthy for its clinical application is the lack or limited side effects of vaccination observed in HuDo-CSPG4 vaccinated dogs. These data parallel the lack of

toxicity described in human melanoma patients and rats with chemically induced chondrosarcoma immunized with CSPG4 mimics.[48–51] These results altogether argue against the broad distribution of CSPG4 in normal tissues reported in the Protein Atlas and support the validity of the studies which have shown that the expression of CSPG4 in normal tissues is restricted to activated pericytes in the tumor microenvironment.[12 14 52]

Some limitations of this study need to be surmised. As mentioned above, these include the lack of randomization, with the inherent potential problems of selection bias and not-blinded outcome evaluation. Unfortunately, a randomization was not possible in this study. In view of the promising results of our previous veterinary trials, using a Hu-CSPG4 vaccine for the treatment of canine melanoma patients, for clinicians it would have been difficult for ethical reasons to randomly assign patients; concurrently, it should be noted that the dog owners always refuse to accept the possibility that their dogs may enter the non-vaccinated arm. Unlike what is expected in the human clinics, the veterinary medicine scenario is different and specifically in this case no dedicated funds covering the expenses in both vaccinated and non-vaccinated arms of dogs were available; thus, the non-vaccinated arm was made up of dogs whose owners were not available to proceed with further therapies other than surgery. Nevertheless, it has to be considered that ONCEPT, the first antitumor vaccine licensed for dogs with locally controlled oral melanoma, was USDA-approved starting from the results of similar non-randomized, retrospective studies.[42 53] Also, more recent reports on ONCEPT efficacy are non-randomized, uncontrolled, and retrospective studies.[54–56] Other limitations of our study are the use of a single DNA dose and a single administration procedure as well as the search of anti-CSPG4 IgA in the serum rather than in the mucosal compartment. The small number of enrolled dogs affected by metastatic melanoma is another limitation. Enrollment of other melanoma-bearing dogs with either local or distant metastasis is warranted to evaluate the potential benefit of HuDo-CSPG4 vaccination also in a metastatic setting. Finally, a deeper characterization of circulating and tumor-infiltrating cells at diagnosis and after HuDo-CSPG4 vaccination, including T-regulatory cells, as well as a better dissection of the vaccine-induced cellular immunity, is needed.

By contrast, the relatively high number of dogs enrolled in the prospective study, the long-term follow-up, and the chimeric structure of the proposed HuDo-CSPG4 vaccine that we showed herein to be effective at breaking tolerance to self-CSPG4 in dogs, represent important strengths of this study.

Nevertheless, since disease progression has been observed in some vaccinated dogs, possibly also owed to the escape of CSPG4-negative clones as the result of antigen loss due to the immunological pressure exerted by the vaccine, the identification of other key targetable antigens may be relevant to the design of more effective and multimodal treatments. In addition, since the expression of programmed cell death 1 and programmed death ligand 1 has also been detected in canine melanoma patients, combinatorial approaches using ICIs plus anti-CSPG4 vaccination in this comparative oncology model should be investigated.[57 58]

Lastly, it should be noted that no BRAF mutations, which occur in approximately 50% of human cutaneous melanomas,[3] have been detected in melanoma-bearing dogs. Still, a significant proportion of human cutaneous melanoma, as well as almost all uveal and mucosal melanomas, do not show any BRAF alterations but overexpress CSPG4.[22 24]

The results from this veterinary trial suggest that the anti-CSPG4 therapy may represent a new therapeutic possibility for the treatment of these tumor subtypes that behave more aggressively and have less favorable prognosis.

In summary, the application of a novel anti-CSPG4-mimicry strategy, based on the use of a hybrid DNA vaccine coding for a human/dog CSPG4 (HuDo-CSPG4) chimera, resulted safe and immunogenic, displaying a potential clinical benefit in prolonging the survival of CSPG4-positive oral melanoma-affected dogs. Ultimately, thanks to the highly recognized predictive power of comparative veterinary studies and to the structure of the vaccine, these findings justify exploring the possibility to translate the chimeric CSPG4 treatment also to the human clinics. Finally, it should be noted that HuDo-CSPG4 vaccination could be extended to other CSPG4-positive cancers in both canine and human patients.

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Supplementary material is available at the following link: **https://jitc.bmj.com/content/jitc/suppl/2022/05/17/jitc-2021-004007.DC1/jitc-2021-004007supp001_data_supplement.pdf**

Chapter II

Beyond current OSA treatments: CSPG4 as a novel targetable OSA antigen

(For detailed materials and methods and results, please refer to Paper II and Paper III**)**

The complex interaction between TME components and OSA cells appears to influence clinical outcomes and therapeutic responses by regulating tumor chemoresistance, which in fact represents one of the main reasons for treatment failure in patients with OSA (44, 401). As reported in the *Introduction* of this thesis, due to the high toxicity and the poor response rate to chemotherapy, a flurry of new research is exploring the role of immunotherapy in the management of OSA (402). Currently, there are many clinical trials exploring immunotherapeutic approaches, especially ICIs, in patients with OSA (403), but none of them have been approved due to poor clinical responses. Hence, finding alternative innovative therapies that could positively affect patients' outcomes is still an unmet need.

One limitation of the lack of therapeutic progress for OSA, is ascribed to the lack of a common oncogenic targetable driver. Therefore, in the search for novel potential treatment opportunities for OSA management, with my coworkers I wondered whether CSPG4 could represent a relevant tumor antigen to target through DNA vaccination in OSA. Hence, this project started with the purpose of validating CSPG4 as a potential immunotherapeutic target for human patients, using pet dogs naturally developing OSA as pre-clinical model.

At first, we demonstrated CSPG4 expression in human OSA. We found CSPG4 mRNA upregulation in human high-grade OSA as compared to normal OSB, while we found a mild expression in MSCs considered precursors of OSA. In human OSA biopsies, CSPG4 overexpression was independent from the tumor histological grade, but correlated with the patients' poor prognosis. To use spontaneous canine OSA as a pre-clinical model to test our anti-CSPG4 immunotherapy, we therefore validated CSPG4 expression in canine tumors. We found CSPG4 expression in a high percentage of canine OSA biopsies. As for humans, CSPG4 overexpression in canine patients was correlated with poor prognosis. Thus, these initial results highlighted the potential clinical relevance of evaluating anti-CSPG4 strategies in canine OSA patients, with a high translational value.

In the *in vitro* evaluation of the consequence of CSPG4 immune-targeting, we used anti-CSPG4 mAbs, and exploited both human and canine OSA cell lines and their derived osteospheres, enriched in cancer stem cells (CSC). Anti-CSPG4 treatment significantly impaired tumor cell malignant properties, such as cell proliferation, migration and osteospheres generation.

We also found that anti-CSPG4 mAbs potentiated the anti-proliferative effect of Doxorubicin, and significantly inhibited both human and canine OSA cell proliferation, migration and osteospheres

generation *in vitro.* Finally, to evaluate the possibility of applying our HuDo-CSPG4 vaccination for the treatment of CSPG4-positive OSA, we used sera from OMM canine patients enrolled in our previous clinical veterinary trial (281, 282). Anti-CSPG4 antibodies induced by the xenogeneic Hu-CSPG4 vaccination were effective in impairing both human and canine OSA cells growth *in vitro*.

Our first results showed that CSPG4 overexpression in OSA may have clinical implication in oncological patients, and that its targeting through immunotherapy could represent an adjuvant option for the first-line standard of care. Taken together, these findings provided the rationale for testing the clinical effectiveness of our anti-CSPG4 immunotherapy in dogs with spontaneous OSA.

Paper II

Therapeutic Advances in Medical Oncology *Original Research*

Identification of CSPG4 as a promising target for translational combinatorial approaches in osteosarcoma

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Abstract

Background: Osteosarcoma (OSA) is a highly metastatic pediatric bone tumor. Adjuvant chemotherapy and surgical resection represent standard treatments; however, the prognosis is still poor. Effective strategies are urgently needed. Chondroitin sulfate proteoglycan (CSPG)4 is a transmembrane proteoglycan with a low expression in normal tissues but high expression in several solid tumors, where it plays a central tumorigenic role. Therefore, it represents a promising therapeutic target. The high homology between human and canine CSPG4 and the recognized translational power of canine tumors as preclinical models for human malignancies prompted us to evaluate CSPG4 expression and the consequences of its immune-targeting for both human and canine OSA treatment.

Methods: We analyzed CSPG4 overexpression in human and canine OSA samples and its significance for the survival of OSA patients. We exploited functional *in vitro* experiments to assess the antitumor potential of CSPG4 immune-targeting.

Results: CSPG4 is overexpressed in OSA and has possible clinical implications as suggested by an evident correlation between CSPG4 overexpression and a shorter survival for both OSA-affected humans and dogs. The potential of CSPG4 immune-targeting for OSA treatment came from the ability of anti-CSPG4 monoclonal antibodies and sera, derived from human-CSPG4-DNA vaccinated canine patients, to significantly inhibit human and canine CSPG4-positive OSA cell proliferation, migration, and osteospheres generation. Moreover, CSPG4 immune-targeting has been shown to potentiate the effect of doxorubicin.

Conclusions: Overall, these results provide the rationale to investigate the CSPG4 immune-targeting as a promising weapon for the treatment of CSPG4-positive OSA canine patients, to be successfully translated to a human setting.

Keywords: antibodies, comparative oncology, CSPG4, osteosarcoma

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Introduction

Osteosarcoma (OSA) is the most common primary malignant tumor of the bones. Of note, OSA is the sixth most-frequent pediatric cancer and represents the second most common cause of cancer-related death in this age group; a second peak is observed in adults after the sixth decade of life.[1,2] Currently, primary OSA is classified into low- (Grade I) and high-grade subtypes (Grade II and III in the presence of metastasis), high-grade OSA being the most prevalent and aggressive variant.[3]

At initial diagnosis, almost 20% of OSA patients present evidence of metastatic spreading commonly involving lungs (90%), sites within the same affected bones (8–10%) or, more rarely, lymph nodes.[4,5] However, considering that the

vast majority of patients without overt metastasis at diagnosis develop lung metastasis within 6–36 months, it is presumed that these apparently non-metastatic patients actually have micrometastases already at diagnosis.[6,7]

Conventional treatments consist mainly in the surgical resection of the primary tumor, in association with neoadjuvant and adjuvant chemotherapy with doxorubicin, methotrexate, and cisplatin. On the basis of the percentage of tumor necrosis after neoadjuvant chemotherapy, patients can be classified as poor responders (Huvos Grades I–II), responders (Huvos Grade III), or good responders (Huvos Grade IV). A strong correlation between the Huvos Grade and the subsequent effectiveness of postoperative chemotherapy and disease-free survival has been observed. This standard of care treatment is quite effective in the setting of localized OSA. Indeed, nowadays, the 5-year survival rate for localized OSA is about 65–70%, while only 20% of patients with multifocal disease or metastasis at diagnosis are still alive after 5 years.[2,7,8]

Therefore, it is evident that OSA is still a very aggressive and fatal disease for which no significant therapeutic advances have been achieved in the last 30 years and for which the identification of novel and more effective approaches is needed to improve patient survival.

As a step forward, during the last few decades, researchers have addressed to the identification of potentially therapeutically targetable OSA-associated antigens. Several tyrosine kinase receptors have been identified as over-expressed in OSA, including KIT, vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3, platelet-derived growth factor (PDGFR)-β, and MET, and found to be correlated with metastasis development and poor survival.[9–12] As a consequence, in the last few years several targeted therapies have been investigated, which unfortunately showed only limited efficacy in advanced OSA.[11,13] The need for novel and more clinically relevant targets is therefore critically evident.

The chondroitin sulfate proteoglycan (CSPG)4 is a cell surface proteoglycan, considered as an ideal tumor-associated antigen, that is, an oncoantigen,[14] because it is poorly expressed in healthy tissues,[15–23] whereas in a vast range of human neoplasms it is in overexpressed by tumor cells, the tumor microenvironment and, cancer stem cells (CSCs). It has been widely described that CSPG4 has a key role in several oncogenic pathways required for malignant progression and metastasization.[24] We have already demonstrated the clinical relevance of CSPG4 immune-targeting by means of DNA vaccination for the treatment of canine malignant melanoma (MM);[25,26] however, to the best of the authors' knowledge very few investigations have been undertaken on the involvement of CSPG4 in OSA.[27,28]

The aim of this study was to evaluate CSPG4 as a potential immunotherapeutic target for both human and canine OSA patients. This could offer an appealing opportunity to exploit spontaneous occurring OSA in pet dogs as model of human OSA tumors and for predicting the clinical efficacy of therapeutic approaches targeting CSPG4. Indeed, canine OSA is nearly indistinguishable from the human disease, presenting the same risk factors, histological tumor grading, similar standard treatments, and clinical responses.[29,30] All these shared features make OSA-bearing dogs a valuable translational model of human OSA for the investigations of novel immunotherapies that could benefit both species.

Based on these considerations, we investigated CSPG4 expression in both human and canine OSA. We aimed to evaluate the effects of anti-CSPG4 targeting, alone or in combination with doxorubicin, on both human and canine OSA tumor cells and on osteospheres, enriched in CSC, which are considered responsible for recurrences and metastasis. Overall, our results provide the rationale for testing the clinical effectiveness of an anti-CSPG4 immunotherapy in dogs affected by spontaneous OSA, with the final aim of its translation for the treatment of human OSA patients.

Material and methods

Sample collection and clinical follow up

Tissue samples from 50 cases of spontaneous canine appendicular OSA collected *via* routine care between 2008 and 2014 at the Veterinary Teaching Hospital, Department of Veterinary Science of the University of Turin, were examined in this study.

Client-owned dogs affected by spontaneous OSA were treated according to the European guidelines established in the Principles of Laboratory

Animal Care (directive 86/609/EEC). The Ethical Committee of the Department of Veterinary Science (University of Turin) approved the experimental protocol, which follows the best practice of veterinary care; written consent for entry into the study was obtained from dog owners. For all canine OSA patients, the initial data collected included history, physical examination, complete blood count, serum biochemical profile, urinalysis and abdominal ultrasound. Limb [lateral–lateral (LL) and anterior–posterior (AP) views] and chest [LL, right and left, and dorso–ventral (DV) views] radiographic evaluation was performed to examine the features and the extent of the tumor in addition to the presence of lung metastasis. From 2010 evaluation with computer tomography (CT) was included. In cases where regional lymph nodes were enlarged, they were aspirated, cytologically examined, and then removed for histological evaluation. All dogs included in this study were surgically treated (amputation or limb sparing) before receiving adjuvant chemotherapy. The Kaplan–Meier method was used to estimate overall and disease-free survival times. Differences in survivals were tested for significance using log-rank tests.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed as described previously[24,31] on collected canine OSA. Samples were fixed in 4% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm. Then, samples were stained with hematoxylin and eosin to establish the histological diagnosis. The histological grade was determined according to the systems proposed by Loukopoulos and Robinson.[32] The grading was evaluated as I (low), II, or III (high). IHC for CSPG4 in the 50 samples was performed on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Sections were exposed to high-temperature for antigen unmasking by incubation at 98°C with citric acid buffer, pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Tissue sections were incubated for 12 h at room temperature with a polyclonal anti-CSPG4 antibody (diluted 1:40, Sigma Aldrich, St. Louis, MO, USA), then 30 min with biotinylated-secondary antibody (Vectastain Elite ABC) and revealed with the ImmPACT DAB kit for peroxidase, both from Vector Laboratories Inc. (Burlingame, CA). A total score considering the proportion of positively stained tumor cells and the average staining intensity was assigned as previously described.[31] Briefly, the score indicating the positivity of tumor cells was assigned as follows: 0 (none); 1 (<1/100 or <1%); 2 (1/100–1/10 or 1–10%); 3 (1/10–1/3 or 10–30%); 4 (1/3–2/3 or 30–70%); and 5 (>2/3 or >70%). The score representing the estimated average staining intensity of positive tumor cells encompass 0 if none, 1 weak, 2 intermediate, or 3 strong. The two scores were then added to each other to obtain a final score of CSPG4 expression ranging from 2 to 8. A total score ⩾4 was used as cut-off.

Cell lines and osteospheres

Human OSA cell lines (MG-63, SAOS-2, U2OS) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich) or RPMI (Sigma Aldrich) supplemented with 20% fetal bovine serum (FBS, Sigma Aldrich). Penny cells, derived from the tail biopsy of a primary grade III canine OSA, were grown in ISCOVE Modified Dulbecco's Medium (Sigma Aldrich) supplemented with 10% FBS. All cells were grown in medium supplemented with penicillin/streptomycin (Sigma Aldrich) and maintained at 37°C and 5% carbon dioxide in a humidified incubator.

Both human and canine osteospheres were generated following the protocol described in Conti *et al.*[33] Briefly, cells were detached and plated in ultra-low-attachment 75 cm flasks (Sigma Aldrich) at 6×10^4 viable cells/ml in serum-free DMEM-F12 (Sigma Aldrich) supplemented with 0.4% bovine serum albumin (BSA), 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 5 mg/ml insulin, all from Sigma Aldrich. Non-adherent spherical cells' clusters, named osteospheres, were collected after 48 h or 5 days for further analysis. Photographs of osteospheres were taken using a CCD-300-RC camera, and images were processed using Fiji Software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD) and PowerPoint (Microsoft, Redmond, WA).

MTT cell proliferation assay

3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol (MTT; Merck Millipore, Burlington, MA, USA)

assay was used to assess proliferation of human MG-63 and canine Penny OSA cells. Briefly, epithelial cells were seeded in triplicate in 96-well plates (5×10^3 cells and 8×10^3 cells/100 µl well, respectively) in serum-free medium and allowed to adhere overnight. To evaluate the inhibition of the CSPG4-mediated cell proliferation, four-selected anti-CSPG4 purified mAbs, 225.28, TP32, TP49, and VF20-VT87.41, were mixed in a pool or used as single agents. All the mAbs were produced in the laboratory of Prof. Ferrone (Massachusetts General Hospital, Harvard Medical School, Boston, MA) and are secreted by hybridomas generated from BALB/c mice immunized with human melanoma cells. Specifically, the mAb 225.28 is a mouse IgG2a, while the others (TP32, TP49, and VF20-VT87.41) are all mouse IgG1, which recognize distinct and spatially distant epitopes of human CSPG4. The specificity of mAbs was characterized as described elsewhere.[34–36]

Cells were incubated with different treatments for 48 h at the following final concentrations or dilutions: control isotypes (Sigma Aldrich; 25 µg/ml), anti-CSPG4 mAbs (225.28, TP32, TP49, and VF20-VT87.41, tested individually at 25 µg/ml or mixed in a pool to a final total concentration of 25 µg/ml), doxorubicin (Sigma Aldrich; 100 nM, 1 µM, or 10 µM), melanoma canine patients' sera collected pre- and post-vaccination (1:100) with Hu-CSPG4 DNA plasmid.[25,26] Cells grown in medium alone were used as control. MTT solution (5 mg/ml) was added to each well following manufacturer's instruction. Following 4 h incubation at 37°C, 100 µl of dimethyl sulfoxide (DMSO, Sigma Aldrich) were added to dissolve formazan crystals and absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, CA) with a wavelength of 570 nm.

To assess viability of human MG-63 and canine Penny osteosphere-derived cells, 6×10^4 cells/ml were seeded in 96-well plates in non-adherent conditions and incubated with different stimuli as described above and spheres were allowed to generate for 48 h. Following overnight incubation at 37°C, formazan crystals were dissolved by adding 100 µl isopropanol with HCl 0.04 N to each well. Optical density was measured on an ELISA plate reader (Bio-Rad) with a test wavelength of 570 nm and a reference wavelength of 630 nm. Difference between 570 nm and 630 nm readings represents the output value.

Flow cytometry analysis

Human and canine OSA epithelial cells and osteospheres were detached and dissociated by using Cell Dissociation Non Enzymatic 1X solution (Sigma Aldrich) and then resuspended in the culture medium with a concentration of 1×10^5 cells in 100 µl. Cytofluorimetric analysis were performed on cells for the detection of CSPG4 antigen using a mixed pool of the following mAbs (225.28, TP32, TP49, and VF20-VT87.41; 25 µg/ml final concentration) directed towards different epitopes of CSPG4 and produced in the lab of Prof. Ferrone (refer to MTT cell proliferation assay). Pooled mAbs were incubated on OSA cells for 30 min at 4°C and antibody binding was evaluated using a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (Dako-Cytomation, Glostrup, Denmark). Flow cytometry was performed with a FACS Verse (BD Biosciences, San Jose, CA, USA) and the results were expressed as percentage of positive cells and mean fluorescence intensity (MFI) and analyzed with BDFacs Suite software.

Western blotting

Human and canine OSA cells were incubated in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM NaF, and protease inhibitors, all from Sigma Aldrich) for 30 min at 4°C. Cell lysates were centrifuged for 5 min at 15,000*g*. Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific, Rockford, IL, USA). Following 5' denaturation at 95°C in 2-mercaptoethanol-containing Laemmli sample buffer (Bio-Rad, Hercules, CA), equal amounts of protein (70 µg) were separated through electrophoresis in an Any kDa Mini-Protean TGX precast gel (Bio-Rad) and then transferred onto an Immobilion-P PVDF membrane (Merck Millipore, Billerica, MA). Following blocking with 5% nonfat dry milk (Santa Cruz Biotechnology, Dallas, TX) in wash buffer (Tris Buffered Saline - TBS - supplemented with 0.05% Tween-20 from Sigma Aldrich), the membrane was incubated overnight at 4°C with a pool of anti-CSPG4 mAbs (225.28, TP32, TP49, and VF20-VT87.41, 5 µg/ml each), washed three times in TBS and 0.05% Tween-20, and then incubated for 1 h at room temperature with HRP-conjugated rabbit antimouse Ig (Sigma Aldrich). Actin was used as control for equal protein loading. Immunoreacting bands were detected using ECL (Thermo Scientific) according to the manufacturer's instructions. Band relative intensity

was acquired using a ChemiDoc™ Touch Imaging System (Bio-Rad).

Cell migration assay

To measure cell migration, human MG-63 and canine Penny OSA cells were pre-incubated with different treatments at the following final concentrations: control isotypes (Sigma Aldrich; 100 μg/ml), anti-CSPG4 mAbs (225.28, TP32, TP49, and VF20-VT87.41 mixed in a pool to a final total concentration of 100 μg/ml), alone or in combination with doxorubicin (Sigma Aldrich; 10 μM or 100 nM); then seeded (5×10^4 and 3×10^4, respectively, per well) in 100 μl of serum-free medium in the top chamber of a 24-Transwell plate (8-μm pore size; Corning, Amsterdam, Netherlands). Cells incubated with medium alone were used as control. All bottom chambers of the Transwell plates were filled with 10% FBS-supplemented medium (600 μl per well) and cells were incubated at 37°C in a 5% CO_2 atmosphere. After 48 h, the nonmigrated cells on the top side of the filter were removed by scrubbing twice with cotton tipped swab. Migrated cells on the bottom side of the filter were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) and stained with 0.2% crystal violet (Sigma-Aldrich). After washing, the migrated cells of four randomly selected fields per well were imaged using an Olympus BX41 microscope (Olympus Corp., Tokyo, Japan) and analyzed using Fiji and Imagej Softwares (Rasband, W.S., ImageJ, US National Institutes of Health).

Dataset

Genome-wide gene expression analysis was based on previously deposited microarray experiments and data preprocessing, described in Kuijjer *et al.*[1,37] The dataset included genome-wide gene expression data of osteoblasts (GEO accession number GSE33382), mesenchymal stem cells (MSCs; GEO accession number GSE28974), and 84 high-grade OSA pre-treatment biopsies (GEO accession number GSE33382). All data were processed together as described by Kuijjer *et al.*[1]

Meta-analysis on patient databases

The mRNA expression of CSPG4 in human OSA samples was determined by querying the R2 Kaplan–Meier scanner (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). For prognostic studies, R2 analysis software was used and patients were stratified by expression of the gene of interest. Overall survival and metastasis-free survival data were presented as Kaplan–Meier plots and tested for significance using log-rank tests. To define the cutoff between high and low gene expression, all percentiles between the lower and upper quartiles were computed, and the best performing threshold was used as a cutoff.

Statistical analysis

Normally distributed data are reported as means ± standard deviation (SD) unless otherwise stated. Other variables are expressed as percentages. Quantitative evaluations were carried out using the Student's *t* test. The Kaplan–Meier method was used to estimate survival times and differences in survival distribution were analyzed using the log-rank test. Values of $p < 0.05$ were considered significant. All analyses were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Results

CSPG4 is overexpressed in human high-grade OSA and is related to a poor prognosis.

In order to evaluate the potentiality of CSPG4 as a novel target for human OSA, we used a publicly available comprehensive microarray dataset (superseries accession number GSE42352), including normalized gene expression data of osteoblasts ($n = 3$; GEO accession number GSE33382), MSCs ($n = 12$, GEO accession number GSE28974), and high-grade OSA pretreatment biopsies ($n = 84$, GEO accession number GSE33382).[1,37] CSPG4 mRNA expression resulted significantly upregulated in MSC, considered potential precursors for OSA development, as compared with normal osteoblasts. CSPG4 mRNA expression was further significantly increased in high-grade OSA biopsies (Figure 1(a)). Taking advantage of the clinicopathological details regarding the high-grade OSA samples included in the analyzed dataset,[37] we sought to evaluate whether the CSPG4 expression level in pretreatment OSA biopsies could be related to their response to neoadjuvant chemotherapy, expressed in Huvos Grades. As shown in Figure 1(b), CSPG4 mRNA in pretreatment OSA samples is not related to the Huvos Grade, therefore it cannot be considered as a prognostic factor implicated in the response to the neoadjuvant treatment. Nevertheless, CSPG4 mRNA expression is quite higher in Huvos Grade I–III samples, which include OSA with a lower necrosis in response to neoadjuvant chemotherapy and therefore with a worse prognosis, as compared with Huvos Grade

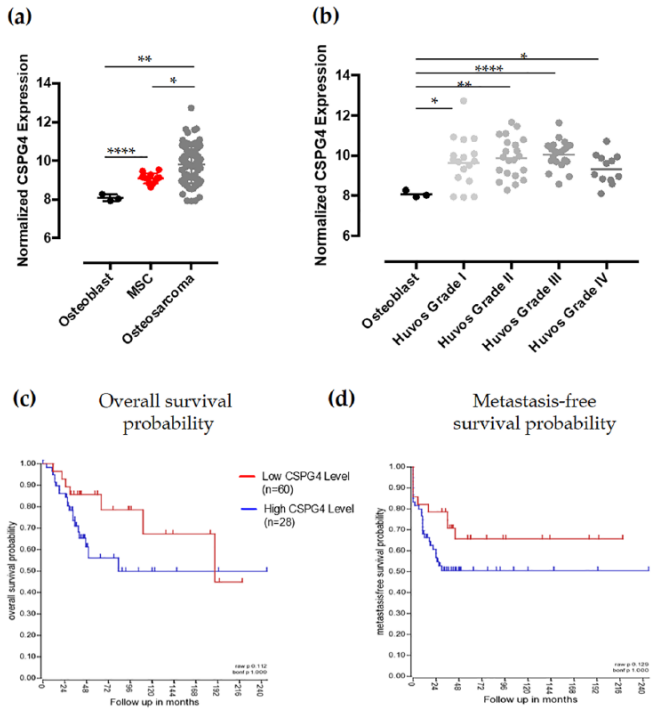

Figure 1. Chondroitin sulfate proteoglycan (CSPG)4 mRNA expression in human osteosarcoma (OSA). (a,b) Normalized gene expression levels of CSPG4, in mesenchymal stem cell (MSC) and OSA biopsies as compared with normal osteoblasts *in toto* (mean ± SD) (a) or divided according to the Huvos system grading (geometric mean) (b). Student's *t* test: *$p < 0.05$; **$p < 0.050$; ****$p < 0.0001$. (c,d) Kaplan–Meier curves depicting overall survival (c) and metastasis-free survival (d) probability, in years, for OSA patients stratified by high (blue) or low (red) mRNA CSPG4 expression.

IV samples, characterized by OSA with a 100% necrosis after neoadjuvant chemotherapy and with a better prognosis. In addition, CSPG4 overexpression showed a trend of correlation with a shorter overall survival (OS; Figure 1(c)) and metastasis-free survival (Figure 1(d)) of OSA patients. Taken together, these data suggest the potential role of CSPG4 in human OSA progression and the possible clinical relevance of its adjuvant targeting for high-grade OSA treatment, representing an even more interesting opportunity for those OSA that are not responsive to neoadjuvant chemotherapy.

CSPG4 is highly expressed in canine OSA

Canine patients affected by spontaneous OSA are considered a highly translational and predictive model to evaluate the clinical response to innovative therapeutic treatments for human OSA, providing several advantages over mouse models.[38] On the basis of these considerations and to confirm the predictivity of the canine OSA model also for CSPG4, first we investigated its expression in canine OSA biopsies. Specifically, we evaluated CSPG4 protein expression using IHC in a total of 50 samples of canine appendicular OSA from patients treated between 2008 and 2014 at the Department of Veterinary Science of the University of Turin. IHC staining for CSPG4 detection was performed on all the collected tissues as previously described[31] at the moment of the surgery, before any treatments. A score of CSPG4 expression was determined as published previously[31] in order to obtain a value of 0 (if negative) or from 2 to 8. There were 38 (76%) CSPG4-positive and 12 (24%) CSPG4-negative primary canine OSA samples (Figure 2(a)). For all CSPG4-positive samples, staining was observed mainly on the membrane surface and in the

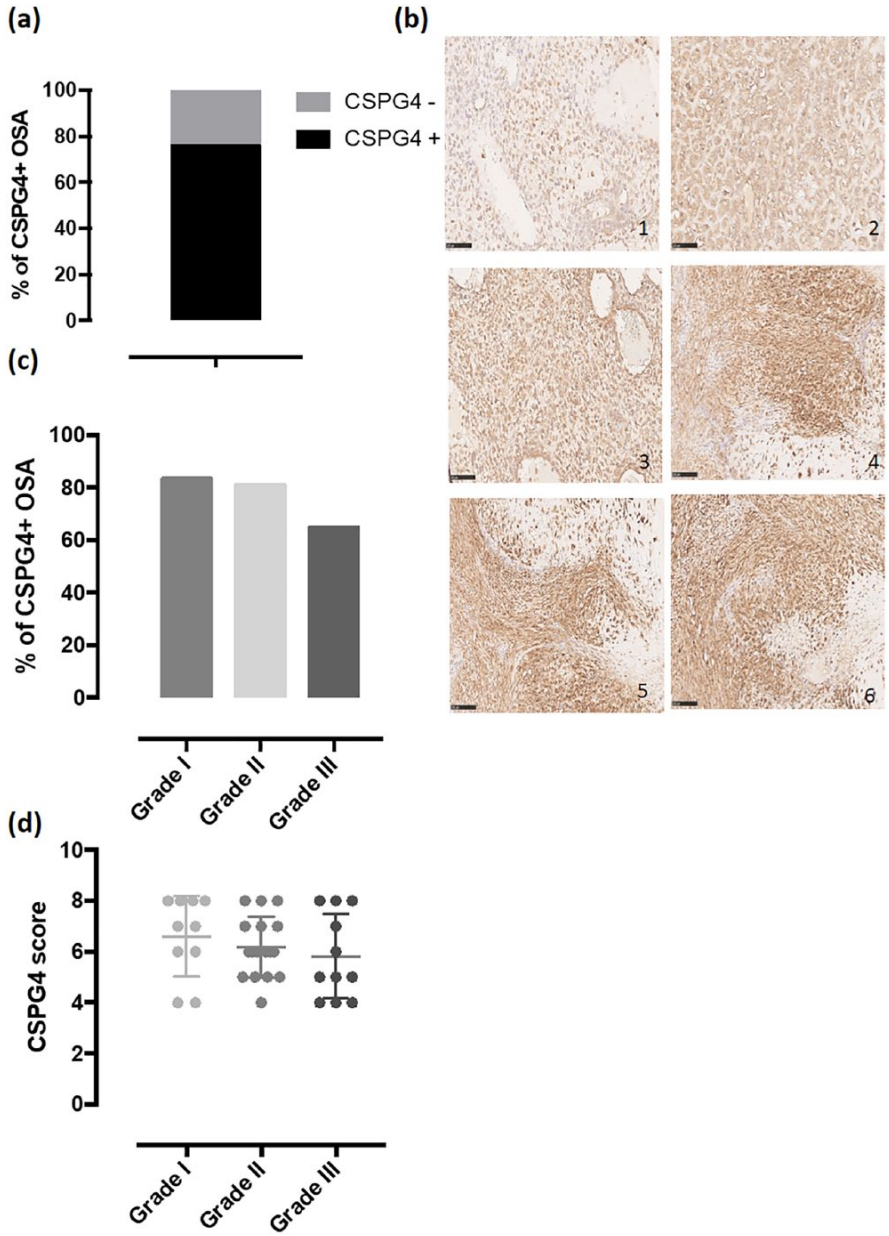

Figure 2. Expression of chondroitin sulfate proteoglycan (CSPG)4 antigen in canine osteosarcoma (OSA) samples. (a) Percentage of primary OSA that scored positive (black bar) or negative (gray bar) for CSPG4 expression. (b) Immunohistochemical (IHC) staining of canine OSA biopsies with an anti-CSPG4 antibody. Representative sections of primary OSA not expressing (1), expressing low levels (2), or expressing high levels (3–6) of the CSPG4 antigen. (c) Percentage of CSPG4-positive primary OSA considering the different histological grade. (d) CSPG4 score of expression (mean ± SD) of CSPG4-positive primary OSA considering the different histological grade.

cytoplasm of neoplastic cells, while no staining was detected in the surrounding healthy tissue (Figure 2(b)). The score of CSPG4 expression was different among positive samples and representative IHC examples are shown in Figure 2(b). Considering the histological classification of OSA biopsies at diagnosis, CSPG4 expression was observed in 10 out of 12 (83%) canine patients of Grade I, 17 out of 21 (81%) of Grade II, and 11 out of 17 (65%) of Grade III (Figure 2(c)). CSPG4 expression score showed to be independent from the tumor grade (Figure 2(d)). Overall, these results labeled the CSPG4 as a tumor-associated antigen overexpressed in canine OSA.

CSPG4 overexpression in canine OSA is related to poor survival

For each of the 50 canine patients included in the study, in addition to the OSA biopsy, we collected clinical data at the diagnosis including history, a physical exam, complete blood count, serum biochemical profile, urinalysis, abdominal ultrasound, and clinical follow up. Limb and chest radiographic (or CT) evaluation was performed to examine the features and the extent of the tumor and to exclude lung metastasis at diagnosis. All dogs included in this study were surgically treated (amputation or limb sparing) before receiving adjuvant chemotherapy using doxorubicin (30 mg/m^2, 4–5 administrations, 21 days apart) or cisplatin (70 mg/m^2, 4–5 administrations, 21 days apart) as single agents or in combination (4 cycles, 21 days apart, each cycle consisting of cisplatin 50 mg/m^2 at day 1 and doxorubicin 15 mg/m^2 at day 2).

Canine patients were clinically and radiographically examined every 3 months during the first year after the conclusion of chemotherapy and then every 6 months. OS was considered as the days between the surgery and death, while the disease-free interval (DFI) was considered as the number of days between surgery and tumor recurrence and/or evidence of metastatic disease. Considering a CSPG4 score $\geqslant$4 as threshold, canine patients affected by CSPG4-positive OSA displayed a significantly shorter survival as compared with CSPG4-negative OSA (Figure 3(a)), with a median survival time (MST) of 271 and 440 days, respectively (Table 1). As far as the DFI is concerned, a trend is evident (Figure 3(b)), with a median DFI of 237 days for canine patients bearing a CSPG4-positive OSA and 339 days for dogs affected by a CSPG4-negative OSA (Table 1).

Overall, these results suggest the potential clinical relevance of CSPG4 for canine OSA progression, highlighting the importance of its targeting for comparative oncology studies for OSA treatment.

CSPG4 immune-targeting significantly inhibits CSPG4-dependent human and canine OSA cells proliferation

As CSPG4 facilitates constitutive activation of signaling pathways, which promote cell proliferation,[24,39] we sought to *in vitro* evaluate the impact of CSPG4 immune-targeting on the growth of OSA cells. First, we evaluated CSPG4 expression in human (MG-63, U2OS, and SAOS-2) and canine (Penny) OSA cell lines. Flow cytometry analyses were performed using a pool of specific anti-CSPG4 mAbs (225.28, TP32, TP49, and VF20-VT87.41). Interestingly, cytofluorimetric results demonstrated that all the tested cell lines expressed high levels of CSPG4 (Supplemental Figure S1(a)) and this was confirmed also by Western blot analysis (Supplemental Figure S1(b)). For the following experiments, we selected the human MG-63 and the canine Penny cell lines. To understand the role of CSPG4 in OSA growth, MG-63 and Penny cells were incubated with control isotypes or anti-CSPG4 mAbs (225.28, TP32, TP49, and VF20-VT87.41), used as a mixed pool or as single agents. The proliferation of both OSA cell lines was significantly inhibited, as compared with control, by anti-CSPG4 mAbs used as a pool (Figure 4(a) and (b)) or as single agents (Supplemental Figure S2(a) and (b)). For this reason, in the following studies the mixed pool of the four anti-CSPG4 mAbs was used. As doxorubicin is a chemotherapeutic drug commonly used for the treatment of both human and canine OSA, we evaluated whether the CSPG4 targeting through mAbs could enhance its antiproliferative effect. At the lowest selected dose of doxorubicin (100 nM), MG-63 cells resulted resistant to the chemotherapeutic agent (Figure 4(a)), therefore the reduction in cell proliferation obtained by the combination of doxorubicin and anti-CSPG4 mAbs was primarily due to CSPG4 immune-targeting alone. As expected, when resistance is overcome using a higher dose of doxorubicin (10 μM), the MG-63 cell proliferation is significantly inhibited (Figure 4(a)). In this case, the combination with anti-CSPG4 mAbs is more effective in inhibiting human OSA cell proliferation compared with both the highest dose of doxorubicin and mAbs alone (Figure 4(a)). Regarding the canine OSA cells, as shown in Figure 4(b), the addition of doxorubicin

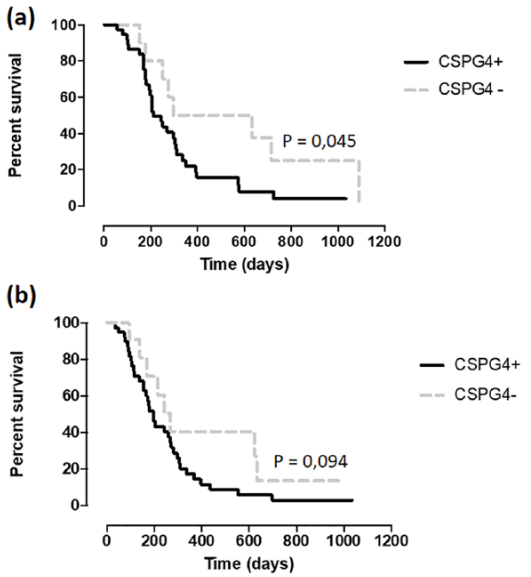

Figure 3. Kaplan–Meier curve comparing median survival times (MSTs) and disease-free intervals (DFIs) in different groups. MST (a) and DFI (b) (in days) in chondroitin sulfate proteoglycan (CSPG)4-positive (black line) and CSPG4-negative (gray dotted line) osteosarcoma (OSA) canine patients (log-rank test *$p = 0.045$, $p = 0.094$; Hazard Ratio (Mantel–Haenszel) = 2.021; 95% confidence intervals of ratio = 1013–4034).

Table 1. Median survival times (MSTs) and disease-free intervals (DFIs) for osteosarcoma canine patients enrolled in the study.

Group	MST (days)	DFI (days)
Overall population ($n = 50$)	312 (241–382)[a]	261 (199–324)[a]
CSPG4 + ($n = 38$)	271 (207–336)	237 (174–300)
CSPG4 - ($n = 12$)	440 (221–659)	339 (158–520)

[a](LCL95% – UCL95%), lower – upper control limits.
CPSG4, chondroitin sulfate proteoglycan 4.

at the lowest selected dose (100 nM) resulted *per se* effective in significantly inhibiting tumor cell proliferation, however a significantly higher inhibition was observed when the combination of mAbs and doxorubicin was applied, being superior to both single agents alone. As we have previously demonstrated the clinical effectiveness of *in vivo* CSPG4 immune-targeting by means of DNA vaccination in dogs affected by MM,[25,26] to evaluate the potential of this strategy for the treatment of OSA, we incubated human and canine OSA cells with sera collected from canine MM patients enrolled in our previous veterinary trial. The sera were collected at the moment of the surgical removal of the primary tumor (pre-Vax) or after the vaccination (post-Vax) with a plasmid coding for the Hu-CSPG4, that induces the production of anti-CSPG4 antibody in the vaccinated dogs, against both the human and the canine CSPG4 protein.[25] Interestingly, post-Vax sera were able to inhibit the proliferation of both human and canine OSA cells as compared with pre-Vax sera (Figure 4(c) and (d)). Together, these results suggest that CSPG4 may have a key role in both human and canine OSA cell proliferation, which could be impaired by its immune-targeting. Of note, our data propose

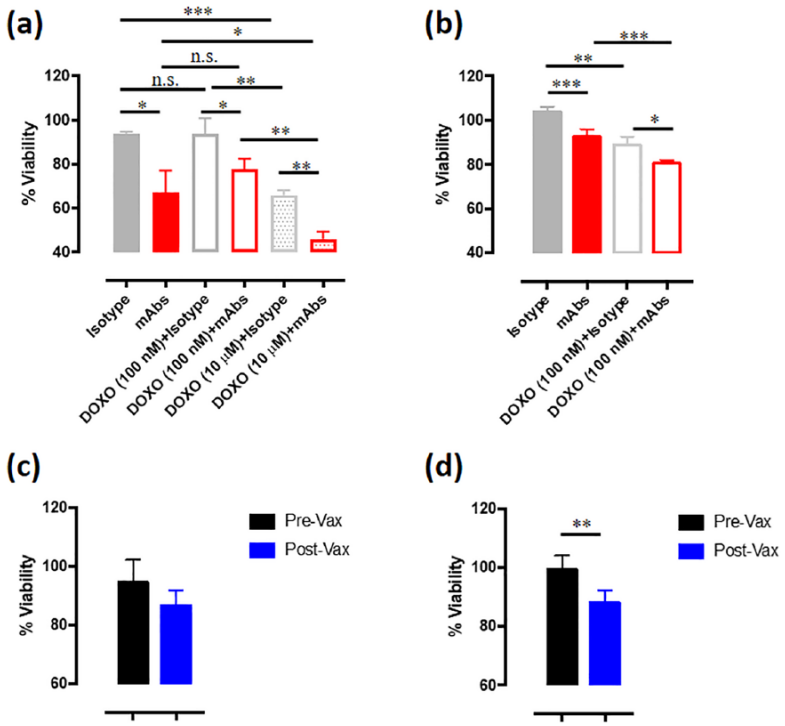

Figure 4. Effects of chondroitin sulfate proteoglycan (CSPG)4 immune-targeting on osteosarcoma (OSA) cell proliferation. Cell proliferation was assessed by using the MTT assay and the results are expressed as the percentage (mean value ± SD) of cell viability in each condition respect to cells grown in the medium alone, considered as 100%. (a) Proliferation of human CSPG4-positive MG-63 cells incubated with medium alone, control isotypes (25 μg/ml final concentration), anti-CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41 mixed to a final concentration of 25 μg/ml), alone or in combination with 100 nM or 10 μM doxorubicin (DOXO), for 48 h. (b) Proliferation of canine CSPG4-positive Penny cells incubated with medium alone, control isotypes (25 μg/ml final concentration), anti-CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41 mixed to a final concentration of 25 μg/ml), alone or in combination with 100 nM doxorubicin (DOXO), for 48 h. (c,d) Proliferation of CSPG4-positive human (c) and canine (d) OSA cells incubated with medium alone, pre-Vax sera (black bars) or post-Vax sera (blue bars) from five canine malignant melanoma (MM) patients after the fourth cycle of vaccination with the Hu-CSPG4 DNA vaccine. Student's *t* test: *$p < 0.03$; **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

the potential impact of adjuvant anti-CSPG4 DNA vaccination, alone or in combination with doxorubicin (Supplemental Figure S3) for the treatment of OSA patients, as we previously demonstrated for another aggressive and CSPG4-positive disease, MM.[25,26]

CSPG4 immune-targeting significantly inhibits CSPG4-positive human and canine osteospheres.

OSA is among the several cancer types in which a relevant role of CSC in tumor initiation and progression is evident. Indeed, CSC are thought to be the main drivers of OSA-related death, being responsible for tumor chemoresistance, finally resulting in recurrence and metastasis.[40] Starting from this assumption, we generated MG-63 (Supplemental Figure S4(a)) and Penny (Supplemental Figure S4(b)) derived osteospheres, following the protocol described by Conti and coworkers.[33] Interestingly human (Supplemental Figure S4(c)) and canine (Supplemental Figure S4(d)) osteosphere-derived cells expressed high levels of CSPG4, making it an even more interesting antigen for the immune-targeting of both differentiated cancer cells and

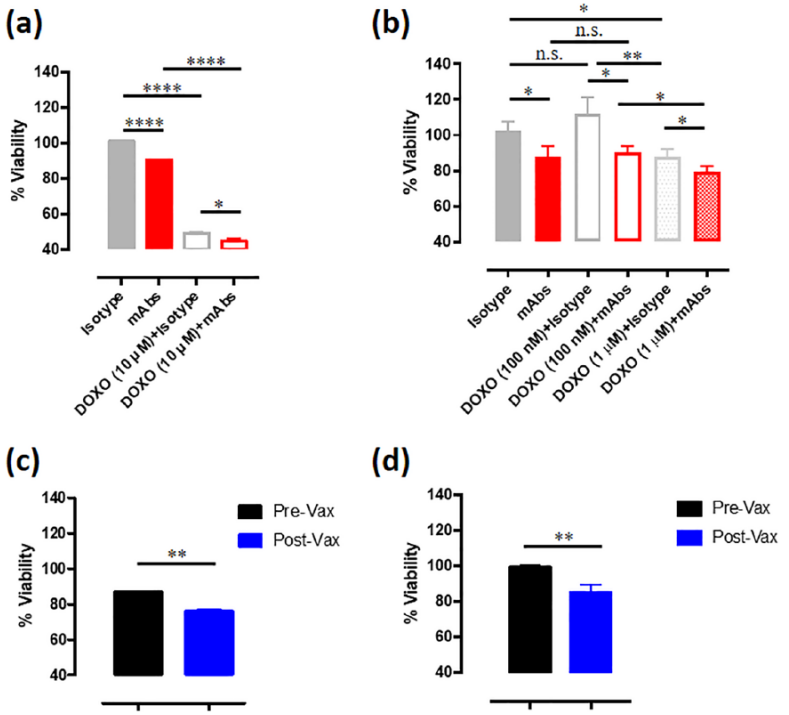

Figure 5. Effects of chondroitin sulfate proteoglycan (CSPG)4 immune-targeting on cancer stem cell (CSC)-enriched osteospheres. Osteospheres viability was assessed by using the MTT assay and the results are expressed as the percentage (mean value ± SD) of cell viability in each condition respect to cells grown in the medium alone, considered 100%. (a) Viability of human MG-63-derived osteospheres incubated with medium alone, control isotypes (25 μg/ml final concentration), or anti-CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41 mixed to a final concentration of 25 μg/ml), alone or in combination with 10 μM doxorubicin (DOXO), for 48 h. (b) Viability of canine Penny-derived osteospheres incubated with medium alone, control isotypes (25 μg/ml final concentration) or anti-CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41 mixed to a final concentration of 25 μg/ml), alone or in combination with 100 nM or 1 μM doxorubicin (DOXO), for 48 h. (c,d) Viability of human MG-63 (c) and canine Penny-derived (d) osteospheres incubated with medium alone, pre-Vax sera (black bar) or post-Vax sera (blue bar) from five canine malignant melanoma (MM) patients after the fourth cycle of vaccination with the Human CSPG4 DNA vaccine. Student's *t* test: *$p < 0.05$; **$p < 0.050$; ****$p < 0.0001$.

CSC in OSA. Of note, both human (Figure 5(a)) and canine (Figure 5(b)) osteospheres incubated with anti-CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41) displayed reduced spheroid viability as compared with control, with each mAb clone showing a similar effect (Supplemental Figure S2(c) and (d)). In addition, we measured the *in vitro* growth of osteospheres when incubated with doxorubicin alone or in combination with anti-CSPG4 mAbs. For the human MG-63-derived osteospheres we selected the 10 μM doxorubicin dose, since the lower dose was not even effective on epithelial cells (Figure 4(a)). CSPG4 targeting with mAbs pool significantly increased the inhibitory effect of doxorubicin on osteospheres viability (Figure 5(a)). Regarding the canine Penny-derived osteospheres, the 100 nM doxorubicin dose, successfully used for epithelial canine Penny cell proliferation studies (Figure 4(b)) was not effective alone against osteospheres (Figure 5(b)), confirming previous findings that CSC avail of increased chemoresistance ability.[41] Using a higher doxorubicin concentration (1 μM) on canine Penny-derived osteospheres, we observed that doxorubicin alone in this case significantly inhibits the sphere viability (Figure 5(b)), however, the combinatorial approach of doxorubicin

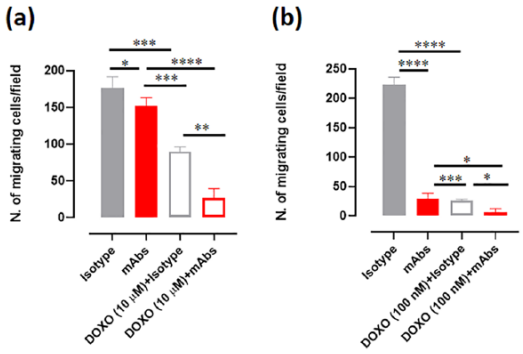

Figure 6. Effects of chondroitin sulfate proteoglycan (CSPG)4 immune-targeting on osteosarcoma (OSA) cell migration. OSA cell migratory ability was assessed by using the Transwell migration assay. Human MG-63 (a) and canine Penny (b) cells were placed in the upper chamber and incubated for 48 h with medium alone, control isotypes (100 μg/ml final concentration) or CSPG4 mAbs pool (225.28, TP32, TP49, and VF20-VT87.41 mixed to a final concentration of 100 μg/ml), alone or in combination with 10 μM or 100 nM doxorubicin (DOXO). Cells migrated to the lower surface of the membrane were stained with crystal violet for microscopical observation. The mean ± SD of the number of migrated cells counted in four different fields were reported in the graph. Student's *t* test: *$p < 0.05$; **$p < 0.001$; ***$p < 0.0004$; ****$p < 0.0001$.

plus anti-CSPG4 mAbs further improved the effect of the single treatments alone (Figure 5(b)). Interestingly, post-Vax sera derived from canine MM patients vaccinated with the Hu-CSPG4 plasmid [25] resulted effective in inhibiting human and canine osteosphere viability, as compared with pre-Vax sera (Figure 5(c) and (d)). Overall, these findings suggest that CSPG4 immune-targeting with a mix of anti-CSPG4 mAbs, and most interestingly, with anti-CSPG4 DNA vaccination, could also have an impact on the CSC compartment, which is considered endowed of a high metastatic potential.

CSPG4 immune-targeting significantly inhibits CSPG4-dependent human and canine OSA cells migration.

Migration is a critical property of cancer cells, which could determine their metastatic behavior. As metastasis is one of the major challenges in OSA treatment, we evaluated the potential of CSPG4 immune-targeting in counteracting the migratory ability of human and canine OSA cells. Interestingly, when cells were pre-incubated with anti-CSPG4 mAbs pool (225.28, TP32, TP49, VF20-VT87.41), a significantly reduced migratory potential, as compared with control, was evident for both human MG-63 (Figure 6(a)) and canine Penny (Figure 6(b)) OSA cells. Moreover, cell motility was also affected by doxorubicin; indeed, a significant migratory reduction was observed when MG-63 (Figure 6(a)) and Penny (Figure 6(b)) cells were treated with 10 μM and 100 nM doxorubicin, respectively. However, the combinatorial approach using doxorubicin plus anti-CSPG4 mAbs pool further improved the effect of the single treatments alone in both human MG-63 (Figure 6(a)) and canine Penny (Figure 6(b)) cells, making the possibility of chemotherapy and adjuvant CSPG4 immune-targeting an even more appealing strategy to fight against the metastatic disease.

Discussion

CSPG4 is a transmembrane protein involved in several protumorigenic signaling pathways. It is expressed in a wide range of highly aggressive tumors, including MM, triple-negative breast carcinomas, leukemia, gliomas, in which it is associated with those hallmarks linked to tumorigenesis including proliferation, invasion, and metastasization.[24,39] The overexpression of CSPG4 on CSC in different tumor histotypes, could suggest its potential implication also in providing a survival advantage to this subpopulation, considered responsible for recurrences and metastasis. Therefore, for all these properties, the CSPG4 is considered an ideal and safe oncoantigen[14] for anticancer targeted therapies, being

barely expressed on normal healthy tissues.[15–23] Indeed, by means of an IHC analysis of a FDA Standard Frozen Tissue Array, including 30 different organs, Rivera *et al*. demonstrated that no CSPG4 expression was found in healthy tissues.[22] In general, a limited CSPG4 expression is associated with stem cells and adult progenitor cells, which however have been suggested to lose its expression during terminal differentiation.[19] Moreover, a heterogeneous CSPG4 expression has been detected on activated pericytes but interestingly, only poorly stabilized vascular structures contain CSPG4 expressing pericytes, whereas CSPG4 is downregulated in pericytes associated with quiescent vessels, and absent or not detectable in pericytes of stable vessels in the adult healthy tissues.[42] On the basis of these considerations, several immunotherapeutic approaches against CSPG4 for the treatment of melanoma and other CSPG4-expressing tumor histotypes have been tested both in preclinical and clinical settings.[24] CSPG4-specific chimeric antigen receptors (CAR) T cells,[27] as well as sophisticated mAb-based approaches have been generated,[27,43] demonstrating the antitumor potential of CSPG4 immune-targeting. An alternative approach is active immunization, which has demonstrated to bring about effective and long-lasting antitumor responses, without the risk of resistance development. In this direction, some evidence of the effectiveness of active immunization against CSPG4 in melanoma patients was found through vaccination with the anti-idiotypic antibody MK2-23, which bears the internal image of the mAb 763.74 against a defined CSPG4 epitope. Interestingly, the induction of CSPG4-specific antibodies in immunized patients was associated with significantly longer survival and metastasis regression.[44,45] However, this approach never ended up in clinics, owing to both the difficulties in standardization of MK2-23 and to side effects associated with Bacille Calmette–Guerin administration, the adjuvant required to break immune tolerance and to induce an efficient immune response.[24,46] However, these encouraging data provided a strong rationale for the development of new strategies of active immunization against CSPG4.

Recently, DNA-based vaccines have raised interest as a concrete and viable anticancer strategy.[47] In this direction, we have recently focused our attention on the antitumor potential of *in vivo* electroporation of a DNA vaccine (electrovaccination) coding for the Hu-CSPG4 protein. To confer a high translational power to our study, we tested the safety, immunogenicity, and clinical efficacy of the vaccine in prospectively enrolled client-owned dogs with *en bloc* surgically resected stage II and III CSPG4-positive spontaneous oral MM.[25,26] The results obtained in our studies demonstrated the ability of the xenogeneic DNA electrovaccination against CSPG4 to break the immune tolerance in dogs and to induce a specific humoral response which relates favorably with a significant prolongation of disease-free and overall survival time in vaccinated dogs with surgically resected MM as compared with controls treated with surgery alone.[25,26] These results lay the foundation for the evaluation of this immunization strategy for the treatment of other CSPG4-expressing tumors.

To date, OSA still represents a critical challenge in the oncology field, because conventional therapies have demonstrated partial effectiveness only in patients affected by localized tumor, while failing in the treatment of advanced patients. Several strategies have been evaluated to improve the survival of OSA patients without encouraging results. Some clinical trials involving tyrosine kinase targeted therapies or checkpoint inhibitors[48] have been assessed, however, considerable improvements in patients' outcome have not been realized at all.[41] Therefore, it is clearly evident the urgent need for novel and effective therapies. In this panorama, the identification of CSPG4 as a potential OSA-associated target could offer new possibilities for the treatment of this disease.

For this purpose, in the present study, we first focused our attention on the evaluation of CSPG4 expression in human OSA. We have analyzed mRNA levels for CSPG4 in previously published[1,37] genome-wide expression data of osteoblasts, MSCs, and 84 high-grade OSA pretreatment biopsies. We detected the overexpression of CSPG4 mRNA in human high-grade OSA biopsies, as compared with the hypothesized OSA progenitors, and validated the CSPG4 protein overexpression in human OSA cell lines. We availed of the collected information regarding the clinical evolution of the disease of the 84 high-grade OSA patients included in the dataset.[37] All patients underwent neoadjuvant chemotherapy, and for all patients, the Huvos necrosis grading system was applied for the assessment of chemotherapy efficacy. Unfortunately, patients with a poor histologic response to neoadjuvant chemotherapy (mostly Huvos Grade I–II) showed no

benefit following distinct postoperative therapies.[49] Interestingly, CSPG4 mRNA was found overexpressed independently from the Huvos Grade and a higher degree of expression resulted in lower grades. We further evaluated whether the CSPG4 expression is related to OSA patients' prognosis. Actually, OSA patients show a shorter overall survival and metastasis-free survival probability when CSPG4 is overexpressed. Therefore, taken as a whole, these results suggest the strong potentiality of adjuvant CSPG4 targeting for both good and poor responders to chemotherapy.

In addition, because of the age of its onset, OSA is socially important and limits the possibility of testing new therapies; the identification of reliable models of human OSA is therefore a critical point. For this reason, we decided to evaluate the feasibility of using spontaneous canine OSA as a preclinical model to test anti-CSPG4 immunotherapies. Indeed, canine patients spontaneously develop tumors as humans do, in a context of an intact immune system, with strong anatomical and physiological similarities with the human counterpart. This is true also for OSA. Moreover, as in humans, treatments for canine OSA includes mainly surgery and chemotherapy, which however are often disappointing with only 20% of canine OSA patients treated using the current standard of care still alive at 1 year and lung metastasis being the most relevant cause of death.[50–52] A tangible example of the importance of comparative oncology in OSA came from the early 1990s, when limb-sparing methods pioneered in canine patients with OSA[53] have become standard of care for human patients, which today clearly benefit from advances made in both surgical treatment and in the provision of supportive care.

With this in mind, we have demonstrated that CSPG4 expression is detectable, with variable expression levels, in a high percentage of canine OSA biopsies by means of IHC. In addition, also canine OSA cell line showed to highly express CSPG4, representing an interesting tool to be exploited for *in vitro* studies. As for humans, the Kaplan–Meyer curves suggest that CSPG4 overexpression is also related to a poor prognosis in canine patients. Thus, with this study we highlighted the potential clinical relevance of evaluating anti-CSPG4 strategies in canine OSA patients, with a high translational value. For this reason, we examined the potentiality of CSPG4 immune-targeting against both human and canine OSA cell lines *in vitro*, in order to consider anti-CSPG4 immunotherapy as a potential new weapon against OSA.

CSPG4 is implicated in several cellular processes,[24,39] therefore its targeting could impair simultaneously different steps in the tumorigenic process. First, our results suggest that CSPG4 is involved in OSA cell proliferation. Indeed, we showed that four anti-CSPG4 selected mAbs (225.28, TP32, TP49, and VF20-VT87.41) are able to significantly impair the proliferation of both human and canine OSA tumor cells. This inhibition is evident when mAbs are used in a mixed pool or when used as single agents, suggesting that the engagement of different antigen epitopes by each clone does not seem to have different effects on cancer cell survival. The effect of CSPG4 immune-targeting *in vitro* is evident although modest. This can be attributed to several reasons: both OSA cell lines are not 100% positive for CSPG4 expression, therefore there will be a CSPG4-negative population able to escape to mAbs treatment; the selected dose of mAbs used is low, so better effects could be achieved increasing the dose. However, an interesting finding is the ability of mAbs treatment to significantly sensitize OSA cells to doxorubicin. Actually, the combination of doxorubicin, one of the most common chemotherapeutic agent used in both human and veterinary setting,[41] with anti-CSPG4 mAbs enhanced the inhibition of cancer cells' growth. Of note, on one side CSPG4 can regulate the AKT–pAKT pathway considered responsible for chemoresistance, on the other side we have previously demonstrated that anti-CSPG4 mAbs can induce a downregulation of the CSPG4 receptor when incubated with CSPG4-positive melanoma cells,[26] and this could consequently impair the downstream signaling, leading to the reduction of the AKT-pAKT axis (unpublished data and Rolih *et al.*[24]). Therefore, the association of chemotherapy and anti-CSPG4 immune-targeting open up the possibility of increasing the antitumor effect of single agents alone, combining standard of care with novel strategies.

Moreover, the intrinsic OSA chemoresistance may be the result also of a privileged survival of a population of tumor cells, that is, CSC to which are associated tumor recurrence and metastasis development following chemotherapy. For this reason, in this study we evaluated the ability of mAbs alone or in combination with doxorubicin to impair not only epithelial cancer cells but also osteospheres enriched in CSC. First, we demonstrated that

subsequent passage of CSC-enriched osteospheres retain the CSPG4 overexpression, making it an interesting antigen to target CSC too. Then, we showed the ability of mAbs, alone or in combination with doxorubicin, to inhibit osteospheres viability. Also in this case, the selected anti-CSPG4 mAbs (225.28, TP32, TP49, VF20-VT87.41) showed a similar effect when used together in a pool or when tested individually. These results propose the potentiality of CSPG4 immune-targeting for the elimination of CSC and the prevention of recurrences and metastasis in OSA. Moreover, our data suggest a potential involvement of CSPG4 in OSA cell migration, because we demonstrated the significant impact of CSPG4 immune-targeting against the migratory ability of both human and canine OSA cell lines, highlighting the pleiotropic effects of anti CSPG4 mAbs. In addition, mAbs treatment significantly increases the antimigratory effect of doxorubicin, as demonstrated by Transwell assays, supporting the relevant clinical consequence of combinatorial anti-CSPG4 immune-targeting and chemotherapy to fight against OSA metastasis. Overall, these results provide an additional step forward in the understanding the impact of CSPG4 in its whole for OSA progression.

Finally, to consider anti-CSPG4 DNA electrovaccination as a new therapy for the adjuvant treatment of OSA, on the basis of our previous positive results obtained for MM, we used sera derived from MM canine patients adjuvantly treated with Hu-CSPG4 DNA plasmid to evaluate the ability of vaccine induced antibodies to inhibit the proliferation of OSA cells and osteospheres *in vitro*. Interestingly post-vaccination sera were effective in inhibiting cell growth and sphere viability alone or in combination with doxorubicin. These results suggest the potential efficacy of our DNA vaccination strategy also for the treatment of canine OSA patients *in vivo*, with a strong translational value for human OSA management.

Acknowledgements
Paolo Buracco and Federica Cavallo contributed equally.

Author contributions
FC, PB, and FR contributed to the conception and design of the study. FC, FR, and LT wrote the manuscript. FR and LT performed the *in vitro* experiments. SI performed the immunohistochemical analyses. DG, FS, EM, MM, FG, and PB contributed to patient recruitment and clinical follow up. MA performed the meta-analysis and data collection from human datasets. RDM and SF provided reagents and cell lines. All authors contributed to collection, analyses and interpretation of data, reviewed, and approved the final submitted version.

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Conflict of interest statement
The authors declare that there is no conflict of interests.

Supplemental material
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Starting from these bases, we have recently explored the potential of our chimeric HuDo-CSPG4 DNA vaccine as a novel therapeutic option for the treatment of OSA in relevant comparative pre-clinical models, such as human OSA xenograft mouse models and canine OSA patients.

To first corroborate the functional role of CSPG4 in human OSA malignant progression, we downmodulated CSPG4 in two different human OSA cell. In both cell lines, CSPG4 transient silencing resulted in decreased tumorigenic potential, suggesting the potential clinical relevance of CSPG4 targeting by means of immunotherapy for OSA treatment.

In human xenotransplant mouse models, we tested the immunogenicity and effects of HuDo-CSPG4 DNA vaccination in influencing both tumor growth and metastasis development by the adoptive transfer of HuDo-CSPG4 induced CD8+ T cells in immunodeficient mice transplanted with a human OSA cell line. We observed a significant delay of tumor growth of human $CSPG4^{+}$ OSA cells. CD8+ T cells were also able to infiltrate the tumors at different levels; nonetheless, in bigger tumors we observed the up-regulation of B7-H3 checkpoint molecule expression. This result led us speculate regarding the possible mechanism of tumor cell escape, resulting in progressive tumor growth. On the other hand, we only observed a trend in the delay of tumor growth in xenografts that were adoptively transferred with sera derived from HuDo-CSPG4 immunized mice. Nonetheless, a significant impairment in metastasis development was observed compared to controls. These results in human xenograft mouse models supported the hypothesis that the direct contribution of both cellular and antibody immune responses could hinder tumor progression.

To exploit the efficacy of our HuDo-CSPG4 DNA vaccine in an immunocompetent pre-clinical model, we enrolled client-owned dogs referred to the Veterinary Teaching Hospital of the University of Turin because affected by spontaneous CSPG4-positive appendicular OSA. Dogs were adjuvantly electrovaccinated with the HuDo-CSPG4 vaccine, after receiving the standard of care (i.e., surgery and chemotherapy). This pilot proof-of-concept study was aimed at evaluating the safety, immunogenicity, and potential therapeutic potential of the HuDo-CSPG4 vaccine in a relevant pre-clinical model of OSA.

As we previously obtained in dogs affected by OMM (399), also in this setting we observed the induction of anti-tumor immunity. Vaccine-induced antibodies were cross-reactive with the canine CSPG4 molecule and were able to exert a functional role on canine OSA cells, inhibiting tumor cell pro-tumoral functions *in vitro*. Regarding the influence of the HuDo-CSPG4 vaccination on cellular immunity, in the vaccinees we observed a decrease in immune-suppressive cells, such as MSDC and Treg, and an increase in CSPG4-specific cytotoxic CD8+ T cells. Together these results highlighted the impact of anti-CSPG4 vaccination in hampering the immune suppressive

mechanisms that persist in canine patients with minimal residual disease, together with the induction of CSPG4-specific cellular immunity. We observed a clinical benefit for canine OSA patients immunized with the HuDo-CSPG4 vaccine, in terms of increased OS and better DFI as compared to dogs treated with conventional treatments alone.

Finally, we performed a human surrogate *in vitro* cytotoxicity assay, using healthy donor-derived dendritic cells transfected with CSPG4 plasmids to stimulate autologous lymphocytes. HuDo-CSPG4 induced a cytotoxic response even in this setting.

These results therefore underline the fundamental role that CSPG4 likely fulfills in OSA biology, and that its immune-targeting through the chimeric HuDo-CSPG4 vaccine is safe and immunogenic, holding the potential to treat both human and canine OSA patients.

Paper III

A chimeric human/dog-DNA vaccine against CSPG4 induces immunity with therapeutic potential in comparative pre-clinical models of osteosarcoma

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Running title. Comparative study of a CSPG4 DNA vaccine against osteosarcoma

Keywords. Osteosarcoma, CSPG4, DNA Vaccination, Comparative Oncology, Metastasis

Abstract

Purpose: The high mortality rate of osteosarcoma (OSA) patients highlights the urgent need for alternative therapies. Having previously shown the over-expression of the chondroitin sulfate proteoglycan (CSPG)4 in OSA, we herein evaluated the potential of a chimeric human/dog (HuDo)-CSPG4 DNA vaccine in comparative models to propose this strategy as a potential novel treatment for human patients.

Experimental design: CSPG4 was first downmodulated in human OSA cells to study the functional consequences. The safety and immunogenicity of HuDo-CSPG4 vaccination were then investigated in human xenograft mouse models and canine OSA patients. Twenty-five OSA-bearing dogs received conventional treatments and of these, 12 were immunized with HuDo-CSPG4. Finally, human surrogate *in vitro* cytotoxic assays were performed using healthy donor-derived dendritic cells transfected with CSPG4 plasmids to stimulate autologous lymphocytes.

Results: CSPG4 down-regulation impaired the proliferation, migration and osteospheres formation of human OSA cells. The adoptive transfer of HuDo-CSPG4 vaccine-induced $CD8^+$ T cells and sera

in immunodeficient human OSA-bearing mice delayed tumor growth and metastasis development. The induction of anti-CSPG4 humoral and cellular responses and a prolongation in survival were observed in OSA-affected dogs vaccinated with HuDo-CSPG4. HuDo-CSPG4 also induced a cytotoxic response in a human surrogate setting.

Conclusions: CSPG4 likely has a functional role in OSA biology and its targeting through the chimeric HuDo-CSPG4 vaccine resulted safe and immunogenic, holding the potential to treat human and canine OSA in pre-clinical models. Considering the high predictive value of spontaneous OSA in dogs, these results lay the foundation for the translation of this approach to humans.

Translational relevance. The management of pediatric osteosarcoma (OSA) has not particularly improved in the recent decades. The young age of patients, together with the rarity and aggressiveness of the disease, limits opportunities for the robust testing of novel therapies. There is, consequently, a clear need for valuable preclinical systems. In this work, human preclinical models and canine patients affected by spontaneous OSA have been exploited to demonstrate that anti-CSPG4 DNA vaccination is safe and that it induces specific immunity that can counteract tumor progression. This study has made use of $CSPG4^+$ OSA-bearing dogs as avatars of the corresponding human tumor, as the two display significant biological and clinical similarities. The promising results observed in this setting, using a chimeric human/dog vaccine, could pave the way for a possible evaluation of this treatment in human clinical trials.

Introduction

Osteosarcoma (OSA) is a rare pediatric cancer of mesenchymal origin that accounts for 56% of bone tumors in the childhood population (1). In the case of localized disease, the surgical excision of the primary tumor and the adoption of multi-agent neoadjuvant/adjuvant chemotherapy is curative in up to 70% of cases (2). However, OSA has a high tendency to recur and metastasize, mainly to the lungs, despite these treatments (2). Patients that bear undetectable micrometastases at diagnosis or develop metastatic lesions after standard treatment are generally characterized by chemotherapy resistance and treatment failure. Advanced disease is usually incurable in these cases, and the only therapeutic option that remains is palliation, with 5-year survival rates dropping to 20-30% (3,4); https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4833631/pdf/nihms759784.pdf).

Immune checkpoint inhibitors (ICIs) have revolutionized the management of cancer patients affected by highly immunogenic solid tumors. Unfortunately, OSA is a cold tumor for which ICIs have limited clinical activity (4,5). Other therapeutic options must therefore urgently be evaluated, even with a view to applications in multimodal approaches.

Both the rarity of the disease and the young age of patients limit opportunities to effectively test new therapeutic approaches, and comparative oncology has recently gained significant prominence in the OSA panorama for this reason (6,7). In dogs, OSA represent 85-90% of all primary malignant bone tumors and, interestingly, has an incidence that is 27-times higher than in humans (8,9). Human and canine OSA display common molecular alterations and signaling pathway dysregulations, resulting in similar, aggressive clinical behavior with superimposable responses to the same conventional therapies (7,10). It can therefore be stated that canine OSA is now established as a good and reliable model for testing innovative therapeutic approaches.

Of the tumor-associated antigens (TAA) that are shared by human and canine OSA, we herein focus on the chondroitin sulfate proteoglycan (CSPG)4, a cell surface proteoglycan with a demonstrated oncogenic role in several tumor histotypes (11,12) and that has also recently been proposed as a clinically relevant target in OSA (13,14). Indeed, CSPG4 is highly expressed in OSA cell lines and in their derived osteospheres, which are enriched in cancer stem cells (CSC), while it is not expressed in healthy osteoblasts or in other normal tissues (14,15). Moreover, CSPG4 overexpression has been found to be related to worse patient prognosis in both human and canine OSA patients (14). These features and its cell-surface localization potentially make CSPG4 an ideal target for anti-cancer immunotherapy, as $CSPG4^{+}$ cancer cells could be simultaneously targeted by both CSPG4-specific humoral and cellular immunity (16,17).

Herein, we report safety and immunogenicity data for the evaluation of a chimeric human/dog CSPG4-targeting DNA vaccine (HuDo-CSPG4, (17) in human OSA xenograft mouse models and in a pilot veterinary trial in client-owned OSA-affected dogs. To the best of our knowledge, this is the first study that investigates *in vivo* CSPG4 targeting for the treatment of OSA. Our results confirm that CSPG4 is an attractive comparative target for the testing of immunotherapy and propose HuDo-CSPG4 vaccination as a possible novel therapeutic option for OSA treatment.

Material and Methods

Cell lines

Human OSA cell lines (U2OS and SaOS2) were obtained from the American Type Culture Collection (ATCC; HTB-96, RRID:CVCL_0042, and HTB-85, RRID:CVCL_0548, respectively) and were cultured in RPMI (Sigma-Aldrich) supplemented with 20% fetal bovine serum (FBS, Sigma Aldrich). Penny cells, derived from a biopsy of a primary grade III canine OSA tumor (14,18), were grown in ISCOVE Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 10% FBS. The canine OSA cell line D22 was obtained from ATCC (ATCC; CRL-6250, RRID:CVCL_3458) and cultured in DMEM F12 (Sigma-Aldrich) supplemented with 20% FBS. The D22 cell line overexpressing dog CSPG4 (D22 Do-CSPG4) was generated by transfecting D22 cells (ATCC; CRL-6250, RRID:CVCL_3458), which are naturally negative for CSPG4 expression, with the canine CSPG4-coding plasmid (obtained from GenScript) using Lipofectamine 2000 (Thermo-Fisher Scientific), according to the manufacturer's instructions. Stable transfected clones were maintained in DMEM F12 supplemented with 20% FBS and 1.5 mg/ml Geneticin (G418, Gibco). CSPG4 expression was confirmed by western blot analysis and flow cytometry using an anti-CSPG4 mAb (TP-49; generated and provided by Prof. S. Ferrone, (14)). Total IgG binding was evaluated using a PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs Cat# 115-116-072, RRID:AB_2338627) secondary antibody, while samples were acquired using a BD FACSVerse (BD BioScience) and analyzed using FlowJO V.10.5.3 (RRID:SCR_008520). All cell lines were grown in medium that was supplemented with penicillin/streptomycin (Sigma-Aldrich) and maintained at 37°C and 5% carbon dioxide (CO_2) in a humidified incubator. The cell lines were regularly monitored for mycoplasma contamination using a commercially available assay kit (Mycoalert from Lonza Inc.).

siRNA transfection

Small interfering RNA (siRNA), specific for human CSPG4 (hCSPG4-siRNA), and the negative control siRNA (Scramble) were purchased from Ambion. Human OSA cells were transfected with

siRNA using Lipofectamine 2000 transfection reagent (Thermo-Fisher Scientific), in accordance with the manufacturer's instructions. Cells were harvested 24h-48h and 72h post-transfection to verify CSPG4 down-regulation by Real-Time PCR and Western Blot analyses. For functional studies, cells were detached 24h post-transfection for plating in proliferation, migration, osteosphere-formation and viability assays.

Real-Time (RT) PCR

Total RNA was isolated from human OSA cells and treated with either hCSPG4-siRNA or the Scramble negative control using the TriZol reagent (Thermo-Fisher Scientific), according to the manufacturer's instructions. Genomic DNA contamination was removed from the RNA samples using the Ambion® DNA-free kit (Thermo-Fisher Scientific). RNA concentration and quality were estimated using a NanoDROP 2000 Spectrophotometer (Thermo-Fisher Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. DNase-treated RNA (1 µg) was retrotranscribed with RETROscript reagents (Ambion), and RT-PCR was carried out using gene-specific primers (QuantiTect Primer Assay), SYBR green and a 7900HT RT-PCR system (Applied Biosystems). Applied Biosystems SDS Software Version 1.3.1 was used to analyze data. Quantitative normalization was performed on the expression of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Relative gene expression levels were calculated using the comparative ΔCt method (19).

MTT cell-proliferation assay

3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol (MTT; Merck Millipore) was used to assess the proliferation of human OSA cells following siRNA transfection. Briefly, 24h post-transfection, the cells were seeded in triplicate in 96-well plates (5 x 10^3 cells/100µl well) in serum-free medium and allowed to adhere overnight. MTT solution (5 mg/ml) was added to each well, in accordance with the manufacturer's instructions, at different time points (24h, 48h, and 72h). After 4h incubation at 37°C, 100µl of dimethyl sulfoxide (DMSO, Sigma Aldrich) were added to dissolve formazan crystals, and absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad) at a wavelength of 570 nm.

Canine OSA Penny cells (5 x 10^3 cells/well) were starved for 4h in 96-well plates. Pooled canine sera (1:50) from vaccinated dogs were then added and incubation was continued for 24, 48 and 72 h. Cell viability was then evaluated using the MTT assay, as previously described.

Migration assay

Scramble- and hCSPG4-siRNA-transfected human OSA cells (2 x 10^4 cells/100µl well) were both added to the top chamber of a Transwell insert (8 µm, Corning) in serum-free medium, while the bottom chambers were filled with complete medium. Penny cells were pre-incubated with a pool of canine sera, collected before the first immunization (Pre-Vax) and after the fourth (Post-Vax), for 1h at 37 °C before being transferred to the top chamber of a Transwell insert. Cells were cultured for 48h at 37°C in a 5% CO_2 atmosphere. The non-migrating cells in the upper chamber were removed using a cotton swab and the migrated cells on the bottom side of the insert were fixed with 2.5% Glutaraldehyde (Sigma-Aldrich) and stained with 0.2% Crystal Violet (Sigma-Aldrich). The migrated cells of four randomly selected fields per well were imaged using an Olympus BX41 microscope (Olympus Corp., Tokyo, Japan) and analyzed using Fiji (RRID:SCR_002285) and ImageJ (RRID:SCR_003070; Rasband, W.S., ImageJ, US National Institutes of Health) softwares.

Sphere-generation assay

Human osteospheres were generated according to the protocol described in (14,20). Briefly, human OSA cells, 24h post-transfection with Scramble- or hCSPG4-siRNA, were harvested and plated (6 x 10^4 cells/ml) in ultra-low-attachment 75 cm^2 flasks (Sigma Aldrich) in serum-free DMEM F12, supplemented with 0.4% bovine serum albumin (BSA), 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF) and 5 µg/ml insulin, all from Sigma-Aldrich. Non-adherent spherical-cell clusters, named osteospheres, were collected after 5 days for further analysis. Photographs of osteospheres were taken using a CCD-300-RC camera, and images were processed using Fiji Software (RRID:SCR_002285; Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD) and PowerPoint (Microsoft, Redmond, WA).

To assess osteospheres viability, MTT solution was added to each well and incubated overnight at 37°C, and formazan crystals were dissolved by adding 100µl isopropanol with HCl 0.04N to each well. Optical density was measured using an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 655 nm. The difference between the 570 nm and 630 nm readings represents the output value.

Mice and DNA immunization

Female C57BL/6 HLA-A2.1 Transgenic (Tg) mice (RRID:IMSR_JAX:003475; from The Jackson Laboratory) and Balb/c mice (RRID:IMSR_APB:4790; from Charles River Laboratory) were maintained at the Molecular Biotechnology Center, University of Turin (Turin, Italy) and treated in accordance with the University's Ethical Committee and European guidelines under Directive 2010/63. All animal studies were approved by the Italian Ministry of Health (Authorization N°

29/2021-PR). Six-weeks-old C57BL/6 HLA-A2.1 Tg and Balb/c mice were vaccinated with HuDo-CSPG4 plasmids, generated as previously described (17). Briefly, the plasmid is a pcDNA3.1 (Cat# V79020; Invitrogen) backbone and includes the cDNA sequence covering the N-terminal portion of Hu- and the C-terminal portion of Do-CSPG4 (17). Large-scale plasmid preparation was carried out using EndoFree Plasmid Giga kits (Qiagen), according to Good Laboratory Practice. Mice were anesthetized and then immunized intramuscularly with either 50 μg of the pcDNA3.1 empty vector or the HuDo-CSPG4 plasmid diluted in 20 μL of saline solution. Immediately after injection, two 25-ms transcutaneous low-voltage electric pulses (amplitude 150 V; interval 300 ms) were administered at the injection site via a multiple-needle electrode connected to a Cliniporator^{TM} (IGEA) (Riccardo *et al.*, 2020). Mice were immunized twice, at a two-week interval, and were sacrificed two weeks after the second immunization in order to collect their spleens and blood for the adoptive-transfer experiment.

Female NOD/SCID/$\gamma c^{-/-}$ (NSG; RRID:BCBC_1262) mice (Charles River Laboratory) were used for the adoptive transfer experiment. Mice were injected subcutaneously with 1×10^6 of the HLA-A2.01-positive U2OS cell line in the right flank, and tumor growth was monitored twice a week using a caliper. NSG mice were adoptively transferred, with either $CD8^+$ T cells or sera derived from previously vaccinated immunocompetent mice, when a tumor volume of 0.5 mm^3 was reached. Mice were assigned to treatment groups by simple random sampling and adoptively transferred with $CD8^+$ T cells or sera derived from previously vaccinated immunocompetent mice. Tumor volume was calculated using the following formula: $V = ½ (Length \times Width^2)$.

Adoptive transfer

$CD8^+$ T cells were isolated, under sterile conditions, from the spleens of C57BL/6 HLA-A2.1 Tg mice (RRID:IMSR_JAX:003475; from The Jackson Laboratory) that were either vaccinated with the pcDNA3.1 empty vector or HuDo-CSPG4. Individual spleens were homogenized in RPMI medium supplemented with 10% FBS. Cell suspensions were centrifuged for 10 min at 1100 rpm and room temperature (RT), and were resuspended in red blood cell lysis buffer for 10 min RT. The single-cell splenocyte suspensions were pooled and $CD8^+$ T cells were isolated using magnetic cell sorting via negative selection ($CD8a^+$ T Cell Isolation Kit, Miltenyi), according to manufacturer's instructions. Three x 10^6 $CD8^+$ T cells were adoptively transferred via tail-vein injection into OSA-bearing NSG mice.

For the adoptive transfer of sera, blood was collected, via intracardiac sampling, from Balb/c mice two weeks after the second immunization. Blood was centrifuged (3000 *g* for 10 min at 4°C) and the serum was isolated. Sera were pooled and stored at -20°C until use. At the time of adoptive-serum

transfer, the recipient OSA-bearing NSG mice received 150 µl of serum intraperitoneally once a week for 5 weeks. The mice were sacrificed at the end of the experiment, and the tumors and lungs were explanted for further analysis.

Western Blot analysis

Human and murine OSA cell lines were incubated in RIPA Lysis buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris pH 8.0, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors, all from Sigma Aldrich). All samples were placed on ice for 30 min and then centrifuged at 12,000 *g* for 5 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific). Equal amounts of protein (50 µg) were separated by electrophoresis in a 4-20% Mini-Protean TGX precast gel (Bio-Rad). Western blotting for the detection of CSPG4 (14), Stro-1 (diluted 1:250; Thermo Fisher Scientific Cat# 39-8401, RRID:AB_2533437) and CD133 (diluted 1:1000; Proteintech Cat# 18470-1-AP, RRID:AB_2172859) was performed as previously described (14). β-Actin (diluted 1:500; Santa Cruz Biotechnology Cat# sc-69879, RRID:AB_1119529) and GAPDH (diluted 1:500; Santa Cruz Biotechnology Cat# sc-32233, RRID:AB_627679) were used as the loading control. Images were acquired using a BioRad ChemiDoc imaging system.

Canine-patient enrolment, eligibility criteria and clinical procedures

According to the study protocol approved by the Italian Ministry of Health (0026167-14/10/2019-DGSAF-MDS-P), canine patients that had been diagnosed with appendicular OSA at the Veterinary Teaching Hospital, University of Turin, Grugliasco (Italy), and the Tyrus Veterinary Clinic, Terni (Italy), over the period from 2011 to 2021, were considered eligible for the study. Inclusion criteria were: (1) confirmed stage I-III, CSPG4 overexpressing appendicular OSA; (2) absence of detectable distant metastasis at presentation and restaging after standard treatments; (3) local tumor control via limb amputation; (4) completion of standard chemotherapy based on 4-6 cycles of adjuvant carboplatin i.v. administrations at 3-week intervals (300 mg/m^2); (5) absence of concurrent life-threatening diseases; and (6) written informed consent signed by the owners.

Full pre-treatment tumor staging included a thorough clinical examination, laboratory tests (complete blood count, extensive biochemical profile and urinalysis), cardiac examination, either cytology or histology after fine-needle aspiration or the incisional biopsy of the primary tumor, fine-needle aspiration of any enlarged regional lymph node, an examination for metastasis using chest radiographs (three views) and abdominal ultrasound or total body Computed Tomography (CT).

Postoperative tumor samples were immunohistochemically tested for CSPG4 expression as previously described. Only OSA with a CSPG4 expression score ≥ 3 were included (14,17).

Twenty-five client-owned dogs were enrolled in the study. The dogs were included in either Arm 1 (standard treatment only, n = 13) or Arm 2 (Standard treatment + adjuvant HuDo-CSPG4 vaccination, n = 12) according to their owners' decisions. Dogs included in Arm 2 were adjuvantly immunized with the HuDo-CSPG4 plasmid as previously described (17), starting from 2 weeks after the last chemotherapy cycle. Immunization was repeated monthly, for a minimum of 4 and a maximum of 24 cycles. Thorough clinical examinations, three-view chest radiographs and/or CT scans were performed before each vaccination, and, in addition, sera and peripheral blood mononuclear cells (PBMC) were collected, whenever possible. Blood workup and/or urinalysis was performed if clinically indicated.

Dogs included in the control group (Arm 1) were monitored via chest radiographs (and abdominal ultrasound, if clinically indicated) every 3 months for the first postoperative year and every 6 months in the second year post-surgery. Dogs were monitored once a year (three-view chest radiographs) thereafter for the rest of their lives.

The primary objectives of this study were safety and immunogenicity. The Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events V.1.1, (21) was used to classify the adverse events. The secondary objective was the clinical monitoring of disease progression in both arms, with overall survival and disease-free interval being considered.

Histological and Immunohistochemical analyses

The tumors and lungs collected from NSG mice that were challenged with U2OS cells (ATCC; HTB-96, RRID:CVCL_0042), and tissue samples from the 25 cases of spontaneous canine appendicular OSA, collected via routine care at the Veterinary Teaching Hospital and the Tyrus Veterinary Clinic, were examined in this study. Sections from mouse primary tumors and lungs were stained with hematoxylin and eosin (H&E) for histological tumor evaluation and the presence of metastatic lesions. Sections from canine primary tumors were stained with H&E for histological tumor evaluation. The histological diagnosis and grading of canine appendicular OSA were defined according to Loukopoulos and Robinson (2007). Canine tumors were histologically classified as grade I (low), II (intermediate) and III (high). Immunohistochemical (IHC) analyses were performed on collected samples, which were fixed in 4% formalin and embedded in paraffin, and then sectioned at 4 μm, as described previously (14,17). IHC staining for CSPG4 was performed on paraffin sections with a primary anti-CSPG4 antibody (MBS716314, diluted 1:40, MyBiosource), and the CSPG4 semi-quantitative scoring system was adopted as previously described (14,17). A CSPG4 total score,

ranging from 0 to 8, was assigned to each OSA sample in accordance with the value assigned to the proportion of CSPG4-positively-stained tumor cells (score from 0 to 5) and the average staining intensity of CSPG4-positive tumor cells (score from 0 to 3). IHC staining for T lymphocytes in mouse tumors was performed using a primary anti-CD3 antibody (SP7, diluted 1:500; Abcam Cat# ab16669, RRID:AB_443425).

ELISA assay

Sera from healthy dogs were obtained from the blood withdrawn for routine blood tests required for anesthesia before a spaying procedure. The sera collected from vaccinated dogs before the first (Pre-Vax) and after the fourth, fifth and sixth (Post-Vax) immunizations were used for ELISA tests, which were performed as described in (17). Briefly, thawed sera (dilution 1:100) were incubated in 96-well plates that were previously coated with different recombinant canine-CSPG4 domains (D1, D2, D3; 50 ng/well; GenScript).

In order to test the avidity of the anti-CSPG4 antibodies, a chaotropic ELISA assay was performed by adding the chaotropic Sodium Thiocyanate agent (Sigma Aldrich) to incubated sera at 5M for 15 min at RT, as previously described (17). Antibody binding was detected using horseradish peroxidase–conjugated goat anti-dog-IgG (diluted 1:10000; Abcam Cat# ab112852; RRID:AB_2927648) on an ELISA Microplate Reader at a wavelength of 470 nm.

Flow-cytometry analysis

For the flow cytometric analysis of the vaccine-induced antibodies, human U2OS (ATCC; HTB-96, RRID:CVCL_0042) and Penny cells were incubated with sera (1:40 dilution), collected from immunized dogs, for 30 min at 4°C in PBS (Sigma-Aldrich). Total IgG binding was evaluated as previously described (17). Samples were acquired using a BD FACSVerse (BD BioScience) and analyzed using FlowJO V.10.5.3 (RRID:SCR_008520).

The PBMC that were separated from vaccinated dogs before the first (Pre-Vax) and after the fourth (Post-Vax) immunization were used for cytofluorimetric analyses, according to (17). For Treg cell detection, thawed PBMC were incubated with dog IgG (Thermo-Fisher Scientific) to block the Fc receptor, and were then stained with LIVE/DEAD Fixable Red Dye (Invitrogen) for 30 min at 4°C. After washing, cells were stained with the following mAbs: rat anti-canine CD4-RPE-cy7 (YKIX302.9, MCA1038GA, AbD Serotec) and mouse anti-dog CD25-FITC (P4A10, 11-0250-042, Thermo-Fisher Scientific). Cells were then fixed/permeabilized and stained with anti-mouse FoxP3-APC (FJK-16s, 14-5773-82, eBiosciences). Samples were acquired using a BD FACSVerse (BD BioScience) and analyzed with FlowJO V.10.5.3 (RRID:SCR_008520).

Human specimens and lymphocyte activation

Human peripheral blood leukocytes (PBL) were isolated via Ficoll-Hypaque (Lonza) gradient centrifugation from the heparinized venous blood of healthy subjects (n = 8) that was provided by the local Blood Bank (Turin, Italy). To determine human leukocyte antigen (HLA)-A2 positivity, the PBL were incubated with anti-HLA-A2-PE mAb (clone BB7.2, BD Pharmingen), and expression was evaluated by flow cytometry. Four healthy donors were found to be HLA-A2 positive and were used for further assays.

The generation of monocyte-derived dendritic cells (DC) was conducted as previously described (22). Briefly, monocytes were isolated from PBL using CD14 MicroBeads (Milteny) and were subsequently cultured with IL-4 and GM-CSF, both from PeproTech, to generate immature DC. TNF-α (50 ng/mL) and IL-1ß (50 ng/mL), both from PeproTech, were added for the final 24 h to induce DC maturation. CD14-depleted PBL were stored in liquid nitrogen until use.

Mature DC (mDC) were resuspended in 100 µL of electroporation buffer (DC transfection kit, Amaxa, Lonza) and mixed with either 5 µg of HuDo-CSPG4, Hu-CSPG4 or empty pCDNA3.1 DNA plasmids. Electroporation was performed using a Nucleofector program U-002 (Amaxa, Lonza). After electroporation, cells were transferred into complete media and co-cultured with thawed lymphocytes for 7 days in RPMI-1640 medium with 10% heat-inactivated human serum AB (Lonza) at 2 x 10^6/mL. Pre-activated lymphocytes were then collected for the cytotoxicity assays using U2OS as the target cells, as previously described (17).

Cytotoxicity assay

Canine D22, canine D22 Do-CSPG4 and human U2OS target cells were labeled with 2µM of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes). Canine OSA cells were then cultured with thawed Pre-Vax and Post-Vax PBMC from dogs included in the vaccination group, based on sample accessibility. Human OSA cells were incubated with pre-activated lymphocytes from healthy donors at an effector:target (E:T) ratio of either 50:1 or 25:1 for 48h at 37°C in a 5% CO_2 atmosphere. After staining with 1µg/mL 7-Amino-ActinomycinD (7-AAD, RRID:AB_2869266; BD BioSciences), cells were acquired using a BD FACSVerse and analyzed with FlowJO V.10.5.3 (RRID:SCR_008520). The percentage of killing was obtained by back-gating the $CFSE^+$ targets and measuring the percentage of dead 7-AAD^+ cells, as previously described (17). Spontaneous death was obtained by culturing target cells without PBMC, and maximal cell death was obtained after treatment with 1% saponin. The percentage of specific lysis was calculated using the

following formula: ((dead targets in sample (%) − spontaneously dead targets (%))/(dead target maximum (%) − spontaneously dead targets (%)) × 100.

Statistical analysis

Two-tailed paired and unpaired Student's t-tests or one-way ANOVA were used to perform the statistical analyses for normally distributed data. The Kaplan-Meier method was used to estimate the overall survival (OS) and disease-free interval (DFI) of dogs enrolled in the study. The OS of dogs was calculated as the number of days from surgery to death. The DFI was calculated as the number of days from the date of surgery to the date in which metastases were first detected. Differences in survival times were analyzed using the Gehan-Breslow-Wilcoxon test and the Log-Rank test. Statistical significance was evaluated using GraphPad V.9 software (GraphPad; RRID:SCR_002798) and values of $p \leq 0.05$ were considered significant**.**

Results

CSPG4 silencing in human OSA cell lines results in significant losses in proliferative, migratory, and osteosphere-generation capacity

CSPG4 was silenced in human U2OS OSA cells by means of hCSPG4-siRNA to corroborate the involvement of CSPG4 in sustaining several tumor-related processes in OSA. Silencing efficiency was monitored at three different time points (24h, 48h and 72h) using RT-PCR and Western Blot analyses. Decreased levels of CSPG4 mRNA (**Figure 1A**) and protein expression (**Figure 1B**) were confirmed from 24h post transfection and were constant at 72h. U2OS cells silenced for CSPG4 expression showed significantly decreased proliferative ability (**Figure 1C**) and migratory potential (**Figure 1D**), compared to cells treated with the scramble siRNA. CSC-enriched osteospheres were then generated (**Figure 1E**). CSPG4-silenced U2OS cells displayed a lower ability to generate osteospheres than cells transfected with the scramble siRNA (**Figure 1F, G**). These results were validated in another human OSA cell line, SaOS2 (**Supplementary figure S1**).

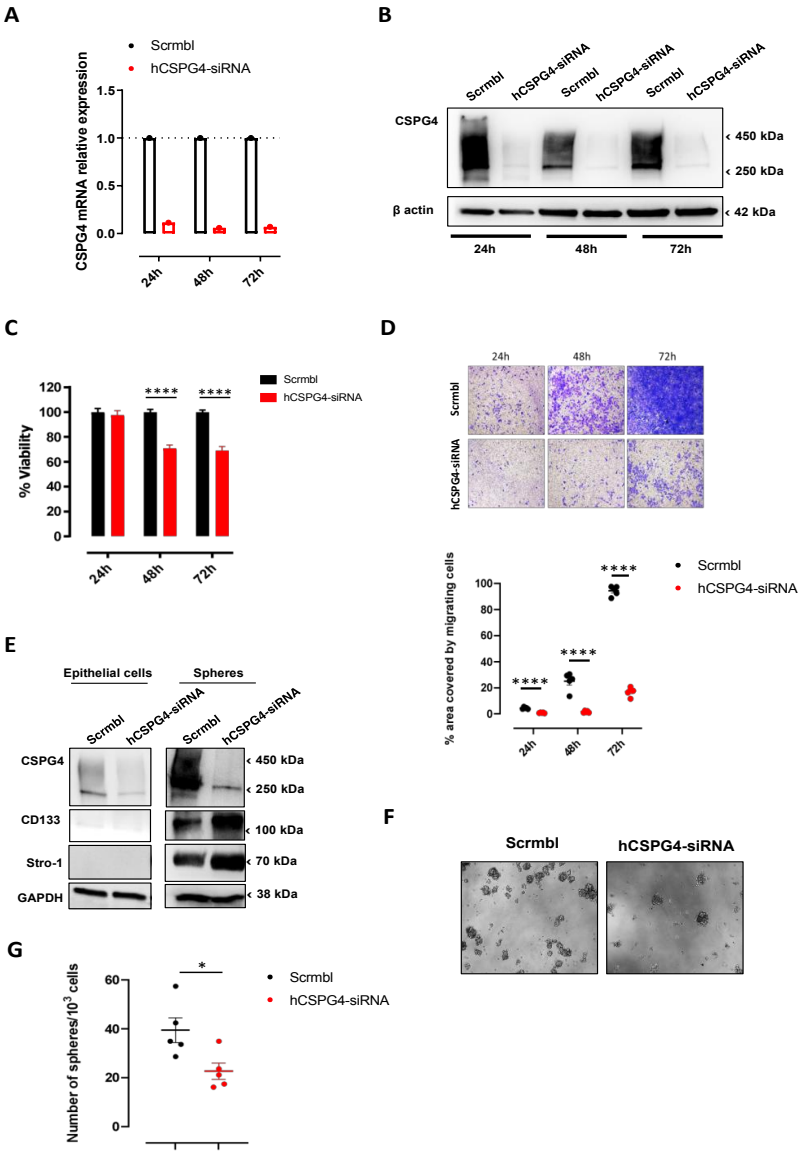

Figure 1. Functional consequences of CSPG4 silencing in human the U2OS OSA cell line. (**A**) Semi-quantitative RT-PCR of CSPG4 mRNA expression in U2OS OSA cells. Results are calculated using the $2^{-\Delta\Delta Ct}$ method and by considering the difference between the Ct of CSPG4 mRNA and the matched Ct of the internal control gene GADPH mRNA, and by then comparing cells treated with scrambled (Scrmbl) siRNA (black) with hCSPG4-siRNA (red). (**B**) Immunoblot of CSPG4 protein expression in U2OS OSA cells, comparing lysates from cells treated with the scramble (Scrmbl) siRNA with those from cells treated with hCSPG4-siRNA. β-actin was used as the protein-loading control. CSPG4 mRNA (**A**) and protein (**B**) expression levels were evaluated 24, 48 and 72h after

siRNA transfection. (**C**) U2OS cell proliferation was assessed using the MTT assay, and the results of three biological replicates are expressed as the percentage (mean value ± SEM) of cell viability at different time points, using the optical density, measured at 570 nm, of cells treated with scramble (Scrmbl) siRNA as 100%. Student's t test, **** $p < 0.0001$. (**D**) U2OS cell migratory ability was assessed using the Transwell migration assay. OSA cells treated with either scramble (Scrmbl) or hCSPG4-siRNA were placed in the upper chamber and incubated for the indicated time points. Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. Representative images of one of at least three experiments, showing Scrmbl- and hCSPG4-siRNA-migrating cells at 24h, 48h and 72h (upper panels). The percentage (mean ± SEM) of the area covered by migrated cells in five different fields are reported in the graphs (lower panels, one of at least three experiments). Student's t test, **** $p < 0.0001$. (**E**) Western blot analysis of CD133 and Stro-1 CSC markers in U2OS epithelial cells (left panel) and derived osteospheres (right panel), comparing lysates from cells treated with scrambled (Scrmbl) siRNA with those treated with hCSPG4-siRNA. GAPDH was used as the protein-loading control. (**F**) Representative images showing osteospheres derived from scramble (Scrmbl)- and hCSPG4-siRNA-treated U2OS OSA cells. (**G**) Number of osteospheres derived from U2OS cells that were previously treated with scramble (Scrmbl)- or hCSPG4-siRNA, counted in five random fields/well. Results are expressed as the number of spheres/1000 plated cells and are representative of three replicate experiments. Student's t-test, * $p<0.0238$.

Overall, these data highlight the functional role that CSPG4 plays in the biological behavior of human OSA cells, indicating that CSPG4 targeting may be a relevant option for the treatment of $CSPG4^+$ OSA.

HuDo-CSPG4 DNA vaccination controls OSA growth and metastatic spread in human xenograft mouse models

The anti-tumor potential of HuDo-CSPG4 vaccination was explored in a xenotransplant mouse model of human OSA. U2OS OSA cells were injected subcutaneously into NSG mice and, once the tumors had reached a volume of 0.5 mm^3, $CD8^+$ T cells that had been isolated from the spleens of either empty vector- or HuDo-CSPG4-immunized HLA-A2.1 Tg mice were adoptively transferred intravenously, and tumor growth was monitored (**Figure 2A**). OSA-bearing NSG mice that received $CD8^+$ T cells from HuDo-CSPG4 vaccinated animals displayed a significant slowdown in tumor growth compared to those that received $CD8^+$ T cells from controls, which were immunized with the empty pCDNA3.1 plasmid (**Figure 2B; Supplementary figure S2A, B**). Only one of the 6 (17%)

mice in the HuDo-CSPG4 vaccinated group showed delayed, but progressive, tumor growth; the stabilization of tumor growth was observed in 4 mice (67%), while the last mouse displayed consistent tumor regression (**Supplementary figure S2B**). Interestingly, the residual lesion collected from this mouse revealed no CSPG4 expression when tested by Western-Blot analyses (HuDo#1), while the other tumors collected from mice that underwent HuDo-CSPG4-CD8$^+$ T-cell transfer displayed lower CSPG4 expression, despite their growth, than the growing tumors collected from control mice (**Supplementary figure S2C**), suggesting that CSPG4-expressing tumor cells were being killed. An IHC analysis of explanted tumors revealed that transferred T lymphocytes derived from HuDo-CSPG4-vaccinated mice infiltrated the tumor mass at different rates, with higher lymphocyte infiltration evident in the more responsive tumors (**Figure 2C** and **Supplementary figure S2D**). On the other hand, T cells derived from the control group resided in the periphery rather than in the tumor core (**Figure 2C** and **Supplementary figure S2D**), while higher levels of circulating, rather than tumor-infiltrating, CD8$^+$ T cells were observed in mice with larger tumors (**Supplementary figure S2D, E**). Overall, these results suggest that CD8$^+$ T cells can hamper tumor growth when they infiltrate the mass, even achieving tumor regression when massive infiltration into the tumor core occurs.

To discover the potential mechanism by which some tumors may grow despite anti-CSPG4-CD8$^+$ T-cell adoptive transfer, the expression of PD-L1 and B7-H3 checkpoint molecules was analyzed. While PD-L1 expression was quite stable in the OSA of the control group, its levels were inversely related to tumor volumes in the HuDo-CSPG4 group, with higher PD-L1 expression in smaller and highly infiltrated tumors. Conversely, B7-H3 expression was higher in larger and less-infiltrated tumors, compared to smaller and highly infiltrated ones (**Supplementary figure S2C**), suggesting that B7-H3 may possibly play a prominent role in hampering tumor regression.

The lungs explanted from mice bearing a tumor of similar volume were then analyzed. Animals that received CD8$^+$ T cells from HuDo-CSPG4 vaccinated mice were found to be free from metastases, while lungs collected from the controls showed CSPG4$^+$ pre-metastatic/metastatic pulmonary lesions (**Figure 2C, D** and **Supplementary figure S2D**). This evidence suggests the possibility of HuDo-CSPG4 induced CD8$^+$ T cells playing a role in hindering the primary tumors, and in halting the metastatic spreading of OSA cells.

Subsequently, NSG mice carrying a U2OS-derived subcutaneous tumor of 0.5 mm^3 in volume were passively transferred with sera collected from either empty vector- or HuDo-CSPG4 immunized mice (**Figure 2E**) to evaluate the anti-tumor potential of vaccine-induced antibodies. According to the literature (23), flow cytometry analyses have revealed that Balb/c mice vaccinated with a HuDo-CSPG4 plasmid developed a higher antibody response against Hu-CSPG4 than C57/BL6 mice

(**Supplementary figure S2F**); Balb/c mice were therefore chosen for this experimental vaccination setting. Two weeks after the last immunization, sera were collected and pooled together for intraperitoneal injection into tumor-bearing NSG mice (**Figure 3E**). Although no significant differences were observed, a clear trend of reduced tumor growth was noted in mice that were treated with sera from HuDo-CSPG4-vaccinated, rather than control, mice (**Figure 2F** and **Supplementary figure S2G, H**). Lower CSPG4 expression was observed in primary tumors after treatment with sera derived from HuDo-CSPG4 vaccinated mice, than in volume-matched control tumors (**Supplementary figure S2I**). Interestingly, mice that received sera from HuDo-CSPG4-vaccinated animals displayed metastasis-free lungs, while control mice displayed pulmonary CSPG4-positive metastases (**Figure 2G, H** and **Supplementary figure S2L**). These results indicate that anti-CSPG4 antibodies may provide a potential benefit against tumor growth and metastasis.

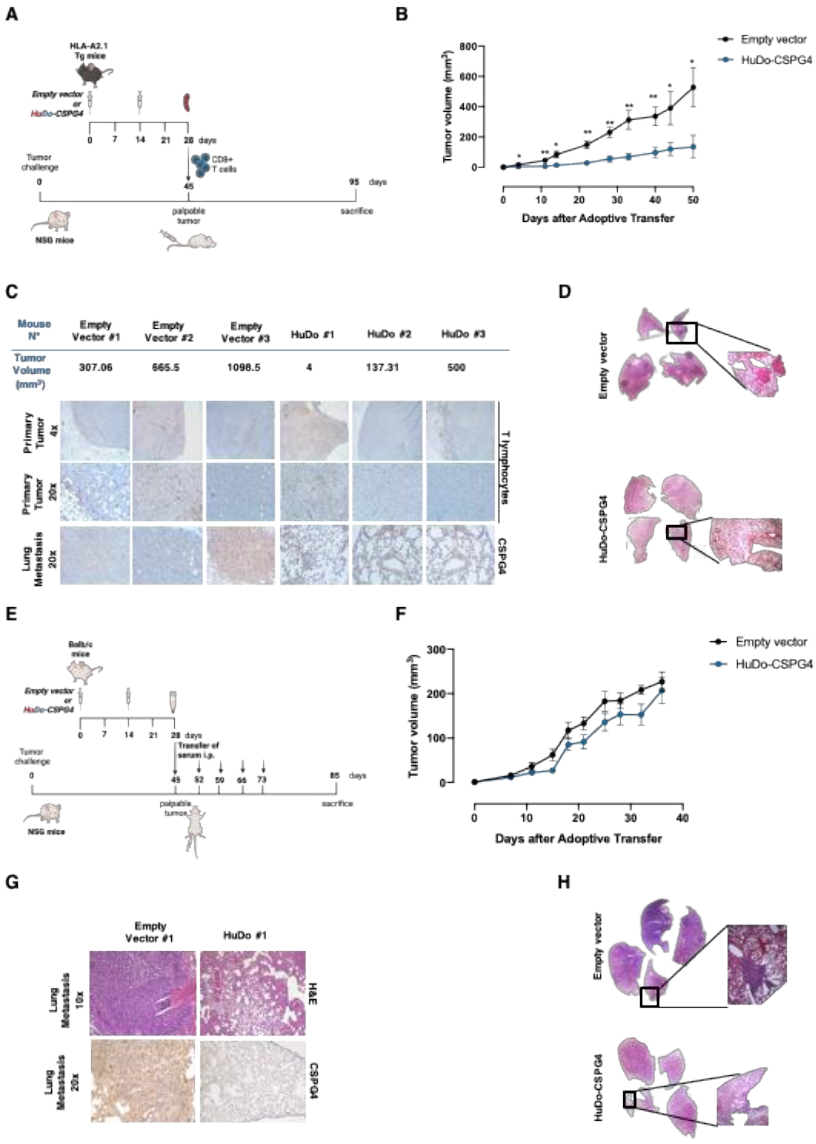

Figure 2. HuDo-CSPG4 vaccination delays human OSA tumor growth and impairs metastatic dissemination *in vivo*. (**A, E**) Experimental protocols of adoptive transfer of isolated $CD8^+$ T cells (**A**) and passive transfer of sera (**E**) in OSA-bearing NSG mice. HLA-A2.1 Tg mice (**A**) and Balb/c mice (**E**) were immunized twice, at a two-week interval, with either the pcDNA3.1 empty vector or HuDo-CSPG4 coding plasmids. Two weeks after the last administration, spleens were harvested and $CD8^+$ T cells were isolated (**A**) and sera were collected (**E**). $CD8^+$ T cells and sera were injected into the tail vein of NSG mice previously challenged with 1 x 10^6 U2OS OSA cell line that displayed a palpable tumor. Illustrations were created using BioRender.com. (**B**, **F**) Tumor growth curves

following the adoptive transfer of $CD8^+$ T cells (**B**) and passive transfer of sera (**F**) collected from pcDNA3.1-empty-vector- (black line) and HuDo-CSPG4- (blue line) vaccinated mice. Subcutaneous tumor volumes (mm^3) were measured at the indicated time points. Student's t-test, * $p<0.03$; ** $p<0.0080$. (**C**) IHC analysis of U2OS-derived tumors (upper and middle panels) and lungs (lower panels) explanted from animals transferred with $CD8^+$ T cells collected from pcDNA3.1 and HuDo-CSPG4 vaccinated mice**.** Representative IHC analysis of T lymphocytes infiltrating the tumors and CSPG4 expression in lung lesions. Images were acquired using a Leica DM750 microscope and an ICC50 camera. (**D**, **H**) Representative H&E images of metastases in the lungs explanted from NSG-OSA-bearing mice treated with $CD8^+$ T cells (**D**) or sera (**H**) derived from empty pcDNA3.1- or HuDo-CSPG4-vaccinated animals. (**G**) Representative H&E (upper panels) and CSPG4 IHC (lower panels) analyses of lungs explanted from animals transferred with sera collected from pcDNA3.1 or HuDo-CSPG4 vaccinated mice**.** Images were acquired using a Leica DM750 microscope and an ICC50 camera.

Pilot veterinary study

The enrolled population included 25 dogs affected by stage I-III $CSPG4^+$ appendicular OSA. The main patient characteristics are listed in **Supplementary Table 1**. All eligible dogs received standard of care treatment. Only dogs free of metastasis at restaging (chest radiographs/abdominal ultrasound and/or total body CT scan) at the end of the chemotherapeutic protocol (4-6 cycles of adjuvant carboplatin) were enrolled in the study. OSA dogs were included in either the vaccination or control arm according to their owners' wishes. Dogs included in the vaccination arm were adjuvantly treated with the HuDo-CSPG4 vaccine (**Supplementary figure S3A**), as previously described (17,24,25). Dogs were assessed for clinical signs of toxicity (i.e., temperature, body weight, lethargy, lameness, etc.) and clinicopathologic parameters (i.e., complete blood count and/or biochemistry panel and/or urinalysis, when clinically indicated). No toxicity, as defined by VCOG- CTCAE V.2., was observed over the entire vaccination period.

HuDo-CSPG4 vaccination is immunogenic in canine OSA patients

Humoral response

The vaccine-induced antibody response was evaluated in the sera of vaccinated dogs, collected before the first (Pre-Vax) and after the fourth vaccination (Post-Vax). A spontaneous and detectable anti-CSPG4 antibody response was present in most $CSPG4^+$ OSA patients even before vaccination, probably as consequence of chemotherapeutic treatment (**Supplementary figure S3B**).

Sera were tested by ELISA for the presence of IgG against three different domains (D1, D2, D3; (17))

of Do-CSPG4 (**Figure 3A; Supplementary figure S4A**). An increase in IgG response following HuDo-CSPG4 vaccination against at least one of the Do-CSPG4 domains was observed in 5 out of 12 vaccinated dogs (41.66%) (**Figure 3A, B; Supplementary figure S4A**). Of these 5 responding dogs, 2 (16.66% of the vaccinees) displayed an increased IgG response against all of the Do-CSPG4 domains in the Post-Vax as compared to Pre-Vax sera. In the Post-Vax sera of the other 3 dogs (23.07%) increased IgG binding was detected only against the D2 domain (partial response, **Figure 3A; Supplementary figure S4A**). The sera from the 7 non-responding dogs (**Figure 3A; Supplementary figure S4A**) were tested in a chaotropic ELISA assay against the Do-D2. After treatment with the chaotropic agent at 5M, a higher percentage (fold change>1) of IgG binding was detected in the Post-Vax than in the Pre-Vax sera of 4 out of 7 dogs (**Figure 3C; Supplementary figure S4A**). Post-Vax sera were then tested for their ability to bind the Do-CSPG4 as it is overexpressed in its natural conformation on canine OSA cells (Penny). In 6 out of 12 (50%) vaccinated dogs, the Post-Vax sera showed a higher capacity to stain Penny than the corresponding Pre-Vax sera (**Figure 3D; Supplementary figure S4A**). Moreover, the antibody level against Penny was also persistent in sera collected at later time points (after the fifth and the sixth immunizations, **Supplementary figure S5A**).

A vaccine-induced anti-Hu-CSPG4 antibody response was detected in 9 out of 12 (75%) immunized dogs after the fourth vaccination (**Supplementary figure S4A; Supplementary figure S5B**) and this modestly increased at sequential time points (**Supplementary figure S5C**), as demonstrated by the ability of sera to stain the human U2OS cell line, which naturally overexpresses CSPG4.

To explore the possible mechanisms of action of vaccine-induced antibodies against OSA, Pre-Vax and Post-Vax sera pools were generated and added to the Penny-cell culture medium. The Post-Vax pool displayed a higher ability to inhibit canine OSA-cell proliferation (**Figure 3E**) and migration (**Figure 3F**) *in vitro* than the Pre-Vax pool.

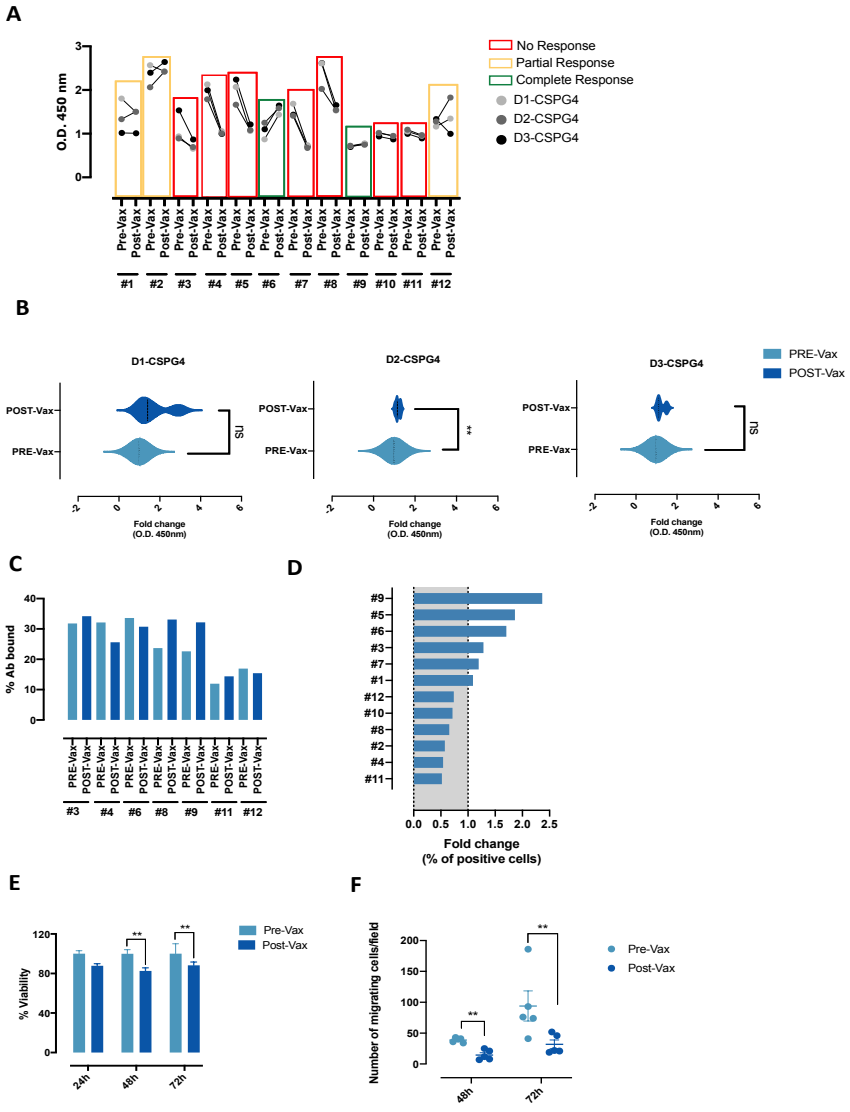

Figure 3. **HuDo-CSPG4 vaccination induces a specific anti-canine CSPG4 antibody response in vaccinated OSA-affected dogs**. (**A**) Analysis, by means of ELISA assay, of the presence of IgG against the D1, D2 and D3 domains of the canine CSPG4 protein in the sera of dogs before the first immunization (Pre-Vax) and after the fourth (Post-Vax). Results express the optical density (O.D.) at the absorbance measured at 450 nm. A fold change between the Post-Vax O.D./Pre-Vax O.D. > 1 is considered as a vaccine-induced IgG response. Green Boxes indicate a complete response (against all tested domains), yellow boxes indicate a partial response (against at least one domain) and red boxes indicate the absence of a response. (**B**) ELISA assay evaluating the presence of IgG against

Do-D2 in the sera of dogs before the first immunization (Pre-Vax) and after the fourth (Post-Vax), measured in "responder" (**A**) dogs. Results are expressed as the ratio (fold change) between the O.D. (measured at 450 nm) of Post-Vax and Pre-Vax sera. (**C**) Chaotropic ELISA assay measuring avidity against the Do-D2 domain of the vaccine-induced antibodies in the sera of "non-responder" (**A**) dogs immunized with the HuDo-CSPG4 vaccine. Results are expressed as the percentage (%) of antibodies (Ab) that remain bound after treatment with the chaotropic agent at a 5M concentration in Post-Vax sera, compared to Pre-Vax sera**.** (**D**) Flow cytometry analysis of canine CSPG4$^+$ OSA cells (Penny) incubated with sera of dogs before immunization and after the fourth HuDo-CSPG4 vaccination. Total IgG binding was evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as the ratio (fold change) between the percentages (%) of stained cells incubated with the Post-Vax/Pre-Vax sera. (**E**) The proliferation of canine OSA CSPG4$^+$ Penny cells was assessed using the MTT assay and the results (from two independent experiments, each including triplicate technical replicates) are expressed as optical density (O.D.; mean value ± SEM) measured at 570 nm. Cells were incubated for 24-48-72h with pooled Pre-Vax or Post-Vax sera collected from canine OSA patients. Student's t-test, ** $p < 0.0066$. (**F**) Penny-cell migratory ability was assessed using the Transwell migration assay. OSA cells treated with pooled Pre-Vax or Post-Vax sera from canine OSA patients were placed in the upper chamber and incubated for the indicated time periods. Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. The number (mean ± SEM) of migrated cells in five different fields, representative of four independent experiments, are reported in the graph. Student's t-test, ** $p < 0.0039$. Representative images showing migrating cells at 48h and 72h were included (right panels).

Cellular response

An immunophenotyping analysis of patient-derived PBMC collected before the first (Pre-Vax) and after the fourth (Post-Vax) immunization was performed in 10 vaccinated dogs to evaluate the vaccine-induced cellular response. While no significant increase in the number of B and CD4$^+$ T cells was observed, a decrease in the number of myeloid-derived suppressor cells (MDSC) and/or T regulatory cells (Treg) and/or an increase in CD8$^+$ T cells was observed in some of the analyzed dogs (**Figure 4A; Supplementary figure S4B**). In detail, 8 out of 10 (80%) dogs displayed an increase in the number of CD8$^+$ T cells after vaccination (**Figure 4B; Supplementary figure S4B**). Three of these (30% of the analyzed dogs) also showed a decrease in MDSC and Treg percentage, whereas another 3 (30%) only showed a decrease in MDSC, and 1 (10%) only showed a decrease in Treg. Of the two dogs that did not display an increase in CD8+ T-cell number, 1 (10%) displayed a decrease in both MDSC and Treg cells, while the other (10%) only showed a reduction in the Treg population.

The anti-tumor effect potentially mediated by the vaccine-induced cellular response was assessed, by means of an *in vitro* cytotoxicity assay, for the 8 dogs in which an increase in $CD8^+$ T cells was observed. A canine OSA cell line that is naturally negative for CSPG4 expression (D22) was stably transfected to overexpress the Do-CSPG4 (D22 Do-CSPG4; **Supplementary figure S6**), which was used as a target. The killing of CFSE-labeled D22 Do-CSPG4 cells was found to be significantly higher after 48h of incubation with Post-Vax PBMC than with Pre-Vax ones (**Figure 4C; Supplementary figure S4B**). This cytotoxic effect was mainly directed against CSPG4, as significantly fewer CSPG4-negative D22 cells were killed (**Figure 4D; Supplementary figure S4B**).

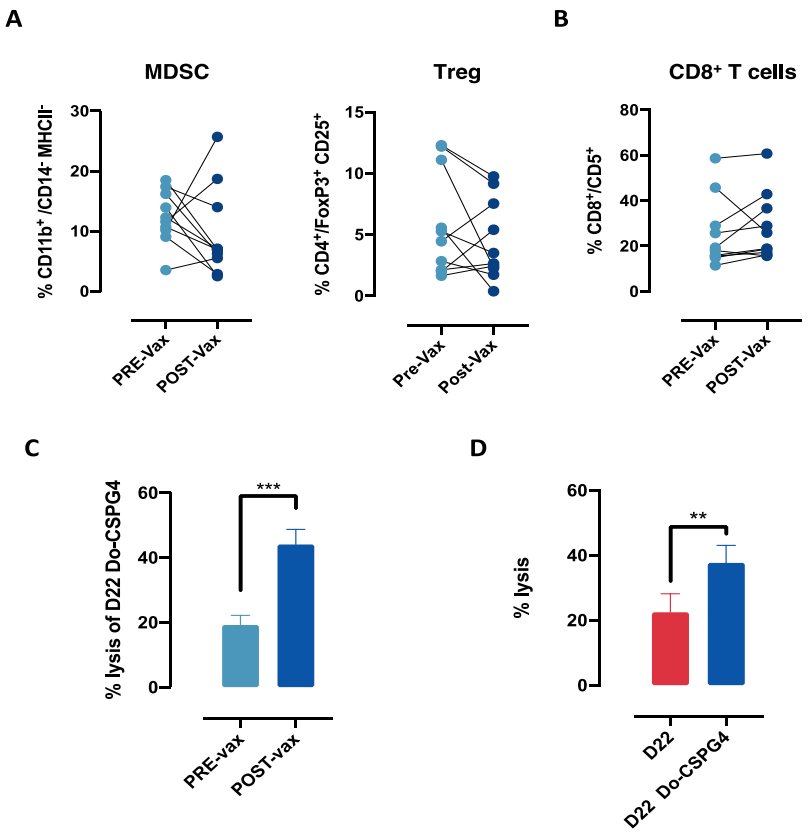

Figure 4. HuDo-CSPG4 induces a specific anti-CSPG4 cellular immune response in vaccinated dogs. (**A, B**) Flow cytometry analysis of the frequencies of circulating MDSC, gated on $CD11b^+/CD14^-MHCII^-$, T regulatory (Treg) cells, gated on $CD4^+/FoxP3^+$ $CD25^+$ cells (**A**), and $CD8^+$ T lymphocytes, gated on $CD5^+$ cells (**B**). Graphs show the percentage of MDSC, Treg and $CD8^+$ T cells circulating in canine patients before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). (**C, D**) Cytotoxicity assays to quantify (**C**) the ability of PBMC to kill D22 Do-CSPG4

cells before (Pre-Vax, light blue bar) and after HuDo-CSPG4 vaccination (Post-Vax, blue bar) and (**D**) the ability of Post-Vax PBMC to specifically kill CSPG4-expressing D22 canine OSA cells (blue bar) compared to the CSPG4-negative D22 counterpart (red bar). Results are shown as the % of lysis of CFSE-labeled tumor cells after 48h of incubation with Post-Vax and Pre-Vax PBMC. Student's t-test, ***p<0.0010; **p=0.0044.

Clinical outcome following HuDo-CSPG4 vaccination in canine OSA patients

The overall survival (OS) and disease-free interval (DFI) of HuDo-CSPG4-adjuvantly-immunized (Vax) and control (Ctrl) dogs (the latter treated with conventional therapies alone) were evaluated for all patients. The clinical information for each enrolled dog is reported in **Supplementary figure S7**. One year after surgery, vaccinated dogs showed significantly longer OS than Ctrl-group dogs (**Figure 5A**), with the OS rates being 66.66% and 30.76%, respectively. Two years after surgery, the benefit of anti-CSPG4 vaccination became more modest, with similar OS rates in the two groups (18.18% vs 15.38% for Vax and Ctrl dogs, respectively). The median survival times (MST) for the vaccinated and control arms were 438 and 202 days, respectively (**Figure 5A**).

The DFI was of 242.5 and 160 days for the vaccinated and the control arms, respectively (**Figure 5B**). Six months after surgery, 25% and 61.53% of vaccinated and control dogs, respectively, developed distant metastasis, while after 1 year, 75% and 76.92% of dogs presented distant metastasis in the vaccinated and control arms, respectively.

Overall, these results suggest that HuDo-CSPG4 vaccination may provide a clinical benefit in initially prolonging OS and delaying metastasis development.

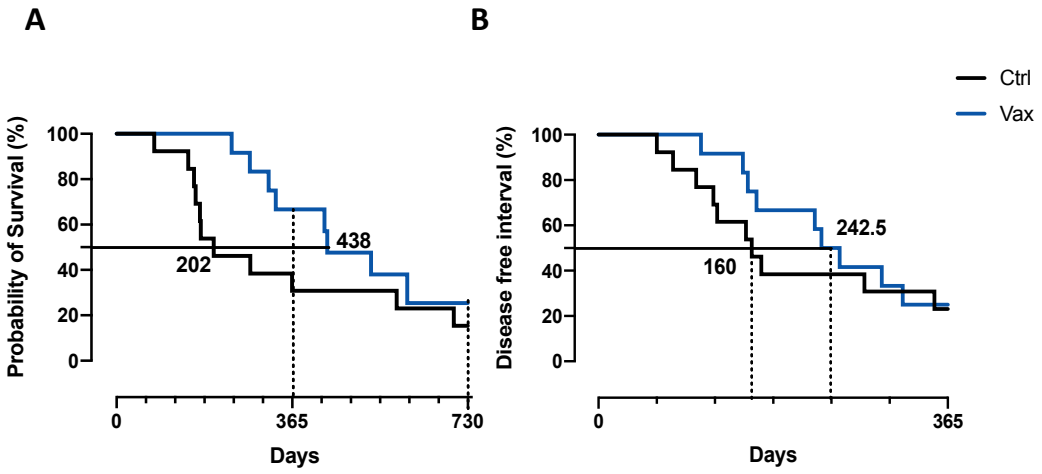

Figure 5. HuDo-CSPG4-vaccinated dogs showed prolonged overall survival (OS) and disease free interval (DFI) compared to conventionally treated controls. (**A**) Kaplan-Meier curve comparing the OS (in days) of HuDo-CSPG4 vaccinated (blue line) and control (black line) dogs, updated to November 2022. OS rate (percentage) and median survival time (in days) are indicated in the graph. 1-year OS, Gehan-Breslow-Wilcoxon test, * p=0.0208, Log-rank test, * p=0.0364; 2-year OS, Gehan-Breslow-Wilcoxon test, p=0.0526, Log-rank test, p=0.1888. (**B**) Kaplan-Meier curve comparing the 1-year DFI (in days) of HuDo-CSPG4-vaccinated (blue line) and control (black line) dogs, updated to November 2022. Gehan-Breslow-Wilcoxon test, p=0.2871, Log-rank test, p = 0.5459.

HuDo-CSPG4 is effective in a human surrogate setting

Pilot investigations in human surrogate models were carried out to assess the potential of translating this chimeric vaccination strategy to a human setting. mDC generated from healthy donors were transfected with either the chimeric HuDo-CSPG4 (HuDo-CSPG4-DC) or fully Hu-CSPG4 (Hu-CSPG4-DC) plasmid to evaluate and compare the potential of chimeric and homologous vaccines in inducing an anti-CSPG4 response in a human system that simulates *in vitro* DNA vaccination. mDC transfected with the empty plasmid (empty-DC) were used as a control. Autologous T cells that were pre-activated by HuDo-CSPG4-DC were more effective in killing the human HLA-A2-matched $CSPG4^+$ U2OS OSA cell line than those pre-activated by Hu-CSPG4-DC and empty-DC in an *in-vitro* cytotoxicity assay (**Figure 6**). These data suggest that the chimeric vaccine may be able to break immune tolerance against the self CSPG4 antigen and induce a cytotoxic response against $CSPG4^+$ OSA cells.

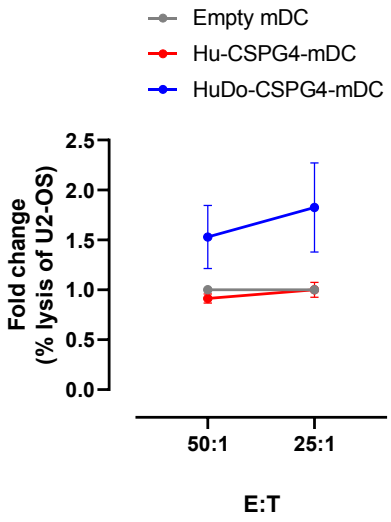

Figure 6. HuDo-CSPG4-stimulated PBL from healthy subjects show potential anti-CSPG4 cytotoxic activity. Cytotoxicity assay performed with healthy-donor PBMC (N = 4) recovered after 7 days of co-culture with autologous mDC transfected with either the pcDNA3.1 empty vector, Hu-CSPG4 or HuDo-CSPG4 plasmids. Pre-activated PBMC were incubated for 48h at 37°C with CFSE-labeled CSPG4^{+} U2OS human OSA cells, at different effector:target (E:T) ratios. Results show the fold change between the percentage (± SEM) of CFSE-labeled tumor cells lysed by HuDo- and Hu-CSPG4-pre-activated PBMC/pcDNA3.1-pre-activated PBMC. One-way ANOVA test, p=0.0660.

Discussion

Its high rates of recurrence and metastasis mean that OSA remains a critical issue in pediatric oncology. The standard of care for OSA patients has remained mostly unchanged since the 1970s, and the introduction of neoadjuvant/adjuvant aggressive chemotherapy, which improves the outcomes of patients with localized tumors, is barely effective in cases of recurrent or advanced disease (26). Therefore, the development of novel therapies is still an unmet clinical need. Significant limitations in the advancement of OSA management include the rarity of the disease, the very young age of patients and the lack of defined common targetable oncogenic drivers. The considerable and recognized similarity between human and canine OSA (6–8,21) has driven interest in using pet dogs with spontaneously occurring OSA to test new therapeutic options, including immune-based therapies, with the final aim of informing human clinical trials (7,27). Indeed, human and canine OSA share many biological and clinical similarities, as they are both highly metastatic and resistant to

conventional treatments. Recent examples of relevant comparative oncology studies in the field include a Listeria monocytogenes (Lm)-based vaccine that expresses a chimeric human epidermal growth factor receptor (HER)2 fusion protein; positive results in a phase I veterinary clinical trial led to the issuing of a conditional license by the United States Department of Agriculture (USDA) in 2017 for the adjuvant treatment of dogs with OSA (28,29). The promising results obtained in canine patients led to a clinical trial in adult patients with $HER2^+$ tumors (NCT02386501) and to a license for its development in the pediatric OSA setting (30). The occurrence of adverse effects, caused by Listeria infections in dogs and the potential hazard of the zoonotic spread of the disease in humans, led to some concerns regarding the safety of this strategy. However, this study has highlighted the potential value of comparative studies for the human clinic. In pursuing this path, here we have exploited the immunogenicity and consequent potential clinical benefit of an immunization strategy against CSPG4 by using a chimeric human/dog (HuDo)-CSPG4 DNA vaccine (17). Of the different immunotherapeutic strategies available, DNA plasmid-based vaccines own the advantages of being stable, easy to manufacture and cost-efficient, as well as safe and effective in stimulating both immune arms of a patient's own immune system, as demonstrated in several clinical trials (31).

CSPG4 has come to prominence as an appealing immunotherapeutic target in the melanoma setting, as demonstrated in human (32,33) and veterinary clinical trials (17,24,25). As a co-receptor/plasma membrane scaffold, CSPG4 can enhance, when over-expressed, the signal intensity and duration of multiple oncogenic pathways that sustain the pro-tumoral attitude of malignant cells (11,12,34,35).

We have previously demonstrated that human and canine OSA cells overexpress CSPG4 and that this overexpression negatively impacts the prognoses of both human and canine OSA patients (14). In addition, we have shown that CSPG4 is overexpressed on OSA-derived CSC (14), considered responsible for chemo-resistance and favoring relapse and metastasis in OSA (36). To functionally support these findings, the present study has induced the transient silencing of CSPG4 in human OSA cell lines, resulting in an impairment of their malignant behavior. Indeed, *in vitro* proliferation, migration, and osteosphere formation was inhibited in CSPG4-silenced OSA cells. The anti-tumor impact of CSPG4 down-regulation was independent of the mutational status of OSA cells, with similar effects observed in U2OS and SaOS2 cells (i.e., p53 and Rb wild-type vs. p53 null and Rb mutated, respectively).

Before the in-dog studies, we performed a pre-clinical evaluation of HuDo-CSPG4 vaccine anti-tumor potential in adoptive-transfer experiments in a human xenotransplant model. Given its recognized higher metastatic potential compared to SaOS2 cells, the U2OS cell line was selected for these experiments as we aimed to investigate the ability of HuDo-CSPG4 to counteract primary OSA growth and lung dissemination (37,38). Adoptively transferred HuDo-CSPG4-induced $CD8^+$ T cells

significantly hampered human CSPG4$^+$ OSA growth in NSG mice. However, some mice showed slowed, but continuous, tumor growth. In an attempt to better characterize CD8$^+$ T-cell activity against established U2OS tumors, we analyzed the levels of infiltrating lymphocytes and the expression of PD-L1 and B7-H3 checkpoint molecules in tumors, in view of their suggested role in mediating immune evasion in several cancer models (39,40). Firstly, in a comparison with controls, only HuDo-CSPG4-induced CD8$^+$ T cells were able to infiltrate the primary tumor core, and this infiltration seemed to be related to better tumor rejection. Furthermore, higher PD-L1 expression was found in smaller, highly infiltrated tumors. This result fits with previous findings in human patients in which PD-L1 expression was associated with the presence of multiple tumor-infiltrating immune cells, with tumor size being larger in PD-L1-negative cases than in PD-L1-positive ones, and negatively correlating to outcomes (5,41,42). Conversely, increased B7-H3 expression correlated with larger, low-infiltrated tumors, suggesting that the up-regulation of B7-H3 by human OSA cells may be a mechanism of tumor escape, in accordance with recent results in human OSA biopsies (43). In line with these findings, the use of Atezolizumab (anti-PD-L1 mAb) as a single agent (5,44) was ineffective in pediatric OSA patients, while Enoblituzumab (anti-B7-H3) was effective (https://clinicaltrials.gov/ct2/show/NCT02982941). These results suggest that anti-CSPG4 vaccination may be used in combination with B7-H3 immune checkpoint blockade to prime T cells against CSPG4, while increasing their activity and infiltration into tumors.

To investigate the contribution of vaccine-induced antibodies, U2OS-bearing NSG mice were adoptively transferred with sera derived from HuDo-CSPG4 immunized mice. Although no significant differences were observed, a clear trend of tumor-growth reduction was noted. It must be mentioned that NSG mice lack NK cells, meaning that antibody-dependent cellular cytotoxicity, one of the relevant mechanisms by which the anti-CSPG4 antibody is effective in cancer cell elimination (17), is defective in these mice. This may lead to an underestimation of the efficacy of anti-CSPG4 antibodies in an immune-competent system. However, a statistically significant impairment of metastasis development was observed compared to controls, both in mice adoptively transferred with immune sera and those with anti-CSPG4 T cells. This result supports the previously hypothesized direct contribution of both the cellular and antibody immune responses in hindering tumor progression (14,17).

These promising data, together with the need to test HuDo-CSPG4 vaccination in OSA immunocompetent models, prompted us to exploit dogs that spontaneously develop CSPG4$^+$ appendicular OSA and that have completed the standard therapeutic protocol. In these dogs, HuDo-CSPG4 vaccination was effective in overcoming immune tolerance to the self (canine) CSPG4 molecule. A significant and overall increase in the antibody level against Do-CSPG4 was observed

following vaccination in the sera of about 42% of dogs, where we detected an increased IgG titer against the canine Do-D2 domain. A high spontaneous humoral response against a CSPG4-overexpressing tumor could be present and amplified following tumor surgical resection and chemotherapy (17,45). This could lead to an underestimation of the percentage of dogs that respond to HuDo-CSPG4 vaccination in evaluations based on a higher anti-CSPG4 antibody response in post- compared to pre-immunization sera. Indeed, dogs that were classified as non-responders in this way showed an increase in the avidity of antibodies against Do-D2 following vaccination. The D2 domain constitutes the core of the CSPG4 protein extracellular portion and is the putative mediator of ligand/ECM binding, promoting pro-tumor signals (34). Interestingly, vaccine-induced antibodies in the sera of immunized dogs exerted a mechanistic effect on the canine CSPG4-expressing OSA cells, Penny, in terms of the increased inhibition of both tumor cell proliferation and migration compared to sera collected before vaccination. These results hint at a possible mechanism of action for vaccine-induced antibodies *in vivo*, and highlight the low effectiveness of spontaneous low-avidity autoantibodies in counteracting growing tumors, which is in line with our recent findings in a veterinary trial in which HuDo-CSPG4 vaccination was applied for the adjuvant treatment of melanoma-affected dogs (17). High CSPG4 expression in tumors has been found to be directly correlated with a lower tumor infiltration and enhanced immunesuppression (46). MDSC are the predominant cells observed within the tumor microenvironment in both human (47) and canine (48) OSA, while high Treg levels have been correlated with worse prognosis (49), with both these issues contributing to immune evasion. We therefore performed an analysis of PBMC, which revealed decreased levels of MDSC and Treg in most samples collected after the HuDo-CSPG4 vaccination, compared to those collected before the immunization cycles were started. In most cases, we also observed an increase in $CD8^+$ T cells that were able to selectively exert cytotoxic activity against CSPG4-expressing OSA cells. These results suggest that anti-CSPG4 vaccination can hamper the immune suppressive mechanisms that persist in canine patients with minimal residual disease, together with the induction of CSPG4-specific cellular immunity.

Even though this study was designed to explore the immunogenicity of HuDo-CSPG4 vaccination in dogs affected by a poorly immunogenic tumor, such as OSA (50), and not to evaluate clinical outcomes, clinical data were collected, highlighting the potential therapeutic benefit of the adjuvant HuDo-CSPG4 vaccination on survival. Canine OSA patients treated with surgery and chemotherapy generally display a 1-year survival rate ranging from around 35 to 40% (7,8). We therefore examined the 1-year survival probability as the first clinically relevant time point and vaccinated dogs showed prolonged OS compared to the matched control group (66.66% vs. 30.76%, respectively; $p = 0.0208$). Indeed, we observed a separation of the Kaplan-Meier curves from about three to twenty months.

Two years after surgery, the effect of adjuvant HuDo-CSPG4 became modest, with similar OS rates being observed in the two groups (18.18% and 15.38% for vaccinated and Ctrl dogs, respectively; p = 0.0526). The OS rate at 2 years is in line with that previously reported for OSA-bearing dogs receiving standard therapies and is a consequence of the development of metastatic disease. The Authors understand that, because of how this study has been designed, an analysis of DFI has intrinsic limits; it may be affected by timing and, potentially, by the different diagnostic imaging procedures adopted in the two groups. Nevertheless, a separation of the Kaplan-Meier curves from about two to ten months can be noted, and this corresponds to median disease-free intervals of 242 days for vaccinated dogs and 160 days for the controls. We can therefore speculate that vaccine-induced antibodies and T cells may be able to impair the beginning of the metastatic cascade, mirroring observations in preclinical mouse models. HuDo-CSPG4 vaccination could ideally lead to the elimination of all CSPG4-positive tumor cells, while sparing the CSPG4-negative clones that can lead to disease progression.

However, the real potential of vaccination when metastases have already been established is still unexplored. Canine patients with distant metastases should be included in a future trial. To further improve the clinical potential of adjuvant vaccination, the immunization protocol should start earlier, before or between chemotherapeutic cycles, to achieve a better curative effect in the first therapeutic window. Moreover, combinatorial strategies should be designed to prolong the benefit to later stages of progression (for example, by exploiting anti-B7-H3 blockade, as previously discussed).

One of the limitations of this pilot veterinary study is a lack of patient randomization and the small sample size. As safety and the induction of an anti-CSPG4 immune response were the main objectives of the present study, a larger randomized controlled study will be necessary to draw conclusions regarding the effective clinical response to the adjuvant vaccine in OSA canine patients (only envisaged here). Finally, we have demonstrated that differentiated DC from healthy human donors that were electroporated with the chimeric HuDo-CSPG4 plasmid were better able to activate autologous T cells towards the killing of human $CSPG4^{+}$ OSA cells than DC transfected with fully xenogeneic Hu-CSPG4 (24) or the empty vector. These results may predict the ability of the chimeric vaccine to break immune tolerance against the (human) self-antigen and to also mount an effective *in vivo* cytotoxic response in a human setting.

Overall, this study demonstrates that CSPG4 could be a relevant comparative target for OSA treatment through chimeric DNA vaccination. Considering the high translational value of spontaneous canine tumors, these promising results will likely promote the translation of this novel immunotherapeutic approach to a human setting, eventually improving the life expectancy of OSA patients that cannot benefit from present therapies.

Author Contributions

L Tarone: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing-original draft, writing-review & editing; **D Giacobino:** Data curation, formal analysis, investigation, writing-review & editing; **MT Camerino:** Data curation, investigation; **L Maniscalco:** Formal analysis, methodology, writing-review & editing; **S Iussich:** Formal analysis, methodology, writing-review & editing; **L Parisi:** Data curation, formal analysis; **G Giovannini**: Data curation, investigation; **A Dentini:** Data curation, investigation; **E Bolli:** Methodology; **E Quaglino:** Methodology; **IF Merighi:** Methodology; **E Morello:** Conceptualization, data curation, investigation, writing-review & editing; **P Buracco:** Conceptualization, data curation, funding acquisition, investigation, project administration, resources, writing-review & editing; **F Riccardo:** Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, project administration, resources, writing-original draft, writing-review & editing; **F Cavallo:** Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, project administration, resources, writing-original draft, writing-review & editing.

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Conflicts of Interest: None of the authors have a conflict of interest to declare.

Data Availability: The data generated in this study are available upon request from the corresponding authors.

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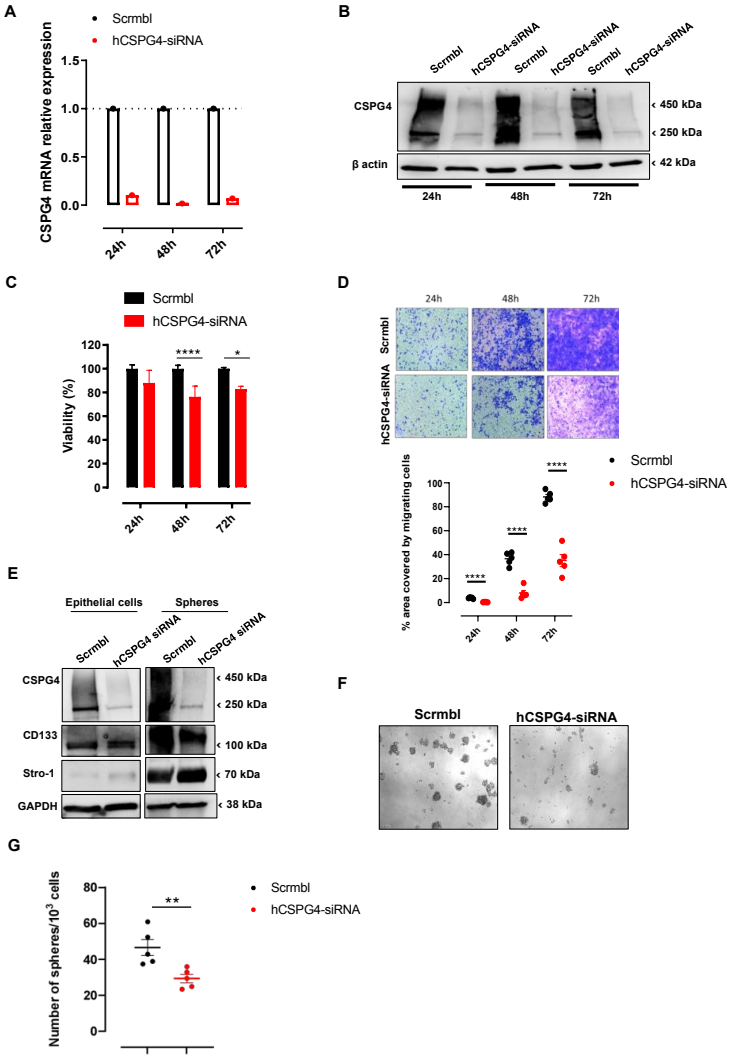
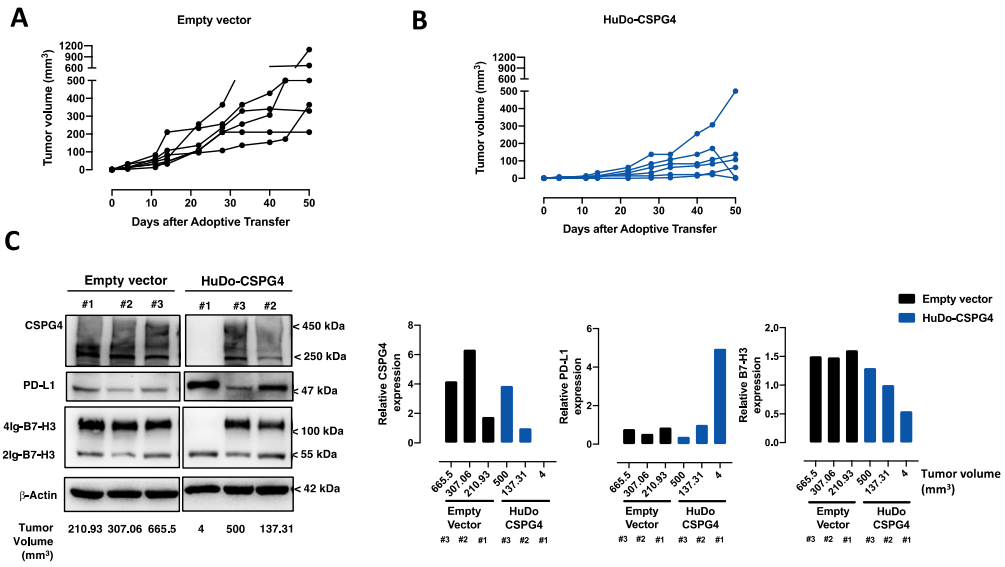
Supplementary Figures and Legends



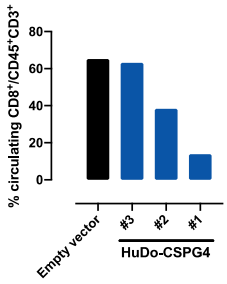
Supplementary Figure S1. Functional consequences of CSPG4 silencing in human SaOS2 OSA cell lines. (**A**) Semi-quantitative RT-PCR of CSPG4 mRNA expression in SaOS2 OSA cells. Results are calculated using the 2^(-ΔΔCt) method by considering the difference between the Ct of CSPG4 mRNA and the matched Ct of the internal control gene GADPH mRNA, and then by comparing cells treated

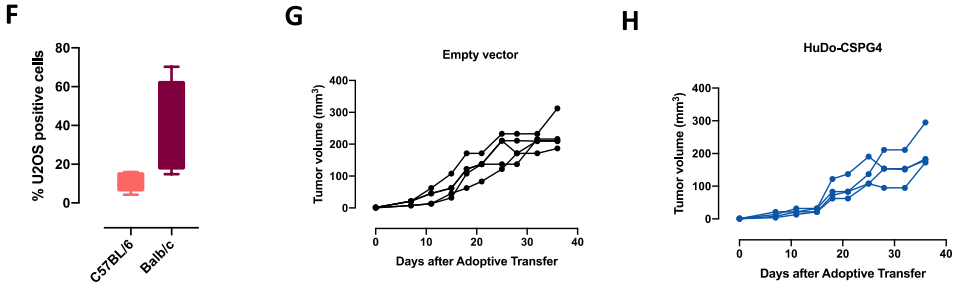
with either scrambled (Scrmbl) siRNA (black) or hCSPG4 siRNA (red). (**B**) Immunoblot of CSPG4-protein expression in SaOS2 OSA cell lines, comparing lysates from cells treated with Scrmbl siRNA with those treated with hCSPG4-siRNA. β-actin was used as the protein-loading control. CSPG4 mRNA (**A**) and protein (**B**) expression were evaluated 24h, 48h and 72h post siRNA transfection. (**C**) SaOS2 cell proliferation was assessed using the MTT assay and results of three biological replicates are expressed as the percentage (mean value ± SEM) of cell viability at different time points, considering the optical density, measured at 570 nm, of cells treated with scramble (Scrmbl) siRNA as 100%. Student's t test, **** $p < 0.0001$; * $p=0.0351$. (**D**) SaOS2 cell migratory ability was assessed using the Transwell migration assay. OSA cells treated with Scrmbl or hCSPG4 siRNA were placed in the upper chamber and incubated for the indicated time points. Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. Representative images of one of at least three experiments, showing Scrmbl- and hCSPG4-siRNA-migrating cells at 24h, 48h and 72h (upper panels). The percentage (mean ± SEM) of the area covered by the migrated cells in five different fields are reported in the graphs (lower panels, one of at least three experiments). Student's t test, **** $p < 0.0001$. (**E**) Western blot analysis of CD133 and Stro-1 CSC markers in SaOS2 epithelial cells (left panel) and derived osteospheres (right panel), comparing lysates from cells treated with scrambled (Scrmbl) siRNA with those treated with hCSPG4-siRNA. GAPDH was used as the protein-loading control. (**F**) Representative images showing osteospheres derived from Scrmbl- and hCSPG4-siRNA-treated SaOS2 OSA cells. (**G**) Number of osteospheres derived from SaOS2 cells that were previously treated with Scrmbl- or hCSPG4-siRNA, counted in five random fields/well. Results are expressed as number of spheres/1000 plated cells and are representative of three replicate experiments. Student's t test, ** $p<0.0091$.

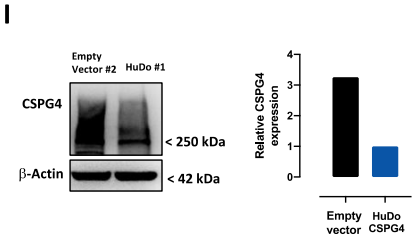


D

Mouse	Tumor volume (mm³)	Infiltrating CD3⁺	CSPG4 score (Lung metastasis)
Empty Vector #3	1098.5	Rare	6
Empty Vector #2	665.5	Abundant in periphery Rare inside the tumor	6 Pre-metastatic lesions
Empty Vector #1	307.06	Intermediate	7 Pre-metastatic lesions
HuDo#3	500	Rare	n/d
HuDo#2	137.31	Intermediate	n/d
HuDo#1	4	Abundant	n/d



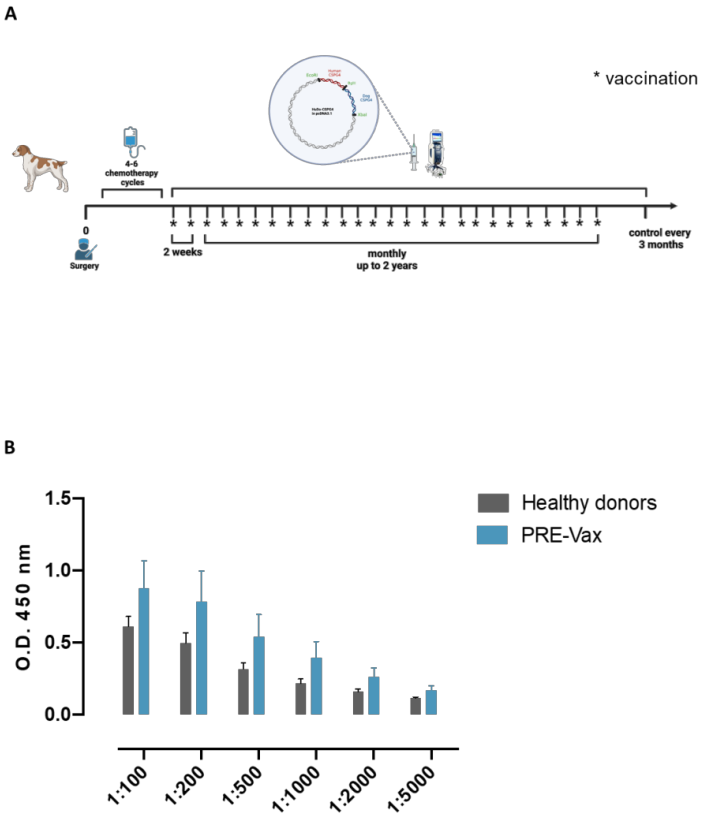




L

Mouse	Tumor volume (mm³)	CSPG4 score (Lung metastasis)
Empty Vector #3	665.5	6
Empty Vector #2	210.93	6
Empty Vector #1	137.31	6
HuDo#1	210.93	n/d

Supplementary Figure S2. Impairment of human OSA tumor growth may be related to CSPG4 downregulation and high $CD8^+$ infiltration. (**A, B**) Tumor growth of U2OS cells injected into NSG mice after adoptive transfer with $CD8^+$ T cells derived from pcDNA3.1 (**A**, Empty vector, N = 6 mice) and HuDo-CSPG4 (**B**, N = 6 mice) vaccinated mice. Each line represents the growth of a single tumor. (**C**) Immunoblot depicting CSPG4, PD-L1 and B7-H3 protein-expression levels in tumors explanted from representative NSG mice following adoptive transfer with $CD8^+$ T cells derived from pcDNA3.1 or HuDo-CSPG4 immunized mice. β-actin was used as the loading control. (**D**) Table summarizing the percentage of $CD3^+$ T cells infiltrating the tumors and the CSPG4 score of expression in lung metastases collected from NSG mice transferred with $CD8^+$ T cells from control or HuDo-CSPG4 immunized mice, considering tumor volume. (**E**) Flow cytometry analysis, showing percentage (%) of $CD8^+$ T cells gated on $CD45^+CD3^+$ cells circulating in the blood of adoptively transferred NSG mice on day 50 after the transfer. (**F**) Flow cytometry analysis of U2OS cells incubated with sera of either C57/BL6 HLA-A2.1 Tg mice or Balb/c mice immunized with the HuDo-CSPG4 plasmid. Total IgG binding was evaluated using a FITC-conjugated goat anti-mouse IgG secondary antibody. Results are expressed as the percentage $\pm$ SEM of CSPG4-positive cells. (**G, H**) Tumor growth of U2OS cells injected into NSG mice and adoptively transferred with pooled sera derived from pcDNA3.1 (**F**, Empty vector, N = 5 mice) and HuDo-CSPG4 (**G**, N = 4 mice) vaccinated mice. Each line represents the growth of a single tumor. (**I**) Representative immunoblot showing CSPG4 protein-expression levels in matched-paired tumors explanted from representative NSG mice following adoptive transfer with sera derived from pcDNA3.1 or HuDo-CSPG4 immunized mice. β-actin was used as the loading control.



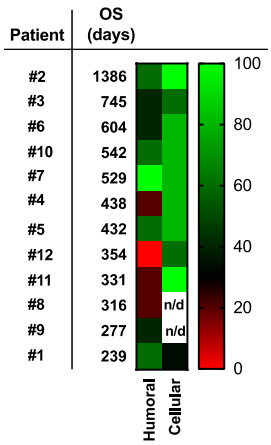
Supplementary Figure S3. Vaccination protocol for OSA-bearing canine patients included in the study. (A) Schematic representation of the study design of adjuvant HuDo-CSPG4 immunization. Illustration was created using BioRender.com. **(B)** Analysis, by means of ELISA assay, of the presence of spontaneous anti-CSPG4 IgG against the D2 of the canine CSPG4 protein in the sera of healthy dogs or in the sera collected from CSPG4^{+} OSA canine patients after the chemotherapeutic protocol with carboplatin and before the starting of the immunization protocol. Sera were analyzed in sequential dilutions, from 1:100 to 1:5000, and results are expressed as optical density (O.D.) measured at 450 nm.

A

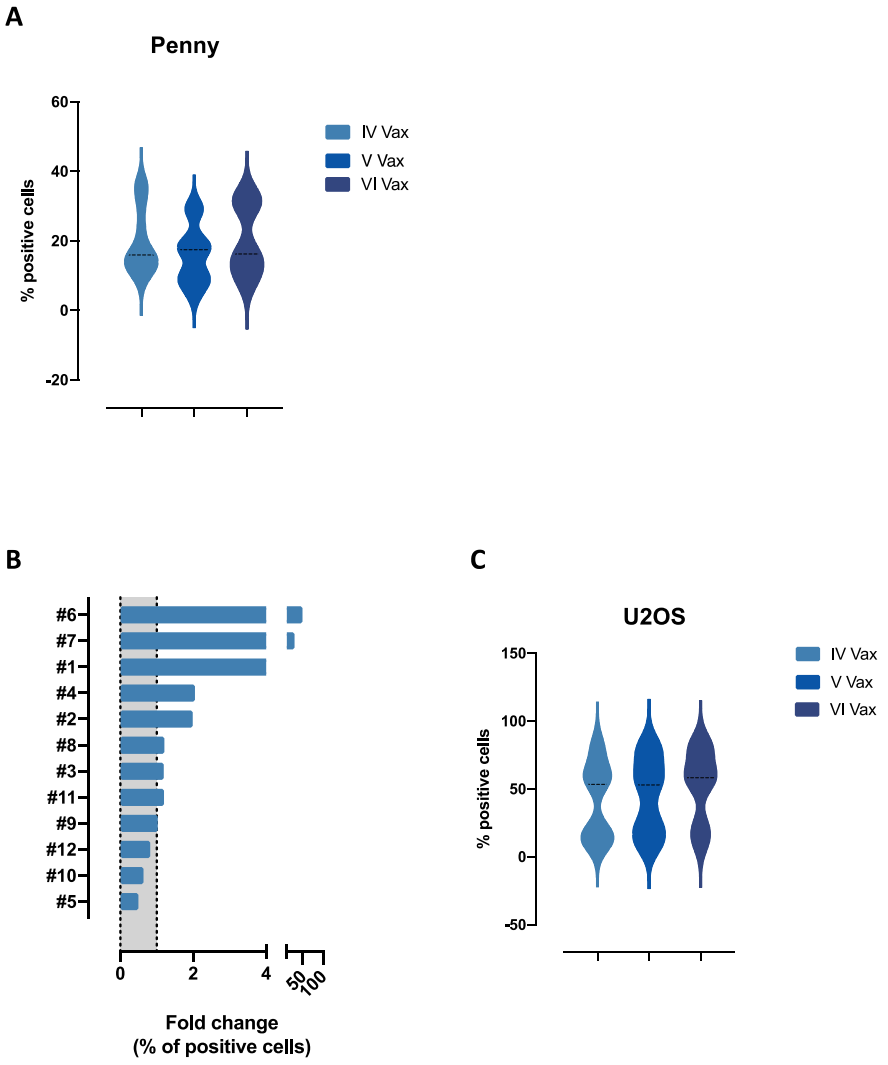
	Humoral response					
Patient	Do-D1 ELISA	Do-D2 ELISA	Do-D3 ELISA	Do-D2 Chaotropic ELISA	Binding to CSPG4+ canine OSA cells	Binding to CSPG4+ human OSA cells
#1	no	yes	no	n/d	yes	yes
#2	no	yes	yes	n/d	no	yes
#3	no	no	no	yes	yes	yes
#4	no	no	no	no	no	yes
#5	yes	yes	no	n/d	yes	no
#6	no	no	no	no	yes	yes
#7	yes	yes	yes	n/d	yes	yes
#8	no	no	no	yes	no	yes
#9	n/d	n/d	n/d	n/d	n/d	yes
#10	yes	yes	yes	n/d	no	no
#11	no	no	no	yes	no	yes
#12	no	no	no	n/d	no	no

	Cellular response				
Patient	MDSC	Treg	$CD8^+$ T cells	In vitro Cytotoxicity (POST-Vax on D22 vs D22-Do-CSPG4)	In vitro Cytotoxicity (PRE-Vax vs POST-Vax on D22 Do-CSPG4)
#1	↑	↓	↓	n/d	n/d
#2	↓	↓	↑	yes	yes
#3	↓	↑	↑	no	yes
#4	↓	↓	↑	yes	no
#5	↓	↓	↓	yes	n/d
#6	↓	↑	↑	yes	yes
#7	↓	↑	↑	yes	yes
#8	n/d	n/d	n/d	n/d	n/d
#9	n/d	n/d	n/d	n/d	n/d
#10	↑	↓	↑	yes	yes
#11	↓	↓	↑	yes	yes
#12	↑	↑	↑	yes	yes

B

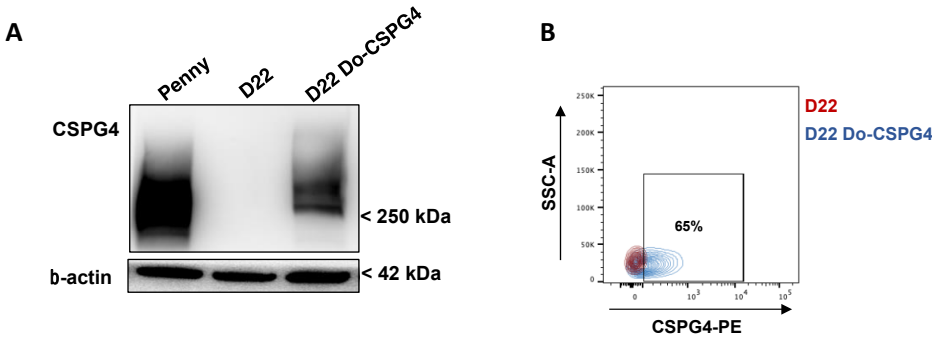


Supplementary Figure S4. Table summarizing HuDo-CSPG4-vaccine-induced immune response. (**A**) Induction of humoral (left panel) and cellular (right panel) immune responses in vaccinated dogs. The arrows indicate an increase or decrease in the percentage of circulating immune cells (myeloid derived suppressor cells, MDSC; T regulatory cells, Treg; and $CD8^+$ T cells). n/d is specified when the analysis was not performed for the indicated sample. (**B**) An immune-response score (between 0 - 100%) has been assigned to each vaccinated canine patient, with 0 indicating the absence of a response and 100 a complete response to the parameters analyzed for both humoral and cellular immunity. The heat map shows the "immune-score" ordered according to the overall survival of vaccinated dogs.

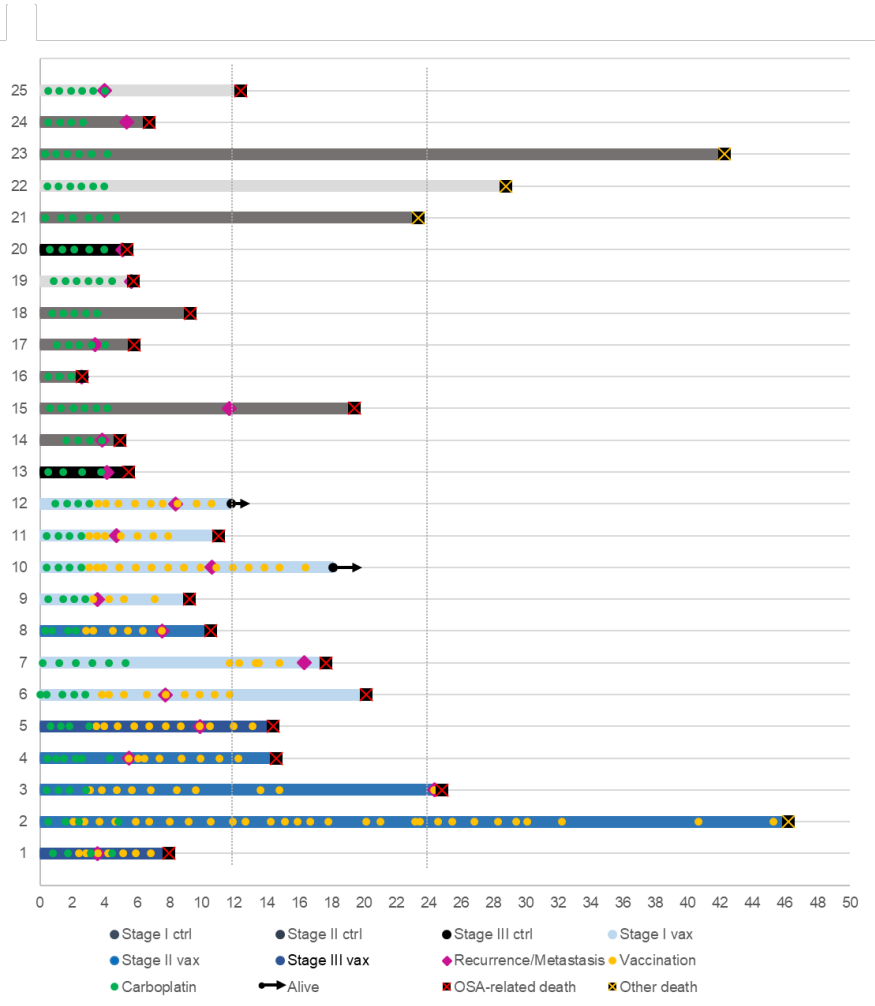


Supplementary Figure S5. HuDo-CSPG4 boosting in dogs induces antibodies that bind the Do- and Hu-CSPG4 proteins. (**A**) Flow cytometry analysis of canine CSPG4$^+$ Penny cells incubated with sera collected after the fourth, fifth and sixth HuDo-CSPG4 vaccinations (IV, V, VI Vax). (**B**, **C**) Flow cytometry analysis of human CSPG4$^+$ U2OS cells incubated with sera collected before vaccination (**B**) and after the fourth (**B**), fifth and sixth (**C**) HuDo-CSPG4 vaccinations (IV, V, VI Vax). Total IgG

binding was evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as the percentage (%) of positive cells (**A**, **C**) and as the ratio (fold change) of the % of stained cells incubated with the Post-Vax (IV)/Pre-Vax sera (**B**).



Supplementary Figure S6. Generation of a canine OSA cell line that stably over-expresses Do-CSPG4. (**A**, **B**) Canine CSPG4^{-} D22 OSA cells were stably transfected with the Do-CSPG4-coding plasmid and resultant CSPG4 over-expression was confirmed by western blot (**A**) and flow cytometry analyses (**B**). (**A**) For western blot assays, naturally CSPG4-over-expressing canine Penny cells were used as the positive control and β-actin was used as the protein loading control. (**B**) D22 (red) and D22 Do-CSPG4 (blue) cells were incubated with anti-CSPG4 mAb (TP-49). Total IgG binding was evaluated using a PE-conjugated goat anti-mouse IgG secondary antibody. Flow cytometry analysis was performed using a FACS Verse and results were analyzed with FlowJo software. A representative plot is shown and the percentages (%) of D22 Do-CSPG4 stained cells are indicated.



Supplementary Figure S7. Swimmer plot. Graph of canine OSA patients included in the study in Arm 1 (grey bars) and Arm 2 (blue bars) depicting survival (in months) and clinical information, including time of surgery, chemotherapy cycles and vaccination treatments. First clinical manifestation of recurrence/metastasis are reported in the graph. Arrows indicate that the patients are still alive at the time of publication. The 1-year and 2-year post-surgery time points have been indicated by a dotted vertical line.

Chapter III

Investigating CSPG4 as a driver gene for OSA-genesis

The findings reported in Chapter II of this thesis, indicate that CSPG4 exerts a key role in sustaining human OSA ((361); Tarone et al., *under revision*).
As a step forward, we wondered whether CSPG4 overexpression could also represent the driver oncogenic event for OSA-genesis. Our hypothesis is that a reduction of CSPG4 expression can occur in pediatric MSCs during their normal differentiation process in mature OSB. However, if the correct OSB maturation doesn't occur, CSPG4 remains overexpressed during the differentiation process allowing the cell to retain a more staminal status and to eventually undergo OSA transformation.

Here, I report very preliminary data that support our hypothesis. We started from the evidence that CSPG4 mRNA is not expressed in normal human OSB that compose the bone, while it is present in pediatric MSCs. A further upregulation in its expression is observed in patients' derived OSA biopsies (361). By means of flow cytometry we have confirmed CSPG4 protein expression in pediatric healthy donors' bone marrow derived (BM)-MSCs, but not in the adults' ones (**Figure 1A**). We then induced MSCs differentiation toward the osteogenic lineage. Differentiation toward an osteoblastic lineage was performed at day 15 by measuring alkaline phosphatase (ALP) activity, a marker of new bone formation, mineralization, and early osteoblasts activity (404). Differentiated cells displayed increased ALP activity as compared to control cells that were maintained in MSCs medium and retained their mesenchymal phenotype (**Figure 1B**). The newly differentiated OSB revealed a consistent decrease of CSPG4 expression, as compared to MSCs, suggesting the loss of the antigen expression during the normal differentiation process (**Figure 1C**). This result suggested the possible role of CSPG4 in sustaining the MSCs phenotype, raising the question whether its retained expression during the differentiation process could be a biomarker of abnormal transformation.

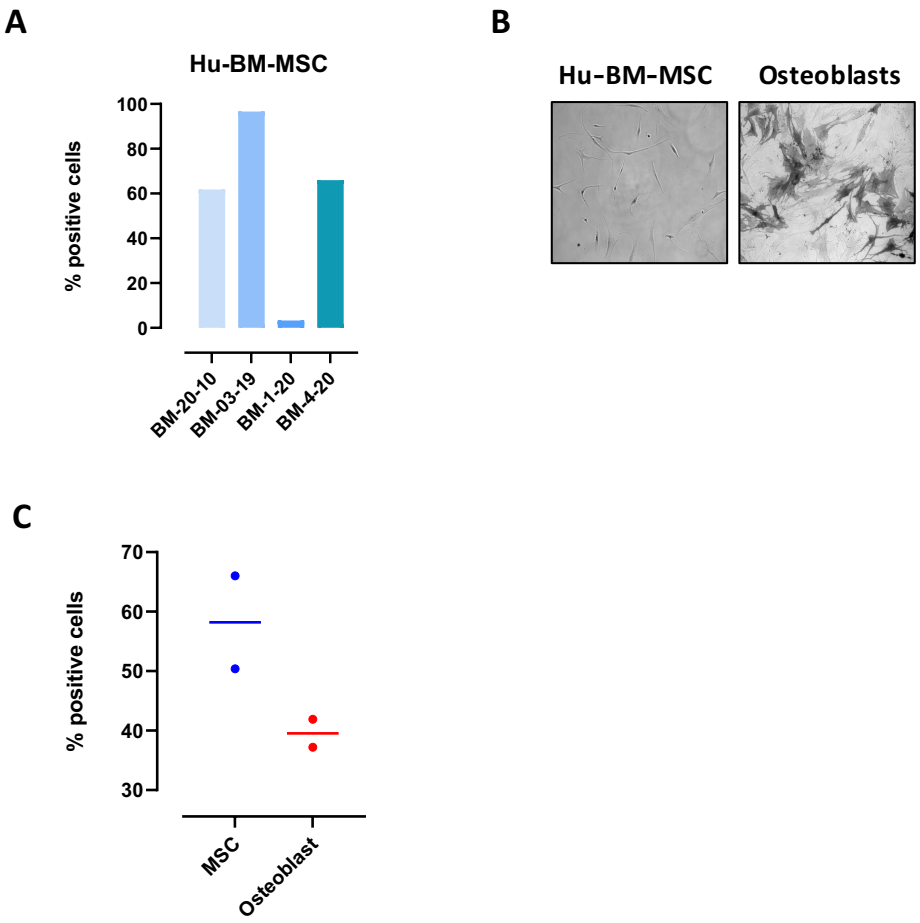


Figure 1. Analysis of CSPG4 expression in human BM-MSC and differentiated OSB. (A) Flow cytometry analysis of human BM-MSC from pediatric (n = 3; BM-20-10, BM-03-19, and BM-4-20) and adult (n = 1; BM-1-20) healthy donors. Results are expressed as the percentage (%) of cells positive for CSPG4 expression. **(B)** Representative images of human BM-MSC (left panel) and differentiated OSB (right panel) after 15-days of culture with osteo-differentiation medium. **(C)** Flow cytometry analysis evaluating CSPG4 expression in human BM-MSC from pediatric patients and the derived differentiated OSB. Results are expressed as the % of cells positive for CSPG4 expression.

Beyond studies for testing novel therapeutic options, canine OSA is a relevant model as well as for studying the implication and consequences of mutations in different driver genes that could trigger OSA development (405). MSCs of canine origin have in fact already been used as a model to identify driver events for OSA initiation, and it has been recently demonstrated that canine MSCs could spontaneously transform after long-term culture, displaying the upregulation of osteogenic markers and the appearance of p53 mutations (406). According to previous findings (reported in the *Introduction* section of this thesis;(90)) suggesting OSB aberrant transformation as the OSA-initiating cells, in this preliminary study we availed of canine OSB as a model to verify our hypothesis.

We stably overexpressed the dog-CSPG4 in primary canine OSB, naturally negative for CSPG4 expression (**Figure 2A**), and the empty vector was used to transfect cells as a control (OSB2-mock). We isolated individual clones, some of which expressed the CSPG4 protein (**Figure 2B,** *left panel*). For subsequent analysis, we selected clone n.1 (Cl.1-CSPG4) and clone n.12 (Cl.12-CSPG4), expressing CSPG4 at different levels (**Figure 2B,** *right panel*)**,** and confirmed its distribution on the cell membrane through immunofluorescence analysis **(Figure 2C).** Of note, Cl.12-CSPG4 expression was close to that of both human and canine OSA cell lines, naturally expressing the antigen **(Figure 2D**).

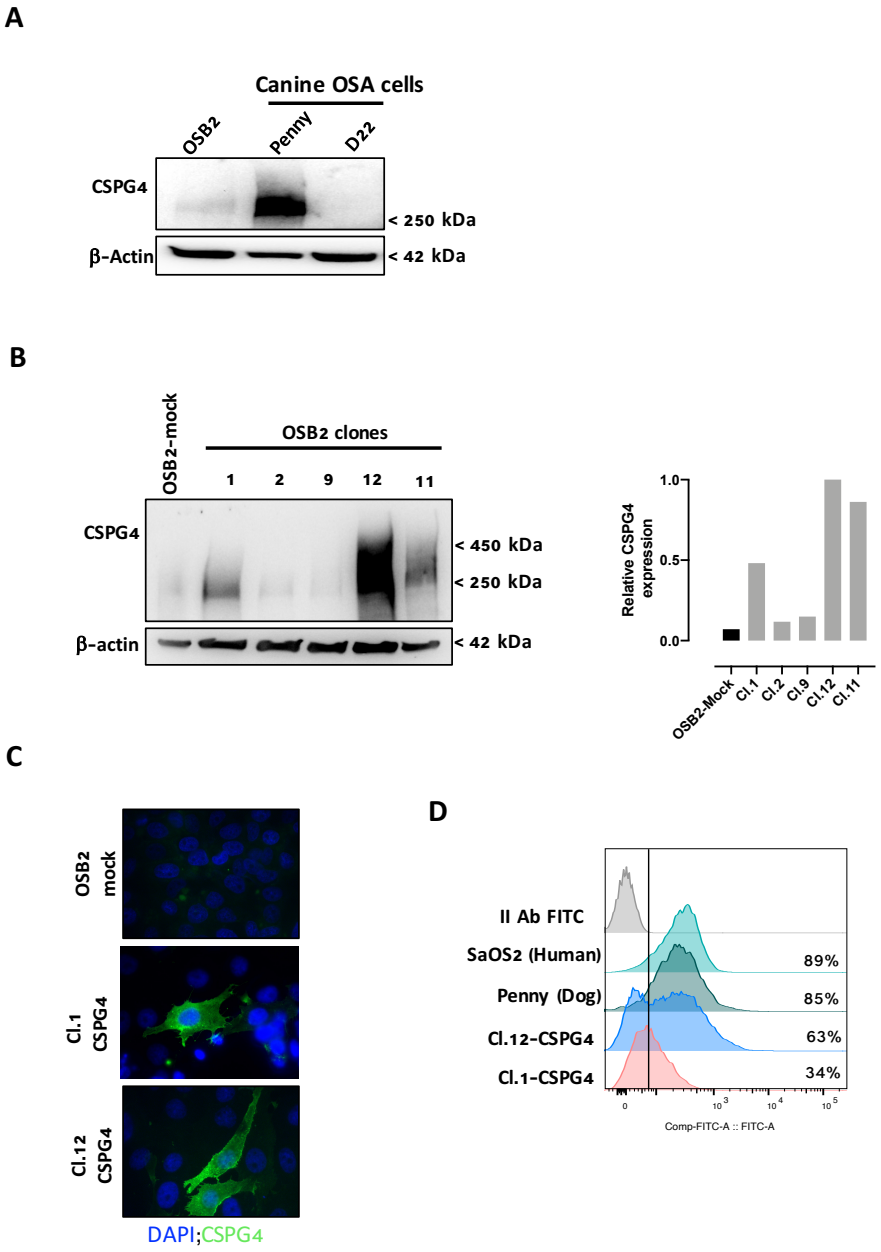


Figure 2. Analysis of CSPG4 expression in OSB-overexpressing clones. (A) Immunoblot of CSPG4 expression in normal canine osteoblasts (OSB2), and two different canine OSA cell lines (Penny, CSPG4-positive; D22 CSPG4-negative). β-actin was used as the protein-loading control. **(B)** Western Blot analysis

of CSPG4 expression in canine OSB2 transfected with the canine CSPG4 protein and obtained by single-cell selection. β-actin was used as the protein-loading control. Relative CSPG4 expression levels were normalized on the β-actin housekeeping protein levels. (**C)** Immunofluorescence analysis of CSPG4 distribution in OSB2-CSPG4 clones Cl.1 CSPG4 and Cl.12 CSPG4 and OSB2 mock negative control. DAPI (blue fluorescence) was used for staining cell nuclei, anti-CSPG4 mAbs were used for staining the canine CSPG4 molecule (green fluorescence). Anti-mouse Alexa Fluor 488 was used as secondary antibody. **(D)** Flow cytometry histograms showing CSPG4 expression in OSB2-overexpressing clones compared to human (SaOS2) and canine (Penny) OSA cells. Results represent the % of cells positive for CSPG4 expression.

Even though we didn't observe any morphological change following CSPG4 overexpression in OSB2, we investigated whether they would have eventually acquired a transformed malignant phenotype by performing *in vitro* functional assays. CSPG4 overexpression induced the loss of osteogenic phenotype, as suggested by a decreased ALP activity in OSB2 clones (**Figure 3A**).
Hence, we analyzed the expression of both epithelial (E-cadherin) and mesenchymal (N-cadherin) markers in CSPG4 overexpressing clones compared to OSB2 wild type and OSB2-mock cells (**Figure 3B**). We observed the loss of E-cadherin, while an increase of the N-cadherin expression (**Figure 3B**) in OSB2 clones, suggesting that CSPG4 expression would sustain a more mesenchymal status. Levels of expression of EMT markers were compared to an established canine primary OSA cell line (Sky;(407)). These results therefore could indicate that CSPG4 retained expression in mature OSB could initiate their transformation, likely sustaining OSA-genesis. As a demonstration, we hence performed functional *in vitro* assays to evaluate if CSPG4 overexpression in normal OSB would have conferred a tumorigenic behavior to this cell type. The CSPG4-overexpressing clones didn't show enhanced proliferative ability, as compared to OSB2-mock controls (**Figure 3C**). However, we observed increased colony formation ability in the clone expressing the higher amount of CSPG4 (**Figure 3D**).
Since we have recently demonstrated that CSPG4 could exert a major role in regulating OSA cell migration, and consequently, lung metastasis colonization in OSA setting (Tarone et al., *under revision*), we also evaluated the impact of its overexpression in promoting these functional properties. Results from transwell migration and invasion assays revealed that only the Cl.12-CSPG4 clone acquired an increased migratory and invasive ability as compared to OSB-mock controls (**Figure 3E; F**), while Cl.1-CSPG4 didn't show the same behavior despite CSPG4 overexpression. We could therefore suppose that only OSB expressing CSPG4 over a critical threshold of expression would have acquired the transformed phenotype.

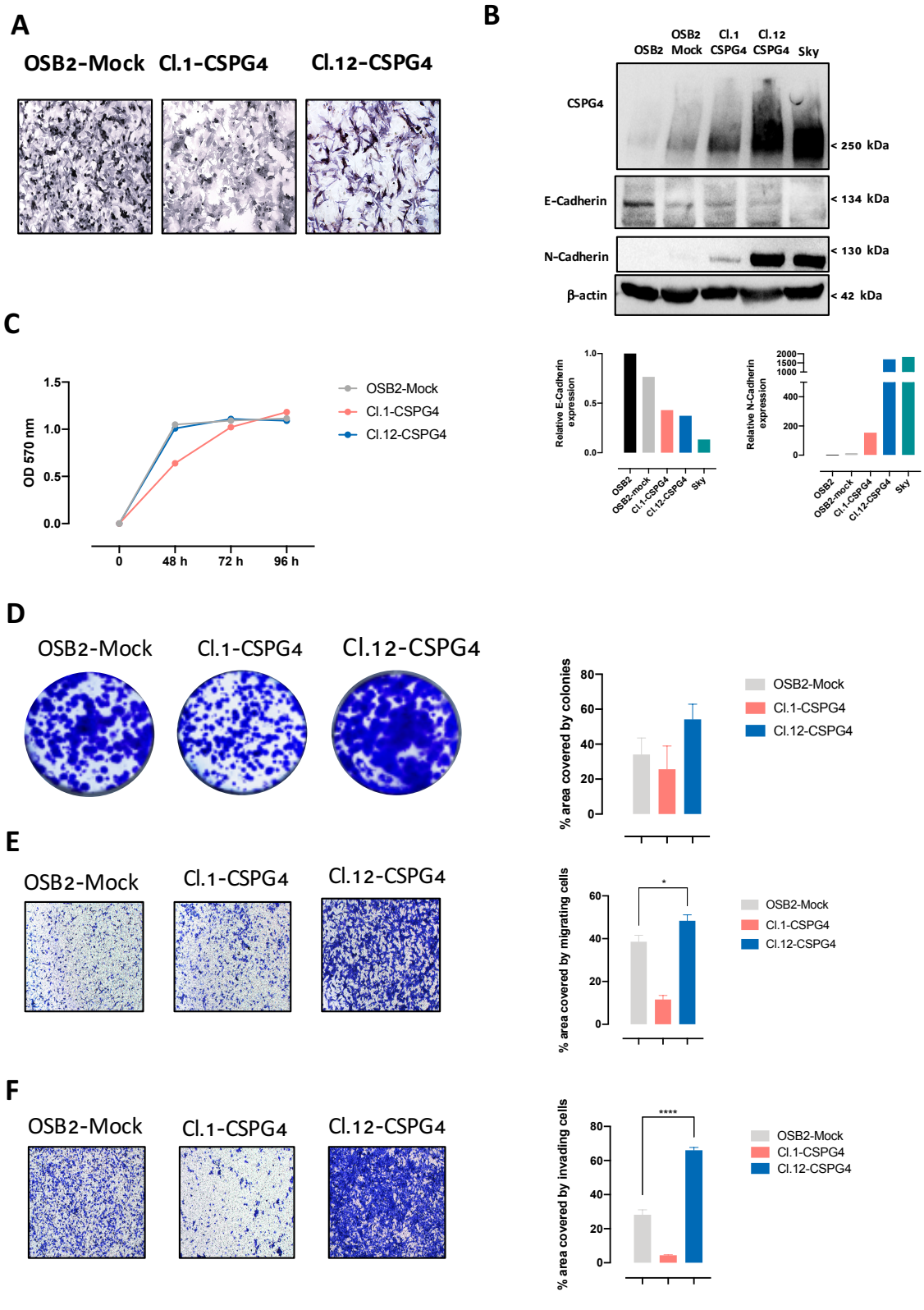


Figure 3. Functional consequences of CSPG4 expression in normal OSB. (A) Representative images showing ALP activity, evaluated through SIGMA FAST BCIP/NBT reagent, in OBS2-overexpressing clones compared to OSB2-mock controls. Images were acquired using an Olympus BX41 microscope. **(B)** Immunoblot of CSPG4 expression and EMT markers in normal canine osteoblasts (OSB2), OSB wild type cells, and OSB2-mock control. Sky cell line was used as positive control for the expression of mesenchymal markers. β-actin was used as protein-loading control. Relative E/N-cadherin expression

levels were normalized on the β-actin housekeeping protein levels. **(C)** OSB2 cell proliferation was assessed using the MTT assay, and the results of three biological replicates are expressed as the optical density (O.D.), measured at 570 nm**. (D)** OSB2 colony-forming ability was assessed by plating 3000 cells/well for 15 days, then the cells were fixed with 2.5% Glutheraldehyde (Sigma-Aldrich) and stained with 0.2% Crystal Violet (Sigma-Aldrich). The number of colonies were counted by using Fiji Software. **(E; F)** OSB2 migratory **(E)** and invasive **(F)** ability were assessed using Transwell assay. Invasion assay was performed by previously coating the transwell insert with Matrigel**.** Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. Representative images of one of at least three experiments. The percentage (mean ± SEM) of the area covered by migrated cells in five different fields are reported in the graphs. Student's t test, *p = 0.0317; **** p< 0.0001

These first *in vitro* results confirmed the involvement of CSPG4 in directing key malignant tumor-related processes, especially cancer cells migration and invasion, envisaging a possible tumorigenic behavior also *in vivo*.

Other experiments to define whether CSPG4 could be a first driver of OSA-genesis, or if somehow the pairing of CSPG4 expression together with common alterations such as p53 and/or Rb inactivation could have a synergistic effect in driving OSA onset should be performed. Nonetheless, these first results could likely suggest that the retained CSPG4 expression on MSC toward lineage differentiation into mature OSB could lead to the generation of OSA, opening the possibility of investigating early therapeutic interventions for OSA management.

Concluding remarks

I have always wanted to study cancer, and in my mind, I'd grow up as a scientist, contributing to the discovery of novel therapies to its cure. Unfortunately, this achievement is harder than I thought when I was a young girl, but since I am working in this laboratory, I have had the opportunity to contribute to projects that foresee the development of an effective anti-tumor therapy.

Immunotherapy really represents a breakthrough in cancer management, and this field is constantly evolving. When speaking about immunotherapy, general attention is directed toward ICIs; however, the immunotherapy field is wider and comprises different valuable anti-cancer strategies. Among these, DNA vaccines emerge with several advantages, as discussed in this thesis; nevertheless, their potential has not totally been exploited for cancer management yet. Although DNA vaccines have been widely investigated in the past, even demonstrating positive results in pre-clinical models of different cancers, interest is slowly diminished to make place to more complex tools. Though, in their simplicity, DNA vaccines for anti-tumor purposes are still promising; hence, as a redeem, the papers and preliminary data presented in this thesis suggest the potential of using the chimeric HuDo-CSPG4 DNA vaccine for the treatment of two aggressive cancers, MM and OSA.

The results presented in this thesis demonstrate that DNA vaccine targeting CSPG4 is safe and able to induce anti-tumor immunity in relevant pre-clinical models of human cancers. In these studies, we availed of pet dogs spontaneously developing these tumors for testing anti-CSPG4 immunotherapy, to underline the strength of this approach, with the hope of its future translation for the treatment of human patients.

Of course, despite the positive achievements reported, further improvements are required.

As we have robustly demonstrated the immunogenicity and the efficacy of the HuDo-CSPG4 vaccine for treating canine OMM, a further important step would be the testing of different administration routes for the vaccine delivery. The current standard vaccination protocol includes the use of electroporation with the need of general anesthesia in treated animals. The possibility of using transdermal and/or intradermal needle-free injection devices, would open the possibility of avoiding anesthesia and pave the way to develop a suitable vaccination protocol that could be more easily and broadly applied to a wider canine population and more straightforwardly translated in a human setting.

Concerning OSA, there is still room for improvement in the probability of achieving better therapeutic results in canine patients. The evaluation of a different vaccination schedule, as for example starting the vaccination alongside chemotherapy treatment, would allow better identification of the best therapeutic window in which operate to counteract early metastatic dissemination. Indeed, in the current protocol that we have tested, canine patients started the vaccination one month or more after the surgical removal of the tumor, at the end of chemotherapy cycles. Despite a significant efficacy of the vaccine in the first year, its potential is becoming modest at later time points. The potential of the HuDo-CSPG4 vaccine to counteract metastatic spread could be exploited earlier in the therapeutic interventions to achieve a better long-term cure. Since the potential of our vaccination has not been tested in the setting advanced disease, in both MM and OSA, we also envisage to extend our inclusion criteria to metastatic canine patients to investigate the ability of HuDo-CSPG4 vaccination in the treatment of already established metastatic lesions.

Also, we are currently evaluating the possibility of extending our anti-CSPG4 DNA vaccination for the treatment of other CSPG4+ tumors. We are starting to investigate CSPG4 expression in canine patients affected by hemangiosarcoma, a very common and aggressive cancer in dogs that develops from blood vessels. The possibility of applying our vaccine to other tumor histotypes could represent a priceless opportunity to treat a still wide portion of oncological patients who cannot benefit from the standard available treatments. As a step forward, in the future we could even explore our anti-CSPG4 vaccine in combination with other (immune)therapies such as ICIs, or using adjuvants, to potentiate the anti-tumor efficacy of our strategy.

Finally, *in vivo* experiments by injecting CSPG4 overexpressing OSB in immunodeficient mice will be performed, to allow us to understand if CSPG4 represents a driver gene of OSA-genesis. Based on our preliminary results obtained in canine OSB, we could suppose that the same mechanisms could be observed in the human setting, and this will be investigated in the near future.

My work always makes me feel grateful for contributing to this research, and I hope that these findings will lead to advancing cancer patients' lives in the future.

Abbreviations

ACT Adoptive T-cell treatment

ADCC Antibody-dependent cellular cytotoxicity

ADCP Antibody-dependent cellular phagocytosis

ALP Alkaline phosphatase

APC Antigen presenting cell

ASC Adult stem cells

B7H3 B7 homolog 3 protein

BCG Bacillus Calmette-Guerin

BM-MSCs Bone marrow derived-mesenchymal stem cells

BMSC Bone marrow stromal cell

BovH4 Bovine herpesvirus 4

CAR Express Chimeric Antigen Receptors

CDC Complement-dependent cytotoxicity

CDKN Cyclin-Dependent Kinase Inhibitor

CDX Cell line-derived xenograft

CNS Central nervous system

CS Chondroitin sulfate

CSPG4 Chondroitin suplhate proteoglycan 4

CT Cancer-testis

CTL Cytotoxic T cells

CTLA-4 Cytotoxic T-lymphocyte-associated antigen-4

DAMP Damage-associated molecular pattern

DC Dendritic cells

DFI Disease-free interval

DMBA 1,3-dimethylbutylamine

ECM Extracellular matrix

ESC Embryonic stem cells

FDA Food and Drug Administration

GEMMs Genetically engineered mouse models

HBV Hepatitis B virus

HER 2 Human epidermal growth factor receptor 2

HGF Hepatocyte growth factor

HMW-MAA High Molecular Weight-Melanoma Associated Antigen

HPV Human papillomavirus

HSV-1 Herpes simplex type-1 virus

HSVtk Herpes simplex thymidine kinase

ICIs Immune checkpoint inhibitors

IFN Interferon

IGF-1R Insulin- like growth factor receptor-1

IL Interleukin

iPSC Induced pluripotent stem cells

KLH Keyhole limpet hemocyanin

L-MTP-PE Liposome-encapsulated lipophilic derivative of muramyl dipeptide

LAG-3 Lymphocyte activation gene-3

Lm Listeria monocytogenes

mAb Monoclonal antibody

MAPK Mitogen-activated protein kinases

MCSP Melanoma chondroitin sulfate proteoglycan

MDSC Myeloid derived suppressor cells

MEK1/2 MAPK enzymes

MEKi MEK inhibitors

MHC Major Histocompatibility Complex

MM Malignant melanoma

MSC Mesenchymal stem cells

MST Median survival time

NCI National Cancer Institute

NG2 Neuro-glia antigen 2

NK Natural killer

OMM Oral malignant melanoma

OS Overall survival

OSA Osteosarcoma

OSB Osteoblasts

OV Oncolytic viruses

OVA Ovalbumin

PAMP Pathogen-associated molecular pattern

PAP prostatic acid phosphatase

PD-1 Programmed death-1

PD-L1 Programmed death-ligand 1

PDGFR Platelet-derived growth factor receptor

PDX Patients-derived xenograft

PPTP Pediatric Preclinical Testing Program

PSA Prostate-specific antigen

RTK Receptor tyrosine kinase

RUNX2 RUNX Family Transcription Factor 2

SF Scatter factor

SG Suicide gene

STING Stimulators of interferon genes

SV40 Simian virus 40

TAA Tumor associated antigen

TBK-1 TANK-Binding Kinase-1

Th T helper

TIGIT T cell immunoglobulin and ITIM domain

TIL Tumor infiltrating lymphocytes

TIM-3 T cell immunoglobulin and mucin-domain containing-3

TLR Toll like receptor

TME Tumor microenvironment

TPA Phorbol ester 12-O-tetradecanoylphorbol-13-acetate

TRAIL Tumor necrosis factor related apoptosis inducing ligand

Treg T regulatory cells

TSA Tumor specific antigen

TWT Triple wild type

Tyr Tyrosinase

USDA United States Department of Agriculture

UV ultra-violet

VLP virus like particles

List of publications related to this thesis

1. **Tarone L.,** Giacobino D, Camerino M, Maniscalco L, Iussich S, Parisi L, Giovannini G, Dentini A, Bolli E, Quaglino E, Merighi IF, Morello E, Buracco P, Riccardo F[#], Cavallo F[#]; A chimeric human/dog-DNA vaccine against CSPG4 induces immunity with therapeutic potential in comparative pre-clinical models of osteosarcoma; *Under revision*

2. **Tarone L.,** Mareschi K., Tirtei E., Giacobino D., Camerino M., Buracco P., Emanuela Morello E., Cavallo F.,*,Riccardo F.*; "Improving Osteosarcoma Treatment: Comparative Oncology in ActionOncology in Action". Life (Basel, Switzerland), 2022 December; Life (Basel, Switzerland), doi: 10.3390/life12122099, 12(12):209. IF 2021/2022 3.251

3. Riccardo F., **Tarone L**., Camerino M., Giacobino D., Iussich S., Barutello G., Arigoni M., Conti L., Bolli E., Quaglino E., Merighi IF., Morello E., Dentini A., Ferrone S.#, Buracco P. #, Cavallo F.# "Antigen mimicry as an effective strategy to induce CSPG4-targeted immunity in dogs with oral melanoma: a veterinary trial". Journal for Immunotherapy of Cancer, 2022 May; 10(5):e004007, doi: 10.1136/jitc-2021-004007. IF 2021/2022: 12.469

4. **Tarone L**., Giacobino D., Camerino M., Ferrone S., Buracco P., Cavallo F.* and Riccardo F.* "Canine Melanoma Immunology and Immunotherapy: Relevance of Translational Research". Frontiers in Veterinary Science. 2022; 9: 803093. Published online 2022 Feb 11. doi: 10.3389/fvets.2022.803093. IF 2021/2022: 3.471

5. Camerino M., Giacobino D., Manassero L., Iussich S., Riccardo F., Cavallo F., **Tarone L**., Olimpo M., Lardone E., Martano M., Del Magno S., Buracco P., Morello E. "Prognostic impact of bone invasion in canine oral malignant melanoma treated by surgery and anti-CSPG4 vaccination: A retrospective study on 68 cases (2010–2020)". Veterinary and Comparative Oncology, 2021, Vol. 2021, p. 1-9, doi: 10.1111/vco.12761. IF 2021: 2.385

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7. **Tarone L**., Barutello G., Iussich S., Giacobino D., Quaglino E., Buracco P., Cavallo F., Riccardo F. "Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy". Cancer Immunology, Immunotherapy, 1 November 2019, Volume 68, Pages 1839-1853. IF 2021/2022: 6.630

8. Riccardo F., **Tarone L**., Iussich S., Giacobino D., Arigoni M., Sammartano F., Morello E., Martano M., Gattino F., De Maria R., Ferrone S., Buracco P., Cavallo F. "Identification of CSPG4 as a promising target for translational combinatorial approaches in osteosarcoma". Therapeutic Advances in Medical Oncology, 1 June 2019, Volume 11. IF 2021/2022: 5.485

9. Barutello G., **Tarone L**., Buracco P., Cavallo F., Riccardo F. "Strengths and weaknesses of pre-clinical models for human melanoma treatment: dawn of dogs' revolution for immunotherapy". International Journal of Molecular Sciences, 10 March 2018, Volume 19, Article number 799. IF 2021/2022: 6.208

Book Chapters

Tarone L., Buracco P., Cavallo F. & Riccardo F. "Canine Melanoma and Osteosarcoma Immunotherapy by Means of In Vivo DNA Electroporation". In: Impellizeri J.A. (eds) Electroporation in Veterinary Oncology Practice. Springer, Cham. ISBN 978-3-030-80667-5 Online ISBN 978-3-030-80668-2

List of publications not included in this thesis

1. Riccardo F., Barutello G., Petito A., **Tarone L**., Conti L., Arigoni M., Musiu C., Izzo S., Volante M., Longo D.L., Merighi I.F., Papotti M., Cavallo F., Quaglino E. "Immunization against ROS1 by DNA electroporation impairs K-ras-driven lung adenocarcinomas". Vaccines, April 2020, Volume 8, Article number 166. IF 2021/2022: 4.961

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List of activities performed during the PhD program

National and international meetings

Poster and oral presentations as first author

1. **Lidia Tarone,** Davide Giacobino, Mariateresa Camerino, Maddalena Arigoni, Selina Iussich, Soldano Ferrone, Emanuela Morello, Paolo Buracco, Federica Riccardo*, Federica Cavallo*, "Evaluation of the efficacy of anti-CSPG4 immune-targeting for the treatment of osteosarcoma: a comparative study". PIVAC 22; 26-28 **September 2022**, Turin, Italy.
2. **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Maddalena Arigoni, Selina Iussich, Soldano Ferrone, Emanuela Morello, Paolo Buracco, Federica Cavallo*, Federica Riccardo*. **"**Investigating the potential of anti-CSPG4 immune-targeting for the treatment of osteosarcoma: a comparative study". EACR 2022; **June 2022**. Seville, Spain.
3. **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Maddalena Arigoni, Selina Iussich, Soldano Ferrone, Emanuela Morello, Paolo Buracco, Federica Cavallo*, Federica Riccardo*. **"**Investigating the potential of anti-CSPG4 immune-targeting for the treatment of osteosarcoma: a comparative study". XII National Congress SIICA; **May 2022,** Napoli, Italy.
4. **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Selina Iussich, Laura Conti, Maddalena Arigoni, Giuseppina Barutello, Elisabetta Bolli, Elena Quaglino, Soldano Ferrone, Paolo Buracco, Federica Cavallo* and Federica Riccardo*. "Targeting the CSPG4 for the treatment of melanoma and osteosarcoma through a chimeric Human/Dog CSPG4 DNA vaccine". 29th IGB Workshop "Targeting the (un)usual suspects in cancer"; **December 2021.** Virtual Conference.
5. **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Selina Iussich, Laura Conti, Maddalena Arigoni, Giuseppina Barutello, Elena Quaglino, Soldano Ferrone, Paolo Buracco, Federica Cavallo* and Federica Riccardo*. "A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma and osteosarcoma". The V Think Tank of the Italian Network for Tumor Bio-Immunotherapy (NIBIT); **October 2021.** Virtual Conference.
6. **Lidia Tarone**, Davide Giacobino, Selina Iussich, Paolo Buracco, Federica Cavallo, Federica Riccardo. "Investigating the role of CSPG4 as a target for immunotherapy in osteosarcoma", CancerTo, Nanoscience in cancer immunotherapy; **March 2021**. Virtual Conference.
7. **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Selina Iussich, Soldano Ferrone, Paolo Buracco, Federica Cavallo*, Federica Riccardo*; Investigating CSPG4 as a novel immunotherapeutic target for the treatment of osteosarcoma; EACR 2021, 27TH CONGRESS OF THE EUROPEAN ASSOCIATION FOR CANCER RESEARCH; **Oral presentation by L. Tarone,** 9th-12th June 2021, Virtual conference
8. **Lidia Tarone**, Maddalena Arigoni, Selina Iussich, Davide Giacobino, Soldano Ferrone, Paolo Buracco, Federica Cavallo* and Federica Riccardo*. "Exploring the efficacy of anti-CSPG4 targeting for the treatment of osteosarcoma: a comparative study". EACR conference, Defence is the Best Attack: Immuno-Oncology Breakthroughs; **February 2021**. Virtual Conference.
9. **Lidia Tarone**, Federica Riccardo, Maddalena Arigoni, Selina Iussich, Davide Giacobino, Francesca Gattino, Soldano Ferrone, Paolo Buracco and Federica Cavallo. "Evaluation of the efficacy of anti-CSPG4 targeting as a promising strategy for the treatment of

osteosarcoma". 15th EACR Conference on A Matter of Life or Death; **'flash talk' poster presentation of top-scoring abstracts by L. Tarone. February 2020**. Bergamo, Italy.

10. **Lidia Tarone**, Federica Riccardo, Selina Iussich, Davide Giacobino, Francesca Gattino, Soldano Ferrone, Paolo Buracco and Federica Cavallo. "CSPG4 IMMUNE-TARGETING AS A PROMISING STRATEGY FOR TRANSLATIONAL STUDIES IN PRE-CLINICAL MODELS OF CSPG4+ TUMORS". PIVAC; **June 2019**. **Oral Presentation by L.Tarone.** Athens, Greece.

As co-author

11. Federica Riccardo, **Lidia Tarone**, Mariateresa Camerino, Davide Giacobino, Selina Iussich, Giuseppina Barutello, Laura Conti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog DNA vaccine against the Chondroitin Sulfate Proteoglycan 4 as an effective strategy to induce CSPG4-targeted immunity for the treatment of melanoma". EACR 2022; **June 2022**. Seville, Spain.
12. Federica Riccardo, **Lidia Tarone**, Mariateresa Camerino, Davide Giacobino, Selina Iussich, Giuseppina Barutello, Maddalena Arigoni, Laura Conti. Elisabetta Bolli, Irene Merighi, Emanuela Morello, Elena Quaglino, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric human/dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma: a veterinary trial". XII National Congress SIICA; **May 2022,** Napoli, Italy.
13. Federica Riccardo, **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Giuseppina Barutello, Elisabetta Bolli, Selina Iussich, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma". EACR 2021 Congress, Innovative Cancer Science: Better Outcomes through Research; **June 2021.** Virtual Conference.
14. Federica Riccardo, **Lidia Tarone**, Mariateresa Camerino, Davide Giacobino, Selina Iussich, Giuseppina Barutello, Maddalena Arigoni, Elisabetta Bolli, Laura Conti, Elena Quaglino, Emanuela Morello, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma". ESVONC; **May 2021. Oral presentation by F.Riccardo.** Virtual Conference.
15. Federica Riccardo, **Lidia Tarone**, Mariateresa Camerino, Davide Giacobino, Selina Iussich, Giuseppina Barutello, Maddalena Arigoni, Elisabetta Bolli, Laura Conti, Elena Quaglino, Emanuela Morello, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog DNA vaccine against the Chondroitin Sulfate Proteoglycan 4 reveals potential therapeutic effects for the treatment of melanoma". XII National Congress SIICA; **May 2021.** Virtual Conference.
16. Federica Riccardo, **Lidia Tarone**, Davide Giacobino, Mariateresa Camerino, Giuseppina Barutello, Maddalena Arigoni, Elisabetta Bolli, Selina Iussich, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma". CancerTo, Nanoscience in cancer immunotherapy; **March 2021**. Virtual Conference.
17. Federica Riccardo, **Lidia Tarone**, Giuseppina Barutello, Maddalena Arigoni, Davide Giacobino, Selina Iussich, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects in canine and human melanoma patients". 15th EACR Conference on A Matter of Life or Death; **February 2020**. Bergamo, Italy.
18. Federica Riccardo, **Lidia Tarone**, Davide Giacobino, Angela Petito, Sergio Occhipinti, Paolo Buracco, Federica Cavallo, "Electroporation of a CSPG4 hybrid plasmid-based

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19. Federica Riccardo, Lidia Tarone, Giuseppina Barutello, Maddalena Arigoni, Davide Giacobino, Selina Iussich, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "Anti-CSPG4 DNA vaccination as a promising strategy for the treatment of CSPG4+ tumors: a comparative oncology trial". ESMO 2019; **September 2019**. Barcelona, Spain.

20. Federica Riccardo, Giuseppina Barutello, **Lidia Tarone**, Maddalena Arigoni, Davide Giacobino, Selina Iussich, Sergio Occhipinti3, Angela Petito, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "Anti-CSPG4 DNA vaccination reveals potential therapeutic effects for the treatment of CSPG4+ tumors: a comparative oncology study". 31th Pezcoller Symposium; **June 2019**. Trento, Italy.

21. Federica Riccardo, Giuseppina Barutello, **Lidia Tarone**, Maddalena Arigoni, Valeria Rolih, Elisabetta Bolli, Sergio Occhipinti, Soldano Ferrone, Elena Quaglino, Paolo Buracco, Federica Cavallo. "Anti-CSPG4 DNA vaccination as a promising immunotherapy for canine and human oncology patients". Scientific Meeting "Scuola Dianzani", MOLECULAR PATHOLOGY IN EXPERIMENTAL AND TRANSLATIONAL MEDICINE". **Oral presentation** by F. Riccardo**. November 2018**, Torino, Italy.

22. Federica Riccardo, Giuseppina Barutello, **Lidia Tarone**, Valeria Rolih, Elisabetta Bolli, Maddalena Arigoni, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "ANTI-CSPG4 DNA VACCINATION FOR THE TREATMENT OF MALIGNANT MELANOMA: A COMPARATIVE ONCOLOGY TRIAL FOR TRANSLATIONAL MEDICINE". IT-Meeting 2018 Designing the next generations of cancer immunotherapies. **Oral presentation** by F. Riccardo. **October-November 2018**, L'Avana, Cuba.

23. Federica Riccardo, Giuseppina Barutello, **Lidia Tarone**, Valeria Rolih, Elisabetta Bolli, Maddalena Arigoni, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "DNA ELECTROVACCINATION WITH A CHIMERIC CSPG4 PLASMID FOR THE TREATMENT OF MALIGNANT MELANOMA IN DOGS: A COMPARATIVE ONCOLOGY TRIAL FOR TRANSLATIONAL MEDICINE". 60th Annual Meeting of the Italian Cancer Society (SIC), **September 2018**, Milano, Italy.

24. Federica Riccardo, Giuseppina Barutello, **Lidia Tarone**, Valeria Rolih, Elisabetta Bolli, Maddalena Arigoni, Sergio Occhipinti, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "ANTI-CSPG4 DNA VACCINATION FOR THE TREATMENT OF MALIGNANT MELANOMA: A COMPARATIVE ONCOLOGY TRIAL FOR TRANSLATIONAL MEDICINE. Pezcoller Symposium; **June 2018**, Trento, Italy.

25. Federica Riccardo, Giuseppina Barutello, Valeria Rolih, Davide Giacobino, Maddalena Arigoni, Sergio Occhipinti, **Lidia Tarone**, Selina Iussich, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "DNA ELECTROVACCINATION WITH A CHIMERIC CSPG4 PLASMID FOR THE TREATMENT OF MALIGNANT MELANOMA IN DOGS". **Oral presentation** by F. Riccardo. 72° CONVEGNO SISVET, **June 2018**, Torino, Italy.

26. Federica Riccardo, Giuseppina Barutello, Valeria Rolih, Elisabetta Bolli, Maddalena Arigoni, Sergio Occhipinti, **Lidia Tarone**, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "DNA electrovaccination with a chimeric CSPG4 plasmid for the treatment of malignant melanoma in dogs: a comparative oncology trial for translational medicine". EFIS-EJI Ruggero Ceppellini Advanced School of Immunology. Course on: Tumour

Immunology: from tissue microenvironment to immunotherapy. **October 2017,** Napoli, Italy.

27. Federica Riccardo, Giuseppina Barutello, Valeria Rolih, Elisabetta Bolli, Maddalena Arigoni, Sergio Occhipinti, **Lidia Tarone**, Soldano Ferrone, Paolo Buracco, Federica Cavallo. "DNA electrovaccination with a chimeric CSPG4 plasmid for the treatment of malignant melanoma in dogs: a comparative oncology trial for translational medicine". The 2nd World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies. **Oral presentation** by F. Riccardo**. September 2017,** Norfolk (VA) – USA.

Awards

- **Immuno-oncology Innovation Award 2022**, Awarded by Milteny Biotech June 2022

Courses

- **20-21 October 2022**, GISM-Gruppo Italiano Staminali Mesenchimali-Conference 2022, Torino
- **1-4 February 2021,** Basics of project writing, held by Doctoral School of the University of Torino
- **November 2018**, Scuola Dianzani, Molecular Biotechnology Center, Torino
- **8-12 June 2019**: Summer School in Immuno-oncology, Athens, Greece
- **March 2019:** Course "Introduction to ImageJ/Fiji", held by Marta Gai, MBC Turin.

Seminars attended during the PhD course

- Department seminar, 4 July 2022, Molecular Biotechnology Center, University of Turin; *Oral Presentation*
- Immunology seminars (on-line) series, year 2021-2022; Organizer: Prof. F. Novelli
- Reacting to inflammatory stimuli: Notch and TLR4 tailor hematopoiesis in the bone marrow niche; Molecular Biotechnology Center, University of Turin; Speaker: Nadia Carlesso, Host: Prof. F. Novelli, online seminar
- **Guido Tarone day,** 16th May 2022, online seminar
- Prof. Tak Mak "Beyond immune checkpoint blockade: emerging strategies"; Prof. Gerry Melino "ON THE CONTROL OF FEMALE FIDELITY-How p63 controls female germline fidelity-"; 7th September 2021
- Bridging the gap between academia and industry, Working with a PhD for NOVONORDISK, Novo Nordisk Good Practices, 18th March 2021
- Basics of project writing (certificate of attendance in attachment), 1st-4th February 2021
- New perspectives in immunology, IISCA school, 16th December 2020
- The role of diacylglycerol kinases in immunity and cancer, Andrea Graziani, 04 December 2020
- The new checkpoint molecules that regulate anti-tumor immunity, Nature research custom media, 13th October 2020
- Carbon-Based Nanomaterials: From Nanosafety to Nanomedicine, Prof. Bengt Fadeel, Ins6tute of Environmental Medicine,Karolinska Institutet, Stockholm. Host: I. Fenoglio
- Undestanding the nanomaterials interaction with biomolecules, Relevance for Nanomedicine and Nanotoxicity, Prof. Marco Monopoli, Royal College of Surgeons in Ireland, Host: I. Fenoglio

- WEBINAR SERIES AT DEPARTMENT OF MOLECULAR BIOTECHNOLOGY AND HEALTHY SCIENCES, 08-15-22 July 2020
- Webinar "Immuno-oncologia e saggi di interazione in ambito oncologico", Perkin Elmer , 04 July 2020 Webinar "Providing a solid foundation for simpler and smarter flow cytometry panel design", BD Bioscience, 28 April 2020
- Pharmacology of pancreatic cancer, novel approaches using solid-liquid models, Elisa Giovannelli, 04 February 2020. Host: F. Novelli
- The genomic R-evolution interplay of coding and non-coding genes in transcrip6onal dynamics, Francesco Nicassio, 25 November 2019. Host: V. Poli
- Nouscom neo-antigen based cancer vaccines, Morena D'Alise from Nouscom, Rome. 30 July 2019. Host: Valeria Poli
- The Development and utility of VLP platform for novel vaccines immunotherapy, Jerri Caldeira from Agilvax, Albuquerque, 20 June 2019. Host: Federica Cavallo
- Modulation of gene expression by therapeutic antibodies, Nasser Salameh from Agilvax, Houston. 20 June 2019. Host: Federica Cavallo
- Aptamers for the selective targeting of therapeutic RNAs, De Franciscis Vittorio from the University of Naples, 31 January 2019. Host: Daniela Taverna
- Roles of ABC transporters: biomarkers of a multi-stress resistant phenotype in cancer cells, Chiara Riganti, 16 January 2019. Host: Paola De Filippi
- Lipid accumulation in the pancreatic tumor microenvironment drives metabolic exhaustion of CD8+ T cells, Teresa Manzo. 14 December 2018. Host: Francesco Novelli

- Unleashing canine comparative oncology, Luca Aresu from Department of Veterinary Sciences, University of Turin. 6 December 2018.
- The role of HMGB1 in anticancer immune responses, Marco Bianchi from San Raffaele Hospital. 27 November 2018. Host: Valeria Poli.