Aus dem Department für Kleintiere und Pferde
der Veterinärmedizinischen Universität Wien
(Departmentsprecher: O.Univ.-Prof. Dr.med.vet. Joerg Aurich Dipl.ECAR)
Fach: Onkologie

INFLUENCE OF CD146 EXPRESSION ON CANINE MELANOMA CELL GROWTH AND MIGRATION

DER EINFLUSS DER EXPRESSION VON CD146 AUF DAS WACHSTUM UND DIE MIGRATION BEI CANINEN MELANOM ZELLEN

DIPLOMARBEIT
zur Erlangung der Würde einer
MAGISTRA MEDINICAE VETERINARIAE
der Veterinärmedizinischen Universität Wien

vorgelegt von
Forte Sarah

Wien, im November 2019

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Abbreviations and units

The following abbreviations and units are used throughout the text:

AA ..... Antibiotic/Antimykotic
BRAF ..... gene that encodes a protein called B-raf
B-raf ..... oncogene, protein kinase (raf- rapidly accelerated fibrosarcoma)
BRAFMAP kinase/ ERK ..... extracellular signal-regulated kinases
BSA ..... bovine serum albumin
CAMs ..... cell adhesion molecules
CD146 ..... cluster of differentiation 146
cells/ml ..... cells per milliliter
CGH ..... comparative genomic hybridization
cm ..... centimeter
CM ..... complete medium
COMM ..... canine oral malignant melanoma
CO_2 ..... carbon dioxide
°C ..... degrees centigrade
DMEM ..... Dulbecco's Modified Eagle Medium
DNA ..... deoxyribonucleic acid
EDTA ..... ethylenediaminetetraacetic
et al. ..... et alii/aliae/alia
FBS ..... fetal bovine serum
FCS ..... fetal calf serum
FDA ..... Food and Drug Administration
Fig. ..... Figure
FISH ..... fluorescence in situ hybridization
FNAB ..... fine needle aspiration biopsy
g/l ..... gram per liter
Gy ..... Gray
h ..... hours
i.e. ..... id est
IgG ..... immunoglobulin

IgSF ..... immunoglobulin superfamily
INK4a/ARF ..... alternate reading frame protein
KIT, c-KIT ..... tyrosine protein kinase
kVp ..... Peak kilovoltage
Melan A ..... melanoma antigen
min ..... minutes
MITF ..... Mikrophthalmia-associated transkription factor
ml ..... milliliter
mg/kg ..... milligram/kilogram
mg/m² ..... milligram per square meter
mM ..... millimolar
NEDD9 ..... NEED9 gene encoding protein
neg. ..... negative
Nm ..... nanometer
NRAS ..... enzyme encoded by NRAS gene
N-RAS ..... protein made by the NRAS gene
PBS ..... phosphate buffered saline
PKC ..... proteinkinase C
pos. ..... positive
PTEN ..... Phosphatase and Tensin homolog
RACK 1 ..... receptor of activated protein C kinase 1
RGO ..... research group oncology
Rpm ..... revolutions per minute
S-100 ..... calcium-binding protein
Tab. ..... Table
TNM ..... tumor, lymph node, metastasis
UVA ..... ultraviolet A
UVB ..... ultraviolet B
UVR ..... UV radiation
WHO ..... World Health Organization
µg/ml ..... micrograms per milliliter
µl ..... microliter

1 Introduction

Every year, about 50.000 humans die from malignant melanoma and the incidence of this cancer disease has continuously increased over the past 30 years [Nishiya et al., 2016]. According to Vail & MacEwen (2000) companion animals with naturally occurring tumors should be used to investigate aspects of malignancy, from etiology to treatment. Cancer is the number one cause of death in dogs. The autopsies of 2000 dogs have shown that 23% of all dogs and 45% of dogs older than 10 years will die of cancer [Vail & MacEwen, 2000].

1.1 Melanoma

Melanoma is a malignant neoplasm arising from activated or genetically altered epidermal melanocytes. Melanocytes are epithelial cells, that derive from neural crest and can be found in vertebrates. They produce the skin-protective pigment, melanin [Sulaimon & Kitchell, 2003]. Many genes that are involved in the development of melanocytes are also co-responsible for the development of melanoma [Uong & Zon, 2010]. Through a complex process melanoma development starts with the transformation of melanocytes to nevi, that can switch to a radial growth phase, and ultimately a vertical growth phase typical of the metastatic phenotype [Zheng et al., 2010].

Melanoma constitutes a significant health problem in humans and its incidence has continued to grow in contrast to most cancers [Hicks & Flaitz, 2000]. As stated by Hicks & Flaitz (2000), of 75 people born in the year 2000, one will develop cutaneous melanoma in his/her lifetime despite the fact that prevention and early diagnosis of precursor melanocytic lesions has improved.

Besides in humans, melanocytic neoplasms occur in many domesticated animals including cats, dogs and horses. As dogs live in close companionship with humans, they are exposed to the same environmental factors, which may in turn lead to similar disease [Nishiya et al., 2016].

According to the World Health Organization nomenclature, melanoma refers to a melanocytic malignancy, whereas melanocytoma refers to a benign tumor of melanocytic origin [Goldschmidt et al., 1998].

In humans, melanoma is the most lethal form of skin cancer [Riker et al., 2010]. Sun exposure, especially to UVB light, mucosal pigmentation characteristics and family history are major risk factors that play a role in its development [Nishiya et al., 2016]. Human

melanomas show high metastatic potential and can spread to virtually any organ, even years after resection of the primary tumor [Lei et al. 2015].

1.2 Biology and Function of Melanocytes

It is important to understand the biology of melanocytes to improve treatment modalities for melanoma and other melanocytic disorders. Melanocytes are dendritic cells of neural crest cell origin, excluding retinal pigment cells which develop from the optic cup of the forebrain. They occur in the dermis and the basal layer of the epidermis [Sulaimon & Kitchell, 2003]. Due the close contact between melanocytes and surrounding keratinocytes via their dendritic processes, melanocytes are able to carry out their main function of producing and delivering melanin-containing melanosomes to these keratinocytes. Therefore, melanin is the main determinant of skin color and assists in protection against damage caused by ultraviolet radiation [Sulaimon & Kitchell, 2003]. Epidermal melanocytes and keratinocytes establish the so-called “epidermal melanin unit”. Melanin is synthesized from tyrosine and is composed of two pigments, eumelanin and pheomelanin [Sulaimon & Kitchell, 2003].

Although eumelanin and pheomelanin are present in various intensities in human skin, eumelanin is the common melanin in people with brown or black hair, whereas pheomelanin is abundant in people with reddish hair [Meredith & Sarna, 2006].

The amount of cysteine in the cell is crucial for eumelanin and pheomelanin production. Most melanin occurs as mixed melanin containing both eumelanin and pheomelanin [Sulaimon & Kitchell, 2003]. After the production of melanin, the melanosomes are transferred to the adjoining keratinocytes. In humans, the pigmentation of skin and hair depends on the size, number and distribution of melanosomes and the chemical nature of melanin [Sulaimon & Kitchell, 2003].

Alpha (α)-melanocyte stimulating hormone (αMSH) and agouti signal protein are factors known to stimulate the quality and quantity of melanin. They adjust the expression of genes encoding melanosomal enzymes, which modulate the eumelanin/pheomelanin switch. αMSH fosters eumelanin synthesis and agouti signal protein promotes pheomelanin synthesis [Sulaimon & Kitchell, 2003].

Gene-related melanogenic metalloenzymes, namely tyrosinase, tyrosinase-related protein 1 and tyrosinase-related protein 2, are involved in the catalytic control of melanogenesis. The different degrees of skin and hair pigmentation are determined by individual constitutive genetic programs and can be facultatively increased by ultraviolet radiation exposure. There

are two categories of facultative pigmentation, i.e. immediate pigment darkening and delayed pigment darkening. The translocation of melanosomes from perinuclear areas to dendrites is responsible for immediate pigment darkening occurring within seconds following UVA (320-400 nm) exposure and resolves in one to three days. In the case of delayed pigment darkening, the number of melanocytes as well as the number of melanosomes increases in melanocytes and keratinocytes. Delayed pigment darkening occurs within two to three days after UVB (290-320 nm) exposure [Sulaimon & Kitchell, 2003]. An important stimulus for facultative pigmentation in humans is UVR. Since UVR is the most potent stimulus regarding growth and differentiation of melanocytes, it seems to be a melanocytic mutagen [Sulaimon & Kitchell, 2003].

Melanin pigment protects against radiation and so prevents sun-induced skin damage and skin cancer development. Melanin has a photoprotective role attributed to its ability to directly absorb ultraviolet photons and reactive oxygen molecules [Sulaimon & Kitchell, 2003]. Kobayashi et al. (1993) found that epidermal cells with supranuclear caps of melanin have less DNA photoproducts than epidermal cells without melanin caps. Another argument that melanin provides epidermal photoprotection is the fact that poorly pigmented skin is far more prone to injury caused by UVR than pigmented skin [Sulaimon & Kitchell, 2003].

1.3 Pathological Aspects of Canine Melanoma

Melanocytic neoplasms can be divided into benign melanocytomas and malignant melanomas. Malignant melanoma arises from melanocytes that normally reside within the basal layer of the epidermis [Nishiya et al., 2016]. Melanocytomas, the benign counterparts, are composed of melanocytes in the basal region of the epithelium, extending into the submucosa or into the sub-epithelial connective tissue underneath normal epithelium (dermal melanocytoma) [Nishiya et al., 2016]. In dogs, benign melanomas usually occur as single, pigmented, firm and moveable structures. Still it is very difficult to differentiate between benign and malignant tumors [Nishiya et al., 2016]. Therefore, cytogenetic methods, including fluorescence in situ hybridization (FISH) analysis as well as comparative genomic hybridization (CGH), have become important methods for discrimination [Zembowicz & Scolyer, 2011].

According to Nishiya et al. (2016) three different tumor cell patterns are encountered in dogs:

- epithelioid—round and polygonal cells
- spindle cell—tumor resembles fibroblasts
- mixed tumors—show both cell types

One third of all melanomas in dogs are amelanotic malignant melanomas and a histopathological diagnosis is not easy if the tumor does not contain melanin. Hence, immunohistochemistry should be used for the diagnosis of canine malignant melanomas [Nishiya et al., 2016].

1.4 Tumor Staging of Canine Melanoma

The size of the primary tumor is an important prognostic factor [Owen, 1980]. The World Health Organization (WHO) published a tumor staging scheme for canine oral melanoma [Owen, 1980].
It considers the size of the tumor: stage I = <2 cm diameter tumor, stage II = 2 - <4 cm diameter tumor, stage III = ≥4 cm diameter tumor and/or lymphnode metastasis, and stage IV = distant metastasis [Owen, 1980].

Stage, size, evidence of metastasis, and a variety of histological criteria are negative prognostic factors [Bergman, 2007; Nishiya et al., 2016].

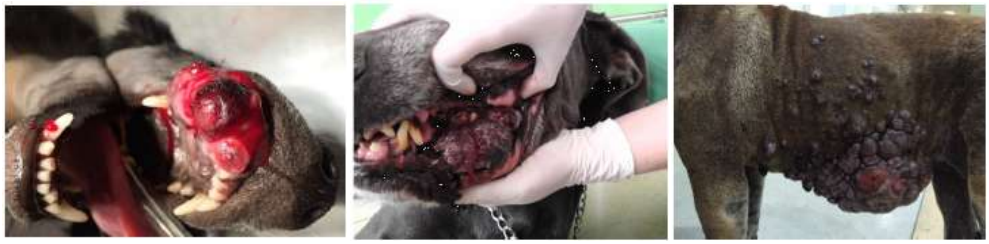
The following modalities can be used for staging:

- FNAB of regional lymphnodes for cytological evaluation
- Biopsy for histological evaluation
- Chest radiography
- Magnetic resonance imaging of the brain
- Ultrasonography for diagnosis of lymph node involvement

- Computed tomography of the chest, abdomen, or pelvis
- Positron emission tomography
[https://www.cancer.org/treatment/understanding-your-diagnosis/advanced-cancer/diagnosis.html; 24.05.2019]

Further diagnostic investigations are recommended, particularly because melanoma has a tendency to grow invasively and will spread to regional lymph nodes which can go undetected by palpation alone [Bostock, 1979; Modiano et al., 1999; Williams & Packer, 2003].

1.5 Melanoma in Dogs



(A) *(B)* *(C)*

Fig. 1 Clinical aspect of malignant oral melanomas (A) in maxilla; (B) in mandible; and (C) in thoracic and abdominal wall [Nishiya et al., 2016].

Melanomas in dogs and humans show many behavioral similarities especially regarding their response to treatment [Vail & MacEwen, 2000; Nishiya et al., 2016]. Therefore, canine melanoma is an excellent model for comparative studies [Westberg et al., 2013].

In dogs, melanomas represent 2 to 9% of all malignant tumors and are common in middle-aged and older dogs >10 years of age [Ritt et al., 1998; Vail & MacEwen, 2000; Westberg et al., 2013]. The main characteristic of these tumors is the cellular pleomorphism [Campagne et al., 2013].

The risk factors for canine melanoma are unknown but consanguinity, trauma, chemical exposure, hormones and genetic susceptibility are believed to play a role [Nishiya et al.,

2016]. Sunlight may promote the development of melanoma on sun-exposed areas, such as the face and ears, but solar radiation is unlikely responsible for mucosal melanoma in the oral cavity [Nishiya et al., 2016]. It seems that genetics may be of importance because prevalence in purebred dogs is higher [Priester, 1973; Gillard et al., 2014].

Canine melanomas are often found in the oral cavity, as well as on the skin, eyes and digits but unlike melanoma in humans, they are probably not associated with sun exposure [Vail & MacEwen, 2000]. Some breeds are considered to be at risk for the development of melanoma and often the location of the neoplasm is specific to the breed [Goldschmidt, 1994; Marino et al., 1995; Bergman et al., 2013].

Breeds in which melanoma is most commonly observed are: Scottish Terrier, Poodle, Golden Retriever, Dachshund, Cocker Spaniel, Miniature Poodle, Chow Chow, Gordon Setter, Anatolian Sheepdog breeds, Airedale Terrier, Boston Terrier, Boxer, Chihuahua, Doberman Pinscher, Rottweiler and Labrador Retriever [Priester, 1973; Gillard et al., 2014; Bergman et al., 2013].
The Schnauzer family, Scottish Terriers and Irish Setters are predisposed to develop digital melanomas [Goldschmidt, 1994; Marino et al., 1995].
In most cases, the prognosis depends on the anatomic location of the primary tumor [Sulaimon & Kitchell, 2003; Esplin, 2008].

Vail & MacEwen (2000) compared several reports regarding the survival time of dogs suffering from oral malignant melanoma. They found that most dogs die 8-10 months after surgery or radiation alone, depending on the stage of melanoma. Dogs with a tumor <2 cm in diameter (stage I, lymph node negative) had a median survival time of 16-18 months.
Patients with a tumor 2-5 cm in diameter (stage II, lymph node negative) had a median survival time of 10 months while patients with metastasis in the regional lymph nodes had a 4 month median survival time [Vail & MacEwen, 2000].

1.6 Therapy

Depending on stage, size and localization of the primary tumor, general health condition and age of the patient and financial status of the owner, there are different types of treatment for

canine melanoma patients, which include surgery, radiation therapy, chemotherapy, immunotherapy and targeted therapy [Nishiya et al., 2016].

1.6.1 Surgery

Early stage melanomas can often be treated with surgical excision alone [Bergman, 2007; Spangler & Kass, 2006; Esplin, 2008]. Adequate excision -margins should be accomplished following known guidelines [Proulx el al., 2003]. In cases where this is not possible due to the anatomical location or because of metastasis, a multimodality treatment should be considered [Bergman, 2007; Brockley et al., 2013]. Whenever possible, invasive melanomas should be removed by radical surgical procedures. This means that the tumor should be excised down to the muscle fascia and also radical procedures such as maxillectomy or mandibulectomy must be considered depending on the localization of the primary tumor [Proulx et al., 2003].
In dogs, where the complete resection was achieved based on histological evaluation, the median survival time is between 354 days and 34 months. The recurrence rate ranges from 3.2% to 8.3% [Esplin, 2008; Boston et al., 2014]. The removal of regional lymph nodes is justified since, as Williams & Packer (2003) showed, 53% of dogs with oral melanomas, cytologic and/or histologic evidence of mandibular lymph node metastases could be found, even if they were not enlarged.

1.6.2 Radiation Therapy

Radiation therapy kills cancer cells and shrinks tumors by damaging their deoxyribonucleic acid (DNA). Damaged DNA includes death of cancer cells but days to weeks of treatment are needed until the DNA is damaged enough for cancer cells to die. [https://www.cancer.gov/about-cancer/treatment/types/radiation-therapy]
Hence, radiation therapy can be used to control local tumors and the involved lymph nodes or as palliative treatment for partial remission of the mass, to control pain, and thus to increase quality of life [Nishiya et al., 2016; Cunha et al., 2018]. Radiation therapy is more effective the smaller the tumor. Furthermore, the presence of osteolysis is a crucial factor [Nishiya et al., 2016]. In a retrospective study by Proulx et al. (2003), it was shown that the rate of distant metastasis in canine oral melanomas remained the significant limiting factor in

terms of treatment, regardless of the radiation therapy protocol used (36 Gy, 9 Gy x 4 fractions; 30 Gy, 10 Gy x 3 fractions; or 245 Gy, 2-4 Gy x 12-19 fractions). In contrast Cunha et al. (2018) describes a difference in the type of radiation equipment used. Orthovoltage machines generate X-rays with an energy of 150-500kVp, while megavoltage machines (cobalt-60 and linear accelerators) radiate photons of energy greater than 1.000.000 volts. The produced photon energy is the most important difference between orthovoltage and megavoltage machines. By using megavoltage machines, a greater amount of energy accumulates below the surface of the skin, while low-energy photons, produced by orthovoltage machines, deposit the dose of the radiation on the surface of the skin. Consequently, megavoltage is recommended for deeper tumors [Cunha et al., 2018].

Nishiya et al. (2016) reported that for melanomas treated with radiation, median time to progression varies from 3.6 to 7.9 months, while median survival time varies from 5.3 to 11.9 months. Several studies have shown that chemotherapy, combined with radiation therapy, may potentially slow local progression and/or improve overall viability [Nishiya et al., 2016].

In a retrospective study, Freeman et al. (2003) described that the median survival time of 39 dogs that received chemotherapy (cisplatin or carboplatin) as adjunctive therapy due to incomplete surgical excision, before radiation, was on average 363 days.

1.6.3 Chemotherapy

Although the real benefit of chemotherapy for canine melanoma is yet not well-established, the use of systemic therapy is recommended, because in spite of correct local therapy like surgery and radiation therapy, the final cause of death is due to metastasis [Nishiya et al., 2016; Cunha et al., 2018].

Commonly used chemotherapeutics for canine melanoma are carboplatin and cisplatin, which both have a broad spectrum of anti-neoplastic properties. Carboplatin and cisplatin stop the proliferation of cancer cells. But also healthy cells are affected, especially those that proliferate quickly, such as blood cells. Thus, the most common side effect of these agents is myelo-suppression.

[https://www.netdoctor.co.uk/medicines/cancer-drugs/a7319/carboplatin/]

Brockley et al. (2013) examined the median survival time in 63 canine patients diagnosed with oral, digital or cutaneous melanoma after treatment with surgical resection, additional carboplatin therapy and palliative therapy using carboplatin combined with piroxicam.

Piroxicam, a non-steroidal anti-inflammatory drug, is known to have an antitumor activity in several tumors in dogs and humans [Boria et al., 2004]. Several studies did not show a significant improvement of survival time by treatment with carboplatin, neither as an addition to surgery nor as palliative treatment, whereas the anatomic site was significantly associated with survival time. [Boria et al., 2004; Rassnick et al., 2001; Proulx et al., 2003; Murphy et al., 2005; Brockley et al., 2013]

1.6.4 Immunotherapy

According to Nishiya et al. (2016) the immune response to therapeutic vaccines seems to be a prospective, efficient method to treat melanoma, since melanoma is a highly immunogenic tumor [Verganti et al., 2017].

Since 2010 there is an approved therapeutic vaccine (OnceptTM, Merial, Duluth, GA, US) available to treat COMM [Bergman & Wolchok, 2008]. This vaccine contains a plasmid - DNA-targeting tyrosinase, a glycoprotein essential for melanin synthesis. This glycoprotein is over-expressed in melanomas [Verganti et al., 2017].

Grosenbaugh et al. (2011) found that canine patients that received the vaccine had a significantly improved median survival time compared with those of historical controls. Verganti et al. (2017) studied the median survival time in 56 dogs with macroscopic disease, melanoma stage I to III, treated with Oncept©. The median survival time was 455 days after diagnosis [Verganti et al., 2017], similarly to the results from Ottnod et al. (2013). In contrast, Ottnod et al. (2013) showed that canine patients that received the Oncept© vaccine, achieved no greater progression free survival time, disease-free interval, or median survival time than patients that did not receive the vaccine.

The Oncept© vaccination is very well tolerated and might be also considered as palliative treatment in patients with macroscopic disease, when surgery or radiation is not possible or for patients with advanced disease. At this time, the vaccine is only licensed for treatment of stages II/III, after achieving local tumor control through surgery or radiation therapy [Verganti et al., 2017].

The use of dendritic cells as a biological adjuvant appears to be promising but, according to different studies, there are limitations such as the cost of vaccine production for veterinary use although thus far there is not enough data available [Nishiya et al., 2016].

1.7 Tumor Markers in Humans

Tumor markers are substances produced by cancer cells or by the body, as a response to cancer [Nagpal et al., 2016]. These are normally proteins and can be found mainly in the urine and blood of patients with cancer [Nagpal et al., 2016].
Tumor markers are rarely helpful for screening purposes, because most markers have not been shown to detect cancer before clinical symptoms are noticeable. Treatment control is one of the most important uses of the markers, in regard to the marker level [Nagpal el al., 2016].

According to Kashani-Sabet (2014), many immunohistochemical markers used to assess melanocytic neoplasms, are unreliable. Several markers are still being usedto differentiate melanocytic from non-melanocytic neoplasms and meanwhile a multimarker assay was developed to assist in distinguishing melanocytic neoplasm from nevi [Kashani-Sabet, 2014]. Kashani-Sabet (2014) stated that molecular markers could be used in the prognostic assessment of patients but further analysis is indicated.

1.7.1 Vimentin

Vimentin is an intermediate filament and is expressed by mesenchymal cells ubiquitously [Satelli & Li, 2011].
Vimentin has been identified as a marker for epithelial-mesechymal transition (EMT) because it is a mesenchymal marker [Thiery, 2002]. Epithelial-mesenchymal transition is a process that allows a polarized epithelial cell to undergo multiple biochemical changes that permit it to take over a mesenchymal cell phenotype, which includes elevated migratory capacity, invasiveness, and increased resistance to apoptosis [Kalluri & Neilson, 2003]. On the one hand, EMT occurs during implantation, embryogenesis, and organ development and on the other hand it is associated with tissue regeneration and organ fibrosis but also with cancer progression and metastasis [Kalluri & Weinberg, 2009]. Vimentin not only serves as a diagnostic marker for the primary tumor but also acts as a predictor of hematogenous metastasis [Satelli & Li, 2001].

1.7.2 CD146

Cluster of differentiation 146 (CD146), also named melanoma cell associated molecule (MCAM/Mucin 18 (MUC18)/S-Endo 1)- due to the characteristic of CD146, was first discovered in metastatic melanomas [Lehmann et al., 1989]. It is a Ca^{2+}-independent cell adhesion molecule and belongs to the immunoglobulin superfamily (IgSF) [Oka et al., 2012; Nodomi et al., 2016].

1.7.2.1 Physiological and Pathological Aspects of CD146

As already mentioned, CD146 is a cell adhesion molecule and is essential for cell-cell and cell-matrix interactions, cell migration, cell cycle, and signaling as well as morphogenesis during development and tissue regeneration [Trzpis et al., 2007; Wang & Yan, 2013]. CD146 can be found in endothelium, smooth muscle, Schwann cells, and ductal and myoepithelial cells of salivary glands in humans [Shih et al., 1998].
Increased CAMs can also be discovered in a variety of pathological processes, such as inflammation, pathogenic infections, autoimmune disease and cancer [Trzpis et al., 2007]. Deregulation of their expression combined with changes in cellular morphology and tissue architecture, results in alterations in intercellular adhesion. These effects are considered indicators for malignancy and contribute to deranged cellular interactions characteristic of cancer [Abou Asa et al., 2016].
According to Zeng et al. (2012), increased CD146 expression was found in different carcinomas, as well as in melanomas in humans. It was reported that the overexpression of CD146 in primary cutaneous melanoma cells enhances adhesion between tumor cells as well as interactions between tumor cells and endothelial cells, and decreases tumor cell adhesion to laminin, thus increasing tumor invasiveness [Xie et al., 1997]. Wang & Yan (2013) proposed a combined treatment strategy of anti-CD146 immunotherapy with other anti-angiogenetic drugs may be a promising anti-cancer modality.

1.8 Molecular Markers for Melanoma in Dogs

In melanocytic tumors melanin could be considered as a marker for melanoma but around one third of all melanoma cases in dogs are amelanotic malignant tumors [Nishiya et al.,

2016]. Thus, diagnosis should be made using immunohistochemistry [Sandusky et al., 1985; Nishiya et al., 2016], especially due to melanoma's microscopic appearance, that resembles carcinomas, sarcomas, lymphomas and osteogenetic tumors [Ramos-Vara et al., 2000].

1.8.1 S-100 Protein

S-100 protein is a highly acidic protein that received its name due to its solubility in saturated ammonium sulfate solution at a neutral pH [Sandusky et al., 1985].
Sandusky et al. (1985) examined the dissemination of S-100 in normal tissue and found that no positive staining was noticed in the liver, heart, kidney, pancreas, spleen, thymus, stomach, duodenum, or colon. Yet in melanocytes of the epidermis, some sweat glands and ducts of the skin, myoepithelial cells of the breast, stellate cells of the pituitary, in Peyer's patches in the ileum, chondrocytes of the trachea and interdigitating reticulum cells in the lymph node, positive staining was observed [Sandusky et al., 1985].
Sandusky et al. (1985) compared different skin tumors (i.e. reticulum cell carcinoma, mast cell tumor, fibrosarcoma, mammary gland adenocarcinoma, histiocytoma, transmissible venereal tumor, and thyreoid adenocarcinoma) regarding the specificity of S-100 for melanoma. Sandusky et al. (1985) considered S-100 an excellent marker for malignant melanoma as it was identified in all melanoma biopsy specimens and was absent in a wide variety of other common skin tumors.
S-100 protein is considered to be very robust and its staining is very sensitive (76%), but its specificity is low, due to its reactivity with different tissues [Ohsie et al., 2008; Ramos-Vara et al., 2000].

1.8.2 Vimentin

According to Smith et al. (2002), almost all, if not all, canine melanomas are vimentin positive whereas Ramos-Vara et al. (2000) reported a reactivitiy to vimentin in 100% of the tumors (122 canine oral melanomas plus seven metastatic melanomas with unknown primary site).

1.8.3 Melan A

Melan A is a specific melanocytic differentiation antigen, a cytoplasmatic protein recognized by cytotoxic T cells. Melan A can be found in more than 92% of melanomas but it is also expressed in non melanotic tumors of the adrenal glands and gonads. Nevertheless, melan A is one of the most commonly used markers for melanoma [Ramos-Vara et al., 2000; Smith et al., 2002,].
Loss of melan A expression in melanomas might influence the immune system because it is an immunodominant antigen [Koenig et al., 2001].

1.8.4 CD146

Many clinicopathological studies have been performed in order to identify factors that may predict the biological behavior of canine cutaneous melanocytic tumor [Smedley et al., 2011]. According to Abou Asa (2017), CD146 is weakly expressed in normal canine skin, vascular smooth muscle, endothelial cells within the dermis and the cytoplasm of epidermal cells, including basal and squamous cells and melanocytes. In addition, it was found in the external and internal root sheath cells of hair follicles whereby the intensity of CD146 labelling was stronger in the internal root sheath cells. Sebocytes and arrector pili muscle cells were moderately positive for CD146 while ductal cells were strongly positive, and pilomatrical cells were negative. CD146 was localized in the cell-membrane and cytoplasm of neoplastic cells. It was significantly upregulated in squamous cell carcinoma. CD146 was significantly overexpressed in 23 of 33 oral melanomas (69.7%) whereas the majority of cutaneous melanomas (4/7) had only weak expression [Abou Asa, 2017]. However, the expression of CD146 is higher in oral and cutaneous melanomas compared to ocular tumors and could be found more often in male dogs than in female dogs [Abou Asa, 2017].

To the best of our knowledge there are no previous studies about the effect of CD146 expression on growth and invasion/migration of canine melanoma cells.
We suggested that in contrast to a CD146 depleted cell population, a CD146 enriched cell population will enhance the migratory and/or invasive potential of COMM cells *in vitro.*

The aim of this study was to analyze whether CD146 enrichment has a positive effect on cellular proliferation and melanoma cell migration/invasion.

2 Materials and Methods

In the present study, five canine melanoma cell lines, established by B. Pratscher at the Research Group Oncology (RGO) of the Department of Companion Animals and Horses, University of Veterinary Medicine, Vienna, were investigated. All specimens were obtained with the owner's permission and in accordance with the institutional guidelines. Another canine melanoma cell line, the control cell line, was kindly provided by Prof. Modiano [Ritt et al., 2000]. All cell lines were deep- frozen (-150°C) early on, i.e. early passages, and then thawed when needed.

2.1 Canine Melanoma Cell Lines

2.1.1 Shadow

This established cell line was kindly provided by Prof. Modiano, College of Veterinary Medicine, Texas A&M University and originated from a male 12 year-old Gordon Setter. The melanoma was located on the gingival. This cell line was used as the reference cell line [Ritt et al., 2000].

2.1.2 cRGO 1

The cells were derived from a primary tumor site of a cross-breed female dog. The dog was neutered at time of clinical presentation and was 11 years old. The melanoma was located in the middle of the palate. Due to difficulties in breathing, an x-ray of the chest was performed but showed no evidence for lung metastasis [Schmid et al., 2019]. Cytologic investigation of the primary tumor exhibited a poorly pigmented, highly pleomorphic malignant melanoma. In addition samples from the left (derived cell line: cRGO1_2) and right (derived cell line: cRGO1_1) mandibular lymph node metastases were excised and provided for cell isolation [Schmid et al., 2019].

The right mandibular lymph node was as large as a chestnut and firm upon palpation (**cRGO 1_1**) [Schmid et al., 2019].

The left mandibular lymph node had the size of a bean (**cRGO 1_2**) [Schmid et al., 2019].

2.1.3 cRGO 2

This cell line was derived from a cross-breed male dog, with 15 years of age at the time of tumor excision. He was presented with a poorly pigmented mass at the caudal mandible. The mass was bleeding at the time of presentation. The excised melanoma was located on left caudal mandible and was diagnosed as an amelanotic, highly aggressive malignant melanoma [Schmid et al., 2019].

2.1.4 cRGO 3

The cells were derived from a Papillon, whose melanoma was located on the right labial angle, that was bleeding at the time of presentation. The female dog was roughly 11 years old at the time of surgery. The histopathological diagnosis was amelanotic melanoma [Schmid et al., 2019].

2.2 Cell Culture and Viability Cell Count

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4,5g/l) and Glutamax supplemented with 10% heat-inactivate fetal bovine serum (FBS) (all from GIBCO – Life Technologies, Carlsbad, CA, USA).
Cells were trypsinized (Trypsin, GIBCO – Life Technologies, Carlsbad, CA, USA) and stained with 0.4% Trypan blue solution (Sigma Aldrich Co., St. Louis, MO, USA), to count and check the viability of the cells. Cell counting was performed on a Leica microscope, using a chamber with a 10x objective. (Fig. 3) To calculate the number of viable cells the average of each of the sets from 16 corner squares were taken, multiplied by 10.000, to get the cells per milliliter, and multiplied by two to correct for dilution 1:2 from the Trypan blue addition. (Fig. 2)

$$concentration\ [cell/ml] = \frac{number\ of\ cells\ x\ 10.000}{number\ of\ square\ x\ dilution}$$

Fig. 2 Formula to account cells/ml

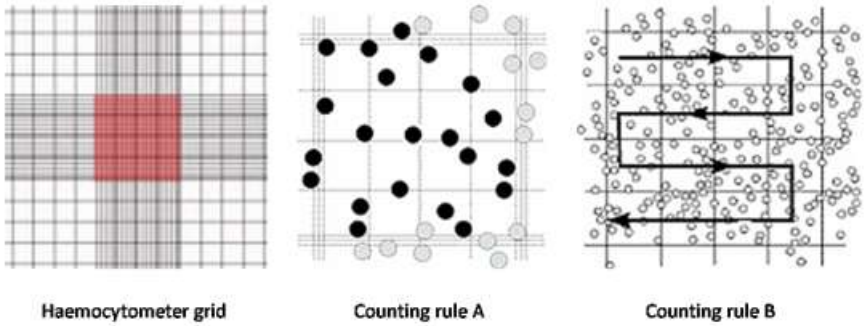


Fig. 3 Neubauer chamber, counting system (http://b110-wiki.dkfz.de/confluence/pages/viewpage.action?pageId=5931811; 07.10.18)

Due to cease manufacturing of the canine specific CD146 antibody during this study, it was not possible to repeat the growth curves for all cell lines in duplicates except for cRGO 1 and cRGO 1_2. To evaluate the optimal cell density 5,000 cells/ml or 10,000 cells/ml were seeded on day 0 and counted every 24 h for 4 days. A seeding density of 10,000 cells/ml proved to be the optimum for the proliferation assay. Cells were seeded in duplicates to verify the results and to calculate the mean.

2.3 Separation of CD146+ Expressing Melanoma Cells by MACS Bead

Cells were grown in T125 flasks and detached with trypsin (GIBCO - Life Technologies, Carlsbad, CA, USA). The number of suspended cells was determined to make sure not to overcharge the column [$<10^8$ cells/ml]. The cell suspension was centrifuged at 1200 rpm and the supernatant was discarded. The cell pellet was resuspended in 5ml phosphate buffered saline (PBS) (GIBCO - Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (GIBCO - Life Technologies, Carlsbad, CA, USA) and incubated for 10 minutes. Cell suspension was centrifuged at 1200 rpm and the supernatant discarded prior to labelling cells with 5 µg/ml mouse anti-CD146 IgG1 antibody clone A32 (5µg/ml PBS; Upstate) in 1 ml PBS containing 0.2% bovine serum albumin (BSA) (Sigma Life Science). Subsequently, cells were incubated on ice for 30 minutes, during which the binding of the antibodies on the CD146+ cells was maximized. The cells were washed twice with 1ml MACS Buffer, which included PBS containing 0.5% BSA and 2mM ethylenediaminetetraacetic acid (EDTA)(Miltenyi Biotec, Bergisch-Gladbach, Germany). The cell pellet was diluted in 80µl MACS Buffer and 20µl of anti-mouse IgG1 Micro Beads were added, mixed and incubated

for 15 minutes on ice. In the meantime, the column (Miltenyi Biotec, Bergisch-Gladbach, Germany) was placed in the magnetic field (Multi-Stand, Miltenyi Biotec) and hydrated by rinsing with 500µl MACS buffer. Suspended cells were washed with 2ml MACS Buffer and afterwards cells were centrifuged for 5 minutes at 1200 rpm and the remaining supernatant discarded. The cell pellet was resuspended in 500µl MACS Buffer. This cell suspension was applied onto the column and the collected flow-through contained unlabelled cells. After three washing steps with the MACS Buffer, the column was removed from the separator and placed on a new collection tube. Subsequently 1ml MACS Buffer was pipetted onto the column and immediately the magnetically labelled cells were flushed out by firmly pushing the plunger into the column. The cells were then counted, checked for viability using an inverted light microscope (Leica Mircosystem GmbH) and seeded on a 24-well plate (#353504 Corning/Falcon/2 cm²) for the growth curve and cell migration/invasion assay. (Fig. 4)

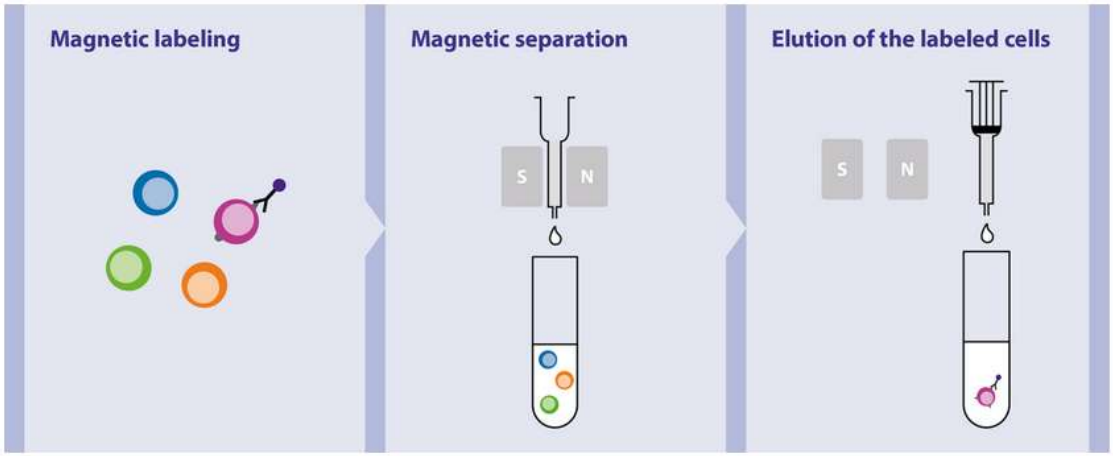


Fig.4 MACS© bead cell separation technology (https://www.miltenyibiotec.com/_Resources/Persistent/b5349effdd595b72195e588aff033be3e24706bd/IM0020021.pdf; 24.05.2019)

2.4 Cell Proliferation of CD146 Positive vs. Negative Cells

For the growth curve the cells were diluted in complete medium (CM) comprised of warm Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4,5g/l) with Glutamax, and 10% heat-inactivated (30 min., 56°C) fetal bovine serum (FBS) (all from GIBCO – Life Technologies, Carlsbad, CA, USA), seeded in duplicates at a density of 10.000 cells/well and incubated at 37°C and 5% CO_2. CD 146- and CD 146+ cell count was monitored 24, 48, 72 and 96 hours after seeding by using a Neubauer chamber.

2.5 Cell Invasion and Migration Assay

After separation of the CD146+ and CD146- cells, some of them were used for the migration/invasion assay. For this purpose inserts (#353097 Corning/Falcon/0.3cm²) were prepared and coated. First of all, gelatin (Sigma- Aldrich) was preheated to 60°C and diluted 1:1000 with PBS. The invasion inserts were coated with the gelatin solution. After coating the transwell-inserts with 100µl diluted gelatin, they were incubated for 10 minutes at 37°C and 5% CO_2. Prior to seeding the cells the excess coating solution was removed. The next step was to add 600µl appropriate medium, i.e. medium containing 10% FCS for migration and invasion or medium without FCS for the negative control, to each well of a 24-well plate and inserts were placed. In each insert 100µl of cell dilution, containing 10.000 cells per insert in serum free medium, were added and incubated at 37°C in a CO_2 incubator. After 24 hours the inserts were removed, and the inside of every insert was gently swabbed with cotton swabs to eliminate the non-migrated cells.

Subsequently the inserts were washed with 200µl PBS and each well with 1ml PBS. For staining of migrated/invaded cells 600µl 0.2% crystal violet solution (Sigma-Aldrich) was added and cells were incubated for 10 minutes. Finally the crystal violet solution was removed and inserts were rinsed with deionised water until water ran clear. Inserts were dried and visualized with a Leica DMIL microscope at 100x magnification. (Fig. 5) To evaluate the number of invaded/migrated cells 5 random fields were counted [Albini & Benelli, 2007].

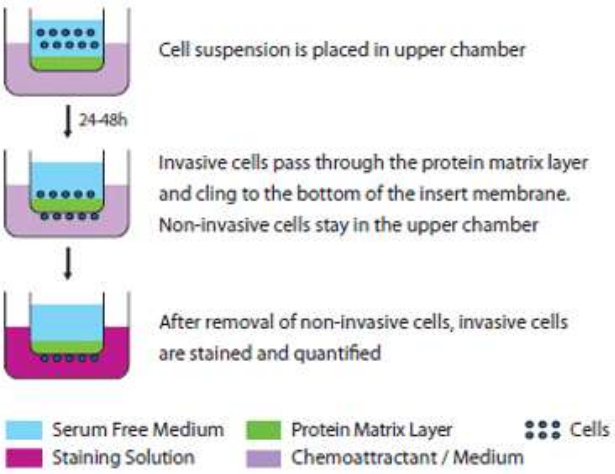


Fig. 5 Invasion assay
(https://www.biocat.com/cell-biology/cell-invasion; 08.10.2018)

2.6 Data Analysis

To evaluate if CD146 expression has an effect on the cell growth, a mixed model was used as statistical model. Furthermore to verify an effect of CD146 expression regarding migration or invasion of the tumor cells, wilcoxon test was used. A p-value < 0.05 was considered statistically significant. Statistic analysis was performed using a commercial software (IBM SPSS statistics).

3 Results

3.1 Growth Curve

Fig. 6-11 shows the growth curves, over a period of time, of the different patients comparing CD146 enriched versus CD146 depleted subpopulations of the cell lines. There was no difference detected between the growth behavior of CD146 enriched cells and CD146 depleted cells at the end of the examination (P=0,976).

3.1.1 Shadow

Shadow is the reference cell line that kindly provided by Prof. Modiano. This cell line proliferated successfully in cell culture. The cells adhered rapidly, and confluency was achieved within 4 days. The CD146 positive cells started more slowly compared to the CD146 negative cells but overtook them on the 4th day.

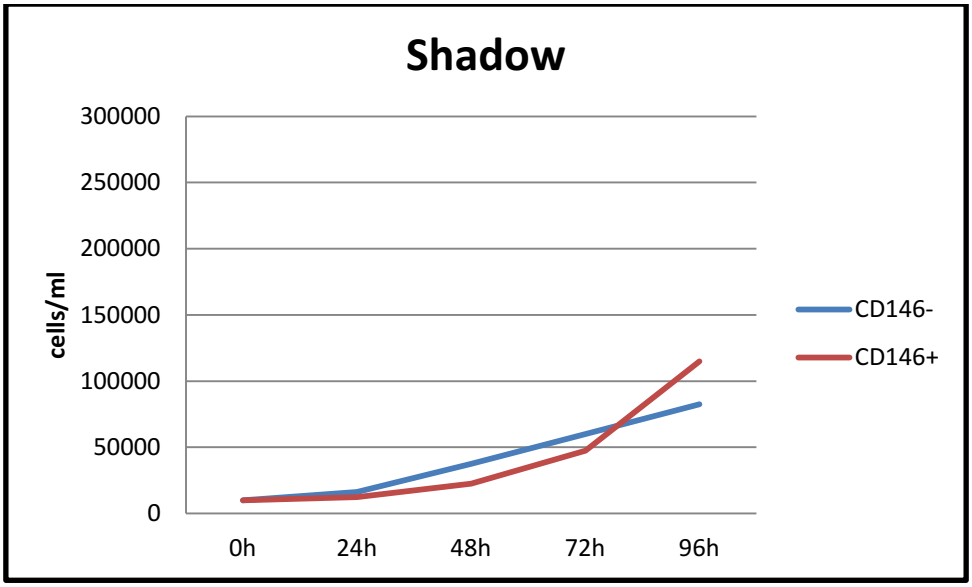


Fig. 6 Growth curve Shadow

	Timepoints				
Cells/ml	0h	24h	48h	72h	96h
CD146-	10000	16250	37500	60000	82500
CD146+	10000	12500	22500	47500	115000

Tab. 1 Data of cell growth, Shadow

3.1.2 cRGO 1

This cell line derived from primary canine oral malignant melanoma was stable and had been in cell culture over an extended time period. cRGO 1 and cRGO 1_2 was examined most commonly throughout the course of this study. Aliquots of cell suspension were deep frozen (-150°C) early on, and then thawed as needed.

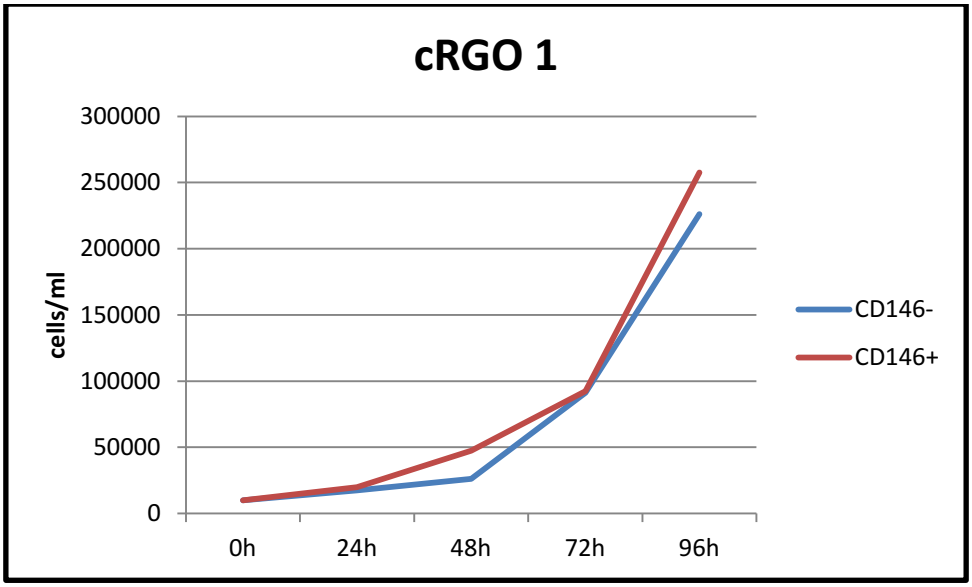


Fig. 7 Growth curve cRGO 1

	Timepoints				
Cells/ml	0h	24h	48h	72h	96h
CD146-	10000	17500	26250	91250	226250
CD146+	10000	20000	47500	92500	257500

Tab. 2 Data of growth curve, cRGO 1

3.1.3 cRGO 1_1

The cell line cRGO 1_1 was derived from a metastasis of cRGO 1. Against all expectations this cell line did not grow predictably, in contrast to cRGO 1 and cRGO 1_2. The cells did not proliferate as quickly as cRGO 1 or cRGO 1_2.

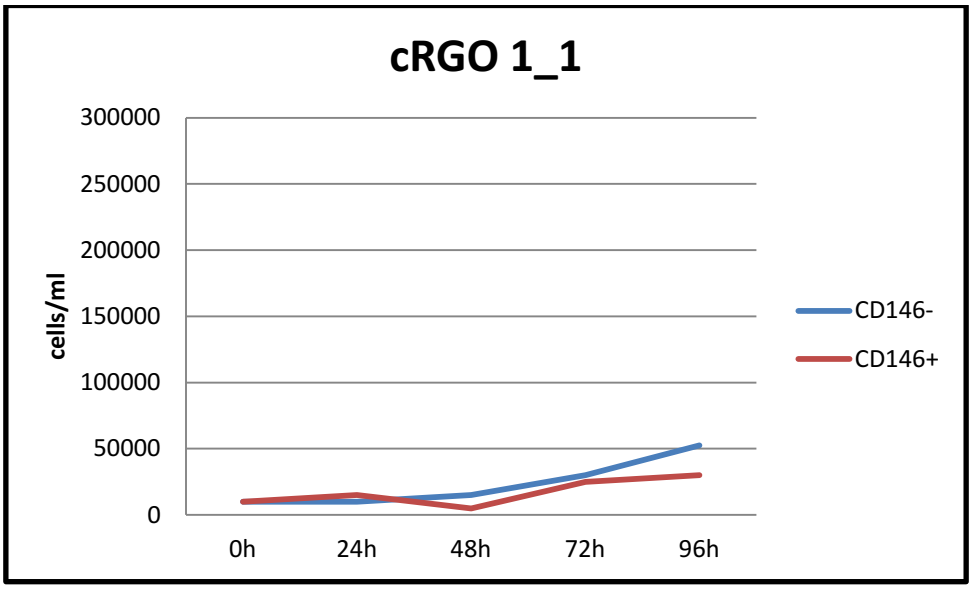


Fig. 8 Growth curve cRGO 1_1

	Timepoints				
Cells/ml	0h	24h	48h	72h	96h
CD146-	10000	10000	15000	30000	52500
CD146+	10000	15000	5000	25000	30000

Tab. 3 Data of growth curve, cRGO 1_1

3.1.4 cRGO 1_2

The cell line named cRGO 1_2 was also derived from a metastasis. The cell line demonstrated stability and predictability. The cRGO 1_2 cell line had been in culture over an extended time period. Aliquots of passages were frozen and thawed when needed. cRGO 1_2 was examined most commonly apart from cRGO 1.

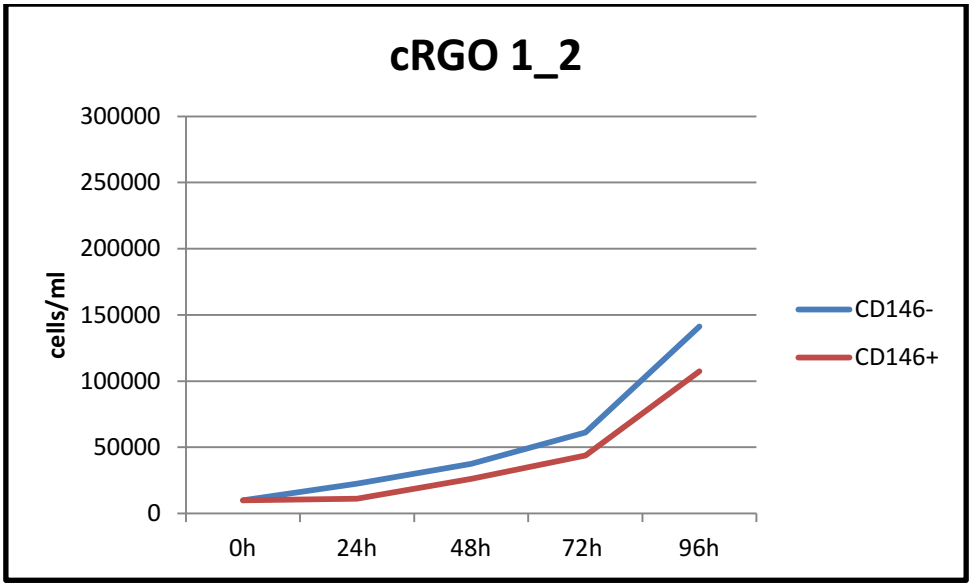


Fig. 9 Growth curve cRGO 1_2

	Timepoints				
Cells/ml	0h	24h	48h	72h	96h
CD146-	10000	22500	37500	61250	141250
CD146+	10000	11250	26250	43750	107500

Tab. 4 Data of growth curve, cRGO 1_2

3.1.5 cRGO 2

cRGO 2 was the first cell line that was examined. This cell line had been in culture over an extended time period.

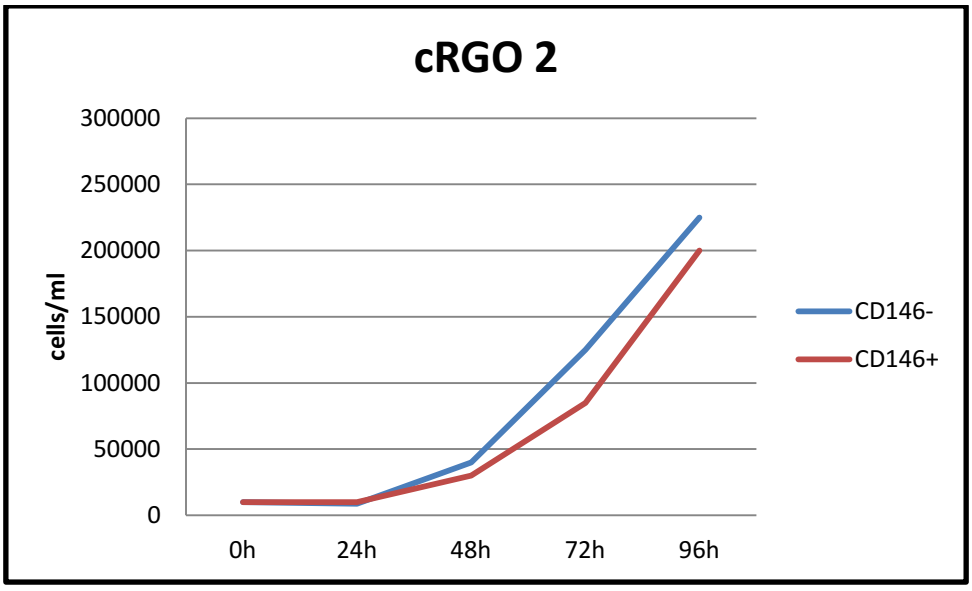


Fig. 10 Growth curve cRGO 2

	Timepoints				
Cells/ml	0h	24h	48h	72h	96h
CD146-	10000	8750	40000	125000	225000
CD146+	10000	10000	30000	85000	200000

Tab. 5 Data of growth curve, cRGO 2

3.1.6 cRGO 3

cRGO 3 did not adhere or proliferate as quickly as the cells of the other cell lines. The cRGO 3 cells were allowed 72h to adhere, recover and proliferate. Neither the CD146 positive cells nor the CD146 negative cells showed a clear tendency.

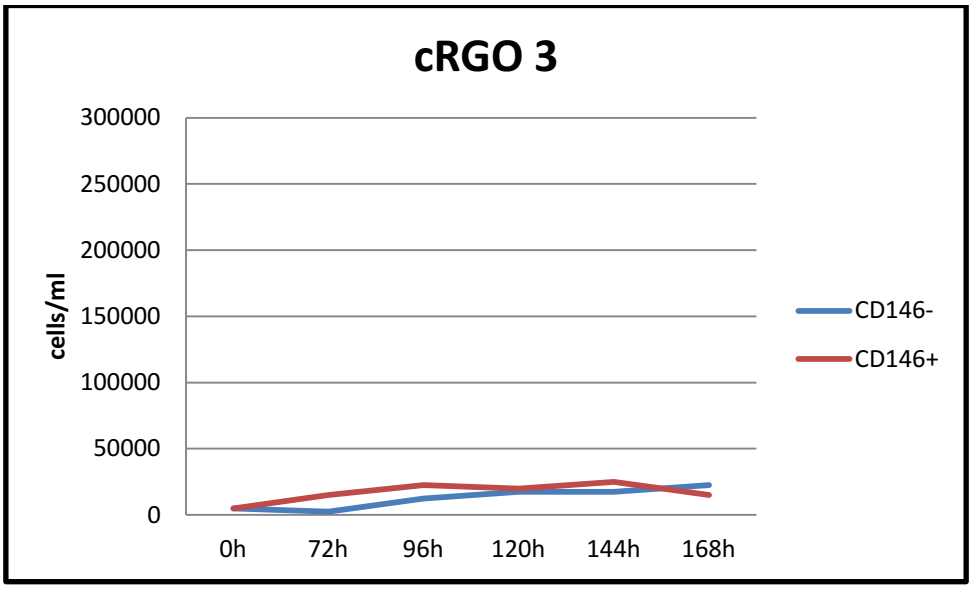


Fig. 11 Growth curve cRGO 3

	Timepoints					
Cells/ml	0h	72h	96h	120h	144h	168h
CD146-	5000	2500	12500	17500	17500	22500
CD146+	5000	15000	22500	20000	25000	15000

Tab. 6 Data of growth curve, cRGO 3

3.2 Migration and Invasion of Canine Melanoma Cell Lines

CD 146 –enriched versus –depleted subpopulations of the cell lines were determined regarding their potential for migration and invasion (Fig. 12-13).

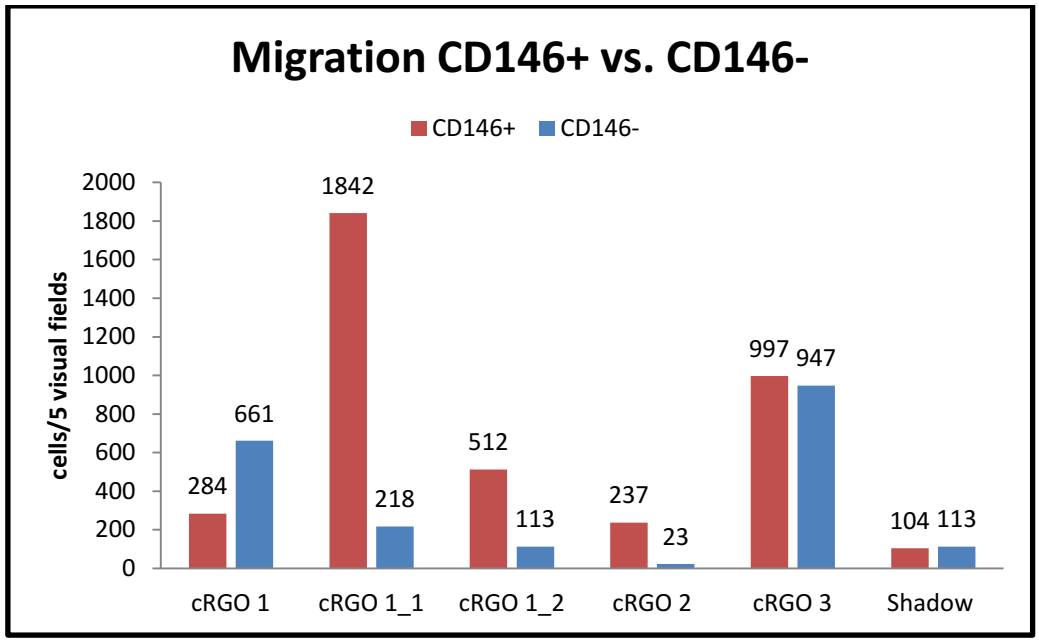


Fig. 12 Comparing CD146 positive cells and CD146 negative cells there was no significant difference regarding their migratory potential (P=0,335).

Migration		
	CD146+	CD146-
Shadow	1,04%	1,13%
cRGO 1	2,84%	6,61%
cRGO 1_1	18,42%	2,18%
cRGO 1_2	5,12%	1,13%
cRGO 2	3,16%	0,31%
cRGO 3	9,97%	9,47%

Tab. 7 Percentage of cells that migrated

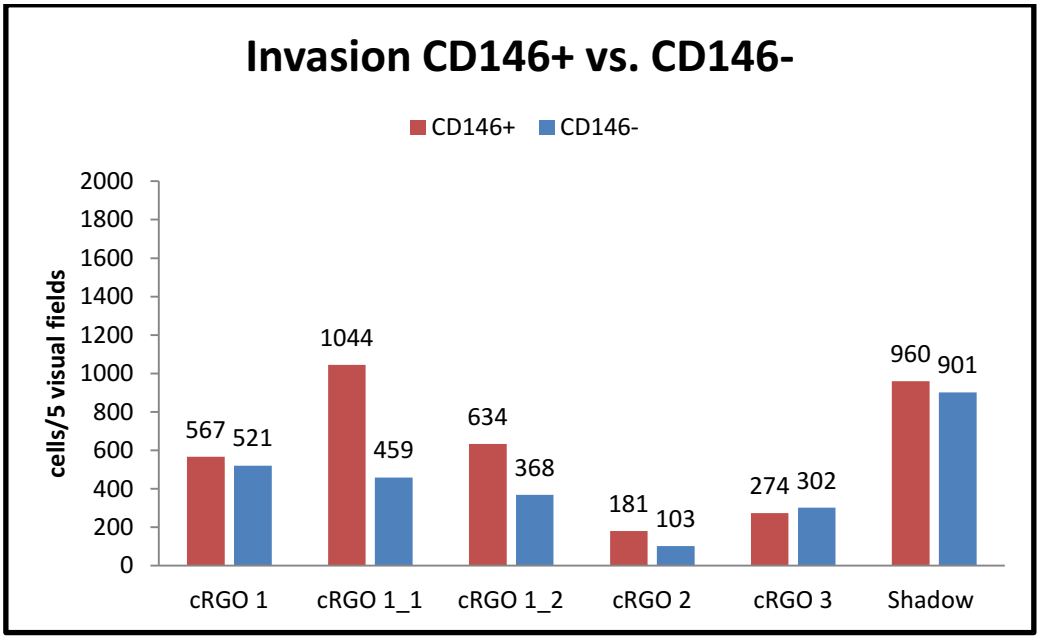


Fig. 13 Comparing CD146 positive cells and CD146 negative cells there was no significant difference regarding their invasive potential (P=0,374).

Invasion		
	CD146+	CD146-
Shadow	9,60%	9,01%
cRGO 1	5,67%	5,21%
cRGO 1_1	10,44%	4,59%
cRGO 1_2	6,34%	3,68%
cRGO 2	2,41%	1,37%
cRGO 3	2,74%	3,02%

Tab. 8 Percentage of cells that invaded

Methode	Expression	Maximum	Percentiles 25	50	75
INVASION	CD146-	901	252,25	413,50	616,00
	CD146+	1044	250,75	600,50	981,00
MIGRATION	CD146-	947	90,50	165,50	732,50
	CD146+	1842	203,75	398,00	1208,25

Tab. 9 Summary of invasion/migration of CD146 positive vs. CD146 negative canine melanoma cells

Methode	Source	Numerator df	Denominator df	F	Sig.
INVASION	Intercept	1	9,360	34,341	,000
	Expression	1	9,360	,872	,374
MIGRATION	Intercept	1	7,896	10,700	,012
	Expression	1	7,896	1,056	,335

a. Dependent Variable: Zellen.

Tab. 10 p-value analysis for migration/invasion of canine melanoma cells

4 Discussion

CAMs are involved in multiple physiological and pathological processes [Abou Asa et al., 2016] such as cell-cell or cell-matrix interactions and these are essential tumor cell properties for metastasis [Xie et al., 1997]. CD146, also known as melanoma cell adhesion molecule (MCAM), is used as a progression marker in human melanoma because it is often over-expressed on the surface of advanced and metastatic melanoma cells. Several studies state that using an antibody against CD146, or another CD146 blocking system, inhibits tumor growth and metastatic potential in humans [Lehmann et al., 1989; Xie et al., 1997; Mills et al., 2002; Kang et al., 2006; Lei et al., 2015]. As described by Jin et al. (2016), high expression of CD146 in human umbilical cord blood- derived mesenchymal stem cells, displayed high growth rates whereas down-regulation leads to cellular senescence.

Improved understanding of the mechanisms of COMM may have a significant impact on a better understanding of the cellular and molecular pathways, which in turn would help developing more efficient therapies in humans and canines.

Hence, the regulatory effects of CD146 on the growth of melanoma cells *in vitro* were examined in this study. Freshly isolated melanoma cells adhered within 24 hours and confluency of the tumor cells was attained within 4-12 days. There was no apparent effect on cell proliferation due to multiple passages (up to 48x). Independent of the passage number, the average population doubling time was one doubling per day [Schmid et al., 2019]. Considering the growth curves of the CD146 negative cells of Shadow, cRGO 1, cRGO 1_1, cRGO 1_2, cRGO 2, cRGO 3 and the growth curves of CD146 positive cells from each cell line there was no statistical, conclusive difference (P=0,976) as also reported for human melanoma cells [Lei et al., 2015].

Resembling results got Mills et al. (2002) determining the effect of a neutralizing anti MUC18 andibody *in vitro*. The proliferation rate of melanoma cells *in vitro* did not alter but *in vivo* the antibody showed an effect on tumorigenicity and metastatic potential [Mills et al., 2002].

Thus Mills et al. (2002) supposed that CD146 is more complex in the progression of human melanoma than a simple autocrine growth stimulation or homotypic interactions.

As observed in humans the expression of CD146 was frequently elevated in metastases then on primary tumors [Lehmann et al., 1987] and according to Shih (1999) tumor cell invasion and metastasis correlates to CD146 expression in melanoma cells. In human prostate carcinoma, CD146 has been associated with malignant transformation and metastatic potential [Wu et al., 2011]. To the best of our knowledge, there is no report investigating the

effect of CD146 on the invasiveness and migratory potential of canine oral melanoma cells. Todorovic et al. (2013) observed a reduction in the metastatic potential of human melanoma cells *in vitro* by downregulation of CD146.

In our study there was no correlation between CD146 expression and migratory (P=0,335) or invasion (P=0,374) potential of the tumor cells. Indeed considering the bar graph, invasion and migration of the metastasic cell lines (cRGO1-1 and cRGO1-2) may have a tendency of increased invasion and migration.

Further investigations with improved methods, evaluating the influence of CD146 on canine melanoma metastatic cells regarding invasion and migration, have to be conducted. In contrast to the methodology used in this study, a long-term downregulation of CD146 using siRNA techniques could provide more significant results as reported for human melanoma [Todorovic et al., 2013].

In addition, a larger sample size of cell lines investigated would clarify the role of CD146 in respect to migration and invasion in canine melanoma metastasis and may improve the understanding of CD146 and its role in canine oral melanoma.

5 Summary

5.1 Objective

The aim of the current study was to examine the influence of CD146 expression in canine melanoma cells regarding their growth, as well as the invasional and migratory potential of a CD146 enriched melanoma cell population *in vitro*.

5.2 Material and Methods

Four canine oral melanoma cell lines and two metastatic canine oral melanoma cell lines, were subjected to a MACS Bead Assay to get CD146 –enriched and CD146 -depleted cells. These cells were cultured over a period of time and their proliferation comparatively assessed by manual cell count each day over a period of 96 hours. Furthermore, CD146 positive and CD 146 negative cells were analyzed by means of a trans-well assay to estimate their migratory or invasion potential.

5.3 Results

There was no significant difference (P=0,976) between the growth curve of CD146 positive and CD146 negative cells. Furthermore the assumption that the expression of CD146 influences on the migration (P=0,335) and invasion (P=0,374) of canine melanoma cells could not be confirmed.

6 Zusammenfassung

6.1 Ziel der Arbeit

Ziel der Arbeit war es, den Einfluss der Expression von CD146 bei caninen Melanomzellen auf deren Wachstum, sowie deren migratorisches und invasives Potential *in vitro,* herauszufinden.

6.2 Material und Methoden

Vier canine, orale Melanom-Zelllinien und zwei canine, orale Melanom-Metastasen wurden einem MACS Bead Assay unterzogen, um CD146 angreicherte – und CD146 dezimierte-Zelllinien zu erhalten. Diese Zellen wurden über einen gewissen Zeitraum kultiviert und ihre Proliferation vergleichend bewertet, durch manuelles Auszählen der Zellen, jeden Tag über einen Zeitraum von 96 Stunden. Desweiteren wurden CD146 positive und CD146 negative Zellen, mittels eines transwell assay, analysiert, um deren migratorisches und invasives Potential zu beurteilen.

6.3 Ergebnisse

Es gab kein signifikanter Unterschied (P=0,976) zwischen der Wachstumskurve von CD146 positiven und CD146 negativen Zellen.
Zudem konnte die Vermutung, dass Expression von CD146 die Migration (P=0,335) und Invasion (P=0,374) von caninen Melanomzellen beeinflusst, nicht bestätigt werden.

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