VARIOUS TECHNIQUES TO ASSESS THE ANTI-VASCULAR EFFECT OF A VASCULAR DISRUPTIVE AGENT ON THE PERFUSION OF A SOLID TUMOR IN A DOG

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Preface

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I would also like to thank you both for the pleasant and professional cooperation during these last two years.

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<tr>
<td>B-mode</td>
<td>brightness-mode</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BSA</td>
<td>body surface area</td>
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<td>CA4P</td>
<td>combretastatin A4-phosphate</td>
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<td>CEUS</td>
<td>contrast-enhanced ultrasound</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>FNA</td>
<td>fine needle aspiration</td>
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<td>HE</td>
<td>haematoxylin and eosin</td>
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<td>MRI</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDUS</td>
<td>power-doppler ultrasound</td>
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<td>PET</td>
<td>positron emission tomography</td>
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<td>PIGF</td>
<td>placental growth factor</td>
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<td>PNS</td>
<td>peripheral nervous system</td>
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<td>SPECT</td>
<td>single proton emission computed tomography</td>
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<td>US</td>
<td>ultrasound</td>
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<td>US FDA</td>
<td>United States food and drug administration</td>
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<td>VDA</td>
<td>vascular disrupting agent</td>
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<td>VEGF-A</td>
<td>vascular endothelial growth factor-A</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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1. **Summary**

The aim of this study is to discuss the different methods that can be used to assess the effect of a vascular disrupting agent (VDA) in a first-line veterinary practice or in a more specialized practice or veterinary clinic. Also, the possible advantages and disadvantages of each technique will be discussed. The VDA, here combretastatin A4 phosphate (CA4P), was injected intravenously to treat a dog with a solid schwannoma in the carpal region. The methods that will be discussed in this thesis are both invasive, such as biopsies, and non-invasive, such as medical imaging techniques. Different techniques can be used in veterinary practice to obtain more information concerning the consistency and vascularization in a solid tumor. Accurate and detailed medical imaging techniques to visualize the vascularization are power-Doppler ultrasound (PDUS) and contrast-enhanced ultrasound (CEUS). Mostly, techniques with a minimal invasive effect are preferable. In some techniques, e.g. biopsies, the patient must undergo sedation or anesthesia. This can be challenging when the patient suffers from clinical conditions that complicate the sedation or anesthesia such as cardiac conditions or breathing problems due to possible lung metastasis of a primary tumor.

This thesis demonstrates that there are different techniques when assessing the effect of a VDA in a veterinary clinical setting. The chosen technique depends on different factors such as the material available, the experience (in both surgery and medical imaging) of the performing vet and the financial resources of the owners. Also, the costs of diagnosis and possible therapy need to be considered in relation to the prognosis. Tumoral processes are often painful and tend to diminish the further life quality of a patient when not treated correctly. In this thesis, the treatment with a VDA will be assessed by the comparison between B-mode US, PDUS, and CEUS as medical imaging techniques. Also, histopathological evaluation of tissue biopsies will be compared to the other techniques to assess their efficiency and accuracy when evaluating the effect of the use of a VDA to treat a solid tumor in a dog.
2. Literature

Dogs and cats develop spontaneous tumors with histopathologic and biologic behavior often similar to tumors that occur in humans (MacEwen, 1990). Animals with cancer often have very advanced (locally invasive and/or metastatic) disease when presented to a veterinarian. Many tumors demand an aggressive surgical approach that can be delivered only by an experienced surgeon (Farese et al, 2012).

In human medicine, surgical oncology is a well-established subspecialty. It is impressive that 60% of human patients who recover from cancer are cured by surgery alone (Poston, 2007). Similarly, in veterinary medicine, surgery is considered the most important component of treatment in dogs and cats suffering from solid tumors (Farese et al, 2012).

In clinical cases where surgery is not an option to treat cancer, other therapies are possible. Apart from established chemotherapy and radiation protocols, sometimes enrollment in clinical trials assessing a novel drug is also an option. One of the drugs that is currently the subject of a veterinary clinical trial in dogs with solid tumors is a vascular disruptive agent (VDA).

2.1 Perfusion and vasculature of a tumor differs from physiological vasculature

As in most other tissues, the presence of vasculature in tumoral tissue is essential for development, growth and survival. Vascularization guarantees the delivery of oxygen, nutrients and different kinds of growth factors to the tissue. Angiogenesis is a normal physiological process which evolves the proliferation, migration and morphogenesis of endothelial cells from existing vessels into new blood vessels (Bielenberg and Zetter, 2015). Angiogenesis can be distinguished from vasculogenesis, which is the de novo formation of first vessels from angioblasts in an embryo (Bielenberg and Zetter, 2015).

Tumor angiogenesis and normal angiogenesis are quite similar regarding the endothelial cells (Bielenberg and Zetter, 2015). When a solid tumor grows beyond a diameter of around 2 mm, the existing vasculature becomes insufficient and to prevent further hypoxia, the need for angiogenesis arises (Bielenberg and Zetter, 2015). To trigger tumor angiogenesis, tumor cells secrete high levels of several important pro-angiogenic factors, including vascular endothelial growth factor-A (VEGF-A), placental growth factor (PIGF) and basic fibroblast growth factor (bFGF) into the tumor micro-environment (Chung et al, 2010). These factors activate endothelial cells of the surrounding blood vessels, which promote endothelial cell proliferation, migration and tube formation via the regulation of cell adhesion molecules (Desgroisellier et al, 2010). Tumoral vessels have a different structure and organization compared to normal vasculature. Whereas physiological vasculature is well organized, tumoral vasculature is heterogenous regarding organization, structure, and function. Tumoral vessels form arterio-venous shunts, branch irregularly and exhibit a serpentine course. The irregularity of tumor vascular bedding occurs due to an imbalance between pro- and anti-vascular mediators (Warren, 1997; Jain, 2003; Goel, 2011).

There is strong evidence that angiogenesis and metastasis can be linked (Bielenberg and Zetter, 2015). This because of the fact that tumor microvessel density correlates with increased metastatic potential and poor survival in nearly all forms of malignancy (Bielenberg and Zetter, 2015).
As angiogenesis is an important rate-limiting step in tumor growth, anti-vascular therapy was conceived as a golden opportunity for cancer treatment (Folkman, 1971).

2.2 Medical imaging of tumors

Imaging of the primary tumor is important for evaluating the location and degree of involvement of adjacent structures, as well as for surgical planning (Farese et al, 2012). A large number of medical imaging techniques are available; some of the most commonly used techniques will be briefly mentioned below.

2.2.1 Radiography

Use of radiography in the diagnostic work-up for malignant tumors involves three-view (ventrodorsal, left lateral and right lateral projections) thoracic radiographs to confirm or to rule out pulmonary metastatic disease (Otoni et al, 2010). Using high-detail screens, metastatic lesions as small as 6 mm can be detected on thoracic radiographs (Demell et al, 2001). Radiography can also be used for the evaluation of bone involvement in the tumoral process (Farese et al, 2012). A disadvantage of radiography is that it doesn’t provide any information on vascularization of a tumoral process.

2.2.2 Ultrasonography

Ultrasonography is a useful and cost-effective tool for the evaluation of tumors and their vasculature. Ultrasound (US) can also be used to accurately guide needles and needle-core biopsy instruments for non-invasive tissue sampling. Recent developments have been proven useful in the evaluation of tumors and include Doppler US to assess tumor vascularity (O’Brien et al, 2004; Farese et al, 2012). A negative aspect of the use of US and color-flow Doppler US is the fact that it cannot differentiate benign and malignant tumors based on their Brightness mode (B-mode) US appearance (Vanderperren et al, 2014). B-mode is often applied when using US as a medical imaging technique. It is a simple, non-invasive technique that can be used with every type of US machine or transducer and can give a lot of information. B-mode forms a two-dimensional image by using the echogenic dots seen on the US display. Each dot represents a reflection of an echogenic wave, sent by the transducer. The brightness of the dots can differ and they each represent a different structure, depending on the intensity of the transduced wave. The difference in echogenicity allows the veterinarian to interpret and clinically evaluate the structure that is being investigated with US.

2.2.3 Computed Tomography and Magnetic Resonance Imaging

Advanced medical imaging techniques such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) have revolutionized the management of animals with neoplasia (Farese et al, 2012). Currently, CT and MRI scans are used to provide accurate three-dimensional information about tumor location for stereotactic CT-guided biopsy, radio-ablation and surgical resection (Lecouteur, 1999).
In general, CT is preferred for evaluation of bones and MRI for soft tissue structures, but considerable overlap has been noted in the information that CT and MRI can provide (Farese et al, 2012). Computed tomography scans are faster than MRI scans, but they have lower contrast resolution and require iodinated contrast agents and ionizing radiation (Alberico et al, 2004). Also, CT is more sensitive than radiography for the detection of metastatic lesions and can delineate lesions as small as 1 mm (Paolini et al, 2006). A CT scan is recommended for evaluation of primary tumors of the axial skeleton, vertebral and pelvic tumors, and primary and metastatic intrathoracic tumors, because the quality of MRI is decreased by respiratory motion. Magnetic Resonance Imaging is preferred for tumors of the central and peripheral nervous system and may provide extra information on intra-abdominal organs (Alberico et al, 2004). A disadvantage of MRI scans is that they take much longer to be performed, metallic implants are a contraindication, and artefacts are common, particularly due to motion (Alberico et al, 2004). Although US can provide similar information to MRI, tumoral characteristics on T1- and T2-weighted MRI provide further information on the consistency and type of the tumor (Clifford et al, 2004).

2.2.4 Positron Emission Tomography and Single Proton Emission Computed Tomography

Two more recent areas of tumor imaging are Positron Emission Tomography (PET) and Single Proton Emission Computed Tomography (SPECT) (Forrest et al, 2000). A PET scan provides in vivo information on biochemical and physiological processes. Although these techniques are not widely available in veterinary medicine, they are increasingly used in human oncology for clinical staging of tumors as well as for the assessment of response to therapy (Jerusalem et al, 2002). The technique of SPECT uses gamma-ray-emitting radionuclides with a gamma camera that reconstructs images in a cross-section, providing improved lesion localization over planar scintigraphy. A PET typically utilizes a radiopharmaceutical called F-fluorodeoxyglucose, which is transported into and trapped inside tumor cells because it is not utilized in the glycolytic pathway. Since tumor cells have a higher uptake of glucose compared to normal cells, a higher signal is detected in neoplastic tissues (Peremans et al, 2007).

Both PET and SPECT are at their infancy in clinical veterinary medicine, but both techniques are regarded with more interest and have shown to be useful in veterinary oncology (LeBlanc and Peremans, 2014). Due to the high cost of both techniques and the use of radiopharmaceutical with PET, it is not (yet) widely used in veterinary medicine.
2.3 General techniques to obtain information on vascularization

There are many ways to obtain information on the vascular structure of a mass or a region in general. The most common techniques that are used in veterinary practice will be discussed below.

2.3.1 Power Doppler ultrasound

Power Doppler sonography is a technique that displays the strength of the Doppler signal in color, rather than the speed and direction information. Power-Doppler US uses the amplitude of a Doppler shift to detect moving matter. Therefore, noise is filtered and the flow is shown as a contrasting color (Hamper et al, 1997). It has three times the sensitivity of conventional color Doppler for the detection of flow and is particularly useful for small vessels and those with low-velocity flow (Babcock et al, 1996). Power Doppler is useful as an adjunct to conventional color and pulsed Doppler sonography (Babcock et al, 1996). The technique of PDUS provides a good and accurate image of the vessels. It is a sensitive technique but PDUS can’t provide assessment of vessels with a diameter less than 200 µm (Rouffiac et al, 2004).

An in vivo study in rodents concluded that quantified PDUS is a decent method for the evaluation of tumor vascularity and blood flow (Donnelly et al, 2001).

2.3.2 Contrast-enhanced ultrasound

To investigate and obtain detailed information on the vascularization of a tumoral mass, contrast-enhanced ultrasound (CEUS) can be used. Contrast-enhanced ultrasound is a very detailed technique to obtain information about blood flow and tissue perfusion because of the use of microbubble contrast agents (Wilson et al, 2009). With CEUS, the microbubbles improve the echogenicity of blood and hereby give a more detailed image of the vessels compared to B-mode and PDUS.

Contrast-enhanced US with microbubble contrast agents has recently been proposed as a new imaging modality to quantify tissue perfusion (Vanderperren et al, 2014). An US contrast agent consist of microbubbles with a mean diameter of 0.5-10 µm. Unlike the contrast agents used in CT and MRI, the microbubbles used in CEUS remain strictly intravascular without any interstitial component (O’Brien and Seiler, 2015). This makes them well suited for the evaluation of blood flow in functional vessels. The visibility of a targeted microbubble contrast after intravenous administration is mostly around five to six minutes (Lamuraglia et al., 2010) although this can be shorter for standard, non-targeted microbubble contrasts.

Contrast-enhanced US is usually performed at acoustic powers as weak as possible in order to avoid destroying the microbubble and to have as little formation of artefacts as possible. It is the selective nonlinear detection of microbubbles that allows their specific and sensitive detection in the microvascularization (Lamuraglia et al., 2010). A study proved that CEUS with microbubbles targeted to αvβ3 integrins could noninvasively detect early tumor angiogenesis. This technique, when coupled with changes in blood volume and velocity, may provide insight in the biology of tumor angiogenesis and can be used for diagnostic applications (Ellegala et al, 2003).
2.4 Combretastatin A4-phosphate: an efficacious vascular disrupting agent

Combretastatin A4-phosphate (CA4P) is a vascular disrupting agent that is originally derived from the tree called Combretum caffrum, a South African willow (Young and Chaplin, 2004). It is a tubulin-binding agent that disrupts the cytoskeleton of immature endothelial cells (Young and Chaplin, 2004). By doing so, CA4P hampers the mitosis and the replication of cells by destabilizing the microtubules. *In vitro*, CA4P destabilizes microtubules and causes endothelial cell death (Kanthou et al, 2004). This destabilization leads to a prolonged mitotic arrest and therefore ischemia and necrosis of the cells (Kanthou et al., 2004). By activating cell death pathways, CA4P, in addition to being an effective anti-vascular agent, may also interfere with regrowth of blood vessels in the tumor (Kanthou et al, 2004).

Vascular disrupting agents such as CA4P are very specific and also selective in their target for vascularization because they only focus on the immature vessels. Immature vasculature, present in the central part of the tumor, is sensitive whereas mature vessels of the periphery and surrounding normal tissues will not be destroyed by CA4P treatment. Following intracellular uptake and dephosphorylation of the prodrug CA4P, the active drug combretastatin A4 (CA4) binds reversibly to tubulin at the colchicine-binding site, leading to tubulin depolymerisation. As a result, microtubule assembly in tumour neovascularisation is inhibited, causing distortion and detachment of the immature proliferating endothelial cells (Griggs et al, 2001). Consequently, the tumoral vessels collapse and the center of the tumor necrotizes (Li et al., 2013). Owing to its selectivity, CA4P has few side-effects on mature vascularization. A VDA is able to destroy the existing vessels and shut down the nutrition and oxygen supply of the tumor, leading to hypoxia and necrosis of the tumor (Siemann et al, 2005). The tumor will not be completely destroyed by using VDAs alone because VDAs target only the immature blood vessels that are situated in the central part of the tumor. Tumor cells at the periphery still receive oxygen and nutrition from the mature vessels originating from the surrounding healthy tissue and will survive the VDA attack. These cells will need to be destroyed by adjuvant therapies (Siemann et al, 2005).

The use of CA4P as a VDA has already been studied many times in several animal models, mainly rodents. When used as monotherapy, CA4P induces necrosis and significant blood flow reductions in tumors studied in preclinical cancer models. Preclinical data in mice with a renal cell carcinoma xenograft further indicate that CA4P can effectively be combined with chemotherapy or radiation therapy (Siemann et al., 2009). In 2016, CA4P became US FDA-approved in a treatment protocol for ovarian cancer in human patients and is currently being evaluated in various Phase II and III clinical trials in combination with chemotherapy or radiation therapy (Mooney et al, 2009; Garon et al, 2010; Ng et al, 2012; Sosa et al, 2014).

Recent studies have shown that the type of tumor plays a role in the sensitivity to CA4P (Yin et al., 2017). Not all tumors react to the same extent to the administration of CA4P, which results in a great variety in the amount of induced necrosis. In a clinical study in rats, which compared hepatic and pancreatic solid tumor response to CA4P treatment, hepatic tumors were more responsive than pancreatic tumors (Yin et al., 2017).
We know that CA4P is already used in human medicine for the treatment of different types of cancer (Mooney et al, 2009; Garon et al, 2010; Ng et al, 2012; Sosa et al, 2014). Possible side-effects of intravenous CA4P have also been studied in human medicine (Dowlati et al, 2002; Rustin et al, 2003; Stevenson et al, 2003). The most common adverse events in human patients are: nausea, vomiting, tumour pain, cardiovascular and neurological toxicity, and are typically mild to moderate in intensity (Abma et al, 2015).

A recent clinical study in healthy dogs demonstrated that in dogs the most common adverse effects after intravenous administration of CA4P are anorexia, nausea, abdominal discomfort and diarrhea (Abma et al, 2017). Additionally, a low-grade neutropenia was observed in all dogs included in the study. The conclusion of this study was that a dose of CA4P up to 75 mg m$^{-2}$ was well tolerated in healthy animals. Keeping in mind that 52 mg m$^{-2}$ is the lowest dose at which changes in parameters associated with tumor blood flow reduction is seen in human cancer patients (Dowlati et al, 2002; Rustin et al, 2003; Stevenson et al, 2003), and on the basis of the toxicity criteria found in this research, the dose recommended for treating canine cancer patients would be 52 to 75 mg m$^{-2}$ (Abma et al, 2017).
3. **Materials and methods: different techniques to assess the anti-vascular effect of CA4P**

3.1 Introduction to the clinical case

A male castrated American Staffordshire Terrier of 15 years old was presented to the Faculty of Veterinary Medicine with complaints of a macroscopically visible and growing mass at the level of the right carpal joint (Figure 1). According to the owner, it had been present for one year. The area around the mass was clearly swollen and the mass itself had a solid, ulcerative and reddish aspect. The dog was not lame and on clinical examination no abnormalities were found apart from the mass. Punch biopsies revealed the histopathological diagnosis of a schwannoma, a malignant peripheral nerve sheath tumor. Because of the extent of the mass, local excision was not possible, and amputation of the affected front limb was not an option for the owner. A general examination and blood analysis of the dog were performed. A complete US examination of the abdomen was performed to detect or rule out metastasis. No metastases were found.

Prior to therapy, different medical imaging techniques were explored to obtain more information about the vascularization of the tumor. In this case B-mode, PDUS, and CEUS were performed. Additionally, biopsies of the tumor were taken and the histopathological slides of the biopsies were stained. The dog was then treated intravenously with a single dose of 75 mg CA4P per m². The amount of CA4P was calculated by using the weight of the dog and converting it to its body surface area (BSA) (Kahn et al, 2010). The weight of the dog was 36 kg so its BSA is 1.101 m². Therefore, 82.5 mg CA4P, dissolved in 10 ml phosphate buffered saline (PBS), was slowly infused over a period of 30 minutes.
After treatment, the dog was hospitalized for one week to monitor potential side effects of the treatment. The different medical imaging techniques were repeated one and three days after treatment, after which new biopsies were taken and sent for histopathological analysis.

The dog was then re-examined at the Faculty of Veterinary Medicine weekly for one month, and then monthly for five months. Two weeks after treatment with CA4P the tumoral mass was macroscopically decreased in size. The overlying skin had a less irritated and ulcerative aspect, as shown in figure 2. Small circular wounds of the regions where the biopsies were taken remained and could still be seen. Additionally, the reddish aspect of the overlying skin was no longer present. Five months after treatment, there was inflammation and infection of the remaining tumoral mass. The dog was painful due to the infection and the ulceration of the remaining schwannoma. Following this clinical decline, the owners decided to euthanize the dog.

### 3.2 B-mode and power-Doppler ultrasound

In this clinical case, a linear transducer was used to obtain both B-mode and PDUS images of the schwannoma. A frequency of 4 Hz was used and the mass was scanned from a dorsal to palmar region.

Figure 3. The schwannoma can be defined as a solid mass, visible in B-mode. The surface is more outspoken and also clearly visible when using B-mode. When using PDUS, both images clearly show the vascularization of the schwannoma, visualized as yellow-red dots. Vasculature is prominent and clearly outspoken in both the central part of the tumor, as well as at the periphery.
Before treatment, and two weeks after the biopsy, vascularization of the schwannoma is demonstrated by using PDUS, as shown in figure 3. Vascularity is prominent and clearly outspoken in the central part of the tumor, as well as at the periphery.

Three days after treatment, the PDUS images show a significant decrease in vascularization. The vascularization in the central part of the tumor is completely absent; however, in the periphery, a small amount of vascularization remains. The decrease in vascularity can clearly be seen in figure 4.

3.3 Contrast-enhanced ultrasound

In addition to B-mode and PDUS, CEUS was performed to visualize the vascularity of the tumor before and after treatment. In this clinical case, a linear transducer (probe) of 12-5 MHz was used. The US machine that was used to obtain the CEUS images was an iU22 (Philips, Bothell, WA) with contrast-specific software. The contrast was injected intravenously into the cephalic vein by using a 22 gauge catheter through a three-way stopcock. The amount of contrast consisted of a bolus of 0.04 ml kg⁻¹ contrast medium and non-targeted sulfur hexafluoride-filled microbubbles (SonoVue®, Bracco Diagnostics Inc., Italy). It was immediately followed by a 2 ml sterile saline injection.
Before treatment, there is a large spectrum of vasculature seen as a Gaussian curve, shown in figure 5. This curve is referred to as the contrast intensity curve. The curve shows that vasculature is very prominent inside the tumor. Before treatment, the vasculature can be visualized with the microbubble contrast. The green line represents the total tumor, the yellow line represents the tumor periphery and the pink line represents the tumor center. There is a large uptake of microbubble throughout the septa of the tumor and the uptake goes from the periphery to the central part of the tumor, shown in the CEUS images in figure 6. In figure 6A, corresponding to the contrast-enhanced image, it is clear that the septa have a larger uptake of contrast than the other parts of the tumor but after a while the central part also has a large uptake. In the B-mode image in figure 6B it is clear that there is still tissue present in the region of the tumor that is scanned. This can be seen as a hyperechogenic zone that contains two septa in the middle.

Contrast-enhanced US of the mass was repeated one day after treatment. The Gaussian distribution is not visible anymore which means that the vasculature is clearly decreased, as demonstrated in figure 7. Figure 8A shows that the uptake of microbubble contrast after treatment is less in the central part of the schwannoma. The central part of the schwannoma is more hypo-echogenic than the rest of the schwannoma. On the B-mode image in figure 8B, there is still tissue present in the same region. This tissue doesn’t take up the contrast anymore after intravenous injection.

Contrast-enhanced image of the schwannoma before treatment. The microbubbles are taken up by the vasculature present in the mass, with a higher uptake in the two septa (red arrowheads). There is also a diffuse uptake of contrast in a large part of the tumor (red star). B-mode image of the same region shows that there is tumoral tissue present, seen as a hyperechogenic zone (blue star).

Contrast intensity curve of the schwannoma one day after treatment. The probe was positioned in the same spot as for the images of figure 5. The Gaussian distribution is almost completely gone which means that the uptake of microbubble contrast is significantly decreased. The three different lines (green, yellow and pink) can barely be seen.
3.4 Biopsies and histopathological sections

Biopsies of the tumor were taken two weeks before and three days after treatment with CA4P. A 6 mm punch-biopsy chamber was used. This technique reveals good quality material for further investigation. Multiple locations in the mass were sampled and a minimum of two biopsies were taken at each occasion. The dog needed to be sedated to reduce movement and pain sensation. The protocol used for this sedation was an intravenous injection of both butorphanol (Dolorex®, MSD, Brussels, Belgium) and dexmedetomidine (Dexdomitor®, Orion Corporation, Espoo, Finland). Butorphanol was administered at a dose of 0.2 mg kg⁻¹ and dexmedetomidine was administered at a dose of 5 µg kg⁻¹. The dog had a weight of 36 kg which means that the dose of butorphanol was 7.2 mg and the dose of dexmedetomidine was 0.180 mg.

The skin needs to be appropriately prepared before taking a biopsy (Tobias and Johnston, 2012). The hairs should be clipped and cleaned to reduce the contamination of the superficial skin. In this way, artefacts on histopathological investigation are reduced to an absolute minimum.

Following the biopsy, histopathological slides were made and stained with haematoxylin and eosin (HE) and anti von Willebrand Factor (vWF). The HE staining is essential for recognizing various tissue types and morphologic changes that form the basis of contemporary cancer diagnosis (Fischer et al, 2008). Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex reaction (Fischer et al, 2008). Eosin is pink and stains proteins nonspecifically. Therefore, in a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have various degrees of pink staining (Fischer et al, 2008). Well-fixed cells show considerable intranuclear detail (Fischer et al, 2008). The vWF staining was made because of its use for identifying vessels in tissue sections (Zanetta et al, 2000). The vWF is a glycoprotein produced uniquely by endothelial cells and megakaryocytes (Zanetta et al, 2000). It is stored in the intracellular granules or constitutively secreted into plasma¹.

Vessel density, in tumor specimens, as determined by immunohistochemical staining for vWF, is a negative prognostic factor for many solid tumors (Zanetta et al., 2000). The brown color in positive staining is due to the fact that the anti-vWF reacts with vWF present in endothelial cells, and is therefore an endothelial cell marker.

On the HE staining slides before treatment, neurologic tissue can be seen as purple mesenchymal cells with a dark purple nucleus. The Schwann cells are well defined and well organized in groups and have a viable character (i.e. the nucleus can still be found and the cells are well stained), as shown in figure 9. The Schwann cells are lying in sheets with no differentiation between tumoral cells and fibrovascular stroma. The cells are spindloid with a basophilic nucleus. The vWF-stained slides, as shown in figure 10, demonstrate a multitude of viable endothelial cells. The endothelial cell nuclei are dark brown (brown arrows), the tumoral cells stain blue.

Figure 9. Histopathological sections of the schwannoma before treatment. The Schwann cells are lying in sheets with no differentiation between tumoral cells and fibrovascular stroma. The cells are spindloid with a basophilic nucleus (Haematoxylin and eosin; Scale bar = 100 µm).

Figure 10. Histopathological sections of the schwannoma before treatment. Immunohistochemical staining for endothelial cells. The endothelial cell nuclei are dark brown (brown arrows), the tumoral cells stain blue (von Willebrand factor; Scale bar = 100 µm).
Biopsies of the schwannoma were repeated three days after treatment. Both HE staining and vWF staining were performed on the histopathological slides. On the HE-stained slides, shown in figure 11, the presence of necrosis is obvious. In comparison with the slides before treatment, the neurologic cells had a less dense nucleus after treatment and in some cells also karyorhexis could be seen. Additionally, the necrotic cells have a lighter purple color compared to the viable cells before treatment, which had a dark purple color. The necrotic regions can be distinguished from viable regions by means of the lighter color and disordered appearance. Necrosis can also be seen as eosinophilic amorphous material consisted of cellular and nuclear debris. Additionally, the necrotic cells have lost their nucleus and are not well organized. The cells have a more pink and amorphous structure. They also have a grainy aspect and core fragments of the nucleus are a sign of karyorhexis.

Figure 11. Histopathological sections of the Schwannoma three days after treatment. Figure 11A, B. There are multiple necrotic regions present in the histopathological slide (red arrowheads). Necrosis can be seen as eosinophilic amorphous material consisted of cellular and nuclear debris. Figure 11C. A big necrotic region can be seen, shown in the red circle (Haematoxylin and eosin; Scale bar = 100 µm).
4. **Discussion**

4.1 **Introduction**

In this clinical case a VDA, here: CA4P, was injected intravenously to treat a dog with a solid schwannoma on the carpal region. The goal of this study was to obtain as much clinical information on the effect of CA4P by using different diagnostic techniques. When regarding the results of the medical imaging techniques and the histopathological evaluation of the biopsies, it is clear that CA4P has a good result regarding the perfusion of the tumoral mass. The perfusion of the schwannoma after administration of CA4P is significantly decreased and necrosis of the tumoral tissue has increased.

4.2 **B-mode and PDUS**

Ultrasonography is already known as a useful and cost-effective tool for the evaluation of intra-abdominal neoplasms, particularly hepatic, adrenal, and urogenital tumors, and sub lumbar node metastasis (Liptak, 2009). Ultrasonography can also be used to guide FNA (fine needle aspiration) and needle-core biopsy for relatively noninvasive tissue sampling (Liptak, 2009). Newer developments in US technology that have proved useful in the evaluation of primary tumors, include Doppler ultrasonography to assess tumor vascularity and CEUS for differentiation of benign from malignant hepatic and splenic tumors (O’Brien et al, 2004).

There are many different options when evaluating the vascularization in a tumoral mass. In a first-line practice, when US is available, the vascularization and thus perfusion of a mass can easily be investigated using PDUS. For the visibility of vasculature, B-mode is not detailed enough because only differences in echogenicity can be seen. Blood flow will not be detected when using B-mode alone. Also in a veterinary practice or a veterinary clinic, both B-mode and PDUS are techniques that are already used when defining or investigating a mass of all sorts of types. In this specific case, a schwannoma was diagnosed in a dog and it was followed-up with both medical imaging techniques and biopsies for histopathological investigation. These techniques can provide us with a clear view of the effect of the administration of CA4P and the structural differences of the tumor before and after treatment. Additionally, these techniques can give detailed information without being too expensive or invasive, providing advantages both for the dog and the owner. Ultrasound can be used to evaluate masses present in a patient without doing a fine-needle aspiration or puncture. It is however, important to remark that US is never a decisive diagnostic option for every tumor. Some tumors are better seen on radiographs than when using B-mode or PDUS. Osteosarcomas for example, are easily detected by using radiographs and can be performed by any veterinarian in possession of an X-ray device. The exact diagnosis of an osteosarcoma always requires a biopsy of the bone mass so that further distinction with other masses can be made (Wittig et al., 2002). Therefore, some tumors should be diagnosed by other techniques than B-mode, PDUS or CEUS.
A suspected diagnosis of a schwannoma can be obtained non-invasively by using US imaging. It is based on the appearance of a solid hypo-echogenic, encapsulated tumor with an indirect continuity with a peripheral nerve at its proximal and distal poles (Reynolds et al., 2004). On PDUS images, a schwannoma has a homogeneously hypochoic echotexture, posterior acoustic enhancement and a hypervascular pattern (Reynolds et al., 2004). When US is used specifically for the evaluation of tumor response to CA4P, PDUS can give a detailed pattern of the vasculature before and after treatment.

A possible disadvantage of using PDUS can be the formation of artefacts and noise (Rubin et al, 1994). Power-Doppler US is relatively angle-dependent and displays background noise in a way that increases the usable dynamic range of an US scanner (Rubin et al, 1994). The artefacts can influence the further interpretation of the images and can lead to false negative or false positive results when evaluating therapy with CA4P.

Before treatment, the vasculature in the schwannoma is outspoken throughout the entire tumor. This can clearly be seen in figure 3. After treatment with CA4P, there are less vessels present. In the post-treatment PDUS images, shown in figure 4, there is a decrease in vasculature noticeable compared to the images before treatment. Given the fact that PDUS can easily give artefacts and false results, it is necessary to repeat the other medical imaging techniques and biopsies as well. In this way, the effect of CA4P can be objectively tested without the risk of obtaining false results.

**4.3 Contrast-enhanced ultrasound**

Contrast-enhanced US imaging is a good technique to obtain information on the vascularization of a tumoral mass. It is a very detailed technique, more detailed than PDUS, and gives clear information on the vascularization of the schwannoma because of the use of microbubble contrast. When administered intravenously, the microbubbles pass the pulmonary circulation and then enhance vascular end organs (Sano and Uemura, 2015). When using CEUS, it is clear that it gives a superior image of the presence and structure of vessels inside a (tumoral) mass (Zhang et al, 2018). It is a relatively new but very detailed technique that can be applied when evaluating tumoral vasculature (Vanderperren et al, 2014). It is however a specific technique that cannot be applied in a first-line practice. The use of CEUS requires a dedicated US machine and the injection of microbubble contrast. Additionally, specific training is required in order to interpret the results, which are obtained only by means of specialized software. Therefore, the use of CEUS is more common in a veterinary clinic or university setting.

Figure 6A shows a clear and detailed image of the vasculature present in the schwannoma. The microbubbles are taken up by the septa, shown as arrows, and the central part of the tumor, shown as a red star. This can be due to two things. Either there is leakage of contrast from the septa through the central part of the tumor, or there is also central vasculature present inside the tumor. Both options are possible although the second option is more likely as there was already central vasculature seen on the power-Doppler images before the CEUS images were taken.
One day after treatment, a difference can already be seen in the CEUS images. Compared to figure 5, which demonstrates the Gaussian curve before treatment, figure 7 shows that the Gaussian distribution is significantly reduced to almost absent. This means that the vasculature is clearly decreased and the uptake of contrast is almost none and therefore there is less vasculature present. However, in the CEUS image, shown in figure 8, we see that there is still an uptake of microbubble contrast. This means that some vasculature is still present, but that the uptake is now concentrated in the periphery of the tumor. In the central part of the tumor, less contrast uptake can be seen. In figure 8, a distinction is made between CEUS (figure 8A) and B-mode (figure 8B) images. Thus, when we compare the left and right image in figure 8, the right image clearly shows the presence of tissue, indicated with a blue star. The fact that there is still soft tissue present but it doesn’t take up the contrast, means that the vascular structures or blood vessels in the tumor are damaged. The fact that this tissue doesn’t take up any microbubbles can mean two things. Either the vasculature is disrupted by the administration of CA4P and the disrupted vessels can’t take up the contrast anymore, or there is less vasculature present and therefore less uptake can be seen. In this case, the first option is most likely. These findings clearly prove that there is a difference in vascular structure after the intravenous administration of CA4P. Therefore, the comparison of the CEUS images before and after treatment provide a good overview on how the vasculature has changed after therapy with a VDA such as CA4P.

A recent study also concludes that the use of CEUS to quantify tumor perfusion could be a promising method for the early detection of tumor response in anti-vascular treatment. This study shows that the Maximum Intensity, a perfusion parameter, is a reliable indicator of tumor perfusion evaluation by CEUS (Zhang et al, 2018).

4.4 Biopsy and histopathological slides

Biopsy provides essential information for diagnosis (neoplastic versus non-neoplastic, benign versus malignant), treatment options, and prognosis. There are four main types of biopsy techniques: FNA, needle-core biopsy, incisional biopsy, and excisional biopsy (Soderstrom and Gilson, 1995). Regardless of the technique performed, the same instruments should not be used to sample multiple masses because of the risk of contamination. Samples of cells or tissue are submitted for cytologic or histopathologic analysis, respectively. The ideal biopsy technique should simply and safely procure an adequate sample of tissue and consistently result in an accurate diagnosis (Soderstrom and Gilson, 1995). Tissue samples from needle-core, incisional, and excisional biopsies should be fixed in 10% buffered formalin at one part tissue to 10 parts formalin (Gilson et al, 1990; Withrow et al, 2001; Kharti et al, 2005). Brain, peripheral nerve, eye, and muscle tissue require special handling techniques and fixatives (Ehrhart, 1998). All samples should be submitted to a veterinary pathologist because the histologic type and grade of tumor are often important in treatment planning.

In this study, biopsy-taking was the most invasive technique that was used to obtain information on the effect of CA4P when treating the schwannoma. In private-practice, biopsies can be taken easily but a disadvantage is that the veterinarian needs an extern laboratory and pathologist to investigate and analyze the biopsy.
The procedure of taking a biopsy in a patient also requires sedation and/or anesthesia and a good preparation of the biopsy site (Tobias and Johnston, 2012). Depending on the sedation or anesthesia needed, it is not always the best and easiest option for the dog. Some patients can be critical during premedication or induction due to clinical conditions that can impede the use of anesthetics. In these patients, it is preferable to use other, and less invasive, diagnostic techniques and to limit the use of biopsies to an absolute minimum.

Multiple histopathological assessments revealed necrotic zones on the post-treatment histopathological slides colored with HE. There is a clear difference between the viable Schwann cells before treatment, which are well-stained with a clear nucleus, well-defined and well organized. After treatment, the cells are necrotic and this can be seen as multiple pink amorphous and grainy regions with core fragments. These fragments are a result of karyorhexis. The fact that there is a big difference in the microscopic aspect of the Schwann cells, proves that necrosis did take place after intravenous administration of CA4P.

In the post-treatment biopsies stained with vWF, the endothelial cells are decreased in number which suggests that the presence of vasculature is decreased. Before treatment, the Schwann cells on the vWF section were viable and had a dark nucleus. The vasculature could be seen as brown structures. The vessel density can be a prognostic factor in general when staining tissue sections. Different investigations have shown that high vWF levels in tumors may be an early sign of activation of the endothelium (Zanetta et al, 2000).

The use of biopsies was the most invasive technique because sedation is necessary and biopsies were taken two times, before and after treatment. In a private or first-line practice biopsies can be taken when a tumoral mass is present. A disadvantage of this technique is that these biopsies need to be sent to a laboratory and interpreted by a veterinary pathologist. This can mean an extra cost for the owner and it can take a couple of days until the results of the biopsies are known. For the interpretation of the decrease in vasculature, histopathological slides are a good technique because they give a clear and subjective image before and after treatment. Histopathological slides can also show the presence of necrosis or difference in cellular structure which cannot be seen when using only PDUS or CEUS. Therefore, biopsies are essential in the diagnosis of the type of tumor and the treatment afterwards. A first-line veterinary clinic can take biopsies if wanted and can work together with a laboratory for the interpretation and follow-up during and treatment. For the specific interpretation of the effectiveness of a VDA, it is better however to combine biopsies with a non-invasive technique such as PDUS or CEUS. The combination of both techniques will give a more complete image of the differences in vasculature and how the vasculature and surrounding structures evolve.
5. **Conclusion**

We can conclude from this study that the intravenous administration of CA4P has good results on the perfusion of the schwannoma. Vasculature is significantly decreased and necrosis of the cells is clearly visible. When we compare the different techniques to obtain information on the vascular structure of a tumoral mass, it is clear that not all of these techniques are applicable in a first-line practice. The use of both PDUS and CEUS require a suitable US machine and some experience when interpreting the images. Nevertheless, US is a low-cost technique that can be easily and non-invasively performed when an US machine is available. The patient doesn’t necessarily need sedation and by using an US machine, images of the mass can be taken and also the surrounding tissue can be easily evaluated.

In a first-line practice, PDUS is the best technique to gain information on the effect of a VDA. In a specialized veterinary clinic or university setting, CEUS is more detailed and gives a better view of the vasculature compared to PDUS. Also, to obtain CEUS images, the use of microbubble contrast is necessary.

This makes US, and more specific PDUS and CEUS, the preferred techniques to gain information when assessing the anti-vascular effect of a VDA.
6. References


