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CURRENT APPROACHES AND FUTURE DIRECTIONS IN THE MANAGEMENT, DIAGNOSIS AND TREATMENT OF CANINE MAMMARY TUMORS

APPROCCI ATTUALI E DIRETTIVE FUTURE NELLA GESTIONE, DIAGNOSI E TERAPIA DEL TUMORE MAMMARIO DEL CANE

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1. Introduction

Mammary gland tumor (MGT) is one of the most common neoplasms both in bitches and women (Frénel & Nguyen, 2023; Sorenmo et al., 2020).

Anatomically, physiologically and histologically, mammary tissues in dogs and humans show remarkable similarities (Ferreira et al., 2023). Canine mammary neoplasms arise spontaneously and resemble those diagnosed in humans in terms of morphology and biological behavior; however, the incidence rate in female dogs is estimated to be three times higher than in women (Pastor et al., 2018). Given these similarities, dogs serve as valuable models for human breast cancer (HBC) research and for comparative studies on prognosis and treatment (Kwon et al., 2023). The challenge in canine studies, however, lies in recruiting suitable patient populations. Many dog owners decline treatment or participation in clinical trials due to the high cost of therapies, lengthy treatment times or concerns about reduced quality of life for their pets (Ferreira et al., 2023; Kwon et al., 2023). Additionally, owners' subjective assessments during follow-up often hinder the collection of consistent and reliable data (Abdelmegeed & Mohammed, 2018).

In human oncology, a multidisciplinary approach—incorporating surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapies—has become standard and contributed to improved outcomes. By contrast, while various therapeutic options are available for dogs, standardized protocols are lacking due to the limited number of clinical trials and restricted access to advanced treatments (Frénel & Nguyen, 2023). Consequently, despite the biological similarities between canine and human MGT, the outcomes for dogs with mammary cancer remain poor (Frénel & Nguyen, 2023), while outcomes for women with breast cancer (BC) have improved significantly over time (Ferreira et al., 2023). This gap underscores an urgent need for enhanced research and infrastructure in veterinary oncology.

There is also a worldwide priority to explore alternative cancer biopathology research methods and to develop low-cost, effective therapies.

Female rats are the most widely used models for mammary cancer research in laboratories (Ferreira et al., 2023); however, they require chemical induction or genetic modification to develop tumors (Frénel & Nguyen, 2023; Kwon et al., 2023). Promising alternatives to animal testing include 2D and 3D in vitro tumor models derived from canine tumor samples (Ferreira et al., 2023), which allow for an in-depth study of the tumor microenvironment (TME). Since the TME is known to contribute to therapy resistance (Guo et al., 2023), developing therapies that target the TME is of critical interest and could be crucial for the long-term management of HBC. Additionally, interest is growing in using bacteria to control cancer progression, with numerous studies highlighting the therapeutic potential of *Salmonella enterica* serovar Typhimurium in cancer treatment (Broadway & Scharf, 2019; Chirullo et al., 2015; Chirullo et al., 2024; Hoffman, 2012; Razzuoli et al., 2022).

This study has two main objectives. First, it aims to review current best practices in the management, diagnosis and treatment of canine mammary tumors, emphasizing common and accessible methods available to most veterinary facilities. Critical points within the diagnostic and therapeutic process will be analyzed, focusing on areas that can be improved with current knowledge and skills. The second objective is to examine methodologies that could represent breakthroughs for the future, particularly in developing new treatments aimed at improving survival times for both dogs and humans affected by mammary tumors.

2. Managing Canine Mammary Tumors: A Practical Guide to Assist Veterinarians

Abstract

Mammary tumor is the most frequently diagnosed neoplasia in non-spayed female dogs. Despite the high incidence, the outcomes for mammary cancer-bearing bitches remain poor. Currently, standardized guidelines for managing canine mammary cancer are lacking, and there is no widely accepted, multidisciplinary therapeutic approach tailored to cancer stage and subtype.

This review aims to equip clinical veterinarians with essential information about canine mammary tumors, to improve disease management. Although significant advancements have been made in our understanding of canine mammary cancer, there is an urgent need for standardized protocols to assist veterinarians in clinical practice. In the absence of such guidelines, effective decision-making relies on clinical judgment and evidence-based knowledge.

2.1 Introduction

Mammary cancer is one of the most prevalent neoplasia affecting both humans and dogs; however, the clinical outcomes between these species differ significantly (Frénel & Nguyen, 2023). In dogs, the prognosis remains poor, characterized by high rates of locoregional recurrence and distant metastasis, along with survival rates of less than 2 years for most bitches following the excision of malignant mammary tumors (Szczubial et al., 2011).

In contrast, advances in breast cancer treatment in humans have led to notable improvements in survival, despite a still considerable mortality rate (Ferreira et al., 2023).

Several key factors contribute to the advancements in human oncology. In the treatment of human breast cancer (HBC), a multidisciplinary, well-established approach is employed, including surgery, chemotherapy, radiation therapy, endocrine therapy for hormone receptor (HR)-positive cancers, as well as targeted and immuno-therapy. This approach is based on a deep understanding of tumor biology, which has enabled the development of standardized guidelines for HBC treatment according to cancer stage and subtype (Frénel & Nguyen, 2023). In dogs, several adjuvant therapeutic options exist although there are no standard procedures for mammary gland tumor (MGT)-bearing dogs. One

reason is the limited number of minimally powered clinical trials conducted in dogs, resulting in a lack of reliable data on the efficacy and safety of treatments (Kwon et al., 2023). Additionally, cancer biology is often not thoroughly investigated in canine treatment plans, leaving many therapies insufficiently assessed (Frénel & Nguyen, 2023). For other treatments, such as radiation therapy, there are limits related to cost and the limited availability of these services in veterinary practices (Larue & Gordon, 2020).

Furthermore, HBC routine screening programs, such as mammograms, enable earlier detection and more effective interventions, with improved prognosis (Coleman, 2017). In dogs, the absence of standardized screening programs often results in delayed diagnosis, limiting treatment efficacy (Frénel & Nguyen, 2023).

It is essential to recognize that the role of companion animals has evolved significantly over time. Today, they are often regarded as true family members, providing companionship, emotional support and enhancing overall well-being (Overgaauw et al., 2020). As pets are increasingly seen as family, owners expect the highest quality medical care for them. This is especially relevant in veterinary oncology, where a cancer diagnosis can heavily strain the human-animal bond. The psychological toll of an animal's illness on the owner must also be considered, as it falls within the broader framework of the One Health concept (Shawn, 2020).

This review aims to assess current practices in the management, diagnosis and treatment of canine mammary tumors, identifying key steps where improvements can be made to enhance both clinical outcomes and the quality of care provided in veterinary oncology.

2.2 Epidemiology, etiology and risk factors

MGT is the most common neoplasm in female dogs, with an estimated annual incidence ranging from 205 to 418 cases per 100,000 bitches (Baioni et al., 2017; Dobson et al., 2002; Sorenmo et al., 2020). However, the incidence of canine mammary tumors (CMT) varies depending on the characteristics of the studied population, as well as the geographical location, since it is influenced by a series of factors including the age of the dogs, breed and spaying habits in the country where the study is

conducted. In fact, in European countries, especially those in the south, there is a higher incidence of cases compared to the United States or Western Europe, where the neutering procedure is usually performed at an early age in dogs (Sleeckx et al., 2011; Vazquez et al., 2023). Spaying performed at an early age is considered to have a protective effect against the development of MT in bitches because ovarian estrogen and progesterone stimulate the proliferation and growth of mammary epithelium (Santos et al. 2010).

In intact females, mammary tumors (MTs) account for an overwhelming 50-70% of all diagnosed tumors (Brønden et al., 2010; Merlo et al., 2008; Salas et al., 2015; Vascellari et al., 2016), while they are extremely rare in male dogs (Saba et al., 2007; Zheng et al., 2022), where most cases are benign and have a good prognosis (Saba et al., 2007). Intact female dogs are 62 times more likely to develop MGTs than males due to their hormonal environment (Salas et al., 2015). Beyond hormonal exposure, several factors such as age, breed, diet and obesity have been associated with an increased risk of developing MTs (Sorenmo et al., 2011).

2.2.1 Hormonal exposure: does spaying prevent mammary tumors?

Spaying has long been recognized as an effective measure to reduce the incidence of MTs in both dogs and cats (Romagnoli et al., 2024). The influential 1969 study conducted by Schneider and colleagues highlighted that a dog's susceptibility to developing MT rises significantly during the initial estrus cycles. This is exemplified by the observation that a dog undergoing ovariohysterectomy before the first estrus cycle has a mere 0.5% likelihood of MT development, contrasting with rates of 8% and 26% in dogs undergoing the procedure between the first and second estrus cycles or after the second estrus cycle, respectively (Schneider et al., 1969). However, after conducting a comprehensive review of the literature, it was concluded that the evidence supporting the role of OHE in reducing the risk of tumor development was limited (Beauvais et al., 2012), making the issue controversial.

Ovarian hormones, primarily estrogen and progesterone, are essential for the growth and development of normal mammary tissue. At the same time, their proliferative effect makes them some of the main actors in carcinogenesis (Vazquez et al., 2023).

However, several studies showed that HRs expression is lower in mammary carcinomas compared to benign epithelial tumors, which respond well to spaying and are usually associated with a good prognosis (Mainenti et al., 2014; Millanta et al., 2005; Nieto et al., 2000). The expression of HRs further decreases with higher degrees of malignancy (Mainenti et al., 2014), which are also associated with a higher risk of metastasis (Nieto et al., 2000).

In the recent study of Gedon and colleagues (2022), authors estimated a 6.5 times greater risk for MGT development for intact female dogs compared to spayed ones but they noted that hormonal ablation at any point in life appears to lead to a higher frequency of more aggressive malignant tumor subtypes and may therefore contribute to a worse prognosis for spayed female dogs with MTs (Gedon et al., 2022).

Analyzing both past and recent studies on CMT, a consistent reduction in the incidence of MTs has been observed over time, likely due to an increased practice of neutering (Gedon et al., 2022; Merlo et al., 2008; Rodriguez et al., 2022). However, the proportion of malignant tumors has remained relatively unchanged, suggesting a higher likelihood of malignancy in the tumors that do develop in neutered dogs (Rodriguez et al., 2022). Some studies suggest that tumors developing in spayed bitches are more frequently of a malignant nature (Gedon et al., 2022) and that spayed females with MGTs have a 4.5 times higher risk of recurrence or metastasis and a shorter overall survival time (OST) compared to intact females (Peña et al., 2013).

A recent study by Hart and colleagues (2020) revealed that, in certain breeds, the incidence of MTs in female dogs spayed after 6 months of age is significantly higher compared to intact females of the same group. These breeds include Golden Retrievers, Australian Shepherds, Border Collies, Boxers, Chihuahuas, Cocker Spaniels, Collies, English Springer Spaniels and Shih Tzus. However, none of the females spayed before 6 months of age developed MT (Hart et al., 2020).

Furthermore, spaying before 12 or 24 months has been associated with an increased risk of other cancers (including lymphoma, mast cell tumors, hemangiosarcoma, and osteosarcoma) as well as debilitating joint disorders (Hart et al., 2020). These findings suggest that it may be advisable to either

leave female dogs intact throughout their lifespan or delay spaying until after 12 or 24 months of age, depending on the breed (Hart et al., 2020).

For mixed-breed dogs, another study by the previously mentioned group found no association between weight categories and cancer development. However, they identified an increased risk of joint disorders in dogs over 20 kg that were spayed at an early age, suggesting that, for dogs of this size, neutering be delayed until 12 months of age or later (Hart et al., 2020).

Although spaying before puberty does appear to be associated with a reduced risk of developing mammary tumors, current guidelines suggest that the decision to spay a female dog—and the optimal timing for this procedure—should not be based solely on the goal of preventing mammary tumours (Romagnol et al., 2024). Instead, a broader range of factors should be considered, as mentioned before.

Therefore, scheduling regular check-ups to monitor for the development of MGTs may be a prudent course of action. Nevertheless, gonadectomy at the time of MT surgical excision, should always be considered (Banchi et al., 2022). Concurrent neutering with mastectomy is likely to significantly reduce the incidence of new MTs in dogs with hyperplastic or benign lesions by almost 50% (Kristiansen et al., 2013). Its beneficial effect doesn't seem to be the same for dogs with malignant lesions; however, it has been observed that some of them could benefit from concurrent spaying, especially subjects with estrogen receptors (ER)-positive grade II tumors or with increased peri-surgical serum estradiol concentration (Kristiansen et al., 2016).

Dogs that develop mammary tumors within 2.5 years of being spayed do not seem to benefit from hormonal ablation; on the contrary, the decrease in hormones following spaying creates a hormone-poor environment, which could favor HR-negative subclones (Sorenmo et al., 2000) which have a worst prognosis if compared to HR-positive tumors (Kim et al., 2014).

Exposure to exogenous or pharmacologic doses of hormones—including progestins and estrogens—raises the risk of MGT development in dogs. Most studies agree that low-dose progestins primarily increase the likelihood of benign tumors, while a combination of estrogens and progestins tends to

promote the formation of malignant tumors (Concannon et al., 1981; Geil & Lamar, 1977; Giles et al., 1978; Kwapien et al., 1980; Misdorp, 1991; Selman et al., 1995). Additionally, dogs treated with progestins not only face a higher tumor risk but also tend to develop these tumors at a younger age (Misdorp, 1988). Repeated episodes of pseudocyesis have been reported as predisposing factors (Romagnoli et al., 2024).

Other factors, such as pregnancy or parity, do not significantly influence the risk of developing MGTs (Brodey et al., 1966; Schneider et al., 1969; Sonnenschein et al., 1991).

2.2.2 Age

CMT primarily affects older or middle-aged dogs (Sorenmo et al., 2011). It is rare in individuals under 6 years old, but beyond that age, it becomes the most common tumor in intact females (Gamlem et al., 2008) with an approximate 60% risk of development between 8 and 13 years old (Vascellari et al., 2016). Additionally, the peak incidence age varies depending on the lifespan of different breeds. Generally, larger breeds have shorter lifespans and thus tend to be younger when diagnosed compared to smaller breeds. These variations are particularly pronounced in high-risk breeds like English Springer Spaniels (median age of onset at 6.9 years) (Egenvall et al., 2005).

Increasing age is also associated with a greater risk of developing malignant forms (Rodriguez et al., 2022; Vascellari et al., 2016). The chance of having a malignant tumor it is likely to increase by 1.19 times with every year of age (Gedon et al., 2021); indeed, dogs with malignant tumors are significantly older compared to dogs with benign tumors (with age ranges typically between 9 to 11 years versus 7 to 9 years, respectively) (Goldschmidt et al., 2001; Sorenmo et al., 2009).

In spayed bitches, the average age of onset for a mammary tumor appears to be higher (Rafalko et al., 2023), along with a greater tendency towards malignancy (Vascellari et al., 2016).

According to Rafalko and colleagues (2023), there in an inverse correlation between weight and age at cancer diagnosis, with purebred dogs being diagnosed at significantly younger ages than mixed-breed dogs.

To enhance the chances of early detection and treatment, it could be reasonable to contemplate annual cancer screening starting at the age of 7 for all dogs, and as early as age 4 for breeds with a lower median age at cancer diagnosis (Rafalko et al., 2023).

2.2.3 Breed

Smaller breeds seem to be more frequently affected by MGTs (Sorenmo et al. 2019); however, CMT can be observed in dogs of any breed, nevertheless several studies have shown a higher MT incidence in purebred than in mixed-breed dogs (Merlo et al., 2008; Sleeckx et al., 2011; Vascellari et al., 2016). In the 2016 research conducted by Vascellari and colleagues, purebred dogs under 7 years of age were approximately twice as likely as mixed-breed dogs to have a malignant neoplasm, whereas for dogs over 7 years of age, no disparities between the two groups were observed.

Among small breed dogs, Poodles, Chihuahuas, Dachshunds, Yorkshire Terriers, Maltese and Cocker Spaniels are considered those at high-risk, whereas English Springer Spaniel, English Setters, Brittany Spaniels, German Shepherds, Pointers, Doberman Pinschers and Boxers are regarded as large high-risk breeds (Burrai et al., 2020). However, there is no consensus on which breeds are at the highest risk of developing MT because incidence can vary depending on study characteristics and geographic location. Providing some examples, in Italy, according to the study of Vascellari and colleagues of 2016, the Samoyed dogs were approximately twice as likely to develop malignant MT than the overall population, followed by Doberman, Schnauzer and Yorkshire Terrier, whereas English Setters, Golden Retrievers and Labrador Retrievers are considered low-risk dogs. In Spain, Retrievers, flushing dogs and water dogs seem to have the higher incidence of MT (Pastor et al., 2018). In Sweden, English Springer Spaniel (ESS), Doberman and Boxer are among the breeds with the highest risk of developing MTs (Egenvall et al., 2005).

The English Springer Spaniel (ESS) is recognized as one of the breeds at the risk for developing CMT. In a study by Rivera and colleagues in 2009, a significant correlation between CMT and breast cancer genes 1 and 2 (BRCA1, BRCA 2) was identified in ESS dogs, with BRCA1 showing a stronger association with malignant cases. Women with inherited mutations in the BRCA1 or BRCA2 genes

have heightened susceptibility to breast cancer (Antoniou et al., 2006; Ford et al., 1998; King et al., 2003); therefore, it is likely that the mutation of the BRCA1 and BRCA2 genes in ESSs contributes to the increased predisposition to MT development in this breed (Rivera et al., 2009).

2.2.4 Obesity and diet

It is now well-established that factors such as diet and obesity are risk factors for cancer incidence in both humans and dogs (Vazquez et al., 2023). Obesity might influence CMTs through inflammation in adipose tissue, leading to cell proliferation and angiogenesis (Lim et al., 2015). A 2020 study found that 49% of these tumors expressed adiponectin (Tesi et al., 2020), which has apoptotic and antiproliferative effects (Laflamme et al., 2006; Lee et al., 2019), but no association with tumor aggressiveness was observed (Tesi et al., 2020). Furthermore, adipose tissue and high levels of cholesterol serve as sources of steroid hormones, including estrogens, progesterone and androgens; the conversion of androgens into estrogens by aromatase seems to be more pronounced in overweight dogs likely due to an increased expression of these enzymes (Lim et al., 2015).

Overweight female dogs have shown to exhibit elevated aromatase expression, earlier onset of CMT and a higher histologic grade (Lim et al., 2015).

The effect of obesity on cancer development appears to be most pronounced when obesity occurs at around 1 year of age, during the period when hormonal impacts on mammary tissue are most detrimental (Romagnoli et al., 2024). Furthermore, high consumption of red meat in young overweight dogs has been shown to increase the risk of MT and dysplasia development (Alenza et al., 1998). From another study, it was found that there is no significant correlation between homemade food and the development of mammary tumors in dogs. However, dogs on a homemade diet tend to have a higher body condition score (BCS) compared to those fed a commercial diet, and weight gain could potentially impact cancer incidence (Tesi et al., 2020).

2.3 Clinical examination

Detection of MGTs is generally straightforward during routine physical exams, wherein both mammary chains and the regional lymph nodes (LNs) should be thoroughly assessed.

In female dogs, the mammary glands (MGs) are anatomically divided into five pairs: cranial thoracic (M1), caudal thoracic (M2), cranial abdominal (M3), caudal abdominal (M4) and inguinal (M5). Variations in anatomy are common, without any association with the breed, with some individuals having more or fewer glands than usual (Ferreira et al., 2023). The axillary and superficial inguinal LNs are recognized as the regional nodes of the cranial and caudal MGs (Pimentel et al., 2023).

MGTs typically present as well-defined nodules with varying sizes, consistency and degrees of mobility relative to the skin and muscles (Vazquez et al., 2023). Ulceration can be caused by invasive tumor growth or large masses trauma (Soares et al. 2023) (Figure 1).

Tumor size, fixation to underlying structures and ulceration are strong predictors of negative prognosis (Hörnfeldt & Mortensen, 2023; Soares et al., 2023). Dogs with tumors ≥ 5 cm in diameter and/or present for more than six months may have already metastasized to LNs (Chang et al., 2005). M4 and M5 are more frequently affected than the thoracic glands. Due to the larger size of these glands and surrounding tissues, meticulous palpation may be required to detect small tumors (Sorenmo et al., 2020). MTs can present as single or multiple nodules; multiple tumors can occur in more than one gland at the same time or coexist in the same unit, as they can be of different histological types and grades simultaneously (Vazquez et al., 2023). Multiple mammary nodules are

Figure 1. A large, ulcerated mammary tumor localized between M3 and M5 in the right mammary chain.

significantly more common in intact bitches than in spayed females (Gedon et al., 2022), suggesting that sexual hormones may influence tumor multiplicity (Chai and Brown, 2009).

Examination of the regional LNs should be incorporated into the standard clinical assessment of dogs with mammary tumors, as the presence of metastasis affects the cancer's clinical staging, thereby influencing prognosis and treatment strategies. Clinically normal nodes can be challenging to detect, as the axillary LNs are often not palpable and the superficial inguinal LNs are located deep within the inguinal fat pad beneath the fifth MG (Sorenmo et al., 2020). However, the regional anatomical LNs may not always be the first to receive drainage because of the potential for aberrant lymphatic pathways originating from the tumor (Liptak & Boston, 2019).

Determining the ratio of benign to malignant mammary neoplasms in dogs is challenging due to various influencing factors, but it is estimated that approximately 50% of these tumors are malignant (Sorenmo et al., 2003; Thomson & Britt, 2022).

Benign tumors are associated with significantly smaller mean sizes compared to their malignant counterparts (Burrai et al., 2020; Gedon et al., 2021; Sorenmo et al., 2009). Small neoplasms (<1 cm in diameter) are more likely to be benign whereas larger ones (>3–5 cm in diameter) are more frequently malignant (Goldschmidt et al., 2001) (Figure 1).

Nonetheless, it is not uncommon for malignant tumors to present at sizes smaller than 1 cm (Burrai et al., 2020; Gedon et al., 2021); therefore, performing post-excisional histological examination is mandatory for all nodules, even the smallest ones. In any case, it is currently believed that there is a progression of lesions from benign to malignant as their size increases, according to the “histological continuum” theory suggested by Sorenmo and colleagues in 2009 for CMT (Gedon et al. 2021). In spayed female dogs, this progression is likely to occur even at smaller tumor sizes, suggesting the protective role of sexual hormones against malignant progression in the histological continuum via non-receptor or immunoregulatory mechanisms (Gedon et al., 2022). Although not all benign tumors acquire malignant characteristics, benign lesions should therefore be considered as precursors and predictors of malignant lesions (Sorenmo et al., 2009).

At the time of diagnosis, most dogs are clinically healthy, and tumors are confined to the MGs (Sorenmo et al., 2020). However, the patient's clinical status primarily depends on the presence of comorbidities or distant metastases. Approximately 8% to 32% of mammary carcinomas spread to LNs (Pimentel et al., 2023). The extent of LN involvement varies and can facilitate distant metastasis, predominantly to the lungs (Vazquez et al., 2023), with liver and bone metastatic lesions possible (Agnoli et al., 2023; Sorenmo et al., 2020). Generally, the most common symptoms associated with metastases include fatigue, lethargy, weight loss, dyspnea, cough, edema or lameness; their presentation and intensity depend on the localization and size of the metastases (Vazquez et al., 2023). Table 1 lists all the clinical factors with negative prognostic value that should be considered during the examination.

In addition to the physical examination, collecting the dog's complete medical history is essential. This should include a detailed reproductive history of the bitch (covering the regularity of heat cycles, number of litters, spaying status, use of hormonal therapies, abortions and history of pseudocyesis), the approximate time when the lesions were first noticed by the owner and any previous occurrences of mammary or other tumors (Cassali et al., 2014).

Table 1. Clinical features which are recognized to influence outcome in canine mammary tumors.

NEGATIVE PROGNOSTIC CLINICAL FACTORS
Tumor size (T>5cm)
Fixation to underlying structures
Ulceration
Tumor present for > 6 months
Lymph node metastasis
Distant metastasis

2.4 Diagnostic work-up and staging

When assessing CMTs, staging prior to the initiation of therapy is mandatory, due to the risk of metastasis. In the presence of a mammary nodule, the minimal preoperative diagnostic work-up and staging should include a complete blood count (CBC), serum biochemistry, fine-needle aspiration cytology (FNAC) of the mammary nodules and regional LNs, three-view thoracic radiographs and

abdominal ultrasound. To accurately characterize LN status, it is highly recommended to perform lymphatic drainage mapping in addition to cytology of the regional LNs (Pimentel et al., 2023).

Biopsy of canine mammary nodules prior to surgery is rarely performed, because the extent of surgery is often determined by the size and number of the lesions (Thomson & Britt, 2022), regardless of the biological features, but FNAC can be helpful in differentiating benign vs malignant tumors (Cassali et al., 2007; Simon et al., 2009) and in excluding alternative diagnoses such as mastitis or non-mammary dermal and subcutaneous tumors (e.g. lipomas or mast cell tumors and various others) (Sorenmo et al. 2019).

Abdominal ultrasound can be instrumental in detecting abdominal metastases (Sorenmo, 2003) and is recommended for dogs with suspected regional LN involvement or alterations in preoperative blood work indicating tumor-related or non-tumor-related serum biochemistry changes (Sorenmo et al., 2020), while the ultrasonographic evaluation of MGTs using traditional B-mode and doppler methods is not reliable in identifying features indicative of tumor malignancy. On the other hand, according to Feliciano and colleagues (2017) acoustic radiation force impulse elastography has proven to be a great accurate predictor of malignancy.

Computed tomography (CT) scans offer greater sensitivity in identifying metastases compared to thoracic radiography. In fact, CT can detect lesions as small as 1 mm, making it more sensitive than radiography. However, the practicality of CT for every patient may be limited due to higher costs and restricted accessibility (Barbagianni & Gouletsou, 2023).

CMT is staged through the TNM system, established by the World Health Organization (WHO) (Owen et al., 1980). Based on this system, the patient is categorized into one of five clinical stages according to the size of the primary lesion (T), the spread to LNs (N) and the presence or absence of distant metastases (M), as shown in Table 2.

Table 2. World Health Organization - TNM system staging for Canine Mammary Tumor

STAGING OF CANINE MAMMARY TUMORS			
Stage	**Tumor Size**	**Lymph Node Status**	**Metastasis**
Stage I	T1 <3 cm	N_0	M_0
Stage II	T2 3-5cm	N_0	M_0
Stage III	T3 >5cm	N_0	M_0
Stage IV	Any	N_1 (positive)	M_0
Stage V	Any	Any	M_1 (metastasis)

This staging system should be utilized for determining prognosis and guiding case management in dogs with non-inflammatory epithelial tumors, but not for sarcomas (Sorenmo et al., 2020), as it can serve as a predictor of survival times (Pimentel et al., 2023).

Tumor diameter is recognized as an independent prognostic factor (Rasotto et al., 2017) and a valuable tool for assessing the biological behavior of CMTs (Ferreira et al., 2009).

Masses greater than 5 cm are mostly malignant and related to worse prognosis (Ferreira et al., 2009). Nevertheless, the incidence of highly malignant tumors measuring < 1 cm provides compelling evidence for the re-evaluation of tumor size in the assessment of TNM-WHO clinical staging (Burrai et al., 2020). Recently, paralleling developments in human oncology, a new histological staging model with prognostic relevance has been put forth (Table 3) (Chocteau et al., 2019).

LN positive disease and distant metastasis are associated with a poor prognosis (Sorenmo et al., 2020). A 2011 study by Szczubial and Lopuszynski found that dogs with LNs involvement (stage IV) had an average survival time of 6-8 months, compared to over 18 months for those with unaffected LNs.

The TNM staging system does not consider the number of metastatic LNs. However, the presence of two or more involved LNs appears to be associated with a worse prognosis compared to having only one metastatic LN (de Araújo et al., 2015).

Table 3. Histological staging system proposed by Chocteau and colleagues (2019)

Stage	Invasiveness	Pathologic tumor size	Lymph vascular invasion	Pathological nodal state
Stage 0	*In situ*	Any pT	LVI-	pN0 or pNX
Stage I	Invasive	pT1 ≤ 20 mm	LVI-	pN0 or pNX
Stage II		pT2 > 20 mm		
Stage IIIA		pT1 ≤ 20 mm	LVI+ and/or pN+	
Stage IIIB		pT2 > 20 mm		

LVI-, absence of lymph vascular invasion.

LVI+, presence of lymph vascular invasion.

pN0, absence of nodal metastasis.

pN+, presence of nodal metastasis.

pNX, nodal stage unknow.

2.4.1 Nodule fine-needle aspiration cytology

While the definitive diagnosis of MGTs must be confirmed by histopathology, FNAC can be considered a valid non-invasive and cost-effective preliminary exam through which valuable information about the mammary nodule can be obtained. Cytology does not provide a complete picture of tissue architecture and may not allow for the distinction between a reactive or neoplastic process, or even between benign and malignant lesions, especially in cases of inflammation, necrosis or hemorrhage. The dog's owner must be informed that FNAC provides limited information and may yield non-diagnostic or inconclusive results. However, Simon and colleagues (2009) demonstrated that cytological examination has a sensitivity of 88% and a specificity of 96% for diagnosing malignant MGTs, with histopathology serving as the gold standard. Other reports further substantiate the efficacy of cytologic examination as a valuable tool in supporting the diagnosis of MGTs (Cassali et al., 2007; Ghisleni et al., 2006; Pierini et al., 2017; Simeonov & Simenova, 2006; Simeonov & Simenova, 2006; Simeonov & Simenova, 2007). The heterogeneity of CMT, along with the variability of cellular morphology in different tumor areas, suggests the necessity of sampling multiple portions of the nodule to increase the likelihood of recognizing the true characteristics of the lesion. Additionally, it is advisable for the slides to be examined by multiple observers to mitigate the error associated with examination subjectivity.

Small-gauge needles (22–25g) are suitable for smaller lesions, reducing hemorrhage. Medium-sized syringes (12–15 cc) provide more suction than smaller ones (3–6 cc). The objective is to draw cells into the needle shaft, not to fill the syringe. After the needle is inserted into the lesion, vacuum is maintained in the syringe while the needle is redirected into the tissue multiple times to collect a broad representation of cells (this is particularly crucial when aspirating LNs for metastasis evaluation). Subsequently, the vacuum is released, the needle is removed from the tissue and syringe, the syringe is filled with air, reattached to the needle and cells are expelled onto a glass slide. An alternative method, known as "fenestration", involves the rapid insertion of the needle into the lesion without aspiration. This method often yields as much cellular material as the aspiration technique and produces less hemorrhage and patient discomfort. Similarly, a syringe is employed to expel material onto a slide, after which a clean slide is placed on top and gently separated to achieve a monolayer of intact cells. Immediate spreading prevents samples from becoming too thick, while applying excessive pressure may rupture cells, resulting in a non-diagnostic specimen (Friedrichs & Young, 2020). Cell smears collected via puncture should be air-dried or promptly fixed in a 70% ethanol solution. It's important to minimize contamination from blood, hair and liquefied material, as this may compromise sample quality (Cassali et al., 2007).

2.4.2 Sentinel lymph node mapping and assessment

The lymphatic system serves as the primary route of metastasis of CMT (Patsikas et al., 2006) and LN staging is essential for both the diagnosis and treatment of CMT, as it significantly influences the prognosis (Pimentel et al., 2023).

FNAC is a safe method for examining LNs and is recommended for peripheral LNs that exhibit changes in size, shape and texture during clinical assessment. However, the size of a LN as determined by palpation does not necessarily indicate the presence of metastasis (Williams & Packer, 2003). Despite reports indicating high sensitivity and specificity of the technique in detecting metastasis (Lagenbach et al., 2001; Ku et al., 2017; Yu et al., 2016), particularly for carcinomas

(Fournier et al., 2018), metastatic disease may still be present even if tumor cells are not identified in a sample collected from a LN.

The lymphatic drainage of the mammary chains in bitches is complex, and cancer-induced lymph angiogenesis further alters the existing lymphatic connections during tumor development (Patsikas et al., 1996; Patsikas et al., 1996; Patsikas et al., 2006; Pereira et al., 2003).

The sentinel LN (SLN) is defined as the first LN in the regional lymphatic system that receives lymphatic drainage from the primary tumor and is frequently different to the regional anatomic LN (Annoni et al., 2023). Conceptually, metastatic progression occurs in an orderly manner: if the SLN is analyzed and found negative for metastasis, the subsequent LNs in the drainage pattern will also be negative, making the hypothesis of distant metastasis almost impossible (Ehrhart, 2020).

In healthy dogs, the lymphatic vessels in the mammary chains are divided into cranial (M1 and M2) and caudal (M4 and M5) groups. The cranial group usually drains into the ipsilateral axillary LNs, while the caudal group primarily drains into the ipsilateral superficial inguinal LNs. The M3 gland has a unique drainage pattern, connecting to both the ipsilateral axillary and superficial inguinal LNs, with a preference for the axillary pathway (Patsikas et al., 1996; Patsikas et al., 1996; Patsikas et al., 2006; Pereira et al., 2003). Furthermore, in healthy mammary glands, ipsilateral lymphatic communications typically occur among M1, M2 and M3, as well as between M4 and M5. In contrast, contralateral anastomoses appear to be present only between contralateral M2 and M5 (Pereira et al., 2003). Conversely, Pereira and colleagues demonstrated in 2003 that neoplastic mammary glands exhibit a higher prevalence of various types of lymphatic anastomoses (40.9%) compared to healthy glands (33.33%). Additionally, an increase in contralateral anastomoses (50%) was observed in neoplastic mammary glands compared to healthy ones (Pereira et al., 2003).

Figure 2 illustrates the common lymphatic drainage under physiological conditions and the potential pathways of communication between neoplastic MGs in dogs and various lymphatic centers. It is important to note that each lymph center is composed of multiple LNs.

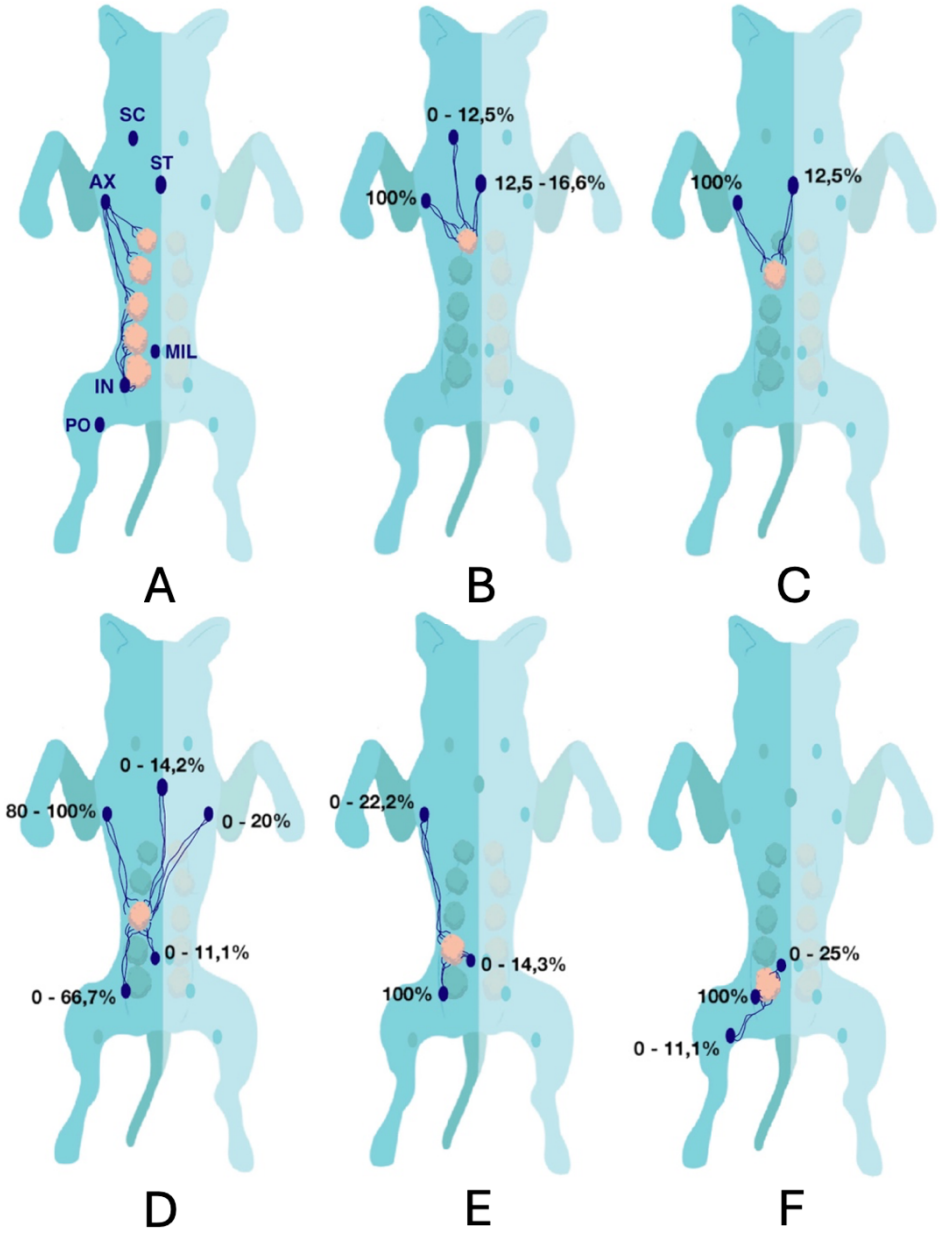

Figure 2. Common lymphatic drainage under physiological conditions (A) and potential pathways of communication between neoplastic M1 (B), M2 (C), M3 (D), M4 (E), M5 (F) and various lymphatic centers in dogs. AX: axillary lymph center; SC: superficial cervical lymph center; ST: sternal lymph center; IN: superficial inguinal lymph center; MIL: medial iliac lymph center; PO: popliteal lymph center.

Analyzing the relevant literature, neoplastic M1 and M2 usually drain into the ipsilateral axillary (100%) and sternal (M1:12,5-16,6%, M2:12,5%) lymph centers (Patsikas et al., 2006; Pereira et al., 2003). Pereira and colleagues (2003) reported the involvement of the ipsilateral superficial cervical lymph center in 12.5% of cases of tumoral M1 drainage. Neoplastic M3 may be often drained by the axillary (80-100%) and the inguinal (0-66.7%) lymph centers (Collivignarelli et al., 2021; Martonos et al., 2019; Patsikas et al., 2006; Pereira et al., 2003). Regarding the axillary lymph center, some authors specified that the accessory axillary LN was involved in the drainage in 20-57.1% of cases

(Collivignarelli et al., 2021; Martonos et al., 2019). Interestingly, in rare cases, neoplastic M3 have demonstrated drainage into the contralateral axillary (12.5-20%) (Collivignarelli et al., 2021; Pereira et al., 2003), ipsilateral medial iliac (11.1%) (Patsikas et al., 2006) and sternal lymph centers (14,2%) (Martonos et al., 2019).

Neoplastic M4 may have its drainage directed to the ipsilateral inguinal (100%) and axillary (0-22,2%) lymph centers (Collivignarelli et al., 2021; Patsikas et al., 2006; Pereira et al., 2003), but also ipsilateral medial iliac lymph center may be involved (0-14.3%) (Collivignarelli et al., 2021). Tumoral M5 may often drain through the inguinal lymph center (100%), but both ipsilateral popliteal (0-11.1%) and medial iliac (0-25%) lymph centers may be involved (Collivignarelli et al., 2021; Patsikas et al., 2006; Pereira et al., 2003). Different lymph centers may be concurrently involved (Collivignarelli et al., 2021; Patsikas et al., 2006; Pereira et al., 2003).

Given the variability and unpredictability of lymphatic drainage, SLN mapping enables the identification of LNs that are the first to receive draining tumor lymph, thereby highlighting those most at risk for metastasis. It is important to emphasize that a positive result for a lymph node on mapping does not necessarily indicate the presence of metastasis. Consequently, surgical excision of the SLN and subsequent histopathological analysis is strongly recommended to establish prognosis and guide therapeutic options (Sorenmo et al., 2003).

Furthermore, the extent of the LN area affected by cancer, as well as the number of involved nodes, are critical factors (de Araújo et al., 2015). Some authors reported that LN macro metastasis (foci of neoplastic cells >2 mm in diameter) were significantly associated with shorter overall survival and disease-free intervals (de Araújo et al., 2015; Szczubial & Lopuszynski, 2011). In contrast, micro metastases (foci ranging from 0.2 to 2 mm) did not significantly impact outcomes compared to cases without LN metastasis (Szczubial & Lopuszynski, 2011). This suggests that in cases of micro metastasis, lymphadenectomy could have therapeutic value, as it is hypothesized that the metastatic cells have not yet spread beyond the LN.

Concerning the controversial role of isolated tumor cells, a poor prognosis appears to be associated only with aggressive histotypes of the primary tumor (Coleto et al., 2018; de Araújo et al., 2015)

Given that lymphatic drainage of canine mammary tumors varies significantly among individual dogs, it is advisable to identify the involved SLN(s) for each new patient encountered.

In human medicine, standard care for SLN identification involves peritumoral injection of radioactive marker and subsequent scintigraphy (Brissot & Edery, 2017; Pereira et al., 2008). Lymphoscintigraphy has also been described in dogs (Hlusko et al., 2020; Pereira et al., 2008; Worley, 2014); however, this equipment is not readily available in most veterinary facilities.

Nowadays there is no standard approach for SLN mapping in veterinary medicine, although many techniques have been described in recent years (Annoni et al., 2023).

Do date, indirect lymphography (IL) is considered an alternative to scintigraphic SLN mapping (Brissot & Edery, 2017; Collivignarelli et al., 2021; Ehrhart, 2020; Hlusko et al., 2020). Imaging agents are instilled into the tissues adjacent to the tumor, leading to preferential accumulation and lymphatic uptake to delineate the lymphatic pathways. Depending on whether LN mapping is intended for staging purposes or to assist in surgical resection, contrast agents, radiopharmaceutical colloids or dyes may be used (Worley, 2022).

For preoperative staging purposes, imaging after contrast uptake can be accomplished through various modalities, with conventional radiology and CT being the most routinely employed (Brissot & Edery, 2017; Collivignarelli et al., 2021; Majeski et al., 2017; Mayer et al., 2013; Rossi et al., 2018; Tuohy et al., 2009).

Given that the positivity of a LN on mapping significantly influences the surgical treatment, recent studies have been conducted to evaluate the prognostic value of IL through CT and ultrasonographic assessments of the SLN (Soultani et al. 2017; Stan et al. 2020).

According to Soultani and colleagues (2017), the size and shape of sentinel LNs as observed in CT-IL, did not show accuracy in the prediction of metastasis; on the contrary, a low degree of iodinated

contrast enhancement, was significantly associated with the presence of metastases, as demonstrated through the following histopathological examination.

As previously mentioned, sentinel LNs ultrasonographic assessment may serve as a valid exam in predicting metastases, and thus preventing their unnecessary excision. In 2020, Stan and colleagues developed an ultrasound examination algorithm for assessing the SLN based on B-mode imaging, Doppler techniques, contrast-enhanced ultrasound and elastography. This method achieved an accuracy of 92.2% in detecting metastasis, with the presence of metastasis confirmed through histopathological examination (Stan et al., 2020).

Below, general practical indications for performing digital radiographic IL will be provided. Radiology has been selected for the description of this technique due to its widespread adoption among veterinarians, attributable to its cost-effectiveness and lack of requirement for highly specialized equipment (Hlusko et al., 2020).

Authors reported the use of both lipid-based (Brissot & Edery, 2017; Collivignarelli et al., 2021) or water-soluble contrast agents (Annoni et al., 2023; Hlusko et al., 2020). Water-soluble agents enable rapid lymphatic uptake and clearance; therefore, imaging should be performed within minutes following administration (Majeski et al., 2017; Soultani et al., 2017). In contrast, optimal images with lipid-based agents are obtained at least 24-48 hours after injection due to their slower clearance (Brissot & Edery, 2017; Collivignarelli et al., 2021; Hlusko et al., 2020; Mayer et al., 2013). Furthermore lipid-based media are more expensive compared to water-soluble agents and may cause adverse reactions at the injection site (Hlusko et al., 2020).

2.4.2.1 How to perform a radiographic indirect lymphography

The contrast medium is injected in four quadrants at the border of the tumor into the subcutis (Annoni et al., 2023; Collivignarelli et al., 2021; Hlusko et al., 2020) or dermis (Brissot & Edery, 2017) using a 22-27 gauge needle (Annoni et al., 2023; Brissot & Edery, 2017; Collivignarelli et al., 2021). Care must be taken to avoid injecting the contrast medium in the blood vessels.

Specifically for MGT, Collivignarelli and colleagues described the injection of 0.8 mL to a maximum of 1.6 mL of Lipiodol Ultra-Fluid® (a lipid-based contrast medium with a concentration of 480 mg/mL), evenly distributed across the four quadrants (Collivignarelli et al., 2021). For lipid-based contrast agents, radiographs should be taken 24–48 hours after administration (Collivignarelli et al., 2021; Hlusko et al., 2020). On the other hand, the use of water-soluble agents like iohexol and iopamidol allows immediately after-injection image evaluation (Annoni et al., 2023; Hlusko et al., 2020). Some authors have described the peritumoral injection of iohexol in volumes ranging from 0.3 to 0.75 mL to detect SLNs of head and neck tumors in dogs through CT scans (Randall et al., 2020). A minimum of two orthogonal radiographic projections should be taken 4 to 5 times within the first 10 minutes following the injection (Annoni et al., 2023; Hlusko et al., 2020) (Figure 3).

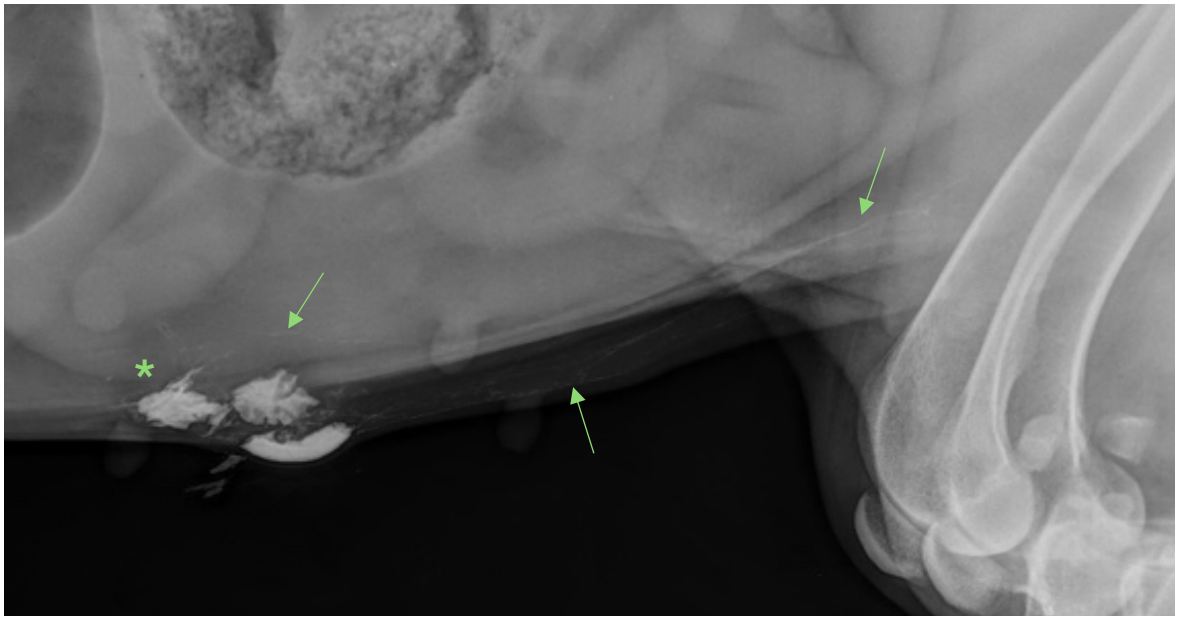

Figure 3. Magnified view of a contrast-enhanced lateral abdominal radiograph illustrating peritumoral marking due to contrast administration (green asterisk) and initial lymphatic uptake toward the caudal lymph centers (green arrows).

2.4.3 Diagnostic imaging

Radiology is the preferred initial screening test for detecting metastases due to its widespread availability and cost-effectiveness. Consequently, it has been selected as the sole method for describing metastasis detection.

It is important to note that in cases with multiple negative prognostic clinical factors or when a high-risk tumor is suspected based on cytological findings, recommending contrast-enhanced CT as the initial diagnostic exam to the dog's owner may be prudent. This technique would offer more accurate information regarding the presence of LN and distant metastases (Barbagianni & Gouletsou, 2023; Liptak & Boston, 2019; Soultani et al., 2017).

Three-view radiographs are always recommended, to allow a complete evaluation of the lungs and to ameliorate lesion conspicuity (Nykamp & Randall, 2019; Ober & Barber, 2006; Rudorf et al., 2008). Furthermore, depending on the recumbency position, there may be varying degrees of pulmonary parenchyma atelectasis. This is particularly relevant in lateral recumbency: when performing thoracic radiographs, prolonged lateral recumbency should be avoided to prevent position-dependent lung atelectasis. The lung in the dependent hemithorax collapses due to the reduced mobility of the thoracic cage and the compression exerted by the heart and the ipsilateral portion of the diaphragm during its cranial excursion. This atelectasis occurs shortly after the animal is positioned in lateral recumbency and is proportional in magnitude to the patient’s body mass (Thrall, 2013).

Although the use of sedation may exacerbate the degree of recumbency-associated atelectasis (Thrall, 2013), it is highly recommended as it induces drowsiness in the animals, making them more manageable and reducing the respiratory rate. This, in turn, minimizes movement artifacts due to thoracic cage excursions (Rudorf et al., 2008).

To minimize artifacts when performing a chest x-ray study, it is advisable to position the sedated animal in sternal recumbency and start the exam performing a dorsoventral (DV) view, because the least amount of recumbency-associated atelectasis occurs in this position (Thrall, 2013). Subsequently, the two lateral projections can be performed. For detection of pulmonary metastasis, the ventrodorsal (VD) projection is highly recommended (Rudorf et al., 2008).

Regardless of the projection performed, the exposure should typically be made at the end of the inspiratory phase, when the lungs are fully expanded allowing for more efficient detection of

metastases. Additionally, a positional marker (right or left) should be always included in the collimated area to double check the positioning.

Lung metastases typically present as nodular interstitial patterns characterized by circular, well-defined radiodensities which require a minimal diameter of 3-5 mm to be visible (Maï et al., 2008) (Figure 4.). Superimposition of soft tissue structures sometimes prevents their visibility or can mimic pulmonary nodules, as in the case of nipples and costochondral junctions, which can be recognized through the observation of their regular distribution. A poorly aerated lung similarly reduces the possibility of recognizing metastases.

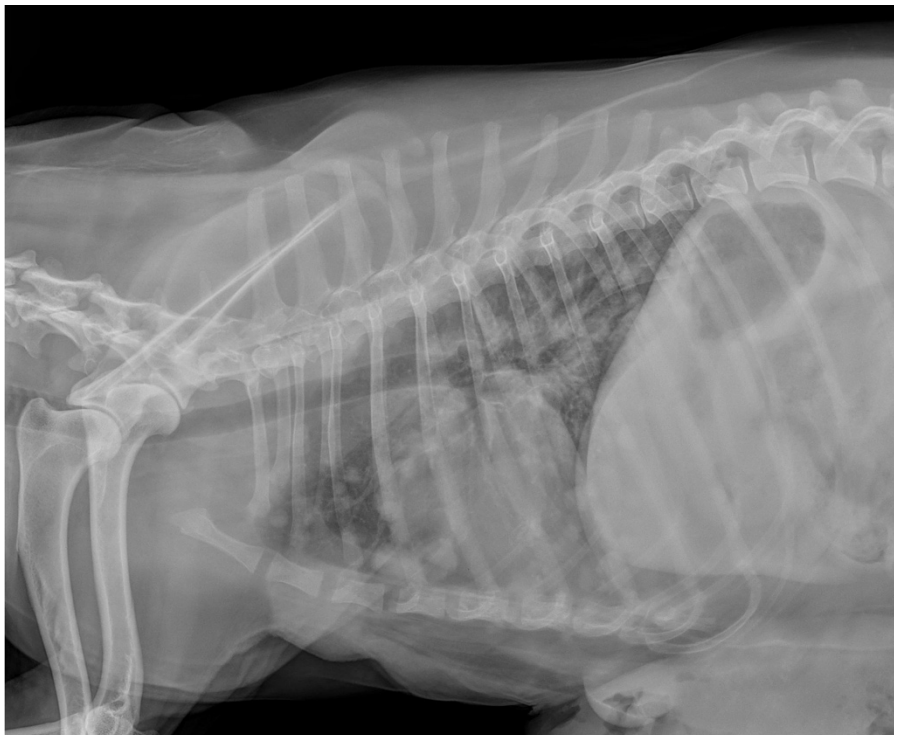

Figure 4. Lateral-view radiograph of the thorax showing disseminated pulmonary metastases. The image also reveals a large mammary tumor located in the abdominal mammary glands, from which the metastases originated. This dog is classified as TNM stage V.

2.4.3.1 Standard radiographic thoracic views positioning

DV: The dog should be positioned in sternal recumbency with the forelimbs extended so that the elbows are at the sides of the thorax and with the hindlimbs flexed in a crouched position. The use of sandbags can help maintain this positioning. The sternum and vertebrae must be perfectly aligned so that the hemi thoraxes are symmetrical. The beam must be centered between the shoulder blades and at their caudal aspect.

VD: The dog must be positioned in dorsal recumbency in a trough or bean bag with the forelimbs pulled forward and the hindlimbs left in a “frog leg” position. The use on sandbags allows the fixation of the forelimbs. The sternum and vertebrae must be perfectly aligned so that the hemithorax’s are symmetrical. The beam must be centered on the center of the sternum.

For DV and VD views, collimated area should include the thoracic inlet, diaphragm and cranial abdomen, and the lateral aspects of the thorax.

Lateral recumbency: The dog must be positioned in lateral recumbency with the head and neck extended and the forelimbs pulled forward and maintained in this position with sandbags. This is very important to avoid the overlap of the triceps muscles with the cranial lung lobes. The neck can be supported by a concave support. To ensure the dog remains in the required position, hindlimbs can be hold by sandbags. A foam wedge positioned under the sternum or spine can be helpful to hold sternum and vertebrae levelled on the same plane. The beam should be centered approximately at the intersection of a vertical line tangent to the caudal edge of the scapula and a line passing through the midpoint of the distance between the spine and the sternum. Collimated area should include the thoracic inlet and whole diaphragm (Rudorf et al., 2008) (Figure 5).

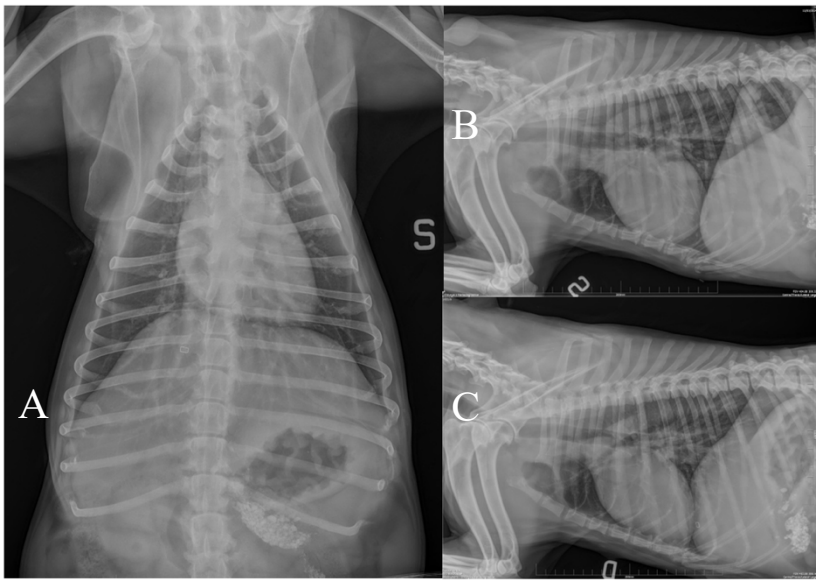

Figure 5. Three-views radiographic study of the thorax: dorsoventral (A), left lateral (B) and right lateral recumbency (C) projections.

2.5 Histotype, grading and prognosis

Histopathological examination is fundamental as it enables the assessment of tumor histotype and grade, the presence of lymphatic invasion and the status of the LNs, all of which are critical in defining prognosis. Tumor histotype has recently been confirmed as an independent prognostic factor (Rasotto et al., 2017).

Regarding the histotype, over the years various classification systems for canine mammary tumors have been proposed (Goldschimdt et al., 2011; Hampe and Misdorp, 1974; Misdorp et al., 1999; Monlux et al., 1977). Authors chose for description the 2019 update (Zappulli et al., 2019) by WHO and Davis-Thomson DVM foundation of the 2011 classification by Goldschmidt and colleagues (Table 4).

The majority of CMTs originate from epithelial tissues; however, some tumors have a mixed origin, involving both epithelial and myoepithelial tissues, as seen in complex carcinoma or complex adenoma. Additionally, certain tumors are of mesenchymal origin, like osteosarcoma, fibrosarcoma and fibroadenoma, while carcinosarcomas or mixed benign tumors arise from both mesenchymal and epithelial tissues.

For epithelial tumors, grading is determined through the assessment of tubules formation, nuclear pleomorphism and mitosis per 10 high-power fields (HPS) (Elston and Ellis, 1991; Peña et al. 2013). The tumor will be given a grade I (low, well differentiated), II (intermediate, moderately differentiated) or III (high, poorly differentiated) based on the score obtained (Peña et al. 2013). A higher grade corresponds to greater malignancy and a worse prognosis (Peña et al. 2013; Rasotto et al., 2017).

Grade III tumors have shorter disease-free intervals (DFI) and overall survival (OS) compared to grade I and II tumors (Nunes et al., 2018; Peña et al. 2013; Soares et al., 2023), which tend to behave similarly and are linked to longer tumor-specific survival (Rasotto et al., 2017; Soares et al., 2023). The risk of death for grade III tumors is 7.1 times higher than for grade I (Rasotto et al., 2017).

Table 4. Davis Thomson Foundation classification of Canine Mammary Tumors. Main categories are indicated in bold.

Canine Mammary Tumors histotype classification
1. Hyperplasia/Dysplasia
1.1 Duct ectasia
1.2 Lobular hyperplasia (adenosis)
1.2.1 Regular
1.2.2 With secretory activity
1.2.3 With fibrosis
1.2.4 With atypia
1.3 Epitheliosis
1.4 Papillomatosis
2. Benign epithelial neoplasms
2.1 Simple benign tumors
2.1.1 Adenoma—simple
2.1.2 Myoepithelioma
2.2 Non-simple benign tumors
2.2.1 Complex adenoma
2.2.2 Benign mixed tumor
2.2.3 Fibroadenoma
2.3 Ductal-associated benign tumors
2.3.1 Ductal adenoma
2.3.2 Intraductal papillary adenoma
3. Malignant neoplasms
3.1 Carcinoma–in situ
3.2 Simple carcinomas
3.2.1 Tubular (including cribriform) carcinoma
3.2.2 Tubulopapillary carcinoma
3.2.3 Solid carcinoma
3.2.4 Invasive micropapillary carcinoma
3.2.5 Comedocarcinoma
3.2.6 Anaplastic carcinoma
3.3 Non-simple carcinoma
3.3.1 Carcinoma arising in complex adenoma/benign mixed tumor
3.3.2 Complex carcinoma
3.3.3 Carcinoma and malignant myoepithelioma
3.3.4 Mixed carcinoma
3.4 Ductal-associated carcinoma
3.4.1 Ductal carcinoma
3.4.2 Intraductal papillary carcinoma
4. Malignant epithelial neoplasms-special types
4.1 Squamous cell carcinoma
4.2 Adenosquamous carcinoma
4.3 Mucinous carcinoma
4.4 Lipid-rich carcinoma
4.5 Spindle cell carcinoma
4.6 Malignant myoepithelioma
5. Malignant mesenchymal neoplasms
5.1 Osteosarcoma
5.2 Chondrosarcoma
5.3 Fibrosarcoma
5.4 Hemangiosarcoma
5.5 Other sarcomas
6. Carcinosarcoma
7. Hyperplasia/dysplasia of the Teat
7.1 Melanosis of the skin of the teat
7.2 Hyperplasia of the teat
8. Neoplasms of the teat
8.1 Benign ductal-associated neoplasms
8.1.1 Ductal adenoma
8.1.2 Intraductal papillary adenoma
8.2 Malignant ductal-associated neoplasms
8.2.1 Ductal carcinoma
8.2.2 Intraductal papillary carcinoma
8.3 Carcinoma with epidermal infiltration (Paget-like disease)

Information regarding stromal and vascular/lymphatic invasion aids in identifying more aggressive tumor behavior, as these factors indicate an increased risk of lymphatic system involvement and consequently the presence of metastases (Rasotto et al., 2012; Rasotto et al., 2017).

Carcinosarcomas and sarcomas are generally not evaluated using this system; however, most are biologically aggressive tumors linked to very poor long-term survival (Sorenmo et al., 2020).

Specifically regarding histotypes, Rasotto and colleagues (2017) evaluated the median survival time (MST) as well as the 1- and 2-year survival rates in dogs undergoing surgical tumor excision (Table 5). They found that carcinomas arising from benign mixed tumors exhibit behavior similar to that of benign tumors, such as complex adenomas, benign mixed tumors and simple adenomas, which showed no recurrence or metastases development.

Histotypes such as carcinosarcoma, solid carcinoma, pleomorphic lobular carcinoma (de Araújo et al., 2015; Gonçalves et al., 2021; Nunes et al., 2018; Rasotto et al., 2017) and invasive micropapillary carcinoma (de Souza et al., 2023; Gonçalves et al., 2021) are associated with a higher likelihood of metastasizing to regional LNs, leading to reduced survival times (de Araújo et al., 2015).

The worst prognosis overall has been observed for anaplastic carcinoma and carcinosarcoma with described MST of 3 months and a respectively 89 and 100% metastatic rate (Rasotto et al., 2017).

Adenosquamous, comedo- and solid carcinoma are associated with a poor prognosis (MST respectively of 18, 14, 8 months), with adenosquamous carcinoma exhibiting the highest local recurrence rate (50%) (Rasotto et al., 2017).

Dogs with complex and simple tubular carcinomas tend to exhibit a prolonged overall survival, whereas those with other types, such as simple tubulopapillary carcinoma, intraductal papillary carcinoma and carcinoma and malignant myoepithelioma, face more than a tenfold increased risk of tumor-related death compared to the former (Rasotto et al., 2017).

Similarly, Nunes and colleagues described shorter MSTs for dogs with invasive micropapillary carcinomas (MST=188 days) and solid carcinomas and carcinosarcomas (MST=193 days), when

compared to those with carcinomas in mixed tumors (MST not reached at 980 days) (Nunes et al., 2014).

Table 5. Median survival time and 1- and 2-year survival rate after surgical resection for specific histotypes of canine mammary tumors from Rasotto et al., 2017.

		Survival Rate	
Subtype	**MST**	**1 Year**	**2 Year**
Complex carcinoma	nr	100%	96%
Simple tubular carcinoma	nr	93%	73%
Simple tubulopapillary carcinoma	nr	75%	67%
Intraductal papillary carcinoma	nr	83%	50%
Carcinoma and malignant myoepithelioma	nr	70%	55%
Adenosquamous carcinoma	18	60%	0%
Comedocarcinoma	14	71%	29%
Solid carcinoma	8	45%	25%
Anaplastic carcinoma	3	0%	0%
Carcinosarcoma	3	0%	0%

MST= median survival time (months); nr= not reached

Mammary gland sarcomas are typically biologically aggressive tumors and are linked to very poor long-term survival outcomes. Common types include osteosarcoma (OSA), fibrosarcoma, chondrosarcoma and hemangiosarcoma. Among these, OSA is by far the most frequent. Metastasis occurs via the hematogenous route, mainly to the lungs (Sorenmo et al., 2020).

Metastases and recurrences in mesenchymal tumors appear to develop in approximately 80–100% of bitches following excision (Szczubial et al., 2011).

In 2019, Sorenmo and colleagues proposed a refined flexible scoring (RFS) system adapted for epithelial carcinomas to identify high-risk dogs based on tumor size, LN involvement, histotype and grading as shown in Table 6. The RFS identifies as high-risk dogs those with a bio-score > 3 (Sorenmo et al., 2019). In cases of mammary tumor multiplicity, prognosis should be determined on the base of the most aggressive tumor.

Furthermore, assessment of tumor markers enables the determination of prognosis and the formulation of effective individualized treatments.

Table 6. The refined flexible scoring system (RFS) for epithelial carcinomas by Sorenmo and colleagues (2019)

Factor	Categories	Score
Tumor Size	Any size	0 (in all grade 1 tumors)
	≥5	1 (all grade 2 tumors)
	Any size	1 (all grade 3 tumors)
Grade	1	0
	2	1
	3	2
Lymph node status	Negative or unknown	0
	positive	0 in grade 1 and 2 tumors 1 in grade 3 tumors
Histological subtypes	1 (carcinoma*)	1
	2 (complex carcinoma)	0
	3 (carcinoma arising in benign mixed tumor)	0
	4 Others**	2
Total bio-score		Maximum possible score: 6 Low-grade: ≤2 High-grade: >3

*Included carcinomas were tubular, tubulopapillary and ductal carcinomas.
**Others: solid carcinomas, comedocarcinoma, carcinoma and malignant myoepithelioma, anaplastic carcinoma, carcinosarcoma.

2.5.1 Tumor Markers

Unlike in human breast cancer (HBC), tumor markers are rarely investigated in dogs. ER and progesterone receptors (PR), epidermal growth factor receptor 2 (HER2) and Ki-67 (proliferation index) immunohistochemical markers are strong predictive factors and their investigation allows for the classification of four immunophenotypes in HBC, which determine treatment options: Luminal-A BCs (ER-positive and/or PR-positive, HER2-negative and low Ki-67 proliferation index), Luminal-B BCs (ER-positive and/or PR-positive, HER2-positive or high Ki-67 proliferation index), HER2-positive BCs (ER-negative, PR-negative, HER2 protein overexpression and/or HER2 gene amplification) and triple-negative BCs (ER−negative, PR−negative, HER2−negative) (Frénel & Nguyen, 2023).

Although tumor markers are not routinely evaluated in dogs, similar immunophenotypes have been described, which retain prognostic significance (Abadie et al., 2018).

The two most common immunophenotypes in CMT appear to be triple-negative (Abadie et al., 2018) and luminal-B-HER2-negative (Frénel & Nguyen, 2023), with the former showing significantly

shorter DFI and OS in comparison to luminal-A tumors (Abadie et al., 2018). ER-/PR- MTs have a worst prognosis if compared to ER+/PR+ and ER-/PR+ tumors (Kim et al., 2014).

Regarding HER-2, its role in CMTs has been debated. In fact, while it may play a part in tumor formation, it does not seem to be directly linked to malignant transformation or it cannot be considered a reliable marker of malignancy (Kaszak et al., 2018).

Several studies confirm Ki-67 proliferation index as a good predictor of high grade of malignancy and poor prognosis in CMTs (Arenas et al., 2016).

Other biomarkers investigated in CMTs are cyclooxygenase-2 (COX-2) and endothelial growth factor (VEGF) (Valdivia et al., 2021). The overexpression of VEGF, Ki-67 and COX-2 markers in the primary tumor is associated with a worse prognosis and an increased likelihood of metastasizing to LNs (Carvalho et al., 2017; Queiroga et al., 2010).

Tumors ≥ 5 cm in diameter proved to be associated with loss of PR and a higher ki-67 proliferation index (Ferreira et al., 2009).

In summary, a thorough understanding of the biological behavior of a specific tumor histotype, as well as its grade and stage, is crucial for veterinarians when advising the owner of a dog suffering from mammary cancer. This knowledge is essential for accurately indicating the prognosis and discussing therapeutic options.

2.6 Therapy

2.6.1 Surgery

Except in cases of inflammatory or stage V carcinoma, surgery remains the preferred treatment option for both benign and malignant MTs (Sorenmo et al., 2020).

Surgery is curative for all cases of benign tumors (Rasotto et al., 2017; Thomson & Britt, 2022) and for most malignant clinical stage I tumors (MacEwen et al., 1985), such as carcinoma arising in benign mixed tumors, complex carcinomas and simple tubular carcinomas (Rasotto et al., 2017).

Surgery cannot be considered curative for all malignant epithelial tumors larger than 5 cm (stage III), as well as for those with regional or distant metastases (stages IV and V), aggressive histotypes,

lymphatic histological invasion or high-grade histological carcinomas. In these cases, medical therapy is recommended due to the increased risk of recurrence or metastasis (Sorenmo et al., 2020). Surgery of high-risk tumors, such as adenosquamous, comedo-, solid, anaplastic carcinoma and carcinosarcoma, always requires support of chemotherapy (Rasotto et al., 2017; Sorenmo et al., 2020).

Given their high tendency to metastasize, surgery may not be completely curative for other tumors such as pleomorphic lobular, micropapillary, squamous cells (Gonçalves et al., 2021) and lipid-rich (Muscatello et al., 2021) carcinomas.

Carcinoma and malignant myoepithelioma, intraductal papillary carcinoma and simple tubulopapillary carcinoma might benefit from adjuvant therapies (Rasotto et al., 2017).

Following the recommendations from the scoring system of Sorenmo and colleagues (2019), dogs diagnosed with a bio-score≤2 have an excellent prognosis following surgical resection. Dogs with a bio-score>3 are at risk of metastasis and require adjuvant systemic therapy (Sorenmo et al., 2019).

Palliative surgery in stage V dogs does not improve prognosis. However, it may be performed to prevent ulceration of the tumor mass, relieve pain or stop bleeding (Cassali et al., 2014).

Currently there are no standard guidelines about the appropriate extent or "dose" of surgical excision. Due to the lack of data standardization, to date none of the published studies shows a clear benefit of choosing one surgical dose over another. Therefore, the decision to perform lumpectomy, simple mastectomy, regional mastectomy or unilateral or bilateral chain mastectomy should be based on known prognostic factors and surgeon's experience (Hörnfeldt & Mortensen, 2023).

Selecting the most appropriate treatment option for the patient necessitates consideration of numerous factors including age, spaying status, tumor size, number and location of nodules, ulceration, fixation to underlying structures, lymphatic drainage from the affected gland, clinical stage as well as previous mammary gland tumors (Lana et al., 2007).

Whenever feasible, the optimal outcome following surgical excision should be the achievement of clean margins and the prevention of new tumors on the remaining mammary glands. Incomplete

tumor excision is predictive of local recurrence (Rasotto et al., 2017) and should be followed by an additional resection (Sorenmo et al., 2020). In a 2021 study by Chocteau and colleagues, resection of locoregional recurrence was associated with prolonged survival (44 months compared to 6 months in untreated dogs.

However, excessive surgical dosing is not advisable, even for prophylactic purposes. Instead, the preferred approach should always be to choose for the least invasive surgery that achieves the best possible outcome.

When unnecessary, performing radical prophylactic surgeries is discouraged as it prolongs the duration of the procedure, increases stress and nociceptive stimulation and makes postoperative complications more frequent (Horta et al., 2015).

To properly plan the extent of surgery, it is crucial to recognize that the lymphatic system serves as the primary route for the spread of metastasis in canine mammary cancer (CMC) and that lymphatic connections exist between several glands (Patsikas et al., 2006; Pereira et al., 2003). Additionally, metastases may be present in mammary gland tissue even if it appears clinically unaffected (Busch & Rudolph, 1995). Therefore, it is strongly advised to remove the LNs and the glands associated with lymphatic drainage during the excision of MTs.

For intact bitches, it is advisable to perform simoultaneously ovariectomy or ovariohysterectomy. Tumor excision should be conducted after spaying and following the closure of the abdominal cavity. This approach helps minimize the risk of tumor cell dissemination in the event of accidental rupture of the tumor capsule (Sorenmo et al., 2020).

Among the various degrees of surgical intervention, the following are recognized:

- Lumpectomy or nodulectomy: this procedure involves the localized removal of a small solitary tumor (<0,5 cm), for which a benign nature is hypothesized. It should be not performed with tumors expressing malignant features. The skin and surrounding mammary tissue are resected along with the nodule, respecting a margin of 1 cm approximately (MacPhail & Fossum, 2019; Thomson & Britt, 2022; van Nimwegen & Kirpensteijn, 2018).

- Simple mastectomy or mammectomy: this technique consists of the complete removal of the entire gland in cases of small tumors (around 1 cm), centrally located in the breast (Sorenmo et al., 2020; Thomson & Britt, 2022).
- Regional mastectomy: this procedure entails the en bloc removal of MGs 1 to 3 for tumors located in the thoracic glands and MGs 3 to 5 for tumors located in the caudal abdominal and/or inguinal glands, based on knowledge of the venous and lymphatic anatomy of canine mammary tissue. It is indicated in case of larger tumors or multiple nodules in adjacent glands which lie in these regions. The inguinal LN is automatically removed during the surgical excision of 3 to 5 glands, as it lies within the fatty tissue beneath the fifth mammary gland. The axillary LN should be removed if it is enlarged or tests positive or uncertain for metastasis, as well as in cases where it is positive on LN mapping (Thomson & Britt, 2022; van Nimwegen & Kirpensteijn, 2018).

  As previously reported, since M3 can often drain into both the ipsilateral axillary and superficial inguinal LNs, cranial or caudal regional mastectomy may not be effective to prevent new tumors formation in the remaining glands. The same applies in cases of caudal regional mastectomy for neoplastic M4 resection (van Nimwegen & Kirpensteijn, 2018).

  Some authors have reported a 'bilateral caudal regional mastectomy,' which consists of the simultaneous bilateral excision of M4 and M5 (Thomson & Britt, 2022). After regional mastectomy, approximately 22% of bitches with benign nodules (Sorenmo et al., 2009) and 48-58% of those with malignant nodules (Sorenmo et al., 2009; Stratmann et al., 2008) have shown the development of new tumors in the remaining ipsilateral MGs.
- Chain mastectomy: this surgical procedure involves the en bloc removal of MGs 1 to 5 and it could be unilateral or bilateral. This procedure is especially recommended in dogs affected with multiple nodules on the same chain (Sorenmo et al., 2020) or with tumors in M3 and M4, draining both cranially and caudally (van Nimwegen & Kirpensteijn, 2018). It is not inadvisable to perform a bilateral chain mastectomy simultaneously due to the numerous

related complications. It is therefore recommended to perform a staged procedure (two unilateral mastectomies 3 to 6 weeks apart) (Thomson & Britt, 2022; van Nimwegen & Kirpensteijn, 2018).

Regardless of the procedure chosen, the patient should be positioned in dorsal recumbency, with the thoracic limbs secured cranially and the pelvic limbs fixed caudally in a relaxed state.

In the case of extensive surgeries, bitches should be clipped from the mid-chest to the caudal inguinal region and prepared for aseptic surgery, as shown in Figure 6 (MacPhail & Fossum, 2019).

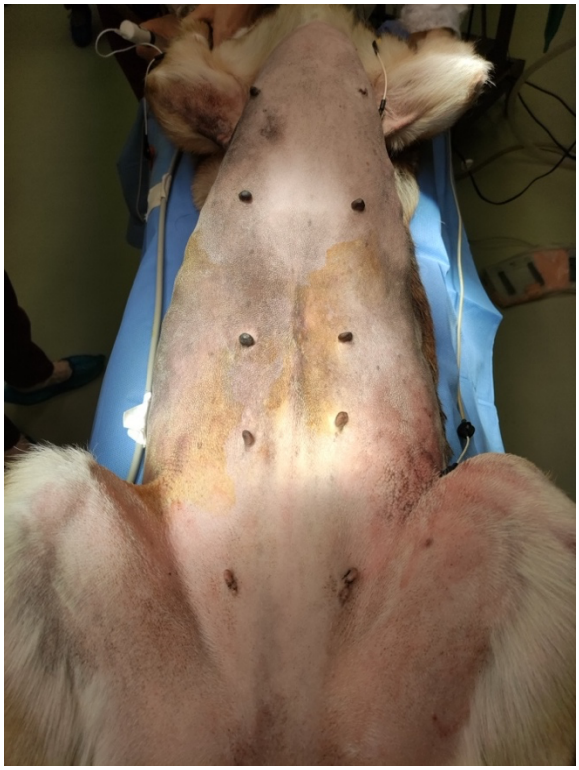

Figure 6. Positioning of the patient for mastectomy. The entire ventral thoracic, abdominal and inguinal areas have been clipped and prepared with a surgical scrub for the unilateral mastectomy

The use of a surgical skin marker to outline the tissue to be resected can be helpful (Thomson & Britt, 2022). Elliptical skin incisions are usually made around the glands to be removed. The skin and surrounding tissue is resected along with the MGs considering a lateral margin of 1-3 cm, while the deep dissection is carried out up to the ventral aspect of the external abdominal fascia. If there are adhesions to the abdominal fascia, this must be removed, along with the first layer of muscle if indicated. Whenever possible, it is advisable to avoid the incision of mammary tissue (Thomson & Britt, 2022; van Nimwegen & Kirpensteijn, 2018).

An en bloc excision can be performed by elevating one edge of the incision (Figure 7 A) and utilizing scissors, along with manual dissection, to separate the subcutaneous tissue from the underlying fascia. The traction applied to the elevated skin segment aids in the dissection process (Figure 7B). If the fascia and/or muscle are adherent, they must also be excised (Figure 7C).

Superficial hemorrhage must be controlled with electrocoagulation, hemostats or ligation as required. It is always advisable to ligate major vessels. These are represented by cranial superficial epigastric, which penetrate the rectus abdominis between the caudal thoracic and cranial abdominal mammary glands, and by caudal superficial epigastric which are identified at the level of M5,

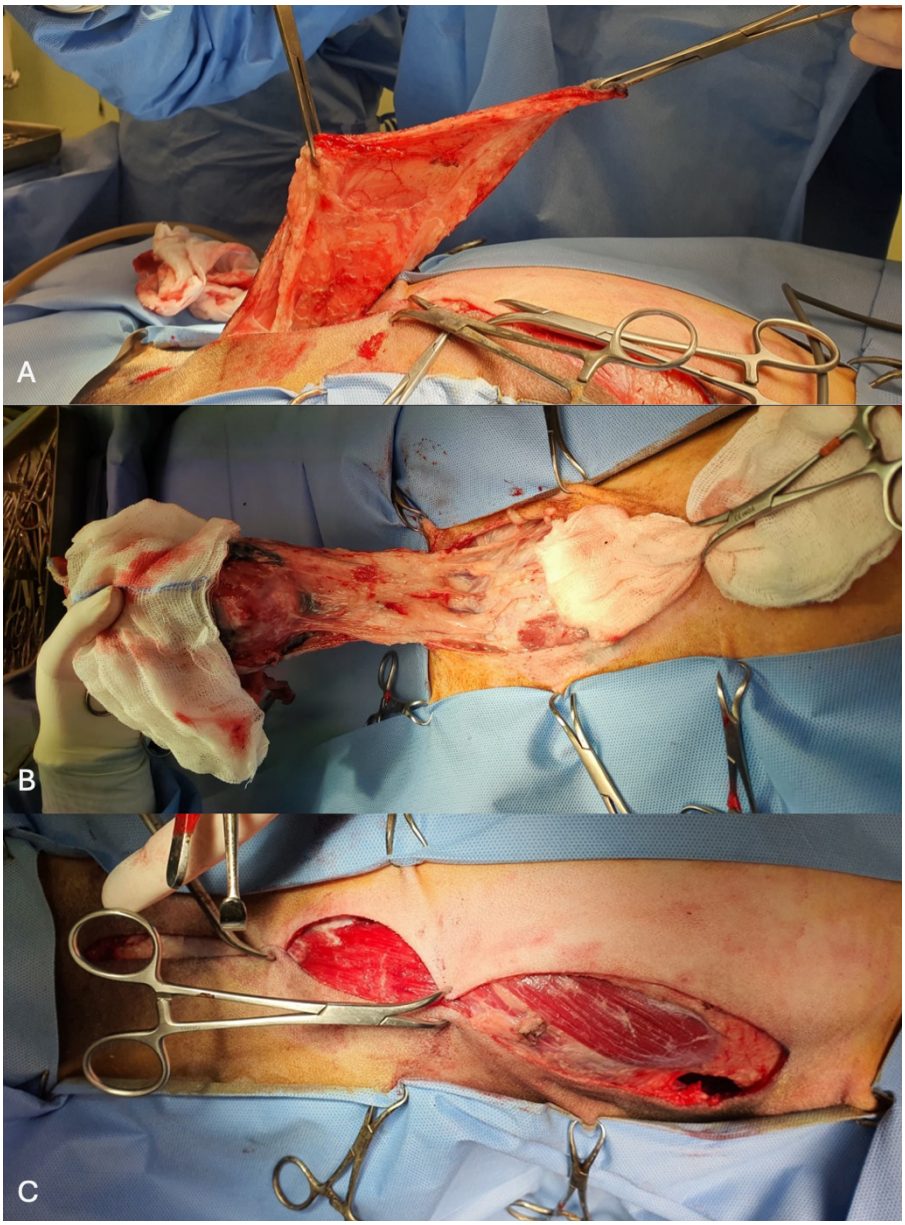

Figure 7. Different stages of mastectomy: lifting the cranial edge for the en-bloc excision (A), traction applied to aid dissection (B) and approximation of the wound edges to assess tension (C). In the final image, note how the fascia has been removed from the underlying muscular plane due to adhesions.

near the inguinal ring. Both arteries and veins must be carefully isolated, ligated and then divided (MacPhail and Fossum, 2019).

After excising the neoplastic tissue, it is important to assess whether the wound edges can be approximated without tension (7C). This is crucial to prevent ischemic necrosis of the tissue and wound dehiscence.

Walking sutures (absorbable sutures placed from the deep dermis to the underlying fascia) can be applied during mastectomy wounds closure to advance skin toward the center of the defect, with the aim of managing tension, reducing seroma formation as well as the acute postoperative pain (MacPhail & Fossum, 2019; Thomson & Britt, 2022; Travis et al., 2018).

When excessive tension is present, performing mesh-releasing incisions (Figure 8A) or achieving closure through advancement flaps or non-linear reconstruction is always advisable (Figure 8B) (Buiks et al., 2013).

In cases of substantial inguinal or sternal defects resulting from the surgical resection of large tumors, transpositional skin flaps from the axillary or flank region may be utilized to facilitate wound closure. Monolateral or bilateral flaps can be created depending on the size of the wound (Hunt 1995; MacPhail & Fossum, 2019; Sadhasivan et al., 2017).

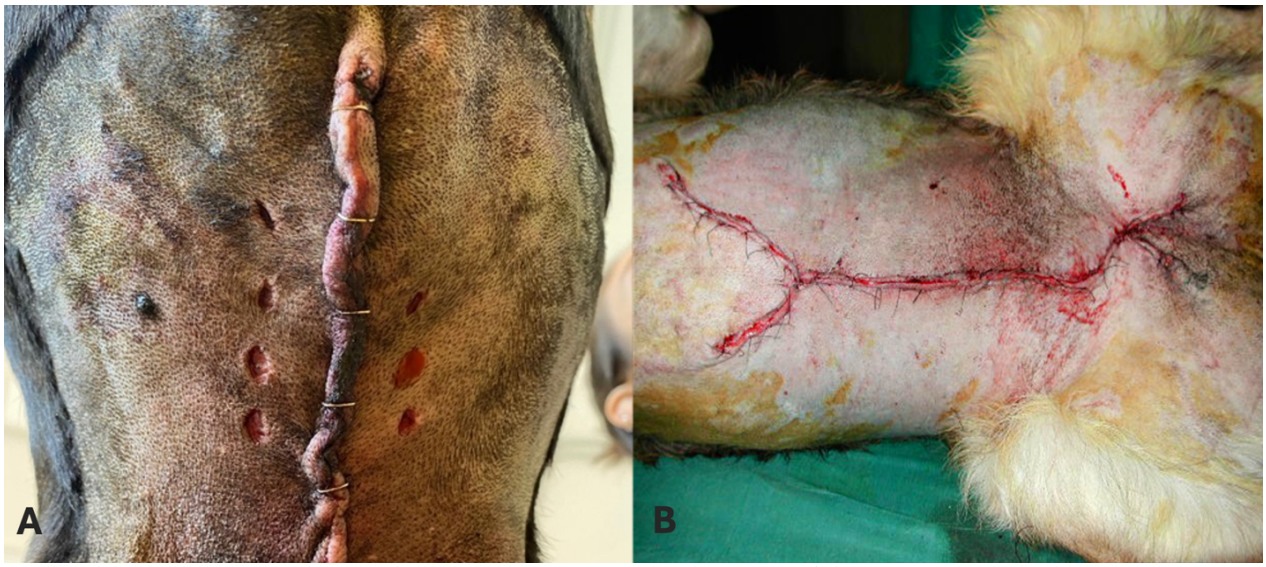

Figure 8. Mesh-releasing incisions made to reduce tension in a localized area of the mastectomy (9A); double Y-reconstruction following an extensive mastectomy (9B).

In case of significant dead space, it is recommended to use a closed suction or Penrose drain to help prevent fluid accumulation, to be removed 3-5 days post-surgery or when drainage diminishes to a minimal amount (MacPhail & Fossum, 2019).

The subcutaneous tissue can be closed using a continuous or interrupted pattern suture in either a single or double layer. A 4/0, 3/0 or 2/0 US Pharmacopeia (USP) sized monofilament absorbable suture material swaged onto a ½ circle tapered needle is recommended for subcutaneous closure, depending on the size of the patient (Fahie, 2018).

Skin closure can be accomplished through various methods. For external cutaneous suture patterns, skin closure is typically done using staples or monofilament nonabsorbable material (such as nylon, polybutester [Novafil], or polypropylene [Prolene]) attached to a cutting or, preferably, reverse-cutting 3/8 circle needle. Simple interrupted or continuous sutures, interrupted or continuous cruciate sutures and continuous Ford interlocking sutures are often used. Buried intradermal suture patterns need more time for their application, but they entail the use of absorbable material suture that is not removed. A continuous intradermal pattern is typically employed, usually oriented horizontally (parallel to the incision), though it can also be applied vertically. Apposition of tissue is usually performed with 3-0 USP or 4-0 USP sized suture material. Regarding the materials most commonly used for subcutaneous or intradermal sutures, these include poliglecaprone 25 (Monocryl), glycomer 631 (Biosyn), polydioxanone (PDS), polyglyconate (Maxon), polyglactin 910 (Vicryl), and polyglycolic acid (Dexon) (Fahie, 2018).

2.6.1.1 Lymphadenectomy

Vital dyes are helpful in the visual identification of LNs during lymphadenectomy, especially when they are not enlarged, since their identification can sometimes be difficult and frustrating. Sterile methylene blue is a commonly used agent since it does not require specialized equipment and it appears to be associated with fewer side effects compared to other analogues, such as patent blue dye or isosulphan blue (Liptak & Boston, 2019).

The use of vital dyes as the sole method for identifying the SLN is discouraged, as it can be challenging and may result in false negatives. Indeed, they do not indicate whether there is drainage to intracavitary LNs or may require a wider surgical exposure to trace the afferent lymphatics leading to distant SLNs. For this reason, it is advisable to use blue dye primarily to enhance the intraoperative visualization of LNs, previously identified through IL techniques (Liptak & Boston, 2019).

Sterile methylene blue (10 mg/mL) should be diluted with an equal volume of sterile saline to achieve a concentration of 5 mg/mL. Four 0.1 mL aliquots are then injected around the tumor into four quadrants, penetrating the skin adjacent to the tumor, as shown in Figure 10A.

Approximately 5 minutes are needed for the lymphatic uptake of the dye (Figure 10B).

Extirpation of the blue staining lymphatic vessels is not typically performed, as the presence of metastases along their path is considered extremely rare. The LNs should be removed only after the excision of the MT, but their removal should be carried out within 45–120 minutes of dye injection to minimize dye washout in the LN. It would be advisable to use new gloves and instruments for the excision of the LNs (Worley, 2022).

Other types of indirect lymphography are used to enhance the intraoperative visualization of LNs, such as those using fluorescein or near-infrared (through indocyanine green fluorescent dye) or

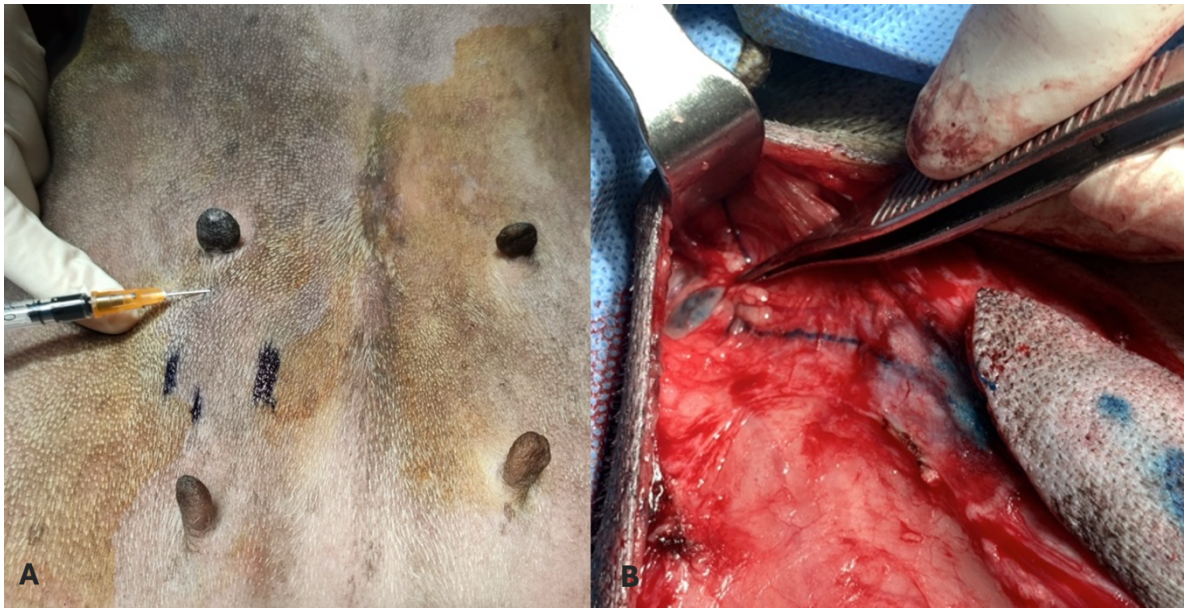

Figure 10. Preoperative peritumoral injection of methylene blue dye (A); lymphatic uptake of methylene blue dye from M1 toward the axillary lymph center. Note how the dye enhances the visualization of the lymphatic vessels and nodes.

scintigraphic techniques. However, these methods require either specialized equipment to detect fluorescence or radiation safety equipment and trained personnel, which naturally limits their accessibility in many veterinary facilities (Worley, 2022).

Among the LNs most identified as SLNs in MTs are the axillary, inguinal and popliteal nodes (Patsikas et al., 2006; Pereira et al., 2003), whose surgical removal will be specifically addressed in this text. The inguinal LN is typically removed en bloc along with M5; other LNs require a specific surgical approach. Removal of the sternal, medial iliac or superficial cervical LNs may also be necessary.

The removal of a peripheral LN is a relatively simple procedure. When possible, it is advisable to palpate and isolate the LN between the fingers, to address the incision directly over it. A skin incision matching the length of the LN, oriented along its longitudinal axis, is sufficient. The perinodal fatty tissue is bluntly and sharply dissected to free the LN. Manipulations should be gentle, avoiding a firm grasp to prevent rupture of the capsule and parenchyma. It is important to control bleeding by ligating or cauterizing the vessels at the hilus. Subcutaneous tissues and skin are closed as usual (Worley, 2022). For removal of LNs within the thorax or abdomen, an exploration of that body cavity is performed, and nodes are removed by careful dissection and maintenance of haemostasias. Since

many LNs are actually lymph centers, care must be taken to identify multiple LNs that may need to be removed in the same area (Culp & Herhart, 2022). Histopathological examination of the excised LNs is mandatory. Below are brief directions for the removal of the most common peripheral SLNs:

- AXILLARY: Following the mastectomy, the patient can be maintained in a dorsal recumbency position, or alternatively, placed in lateral recumbency with the ipsilateral forelimb suspended. A skin incision is made along the caudal border of the triceps muscle. The dorsal border of the deep pectoral muscle is then identified, which may require transecting the tendon of the latissimus dorsi muscle at its ventral aspect and reflecting the muscle dorsally. Blunt dissection through the loose fascia between these muscles leads to the axillary vein, along the caudal border of which the axillary LN is located (Worley, 2022).
- POPLITEAL: The patient can be placed either in dorsal recumbency with the hind limb suspended or in lateral recumbency with the limb relaxed. The incision is made over the popliteal fossa. Care should be taken to avoid deep dissection that could potentially damage the cranial tibial artery or vein (Worley, 2022).

2.6.1.2 Preparation of the specimen for histopathological examination

Once the tumor excision is complete, it is crucial to clearly mark the margins of the resected specimen using inks or sutures. The surgical margin refers to any area of the biopsy specimen that was adjacent to or in contact with the tissue remaining in vivo. Assessment of surgical margins is paramount, as the completeness of the surgical excision influences the outcome and impacts the prognosis (Sorenmo et al., 2020).

Following the guidelines outlined by Kamstock and colleagues in 2011, it is advisable to ink the specimens and allow them to dry thoroughly for 5-10 minutes before placing them in 10% neutral buffered formalin. To accurately apply the ink, use a cotton swab or wooden applicator stick, and when using ink alone, apply different colors to orient the specimen (e.g. deep margin, cranial, and

caudal). Sutures can be used to demarcate surgical margins, and identification is based on either suture number or color (e.g., 1 or blue sutures = cranial margin, 2 or black sutures = caudal margin).

Since the inguinal LNs are typically removed en bloc along with the fifth mammary glands (11A), it is advisable to isolate them (Figure 11B) or clearly mark their position to assist the pathologist in their detection, especially when they are very small in size.

Fixation should always follow a 1:10 tissue-to-formalin volume ratio and should start as soon as possible, ideally within 30 minutes of excision, to minimize tissue artifacts (Kamstock et al., 2011).

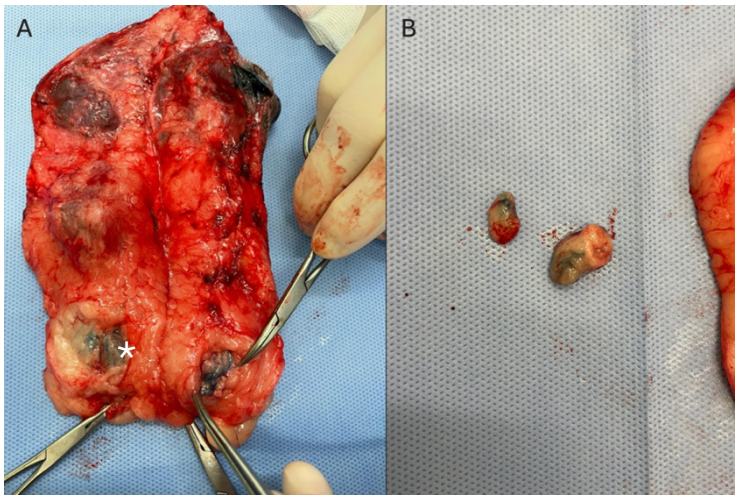

Figure 11. In A: En-bloc resected tissue after bilateral caudal regional mastectomy. The image shows the inspection of the superficial inguinal lymph center in progress (white asterisk). In B: Isolation of two small lymph nodes identified within the superficial inguinal lymph center

2.6.1.3 Postoperative care

Analgesics and supportive therapy should be tailored to the individual patient on a case-by-case basis. Abdominal bandages should be applied and left in place for at least 5 days, especially in cases of large excisions, to provide wound support, compress dead space and absorb fluids. These bandages should be kept as dry as possible and therefore changed daily or as needed. The wound should be regularly inspected for signs of inflammation, swelling, drainage, seroma, dehiscence or necrosis (MacPhail & Fossum, 2019) (Figure 12). Skin sutures are generally removed 10 to 14 days after surgery. However, when delayed wound healing is suspected, sutures are left in place for 14 to 21 days (Fahie, 2018).

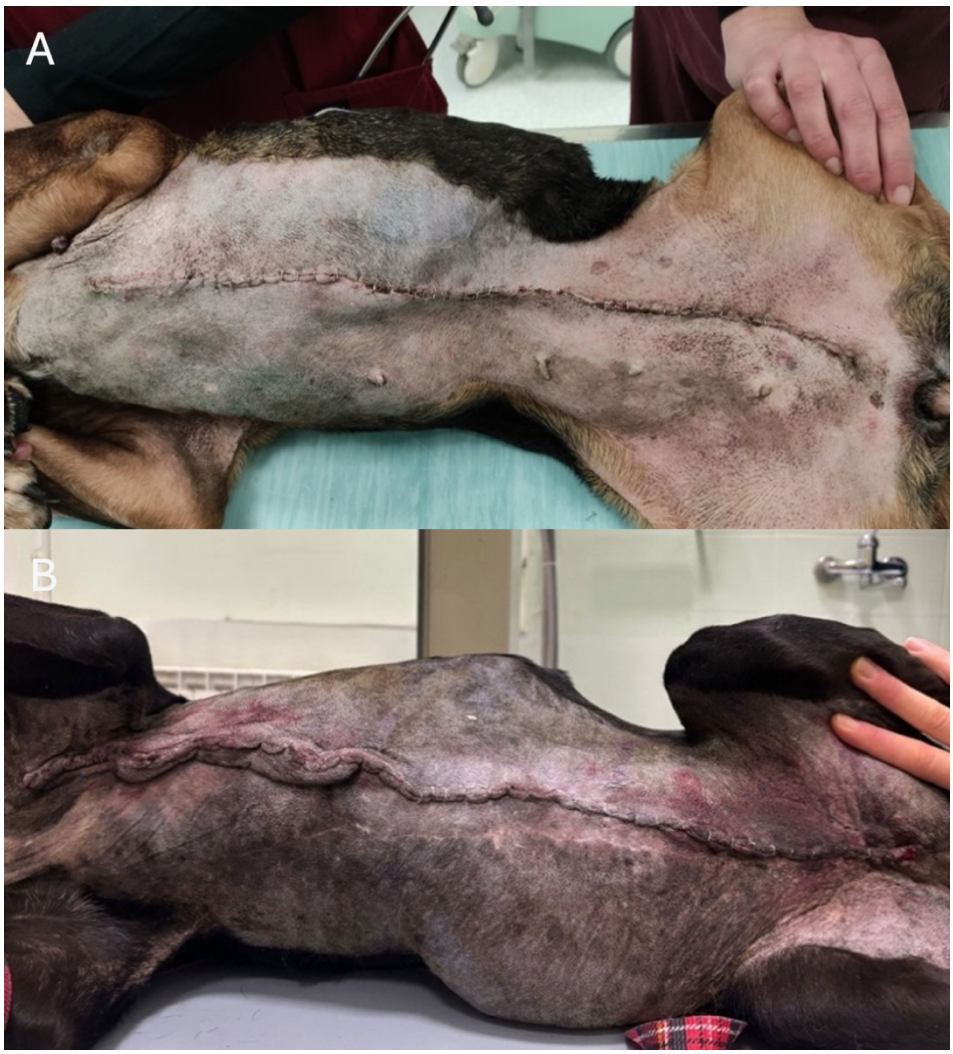

Figure 12. Regular postoperative inspection of the surgical wound after invasive unilateral mastectomy. In A: Good healing of the wound observed on the tenth postoperative day; in B: Large hematomas observed in the thoracic and inguinal ventral regions on the second postoperative day.

Pain, inflammation, hemorrhage, seroma, infection, ischemic necrosis, self-trauma, dehiscence, hind limb edema and tumor recurrence are common complications associated with mastectomy, particularly in invasive or radical surgeries (Evans et al., 2021; MacPhail & Fossum, 2019; Spåre et al., 2021). High body weight and postoperative antimicrobial administration can also influence postoperative complications incidence (Evans et al., 2021; Spåre et al., 2021); interestingly, in the study by Evans and colleagues of 2021, the association between postoperative antibiotics and complications, varied according to mastectomy type, making the issue controversial (Evans et al., 2021).

2.6.1.4 Perioperative antimicrobial and pain management

In 2021, Spare and colleagues evaluated the incidence of postoperative complications in dogs undergoing mastectomy without perioperative prophylactic antimicrobial therapy. Their findings indicated a similar or lower overall incidence of complications compared to previously reported incidences in dogs that underwent the same type of surgery but were administered antibiotic therapy (Evans et al., 2021; Horta et al., 2015). They also found that dogs with two or more glands excised had an increased risk of developing surgical site infections compared to those with only one gland excised. These results suggest that perioperative prophylactic antimicrobial therapy may not be necessary for all patients but could be recommended for those undergoing extensive mastectomies (Evans et al., 2021; Spåre et al., 2021) or for very debilitated patients (Mac Phail & Fossum, 2019). In case of infected or ulcerated neoplasm, it is advisable to administer antibiotics several days prior to surgery (MacPhail & Fossum, 2019).

To improve intraoperative anti-noception and short term post-operative analgesia, the transverse abdominis plane block (also called TAP block) with 0.25% bupivacaine (0.3 to 0.35 mL/kg), combined with intercostal nerve blocks with 0.25% bupivacaine (0.013 to 0.04 mL/kg) in dogs undergoing unilateral radical mastectomy, has been reported (Portela et al., 2014); indeed local anesthesia allows the reduction of the amount of general anesthetics and the perioperative opioid consumption, reducing opioid-related side-effects (Kettner et al. 2011). If the surgery is invasive, it might be beneficial to perform epidural analgesia both preoperatively and postoperatively; a good postoperative analgesia in dogs undergoing unilateral mastectomy, with reduced postoperative opioid requirement, was described for preoperative epidural anesthesia using a combination of ropivacaine and morphine (Becerra et al., 2022; Tayari et al., 2022).

2.6.1.5 Clinical follow-up

Dogs with malignant tumors should be reevaluated every 2 to 3 months for a minimum of 24 months post-surgery. Patients must undergo a complete physical examination and chest and abdominal X-

rays to rule out the presence of local recurrences, LN or distant metastases. Upon detecting any irregularity, it is important to determine whether it is associated with the resected MT. In case of locoregional recurrence or new tumors development, a new diagnostic work-up and staging should be performed. In case of locoregional recurrence, surgical resection is always advisable (Chocteau et al., 2021 (Figure 13).

In the event of the animal's death or euthanasia, it's important to advise the owner to allow a necropsy to assess the presence of metastases (Matos et al., 2012).

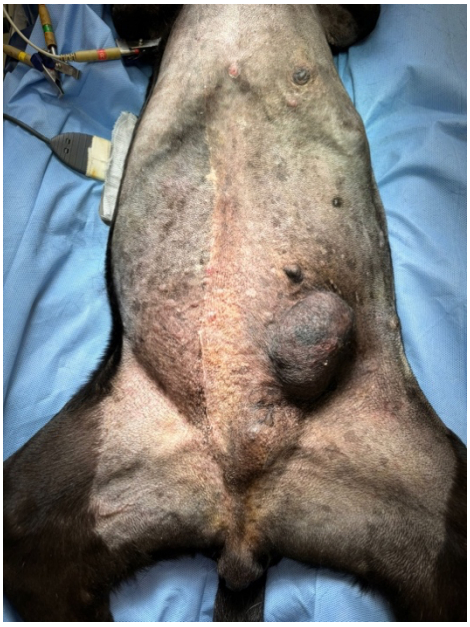

Figure 13. A 10-year-old spayed bitch undergoing resection of a new tumor in the left mammary chain. Note the absence of the right mammary chain and the long scar from the previous unilateral mastectomy.

2.6.2 Chemotherapy

Despite the lack of solid literature confirming the efficacy of chemotherapy, it is still recommended in case of inflammatory (Marconato et al., 2009), metastatic or high-risk MTs where surgery alone may not be effective (Sorenmo et al., 2020).

For specific aggressive histotypes, like adenosquamous carcinoma, comedocarcinoma, solid carcinoma, anaplastic carcinoma and carcinosarcoma, adjuvant chemotherapy is always recommended. Regardless of the histotype, in case of malignant large tumors (diameter >5 cm) or lymphatic involvement, chemotherapy is still recommended due to the high-rate risk of recurrence or metastasis (Sorenmo et al., 2020).

Currently, the chemotherapeutic agents that have been investigated as adjuvant therapy for canine mammary tumors include carboplatin, mitoxantrone, 5-fluorouracil and cyclophosphamide, doxorubicin, docetaxel, gemcitabine, paclitaxel, cisplatin and capecitabine (Agnoli et al., 2024; Arenas et al., 2016; De Campos et al., 2018; Karayannopoulou et al., 2001; Khanna et al., 2015; Lavalle et al., 2012; Marconato et al., 2008; Simon et al., 2006; Suryawanshi 2021; Tran et al., 2016). Some authors have described cyclophosphamide as an effective chemotherapic agent against malignant MTs (Karayannopoulou et al., 2001; Suryawanshi 2021).

In the 2001 study conducted by Karayannopoulou and colleagues, post-surgery combined administration of cyclophosphamide (100 mg/m2) and 5-fluorouracil (150 mg/m2 of body surface area) in bitches carrying invasive stage III or IV tubular adenocarcinoma, papillary adenocarcinoma, solid carcinoma and sarcoma (administered intravenously on the same day starting 7 days post-surgery and continued weekly for four weeks), resulted in extended overall survival compared to dogs receiving surgery alone (24 months versus 6 months, respectively).

The 2016 retrospective study by Tran et al. found that dogs with clinical stage IV carcinoma, or lower stages with confirmed histological lymphatic invasion, showed improved long-term survival when treated with carboplatin and mitoxantrone, though the results were not statistically significant. This improvement was observed only in cases where complete surgical margins were confirmed through histopathological examination. However, the study did not provide information on the tumor's histologic subtype or grade, both of which are recognized as important prognostic factors.

Studies on post-surgery treatments with gemcitabine (Marconato et al., 2008) and mitoxantrone (Arenas et al., 2016), as well as the combination of doxorubicin and docetaxel (Simon et al., 2006), did not show significant differences in OST or time to metastasis compared to surgery alone.

Endovenous paclitaxel showed a 20% response rate but unacceptable toxicity levels in treated dogs (Khanna et al., 2015).

Currently, capecitabine is being studied in veterinary medicine in the treatment of carcinomas of different origins in dogs (Agnoli et al., 2024; Wetzel et al., 2024). In humans, this agent is indicated

in the treatment of various malignant epithelial cancers including metastatic breast cancer. It has the advantage of being able to be administered orally and, in humans, it appears to be eliminated by 75% in the 24 hours following administration (Wetzel et al. 2024). In dogs seems to be well tolerated, with a good safety profile (Agnoli et al., 2024; Wetzel et al., 2024). Once absorbed by the intestinal mucosa, it is then transformed selectively in the tumor site into its only active metabolite, 5-fluorouracil (Agnoli et al., 2024).

The findings from the 2024 study by Agnoli and colleagues suggest that capecitabine is moderately effective in treating dogs with biologically aggressive carcinoma; however, further research is necessary to more accurately define the efficacy of capecitabine in treating mammary carcinomas in dogs. One study reported an overall response rate of 13% in MGT-bearing dogs treated with a combination of carboplatin and gemcitabine (Dominguez et al., 2009).

Adjuvant chemotherapy with doxorubicin or cisplatin are always recommended for mammary OSA, regardless of tumor diameter and LN involvement (Kuntz et al., 1998).

New results regarding chemotherapy survival improvement could potentially be achieved by testing multimodal chemotherapy protocols and opting for neoadjuvant approaches to surgery (Frénel & Nguyen, 2023).

The recommendations for inflammatory carcinoma will be discussed in detail later.

2.6.2.1 Metronomic chemotherapy

Oral metronomic chemotherapy is a treatment strategy that involves administering lower doses of chemotherapy drugs more frequently, as opposed to the higher doses used in conventional chemotherapy. In dogs, chemotherapeutics such as cyclophosphamide, lomustine and chlorambucil have been tested for several neoplasms (Valdivia et al., 2021). To authors knowledge, cyclophosphamide is the most frequently utilized agent in CMTs metronomic protocols (Nosalova et al., 2024), along with thalidomide (De Campos et al., 2018; Rossi et al., 2018). Metronomic strategy reduces toxicity while maintaining continuous drug exposure. It targets cancer cells indirectly by exerting anti-angiogenic and immune-modulatory effects.

In mammary stage V carcinoma-bearing dogs, longer MSTs were observed in patients treated with surgery and metronomic chemotherapy compared to dogs treated with surgery and conventional chemotherapy (De Campos et al., 2018).

Combining metronomic therapy with other agents, such as COX-2 or tyrosine kinase inhibitors (TKIs), is expected to lead to clinical benefits by enhancing the anti-angiogenic, immune-modulatory and anticancer effects (Alonso-Miguel et al., 2022; Elmslie et al., 2008; Rossi et al., 2018). The majority of TKIs are administered orally, offering a significant advantage for animal welfare by reducing stress and making it easier for the owner to administer the medication (Valdivia et al., 2021). In veterinary medicine, toceranib (Palladia®) is a TKI which is registered for canine mast cells tumor treatment but is commonly used for CMTs since some studies reported its anti-tumor activity against this malignancy (Valdivia et al., 2021), especially in case of pulmonary metastasis (London et al., 2003). Nevertheless, evaluation of toceranib in clinical trials is very limited and further studies are required.

As previously noted, in a 2018 study by De Campos and colleagues, the addition of metronomic thalidomide to postoperative carboplatin significantly increased survival in dogs with stage V mammary tumors (MST of 463 days), compared to those treated with surgery alone or surgery plus carboplatin (MST of 140 and 148 days, respectively). A similar survival benefit was observed in stage V dogs receiving post-surgery carboplatin combined with metronomic chemotherapy using cyclophosphamide and firocoxib (MST of 376.5 days). This highlights the clear clinical benefit of adding metronomic antiangiogenic and immunomodulatory therapies for these patients.

2.6.3 Cyclooxigenase-2 Inhibitors

Nonsteroidal anti-inflammatory drugs (NSAIDs), specifically COX-2 inhibitors, may be beneficial in treating malignant CMTs. This class of enzymes is involved in several steps of the carcinogenetic process, being responsible for neoplastic progression by promoting invasion, neo angiogenesis and metastasis and inhibiting apoptosis (Arenas et al., 2016; Valdivia et al., 2021)

It is now understood that COX-2 is expressed in both benign and malignant canine mammary tumors, but overexpression of COX-2 is associated with malignancy and a poor prognosis (Badowska & Malicka, 2010; Doré et al., 2003; Guimarães et al., 2014; Lavalle et al., 2012; Millanta et al., 2016).

In various studies, administration of COX-2 inhibitors showed to be significantly effective in prolonging survival in dogs with advanced or inflammatory carcinomas, combined or not with chemotherapy (Arenas et al., 2016; Lavalle et al., 2012; Marconato et al., 2009; Souza et al., 2009).

The combination of piroxicam or firocoxib with surgery and carboplatin brought to an MST respectively of 390 and 570 days, significantly higher than that observed in dogs treated with surgery alone or combined with chemotherapy (9 days vs "not reached") (Lavalle et al., 2012).

Firocoxib as a single agent was likely to increase overall survival and disease-free survival in dogs with high malignant CMT (defined as a histological malignant grade III and/or a clinical stage IV), according to the study of Arenas and colleagues (2016).

Piroxicam as a single agent brough an improvement in clinical conditions and significantly increased survival rates (mean and median progression-free survival of 171 and 183 days) compared with dogs treated with traditional chemotherapy protocols (doxorubicin, cyclophosphamide and 5-fluorouracil) (Souza et al., 2009).

Nevertheless, further studies are needed to better characterize the therapeutic use of COX-2 inhibitors.

2.6.4 Radiotherapy

Although radiotherapy is commonly used in HBC treatment (Harbeck et al., 2019), it is rarely employed in CMT therapy. (Nosalova et al., 2024). Hypo-fractionated radiotherapy in dogs has been proposed to enhance the control of IMC when combined with toceranib phosphate, piroxicam and thalidomide (Rossi et al., 2018). However, radiotherapy is still not widely used in veterinary medicine due to high associated costs, lack of availability and limited research (Larue & Gordon, 2020).

2.6.5 Endocrine therapy

Since ovarian hormones have a role in tumorigenesis of CMT, they become the target in hormonal therapy. In humans, it is typically achieved through medical means; in dogs, surgical ovarian ablation is a more practical solution, as it eliminates the production of both estrogen and progesterone (Sorenmo et al., 2020).

However, specific ER/PR modulators and luteinizing hormone-releasing hormone (LHRH) agonists have been tested in vivo.

Aglepristone, a PR antagonist, was randomly administered 14 and 7 days prior to surgery to a specific subset of dogs selected based on well-established favorable clinicopathological criteria, including tumor size less than 3 cm, complex or mixed histologic subtype and histologic grade I or II. The treatment demonstrated that neoadjuvant aglepristone in dogs with PR-positive tumors had an antiproliferative effect and resulted in improved DFI (Guil-Luna et al., 2011).

Tamoxifen, a selective ER blocker commonly used in human premenopausal ER-positive breast cancer (Valdivia et al., 2021), is not tolerable in dogs due to its several side effects such as vulvar edema, vaginal purulent discharge and pyometra (Morris et al., 1993; Tavares et al., 2010).

Goserelin, classified as an LHRH agonist, led to decreased levels of estradiol and progesterone, as well as a reduction in tumor size in dogs with CMTs (Lombardi et al., 1999).

Other agents that directly or indirectly interfere with ovarian hormones and their receptors, such as aromatase inhibitors (letrozole), oxytocin, melatonin and indole-3-carbinol have been tested. However, their use for antineoplastic purposes has remained limited to in vitro or laboratory animals research (Valdivia et al., 2021).

Desmopressin is a synthetic hormone that mimics vasopressin, a natural hormone produced by the pituitary gland, also known as antidiuretic hormone (Valdivia et al., 2021). Since desmopressin has been proposed to have anti-metastatic effects (Valdivia et al., 2021), a few clinical trials have been conducted in dogs (Hermo et al., 2008; Hermo et al., 2011; Sorenmo et al., 2020). However, the results have been conflicting, making desmopressin's anti-metastatic effects in canines controversial.

2.6.6 Other therapies

There are several promising and innovative treatment strategies for mammary tumors. However, the majority have only been investigated through in vitro studies or experimental models using laboratory animals (Nosalova et al., 2024; Valdivia et al., 2021).

Immunotherapy for dogs with mammary carcinoma is an emerging field aimed at enhancing the immune system's ability to recognize and attack cancer cells. Although immunotherapy in canine oncology is still in the early stages, it holds promise as a treatment for CMC, especially in those cases where traditional therapies have limited success (Nosalova et al., 2024).

Some strategies used in immunotherapy for CMC include cancer vaccines, monoclonal antibodies (mAbs), cellular immunotherapy, oncolytic viruses (Nosalova et al., 2024; Valdivia et al., 2021) and bacteria (Razzuoli et al., 2022).

Given the lack of clinical trials testing these therapies, no validated protocols currently exist. Therefore, the detailed discussion of these approaches has not been included in this work.

2.7 Inflammatory carcinoma

Inflammatory mammary carcinoma (IMC) is the most aggressive form of mammary tumor either in humans or dogs characterized by a locally aggressive behavior and a high rate of early regional and distant metastases (Alenza et al., 2001; Marconato et al., 2009; Singletary et al., 2008), primarily to the urinary bladder and other organs of the reproductive tract (Clemente et al., 2010).

In dogs it seems to be uncommon, but the true incidence is unknown due to the fact it can be misdiagnosed with severe dermatitis or mastitis, especially in the earliest phase. In fact, clinical signs are compatible with cutaneous erythema, local edema and pain, firmness and warmth of the MGs with or without palpable nodules (Alenza et al. 2001; Clemente et al. 2010) (Figure 14).

Bitches with IMC frequently present systemic signs such as weakness, anorexia and weight loss (Alenza et al. 2001).

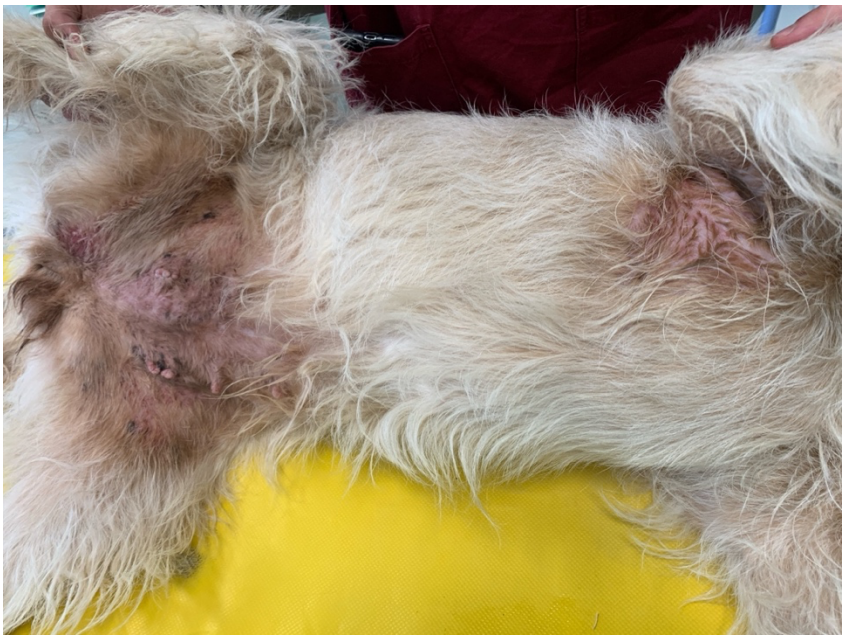

Figure 14. Inflammatory mammary carcinoma with axillary lymph center involvement in an old bitch. Wide areas of inflammation with erythema, local edema and exudation are recognized in the inguinal and right axillary region.

It is distinguished from other forms of mammary cancer by its sudden presentation and fulminant progression, which are related to a poor prognosis and a median OST from 25 to 60 days (Alenza et al., 2001; Clemente et al., 2009; Marconato et al., 2009; Souza et al., 2009). Intact female dogs are typically affected, with no breed predisposition (Peña et al., 2003). However, IMC has been described in one male dog (da Silva et al., 2019).

Bitches with IMC are typically older than those with other types of mammary carcinomas (Alenza et al., 2001). While the etiology of IMC remains unknown, several predisposing factors have been identified, the most notable being elevated progesterone levels during diestrus (Peña et al., 2003).

Histologically, it cannot be considered as a specific entity. The histologic hallmark of IMC is the presence of embolization of dermal lymphatic vessels by neoplastic cells, along with marked pleomorphism and loss of cellular differentiation and phagocytic activity (Alenza et al 2001; Peña et al., 2003; Tavassoli et al., 1999).

The combination of both clinical signs and histopathological features is used to confirm the diagnosis. Although several types of highly malignant carcinoma have been described, poorly differentiated or anaplastic carcinoma is usually diagnosed (Alenza et al., 2001).

FNAC can aid in diagnosing IMC and may help distinguish it from other mammary carcinomas (Pîrvu et al., 2024). However, histopathology remains the gold standard, as it uniquely reveals dermal involvement by neoplastic emboli.

Two subtypes of IMC in dogs are recognized, which have been described in humans: secondary IC can present as a strong local inflammation after surgical excision of mammary lesions (Taylor & Meltzer, 1938; Richards & Lewison, 1961; Nishimura et al., 1998) or as palpable masses with signs of inflammation beneath nodules that have been present for at least 4 months (Attia-Sobol et al., 1993). In women, secondary IMC can develop following the surgical removal of "occult IMC", defined as a mass with histological dermal lymphatic infiltration but no clinical signs of inflammation (Saltzstein 1974). Primary and secondary IMC are clinically different. The former is likely to be more aggressive, related to a faster growth rate and a poorer general condition (Alenza et al., 2001). In 2003, Peña and colleagues found a correlation between the overexpression of P53 and the worse clinical and pathological aggressive behavior in cases of primary IMC. The immunochemical expression of P53 has been associated with poor prognosis both in human (Falette et al., 1998) and canine (Wakui et al., 2001) mammary malignancies.

According to Marconato et al. (2009), the classification of IMC did not impact the outcome. The only factors associated with longer survival times were the use of medical therapy and the absence of coagulopathies, a condition found more frequently in animals with advanced-stage mammary carcinomas (distant metastases) or IMC (Stockhaus et al., 1999).

IMC is characterized by high COX-2 expression, associated with pronounced angiogenesis and lymph angiogenesis (Clemente et al., 2013; de Souza et al., 2018; Queiroga et al., 2005; Raposo et al., 2017), as well as vascular endothelial growth factors (Clemente et al., 2013; Millanta et al., 2010). Indeed, these are some of the targets of IMC treatment.

Surgical excision alone is not considered the proper treatment in dogs with IMC (Lana et al., 2007), while the role of chemotherapy is controversial and there are few reports about. Traditional dose-

intense chemotherapy could expose animals to unnecessary toxicity agents without ensuring either an efficient locoregional disease control or metastasis prevention (Clemente et al., 2009).

In women, multimodal therapy has considerably improved the OST, and it involves the combination of neoadjuvant chemotherapy, surgical excision and postoperative radiotherapy (Rossi et al., 2018).

To date, treatments proposed for canine IMC include traditional and metronomic chemotherapy, COX-2 inhibitors, TKIs and radiotherapy. Chemotherapy and COX-2 inhibitors have proven to be effective in increasing survival rates compared to palliative care alone (Clemente et al., 2010; Marconato et al., 2009; Souza et al., 2009). However, the higher OST (480 days) and MST (180 days) have been achieved in dogs which received hypo fractionated radiation therapy combined with metronomic chemotherapy (thalidomide), toceranib and piroxicam (Rossi et al., 2018). Authors chose to combine the three molecules due to their anti-angiogenetic and immune-modulatory activity, hypothesizing a potential clinical benefit in the treatment of IMC.

Thalidomide and toceranib are known for their angio-genetic and immuno-modulatory effects and take part of metronomic strategies (Kareva et al., 2015; Rossi et al., 2018), while piroxicam is a selective COX-2 inhibitor NSAID which activity against tumor growth has been shown.

In a recent study by Alonso-Miguel and colleagues (2022), the addition of toceranib and oral cyclophosphamide to COX-2 inhibitors was shown to enhance tumor growth control and extend survival times compared to treatment with COX-2 inhibitors alone.

In the 2009 study by Marconato and colleagues, the longest survival times were observed in two dogs treated with surgery and medical therapy (either piroxicam alone or in combination with carboplatin), surviving 97 and 300 days, respectively, after being diagnosed with IMC. Both dogs had limited cutaneous involvement and were free from pulmonary metastasis and coagulopathies. Although these findings are preliminary and based on a small sample, they suggest that selected dogs with early-stage IMC may benefit from multidisciplinary therapeutic protocols which include surgery.

Table 7 summarizes the main therapeutic indications for the different types of mammary tumors based on current knowledge.

Table 7. Main therapeutic indications for the different types of mammary tumors based on current knowledge.

TUMOR TYPE	INDICATIONS FOR TREATMENT
BENIGN TUMORS	**SURGERY**[1]
STAGE I/II LHG CARCINOMA **STAGE I/II LHG CARCINOMA (PR+)**	**SURGERY**[1,2] **NEOADJ. AGLEPRISTONE + SURGERY**[3]
STAGE I/II -AGGRESSIVE HISTOTYPE CARCINOMA **-HHG CARCINOMA** **-CARCINOMA WITH HLI or HVI**	**SURGERY + ADJUVANT THERAPIES (CHEMOTHERAPY + COX-2 I)** [2,4]
STAGE III CARCINOMA	**SURGERY + ADJUVANT THERAPIES (CHEMOTHERAPY + COX-2 I)** [2,4,5]
STAGE IV CARCINOMA	**SURGERY + ADJUVANT THERAPIES (CHEMOTHERAPY + COX-2 I)** [2,4]
STAGE V CARCINOMA	**CHEMOTHERAPY + METRONOMIC CHEMOTHERAPY + COX-2 I + TKI** [5,6,7]
MAMMARY OSTEOSARCOMA	**SURGERY + CHEMOTHERAPY** [8]
INFLAMMATORY CARCINOMA	**RADIOTHERAPY + CHEMOTHERAPY + COX-2 I + TKI** [9,10,11,12]

PR+: progesterone receptors positive; LHG: low histological grade; HHG: high histological grade; HLI: histological lymphatic invasion; HVI: histological vascular invasion; NEOADJ: neoadjuvant; COX-2 I: COX-2 inhibitors.
1: Rasotto et al., 2017; 2: Sorenmo et al., 2020; 3: Guil-Luna et al., 2011; 4: Arenas et al., 2016; 5: Karayannopoulou et al., 2001; 5: Lavalle et al., 2012; 6: De Campos et al., 2018; 7: London et al., 2003; 8: Kuntz et al., 1998; 9: Clemente et al., 2010; 10: Rossi et al., 2018; 11: Marconato et al., 2009; 12: Souza et al., 2009.

2.6 Conclusions

The findings from past and recent studies offer valuable insights into the biological behavior of CMT and its response to various treatment modalities. By synthesizing this evidence, we can develop comprehensive recommendations that encompass risk assessment, diagnostic imaging, surgical techniques and adjuvant therapies.

As we work to enhance the management of CMT, the implementation of standardized screening programs will be essential for achieving early diagnosis and improved outcomes.

It is crucial to follow the correct sequence in the diagnostic and therapeutic process, as this will help prevent the omission of critical information that could influence prognosis and treatment options.

Additionally, this approach can avoid "overstaging," which may be considered time and money consuming for owners.

To improve knowledge on the safety, efficacy and potential combinations of therapies, it is essential to expand clinical trials and ensure they include a statistically valid number of cases for reliable, broader application. Implementing standardized, multidisciplinary therapeutic protocols may enhance prognosis accuracy. Greater insight into treatment efficacy, safety, costs, administration timelines and impacts on recurrence risk, metastasis and survival rates would empower veterinarians and pet owners to make more informed decisions.

As we continue to obtain new results from ongoing and future clinical studies, these recommendations are likely to be subject to change. Therefore, it is the veterinarian's responsibility to update and adapt new knowledge to existing practices. Emphasizing evidence-based practices will enhance our ability to manage CMTs more effectively, paving the way for improved care and outcomes in veterinary oncology.

2.7 References

Abadie, J., Nguyen, F., Loussouarn, D., Peña, L., Gama, A., Rieder, N., Belousov, A., Bemelmans, I., Jaillardon, L., Ibisch, C., & Campone, M. (2018). Canine invasive mammary carcinomas as models of human breast cancer. Part 2: immunophenotypes and prognostic significance. *Breast cancer research and treatment*, *167*(2), 459–468. https://doi.org/10.1007/s10549-017-4542-8

Agnoli, C., Rimondi, S., Ghisoni, G., Guerra, D., Tumbarello, M., Perfetti, S., Tirolo, A., & Marconato, L. (2024). Spotlight on capecitabine for the treatment of unresectable or metastatic carcinoma of various origin: A retrospective study of 25 dogs. *Veterinary and comparative oncology*, *22*(1), 42–48. https://doi.org/10.1111/vco.12945

Agnoli, C., Sabattini, S., Ubiali, A., Battisti, E., Rossi, F., Diana, A., Camerino, M. T., Perfetti, S., Ciammaichella, L., Stefanello, D., Papa, M., Zaccone, R., & Marconato, L. (2023). A retrospective study on bone metastasis in dogs with advanced-stage solid cancer. *The Journal of small animal practice*, *64*(9), 561–567. https://doi.org/10.1111/jsap.13621

Alonso-Miguel, D., Valdivia, G., García-San José, P., Alonso-Diez, Á., Clares, I., Portero, M., Peña, L., & Pérez-Alenza, M. D. (2022). Clinical outcome of dogs diagnosed with canine inflammatory mammary cancer treated with metronomic

cyclophosphamide, a cyclooxygenase-2 inhibitor and toceranib phosphate. *Veterinary and comparative oncology*, *20*(1), 179–188. https://doi.org/10.1111/vco.12760

Annoni, M., Borgonovo, S., & Aralla, M. (2023). Sentinel lymph node mapping in canine mast cell tumors using a preoperative radiographic indirect lymphography: Technique description and results in 138 cases. *Veterinary and comparative oncology*, *21*(3), 469–481. https://doi.org/10.1111/vco.12906

Antoniou, A. C., Pharoah, P. D., Easton, D. F., & Evans, D. G. (2006). BRCA1 and BRCA2 cancer risks. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *24*(20), 3312–3314. https://doi.org/10.1200/JCO.2006.06.7934

Arenas, C., Peña, L., Granados-Soler, J. L., & Pérez-Alenza, M. D. (2016). *Adjuvant therapy for highly malignant canine mammary tumors: Cox-2 inhibitor versus chemotherapy: a case-control prospective study. The Veterinary record, 179(5), 125. https://doi.org/10.1136/vr.103398*

Attia-Sobol, J., Ferrière, J. P., Curé, H., Kwiatkowski, F., Achard, J. L., Verrelle, P., Feillel, V., De Latour, M., Lafaye, C., & Deloche, C. (1993). Treatment results, survival and prognostic factors in 109 inflammatory breast cancers: univariate and multivariate analysis. *European journal of cancer (Oxford, England : 1990)*, *29A*(8), 1081–1088. https://doi.org/10.1016/s0959-8049(05)80292-8

Badowska-Kozakiewicz, A. M., & Malicka, E. (2010). Expression of cyclooxygenase-2 in neoplasms of the mammary gland in bitches. *Polish journal of veterinary sciences*, *13*(2), 337–342.

Baioni, E., Scanziani, E., Vincenti, M. C., Leschiera, M., Bozzetta, E., Pezzolato, M., Desiato, R., Bertolini, S., Maurella, C., & Ru, G. (2017). Estimating canine cancer incidence: findings from a population-based tumour registry in northwestern Italy. *BMC veterinary research*, *13*(1), 203. https://doi.org/10.1186/s12917-017-1126-0

Banchi, P., Morello, E. M., Bertero, A., Ricci, A., & Rota, A. (2022). A retrospective study and survival analysis on bitches with mammary tumors spayed at the same time of mastectomy. *Veterinary and comparative oncology*, *20*(1), 172–178. https://doi.org/10.1111/vco.12759

Barbagianni, M. S., & Gouletsou, P. G. (2023). Modern Imaging Techniques in the Study and Disease Diagnosis of the Mammary Glands of Animals. *Veterinary sciences*, *10*(2), 83. https://doi.org/10.3390/vetsci10020083

Beauvais, W., Cardwell, J. M., & Brodbelt, D. C. (2012). The effect of neutering on the risk of mammary tumors in dogs--a systematic review. *The Journal of small animal practice*, *53*(6), 314–322. https://doi.org/10.1111/j.1748-5827.2011.01220.x

Brissot, H. N., & Edery, E. G. (2017). Use of indirect lymphography to identify sentinel lymph node in dogs: a pilot study in 30 tumors. *Veterinary and comparative oncology*, *15*(3), 740–753. https://doi.org/10.1111/vco.12214

Brodey, R. S., Fidler, I. J., & Howson, A. E. (1966). The relationship of estrous irregularity, pseudopregnancy, and pregnancy to the development of canine mammary neoplasms. *Journal of the American Veterinary Medical Association*, *149*(8), 1047–1049.

Brønden, L. B., Nielsen, S. S., Toft, N., & Kristensen, A. T. (2010). Data from the Danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in Denmark. *The Veterinary record*, *166*(19), 586–590. https://doi.org/10.1136/vr.b4808

Buiks, S.C., van Delden, M. and Kirpensteijn, J. (2013). General reconstructive techniques. In *Reconstructive surgery and wound management of the dog and cat* (pp. 49-76). Manson Pub.

Burrai, G. P., Gabrieli, A., Moccia, V., Zappulli, V., Porcellato, I., Brachelente, C., Pirino, S., Polinas, M., & Antuofermo, E. (2020). A Statistical Analysis of Risk Factors and Biological Behavior in Canine Mammary Tumors: A Multicenter Study. *Animals : an open access journal from MDPI*, *10*(9), 1687. https://doi.org/10.3390/ani10091687

Busch, U., & Rudolph, R. (1995). Mammakarzinom der Hündin: Eine neue Routinediagnostik zum Nachweis okkulter Mikrometastasen in den regionären Lymphknoten [Mammary carcinoma in female dogs: a new routine diagnosis by the detection of occult micrometastases in the regional lymph nodes]. Berliner und Munchener tierarztliche Wochenschrift, 108(2), 43–46.

Carvalho, M.I., Pires, I., Prada, J., Raposo, T.P., Greg´ orio, H., Lobo, L., Queiroga, F.L., 2017. High COX-2 expression is associated with increased angiogenesis, proliferation and tumoural inflammatory infiltrate in canine malignant mammary tumors: a multivariate survival study. Vet. Comp. Oncol. 15, 619–631. https://doi.org/10.1111/vco.12206

Cassali, G. D., Gobbi, H., Malm, C., & Schmitt, F. C. (2007). Evaluation of accuracy of fine needle aspiration cytology for diagnosis of canine mammary tumors: comparative features with human tumors. *Cytopathology : official journal of the British Society for Clinical Cytology*, *18*(3), 191–196. https://doi.org/10.1111/j.1365-2303.2007.00412.x

Cassali, G.D., Lavalle, G.E., Ferreira, E., Estrela-Lima, A., De Nardi, A.B., Ghever, C., Sobral, R.A., Amorim, R.L., Oliveira, L.O., Sueiro, F.A.R., Beserra, H.E.O., Bertagnolli, A.C., Gamba, C.O., Damasceno, K.A., Campos, C.B., Araujo, M.R., Campos, L.C., Monteiro, L.N., Nunes, F.C., Horta, R.S., Reis, D.C., Luvizotto, M.C.R., Magalhaes, G.M., Raposo, J.B., Ferreira, A.M.R., Tanaka, N.M., Grandi, F., Ubukata, R., Batschinski, K., Terra, E.M., Salvador, R.C.L., Jark, P.C., Delecrodi, J.E.R., Nascimento, N.A., Silva, D.N., Silva, L.P., Ferreira, K.C.R.S., Frehse, M.S., Di Santis,G.W., Silva, E.O., Guim, T.N., Kerr, B., Cintra, P.P., Silva, F.B.F., Leite, J.S., Mello, M.F.V., Ferreira, M.L.G., Fukumasu, H., Salgado, B.S., Torres, R. (2013). Consensus for the diagnosis, prognosis and treatment of canine mammary tumours. *Brazilian Journal of Veterinary Pathology*, 7(2), 38-69.

Chai, H., & Brown, R. E. (2009). Field effect in cancer-an update. *Annals of clinical and laboratory science*, *39*(4), 331–337.

Chang, S. C., Chang, C. C., Chang, T. J., & Wong, M. L. (2005). Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002). *Journal of the American Veterinary Medical Association*, *227*(10), 1625–1629. https://doi.org/10.2460/javma.2005.227.1625

Chocteau, F., Abadie, J., Loussouarn, D., & Nguyen, F. (2019). Proposal for a Histological Staging System of Mammary Carcinomas in Dogs and Cats. Part 1: Canine Mammary Carcinomas. *Frontiers in veterinary science*, *6*, 388. https://doi.org/10.3389/fvets.2019.00388

Chocteau, F., Mordelet, V., Dagher, E., Loussouarn, D., Abadie, J., & Nguyen, F. (2021). One-year conditional survival of dogs and cats with invasive mammary carcinomas: A concept inspired from human breast cancer. *Veterinary and comparative oncology*, *19*(1), 140–151. https://doi.org/10.1111/vco.12655

Clemente, M., De Andrés, P. J., Peña, L., & Pérez-Alenza, M. D. (2009). Survival time of dogs with inflammatory mammary cancer treated with palliative therapy alone or palliative therapy plus chemotherapy. *The Veterinary record*, *165*(3), 78–81. https://doi.org/10.1136/vetrec.165.3.78

Clemente, M., Pérez-Alenza, M. D., Illera, J. C., & Peña, L. (2010). Histological, immunohistological, and ultrastructural description of vasculogenic mimicry in canine mammary cancer. *Veterinary pathology*, *47*(2), 265–274. https://doi.org/10.1177/0300985809353167

Clemente, M., Sánchez-Archidona, A. R., Sardón, D., Díez, L., Martín-Ruiz, A., Caceres, S., Sassi, F., Dolores Pérez-Alenza, M., Illera, J. C., Dunner, S., & Peña, L. (2013). Different role of COX-2 and angiogenesis in canine inflammatory

and non-inflammatory mammary cancer. *Veterinary journal (London, England : 1997)*, *197*(2), 427–432. https://doi.org/10.1016/j.tvjl.2013.02.009

Coleman C. (2017). Early Detection and Screening for Breast Cancer. *Seminars in oncology nursing*, *33*(2), 141–155. https://doi.org/10.1016/j.soncn.2017.02.009

Coleto, A. F., Wilson, T. M., Soares, N. P., Gundim, L. F., Castro, I. P., Guimarães, E. C., Bandarra, M. B., & Medeiros-Ronchi, A. A. (2018). Prognostic Value of Occult Isolated Tumour Cells within Regional Lymph Nodes of Dogs with Malignant Mammary Tumours. *Journal of comparative pathology*, *158*, 32–38. https://doi.org/10.1016/j.jcpa.2017.11.001

Collivignarelli, F., Tamburro, R., Aste, G., Falerno, I., Del Signore, F., Simeoni, F., Patsikas, M., Gianfelici, J., Terragni, R., Attorri, V., Carluccio, A., & Vignoli, M. (2021). Lymphatic Drainage Mapping with Indirect Lymphography for Canine Mammary Tumors. *Animals : an open access journal from MDPI*, *11*(4), 1115. https://doi.org/10.3390/ani11041115

Concannon, P. W., Spraker, T. R., Casey, H. W., & Hansel, W. (1981). Gross and histopathologic effects of medroxyprogesterone acetate and progesterone on the mammary glands of adult beagle bitches. *Fertility and sterility*, *36*(3), 373–387.

Culp, W.T.N. and Ehrhart, N. (2022). Principles of Surgical Oncology. In *Veterinary Surgical Oncology*.(pp 1-13). John Wiley & Sons, Inc.

De Andrés, P. J., Cáceres, S., Clemente, M., Pérez-Alenza, M. D., Illera, J. C., & Peña, L. (2016). Profile of Steroid Receptors and Increased Aromatase Immunoexpression in Canine Inflammatory Mammary Cancer as a Potential Therapeutic Target. *Reproduction in domestic animals = Zuchthygiene*, *51*(2), 269–275. https://doi.org/10.1111/rda.12676

de Araújo, M. R., Campos, L. C., Ferreira, E., & Cassali, G. D. (2015). Quantitation of the Regional Lymph Node Metastatic Burden and Prognosis in Malignant Mammary Tumors of Dogs. *Journal of veterinary internal medicine*, *29*(5), 1360–1367. https://doi.org/10.1111/jvim.13576

De Campos, C. B., Lavalle, G. E., Monteiro, L. N., Pêgas, G. R. A., Fialho, S. L., Balabram, D., & Cassali, G. D. (2018). Adjuvant Thalidomide and Metronomic Chemotherapy for the Treatment of Canine Malignant Mammary Gland Neoplasms. *In vivo (Athens, Greece)*, *32*(6), 1659–1666. https://doi.org/10.21873/invivo.11429

de M Souza, C. H., Toledo-Piza, E., Amorin, R., Barboza, A., & Tobias, K. M. (2009). Inflammatory mammary carcinoma in 12 dogs: clinical features, cyclooxygenase-2 expression, and response to piroxicam treatment. The Canadian veterinary journal = La revue veterinaire canadienne, 50(5), 506–510.

de Souza, M. C. C., Flecher, M. C., Arrais, F. M., de Sena, B. V., Giuliano, A., & Horta, R. D. S. (2023). Comparison of surgical resection of Axillary Lymph Nodes in Dogs with Mammary Gland Tumors with or without sentinel lymph node visualization with patent blue dye. Frontiers in veterinary science, 10, 1149315. https://doi.org/10.3389/fvets.2023.1149315

de Souza, T. A., de Campos, C. B., De Biasi Bassani Gonçalves, A., Nunes, F. C., Monteiro, L. N., de Oliveira Vasconcelos, R., & Cassali, G. D. (2018). Relationship between the inflammatory tumor microenvironment and different histologic types of canine mammary tumors. *Research in veterinary science*, *119*, 209–214. https://doi.org/10.1016/j.rvsc.2018.06.012

Diessler, M. E., Castellano, M. C., Portiansky, E. L., Burns, S., & Idiart, J. R. (2017). Canine mammary carcinomas: influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. *Veterinary and comparative oncology*, *15*(2), 450–461. https://doi.org/10.1111/vco.12189

Dobson, J. M., Samuel, S., Milstein, H., Rogers, K., & Wood, J. L. (2002). Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. *The Journal of small animal practice*, *43*(6), 240–246. https://doi.org/10.1111/j.1748-5827.2002.tb00066.x

Dominguez, P. A., Dervisis, N. G., Cadile, C. D., Sarbu, L., & Kitchell, B. E. (2009). Combined gemcitabine and carboplatin therapy for carcinomas in dogs. *Journal of veterinary internal medicine*, *23*(1), 130–137. https://doi.org/10.1111/j.1939-1676.2008.0248.x

Doré, M., Lanthier, I., & Sirois, J. (2003). Cyclooxygenase-2 expression in canine mammary tumors. *Veterinary pathology*, *40*(2), 207–212. https://doi.org/10.1354/vp.40-2-207

Egenvall, A., Bonnett, B. N., Ohagen, P., Olson, P., Hedhammar, A., & von Euler, H. (2005). Incidence of and survival after mammary tumors in a population of over 80,000 insured female dogs in Sweden from 1995 to 2002. *Preventive veterinary medicine*, *69*(1-2), 109–127. https://doi.org/10.1016/j.prevetmed.2005.01.014

Ehrhart, N.P. (2020). Biopsy and Sentinel Lymph Node Mapping Principles. In *Withrow and MacEwen's Small Animal Clinical Oncology (*pp. 158-163). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Elmslie, R. E., Glawe, P., & Dow, S. W. (2008). Metronomic therapy with cyclophosphamide and piroxicam effectively delays tumor recurrence in dogs with incompletely resected soft tissue sarcomas. *Journal of veterinary internal medicine*, *22*(6), 1373–1379. https://doi.org/10.1111/j.1939-1676.2008.0179.x

Elston, C. W., & Ellis, I. O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*, *19*(5), 403–410. https://doi.org/10.1111/j.1365-2559.1991.tb00229.x

Evans, B. J., Holt, D. E., Stefanovski, D., & Sorenmo, K. U. (2021). Factors influencing complications following mastectomy procedures in dogs with mammary gland tumors: 140 cases (2009-2015). *Journal of the American Veterinary Medical Association*, *258*(3), 295–302. https://doi.org/10.2460/javma.258.3.295

Fahie, M.A. (2018). Primary wound closure. In *Veterinary Surgery: Small Animal* (pp 1397-1410). Elsevier.

Falette N, Paperin MP, Treilleux I, Gratadour AC, Peloux N, Mignotte H, Tooke N, Lofman E, Inganas M, Bremond A, Ozturk M, Puisieux A: Prognostic value of p53 gene mutation in a large series of node-negative breast cancer patients. Cancer Res 58: 1451–1455, 1998.

Feliciano, M. A. R., Uscategui, R. A. R., Maronezi, M. C., Simões, A. P. R., Silva, P., Gasser, B., Pavan, L., Carvalho, C. F., Canola, J. C., & Vicente, W. R. R. (2017). Ultrasonography methods for predicting malignancy in canine mammary tumors. *PloS one*, *12*(5), e0178143. https://doi.org/10.1371/journal.pone.0178143

Ferreira, E., Bertagnolli, A. C., Cavalcanti, M. F., Schmitt, F. C., & Cassali, G. D. (2009). The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumors. *Veterinary and comparative oncology*, *7*(4), 230–235. https://doi.org/10.1111/j.1476-5829.2009.00193.x

Ferreira, T., Gama, A., Seixas, F., Faustino-Rocha, A. I., Lopes, C., Gaspar, V. M., Mano, J. F., Medeiros, R., & Oliveira, P. A. (2023). Mammary Glands of Women, Female Dogs and Female Rats: Similarities and Differences to Be Considered in Breast Cancer Research. *Veterinary sciences*, *10*(6), 379. https://doi.org/10.3390/vetsci10060379

Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., … Zelada-Hedman, M. (1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *American journal of human genetics*, *62*(3), 676–689. https://doi.org/10.1086/301749

Fournier, Q., Cazzini, P., Bavcar, S., Pecceu, E., Ballber, C., & Elders, R. (2018). Investigation of the utility of lymph node fine-needle aspiration cytology for the staging of malignant solid tumors in dogs. *Veterinary clinical pathology*, *47*(3), 489–500. https://doi.org/10.1111/vcp.12636

Frénel, J. S., & Nguyen, F. (2023). Mammary carcinoma: Comparative oncology between small animals and humans-New therapeutic tools. *Reproduction in domestic animals = Zuchthygiene*, *58 Suppl 2*, 102–108. https://doi.org/10.1111/rda.14408

Friedrichs, K.R. & Young, K.M. (2020). Diagnostic cytopathology in clinical oncology. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp. 126-145). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Gamlem, H., Nordstoga, K., & Glattre, E. (2008). Canine neoplasia--introductory paper. *APMIS. Supplementum*, (125), 5–18. https://doi.org/10.1111/j.1600-0463.2008.125m2.x

Gedon, J., Wehrend, A., & Kessler, M. (2022). Ovariectomy reduces the risk of tumour development and influences the histologic continuum in canine mammary tumors. *Veterinary and comparative oncology*, *20*(2), 476–483. https://doi.org/10.1111/vco.12793

Gedon, J., Wehrend, A., Failing, K., & Kessler, M. (2021). Canine mammary tumors: Size matters-a progression from low to highly malignant subtypes. *Veterinary and comparative oncology*, *19*(4), 707–713. https://doi.org/10.1111/vco.12649

Geil, R. G., & Lamar, J. K. (1977). FDA studies of estrogen, progestogens, and estrogen/progestogen combinations in the dog and monkey. *Journal of toxicology and environmental health*, *3*(1-2), 179–193. https://doi.org/10.1080/15287397709529557

Ghisleni, G., Roccabianca, P., Ceruti, R., Stefanello, D., Bertazzolo, W., Bonfanti, U., & Caniatti, M. (2006). Correlation between fine-needle aspiration cytology and histopathology in the evaluation of cutaneous and subcutaneous masses from dogs and cats. *Veterinary clinical pathology*, *35*(1), 24–30. https://doi.org/10.1111/j.1939-165x.2006.tb00084.x

Giles, R. C., Kwapien, R. P., Geil, R. G., & Casey, H. W. (1978). Mammary nodules in beagle dogs administered investigational oral contraceptive steroids. *Journal of the National Cancer Institute*, *60*(6), 1351–1364. https://doi.org/10.1093/jnci/60.6.1351

Goldschmidt, M., Peña, L., Rasotto, R., & Zappulli, V. (2011). Classification and grading of canine mammary tumors. *Veterinary pathology*, *48*(1), 117–131. https://doi.org/10.1177/0300985810393258

Gonçalves, A. D. B. B., de Pinho, T. C. R., Toledo, F. A. O., Balabram, D., Ferreira, E., Campos, M. R. A., & Cassali, G. D. (2021). Extracapsular extension and tumor implants in lymph nodes with canine mammary carcinoma metastasis: Study of the impact on survival. *Research in veterinary science*, *139*, 87–93. https://doi.org/10.1016/j.rvsc.2021.07.010

Guil-Luna, S., Sánchez-Céspedes, R., Millán, Y., De Andrés, F. J., Rollón, E., Domingo, V., Guscetti, F., & Martín de Las Mulas, J. (2011). Aglepristone decreases proliferation in progesterone receptor-positive canine mammary carcinomas. *Journal of veterinary internal medicine*, *25*(3), 518–523. https://doi.org/10.1111/j.1939-1676.2011.0723.x

Guimarães, M. J., Carvalho, M. I., Pires, I., Prada, J., Gil, A. G., Lopes, C., & Queiroga, F. L. (2014). Concurrent expression of cyclo-oxygenase-2 and epidermal growth factor receptor in canine malignant mammary tumors. *Journal of comparative pathology*, *150*(1), 27–34. https://doi.org/10.1016/j.jcpa.2013.07.005

Hampe, J. F., & Misdorp, W. (1974). Tumours and dysplasias of the mammary gland. Bulletin of the World Health Organization, 50(1-2), 111–133.

Harbeck, N., Penault-Llorca, F., Cortes, J., Gnant, M., Houssami, N., Poortmans, P., Ruddy, K., Tsang, J., & Cardoso, F. (2019). Breast cancer. *Nature reviews. Disease primers*, *5*(1), 66. https://doi.org/10.1038/s41572-019-0111-2

Hart, B. L., Hart, L. A., Thigpen, A. P., & Willits, N. H. (2020). Assisting Decision-Making on Age of Neutering for 35 Breeds of Dogs: Associated Joint Disorders, Cancers, and Urinary Incontinence. *Frontiers in veterinary science*, *7*, 388. https://doi.org/10.3389/fvets.2020.00388

Hart, B. L., Hart, L. A., Thigpen, A. P., & Willits, N. H. (2020). Assisting Decision-Making on Age of Neutering for Mixed Breed Dogs of Five Weight Categories: Associated Joint Disorders and Cancers. *Frontiers in veterinary science*, *7*, 472. https://doi.org/10.3389/fvets.2020.00472

Hermo, G. A., Torres, P., Ripoll, G. V., Scursoni, A. M., Gomez, D. E., Alonso, D. F., & Gobello, C. (2008). Perioperative desmopressin prolongs survival in surgically treated bitches with mammary gland tumors: a pilot study. *Veterinary journal (London, England : 1997)*, *178*(1), 103–108. https://doi.org/10.1016/j.tvjl.2007.06.015

Hermo, G. A., Turic, E., Angelico, D., Scursoni, A. M., Gomez, D. E., Gobello, C., & Alonso, D. F. (2011). Effect of adjuvant perioperative desmopressin in locally advanced canine mammary carcinoma and its relation to histologic grade. *Journal of the American Animal Hospital Association*, *47*(1), 21–27. https://doi.org/10.5326/JAAHA-MS-5509

Herrera Becerra, J. R., Monteiro, E. R., Martins, L. G., Baier, M. E., Santos, E. A., & Bianchi, S. P. (2022). Epidural administration of combinations of ropivacaine, morphine and xylazine in bitches undergoing total unilateral mastectomy:

a randomized clinical trial. *Veterinary anaesthesia and analgesia*, *49*(1), 126–134. https://doi.org/10.1016/j.vaa.2021.10.001

Hlusko, K. C., Cole, R., Tillson, D. M., Boothe, H. W., Almond, G., Coggeshall, W. S., & Matz, B. M. (2020). Sentinel lymph node detection differs when comparing lymphoscintigraphy to lymphography using water soluble iodinated contrast medium and digital radiography in dogs. *Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association*, *61*(6), 659–666. https://doi.org/10.1111/vru.12908

Hörnfeldt, M. B., & Mortensen, J. K. (2023). Surgical dose and the clinical outcome in the treatment of mammary gland tumors in female dogs: a literature review. *Acta veterinaria Scandinavica*, *65*(1), 12. https://doi.org/10.1186/s13028-023-00674-1

Horta, R. S., Figueiredo, M. S., Lavalle, G. E., Costa, M. P., Cunha, R. M., & Araújo, R. B. (2015). Surgical stress and postoperative complications related to regional and radical mastectomy in dogs. *Acta veterinaria Scandinavica*, *57*(1), 34. https://doi.org/10.1186/s13028-015-0121-3

Hunt G. B. (1995). Skin fold advancement flaps for closing large sternal and inguinal wounds in cats and dogs. *Veterinary surgery : VS*, *24*(2), 172–175. https://doi.org/10.1111/j.1532-950x.1995.tb01312.x

Kamstock, D. A., Ehrhart, E. J., Getzy, D. M., Bacon, N. J., Rassnick, K. M., Moroff, S. D., Liu, S. M., Straw, R. C., McKnight, C. A., Amorim, R. L., Bienzle, D., Cassali, G. D., Cullen, J. M., Dennis, M. M., Esplin, D. G., Foster, R. A., Goldschmidt, M. H., Gruber, A. D., Hellmén, E., Howerth, E. W., … American College of Veterinary Pathologists' Oncology Committee (2011). Recommended guidelines for submission, trimming, margin evaluation, and reporting of tumor biopsy specimens in veterinary surgical pathology. *Veterinary pathology*, *48*(1), 19–31. https://doi.org/10.1177/0300985810389316

Karayannopoulou, M., Kaldrymidou, E., Constantinidis, T. C., & Dessiris, A. (2001). Adjuvant post-operative chemotherapy in bitches with mammary cancer. *Journal of veterinary medicine. A, Physiology, pathology, clinical medicine*, *48*(2), 85–96. https://doi.org/10.1046/j.1439-0442.2001.00336.x

Kareva I, Waxman DJ, Klement GL. Metronomic chemotherapy: an attractive alternative to maximum tolerated dose therapy that can activate anti-tumour immunity and minimize therapeutic resistance. Cancer Lett. 2015;358:100-106.

Kaszak, I., Ruszczak, A., Kanafa, S., Kacprzak, K., Król, M., & Jurka, P. (2018). Current biomarkers of canine mammary tumors. *Acta veterinaria Scandinavica*, *60*(1), 66. https://doi.org/10.1186/s13028-018-0417-1

Kettner, S. C., Willschke, H., & Marhofer, P. (2011). Does regional anaesthesia really improve outcome?. *British journal of anaesthesia*, *107 Suppl 1*, i90–i95. https://doi.org/10.1093/bja/aer340

Khanna, C., Rosenberg, M., & Vail, D. M. (2015). A Review of Paclitaxel and Novel Formulations Including Those Suitable for Use in Dogs. *Journal of veterinary internal medicine*, *29*(4), 1006–1012. https://doi.org/10.1111/jvim.12596

Kim, N. H., Lim, H. Y., Im, K. S., Shin, J. I., Kim, H. W., & Sur, J. H. (2014). Evaluation of clinicopathological characteristics and oestrogen receptor gene expression in oestrogen receptor-negative, progesterone receptor-positive canine mammary carcinomas. *Journal of comparative pathology*, *151*(1), 42–50. https://doi.org/10.1016/j.jcpa.2014.04.001

King, M. C., Marks, J. H., Mandell, J. B., & New York Breast Cancer Study Group (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science (New York, N.Y.)*, *302*(5645), 643–646. https://doi.org/10.1126/science.1088759

Kristiansen, V. M., Nødtvedt, A., Breen, A. M., Langeland, M., Teige, J., Goldschmidt, M., Jonasdottir, T. J., Grotmol, T., & Sørenmo, K. (2013). Effect of ovariohysterectomy at the time of tumor removal in dogs with benign mammary tumors and hyperplastic lesions: a randomized controlled clinical trial. *Journal of veterinary internal medicine*, *27*(4), 935–942. https://doi.org/10.1111/jvim.12110

Kristiansen, V. M., Peña, L., Díez Córdova, L., Illera, J. C., Skjerve, E., Breen, A. M., Cofone, M. A., Langeland, M., Teige, J., Goldschmidt, M., & Sørenmo, K. U. (2016). Effect of Ovariohysterectomy at the Time of Tumor Removal in Dogs with Mammary Carcinomas: A Randomized Controlled Trial. *Journal of veterinary internal medicine*, *30*(1), 230–241. https://doi.org/10.1111/jvim.13812

Ku, C. K., Kass, P. H., & Christopher, M. M. (2017). Cytologic-histologic concordance in the diagnosis of neoplasia in canine and feline lymph nodes: a retrospective study of 367 cases. *Veterinary and comparative oncology*, *15*(4), 1206–1217. https://doi.org/10.1111/vco.12256

Kuntz, C. A., Dernell, W. S., Powers, B. E., & Withrow, S. (1998). Extraskeletal osteosarcomas in dogs: 14 cases. *Journal of the American Animal Hospital Association*, *34*(1), 26–30. https://doi.org/10.5326/15473317-34-1-26

Kwapien, R. P., Giles, R. C., Geil, R. G., & Casey, H. W. (1980). Malignant mammary tumors in beagle dogs dosed with investigational oral contraceptive steroids. *Journal of the National Cancer Institute*, *65*(1), 137–144.

Kwon, J. Y., Moskwa, N., Kang, W., Fan, T. M., & Lee, C. (2023). Canine as a Comparative and Translational Model for Human Mammary Tumor. *Journal of breast cancer*, *26*(1), 1–13. https://doi.org/10.4048/jbc.2023.26.e4

Laflamme D. P. (2006). Understanding and managing obesity in dogs and cats. *The Veterinary clinics of North America. Small animal practice*, *36*(6), 1283–vii. https://doi.org/10.1016/j.cvsm.2006.08.005

Lana, S.E., Rutteman, G.R., Withrow, S.J. (2007). Tumours of the mammary gland. In *Withrow & MacEwen's small animal clinical oncology* (pp 619–636). Saunders Elsevier.

Langenbach, A., McManus, P. M., Hendrick, M. J., Shofer, F. S., & Sorenmo, K. U. (2001). Sensitivity and specificity of methods of assessing the regional lymph nodes for evidence of metastasis in dogs and cats with solid tumors. *Journal of the American Veterinary Medical Association*, *218*(9), 1424–1428. https://doi.org/10.2460/javma.2001.218.1424

Larue S.M., Gordon I.K. (2020). Radiation Oncology. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp 209-224). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Lavalle, G. E., De Campos, C. B., Bertagnolli, A. C., & Cassali, G. D. (2012). Canine malignant mammary gland neoplasms with advanced clinical staging treated with carboplatin and cyclooxygenase inhibitors. *In vivo (Athens, Greece)*, *26*(3), 375–379.

Lee, K., Kruper, L., Dieli-Conwright, C. M., & Mortimer, J. E. (2019). The Impact of Obesity on Breast Cancer Diagnosis and Treatment. *Current oncology reports*, *21*(5), 41. https://doi.org/10.1007/s11912-019-0787-1

Lim, H. Y., Im, K. S., Kim, N. H., Kim, H. W., Shin, J. I., Yhee, J. Y., & Sur, J. H. (2015). Effects of Obesity and Obesity-Related Molecules on Canine Mammary Gland Tumors. *Veterinary pathology*, *52*(6), 1045–1051. https://doi.org/10.1177/0300985815579994

Liptak, J. M., & Boston, S. E. (2019). Nonselective Lymph Node Dissection and Sentinel Lymph Node Mapping and Biopsy. *The Veterinary clinics of North America. Small animal practice*, *49*(5), 793–807. https://doi.org/10.1016/j.cvsm.2019.04.003

Lombardi, P., Florio, S., Pagnini, U., Crispino, A., & Avallone, L. (1999). Ovarian function suppression with a GnRH analogue: D-ser(But[t])[6]-Arzgly[10]-LHRH (Goserelin) in hormone dependent canine mammary cancer. *Journal of veterinary pharmacology and therapeutics*, *22*(1), 56–61. https://doi.org/10.1046/j.1365-2885.1999.00184.x

London, C. A., Hannah, A. L., Zadovoskaya, R., Chien, M. B., Kollias-Baker, C., Rosenberg, M., Downing, S., Post, G., Boucher, J., Shenoy, N., Mendel, D. B., McMahon, G., & Cherrington, J. M. (2003). Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clinical cancer research : an official journal of the American Association for Cancer Research*, *9*(7), 2755–2768.

MacEwen, E. G., Harvey, H. J., Patnaik, A. K., Mooney, S., Hayes, A., Kurzman, I., & Hardy, W. D., Jr (1985). Evaluation of effects of levamisole and surgery on canine mammary cancer. *Journal of biological response modifiers*, *4*(4), 418–426.

MacPhail, C. & Fossum, T.W. (2019). Surgery of the Female Reproductive Tract. In *Small Animal Surgery* (pp 746-751). Elsevier

Maï, W., O'Brien, R., Scrivani, P., Porat-Mosenco, V., Tobin, E., Seiler, G., McConnell, F., Schwarz, T. & Zwingenberger, A. (2008). The lung parenchyma. In *BSAVA manual of canine and feline thoracic imaging (*pp. 242-319). British Small Animal Veterinary Association.

Mainenti, M., Rasotto, R., Carnier, P., & Zappulli, V. (2014). Oestrogen and progesterone receptor expression in subtypes of canine mammary tumors in intact and ovariectomised dogs. *Veterinary journal (London, England : 1997)*, *202*(1), 62–68. https://doi.org/10.1016/j.tvjl.2014.06.003

Majeski, S. A., Steffey, M. A., Fuller, M., Hunt, G. B., Mayhew, P. D., & Pollard, R. E. (2017). INDIRECT COMPUTED TOMOGRAPHIC LYMPHOGRAPHY FOR ILIOSACRAL LYMPHATIC MAPPING IN A COHORT OF DOGS WITH ANAL SAC GLAND ADENOCARCINOMA: TECHNIQUE DESCRIPTION. *Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association*, *58*(3), 295–303. https://doi.org/10.1111/vru.12482

Marconato, L., Lorenzo, R. M., Abramo, F., Ratto, A., & Zini, E. (2008). Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumors in dogs. *Veterinary and comparative oncology*, *6*(2), 90–101. https://doi.org/10.1111/j.1476-5829.2007.00143.x

Marconato, L., Romanelli, G., Stefanello, D., Giacoboni, C., Bonfanti, U., Bettini, G., Finotello, R., Verganti, S., Valenti, P., Ciaramella, L., & Zini, E. (2009). Prognostic factors for dogs with mammary inflammatory carcinoma: 43 cases (2003-2008). *Journal of the American Veterinary Medical Association*, *235*(8), 967–972. https://doi.org/10.2460/javma.235.8.967

Martín-Ruiz, A., Peña, L., González-Gil, A., Díez-Córdova, L. T., Cáceres, S., & Illera, J. C. (2018). Effects of indole-3-carbinol on steroid hormone profile and tumor progression in a mice model of canine inflammatory mammarycancer. *BMC cancer*, *18*(1), 626. https://doi.org/10.1186/s12885-018-4518-z

Martonos, C., Alexandru, G., Cristian, D., Aurel, D., Melania, C., Ioan, B.U.D., Marius, P., (2019). Morphological description of sentinel lymphatic channels in canine mammary tumors. In *Proceedings of the Multidisciplinary Conference on Sustainable Development* (pp. 637–643). Filodiritto.

Matos, A. J., Baptista, C. S., Gärtner, M. F., & Rutteman, G. R. (2012). Prognostic studies of canine and feline mammary tumors: the need for standardized procedures. *Veterinary journal (London, England : 1997)*, *193*(1), 24–31. https://doi.org/10.1016/j.tvjl.2011.12.019

Mayer, M. N., Silver, T. I., Lowe, C. K., & Anthony, J. M. (2013). Radiographic lymphangiography in the dog using iodized oil. *Veterinary and comparative oncology*, *11*(2), 151–161. https://doi.org/10.1111/j.1476-5829.2012.00334.x

Merlo, D. F., Rossi, L., Pellegrino, C., Ceppi, M., Cardellino, U., Capurro, C., Ratto, A., Sambucco, P. L., Sestito, V., Tanara, G., & Bocchini, V. (2008). Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *Journal of veterinary internal medicine*, *22*(4), 976–984. https://doi.org/10.1111/j.1939-1676.2008.0133.x

Millanta, F., Asproni, P., Canale, A., Citi, S., & Poli, A. (2016). COX-2, mPGES-1 and EP2 receptor immunohistochemical expression in canine and feline malignant mammary tumors. *Veterinary and comparative oncology*, *14*(3), 270–280. https://doi.org/10.1111/vco.12096

Millanta, F., Calandrella, M., Bari, G., Niccolini, M., Vannozzi, I., & Poli, A. (2005). Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. *Research in veterinary science*, *79*(3), 225–232. https://doi.org/10.1016/j.rvsc.2005.02.002

Millanta, F., Caneschi, V., Ressel, L., Citi, S., & Poli, A. (2010). Expression of vascular endothelial growth factor in canine inflammatory and non-inflammatory mammary carcinoma. *Journal of comparative pathology*, *142*(1), 36–42. https://doi.org/10.1016/j.jcpa.2009.06.004

Misdorp W. (1988). Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins. *The veterinary quarterly, 10(1), 26–33. https://doi.org/10.1080/01652176.1988.9694142*

Misdorp W. (1991). Progestagens and mammary tumours in dogs and cats. *Acta endocrinologica,* 125 Suppl 1, 27–31.

Misdorp, W. (1999). *Histological classification of mammary tumors of the dog and the cat.* Armed Forces Institute of Pathology, Washington.

Monlux, A. W., Roszel, J. F., MacVean, D. W., & Palmer, T. W. (1977). Classification of epithelial canine mammary tumors in a defined population. *Veterinary pathology, 14(3), 194–217. https://doi.org/10.1177/030098587701400303*

Morris, J. S., Dobson, J. M., & Bostock, D. E. (1993). Use of tamoxifen in the control of canine mammary neoplasia. *The Veterinary record*, *133*(22), 539–542. https://doi.org/10.1136/vr.133.22.539

Mpekris, F., Voutouri, C., Panagi, M., Baish, J. W., Jain, R. K., & Stylianopoulos, T. (2022). Normalizing tumor microenvironment with nanomedicine and metronomic therapy to improve immunotherapy. *Journal of controlled release: official journal of the Controlled Release Society*, *345*, 190–199. https://doi.org/10.1016/j.jconrel.2022.03.008

Muscatello, L. V., Papa, V., Millanta, F., Sarli, G., Bacci, B., Cenacchi, G., Poli, A., Giudice, C., & Brunetti, B. (2021). Canine Mammary Carcinoma With Vacuolated Cytoplasm: Glycogen-Rich Carcinoma, a Histological Type Distinct From Lipid-Rich Carcinoma. *Veterinary pathology*, *58*(1), 63–70. https://doi.org/10.1177/0300985820969962

Nieto, A., Peña, L., Pérez-Alenza, M. D., Sánchez, M. A., Flores, J. M., & Castaño, M. (2000). Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. *Veterinary pathology*, *37*(3), 239–247. https://doi.org/10.1354/vp.37-3-239

Nishimura, R., Koyama, H., Kasumi, F., Takashima, S., Kobayashi, S., Komaki, K., Ohkawa, T., Shin, E., Kodama, H., Fukutomi, T., Nishi, T., Sonoo, H., Sano, S., Kimishima, I., Nakaue, K., Nakamura, S., Kusama, M., & Okumura, K. (1998). A case control study on risk factors involved in inflammatory breast recurrence after breast-conserving surgery. *Oncology*, *55*(5), 391–399. https://doi.org/10.1159/000011884

Nosalova, N., Huniadi, M., Horňáková, Ľ., Valenčáková, A., Horňák, S., Nagoos, K., Vozar, J., & Cizkova, D. (2024). Canine Mammary Tumors: Classification, Biomarkers, Traditional and Personalized Therapies. *International journal of molecular sciences*, *25*(5), 2891. https://doi.org/10.3390/ijms25052891

Nunes, F.C., Campos, C.B., Teixeira, S.V., Bertagnolli, A.C., Lavalle, G.E., Cassali, G.D., 2018. Epidemiological, clinical and pathological evaluation of overall survival in canines with mammary neoplasms. Arq Bras. Med. Vet. Zootec. 70, 1714–1722. https://doi.org/10.1590/1678-4162-10217.

Nunes, F.C., Gamba, C.O., Damasceno, K.A., Campos, C.B., Horta, R.S., Araujo, M.R., Monteiro, L.N., Lavalle, G.E., Ferreira, E. and Cassali, G.D. (2014). Analysis of clinico-pathological data, therapeutical conduct and overall survival of

canine mammary lesions attended at the veterinary hospital of the Federal University of Minas Gerais (UFMG). Brazilian Journal of Veterinary Pathology, 7, 122-126.

Nykamp, S. & Randall, E. (2020). Diagnostic Imaging in Oncology. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp. 113-122). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Ober, C. P., & Barber, D. (2006). Comparison of two- vs. three-view thoracic radiographic studies on conspicuity of structured interstitial patterns in dogs. *Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association*, *47*(6), 542–545. https://doi.org/10.1111/j.1740-8261.2006.00183.x

Overgaauw, P. A. M., Vinke, C. M., Hagen, M. A. E. V., & Lipman, L. J. A. (2020). A One Health Perspective on the Human-Companion Animal Relationship with Emphasis on Zoonotic Aspects. *International journal of environmental research and public health*, *17*(11), 3789. https://doi.org/10.3390/ijerph17113789

Owen LN. (1980). *TNM Classification of tumors in domestic animals*. Geneva: World Health Organization.

Pastor, N., Caballé, N. C., Santella, M., Ezquerra, L. J., Tarazona, R., & Duran, E. (2018). Epidemiological study of canine mammary tumors: age, breed, size and malignancy. *Austral journal of veterinary sciences*, *50*(3), 143-147. https://dx.doi.org/10.4067/S0719-81322018000300143

Patsikas, M. N., & Dessiris, A. (1996). The lymph drainage of the mammary glands in the bitch: a lymphographic study. Part I: The 1st, 2nd, 4th and 5th mammary glands. *Anatomia, histologia, embryologia*, *25*(2), 131–138. https://doi.org/10.1111/j.1439-0264.1996.tb00071.x

Patsikas, M. N., & Dessiris, A. (1996). The lymph drainage of the mammary glands in the Bitch: a lymphographic study. Part II: The 3rd mammary gland. *Anatomia, histologia, embryologia*, *25*(2), 139–143. https://doi.org/10.1111/j.1439-0264.1996.tb00072.x

Patsikas, M. N., Karayannopoulou, M., Kaldrymidoy, E., Papazoglou, L. G., Papadopoulou, P. L., Tzegas, S. I., Tziris, N. E., Kaitzis, D. G., Dimitriadis, A. S., & Dessiris, A. K. (2006). The lymph drainage of the neoplastic mammary glands in the bitch: a lymphographic study. *Anatomia, histologia, embryologia*, *35*(4), 228–234. https://doi.org/10.1111/j.1439-0264.2005.00664.x

Peña, L., De Andrés, P. J., Clemente, M., Cuesta, P., & Pérez-Alenza, M. D. (2013). Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship

with clinical and histological characteristics. *Veterinary pathology*, *50*(1), 94–105. https://doi.org/10.1177/0300985812447830

Peña, L., Perez-Alenza, M. D., Rodriguez-Bertos, A., & Nieto, A. (2003). Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. *Breast cancer research and treatment*, *78*(2), 141–148. https://doi.org/10.1023/a:1022991802116

Pereira, C. T., Luiz Navarro Marques, F., Williams, J., Wlademir De Martin, B., & Primo Bombonato, P. (2008). 99mTc-labeled dextran for mammary lymphoscintigraphy in dogs. *Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association*, *49*(5), 487–491. https://doi.org/10.1111/j.1740-8261.2008.00414.x

Pereira, C. T., Rahal, S. C., de Carvalho Balieiro, J. C., & Ribeiro, A. A. (2003). Lymphatic drainage on healthy and neoplasic mammary glands in female dogs: can it really be altered?. *Anatomia, histologia, embryologia*, *32*(5), 282–290. https://doi.org/10.1046/j.1439-0264.2003.00485.x

Pérez Alenza, D., Rutteman, G. R., Peña, L., Beynen, A. C., & Cuesta, P. (1998). Relation between habitual diet and canine mammary tumors in a case-control study. *Journal of veterinary internal medicine*, *12*(3), 132–139. https://doi.org/10.1111/j.1939-1676.1998.tb02108.x

Pérez Alenza, M. D., Tabanera, E., & Peña, L. (2001). Inflammatory mammary carcinoma in dogs: 33 cases (1995-1999). *Journal of the American Veterinary Medical Association*, *219*(8), 1110–1114. https://doi.org/10.2460/javma.2001.219.1110

Pierini, A., Millanta, F., Zanforlin, R., Vannozzi, I., & Marchetti, V. (2017). Usefulness of cytologic criteria in ultrasound-guided fine-needle aspirates from subcentimeter canine mammary tumors. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, *29*(6), 869–873. https://doi.org/10.1177/1040638717718886

Pimentel, P. A. B., da Silva, P. H. S., de Sena, B. V., Flecher, M. C., Cassali, G. D., & Horta, R. D. S. (2024). The role of lymph nodes and their drainage in canine mammary gland tumors: Systematic review. *Research in veterinary science*, *168*, 105139. https://doi.org/10.1016/j.rvsc.2024.105139

Pîrvu, A. M., Caniatti, M., Pieri, M., Roccabianca, P., & Militaru, M. (2024). Cytological Features of Inflammatory Mammary Carcinoma in Dogs. *Veterinary sciences*, *11*(9), 389. https://doi.org/10.3390/vetsci11090389

Portela, D. A., Romano, M., & Briganti, A. (2014). Retrospective clinical evaluation of ultrasound guided transverse abdominis plane block in dogs undergoing mastectomy. *Veterinary anaesthesia and analgesia*, *41*(3), 319–324. https://doi.org/10.1111/vaa.12122

Queiroga, F. L., Perez-Alenza, M. D., Silvan, G., Peña, L., Lopes, C., & Illera, J. C. (2005). Cox-2 levels in canine mammary tumors, including inflammatory mammary carcinoma: clinicopathological features and prognostic significance. *Anticancer research*, *25*(6B), 4269–4275.

Queiroga, F. L., Pires, I., Lobo, L., & Lopes, C. S. (2010). The role of Cox-2 expression in the prognosis of dogs with malignant mammary tumors. *Research in veterinary science*, *88*(3), 441–445. https://doi.org/10.1016/j.rvsc.2009.10.009

Rafalko JM, Kruglyak KM, McCleary- Wheeler AL, Goyal V, Phelps-Dunn A, Wong LK, et al. (2023) Age at cancer diagnosis by breed, weight, sex, and cancer type in a cohort of more than 3,000 dogs: Determining the optimal age to initiate cancer screening in canine patients. PLoS ONE 18(2): e0280795. https://doi.org/10.1371/ journal.pone.0280795

Randall, E. K., Jones, M. D., Kraft, S. L., & Worley, D. R. (2020). The development of an indirect computed tomography lymphography protocol for sentinel lymph node detection in head and neck cancer and comparison to other sentinel lymph node mapping techniques. *Veterinary and comparative oncology*, *18*(4), 634–644. https://doi.org/10.1111/vco.12585

Raposo, T. P., Pires, I., Prada, J., Queiroga, F. L., & Argyle, D. J. (2017). Exploring new biomarkers in the tumour microenvironment of canine inflammatory mammary tumours. *Veterinary and comparative oncology*, *15*(2), 655–666. https://doi.org/10.1111/vco.12209

Rasotto, R., Berlato, D., Goldschmidt, M. H., & Zappulli, V. (2017). Prognostic Significance of Canine Mammary Tumor Histologic Subtypes: An Observational Cohort Study of 229 Cases. *Veterinary pathology*, *54*(4), 571–578. https://doi.org/10.1177/0300985817698208

Rasotto, R., Zappulli, V., Castagnaro, M., & Goldschmidt, M. H. (2012). A retrospective study of those histopathologic parameters predictive of invasion of the lymphatic system by canine mammary carcinomas. *Veterinary pathology*, *49*(2), 330–340. https://doi.org/10.1177/0300985811409253

Razzuoli, E., De Ciucis, C. G., Chirullo, B., Varello, K., Zoccola, R., Guardone, L., Petrucci, P., Rubini, D., Bozzetta, E., Goria, M., Fruscione, F., & Modesto, P. (2022). Molecular Characterization of CF33 Canine Cell Line and Evaluation of Its Ability to Respond against Infective Stressors in Sight of Anticancer Approaches. *Veterinary sciences*, *9*(10), 543. https://doi.org/10.3390/vetsci9100543

Richards GJ, Lewison EF. (1961). Inflammatory carcinoma of the breast. *Surg Gynecol Obstet*, 113, 729–733.

Rivera, P., Melin, M., Biagi, T., Fall, T., Häggström, J., Lindblad-Toh, K., & von Euler, H. (2009). Mammary tumor development in dogs is associated with BRCA1 and BRCA2. *Cancer research*, *69*(22), 8770–8774. https://doi.org/10.1158/0008-5472.CAN-09-1725

Rodríguez, J., Santana, Á., Herráez, P., Killick, D. R., & de Los Monteros, A. E. (2022). Epidemiology of canine mammary tumors on the Canary Archipelago in Spain. *BMC veterinary research*, *18*(1), 268. https://doi.org/10.1186/s12917-022-03363-9

Romagnoli, S., Krekeler, N., de Cramer, K., Kutzler, M., McCarthy, R. and Schaefer-Somi, S. (2024), WSAVA guidelines for the control of reproduction in dogs and cats. J Small Anim Pract, 65: 424-559. https://doi.org/10.1111/jsap.13724

Rossi, F., Sabattini, S., Vascellari, M., & Marconato, L. (2018). The impact of toceranib, piroxicam and thalidomide with or without hypofractionated radiation therapy on clinical outcome in dogs with inflammatory mammary carcinoma. *Veterinary and comparative oncology*, *16*(4), 497–504. https://doi.org/10.1111/vco.12407

Rudorf, H., Taeymans, O. (2008). Basics of thoracic radiography and radiology. In *BSAVA manual of canine and feline thoracic imaging (*pp. 1-19)*,* British Small Animal Veterinary Association.

Saba, C. F., Rogers, K. S., Newman, S. J., Mauldin, G. E., & Vail, D. M. (2007). Mammary gland tumors in male dogs. *Journal of veterinary internal medicine*, *21*(5), 1056–1059. https://doi.org/10.1892/0891-6640(2007)21[1056:mgtimd]2.0.co;2

Sadhasivan, S. B. M., Shafiuzama, M., Shammi, M., Rao, G. V. S., Souza, N. J. D., Senthilnayagam, H., George, R. S., & Prabhakar, P. M. (2017). Studies on reconstruction of large skin defects following mammary tumor excision in dogs. *Veterinary world*, *10*(12), 1521–1528. https://doi.org/10.14202/vetworld.2017.1521-1528

Salas, Y., Márquez, A., Diaz, D., & Romero, L. (2015). Epidemiological Study of Mammary Tumors in Female Dogs Diagnosed during the Period 2002-2012: A Growing Animal Health Problem. *PloS one*, *10*(5), e0127381. https://doi.org/10.1371/journal.pone.0127381

Saltzstein, S.L. (1974). Clinically occult inflammatory carcinoma of the breast. *Cancer*, 34, 382–388.

Santos, M., Marcos, R., & Faustino, A. M. (2010). Histological study of canine mammary gland during the oestrous cycle. *Reproduction in domestic animals = Zuchthygiene*, *45*(5), e146–e154. https://doi.org/10.1111/j.1439-0531.2009.01536.x

Schneider, R., Dorn, C. R., & Taylor, D. O. (1969). Factors influencing canine mammary cancer development and postsurgical survival. *Journal of the National Cancer Institute*, *43*(6), 1249–1261.

Selman, P. J., van Garderen, E., Mol, J. A., & van den Ingh, T. S. (1995). Comparison of the histological changes in the dog after treatment with the progestins medroxyprogesterone acetate and proligestone. *The veterinary quarterly*, *17*(4), 128–133. https://doi.org/10.1080/01652176.1995.9694551

Shawn, J.R. (2020). Supportive Care for the Cancer Patient. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp 310-319). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Silva, D. M. D., Kluthcovsky, L. C., Jensen de Morais, H., Pallú, G. M., Dos Santos, G. C., Costa Castro, J. L., & Engracia Filho, J. R. (2019). Inflammatory Mammary Carcinoma in a Male Dog-Case Report. *Topics in companion animal medicine*, *37*, 100357. https://doi.org/10.1016/j.tcam.2019.100357

Simeonov, R., & Simeonova, G. (2006). Computerized morphometry of mean nuclear diameter and nuclear roundness in canine mammary gland tumors on cytologic smears. *Veterinary clinical pathology*, *35*(1), 88–90. https://doi.org/10.1111/j.1939-165x.2006.tb00093.x

Simeonov, R., & Simeonova, G. (2006). Fractal dimension of canine mammary gland epithelial tumors on cytologic smears. *Veterinary clinical pathology*, *35*(4), 446–448. https://doi.org/10.1111/j.1939-165x.2006.tb00162.x

Simeonov, R., & Simeonova, G. (2007). Computerized cytomorphometric analysis of nuclear area, nuclear perimeter and mean nuclear diameter in spontaneous canine mammary gland tumors. *Veterinary research communications*, *31*(5), 553–558. https://doi.org/10.1007/s11259-007-3562-y

Simon, D., Schoenrock, D., Baumgärtner, W., & Nolte, I. (2006). Postoperative adjuvant treatment of invasive malignant mammary gland tumors in dogs with doxorubicin and docetaxel. *Journal of veterinary internal medicine*, *20*(5), 1184–1190. https://doi.org/10.1892/0891-6640(2006)20[1184:patimm]2.0.co;2

Simon, D., Schoenrock, D., Nolte, I., Baumgärtner, W., Barron, R., & Mischke, R. (2009). Cytologic examination of fine-needle aspirates from mammary gland tumors in the dog: diagnostic accuracy with comparison to histopathology and

association with postoperative outcome. *Veterinary clinical pathology*, *38*(4), 521–528. https://doi.org/10.1111/j.1939-165X.2009.00150.x

Singletary, S. E., & Cristofanilli, M. (2008). Defining the clinical diagnosis of inflammatory breast cancer. *Seminars in oncology*, *35*(1), 7–10. https://doi.org/10.1053/j.seminoncol.2007.11.010

Sleeckx, N., de Rooster, H., Veldhuis Kroeze, E. J., Van Ginneken, C., & Van Brantegem, L. (2011). Canine mammary tumors, an overview. *Reproduction in domestic animals = Zuchthygiene*, *46*(6), 1112–1131. https://doi.org/10.1111/j.1439-0531.2011.01816.x

Soares, E. D. S., Valente, F. L., Rocha, C. C., Real Pereira, C. E., Sarandy, T. B., de Oliveira, F. L. D., de Morais Calado, S. L., & Borges, A. P. B. (2023). Prognostic Factors for Cancer-Specific Survival and Disease-Free Interval of Dogs with Mammary Carcinomas. *Veterinary medicine international*, *2023*, 6890707. https://doi.org/10.1155/2023/6890707

Sonnenschein, E. G., Glickman, L. T., Goldschmidt, M. H., & McKee, L. J. (1991). Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study. *American journal of epidemiology*, *133*(7), 694–703. https://doi.org/10.1093/oxfordjournals.aje.a115944

Sorenmo, K. (2003). Canine mammary gland tumors. *The Veterinary clinics of North America. Small animal practice*, *33*(3), 573–596. https://doi.org/10.1016/s0195-5616(03)00020-2

Sorenmo, K. U., Durham, A. C., Kristiansen, V., Peña, L., Goldschmidt, M. H., & Stefanovski, D. (2019). Developing and testing prognostic bio-scoring systems for canine mammary gland carcinomas. *Veterinary and comparative oncology*, *17*(4), 479–488. https://doi.org/10.1111/vco.12509

Sorenmo, K. U., Kristiansen, V. M., Cofone, M. A., Shofer, F. S., Breen, A. M., Langeland, M., Mongil, C. M., Grondahl, A. M., Teige, J., & Goldschmidt, M. H. (2009). Canine mammary gland tumors; a histological continuum from benign to malignant; clinical and histopathological evidence. *Veterinary and comparative oncology*, *7*(3), 162–172. https://doi.org/10.1111/j.1476-5829.2009.00184.x

Sorenmo, K. U., Rasotto, R., Zappulli, V., & Goldschmidt, M. H. (2011). Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. *Veterinary pathology*, *48*(1), 85–97. https://doi.org/10.1177/0300985810389480

Sorenmo, K. U., Shofer, F. S., & Goldschmidt, M. H. (2000). Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *Journal of veterinary internal medicine*, *14*(3), 266–270. https://doi.org/10.1892/0891-6640(2000)014<0266:eosato>2.3.co;2

Sorenmo, K., Durham, A. C., Evans, B., Scavello, H., & Stefanovski, D. (2020). A prospective randomized trial of desmopressin in canine mammary carcinoma. *Veterinary and comparative oncology*, *18*(4), 796–803. https://doi.org/10.1111/vco.12619

Sorenmo, K.U.; Worley, D.R.; Zappulli, V. (2020). Tumours of the Mammary Gland. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp. 604–625). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Soultani, C., Patsikas, M. N., Karayannopoulou, M., Jakovljevic, S., Chryssogonidis, I., Papazoglou, L., Papaioannou, N., Papadopoulou, P., Pavlidou, K., Ilia, G. M., Kaitzis, D. G., & Ilia, T. M. (2017). ASSESSMENT OF SENTINEL LYMPH NODE METASTASIS IN CANINE MAMMARY GLAND TUMORS USING COMPUTED TOMOGRAPHIC INDIRECT LYMPHOGRAPHY. *Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association*, *58*(2), 186–196. https://doi.org/10.1111/vru.12460

Spåre, P., Ljungvall, I., Ljungvall, K., & Bergström, A. (2021). Evaluation of post-operative complications after mastectomy performed without perioperative antimicrobial prophylaxis in dogs. *Acta veterinaria Scandinavica*, *63*(1), 35. https://doi.org/10.1186/s13028-021-00600-3

Stan, F., Gudea, A., Damian, A., Gal, A. F., Papuc, I., Pop, A. R., & Martonos, C. (2020). Ultrasonographic Algorithm for the Assessment of Sentinel Lymph Nodes That Drain the Mammary Carcinomas in Female Dogs. *Animals : an open access journal from MDPI*, *10*(12), 2366. https://doi.org/10.3390/ani10122366

Stockhaus, C., Kohn, B., Rudolph, R., Brunnberg, L., & Giger, U. (1999). Correlation of haemostatic abnormalities with tumour stage and characteristics in dogs with mammary carcinoma. *The Journal of small animal practice*, *40*(7), 326–331. https://doi.org/10.1111/j.1748-5827.1999.tb03090.x

Suryawanshi R. V. (2021). Assessment of Efficacy and Toxicity of Cyclophosphamide Chemotherapy in Canines with Malignant Mammary Tumor: A Retrospective Study. *Veterinary medicine international*, *2021*, 5520603. https://doi.org/10.1155/2021/5520603

Szczubiał, M., & Łopuszynski, W. (2011). Prognostic value of regional lymph node status in canine mammary carcinomas. *Veterinary and comparative oncology*, *9*(4), 296–303. https://doi.org/10.1111/j.1476-5829.2011.00268.x

Szczubiał, M., & Łopuszynski, W. (2011). Prognostic value of regional lymph node status in canine mammary carcinomas. *Veterinary and comparative oncology*, *9*(4), 296–303. https://doi.org/10.1111/j.1476-5829.2011.00268.x

Tavares, W. L., Lavalle, G. E., Figueiredo, M. S., Souza, A. G., Bertagnolli, A. C., Viana, F. A., Paes, P. R., Carneiro, R. A., Cavalcanti, G. A., Melo, M. M., & Cassali, G. D. (2010). Evaluation of adverse effects in tamoxifen exposed healthy female dogs. *Acta veterinaria Scandinavica*, *52*(1), 67. https://doi.org/10.1186/1751-0147-52-67

Tavassoli, F.A. (1999). Infiltrating carcinoma: special types. Inflammatory carcinoma. In *Pathology of the* breast (pp. 538–541). McGraw-Hill Book Co.

Tayari, H., Otero, P. E., D'Agostino, M., Bartolini, F., & Briganti, A. (2022). Epidural Volume of Injectate Using a Dose Regimen Based on Occipito-Coccygeal Spinal Length (OCL): Randomized Clinical Study Comparing Different Ropivacaine Concentrations, with or without Morphine, in Bitches Undergoing Total Unilateral Mastectomy. *Animals : an open access journal from MDPI*, *12*(5), 587. https://doi.org/10.3390/ani12050587

Taylor, G.W. & Meltzer, A. (1938). 'Inflammatory carcinoma' of the breast. *The American Journal of Cancer,* 33, 33–49.

Tesi, M., Millanta, F., Poli, A., Mazzetti, G., Pasquini, A., Panzani, D., Rota, A., & Vannozzi, I. (2020). Role of body condition score and adiponectin expression in the progression of canine mammary carcinomas. *Veterinary medicine and science*, *6*(3), 265–271. https://doi.org/10.1002/vms3.238

Thomson, M.J. & Britt, T.A. (2022). Reproductive system. In *Veterinary Surgical Oncology* (pp 484-510). John Wiley & Sons, Inc. https://doi.org/10.1002/9781119089124

Thrall, D.E. (2013). Principles of Radiographic Interpretation of the Thorax. In *Textbook of Veterinary Diagnostic Radiology* (pp 474-488). Saunders Elsevier.

Tran, C. M., Moore, A. S., & Frimberger, A. E. (2016). Surgical treatment of mammary carcinomas in dogs with or without postoperative chemotherapy. *Veterinary and comparative oncology,* 14(3), 252–262. https://doi.org/10.1111/vco.12092

Travis, B. M., Hayes, G. M., Vissio, K., Harvey, H. J., Flanders, J. A., & Sumner, J. P. (2018). A quilting subcutaneous suture pattern to reduce seroma formation and pain 24 hours after midline celiotomy in dogs: A randomized controlled trial. *Veterinary surgery : VS*, *47*(2), 204–211. https://doi.org/10.1111/vsu.12754

Tuohy, J. L., Milgram, J., Worley, D. R., & Dernell, W. S. (2009). A review of sentinel lymph node evaluation and the need for its incorporation into veterinary oncology. *Veterinary and comparative oncology*, *7*(2), 81–91. https://doi.org/10.1111/j.1476-5829.2009.00183.x

Valdivia, G., Alonso-Diez, Á., Pérez-Alenza, D., & Peña, L. (2021). From Conventional to Precision Therapy in Canine Mammary Cancer: A Comprehensive Review. *Frontiers in veterinary science*, *8*, 623800. https://doi.org/10.3389/fvets.2021.623800

van Nimwegen, S. & Kirpensteijn, J. (2018). Specific disorders of the skin and subcutaneous tissues. In *Veterinary Surgery: Small Animal* (pp 1508-1550). Elsevier.

Vascellari, M., Capello, K., Carminato, A., Zanardello, C., Baioni, E., & Mutinelli, F. (2016). Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. *Preventive veterinary medicine*, *126*, 183–189. https://doi.org/10.1016/j.prevetmed.2016.02.008

Vazquez, E., Lipovka, Y., Cervantes-Arias, A., Garibay-Escobar, A., Haby, M. M., Queiroga, F. L., & Velazquez, C. (2023). Canine Mammary Cancer: State of the Art and Future Perspectives. *Animals : an open access journal from MDPI*, *13*(19), 3147. https://doi.org/10.3390/ani13193147

Wakui, S., Muto, T., Yokoo, K., Yokoo, R., Takahashi, H., Masaoka, T., Hano, H., & Furusato, M. (2001). Prognostic status of p53 gene mutation in canine mammary carcinoma. *Anticancer research*, *21*(1B), 611–616.

Wetzel, S., Fidel, J., Whittington, D., & Villarino, N. F. (2024). A pilot study investigating plasma pharmacokinetics and tolerance of oral capecitabine in carcinoma-bearing dogs. *BMC veterinary research*, *20*(1), 36. https://doi.org/10.1186/s12917-023-03805-y

Williams, L. E., & Packer, R. A. (2003). Association between lymph node size and metastasis in dogs with oral malignant melanoma: 100 cases (1987-2001). *Journal of the American Veterinary Medical Association*, *222*(9), 1234–1236. https://doi.org/10.2460/javma.2003.222.1234

Worley D. R. (2014). Incorporation of sentinel lymph node mapping in dogs with mast cell tumors: 20 consecutive procedures. *Veterinary and comparative oncology*, *12*(3), 215–226. https://doi.org/10.1111/j.1476-5829.2012.00354.x

Worley, D.R. (2022). Hemolymphatic system. In *Veterinary Surgical Oncology* (pp 624-658). John Wiley & Sons, Inc. https://doi.org/10.1002/9781119089124

Yu, Y. H., Mo, Q. G., Zhu, X., Gao, L. Q., Liang, C., Huang, Z., Qin, Q. H., Wei, W., Jiang, Y., Bu, K. P., & Wei, C. Y. (2016). Axillary fine needle aspiration cytology is a sensitive and highly specific technique for the detection of axillary lymph node metastasis: a meta-analysis and systematic review. *Cytopathology : official journal of the British Society for Clinical Cytology*, *27*(1), 59–69. https://doi.org/10.1111/cyt.12224

Zappulli, V., Peña, L., Rasotto, R., Goldschmidt, M., Gama, A., Scruggs, J., et al. (2019). *Surgical Pathology of Tumours of Domestic Animals*. Davis-Thompson DVM Foundation

Zheng, H. H., Du, C. T., Yu, C., Zhang, Y. Z., Huang, R. L., Tang, X. Y., & Xie, G. H. (2022). Epidemiological Investigation of Canine Mammary Tumors in Mainland China Between 2017 and 2021. *Frontiers in veterinary science*, *9*, 843390. https://doi.org/10.3389/fvets.2022.843390

3. Evaluation of attenuated Salmonella Typhimurium (STMΔznuABC) anticancer activity on canine mammary cancer-associated fibroblasts

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Evaluation of attenuated *Salmonella Typhimurium* (STMΔznuABC) anticancer activity on canine mammary cancer-associated fibroblasts

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ABSTRACT

Bacteria-mediated treatments gained increasing attention as alternative therapies against tumors. An attenuated mutant strain of *Salmonella enterica* serovar Typhimurium (STMΔznuABC) has recently been considered as a potential new anti-cancer strategy. However, it is unclear whether this activity is tumor-induced or species-specific, and no data are available regarding STMΔznuABC on canine mammary tumors (CMTs). This study aimed to investigate the ability of STMΔznuABC in modulating the response of CMTs, focusing on cancer-associated fibroblasts. Four CMT cell lines (CF33, TM51, TM52 TM53) were treated with STMΔznuABC. Then, antiproliferative activity (MTT assay), bacterial invasion, and CMT cell lines gene expression analysis (RT-qPCR) of genes involved in immune response and cancer aggressiveness were evaluated. STMΔznuABC penetrated in TM51, TM52, TM53, and CF33 cell lines, causing a significant reduction of cell viability. Moreover, the expression of several genes was significantly modulated in all CMT cell lines: STMΔznuABC infection determined a significant up-regulation of *CXCL8*, *IL18*, *IL10*, *TLR4* and *RAD51*, while *CD14*, *IL6*, *CXCR4*, *P53*, *PTEN*, *STAT5*, *TLR5* and *TGFB1* were downregulated in TM53. In CF33, *CXCL8* and *P53* were upregulated, while *MYD88*, *MD2*, *IL18*, *TLR4,5*, *TGFB1* were downregulated. In TM52, *CXCL8*, *CD44* and *MD2* were upregulated and *PTEN* was downregulated, while in TM51 *CXCL8*, *CD44* and *ErbB2* were downregulated. We demonstrated the anti-proliferative and immuno-modulatory activity of STMΔznuABC in CMTs, paving the way for potential new anti-cancer treatments.

1. Introduction

Cancer is a major cause of death worldwide. The incidence of cancer in pet animals is similar to that observed in humans, especially for the following tumors: non-Hodgkin's lymphoma, breast cancer, head and neck carcinoma, melanoma, soft tissue sarcoma and osteosarcoma (Oh and Cho, 2023; Schiffman and Breen, 2015). Furthermore, cancers diagnosed in pets present similarities to their human counterparts in terms of risk factors and biological and pathological characteristics. In particular, the dog osteosarcoma and the feline and canine mammary

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adenocarcinoma present the highest degree of correlation with the analogous human cancers (Abdelmegeed and Mohammed, 2018; Simpson et al., 2017). Due to these similarities, several studies proposed pets as a natural model of disease in comparative oncology (Rowell et al., 2011; Tarone et al., 2019). As for therapy, the most frequently adopted strategies in human medicine (chemo/radiotherapy and surgery) have limited practicability in pets and generally very high costs for veterinary standards. However, the reduction of oncological treatment costs is a goal pursued by both human and veterinary medicine, and the development of new low-cost and effective therapies is a priority worldwide. In this context, it is essential to investigate the role of the tumor microenvironment (TME), as several studies showed that therapy resistance is not only determined by cancer cells, but also is regulated by the TME (Guo et al., 2023). In solid cancers, the TME contains several types of stromal cells. Both in human and in canine mammary cancer, a major component of the tumor stroma are cancer-associated fibroblasts (CAFs), which are known to be involved in the growth, invasion, and chemoresistance of cancer cells (Guo et al., 2023; Kudo et al., 2022). Indeed, CAFs can promote cancer progression by modulating the production of unique cytokines (Michishita, 2020).

A renewed interest in controlling cancer progression involves the use of bacteria, whose ability to selectively target cancer cells has been known for more than a century (Coley, 1891; Coley, 1893; Cook and Hagemann, 2013). More recently, various bacteria (obligate or facultative anaerobic) showed to be able to target cancer cells and to induce regression (Chen et al., 2011). Indeed, both the anoxic environment and the high concentration of nutrients within the necrotic area of the tumor, create a perfect niche for bacterial growth (Broadway and Scharf, 2019). Several studies highlighted the great potentialities of *Salmonella enterica* serovar Typhimurium for cancer therapy (Broadway and Scharf, 2019; Hoffman, 2012).

In this study we selected an attenuated mutant strain of *S. typhimurium* (STMΔznuABC) characterized by a deletion of the whole operon znuABC, coding for the high-affinity zinc transporter (Ammendola et al., 2007), which plays an important role for the virulence of different gram-negative pathogens (Cerasi et al., 2013). The deletion of this virulence factor made it possible to obtain attenuated *Salmonella* strains for vaccination purposes. An induced infection with such attenuated strains proved to be protective against both systemic and enteric salmonellosis in mice and swine, causing a short-term but still immunogenic infection (Pasquali et al., 2008; Gradassi et al., 2013). A more recent study highlighted the great potential of STMΔznuABC for the development of new therapeutic strategies for the treatment of both primary and metastatic cancers (Chirullo et al., 2015). Indeed, the in vivo results obtained up to now show that a subcutaneous administration of low doses of STMΔznuABC in the area close to the tumor determines the reduction of the tumor mass of about 9 times and an increase in life expectancy in immunocompetent tumor-bearing mice (Chirullo et al., 2015). Investigations on the host-pathogen interaction showed that STMΔznuABC stimulate a powerful pro-inflammatory response and the recruitment of many immune cells involved in both innate and adaptive responses. Moreover, it was shown that mutant strains can counteract the immunosuppressive environment generated by the tumor (Chirullo et al., 2015). However, it is not clear whether this activity is tumor- or species-specific and what is the mechanism of bacteria-host-tumor interaction in animal species other than mice and pigs. The aim of the present study was to investigate the potential antitumor effects of STMΔznuABC on canine mammary cancer through in vitro models based on different canine cell lines: a continuous cell line (CF33), characterized in terms of gene expression by pivotal molecules for the innate immune response and cell cycle regulation (Razzuoli et al., 2022), and three primary cell lines (TM51, TM52 and TM53) obtained from the resection of tumors of three animals with mammary cancer. Animals were selected based on the presence of a single mammary mass, not yet metastasized, that was chirurgical resected. The ability of these four different cell lines to respond to STMΔznuABC was investigated to test such bacterial strains' anticancer properties, including their ability to control tumor growth and viability.

2. Materials and methods

2.1. CF33 cell culture

We selected a continuous cell line (CF33) (tumor, IZSLER biobank OIE codex BS TCL 1) previously characterized by our group in terms of gene expression, pivotal molecules in the innate immune response, and cell cycle regulation (Razzuoli et al., 2022). CF33 cells were grown until confluence at 37 °C with Dulbecco's Modified Eagle's medium (DMEM, Euroclone, ECM0101L, Milan, Italy) enriched with 10 % (*v*/v) of fetal bovine serum (FBS, Euroclone, ECS5000L), Penicillin/Streptomycin solution 1 % (Gibco, 15,140,122, Thermofisher scientific) and L-Glutamine 4 mM (Euroclone, ECB3004D, Milan, Italy). Cells were used at 37th, 39th and 42nd passages. For the experiments, 12-well culture plates (2.5×10^5 cells/mL, 2 ml per well) were incubated at 37 °C in 5 % CO_2 until confluence (18–24 h).

2.2. Characterization and isolation of primary cell cultures

2.2.1. Case selection

This study was approved by the animal studies subcommittee of IZSPLV review board (25.05.22). During the tumor resection carried out at the Surgery Section of the Department of Veterinary Medicine, University of Perugia, three animals with mammary cancer were selected and tumors were sampled as described in the following sections.

2.2.2. Mammary tumor (TM) 51

A 10-year-old female, intact, mixed breed dog was presented to the Veterinary Teaching Hospital of the University of Perugia. The owner reported a mass on the left inguinal mammary gland ($5 \times 2 \times 5$ cm). The clinical examination did not show alterations of the clinical parameters but confirmed the presence of a single mammary mass on the left inguinal mammary gland. Clinical staging included cytological examination of the mammary lesion, that revealed cells referable to a complex mammary tumor, with mild atypia. A three-view thoracic X-ray examination was performed, and no detectable pulmonary metastases were seen; complete blood test showed unremarkable results. Considering the TNM system, the tumor was classified as T2N0M0 (Sorenmo, 2003). The dog underwent a caudal unilateral mastectomy. After 2 days of hospitalization, the dog was discharged with no perioperative complications. A 10-days follow-up to assess the surgical wound was programmed; skin sutures were removed 10 days after the surgery and wound healing was complete. The clinical and radiographical restaging after 90 days showed good clinical conditions and no detectable lung metastases.

2.2.3. Mammary tumor (TM) 52

A 9-years-old female, intact, German shepherd was presented to the Veterinary Teaching Hospital of the University of Perugia, for the presence of a mass on the mammary gland. The clinical examination did not show any alterations of the clinical parameters but confirmed the presence of a single mammary mass ($4 \times 2 \times 4$ cm) on the left abdominal mammary gland. Clinical staging included cytological examination of the lesion, that revealed cells referable to a complex mammary tumor. A three-view thoracic X-ray examination was performed, and no detectable pulmonary metastases were seen. Complete blood test showed unremarkable results. Considering the TNM system, the tumor was classified as T3N0M0 (Sorenmo, 2003). The dog underwent a radical, uni-lateral mastectomy, with lymphadenectomy, and concurrent gonadectomy. After 2 days of hospitalization, the dog was discharged from the hospital with no perioperative complications.

A follow-up to assess the surgical wound was programmed after 10 days and skin sutures were removed 15 days after the surgery, when wound healing was complete. A 30-days clinical follow-up assessed the

good clinical conditions of the dog, and after 180 days clinical conditions were good and no detectable lung metastases were present at radiologic examination.

2.2.4. Mammary tumor (TM) 53

A 12-years-old female, intact, mixed breed dog was presented to the Veterinary Teaching Hospital of the University of Turin, for the presence of a mass on the mammary gland. Following the clinical examination, a single mammary mass (3.5 × 2 × 4 cm) was detected on the right inguinal mammary gland. Cytological examination of the lesions revealed cells referable to a mammary tumor.

Pulmonary metastases were not detected through thoracic X-ray; complete blood test showed unremarkable results. Considering the TNM system, the tumor was classified as T2N0M0 (Sorenmo, 2003). The dog underwent unilateral mastectomy with concurrent gonadectomy. At day 2 of hospitalization, the patient was discharged from the hospital with no perioperative complications. Skin sutures were removed 15 days after the surgery, when wound healing was complete.

2.2.5. Isolation and characterization of the primary cell lines

The mammary tissue was collected from the three cases described in Section 2.2.1 and delivered to the laboratory in DMEM supplemented with Penicillin/Streptomycin (P/S) (500 U/mL) at 4 °C.

Primary cell cultures were obtained using tissue explant culture and adhesion method, which allowed to isolate CAFs as described in Vangipuram et al. (2013). Briefly, the biopsy was immersed in sterile saline solution supplemented with penicillin/streptomycin (P/S) (10,000 U/mL) for 10 min and minced into small explants measuring 1 cm^2 in size. After washing three times with saline solution supplemented with P/S (10,000 U/mL), the pieces were cut into smaller ones (2 mm^2 in size). Then, 2–3 biopsy pieces were seeded into each 25 cm^2 cell culture flask and added with 1 mL of pre-warmed complete culture medium consisting of RPMI-1640 (Euroclone, ECM2001L, Milan, Italy) with 2 mM L-glutamine, 20 % FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C for 7 days and sub-cultured as described in Vangipuram et al. (2013) then, cultured cells near confluence were washed with phosphate buffered saline (PBS, home-made) and treated with Trypsin-EDTA (Euroclone ECB3052D) at 37 °C for 5 min. Trypsinization was stopped using RPMI-1640 with 20 % FBS. After this step cells were reseeded to a new culture flask for subculture. The same procedure was repeated continuously for over 20 passages, and the established primary cell lines consisting of CAFs were designated as "TM51", "TM52" and "TM53" cells.

2.2.6. Immunofluorescence

Immunofluorescence was used for the characterization of fibroblasts (Supplementary Fig. S1) and for the purity analysis. The cultured fibroblasts were fixed with 4 % paraformaldehyde (Cell Signalling, 12606S) for 15 min, permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich, Merk, 93,443) for 20 min, and finally blocked with 3 % Bovine Serum Albumin (Sigma-Aldrich, Merk, B6917) for 60 min at room temperature. The cells were incubated with primary antibodies against vimentin (clone V-9; DAKO, M0725, 1:50 dilution), calponin (clone EP798Y, Ventana Roche, 05435684001, ready to use), α-SMA (clone 1A4, Dako, M0851, 1:50 dilution), cytokeratin (clone AE1/AE3, Dako, M3515, 1:50 dilution), and p63 (clone 4A4, Ventana-Roche, 05867061001, ready to use). They were then incubated with a goat anti-mouse IgG Alexa Fluor™ 555 or a goat anti-rabbit IgG Alexa Fluor™ 488 secondary antibody (ThermoFisher Scientific, Milan, Italy; 1:500 dilution) for 30 min."

The slides were mounted in Prolong Gold Antifade Mountant with DAPI (ThermoFisher Scientific, Milan, Italy). Fluorescence images were obtained with a Nexcope NE920 fluorescence microscope (TiesseLab, Milan, Italy). The results were analyzed using MOSAIC Software (v2.4-Tucsen, Fujian, PRC) (Supplementary Fig. S1).

2.2.7. Flow cytometry

Several detachment techniques were tried (i.e. Accutase, Trypsin-EDTA, EDTA 5 %) to obtain a single cell suspension. Differently from TM51 and TM52 that were detachable after an overnight incubation at 4 °C with Accutase, TM53 cells were too tightly attached to the tissue culture plate and only trypsin was able to detach them. So, to obtain comparable results we used Trypsin-EDTA detachment for all the analyzed cell lines. In detail, each cell line was enzymatically detached with Trypsin-EDTA solution w/o phenol red at 37 °C for 5 min, counted and resuspended in PBS w/o Ca + Mg+ +FCS 2 % at 2 × 10^6/mL. Briefly, we prepared three sample tubes for each cell line (each one containing 3 × 10^5 cells) and relative controls (negative and isotype). We added one of the following mAbs: CD44 FITC (clone J.173, IgG1, Beckman Coulter, CA, US), CCR3 PE (clone 5E8, IgG1, Becton Dickinson, NJ, US) and CCR5 PE (clone T218, IgG1, Biolegend, CA, US) to each tube. All tubes were incubated for 30 min at 4 °C. Antibody titration was performed, following the international guidelines (Cossarizza et al., 2021), to determine the optimal concentration. After incubation, samples were washed and then resuspended in 400 μl of PBS + FSC for flow cytometric acquisition. For each sample at least 10,000 events were acquired on a BD LSR Fortessa X20™ Cell Analyzer (BD Bio-sciences, CA, US). FCS files were analyzed using Kaluza Software (v.2.2 - Beckman Coulter, CA, US). A doublet discrimination gate strategy was adopted in order to analyze only single cells (Cossarizza et al., 2021), (Supplementary Fig. S2a).

2.2.8. Basal gene expression

Basal gene expression of 20 genes involved in immune response and cancer aggressivity was evaluated using primer sets already described by our group (Razzuoli et al., 2022). In particular, we checked the expression of the following genes: Transforming growth factor beta (*TGFB1*), Nitric oxide synthase 2 (*iNOS*), Interleukins (*IL6*, *IL8*, *IL10*, *IL15*, *IL18*), C-X-C chemokine receptor type 4 (*CXCR4*), Lymphocyte antigen 96 (MD2), Myeloid differentiation primary response 88 (MyD88), Erb-B2 Receptor Tyrosine Kinase 2 (*ErbB2*), Phosphatase and tensin homolog (*PTEN*), Signal transducer and activator of transcription 5 (*STAT5*), Toll-like receptor 4 and 5 (*TLR4* and *TLR5*), Nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB/p65*), tumor suppressor *p53*, Cluster of Differentiation 14 and 44 (*CD14* and *CD44*) and DNA repair *RAD51*. Ribosomal protein S5 was used as reference gene (Razzuoli et al., 2022). RNA extraction, reverse transcription and RT-qPCR were performed as previously described (Razzuoli et al., 2022).

To evaluate the expression of the basal level in each sample, the relative expression of the selected genes was calculated using the formula 2-ΔΔCq, where ΔΔCq = ΔCq (treated) - ΔCq (control), the ΔCq values were obtained with the formula ΔCq = Cq (target gene) - Cq (reference gene). A Cq value of 39 was used as threshold in PCR tests (samples were considered positive if Cq values <39).

2.3. Response to infective stressors

To evaluate the anti-cancer ability of attenuated *S. typhimurium* (STMΔznuABC) we used an in vitro model developed in our previous studies (Chirullo et al., 2015; Razzuoli et al., 2017). Briefly, STMΔznuABC was grown to obtain a mid-log phase culture and it was resuspended at 10^5 CFU/mL. Hence CF33, TM51, TM52 and TM53 cells were treated with a MOI of 100 CFU/cells for 1 h at 37 °C in 5 % CO_2. Treated cells were used to assess the STMΔznuABC effects through the evaluation of 1) immunomodulation, 2) cells vitality and 3) invasiveness.

2.3.1. Experiment 1: Evaluation of innate immune response modulation by STMΔznuABC

As reported above, cells were treated with STMΔznuABC, incubated at 37 °C in 5 % CO_2 for 1 h and then washed three times with PBS. After

this procedure, cells were resuspended in fresh completed medium only and incubated at 37 °C in 5 % CO_2 for 3 h. The experiment was repeated three times in triplicate; untreated control cells were incubated in complete medium under the same experimental conditions. The gene expression analysis was performed as described in Section 2.2.5.

2.3.2. Experiment 2: Evaluation of STMΔznuABC anti-proliferative activity

Proliferation was evaluated by methylthiazolyldiphenyl-tetrazolium bromide (MTT) test. Briefly, cell lines (CF33, TM51, TM52 and TM53) were seeded on 96-well plates to a density of 1×10^5 cells in 200 µL of RPMI-1640 with 10 % FBS. STMΔznuABC was then diluted in RPMI, added to the tumor cells at a MOI of 100:1 and incubated at 37 °C in 5 % CO_2. After 1 h, the cell cultures were rinsed and incubated in a medium containing gentamycin sulfate (100 µg/mL; Sigma Aldrich) and incubated for 24 h. Cells were then re-suspended in 100 L/well of complete RPMI medium, 10 L/well of MTT solution (TACS MTT Cell Proliferation Assay, TREVIGEN, MD) was added and incubated for 3 h. 100 L of detergent reagent, following the producer's instruction, were added and after 3 h the absorbance at 570 nm was measured. This experiment was repeated three times for each cell line, in triplicate.

2.3.3. Experiment 3: Evaluation of STMΔznuABC invasion

Cells at confluence were infected with 1 mL of bacterial suspension (see Section 2.3) at 10^5 CFU/mL and incubated at 37 °C in 5 % CO_2 for 1 h in agreement with previously described protocols (Cossarizza et al., 2021). Briefly, after STMΔznuABC exposure, cells were washed and treated with PBS containing 300 µg/mL colistin sulphate (Microbiol & C. s.n.c., Cagliari, Italy) to remove all extracellular bacteria. Then, cells were lysed, and the resulting cell suspension was serially diluted and seeded on Xylose Lysine Deoxycholate (XLD; Sigma Aldrich) agar plates and incubated at 37 °C for 24–48 h. Cells treated with medium only were used as negative control. The number of bacteria colonies was evaluated for each cell line. The experiment was performed three times.

2.4. Statistical analyses

For the viability and intracellular colonization tests, the significance threshold was set at $P < 0.05$ (Prism 5, GraphPad Software). As first step we checked Gaussian distributions. To this purpose data were submitted to a Kolmogorov-Smirnov test. Then, significant differences were evaluated by Student's *t*-test. Statistical differences in the gene expression evaluation of STMΔznuABC infection of TM51, TM52, TM53 and CF33 cell lines (Supplementary Fig. S3) were evaluated through the Kruskal-Wallis test.

3. Results

3.1. Primary cell culture isolation and cell characterization

3.1.1. Histopathologic diagnosis of selected cases

TM51. Histologically, the tumor was well-demarcated, multilobulated and moderately cellular. A partial fibrous capsule was present. Neoplastic cells originated from two different populations: luminal epithelium and myoepithelium (Fig. 1a). The luminal epithelium was arranged in monolayered tubules and papillae (Fig. 1b). Cellular and nuclear atypia was mild, and the mitotic count was 1 on 2.37 mm^2 (Meuten et al., 2016). The Myoepithelial cell population was arranged in short bundles and whorls and was frequently embedded in myxoid matrix. In this second population, mild atypia was present, and no mitosis were seen. Therefore, the final histological diagnosis was "complex mammary carcinoma (grade I)" (Goldschmidt et al., 2011; Peña et al., 2013).

TM52. Histologically, the tumor was multilobulated, well-demarcated, focally infiltrative, and densely cellular. A partial and thin capsule was present. Neoplastic cells originated from two different populations: luminal epithelium and Myoepithelium (Fig. 1c). The luminal epithelium showed tubular and focally solid growth (Fig. 1d). Anisocytosis and anisokaryosis were moderateand the mitotic count

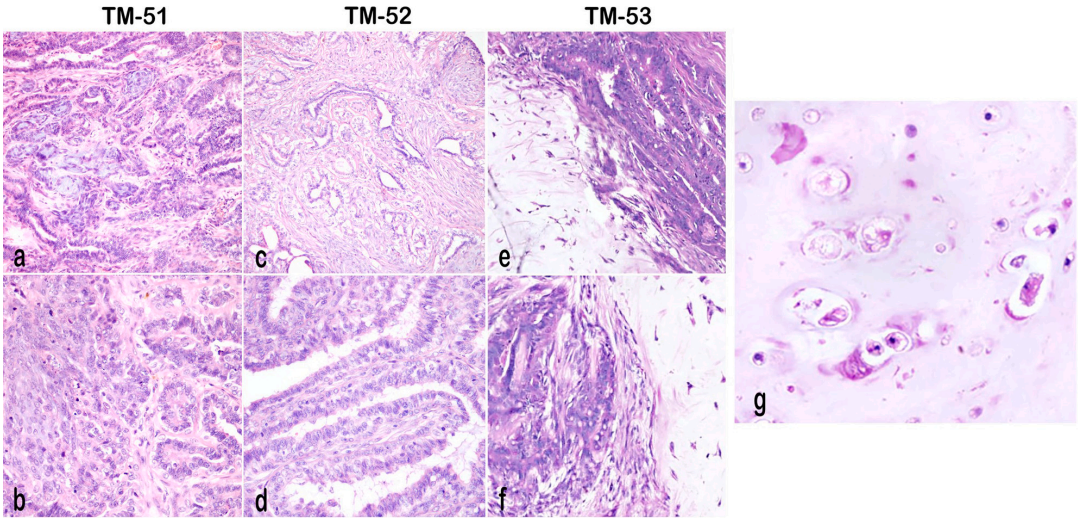

Fig. 1. a) Dog, F, 10 yo, mixed breed. Complex mammary carcinoma (grade I). The tumor was highly cellular and composed of both luminal epithelium and myoepithelium (H&E; 200× magnification). b) Dog, F, 10 yo, mixed breed. Complex mammary carcinoma (grade I). Luminal epithelium was often arranged in multilayer and atypia was prominent. (H&E; 400× magnification). c) Dog, F, 9 years old (yo), German shepherd. Complex mammary adenoma. The tumor showed both a luminal epithelial and a myoepithelial component (Hematoxylin and eosin; 200× magnification). d) Dog, F, 9 yo, German shepherd. Complex mammary adenoma. Luminal epithelium was monolayered, occasionally arranged in papillae and with mild nucleocytoplasmic atypia (H&E; 400× magnification). e) Dog, F, 12 yo, mixed breed. Benign mixed tumor with variable cellularity (H&E; 200× magnification). f) Dog, F, 12 yo, mixed breed. Benign mixed tumor. The luminal epithelium was arranged in tubules, mostly at the periphery of the tumor. (H&E; 400× magnification). g) Example of areas of chondroid differentiation in TM53 (H&E; 400× magnification).

were 6 on 2.37 mm^2 (Meuten et al., 2016). The myoepithelial population was arranged in small whorls and bundles and was frequently associated to a scant myxoid matrix. In this second population, cellular atypia was mild, and no mitosis was seen. The final histological diagnosis was "complex mammary adenoma" (Goldschmidt et al., 2011; Peña et al., 2013).

TM53. Histologically, the tumor was well-demarcated, multi-lobulated and presented a variably dense cellular growth. A partial, thick, fibrous capsule was present. Neoplastic cells were from two different populations: luminal epithelium and myoepithelium (Fig. 1e). The luminal epithelium showed tubular and monolayered growth (Fig. 1f). Anisocytosis and anisokaryosis were mild and the mitotic count was <1 on 2.37 mm^2 (Meuten, 2016). The myoepithelial population was haphazardly admixed with abundant myxoid matrix. In this population, cellular atypia was mild, and no mitosis was seen. Multifocally, areas of chondroid (Fig. 1e, inset) and osseous metaplasia were present. The final histological diagnosis was "benign mixed tumor" (Goldschmidt et al., 2011).

3.1.2. Isolation and characterization of the primary cell lines

We obtained three different cell lines from three different mammary cancers: TM51 (complex carcinoma grade I), TM52 (complex mammary adenoma), TM53 (benign mixed tumor) (see Section 2.2). TM51 showed a fibroblastic-like morphology and was positive for the mesenchymal marker vimentin, the myofibroblast marker α-SMA, and calponin, a regulatory protein associated with actin filament. TM 51 was negative for p63 and the epithelial marker cytokeratin (CK) (Supplementary Fig. S1). Additionally, this cell line showed the expression of CD44 surface marker, CD193 (CCR3) and CD195 (CCR5) (Supplementary Fig. S2b). TM52, obtained from a complex mammary adenoma, also showed a fibroblastic-like morphology (Supplementary Fig. S1) and was positive for calponin, vimentin, and α-SMA, but negative for p63 and CK. Moreover, it showed expression of CD44 surface marker and was negative for CD193 (CCR3) and CD195 (CCR5) (Supplementary Fig. S2b). TM53, obtained from mixed breast cancer (benign), also showed a fibroblastic-like morphology and was positive for calponin, vimentin, and α-SMA, but negative for p63 and CK (Supplementary Fig. S1). Furthermore, it was positive for CD44, and negative for CD193 (CCR3) and CD195 (CCR5) (Supplementary Fig. S2b).

3.1.3. Basal gene expression

TM51. In TM51 we highlighted the expression of all genes under study with the exception of *CXCR4* (Supplementary Table S1). *CD44*, *ErbB2*, *MD2*, *IL6*, *CXCL8*, *IL15*, *iNOS*, *MYD88*, *PTEN*, *NF-kB/p65*, *P53*, *STAT5*, *RAD51*, *TGFB1* and *TLR5* expression was observed in all tested samples. The other genes, *IL10*, *IL18*, *CD14* and TLR4, were expressed in 66.7 %, 83.3 %, 33.3 % and 66.7 % of the samples, respectively.

TM52. All target genes were found to be expressed in TM52 except for *IL10*, *IL15* and *TLR4* (Supplementary Table S1). *CD44*, *CD14*, *ErbB2*, *MD2*, *CXCL8*, *CXCR4*, *NF-kB/p65*, *P53*, *RAD51*, *PTEN*, *STAT5*, *TLR5* and *TGFB1* expression was observed in all tested samples, while *IL6*, *IL18*, *MYD88* and *iNOS* were expressed in 83.3 %, 16.7 %, 83.3 % and 33.3 % of the samples, respectively.

TM53. In TM53 all genes were expressed with the exception for *IL10*, *IL18* and *TLR4* (Supplementary Table S1). *CD44*, *MYD88*, *CD14*, *ErbB2*, *MD2*, *IL6*, *RAD51*, *STAT5*, *CXCL8*, *CXCR4*, *PTEN*, *NF-kB/p65*, *P53*, *TGFB1* and *TLR5* expression was observed in all tested samples. The remaining genes, *IL15* and *iNOS*, were expressed in 83.3 % and 33.3 % of the samples, respectively.

3.2. Interaction between STMΔznuABC and breast cancer cell lines

3.2.1. CF33

STMΔznuABC penetrated the CF33 cells (Log 2 10.6 ± 2.7) causing a significant ($P = 0.0022$) reduction of cell viability (− 40 %) (Fig. 2a, b). Moreover, STMΔznuABC penetration caused an increase of *IL6* ($P < 0.0001$), *CXCL8* ($P < 0.0001$) and *P53* ($P = 0.0022$) gene expression, while *MYD88* ($P = 0.0242$), *MD2* ($P = 0.025$), *IL18* ($P = 0.0041$), *TLR4*, *TLR5* ($P = 0.0002$) and *TGFB1* ($P = 0.0012$) expression was decreased (Supplementary Fig. S3). The expression of the other genes under study was not significantly modulated.

3.2.2. TM51

STMΔznuABC penetrated the TM51 cells (Log2 33.4 ± 10.3) (Fig. 3) causing a significant ($P = 0.0032$) reduction in cell viability (Fig. 4). Moreover, STMΔznuABC infection caused down-regulation of *CXCL8* ($P = 0.0104$), *CD44* ($P = 0.0039$) and *ErB2* ($P = 0.0087$) gene expression (Fig. 5). Other genes under study were not significantly modulated.

3.2.3. TM52

STMΔznuABC penetrated the TM52 cells (Log2 32.2 ± 7.1) (Fig. 3) causing a significant ($P = 0.0047$) reduction in cell viability (Fig. 4). Moreover, STMΔznuABC penetration caused increase of *CXCL8* ($P = 0.0152$), *CD44* ($P = 0.0043$) and *MD2* ($P = 0.022$) gene expression, while *PTEN* ($P = 0.0260$) expression was decreased (Fig. 5). Other genes under study were not significantly modulated.

3.2.4. TM53

STMΔznuABC penetrated TM53 cell line (Log2 29.8 ± 08.5) (Fig. 3) causing a significant ($P = 0.0005$) reduction in cell viability (Fig. 4). Moreover, STMΔznuABC infection caused up-regulation of *CXCL8* ($P = 0.0022$), *IL18* ($P = 0.0260$), *IL10* ($P = 0.0022$), *TLR4* ($P = 0.0022$) and *RAD51* ($P = 0.0411$) gene expression, while *CD14* ($P = 0.0022$), *IL6* ($P = 0.0022$), *CXCR4* ($P = 0.0260$), *P53* ($P = 0.043$), *PTEN* ($P = 0.0087$), *STAT5* ($P = 0.0087$), *TLR5* ($P = 0.0022$) and *TGFB1* ($P = 0.0087$) were down-regulated (Fig. 5). Other genes under study were not significantly modulated.

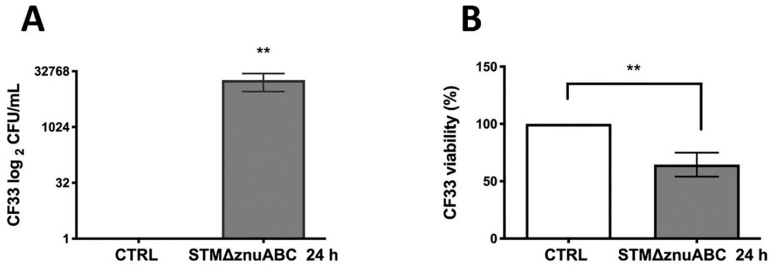

Fig. 2. (A) Intracellular colonization of STMΔznuABC on CF33 cells at 24 h compared to CTRL (control, untreated). Data are expressed as number of CFU/mL represented in log2 scale. Differences were evaluated through Student's *t*-test. (B) Viability of CF33 cells after STMΔznuABC exposure at 24 h. Data are expressed as percentage (%) of viable cells. Differences were evaluated through Student's t-test. Data of both Fig. A and B are expressed as mean of three independent experiments +/-SD. Asterisks represent the statistical significance: $^{**}p < 0{,}01$.

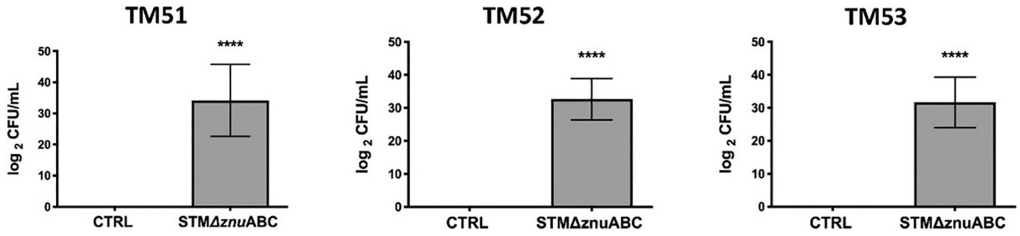

Fig. 3. Intracellular colonization of STMΔznuABC on TM51, TM52, TM53 cells at 24 h compared to untreated ones. Data are expressed as number of CFU/mL represented in log2 scale. Differences were evaluated through Student's t-test. Data are expressed as mean of three independent experiments +/-SD. Asterisks represent the statistical significance: ****p < 0,0001.

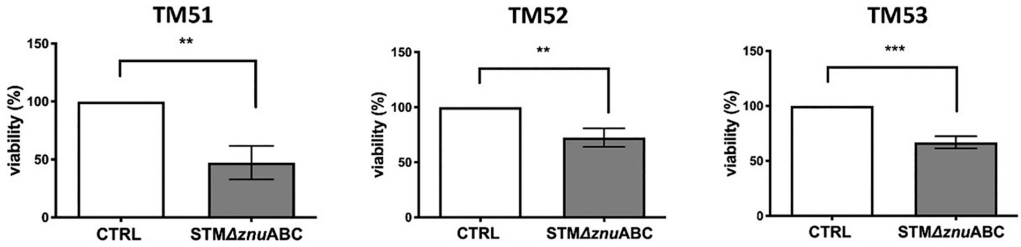

Fig. 4. Viability of TM51, TM52, TM53 cells after STMΔznuABC exposure at 24 h. Data are expressed as percentage (%) of viable cells. Differences were evaluated through Student's t-test. Data are ex-pressed as mean of three independent experiments +/-SD. Asterisks represent the statistical significance: **p < 0,01 and *** p < 0,001.

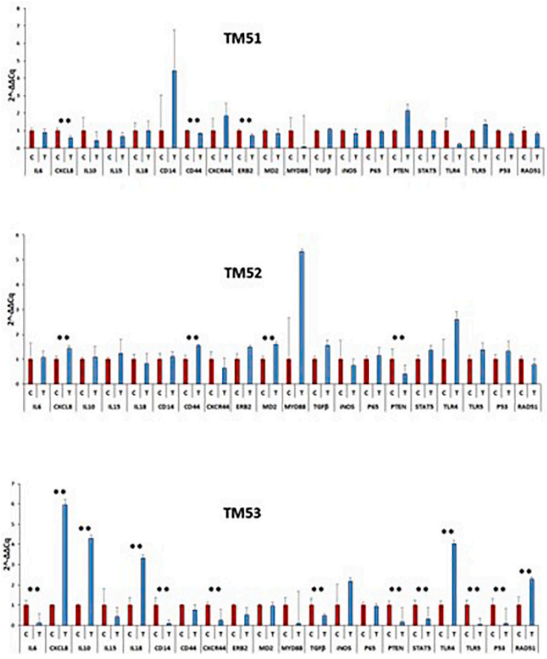

Fig. 5. Evaluation of STMΔznuABC infection on TM51, TM52 and TM53 cells gene expression (blue). Untreated cells were used as control (red). Significant differences are reported with respect to control. Differences were evaluated through the Kruskal-Wallis test. The asterisks indicate the statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4. Discussion

We focused this study on canine mammary tumors (CMTs), which are the most common neoplasms occurring in dogs and are malignant in about 50 % of the cases (Salas et al., 2015). Recent studies have shown an increase in malignant vs. benign tumors over the last years. A similar trend was observed in human medicine (Valdivia et al., 2021). To date, the gold standard therapy for treatment and control of CMTs is surgery; the objective is to remove the tumor and prevent the recurrence in the remaining glands (Sorenmo, 2003). To lower this risk, an additional treatment (adjuvant therapy) can be considered after surgery. Adjuvant therapy may include chemotherapy, radiotherapy, and individualized therapy. However, at least in veterinary medicine, routine adjuvant therapies have a low success rate, thus there is a great need for new advanced therapies. In recent years, many studies, both in human and in veterinary cancer research, are trying to find strategies which act to modify the TME (Elwakeel and Weigert, 2021; Kudo et al., 2022; Schoenfeld and Hellmann, 2020). The development of new therapies targeting TME could turn out to be important also for the long-term management of breast cancer. CAFs are an essential component of TME. Since recent findings outlined the importance CAFs in cancer development, progression, and therapy resistance (Amornsupak et al., 2014; Boesch et al., 2018; Czekay et al., 2022; Glabman et al., 2022; Hu et al., 2022; Schoenfeld and Hellmann, 2020; Zhao et al., 2017), in this study we tried to isolate CAFs from canine mammary tumors with the goal of improving our knowledge on their biology and on the way it could be modulated.The positivite expression of vimentin and the downregulation of cytokeratin in the three cell lines confirm their mesenchymal origin. Additionally, the high expression of α-SMA and calponin markers suggests that these cells are myofibroblast-like CAFs (myCAFs) (Li et al., 2023; Yang et al., 2023). The high expression of CD44 was indicative of their stemness (Kinugasa et al., 2014). Neoplastic epithelial cells in canine mammary tumors express CD44, and myoepithelial components (Rogez et al., 2019). Cell cultures have been isolated using a complex mammary carcinoma, grade 1 (TM51), a

complex mammary adenoma (TM52), and a benign mixed tumor (TM53) and we observed a different expression of CCR3 and CCR5. CCR3 and CCR5 are observed in breast carcinoma cells, while CCL5 as ligand is expressed in various types of stromal cells, including fibroblasts in several types of cancer including breast cancer (Yamaguchi et al., 2021).

The anti-cancer activity of STMΔznuABC, which reduces cancer cells' proliferation, was already tested by the authors on several human and murine cell lines (es: 4 T1, SiHa and TC1) (Chirullo et al., 2015), but no data were available for canine mammary cancers. Besides the three primary canine cell lines (TM51, 52, 53), in this study we also used CF33 canine tumor cell line, due to its retainment of mammary cancer parameters in gene expressions and to its ability to interact with *S. typhimurium*, as clearly demonstrated by our group in a previous work (Razzuoli et al., 2022). In the present study we investigated the modulation by STMΔznuABC on markers essential for bacterium-cell interactions, as well as the expression of some TLRs. We specifically focused the attention on TLR4 and TLR5 that, together with MD2 and CD14, are expressed by many cell types and have a pivotal role in bacterial recognition. Moreover, their interaction with bacteria can trigger an innate immune response (Miyake, 2003) characterized by an inflammatory response, including the secretion of cytokines and chemokines. The expression in all samples of *NF-kB/p65*, one of the major members of the NF-kB protein family, along with *MYD88*, *CXCL8* and *IL-6*, implies the ability of this cell line to trigger an inflammatory response. Indeed, treatment with STMΔznuABC determined an increase of some important proinflammatory cytokines and chemokines (IL8 and IL18), demonstrating the activation of an inflammatory response. In line with our previous findings (Razzuoli et al., 2022), the expression of these molecules shows that the treatment of CF33 with bacteria induced IL8 expression, which allowed STMΔznuABC to penetrate the cells, demonstrating once again the ability of this cell line to interact with bacterial activity. Moreover, we also proved, through an in vitro treatment, the ability of STMΔznuABC to significantly penetrate in canine CAFs 24 h post-treatment and to induce tumor cell cytotoxicity, as demonstrated by a significant reduction of cells viability compared to untreated cells. Our results indicated that STMΔznuABC had a direct action on CAFs viability, leading to a significant reduction in the number of viable cells compared to the untreated controls (Fig. 4).

Our results confirmed, on a model which had never been studied before, the potential of STMΔznuABC in contrasting tumor cells proliferation, highlighting once again their ability to influence cell lines of various embryologic nature and deriving from different animal species. These data suggest that STMΔznuABC could be a promising candidate for unconventional anti-cancer treatment also in dogs. Indeed, STMΔznuABC was already shown to significantly penetrate and reduce the viability of many different tumors both in vivo and in vitro models (es: murine mammary adenocarcinoma 4 T1, SiHa and TC1 cells, grade II of human squamous carcinoma cell of cervix and mouse lung tumor cell line co-transformed with human papillomavirus 16 E6/E7 and c-Ha-Ras cells) (Chirullo et al., 2015). Our data highlight a different interaction between STMΔznuABC and the three different cancer cell lines in terms of gene expression. This phenomenon should be studied in depth since the increase of *CXCL8*, and *IL18* in TM53 could indicate a recruitment of monocytes, macrophages, NK cells and dendritic cells to promote recruitment of effector CD8+ T cells to the TME, suggesting the benefit and value of knowing the type of tumor before choosing a therapy.

5. Conclusions

This study demonstrated the anti-proliferative and immunomodulatory activity of STMΔznuABC in canine CMTs, expanding the knowledge on tumor-host interactions. We outline the ability of this microorganism to cause a reduction in CAFs replication. This is an important finding considering CAFs involvement in mammary cancer progression and therapy response. This suggests that the STMΔznuABC may represent a promising tool for innovative anti-cancer therapies that could be further optimized and developed. Moreover, alternative in vitro models to animal testing are needed to overcome the drawbacks associated with animal experiments and avoid un-ethical procedures, in line with the 3Rs strategy. Indeed, besides the major concern of ethics, there are other disadvantages of animal experimentation, such as requirement of skilled manpower, time consuming protocols and high cost.

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CRediT authorship contribution statement

Barbara Chirullo: Writing - review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Floriana Fruscione:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Genny Del Zotto:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Filippo Dell'Anno:** Writing – review & editing, Formal analysis, Data curation. **Michela Tarantino:** Writing – review & editing, Methodology. **Ilaria Porcellato:** Writing – review & editing, Formal analysis. **Paola Petrucci:** Writing – review & editing, Formal analysis. **Chiara Grazia De Ciucis:** Writing – review & editing, Methodology, Formal analysis. **Antonello Bufalari:** Writing – review & editing, Visualization, Resources. **Lisa Guardone:** Writing – review & editing, Writing – original draft, Visualization. **Katia Cappelli:** Writing – review & editing, Resources. **Giulia Moretti:** Writing – review & editing, Investigation. **Samanta Mecocci:** Writing – review & editing, Formal analysis. **Eleonora Monti:** Writing – review & editing, Investigation. **Livia De Paolis:** Writing – original draft, Methodology, Data curation. **Elisabetta Razzuoli:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors have no competing interests to declare. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105438.

References

Abdelmegeed, S.M., Mohammed, S., 2018. Canine mammary tumors as a model for human disease. Oncol. Lett. 15 (6), 8195–8205.

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., Battistoni, A., 2007. High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of Salmonella enterica. Infect. Immun. 75 (12), 5867–5876.

Amornsupak, K., Insawang, T., Thuwajit, P., O-Charoenrat, P., Eccles, S.A., Thuwajit, C., 2014. Cancer-associated fibroblasts induce high mobility group box 1 and contribute to resistance to doxorubicin in breast cancer cells. BMC Cancer 14 (1), 955.

Boesch, M., Onder, L., Cheng, H.-W., Novkovic, M., Mörbe, U., Sopper, S., Gastl, G., Jochum, W., Ruhstaller, T., Knauer, M., Ludewig, B., 2018. Interleukin 7-expressing fibroblasts promote breast cancer growth through sustenance of tumor cell stemness. Oncoimmunology 7 (4), e1414129.
Broadway, K.M., Scharf, B.E., 2019. *Salmonella typhimurium* as an anticancer therapy: recent advances and perspectives. Curr. Clin. Micro. Rpt. 6 (4), 225–239.
Cerasi, M., Ammendola, S., Battistoni, A., 2013. Competition for zinc binding in the host-pathogen interaction. Front. Cell. Infect. Microbiol. 3, 108.
Chen, J., Wei, D., Zhuang, H., Qiao, Y., Tang, B., Zhang, X., Wei, J., Fang, S., Chen, G., Du, P., Huang, X., Jiang, W., Hu, Q., Hua, Z.C., 2011. Proteomic screening of anaerobically regulated promoters from Salmonella and its antitumor ap-plications. Mol. Cell. Proteomics 10 (6), M111.009399.
Chirullo, B., Ammendola, S., Leonardi, L., Falcini, R., Petrucci, P., Pistoia, C., Vendetti, S., Battistoni, A., Pasquali, P., 2015. Attenuated mutant strain of *Salmonella typhimurium* lacking the ZnuABC transporter contrasts tumor growth promoting anti-cancer immune response. Oncotarget 6 (19), 17648–17660.
Coley, W.B., 1891. II. Contribution to the knowledge of sarcoma. Ann. Surg. 14 (3), 199–220.
Coley, W.B., 1893. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. Am. J. Med. Sci. (1827-1924) 105 (6), 487.
Cook, J., Hagemann, T., 2013. Tumour-associated macrophages and cancer. COPHAR 13 (4), 595–601.
Cossarizza, A., Chang, H.D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., Andrä, I., Andreata, F., Annunziato, F., Arranz, E., Bacher, P., Bari, S., Barnaba, V., Barros-Martins, J., Baumjohann, D., Beccaria, C.G., Bernardo, D., Boardman, D.A., Borger, J., Yang, J., 2021. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). Eur. J. Immunol. 51 (12), 2708–3145.
Czekay, R.P., Cheon, D.J., Samarakoon, R., Kutz, S.M., Higgins, P.J., 2022. Cancer-associated fibroblasts: mechanisms of tumor progression and novel therapeutic targets. Cancers 14 (5), 1231.
Elwakeel, E., Weigert, A., 2021. Breast Cancer CAFs: Spectrum of phenotypes and promising targeting avenues. Int. J. Mol. Sci. 22 (21), 11636.
Glabman, R.A., Choyke, P.L., Sato, N., 2022. Cancer-associated fibroblasts: Tumorigenicity and targeting for Cancer therapy. Cancers 14 (16), 3906.
Goldschmidt, M., Peña, L., Rasotto, R., Zappulli, V., 2011. Classification and grading of canine mammary tumors. Vet. Pathol. 48 (1), 117–131.
Gradassi, M., Pesciaroli, M., Martinelli, N., Ruggeri, J., Petrucci, P., Hassan, W.H., Raffatellu, M., Scaglione, F.E., Ammendola, S., Battistoni, A., Alborali, G.L., Pasquali, P., 2013. Attenuated Salmonella enterica serovar typhimurium lacking the ZnuABC transporter: an efficacious orally-administered mucosal vaccine against salmonellosis in pigs. Vaccine 31 (36), 3695–3701.
Guo, Z., Zhang, H., Fu, Y., Kuang, J., Zhao, B., Zhang, L., Lin, J., Lin, S., Wu, D., Xie, G., 2023. Cancer-associated fibroblasts induce growth and radioresistance of breast cancer cells through paracrine IL-6. Cell Death Discov. 9 (1), 6.
Hoffman, R.M., 2012. The preclinical discovery of bacterial therapy for the treatment of metastatic cancer with unique advantages. Expert Opin. Drug Discovery 7 (1), 73–83.
Hu, D., Li, Z., Zheng, B., Lin, X., Pan, Y., Gong, P., Zhuo, W., Hu, Y., Chen, C., Chen, L., Zhou, J., Wang, L., 2022. Cancer-associated fibroblasts in breast cancer: challenges and opportunities. Cancer Commun. 42 (5), 401–434.
Kinugasa, Y., Matsui, T., Takakura, N., 2014. CD44 expressed on cancer-associated fibroblasts is a functional molecule supporting the stemness and drug resistance of malignant cancer cells in the tumor microenvironment. Stem Cells 32 (1), 145–156.
Kudo, A., Yoshimoto, S., Yoshida, H., Izumi, Y., Takagi, S., 2022. Biological features of canine cancer-associated fibroblasts and their influence on cancer cell invasion. J. Vet. Med. Sci. 84 (6), 784–791.
Li, Y., Wang, C., Huang, T., Yu, X., Tian, B., 2023. The role of cancer-associated fibroblasts in breast cancer metastasis. Front. Oncol. 13, 1194835.
Meuten, D.J., 2016. Tumors in Domestic Animal, 5Th edition. Wiley-Blackwell.
Meuten, D.J., Moore, F.M., George, J.W., 2016. Mitotic count and the field of view area: time to standardize. Vet. Pathol. 53 (1), 7–9.
Michishita, M., 2020. Understanding of tumourigenesis in canine mammary tumours based on cancer stem cell research. Vet. J. 265, 105560.
Miyake, K., 2003. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. Int. Immunopharmacol. 3 (1), 119–128.
Oh, J.H., Cho, J.Y., 2023. Comparative oncology: overcoming human cancer through companion animal studies. Exp. Mol. Med. 55 (4), 725–734.
Pasquali, P., Ammendola, S., Pistoia, C., Petrucci, P., Tarantino, M., da Silva Valente, C., Marenzoni, M.L., Rotilio, G., Battistoni, A., 2008. Attenuated *Salmonella enterica* serovar typhimurium lacking the ZnuABC transporter confers immune-based protection against challenge infections in mice. Vaccine 26, 3421–3426.
Peña, L., De Andrés, P.J., Clemente, M., Cuesta, P., Pérez-Alenza, M.D., 2013. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Vet. Pathol. 50 (1), 94–105.
Razzuoli, E., Amadori, M., Lazzara, F., Bilato, D., Ferraris, M., Vito, G., Ferrari, A., 2017. *Salmonella* serovar-specific interaction with jejunal epithelial cells. Vet. Microbiol. 207, 219–225.
Razzuoli, E., De Ciucis, C.G., Chirullo, B., Varello, K., Zoccola, R., Guardone, L., Petrucci, P., Rubini, D., Bozzetta, E., Goria, M., Fruscione, F., Modesto, P., 2022. Molecular characterization of CF33 canine cell line and evaluation of its ability to respond against infective stressors in sight of anticancer approaches. Vet. Sci. 9 (10), 543.
Rogez, B., Pascal, Q., Bobillier, A., Machuron, F., Lagadec, C., Tierny, D., Le Bourhis, X., Chopin, V., 2019. CD44 and CD24 expression and prognostic significance in canine mammary tumors. Vet. Pathol. 56 (3), 377–388.
Rowell, J.L., McCarthy, D.O., Alvarez, C.E., 2011. Dog models of naturally occurring cancer. Trends Mol. Med. 17 (7), 380–388.
Salas, Y., Márquez, A., Diaz, D., Romero, L., 2015. Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: A growing animal health problem. PloS One 10 (5), e0127381.
Schiffman, J.D., Breen, M., 2015. Comparative oncology: what dogs and other species can teach us about humans with cancer. Philos. Trans. R. Soc. Lond. B Biol. Sci. 370 (1673), 20140231.
Schoenfeld, A.J., Hellmann, M.D., 2020. Acquired resistance to immune checkpoint inhibitors. Cancer Cells 37 (4), 443–455.
Simpson, S., Dunning, M.D., de Brot, S., Grau-Roma, L., Mongan, N.P., Rutland, C.S., 2017. Comparative review of human and canine osteosarcoma: morphology, epidemiology, prognosis, treatment and genetics. Acta Vet. Scand. 59 (1), 71.
Sorenmo, K., 2003. Canine mammary gland tumors. Vet. Clin. North Am. Small Anim. Pract. 33 (3), 573–596.
Tarone, L., Barutello, G., Iussich, S., Giacobino, D., Quaglino, E., Buracco, P., Cavallo, F., Riccardo, F., 2019. Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy. Cancer Immunol. Immunother. 68 (11), 1839–1853.
Valdivia, G., Alonso-Diez, Á., Pérez-Alenza, D., Peña, L., 2021. From conventional to precision therapy in canine mammary cancer: a comprehensive review. Front. Vet. Sci. 8, 623800.
Vangipuram, M., Ting, D., Kim, S., Diaz, R., Schüle, B., 2013. Skin punch biopsy explant culture for derivation of primary human fibroblasts. JoVE 77, e3779.
Yamaguchi, M., Takagi, K., Narita, K., Miki, Y., Onodera, Y., Miyashita, M., Sasano, H., Suzuki, T., 2021. Stromal CCL5 promotes breast Cancer progression by interacting with CCR3 in tumor cells. Int. J. Mol. Sci. 22 (4), 1918.
Yang, D., Liu, J., Qian, H., Zhuang, Q., 2023. Cancer-associated fibroblasts: from basic science to anticancer therapy. Exp. Mol. Med. 55 (7), 1322–1332.
Zhao, X.L., Lin, Y., Jiang, J., Tang, Z., Yang, S., Lu, L., Liang, Y., Liu, X., Tan, J., Hu, X.G., Niu, Q., Fu, W.J., Yan, Z.X., Guo, D.Y., Ping, Y.F., Wang, J.M., Zhang, X., Kung, H.F., Bian, X.W., Yao, X.H., 2017. High-mobility group box 1 released by autophagic cancer-associated fibroblasts maintains the stemness of luminal breast cancer cells. J. Pathol. 243 (3), 376–389.

4. Discussion

Despite the high incidence of mammary gland tumors MGTs in dogs, to date the outcomes for dogs with mammary carcinoma remain unsatisfactory.

Analysis of both recent and past studies reveals a lack of standardized protocols in canine mammary cancer management, along with a delay in scientific research on targeted therapies that have been extensively studied and tested in human oncology (Frénel & Nguyen, 2023).

Given the current uncertainty regarding the best therapeutic options, it becomes crucial to emphasize prevention and proper management, particularly during the initial diagnostic and therapeutic stages. By focusing on early intervention, some of the challenges posed by the absence of established therapeutic guidelines could be mitigated and the overall approach to treating mammary carcinoma in dogs could be improved.

From the outset, veterinarians should be prepared to manage those primary risk factors that contribute most to the development of MGTs.

Although the exposition to ovarian hormonal environment plays a key role in the process (Vazquez et al., 2023), given the current knowledge, it is recommended not to spay dogs prophylactically but rather to implement a proper cancer screening program (Hart et al., 2020; Pena et al., 2013; Rodríguez et al., 2022; Sorenmo et al., 2000). This screening should be tailored to the individual dog's characteristics, beginning at 4 to 7 years of age according to breed, size and spay status (Rafalko et al., 2023). Owners must be aware that early intervention leads to better outcomes. To this end, it would be beneficial to educate dog owners on how to regularly palpate their dog's mammary tissue, enabling them to identify any suspicious lesions and promptly contact their veterinarian.

Once a mammary nodule has been detected, no steps in the diagnostic process should be overlooked because each of them is essential for obtaining information that guides therapeutic decisions. Staging is mandatory before initiating therapy when managing a cancer-bearing patient, not only because it is ethically correct for the patient, but also because the stage will significantly influence the prognosis and the types of treatments available (Sorenmo et al., 2020). Owners must always be informed and

guided throughout this process; as veterinarians, it is our duty to provide them with all our knowledge, explain the potentials and limitations of the therapies, so to empower them to make informed decisions.

Regarding screening imaging diagnostics, radiology is unable to detect nodules smaller than 4 mm. However, although CT scans have a higher sensitivity for detecting distant metastases as small as 1 mm (Barbagianni & Gouletsou, 2023), subjecting dogs to CT always as the initial screening test should be considered as "overstaging". Furthermore, CT is still not available in all veterinary facilities and results in higher costs for pet owners (Sorenmo et al., 2020).

A good practice would be to recommend a CT scan if multiple negative clinical prognostic factors are recognized or metastases are detected during the histopathological examination of excised SLNs, as this raises suspicion of distant metastases.

The introduction of the SLN concept marks a significant progress in veterinary medicine, as it helps avoid the unnecessary removal of non-draining lymph nodes (LNs).

Considering that the positivity of a LN on mapping significantly alters the surgical treatment, recently studies have been conducted to evaluate the prognostic value of this technique through CT and ultrasonographic assessments of sentinel LNs (Soultani et al. 2017; Stan et al. 2020).

According to Soultani and colleagues (2017), the size and shape of sentinel LNs as observed in CT indirect lymphography, did not show accuracy in the prediction of metastasis; on the contrary, a low degree of iodinated contrast enhancement, was significantly associated with the presence of metastasis, as demonstrated through the histopathological examination.

Regarding ultrasonographic assessment, in 2020 Stan and colleagues created an ultrasound examination algorithm based on B-mode, Doppler technique, contrast-enhanced ultrasound and elastography sentinel LNs assessment, achieving a 92,2% accuracy of the method in detecting metastasis, which presence was confirmed through histopathological examination. According to the authors, a single ultrasound method would not have been sufficient to diagnose the presence of metastases (Stan et al., 2020).

Regarding the extent of MGTs resection, there is a clear difference in the approach between humans and dogs. In humans, there is a trend toward breast-conserving surgery (BCS) whenever possible, emphasizing on preserving quality of life and cosmetic outcomes. In Western Europe, 60-80% of newly diagnosed breast cancers are likely to be treated with BCS, along with adjuvant therapies such as radiotherapy and endocrine therapy, particularly for early-stage cancers (tumor ≤2 cm, negative nodes and less aggressive histotype). In case of tumor >2 cm, positive node or aggressive histotype, more intensive protocols are recommended, including neoadjuvant systemic therapy, mastectomy with breast reconstruction, postoperative chemotherapy and targeted therapy (Frénel & Nguyen, 2023). In dogs, surgical resection is less focused on cosmetic considerations and tends to be more aggressive, especially in case of multiple nodules.

Certainly, one of the reasons behind the extensive surgical resection in dogs is because they have five pairs of mammary glands, which are interconnected under normal conditions by numerous vascular and lymphatic structures (Ferreira et al., 2023). However, in cancer-bearing bitches, lymphatic connections between glands may further increase and change, making the spread of metastatic cells unpredictable.

On the other hand, no treatment plans are specifically tailored to systematically defined cancer stages based on tumor size, node involvement and medical imaging for distant metastases (Barbagianni & Gouletsou, 2023; Chocteau et al., 2019), although an evaluation system has recently been proposed to create bio-scores, aimed at identifying high-risk canine patients (Sorenmo et al., 2019).

For these reasons, the tendency to opt for more radical surgeries in dogs is justified, as it helps minimize the risk of local recurrence or the development of new tumors in adjacent, non-excised mammary glands (Stratmann et al., 2008).

Nevertheless, performing unnecessary radical prophylactic surgeries is discouraged because they are associated with more pain, stress and postoperative complications (Evans et al., 2021; Horta et al., 2015).

Therefore, the appropriate approach would be to plan the extent of surgical resection based on the number and size of the nodules, their location and the LNs identified through preoperative mapping and imaging techniques, starting with the most conservative surgery possible. The patient should then undergo frequent postoperative checks every 2-3 months for at least 24 months to identify local recurrences, new tumors or the emergence of metastases (Matos et al., 2012). In case of recurrence or new tumors development, a re-staging and eventually a new resection should be performed.

The limitation in veterinary practice is that the owner may not understand or accept a new surgery, either for emotional attachment and anxiety or economic reasons. Thus, it is essential to always discuss the potential scenarios with the owner before making any decisions regarding the management of cancer-bearing dogs. If the veterinarian forces the client to make decisions, he is likely to be held accountable if the outcome is unsatisfactory, even if the treatment was the best option. On the other hand, leaving the decision entirely to the client can cause confusion and lead to suboptimal outcomes. The veterinarian should not only provide information about diagnoses and treatment options but also gather the client's preferences and concerns, including financial considerations. Together, they can discuss the pros and cons and reach a joint decision, fostering shared responsibility for the treatment outcome (Knesl et al., 2016).

Unfortunately, there are several limitations in veterinary medicine. Veterinarians frequently find themselves reaching a compromise with owners during treatment, whether due to high costs, lengthy treatment durations or the lack of availability of certain methods in specific regions. As a result, the treatments provided do not always reflect what would be ideal for the animal or its condition. For this reason, one of the primary goals of veterinary oncology is the pursuit of new therapies that are more accessible, affordable and easier to administer.

New treatments have been developed for women with breast cancer, focusing on targeted and personalized molecular therapies. The reason is because standard treatments are not effective for all patients and are related to several side effects, especially when considering chemotherapy, which attacks both cancerous and healthy cells (Nosalova et al., 2024; Vazquez et al., 2023).

Targeted therapies aim to focus specifically on cancer cells, reducing damage to healthy tissue and minimizing side effects, and are designed based on the specific molecular characteristics of the tumor, increasing the chances of treatment success. Additionally, since all tumors exhibit different genetic and molecular profiles, personalized therapies ensure treatment plans are tailored to the specific biology of the patient's cancer (Nosalova et al., 2024).

In veterinary oncology, an example of targeted therapy is tyrosine kinase inhibitors (TKIs), which specifically aim to block certain cellular signaling pathways involved in tumor proliferation and growth.

One of the most used drugs is toceranib (marketed as Palladia®), primarily prescribed for the treatment of tumors in dogs, particularly mast cell tumors, for which it is specifically approved (Valdivia et al., 2021).

Although current research is primarily focused on in vitro studies or murine tumor models, immunotherapy holds promise as a treatment for dogs with mammary carcinoma. During tumor development, tumor cells become capable to evade host’s immune system through several mechanisms. Immunotherapy uses the body’s immune system to fight cancer by boosting or modulating the immune system's natural ability to recognize and destroy cancer cells (Valdivia et al., 2021).

Among the various types of immunotherapy are: immune checkpoint inhibitors, which block proteins that suppress immune responses; chimeric antigen receptor (CAR) T-cell therapy, where T-cells are engineered to better recognize and attack cancer cells; cancer vaccines, designed to stimulate the immune system to target specific cancer antigens; monoclonal antibodies, laboratory-made proteins that bind to cancer cells, flagging them for immune attack; oncolytic virus therapy, which uses genetically modified viruses to infect and destroy cancer cells while activating an anti-tumor immune response (Nosalova et al., 2024; Valdivia et al., 2021).

While immunotherapy for canine cancers is still developing, it holds significant potential, particularly for difficult-to-treat cancers like melanoma, lymphoma, osteosarcoma and mast cell tumors (Pellin et al., 2022; Valdivia et al., 2021).

Currently there are no commercially available vaccines specifically designed to prevent or treat canine mammary cancer (CMC). However, research is ongoing to explore the development of therapeutic cancer vaccines that could target specific tumor antigens and stimulate an immune response against mammary cancer cells in dogs (Valdivia et al., 2021).

Among vaccine technologies, two prominent approaches include cellular immunotherapy and DNA vaccines.

Cellular immunotherapy involves using live or modified immune cells, such as dendritic cells or T-cells, which are designed to target and destroy cancer cells. These cells are often collected from the patient, modified to recognize tumor antigens, and reintroduced into the body to trigger a targeted immune response (Valdivia et al., 2021). In the 2019 study by Bird and colleagues, a clinical trial was conducted using autologous hybrid-cell vaccines to treat CMC, with the aim of using CMC as an intermediate model for HBC. The vaccine therapy was combined with immunostimulatory oligonucleotides and gemcitabine. This combination therapy led to a 3.3-fold increase in median survival times compared to the control group. However, for inflammatory mammary carcinoma (IMC), the treatment was less effective, resulting in a median survival of only 42 days (Bird et al., 2019).

DNA vaccines work by delivering small pieces of DNA that encode specific tumor antigens to the target cells. These cells then produce the antigen, prompting the immune system to recognize and attack cancer cells displaying those antigens. The delivery of DNA for vaccines can be either biological-using vectors like viruses or bacteria to carry the DNA into cells—or non-biological, which includes both physical methods (such as electroporation, where electrical pulses open the cell membrane to allow DNA entry) and chemical methods (such as nanoparticles or liposomes, which encapsulate the DNA and deliver it to cells more efficiently) (Valdivia et al., 2021).

In a clinical trial involving nine dogs, including one with a mammary tumor, IL-12 (delivered via plasmid DNA using electroporation) was administered intratumorally as an immunostimulatory cytokine. The treatment resulted in temporary increases in serum and tumor IL-12 and IFN-gamma levels, indicating an immune response. However, despite this immune activation, the study did not show any clinically significant outcomes or substantial benefits for the treated dogs (Cicchelero et al., 2017).

An anticancer DNA vaccine targeting p62 was tested in both mice xenografted with CMC and dogs with mammary tumors. When administered intramuscularly, the p62 DNA vaccine resulted in either a partial response or stable disease in the treated subjects, all without significant secondary effects (Gabai et al., 2014).

In a clinical trial for dogs with MGT, researchers utilized nanoparticles carrying DNA plasmids for canine interferon-β and the HSV thymidine kinase (a suicide gene). These nanoparticles were injected into the tumor bed during mastectomy. Subsequently, dogs received subcutaneous injections of these nanoparticles combined with human granulocyte–macrophage colony-stimulating factor and interleukin-2, along with mammary carcinoma extracts. The treatment was well-tolerated, resulting in only one recurrence among 26 dogs, with no distant metastases observed, and overall survival rates improved (Finocchiaro et al., 2018).

Another clinical trial investigated the use of weekly neoadjuvant intratumorally injections of empty cowpea mosaic virus (eCPMV) as an immunotherapy for companion dogs diagnosed with IMC. Preliminary results showed that the treatment was well-tolerated, with minimal side effects reported. Additionally, the eCPMV immunotherapy demonstrated promise in inducing a local immune response, showcasing anti-tumor efficacy and contributing to improved survival rates in dogs with IMC (Alonso-Miguel et al., 2022).

In summary, while a specific vaccine for CMC is not currently available, ongoing research in cancer immunotherapy holds promise for future advancements in this field. As scientists continue to explore

innovative therapeutic strategies, there is potential for developing effective vaccines tailored to this condition.

Using bacteria as an immunotherapy tool for treating cancer in dogs is an innovative approach that leverages the immune-stimulating properties of certain bacteria to boost the body's natural defense mechanisms against tumors. This method involves the use of live or modified bacteria, bacterial components or bacterial toxins to provoke a targeted immune response, helping to eliminate cancer cells (Chirullo et al., 2015; Chirullo et al., 2024; Razzuoli et al., 2022; Zhou et al., 2018).

Bacterial immunotherapy works by activating the immune system to fight cancer. Certain bacteria or their products can stimulate immune cells like macrophages, dendritic cells and T-cells to recognize and attack cancer cells. Some bacteria can also thrive in oxygen-poor tumor regions, an environment where many conventional treatments are less effective, where they directly kill tumor cells or release toxins, while triggering a strong immune response. This targeted immune activation focuses specifically on cancer cells, sparing healthy tissues and reducing side effects compared to traditional treatments like chemotherapy (Zhou et al., 2018).

Salmonella typhimurium is a facultative anaerobe, meaning it can survive in both oxygen-rich and oxygen-poor environments. This allows it to infect a wide variety of cancer types. It can colonize aerobic environments, such as highly vascularized tumors, and thrive in the hypoxic areas of solid tumors (Broadway & Sharf, 2019), including mammary tumors in dogs (Chirullo et al., 2015), where oxygen levels are too low for conventional chemotherapy to be effective (Broadway & Sharf, 2019). This adaptability makes S. typhimurium particularly useful in targeting different tumor environments. S. Typhimurium selectively targets tumors rather than normal host tissue through several intrinsic mechanisms: bacteria are capable to accumulate at the tumor site, which helps activate the immune system to respond more aggressively to cancer cells; there, they can prevent the growth of cancer cells by competing for nutrients, thereby depriving the tumor of essential resources needed for its survival and proliferation. Furthermore, Salmonella is capable of infiltrating necrotic areas of tumors

that are typically inaccessible to conventional drugs. This allows the bacteria to target and disrupt regions where traditional therapies may be ineffective (Broadway & Sharf, 2019).

A murine in vitro and in vivo study (Chirullo et al., 2015) showed that attenuated mutant strain of S. Typhimurium (STMΔznuABC) is capable to penetrate and replicate into tumor cells, reducing their viability. The strains were devoid of the entire operon that encodes the high-affinity zinc transporter ZnuABC (Ammendola et al., 2007). This transporter is essential for bacterial growth in zinc-deficient environments and for providing full virulence to various Gram-negative pathogens (Cerasi et al., 2013).

In the 2015 study conducted by Chirullo and colleagues, the subcutaneous therapeutic administration of live STMΔznuABC resulted in the reduction of the tumor mass of about 9 times and an increase in life expectancy in immunocompetent tumor-bearing mice (Chirullo et al., 2015).

It has been observed that the CMT cell line CF33 can interact with Salmonella Typhimurium as an infectious stressor (Razzuoli et al., 2022). In the 2022 study of Razzuoli and colleagues, this interaction triggered an inflammatory response in the continuous cell line CF33, evidenced by the up-regulation of IL-6 and IL-8, both of which are pro-inflammatory cytokines. Furthermore, it was demonstrated that Salmonella Typhimurium can penetrate CF33 cells, leading to a reduction in cell viability.

These findings suggest a dual mechanism where Salmonella can induce inflammation while simultaneously impacting the cancer cell's survival, highlighting its potential role in immunotherapy strategies for treating CMC.

Since it remains unclear whether STMΔznuABC activity is specific to certain tumors or species, as well as the mechanisms behind bacteria-host-tumor interactions in dogs, our study aimed to evaluate the potential anti-tumor effects of STMΔznuABC on CMC through in vitro models based on different canine cell lines: the continuous cell line CF33, characterized in terms of gene expression by pivotal molecules for the innate immune response and cell cycle regulation (Razzuoli et al., 2022), and three primary cell lines consisting of CAFs, designated as TM51, TM52 and TM53 cells and obtained from

the resection of tumors of three animals with mammary cancer. Specifically, the investigation focused on the ability of STMΔznuABC to control tumor growth and viability; furthermore, cancer-associated fibroblasts (CAFs) were isolated with the aim of improving knowledge of their biology to understand how they could be modulated.

Indeed, CAFs are a key component of the tumor microenvironment (TME) and a major component of the tumor stroma both in HBC and CMC (Guo et al., 2023; Kudo et a., 2022); furthermore, they play a significant role in cancer progression by modulating the production of unique cytokines (Michishita, 2020).

The TME refers to the complex and dynamic ecosystem that surrounds and interacts with a tumor within the body. It includes a variety of non-cancerous cells, molecules and structures that are essential for the tumor's growth, survival and progression. The assessment of TME is one of the fundamental aspects of precision medicine because it is known to play a critical role in shaping how the tumor evolves, responds to treatment and metastasizes (Cheng et al., 2020).

CAFs are a type of stromal cell that, while not cancerous themselves, contribute to the growth, survival, invasion and metastasis of cancer cells. Unlike normal fibroblasts, which typically help maintain the structure and integrity of tissues, CAFs are reprogrammed by cancer cells to support the tumor's development, playing a role also in chemoresistance (Guo et al., 2023; Kudo et a., 2022).

Based on our results, STMΔznuABC effectively penetrated and colonized all the four cell lines analyzed. Notably, after 24 hours of exposure to STMΔznuABC, cell viability was significantly reduced compared to the untreated control group.

By evaluating gene expression, the study demonstrated the ability of these cell lines to initiate an inflammatory response. Following infection with STMΔznuABC, a marked increase in key pro-inflammatory cytokines and chemokines, specifically IL-8 and IL-18, confirmed the activation of this response. Additionally, we demonstrated the ability of STMΔznuABC to significantly penetrate canine CAFs 24 hours post-treatment. This resulted in notable tumor cell cytotoxicity, as evidenced by a significant reduction in cell viability compared to untreated controls. Understanding the

interaction between STMΔznuABC and CAFs is crucial, because the disruption of the tumor-supportive stroma, weak the cancer cells' survival mechanisms.

These combined effects suggest that STMΔznuABC holds potential as a therapeutic agent for canine cancers, offering both direct bacterial cytotoxicity and the ability to harness the immune system against the tumor. These findings suggest that STMΔznuABC may represent a promising tool for innovative anti-cancer therapies, with further optimization needed to fully realize its clinical potential.

It is obvious that in vitro models cannot fully mimic the complexity of living systems (Pinho et al., 2012). In vitro models are powerful tools for isolating and analyzing specific biological processes in controlled conditions, but they often lack the complexity, systemic interactions and predictive power provided by in vivo models. The need to replicate the intricate cellular interactions and biology of heterogeneous tumors has driven the development of 3D culture systems for in vitro cell growth. These systems often incorporate tumor-associated scaffolds and matrices that simulate the TME more closely than traditional 2D cultures. By using these scaffolds, researchers attempt to mimic the spatial and cellular complexity found in actual tumors, making these models more relevant for certain biological studies. However, while these 3D in vitro models provide valuable insights, they are still insufficient for addressing critical aspects such as drug distribution within tumors, pharmacokinetics, pharmacodynamics and toxicity. These parameters are complex and require in vivo systems, as the dynamics of drug absorption, metabolism, and elimination are closely tied to the physiological conditions present in a whole organism. Thus, despite advancements in 3D culture technology, preclinical testing often still relies heavily on animal models to provide a complete understanding of how a drug will behave in a living system (Gordon & Khanna, 2010). The two approaches are complementary: in vitro models are useful for early, hypothesis-generating research, while in vivo models are essential for testing these hypotheses in the context of the whole organism (Gordon & Khanna, 2010; Nguyen et al., 2018; Pinho et al., 2012).

However, the ethical issues surrounding the use of laboratory animals have sparked debates for many years. The primary concern revolves around the moral justification for subjecting animals to

potentially harmful procedures, often for the benefit of advancing human medicine. While laboratory animals are crucial in providing insights into complex biological processes, their use raises significant ethical dilemmas, particularly regarding animal suffering and welfare. One potential solution to this issue is the reduction in the number of animals used in research by enhancing in vitro studies. These methods are more cost-effective and time-efficient and avoid harming living beings (Gordon & Khanna, 2010). However, they cannot fully replicate the complexity of living organisms, so animal testing remains necessary in many cases. The challenge remains in finding a balance, using in vitro methods to their fullest potential while still acknowledging the limitations they present when compared to the intricacies of in vivo systems. The 3Rs principle—Replacement, Reduction and Refinement—guides researchers in balancing ethics and scientific progress, by reducing animal use and improving conditions in labs (Louis-Maerten et al., 2024).

Since there is a growing necessity to better understand the efficacy and safety of therapies for dogs, enhancement of preclinical studies is required. Such improvements will significantly aid in the development of clinical trials tailored to canine patients. By focusing on rigorous preclinical research, researchers can gather critical data that informs treatment protocols and improves outcomes for canine patients. This approach not only benefits veterinary medicine but also has implications for translational research in human oncology. In fact, preclinical models (both in vitro and in vivo) are essential for the development of anti-cancer drugs and their subsequent use in human clinical trials (Gordon & Khanna, 2010). The prevention of HBC and the development of innovative and effective therapies are the primary goals of researchers; however, the limited availability of tumor tissue samples, along with ethical challenges, pose significant constraints (Abdelmegeed & Mohammed, 2018).

Rodent models are the most widely used animal model in mammary gland cancer research (Ferreira et al., 2023), but they require chemical induction or genetic manipulation to develop tumors (Kwon et al., 2023; Frénel & Nguyen, 2023). In fact, they exhibit significant biological and physiological

differences from humans, which may limit their ability to fully replicate human conditions (Gordon & Khanna, 2010).

In this context, spontaneous CMC is regarded as a valuable model of HBC due to cancer's clinical and biological similarities between both species (Pinho et al., 2012). They share similar high prevalence, molecular subtypes, mutation profiles, histological traits and biomarkers (Kwon et al., 2023). Compared to HBC, CMC provides enhanced access to tissue samples, thereby strengthening resources for conducting in vitro studies (Gordon & Khanna, 2010). Furthermore, the fast progression of spontaneous invasive mammary carcinomas in dogs enables preclinical investigations to be concluded quickly compared to humans (Nguyen et al., 2018). All these features make pet dogs a valuable model for developing translational HBC therapies, and this should encourage the enhancement of research into canine mammary cancer.

5. Conclusions

Currently, there are no established guidelines for managing mammary tumors in dogs. Nonetheless, the incidence and mortality rates remain high. In the absence of standard procedures, the veterinarian's management should be guided by evidence-based knowledge.

Looking ahead, it is important to strengthen prevention efforts and optimize traditional adjuvant therapies such as chemotherapy, radiation therapy, and hormonal therapy.

A multimodal therapeutic approach, along with the implementation of neoadjuvant treatments, could allow for lower doses of individual therapies and the use of more conservative surgeries, thereby enhancing treatment effectiveness and improving patient outcome. Furthermore, this may lead to reduction of side effects and a better overall quality of life for patients and their owners.

Conversely, developing innovative therapies that are more cost-effective, easier to administer and well tolerated by cancer bearing dogs is essential.

Exploring new therapeutic approaches is crucial for future studies in both human and veterinary oncology. Progress in targeted and personalized therapies brings hope for improving outcomes in cancer patients. Interest in personalized medicine continues to rise and it is expected to play a vital

role in research for both human cancer treatment and veterinary oncology. Additionally, immunotherapy could serve as a novel and promising treatment option for CMTs. In this context, it would be beneficial to expand research on the use of bacteria in cancer therapies, as preclinical studies have shown promising results. Since spontaneous CMC is considered a valuable model for HBC due to the clinical and biological similarities between the two species, advancing research in the veterinary field would yield significant benefits for human oncology as well.

6. References

Abdelmegeed, S. M., & Mohammed, S. (2018). Canine mammary tumors as a model for human disease. *Oncology letters*, *15*(6), 8195–8205. https://doi.org/10.3892/ol.2018.8411

Alonso-Miguel, D., Valdivia, G., Guerrera, D., Perez-Alenza, M. D., Pantelyushin, S., Alonso-Diez, A., Beiss, V., Fiering, S., Steinmetz, N. F., Suarez-Redondo, M., Vom Berg, J., Peña, L., & Arias-Pulido, H. (2022). Neoadjuvant in situ vaccination with cowpea mosaic virus as a novel therapy against canine inflammatory mammary cancer. *Journal for immunotherapy of cancer*, *10*(3), e004044. https://doi.org/10.1136/jitc-2021-004044

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., & Battistoni, A. (2007). High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of Salmonella enterica. *Infection and immunity*, *75*(12), 5867–5876. https://doi.org/10.1128/IAI.00559-07

Barbagianni, M. S., & Gouletsou, P. G. (2023). Modern Imaging Techniques in the Study and Disease Diagnosis of the Mammary Glands of Animals. *Veterinary sciences*, *10*(2), 83. https://doi.org/10.3390/vetsci10020083

Bird, R. C., DeInnocentes, P., Church Bird, A. E., Lutful Kabir, F. M., Martinez-Romero, E. G., Smith, A. N., & Smith, B. F. (2019). Autologous hybrid cell fusion vaccine in a spontaneous intermediate model of breast carcinoma. *Journal of veterinary science*, *20*(5), e48. https://doi.org/10.4142/jvs.2019.20.e48

Bird, R. C., Deinnocentes, P., Church Bird, A. E., van Ginkel, F. W., Lindquist, J., & Smith, B. F. (2011). An autologous dendritic cell canine mammary tumor hybrid-cell fusion vaccine. *Cancer immunology, immunotherapy : CII*, *60*(1), 87–97. https://doi.org/10.1007/s00262-010-0921-2

Broadway, K.M., & Scharf, B.E. (2019). Salmonella Typhimurium as an Anticancer Therapy: Recent Advances and Perspectives. *Current Clinical Microbiology Reports, 6*, 225 - 239. https://doi.org/10.1007/s40588-019-00132-5

Cerasi, M., Ammendola, S., & Battistoni, A. (2013). Competition for zinc binding in the host-pathogen interaction. *Frontiers in cellular and infection microbiology*, *3*, 108. https://doi.org/10.3389/fcimb.2013.00108

Cheng, Y. Q., Wang, S. B., Liu, J. H., Jin, L., Liu, Y., Li, C. Y., Su, Y. R., Liu, Y. R., Sang, X., Wan, Q., Liu, C., Yang, L., & Wang, Z. C. (2020). Modifying the tumour microenvironment and reverting tumour cells: New strategies for treating malignant tumors. *Cell proliferation*, *53*(8), e12865. https://doi.org/10.1111/cpr.12865

Chirullo, B., Ammendola, S., Leonardi, L., Falcini, R., Petrucci, P., Pistoia, C., Vendetti, S., Battistoni, A., & Pasquali, P. (2015). Attenuated mutant strain of Salmonella Typhimurium lacking the ZnuABC transporter contrasts tumor growth promoting anti-cancer immune response. *Oncotarget*, *6*(19), 17648–17660. https://doi.org/10.18632/oncotarget.3893

Chirullo, B., Fruscione, F., Del Zotto, G., Dell'Anno, F., Tarantino, M., Porcellato, I., Petrucci, P., De Ciucis, C.G., Bufalari, A., Guardone, L., Cappelli, K., Moretti, G., Mecocci, S., Monti, E., De Paolis, L., Razzuoli, E. (2024). Evaluation of attenuated Salmonella Typhimurium (STMΔznuABC) anticancer activity on canine mammary cancer-associated fibroblasts. *Research in Veterinary Science*, *Volume 180.* https://doi.org/10.1016/j.rvsc.2024.105438

Chocteau, F., Abadie, J., Loussouarn, D., & Nguyen, F. (2019). Proposal for a Histological Staging System of Mammary Carcinomas in Dogs and Cats. Part 1: Canine Mammary Carcinomas. *Frontiers in veterinary science*, *6*, 388. https://doi.org/10.3389/fvets.2019.00388

Cicchelero, L., Denies, S., Haers, H., Vanderperren, K., Stock, E., Van Brantegem, L., de Rooster, H., & Sanders, N. N. (2017). Intratumoural interleukin 12 gene therapy stimulates the immune system and decreases angiogenesis in dogs with spontaneous cancer. *Veterinary and comparative oncology*, *15*(4), 1187–1205. https://doi.org/10.1111/vco.12255

Evans, B. J., Holt, D. E., Stefanovski, D., & Sorenmo, K. U. (2021). Factors influencing complications following mastectomy procedures in dogs with mammary gland tumors: 140 cases (2009-2015). *Journal of the American Veterinary Medical Association*, *258*(3), 295–302. https://doi.org/10.2460/javma.258.3.295

Ferreira, T., Gama, A., Seixas, F., Faustino-Rocha, A. I., Lopes, C., Gaspar, V. M., Mano, J. F., Medeiros, R., & Oliveira, P. A. (2023). Mammary Glands of Women, Female Dogs and Female Rats: Similarities and Differences to Be Considered in Breast Cancer Research. *Veterinary sciences*, *10*(6), 379. https://doi.org/10.3390/vetsci10060379

Finocchiaro, L. M. E., Spector, A. I. M., Agnetti, L., Arbe, M. F., & Glikin, G. C. (2018). Combination of Suicide and Cytokine Gene Therapies as Surgery Adjuvant for Canine Mammary Carcinoma. *Veterinary sciences*, *5*(3), 70. https://doi.org/10.3390/vetsci5030070

Frénel, J. S., & Nguyen, F. (2023). Mammary carcinoma: Comparative oncology between small animals and humans-New therapeutic tools. *Reproduction in domestic animals = Zuchthygiene*, *58 Suppl 2*, 102–108. https://doi.org/10.1111/rda.14408

Gabai, V., Venanzi, F. M., Bagashova, E., Rud, O., Mariotti, F., Vullo, C., Catone, G., Sherman, M. Y., Concetti, A., Chursov, A., Latanova, A., Shcherbinina, V., Shifrin, V., & Shneider, A. (2014). Pilot study of p62 DNA vaccine in dogs with mammary tumors. *Oncotarget*, *5*(24), 12803–12810. https://doi.org/10.18632/oncotarget.2516

Gordon, I. K., & Khanna, C. (2010). Modeling opportunities in comparative oncology for drug development. *ILAR journal*, *51*(3), 214–220. https://doi.org/10.1093/ilar.51.3.214

Guo, Z., Zhang, H., Fu, Y., Kuang, J., Zhao, B., Zhang, L., Lin, J., Lin, S., Wu, D., & Xie, G. (2023). Cancer-associated fibroblasts induce growth and radioresistance of breast cancer cells through paracrine IL-6. *Cell death discovery*, *9*(1), 6. https://doi.org/10.1038/s41420-023-01306-3

Hart, B. L., Hart, L. A., Thigpen, A. P., & Willits, N. H. (2020). Assisting Decision-Making on Age of Neutering for 35 Breeds of Dogs: Associated Joint Disorders, Cancers, and Urinary Incontinence. *Frontiers in veterinary science*, *7*, 388. https://doi.org/10.3389/fvets.2020.00388

Hart, B. L., Hart, L. A., Thigpen, A. P., & Willits, N. H. (2020). Assisting Decision-Making on Age of Neutering for Mixed Breed Dogs of Five Weight Categories: Associated Joint Disorders and Cancers. *Frontiers in veterinary science*, *7*, 472. https://doi.org/10.3389/fvets.2020.00472

Hoffman R. M. (2012). The preclinical discovery of bacterial therapy for the treatment of metastatic cancer with unique advantages. *Expert opinion on drug discovery*, *7*(1), 73–83. https://doi.org/10.1517/17460441.2012.644534

Holleczek, B., Stegmaier, C., Radosa, J. C., Solomayer, E. F., & Brenner, H. (2019). Risk of loco-regional recurrence and distant metastases of patients with invasive breast cancer up to ten years after diagnosis- results from a registry-based study from Germany. *BMC Cancer*, *19*(1), 520. https://doi.org/10.1186/s12885-019-5710-5

Horta, R. S., Figueiredo, M. S., Lavalle, G. E., Costa, M. P., Cunha, R. M., & Araújo, R. B. (2015). Surgical stress and postoperative complications related to regional and radical mastectomy in dogs. *Acta veterinaria Scandinavica*, *57*(1), 34. https://doi.org/10.1186/s13028-015-0121-3

Knesl, O., Hart, B. L., Fine, A. H., & Cooper, L. (2016). Opportunities for incorporating the human-animal bond in companion animal practice. *Journal of the American Veterinary Medical Association*, *249*(1), 42–44. https://doi.org/10.2460/javma.249.1.42

Kudo, A., Yoshimoto, S., Yoshida, H., Izumi, Y., & Takagi, S. (2022). Biological features of canine cancer-associated fibroblasts and their influence on cancer cell invasion. *The Journal of veterinary medical science*, *84*(6), 784–791. https://doi.org/10.1292/jvms.22-0041

Kwon, J. Y., Moskwa, N., Kang, W., Fan, T. M., & Lee, C. (2023). Canine as a Comparative and Translational Model for Human Mammary Tumor. *Journal of breast cancer*, *26*(1), 1–13. https://doi.org/10.4048/jbc.2023.26.e4

Louis-Maerten, E., Milford, A., Shaw, D. M., Geneviève, L. D., & Elger, B. S. (2024). Perceptions of 3R implementation in European animal research: A systematic review, meta-analysis, and meta-synthesis of barriers and facilitators. *PloS one*, *19*(3), e0300031. https://doi.org/10.1371/journal.pone.0300031

Matos, A. J., Baptista, C. S., Gärtner, M. F., & Rutteman, G. R. (2012). Prognostic studies of canine and feline mammary tumors: the need for standardized procedures. *Veterinary journal (London, England : 1997)*, *193*(1), 24–31. https://doi.org/10.1016/j.tvjl.2011.12.019

Michishita, M. (2020). Understanding of tumourigenesis in canine mammary tumors based on cancer stem cell research. *Veterinary journal (London, England : 1997)*, *265*, 105560. https://doi.org/10.1016/j.tvjl.2020.105560

Millanta, F., Calandrella, M., Bari, G., Niccolini, M., Vannozzi, I., & Poli, A. (2005). Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. *Research in veterinary science*, *79*(3), 225–232. https://doi.org/10.1016/j.rvsc.2005.02.002

Nguyen, F., Peña, L., Ibisch, C., Loussouarn, D., Gama, A., Rieder, N., Belousov, A., Campone, M., & Abadie, J. (2018). Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. *Breast cancer research and treatment*, *167*(3), 635–648. https://doi.org/10.1007/s10549-017-4548-2

Nieto, A., Peña, L., Pérez-Alenza, M. D., Sánchez, M. A., Flores, J. M., & Castaño, M. (2000). Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. *Veterinary pathology*, *37*(3), 239–247. https://doi.org/10.1354/vp.37-3-239

Nosalova, N., Huniadi, M., Horňáková, Ľ., Valenčáková, A., Horňák, S., Nagoos, K., Vozar, J., & Cizkova, D. (2024). Canine Mammary Tumors: Classification, Biomarkers, Traditional and Personalized Therapies. *International journal of molecular sciences*, *25*(5), 2891. https://doi.org/10.3390/ijms25052891

Pastor, N., Caballé, N. C., Santella, M., Ezquerra, L. J., Tarazona, R., & Duran, E. (2018). Epidemiological study of canine mammary tumors: age, breed, size and malignancy. *Austral journal of veterinary sciences*, *50*(3), 143-147. https://dx.doi.org/10.4067/S0719-81322018000300143

Pellin, M. A. (2022). The Use of Oncept Melanoma Vaccine in Veterinary Patients: A Review of the Literature. *Veterinary sciences*, *9*(11), 597. https://doi.org/10.3390/vetsci9110597

Peña, L., De Andrés, P. J., Clemente, M., Cuesta, P., & Pérez-Alenza, M. D. (2013). Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. *Veterinary pathology*, *50*(1), 94–105. https://doi.org/10.1177/0300985812447830

Pereira, C. T., Rahal, S. C., de Carvalho Balieiro, J. C., & Ribeiro, A. A. (2003). Lymphatic drainage on healthy and neoplasic mammary glands in female dogs: can it really be altered?. *Anatomia, histologia, embryologia*, *32*(5), 282–290. https://doi.org/10.1046/j.1439-0264.2003.00485.x

Pinho, S. S., Carvalho, S., Cabral, J., Reis, C. A., & Gärtner, F. (2012). Canine tumors: a spontaneous animal model of human carcinogenesis. *Translational research : the journal of laboratory and clinical medicine*, *159*(3), 165–172. https://doi.org/10.1016/j.trsl.2011.11.005

Rafalko JM, Kruglyak KM, McCleary- Wheeler AL, Goyal V, Phelps-Dunn A, Wong LK, et al. (2023) Age at cancer diagnosis by breed, weight, sex, and cancer type in a cohort of more than 3,000 dogs: Determining the optimal age to initiate cancer screening in canine patients. PLoS ONE 18(2): e0280795. https://doi.org/10.1371/ journal.pone.0280795

Razzuoli, E., De Ciucis, C. G., Chirullo, B., Varello, K., Zoccola, R., Guardone, L., Petrucci, P., Rubini, D., Bozzetta, E., Goria, M., Fruscione, F., & Modesto, P. (2022). Molecular Characterization of CF33 Canine Cell Line and Evaluation of Its Ability to Respond against Infective Stressors in Sight of Anticancer Approaches. *Veterinary sciences*, *9*(10), 543. https://doi.org/10.3390/vetsci9100543

Sorenmo, K. U., Durham, A. C., Kristiansen, V., Pena, L., Goldschmidt, M. H., & Stefanovski, D. (2019). Developing and testing prognos- tic bio-scoring systems for canine mammary gland carcinomas. *Veterinary and Comparative Oncology*, *17*(4), 479–488. https://doi. org/10.1111/vco.12509

Sorenmo, K. U., Shofer, F. S., & Goldschmidt, M. H. (2000). Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *Journal of veterinary internal medicine*, *14*(3), 266–270. https://doi.org/10.1892/0891-6640(2000)014<0266:eosato>2.3.co;2

Sorenmo, K.U.; Worley, D.R.; Zappulli, V. (2020). Tumours of the Mammary Gland. In *Withrow and MacEwen's Small Animal Clinical Oncology* (pp. 604–625). Elsevier. https://doi.org/10.1016/C2016-0-01939-3

Valdivia, G., Alonso-Diez, Á., Pérez-Alenza, D., & Peña, L. (2021). From Conventional to Precision Therapy in Canine Mammary Cancer: A Comprehensive Review. *Frontiers in veterinary science*, *8*, 623800. https://doi.org/10.3389/fvets.2021.623800

van der Meer, D. J., Kramer, I., van Maaren, M. C., van Diest, P. J., Linn, S. C., Maduro, J. H., Strobbe, L. J. A., Siesling, S., Schmidt, M. K., & Voogd, A. C. (2021). Comprehensive trends in incidence, treat- ment, survival and mortality of first primary invasive breast cancer stratified by age, stage and receptor subtype in the Netherlands be- tween 1989 and 2017. *International Journal of Cancer*, *148*(9), 2289– 2303. https://doi.org/10.1002/ijc.33417

Vazquez, E., Lipovka, Y., Cervantes-Arias, A., Garibay-Escobar, A., Haby, M. M., Queiroga, F. L., & Velazquez, C. (2023). Canine Mammary Cancer: State of the Art and Future Perspectives. *Animals : an open access journal from MDPI*, *13*(19), 3147. https://doi.org/10.3390/ani13193147

Vendrell, A., Gravisaco, M. J., Goin, J. C., Pasetti, M. F., Herschllik, L., De Toro, J., Rodríguez, C., Larotonda, G., Mongini, C., & Waldner, C. I. (2013). Therapeutic effects of Salmonella typhi in a mouse model of T-cell lymphoma. *Journal of immunotherapy (Hagerstown, Md. : 1997)*, *36*(3), 171–180. https://doi.org/10.1097/CJI.0b013e3182886d95

Zhou, S., Gravekamp, C., Bermudes, D., & Liu, K. (2018). Tumour-targeting bacteria engineered to fight cancer. *Nature reviews. Cancer*, *18*(12), 727–743. https://doi.org/10.1038/s41568-018-0070-z