Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE MEDICHE VETERINARIE

Ciclo XXX

Settore Concorsuale: Area 07 - Scienze agrarie e veterinarie > 07/H - Medicina veterinaria > 07/H2 Patologia veterinaria e ispezione degli alimenti di origine animale
Settore Scientifico Disciplinare: VET/03 Patologia generale e anatomia patologica veterinaria

Expression of P-glycoprotein and Breast Cancer Resistance Protein in Canine Mammary Tumors and in a Chemoresistant Mast Cell Tumor

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Esame finale anno 2018

ABSTRACT

Multidrug resistance (MDR) consists in the ability of cancer cells to become resistant towards different drugs and is frequently mediated by ABC-transporters efflux pumps, such as P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP), which are also infamous for conferring cancer cell stemness and aggressiveness, thereby imparting a poor prognosis. MDR has been extensively studied in human oncology, but less is known in veterinary medicine. The aims of the past three years of investigation on canine mammary tumors have been to determine the distribution of P-gp and BCRP in the different cellular components of hyperplasia and neoplasia, to compare P-gp and BCRP expression in the histological stages and grades of canine mammary carcinomas (CMSs), to describe P-gp and BCRP expression in the stroma associated with neoplasia, and to examine P-gp and BCRP expression in two aggressive types of CMSs, namely canine inflammatory mammary cancer and histological grade 3 non-inflammatory carcinomas.

P-gp and BCRP immunohistochemical expression was significantly higher in malignant vs benign epithelial cells and hyperplastic epithelium of the mammary gland, in aggressive histotypes (simple vs complex carcinomas; inflammatory carcinoma vs non-inflammatory carcinoma, only for P-gp), and in histological grade 2 and 3 carcinomas vs grade 1. Neoplasia-associated fibroblasts showed an increased expression in stage II and grade 2 and 3 carcinomas compared with stage I and grade 1.

An increased expression of P-gp and BCRP was found in a canine relapsing and chemoresistant cutaneous mast cell tumor after chemotherapy with Vinblastine e Prednisolone. Chemoresistance in this case could be related to an increased efflux of the drugs mediated by these transmembrane pumps.

Evaluation of P-gp and BCRP could help in the identification of aggressive, invasive and chemoresistant canine tumors, and the dog could provide a useful spontaneous model for chemoresistant human tumors.

RIASSUNTO

La resistenza multifarmaco (MDR) conferisce alle cellule neoplastiche resistenza verso diversi composti chemioterapici ed è frequentemente dovuta all'azione di pompe di efflusso transmembrana (ABC-transporters), tra le quali la glicoproteina-P (P-gp) e la Breast Cancer Resistance Protein (BCRP), conosciute inoltre, per conferire caratteristiche di malignità e "staminalità" associate ad una prognosi infausta. La MDR è oggetto di molteplici studi in oncologia umana, mentre poco è noto in veterinaria. Gli obbiettivi di questi tre anni di ricerca sui tumori mammari della cagna sono stati: determinare l'espressione di P-gp e BCRP nelle componenti cellulari della mammella iperplastica e neoplastica, confrontarne l'espressione tra i diversi gradi e stadi istologici dei carcinomi, descriverne l'espressione nello stroma associato alla neoplasia, ed esaminarne e confrontarne l'espressione in due gruppi di neoplasie mammarie aggressive quali il carcinoma infiammatorio e il carcinoma di grado istologico 3.

Mediante l'immunoistochimica è emerso che l'espressione di P-gp e BCRP era significativamente più elevata nei tumori mammari maligni (nelle cellule epiteliali maligne rispetto all'epitelio iperplastico), negli istotipi più aggressivi (nei carcinomi semplici rispetto ai complessi e nei carcinomi infiammatori rispetto ai carcinomi non-infiammatori, per P-gp), e nei carcinomi di grado istologico 2 e 3 rispetto al grado 1. I fibroblasti esprimevano maggiormente P-gp e BCRP nello stroma associato ai carcinomi di stadio II e di grado 2 e 3, rispetto a quelli di stadio I e grado 1.

Un aumento dell'espressione di P-gp e BCRP è stato riscontrato in un cane con mastocitoma cutaneo recidivante dopo chemioterapia con Vinblastina e Prednisolone. La chemioresistenza sviluppata potrebbe essere dovuta all'aumento dell'efflusso dei farmaci dal comparto intracellulare mediato da P-gp e BCRP. Determinare l'espressione di P-gp e BCRP potrebbe essere utile ad identificazione le neoplasie aggressive e chemioresistenti, ed il cane potrebbe fornire un valido modello spontaneo per lo studio della chemioresistenza nei tumori dell'uomo.

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Quando la vita scorreva lentamente come un pigro fiume, la complessità esisteva ma non veniva percepita.
Oggi tutti se la sentono addosso come un torrente vorticoso.

De Toni, Comello
Prede o ragni. Uomini e organizzazioni nella ragnatela della complessità.

Ringraziamenti

Ringrazio la Prof.ssa Cinzia Benazzi e il Prof Giuseppe Sarli per l'opportunità e la fiducia datami nell'intraprendere questo percorso, per la grande esperienza e professionalità.

La Prof.ssa Laura Peña per la calorosa accoglienza ricevuta e la proficua collaborazione intrapresa.

Barbara Brunetti e Giancarlo Avallone per essere i miei maestri in questo mestiere, per la loro competenza e comprensione. Siete, per me, infallibili.

Luisa Vera Muscatello che rappresenta la ricercatrice ideale: incorrotta, abile e generosa.

Valeria Pellegrino per portarmi sorrisi, calore, consigli e per la solare operosità.

I miei genitori per sostenermi stabilmente e assolutamente da 30 anni ormai.

Tutte le persone grazie alle quali in questi anni ho appreso ad esprimermi in un nuovo linguaggio, sia esso una descrizione ed una diagnosi istologica, un articolo scientifico o una lingua straniera.
Chi ha sopportato il mio spirito critico.
Le persone che amo e che ho amato.

Dedico questa tesi, con la quale termino il mio percorso da studentessa, al mio gruppo di amiche veterinarie, compagne negli anni universitari ed oggi professioniste disperse per il mondo, che trovo sempre vicine.

GENERAL INTRODUCTION

ABBREVIATIONS

ABC, ATP-binding Cassette Transporters
ATP, Adenosine Triphosphate
BBB, Blood Brain Barrier
BCRP, Breast Cancer Resistance Protein
IC, Canine Inflammatory Mammary Cancer
CK, Cytokeratin
CMC, Canine Mammary Carcinoma
CMT, Canine Mammary Tumor
Cox-2, Cyclo-oxygenase-2
CSC, Cancer stem cells
EGFR, Epidermal Growth Factor Receptor
ER, Estrogen Receptor
FF PE, Formalin Fixed and Paraffin Embedded
GH, Growth Hormone
HE, Hematoxilin and Eosin
HPF, High Power Field
IC-50, 50% Inhibitory Concentration
IGF-I, Insulin-Like Growth Factor-1
IHC, Immunohistochemistry
MDR, Multidrug Resistance
MRP1, Multidrug Resistance-associated Protein 1
MTD, Maximum Tolerated Dose
P-gp, P-glycoprotein
PXR, Pregnane X Receptor
qRT, quantitative real-time
RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction

MULTIDRUG RESISTANCE IN HUMAN NEOPLASIA

"When will there be a cure for cancer?"

It is so difficult to find an answer to this question because cancer is not one disease but many disorders with widely different natural histories and responses to treatments (Kumar et al., 2014; Nunney et al., 2015). Cancer cells fail to contribute to the tissue function in a multicellular being and behave selfishly with uncontrolled reproduction of themselves, until they overwhelm the complexity of the organism, often leading to the death of the individual (Nunney et al., 2015).

Paul Ehrlich was a pioneer in the field of chemotherapy introducing about 110 years ago this cure as a "magic bullet" against infectious diseases using trypan red as the drug to target African trypanosomes (Kaufmann, 2008). Soon from that moment, sadly, drug-resistant bacterial strains and neoplastic cells promptly arose as major therapeutic obstacles (Lage, 2008).

Patients with cancer must be treated with a chemotherapeutic drug maximizing its efficacy and minimizing the adverse effects of the treatment (Holohan et al., 2013; Lage, 2008).

At first patients use to respond positively to chemotherapy but, still, there is a subgroup that from the beginning does not show a remission of cancer (intrinsic chemoresistant) and another that after an initial remission no longer responds to further treatments (acquired chemoresistant) (Bonavida, 2013).

Conventional chemotherapy also presents the side effect of killing rapidly dividing cells, mainly of the bone marrow and gastrointestinal tract thus a break between treatments is necessary to prevent serious toxicity. Without these pauses severe drug toxicities occur but this also permits the development of neoplastic cell clones characterized by acquired drug-resistance capacities often followed by tumor recurrence and metastases (Biller, 2014; Holohan et al., 2013).

Moreover, neoplastic cells often gain cross resistance to several agents by developing the so called multidrug-resistance (MDR) phenotype (Chen et al., 2016; Ferreira et al., 2015; Lage, 2008). MDR is thus defined as the resistance of cancer cells to structurally and mechanistically unrelated classes of anticancer drugs (Gottesman et al., 2002).

The complex phenomenon of MDR is extensively studied and some of the major mechanisms concerning in vitro MDR have been characterized, nevertheless put this knowledge into the clinic practice still represents a major challenge (Gillet and Gottesman, 2010).

Delineating the biochemical, molecular and genetic mechanisms that regulate chemotherapy resistance of neoplastic will be at the basis of the development of strategies in order to answer the immediate need for an effective cure against cancer (Bonavida, 2013).

The mechanisms of drug resistance can be classified into (1) pharmacological mechanisms of drug resistance, strictly related to pharmacological aspects, and (2) cellular mechanisms of drug resistance which involve the cellular capacity to interact with the drug molecule (Colmegna et al., 2017; Ferreira et al., 2015; Lage, 2008).
In this introductive part we will briefly list the former, and then we will focus on the cellular mechanisms of drug resistance, specifically on ATP-binding cassette (ABC) transporters with a special emphasis on P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) efflux transporters, which are the objects of this study.

Pharmacological and Cellular Mechanisms of Drug Resistance

Many tumors show resistance to drugs because of pharmacokinetic reasons, as inefficient and heterogeneous tumor drug distribution, related to a deficient vascularization and high interstitial pressure resulting in an inadequate exposure to anticancer drugs (Colmegna et al., 2017).
In order to eliminate the highest number of neoplastic cells traditional chemotherapeutic protocols assume that the anti-proliferative drug must be administered at the maximum tolerated dose (MTD). The tumor is considered clinically resistant to the MTD if the drug concentration during the time of drug exposure at MDT, is not sufficient to achieve a clinically ascertained complete or partial response (Lage, 2008).
The pharmacological mechanisms of drug resistance include:

1. The application of the drugs, e.g. inadequate infusion or, in the case of orally administered drugs, the bioavailability can be insufficient because of inconstant intestinal absorption, or because of the “first pass effect” in the liver or in the gut;
2. low metabolic activation/drug biotransformation when prodrugs are administered, e.g. cyclophosphamide must be converted into the active metabolite 4-hydroxycyclophosphamide by cytochrome P450 oxidases;

3. the pharmacokinetics of the drug molecule (e.g. molecular weight, lipid solubility, total net charge) and the pharmacokinetics in the plasma, i.e. metabolisms and excretion of the drugs, and binding to plasma proteins;
4. the tumor microenvironment, e.g. tumor fibrovascular stroma characterized by structural and functional anomalies, intermittent hypoxia, compression of intratumoral microvessels by growing neoplastic mass, disorganized lymphatic network, high tumor interstitial fluid pressure, diffusion across the so called "desmoplastic fibroinflammatory stroma" that can hinder the penetration of drugs through the tumor bulk. The fibrovascular stroma can be subjected to the action of the drug molecule as well as the tumor (Fuso Nerini et al., 2014; Minchinton and Tannock, 2006; Padera et al., 2004; Tlsty and Coussens, 2006). The acidification of the extracellular compartment also has important effects on the success of chemotherapy (Gillet and Gottesman, 2010; Trédan et al., 2007);
5. the availability in a specific tissue district, i.e. the peculiar structure of barriers such as the blood brain barrier (BBB) (Chen et al., 2016; Colmegna et al., 2017; Fuso Nerini et al., 2014; Gillet and Gottesman, 2010; Lage, 2008).

Cellular drug resistance is based on various mechanisms that can be classified as:

- drug activation and inactivation
- expression of drug efflux pumps
- deregulation of apoptosis
- alteration of drug target.

Cellular mechanisms of drug resistance are considered a pleiotropic phenomenon involving countless mechanisms that take place directly into neoplastic cells and are closely interconnected to the cell response to drug molecules (Gillet and Gottesman, 2010; Lage, 2008).

A first type of resistance directly connected to the neoplastic cell is the so called kinetic resistance. It occurs when in a tumor just a small growth fraction of neoplastic cell is present and can be targeted by the anti-proliferative drug. Often in large primary solid tumors a large proportion of cells are in the G0 phase/ quiescent state which is especially a barrier for the action of cell cycle phase-specific agents (for example alkylating agents) (Foo and Michor, 2009).

1. Drug entry. The plasma membrane is the first structure that a drug must cross to reach the intracellular compartment. Therapeutic species can enter by passive diffusion, endocytosis, or facilitated transport (uptake transporters); the uptake of drug molecules can be reduced by various mechanisms involving alteration in lipid metabolism (e.g. the ceramide pathway) and by the overexpression of ATP-dependent drug efflux pumps (Gillet and Gottesman, 2010; Lage, 2008).
2. Drug metabolism. Inside the cell three phases of enzymatic reaction can alter drug molecules: Phase I enzyme or oxidative metabolism that is mainly mediated by cytochrome P450 enzymes (CYPs) and epoxide hydrolases and consist of conversion of drug species into highly mutagenic aromatic metabolites (epoxide). Phase II enzymes including glutathione-S-transferases and UDP-glucuronosyltransferases, conjugate with epoxides. In the third phase ABC-transporters efflux pumps eject these conjugated metabolites (Deeley et al., 2006).
3. Drug sequestration. Cytoplasmic organelles as endosomes, lysosomes, golgi, and secretory compartments can entrap drugs molecules thanks to the influx mediated by ABC-transporters (Zapf et al., 2008). Moreover metallothioneins that are cysteine-rich molecules and have high affinity for metal ions and reactive oxygen species, can lead to resistance to metal-based therapy and radiation (Theocharis et al., 2004). Drug sequestration is an important phenomenon but does not seems to confer the same extent of resistance to drugs as mechanisms mediated by efflux transporters do (Gillet and Gottesman, 2010).
4. Even after nuclear entry many mechanisms can be activated in order to evade drug effects. Efflux from the nucleus can be achieved through the action of vault ribonucleoprotein particles, which have shown an important role in non-P-glycoprotein multidrug-resistant cells (Kickhoefer et al., 1998). When, within the nucleus, drugs molecules form damaging adducts with DNA, a complex network of interacting pathways is initiated. The efficacy of therapeutic agents can be significantly reduced by DNA repair. The intricate network of repair systems includes: the direct reversal pathway (MGMT, ABH2, ABH3), the mismatch repair (MMR) pathway, the nucleotide excision repair (NER) pathway, the base excision repair (BER) pathway, the homologous recombination (HR) pathway, and the nonhomologous end joining (NHEJ) pathway. The upregulation of these pathways can repair DNA damage induced by some chemotherapeutic agents preventing cancer cell death (Hakem, 2008).

5. Evasion of drug-induced apoptosis. Disruption of apoptotic pathways is one of the hallmark of cancer and is a major obstacle to the success of chemotherapy. If the damage of DNA caused by the drug is extensive, rather than repair itself the cell will enter one of these states: senescence, apoptosis, or necrosis (Kumar et al., 2014). Moreover, other nonapoptotic mechanisms, including autophagy, mitotic catastrophe, necrosis, and senescence can lead to the cell death and can be altered in a drug resistant cell (Okada and Mak, 2004).
6. Altered signal transduction pathways, governed via integrin receptors, growth factor receptors, frizzled receptors, and smoothened-patched receptors as well as chromosomal abnormalities can lead to the blockage of apoptosis and expression of MDR-linked genes (Gillet and Gottesman, 2010).
7. Cancer stem cells (CSCs) also known as cancer-initiating cells have the capacity to initiate and sustain the growth of a heterogeneous cancer through self-renewal and differentiation (Alkatout et al., 2008; Gillet and Gottesman, 2010). An important feature of stem cells is that they have most of the MDR mechanisms previously discussed. ABCB1 and ABCG2 are well-characterized ABC-transporters expressed in both cancer and normal stem cells and are considered markers associated with immature cell types (Bunting, 2002; Hirschmann-Jax et al., 2004; Holohan et al., 2013; Moitra, 2015; Zhou et al., 2001).
8. Hypoxia induces in mammalian cells the expression of multidrug transporters, upregulating the expression of numerous MDR-linked genes such as ABC-transporters, Bcl2 family genes, glutathione etc, mainly through the activation of the transcription factor Hypoxia Inducible Factor-1(Colmegna et al., 2017).

Multidrug Resistance Mediated by ATP-binding Cassette Transporters Efflux Pumps

One of the most important mechanisms underlying MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters. This super-family of protein complexes, at present, consists of 48 members and is classified into 7 subfamilies from ABC-A through to ABC-G based on their sequence similarities (Borst and Elferink, 2002; Chen et al., 2016). ABC-transporters are transmembrane pumps which efflux both cytotoxic agents and targeted anticancer drugs pumping them out of the cell using ATP driven energy (Chen et al., 2016; Fletcher et al., 2010; Gillet and Gottesman, 2010; Kathawala et al., 2015). Their function in drug resistance is to lower intracellular drug concentrations and compromise the success of

chemotherapeutic regimen (Ferreira et al., 2015).They are one of the largest and oldest families of membrane proteins, their abundance varies between species, but they are highly conserved in sequence and often demonstrate similar structure and functions across prokaryotic and eukaryotic organisms. At the base of their function there is a drug-binding site that can shift from a high-affinity state upon drug binding into a low-affinity state that, with the conformational changes driven by ATP hydrolysis, releases the substrate into the extracellular medium (Ferreira et al., 2015; Glavinas et al., 2004; Kathawala et al., 2015).

The nucleotide-binding domains (NBDs) define the membership to the ABC-protein superfamily, while substrate recognition is a function of the trans-membrane domains (TMDs), and sequence and protein homologies in this region define which subfamily the ABC-proteins belong to (Leitner et al., 2007). Their structure consists of the nucleotide-binding domains (NBDs) and transmembrane binding domains (TMDs). TMDs have the function of substrate recognition. On the NBDs, also known as ABC-domains, is based the topological classification of these pumps (Borst and Elferink, 2002; Kathawala et al., 2015; Leitner et al., 2007).

ABC-pumps transport important substrates across extracellular and intracellular membranes, such as amino acids, cholesterol and its derivatives, sugars, vitamins, peptides, lipids, some important proteins, hydrophobic drugs and antibiotics (Dean and Annilo, 2005; Gottesman and Ambudkar, 2001; Ifergan et al., 2004). Secretory epithelial cells use ABC-transporters to excrete many substances, sometimes against a steep concentration gradient. Several human diseases involving errors in liver metabolism are related to mutations in one of the genes encoding these pumps (Borst and Elferink, 2002).

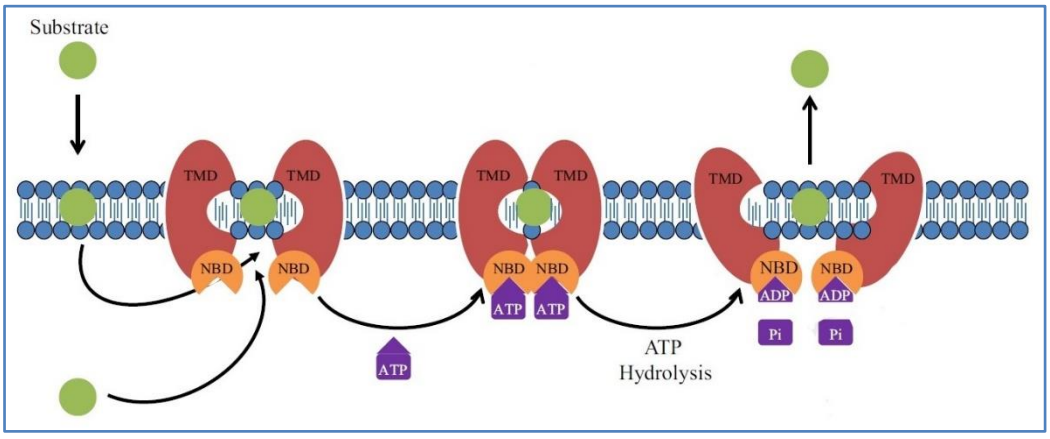
Fifteen members of the ABC-transporter family have been named “infamous” (Kumar et al., 2014), because they have shown the capacity of efflux cancer chemotherapeutic molecules and render cancer cells resistant to treatment (Fletcher et al., 2010).

MDR development in neoplastic cells is explained by various mechanisms. Firstly, cells natively expressing drug-efflux proteins retain their phenotype throughout the process of malignant transformation (Chen et al., 2016). Secondly, chemotherapeutics have been shown to induce genetic PGP expression in non-expressing cells (Ambudkar et al., 2005).

Levchenko and colleagues (2005) have been the first to show that, under the selective pressure of colchicine, the intercellular transfer of functional P-gp

could occur between different tumor cell types, namely co-cultures of sensitive and resistant human neuroblastoma and adenocarcinoma cells. The result was an increase in drug resistance both in vitro and in vivo. The resistance that cells acquired permitted them to survive toxic drug concentrations long enough to develop stable genetic P-gp-mediated resistance (Levchenko et al., 2005).
Cell-to-cell transfer of functional P-gp has been then confirmed by other studies (Pasquier et al., 2011; Zhou et al., 2013). Some authors suggested that the intercellular transfer of P-gp during the course of treatment could be mediated by membrane microparticles (Bebawy et al., 2009; Zhou et al., 2013). In another study trogocytosis was firstly reported to occur between a cancer cell (epithelial ovarian cancer) and an original type of stromal cell ("Hospicells"). This interaction induced autonomous acquisition of chemoresistance to platin and taxanes mediated by multi-drug resistance proteins (Rafii et al., 2008).
These findings introduce a new modality of chemoresistance onset and possible spread in a neoplastic population that could involve the fibrovascular stroma and may have important implications in the diagnostic value of PGP expression.

Figure 1. Function of ABC-transporters. ABC-transporters are energy-dependent transporters; they exhibit a conformational change upon substrate binding and ATP hydrolysis which drives the transport process of the substrate. Modified from (Chen et al., 2016).



Induction and Regulation of ABC-Transporters Expression

In human oncology the induction of ABC-transporters has been extensively investigated. Exposure to xenobiotics (as carcinogens and cytotoxic drugs), hypoxia, heat shock, irradiation and inflammation has been correlated to the upregulation of ABC-transporter in many cells types, normal and neoplastic (Scotto, 2003).
Two members of the nuclear receptor superfamily, the Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR), have been recognized to up-regulate multiple target genes including Phase I (CYP3A4, CYP2B6) and Phase II enzymes (UDP-glucuronosyltransferases, sulfotransferases), as well as various ABC-transporters including P-gp (Urquhart et al., 2007).
The function of PXR and CAR is to sense xenobiotics and to regulate their degradation and clearance. The activation of PXR is induced by many compounds including a wide variety of drugs (i.e. dexamethasone, rifampicin, spironolactone tamoxifen, vinca alkaloids, taxanes and alkylating agents) (Wang et al., 2012).

ABC-transporter expression has also been linked to well-known oncogenes and tumor suppressor (Zandvliet and Teske, 2015).

- P-gp expression id modulated by p53, both mutant and wild type (Chin et al., 1992), by the Ras/Raf signaling pathway (Miltenberger et al., 1995) and the APC (adenomatous polyposis coli) gene (Yamada et al., 2000).
- A decreased BCRP expression is correlated to the activation of the MAPK/ERK and JNK pathway (Tomiyasu et al., 2014).
  Nevertheless, epigenetic regulation and microRNA appear to be of importance in the expression of ABC-transporters (Scotto, 2003).

Three main human ABC-transporters primarily associated with the MDR phenomenon are P-gp (*ABCB1* encoded), multidrug resistance-associated protein 1 (MRP1, *ABCC1* encoded), and the most recently described BCRP (*ABCG2* encoded) (Robey et al., 2007).
In this thesis we will focus on P-glycoprotein and Breast Cancer Resistance Protein and we will briefly summarize their significance in humans in the following pages.

P-Glycoprotein in Human

With the discovery of Permeability-glycoprotein or P-glycoprotein (P-gp), also known as ABCB1 and MDR1, in 1976 by Juliano and Ling a clear relation between efflux pumps and MDR was effectively established (Juliano and Ling, 1976; Ueda et al., 1986; Zandvliet and Teske, 2015). This transporter was expressed in Chinese hamster ovary cells selected for colchicine resistance and it was proposed that such protein would modulate some properties of hydrophobic membrane regions (Gillet and Gottesman, 2010; Juliano and Ling, 1976). Later studies demonstrated that P-gp was involved not only in MDR but also in compromising drug access to sensitive compartments such as brain, testes and ovaries, protected by additional barriers with high P-gp expression (Ferreira et al., 2015). ABCB1 is encoded by ABCB1 gene localized to chromosome 7p21, and has a molecular weight of 170-kDa.
P-gp is located in the kidney, placenta, liver, adrenal glands, intestine (where it limits the uptake of compounds from the gastrointestinal tract), and stimulates excretion of compounds in the liver, kidney, and intestine. (Schinkel, 1997). Important endogenous compounds such as nucleotides, folate, steroids and eicosanoids rely on ABC-transporters to be secreted, and at the BBB and other blood-tissue barriers P-gp protects sensitive organs from exposure to toxic compounds present in the blood compartment (Gottesman et al., 2002; Ifergan et al., 2004; Kathawala et al., 2015; Leitner et al., 2007; Schinkel, 1997).

Many neutral and cationic hydrophobic chemotherapeutic molecules are substrates of P-gp, therefore the overexpression of P-gp confers resistance to taxanes (e.g. paclitaxel, docetaxel), epipodophyllotoxins (e.g. etoposide and teniposide), vinca alkaloids (e.g. vinblastine and vincristine), anthracyclines (e.g. doxorubicin and daunorubicin) antibiotics (e.g. actinomycin D), breakpoint cluster region-abelson (BCR-ABL) tyrosine kinase inhibitors (TKIs) (e.g. imatinib), and epidermal growth factor receptor (EGFR) TKIs (e.g. erlotinib) (Gottesman et al., 2002; Kathawala et al., 2015; Marchetti et al., 2008; Peng et al., 2012).
High expression of P-gp has been observed in many tumors including hematopoietic malignancies and numerous carcinomas including renal, colon, hepatocellular, adrenal, mammary and ovarian carcinoma, frequently bearing intrinsic chemoresistance (Cordon-Cardo et al., 1990).

PGP expression in breast cancer, especially when associated with PGP expression in stromal fibroblasts, is suggestive of a particularly malignant phenotype. P-gp expression in tumor cells, and especially when accompanied by P-gp expression in fibroblasts in desmoplastic stroma, has a prognostic value in primary breast cancer patients and is likely to be a marker of a high malignant phenotype. P-gp expression of tumor cells might play a role in tamoxifen resistance. These findings may have important implications for the treatment of breast cancer patients, and warrant further prospective investigation (Linn et al., 1995).

Stem cells, both in normal tissue and in tumors are characterized by a high level of ABC-transporter expression, including P-gp (Fletcher et al., 2010). P-gp has been found to be expressed in practically all hematopoietic progenitor cells, and the highest levels of P-gp are associated with cells displaying characteristics of pluripotent stem cells (Chaudhary and Roninson, 1991).
Normal stem cells have multiple mechanisms to protect them from cytotoxic insults, that include highly active drug-efflux pumps (Klopfleisch et al., 2016; Moitra, 2015).

Breast Cancer Resistance Protein in Humans

Doyle and colleagues discovered BCRP in 1998 examining a MCF7 breast cancer cell line (MCF-7/AdrVp) that showed resistance to doxorubicin. The responsible for chemoresistance in this case was therefore called breast cancer resistance protein (Doyle et al., 1998; Miyake et al., 1999; Staud and Pavek, 2005).
BCRP is encoded by the *ABCG2* gene which is located on chromosome 4q22(Mao and Unadkat, 2015, 2005). It is also known as ABCG2, mitoxantrone resistance protein (MXR) because it has been shown to cause mitoxantrone resistance (Rocchi et al., 2000), or placenta ABC-protein (ABC-P) because of its presence at the blood tissue barriers of the placenta (Litman et al., 2000). BCRP is a 72-kDa plasma membrane glycoprotein and is activated upon homodimerization or oligomerization with itself or other transporters. Differently from P-glycoprotein and MRP1, which are structured in 2 repeated halves, BCRP has just one TMD and one NBD to mediate MDR, and six putative transmembrane domains, suggesting that BCRP is therefore a half-transporter, which may function as a homodimer or heterodimer (Litman et al., 2000; Maliepaard et al., 2001; Mao and Unadkat, 2005; Rocchi et al., 2000).

BCRP has a wide distribution and is expressed mainly at the level of the plasma membrane, in placental syncytio-trophoblasts, the apical surface of small intestines, colon epithelium, liver canalicular membrane, luminal surfaces of microvessel endothelium of human brain and in the veins and capillaries of blood vessels limiting cellular accumulation of various noxious compounds (Diestra et al., 2002; Doyle et al., 1998; Kathawala et al., 2015; Maliepaard et al., 2001). BCRP in lactating mammary glands mediates the transfer of its substrates into milk thereby increasing the exposure to potential noxes of a breastfed newborn (Meyer zu Schwabedissen and Kroemer, 2011).
Substrates of BCRP include topotecan and mitoxantrone, organic anion conjugates, nucleoside analogs, organic dyes, TKIs, anthracyclines, camptothecin-derived topoiso-merase I inhibitors, methotrexate topotecan and flavopiridols (Burger et al., 2004; Kathawala et al., 2015; Mao and Unadkat, 2005; Rocchi et al., 2000).
The majority of the work regarding BCRP significance in human cancer has been done with leukemia, particularly acute myeloid leukemia. Several studies have shown a positive correlation between high levels of BCRP expression and poor clinical outcomes, even if there are some discrepancies among studies that

are still awaiting to be clarified (Mao and Unadkat, 2015). Solid tumors of the gastrointestinal tract, endometrium, lung and melanoma have shown to express this glycoprotein (Diestra et al., 2002). T-cell lymphomas seem to very commonly express BCRP (Saglam et al., 2008).

Importantly, BCRP has been recognized to be upregulated in a subset of stem cells, termed the "side population", characterized by high efflux capability for antimitotic drugs. However the role of ABCG2 in stem cell biology remains to be elucidated (Fletcher et al., 2010; Hirschmann-Jax et al., 2004; Kathawala et al., 2015)

VETERINARY ONCOLOGY – Canine Mammary Tumors

The aim of the following introduction regarding canine mammary tumors is to give the reader a general frame of this wide topic, including the latest updates regarding this important disease in veterinary medicine from the pathologist's point of view. Part of this chapter and the images are taken from a review that my colleagues and I wrote during my first year of PhD research activities and that has been published in the journal *Veterinaria* (Levi et al., 2016).

Incidence

Mammary neoplasia is among the first three most common tumors in the female dog (Sorenmo, 2003) and for this reason canine mammary tumors (CMTs) have been the subject of intense research by histopathologists and oncologists during the last few decades (Goldschmidt et al., 2017).
Two Italian studies have accounted for a prevalence of 56% in female dogs in the provinces of Venice and Vicenza, and 70% of tumors in the registries of the city of Genoa (Merlo et al., 2008; Vascellari et al., 2009).
In the United Kingdom the prevalence has been reported to be 205/100.000 cases considering both male and female dogs every year respectively (Dobson et al., 2002), while in the United States the prevalence was 198/100000 cases accounting for 41.7% of all neoplasms in intact bitches (Dorn et al., 1968).
The geographical incidence of CMT varies depending on the degree of use of early sterilization (Carolyn, 2014). In fact, the role of ovarian hormones in promoting neoplasms, at least initially, has been well established (Goldschmidt et al., 2017; Millanta et al., 2005; Sorenmo et al., 2011) and for this reason early ovariectomy, commonly performed in the USA, greatly reduces the risk of mammary tumors developing in female dogs and cats.

Risk Factors

The incidence of mammary tumors increases with age, which is considered the most important risk factor, and the mean age of development has been reported to be 8.5 years for benign tumors, and 9.5 years for malignant tumors (Chang et al., 2005; Goldschmidt et al., 2017; Sorenmo et al., 2009).
Breed is another risk factor. Large breeds develop mammary neoplasms at a younger age than small breeds (Goldschmidt et al., 2017), but small breeds bear a higher incidence of CMT, namely poodles, English Springer Spaniel, Brittany

Spaniel, Cocker Spaniel, Maltese, Yorkshire Terrier and dachshund are the most represented. An exception is the German Shepherd that is highly represented too (Sleeckx et al., 2011; Sorenmo et al., 2011).

Sexual hormones are of great importance in the development of CMT, especially estrogen and progesterone (Goldschmidt et al., 2017). In fact in young bitches ovariectomy performed before their first heat cycle, the risk of developing a mammary neoplasm is 0.05% compared to the intact ones. The risk is decreased by 92% and 74% if ovariectomy is performed prior to the second and third heat cycles; no statistically significant benefits are reported if ovariectomy occurs after the third cycle (Goldschmidt et al., 2017; Sleeckx et al., 2011; Sorenmo et al., 2011, 2000). However, in a systematic review on this topic, Beauvais and colleagues questioned that early neutering has such a powerful protecting effect on the risk of developing CMT because of limited available evidence and risk of bias in previous studies (Beauvais et al., 2012).

Exogenous hormones, i.e. progestins and estrogens, seem to promote the development of both benign and malignant CMTs (Goldschmidt et al., 2017; Misdorp, 1991).

Obesity at a young age and a diet rich in animal proteins are thought to be correlated with the development of breast cancer (Cleary, 2013), and also CMTs seem to be affected by these factors (Goldschmidt et al., 2017; Pérez Alenza et al., 1998).

In a recent study Lim and colleagues have examined obesity-related molecules, namely aromatase, leptin, and insulin-like growth factor 1 receptor (IGF-1 R) in canine mammary carcinomas on the basis of the body condition score and found a higher expression of aromatase in the overweight or obese group, in correlation with the expression of ER and PR. In addition, they found higher proportion of poorly differentiated tumors in the overweight or obese female dogs (Lim et al., 2015).

Cytologic Examination

Cytological examination of mammary tumors is considered inexpensive and easy to perform in the veterinary practice. It has been recommended that cytological studies are used prior to surgery to distinguish benign from malignant tumors, inflammatory-type mammary carcinoma from mastitis, tumors other than mammary ones (for example, mast cell tumors) and non-neoplastic lesions (Morris, 2013).

However, Cassali and colleagues highlighted that 25% of cytological preparations from dogs were inadequate for making a diagnosis, often because of insufficient cells in the smear as well as technically unsatisfactory sampling or the intrinsic characteristics of the tumor, i.e. necrosis, and osteo-cartilaginous matrix present in the samples (Cassali et al., 2007). Furthermore, although evaluation of an adequate sample can enable to distinguish benign from malignant lesions, based on examination of fine needle aspirates of mammary neoplasms, malignant lesions are often underestimated in dogs because, typically, benign lesions coexist next to focal malignant lesions in this species. For such reason, the finding of a benign lesion in a bitch does not exclude the presence of poorly represented, unsampled malignant lesions (Cassali et al., 2007).

Cytology is also used to look for neoplastic cells in fine needle aspirates of regional lymph nodes, nearby reactive tissue or surgical scars to distinguish local tumor recurrence from an inflammatory process. Given the above-mentioned drawbacks of cytology and the fact that it does not enable histological typing or grading of the neoplasm, tissue examination continues to be essential, particularly if the tumor is malignant, because it helps to determine the prognosis and allows the evaluation of surgical margins of the excised tumor (Cassali et al., 2007). If the margins do not appear to be infiltrated by the neoplasm, it is recommended that the smallest distance between the tumor and the margin is reported; if, on the other hand, an excision margin is positive, the histology report should specify whether this is due to the presence of isolated cells of a continuation of the lesion right up to the margin (Cassali et al., 2011). Over the years various authors have encouraged a more pertinent and standardized method of evaluating canine mammary tumors in order to obtain homogeneous data, which can be more easily compared in scientific research and usefully applied in clinical practice (Cassali et al., 2011; Matos et al., 2012; Peña et al., 2014).

Histologic Classification

The treatment of mammary tumors is still predominantly surgical: the tissue excised is fixed immediately in formalin and processed in the laboratory, then paraffin embedded, and sectioned with a microtome. The sections are stained with hematoxylin and eosin (HE) and are then available for evaluation by the histopathologist, who classifies the lesion according to the currently used

histological classification and collects any other information that could be useful for estimating the prognosis and, if possible, establishing the treatment (Morris, 2010). In fact, the histologic report can provide the veterinarian not only with a diagnosis of the tissue type of the tumor, but also some important indications on the prognosis (Goldschmidt et al., 2011).

Innovative diagnostic techniques are often used in human oncology, although so far they are employed alongside the histopathological examination, which remains indispensable for the diagnosis of most tumors. The purpose of the histological classification is not only to assign the neoplasm to one of the known types of mammary tumor, but also to provide prognostically useful information; in fact the histological classification attributes specific biological behaviors to different types of tumor (Cassali et al., 2007; Kumar et al., 2014)

The alveoli of the normal mammary gland are composed of two layers of cells: secreting or luminal epithelial cells and basal or myoepithelial cells. These components can proliferate alone or together, giving rise to the so-called complex and mixed tumors in the bitch. The elevated frequency of tumors showing myoepithelial and luminal epithelial proliferation is a unique feature of canine mammary tumors (Gama et al., 2003).

Morphologically tumors can form simple (epithelial luminal or myoepithelial cells), complex (epithelial luminal and myoepithelial cells), mixed (epithelial luminal and/or myoepithelial cells, and osseous/cartilaginous metaplastic tissue), and mesenchymal neoplastic proliferations (Peña et al., 2014). Benign mixed tumors are very common and are formed by the proliferation of benign glandular epithelial and myoepithelial cells with mesenchymal metaplastic elements, primarily cartilage and bone (Beha et al., 2012b). The proliferating myoepithelial cells may exhibit a fusiform or stellate appearance, and these cells are often enveloped within an abundant extracellular myxoid matrix (Peña et al., 2014). Mixed tumors are one of the most common tumor types in the female canine mammary glands (Cassali et al., 2012). The neoplasm is characterized by the presence of three or more cell populations supported by a fibrovascular stroma and consisting of the epithelial component and benign mesenchymal component (cartilage and/or bone and/or adipose tissue) (Goldschmidt et al., 2011). The cartilage contains low or moderate numbers of chondrocytes and chondroblasts rarely exhibiting cellular morphological alterations. When bone tissue is present, it comprises osteoid matrix-forming osteoblasts and mineralized bone. Certain cases also exhibit bone marrow (Cassali et al., 2012).

Mixed tumors are characterized by the presence of benign epithelial elements (ductal and/or acinar and myoepithelial cells) and mesenchymal cells with cartilage and/or bone formation eventually combined with myxoid fibrous tissue (Goldschmidt et al., 2011).

In the dog slightly more than 50% of the tumors are malignant (Goldschmidt et al., 2011). Canine neoplasms are characterized by pronounced morphological heterogeneity and the most commonly found malignant tumor is complex carcinoma; in fact, in this species there is frequently proliferation of both the myoepithelial component and luminal epithelial cells (Carolyn, 2014; Hellmén, 2005; Sorenmo et al., 2011).

It has been hypothesized that the myoepithelial proliferation influences the biological behavior of the tumor, inhibiting the replication, invasion and angiogenesis of the neoplastic luminal epithelial cells (Hellmén, 2005). These neoplasms are biologically less aggressive than the other, so-called simple tumors, in which there is no proliferation of the myoepithelial component (Beha et al., 2012a; Hellmén, 2005; Sorenmo et al., 2011).

One of the first classifications of feline and canine mammary tumors was the "International Histological Classification of Tumours of Domestic Animals" by Hampe and Misdorp, published in 1974. This classification was modified in 1999 by Misdorp et al., approved by the World Health Organization and published by the Armed Forces Institute of Pathology (Misdorp et al., 1999). To date, the most recent classification of canine mammary tumors is that published by Goldschmidt et al. in 2011 (Table 1). The innovative concepts of this classification can be summarized as follows:

1) Emphasis is given to separating complex adenomatous neoplasms with carcinomatous microfoci (called carcinoma arising in a complex adenoma or benign mixed tumor) from those in which the luminal epithelial component is wholly carcinomatous (called complex carcinomas), with the former being a precursor of the latter (Goldschmidt et al., 2011).

2) New histological entities are introduced and the marked malignant potential of some, such as micropapillary carcinoma, is highlighted (Goldschmidt et al., 2011).

3) It is pointed out that neoplastic myoepithelium can be malignant and, consequently, malignant myoepithelioma and carcinoma associated with malignant myoepithelioma are recognised as new histological entities (Goldschmidt et al., 2011).

4) For the first time in a classification system for mammary tumors to be used in veterinary medicine, it is suggested the use of IHC markers to identify the

luminal epithelial and myoepithelial components, since there are cases in which it is not easy to differentiate the two components through staining with HE (Goldschmidt et al., 2011).

Luminal epithelial proliferation is identified by the expression of the following IHC markers: cytokeratin (CK)8, CK18, CK19 and CK7; markers of myoepithelial cells are CK5/6, CK14, CK17, smooth muscle -actin, calponin, vimentin, and p63 (Beha et al., 2012b; Peña et al., 2014). Thus, in order to obtain a definitive diagnosis of the histological type of tumor according to this classification system, the use of immunohistochemical markers could be necessary, in addition to the routine histological examination with HE staining, to optimize the identification of the two components (Peña et al., 2014).

Interestingly Rasotto and colleagues have recently investigated the prognostic significance of canine mammary tumor histologic subtypes in a 2-year prospective study (Rasotto et al., 2017). The results pointed out that the currently applied classification of Goldschmidt and colleagues (2011) is an independent prognostic indicator identifying subtype-specific median survival times (MST) and local recurrence/distant metastasis rates. An excellent prognosis was associated with a histologic diagnosis of benign tumors and carcinoma arising in benign mixed tumors, and also complex carcinoma and simple tubular carcinoma were considered to have a good prognosis. The risk of tumor-related death was ten times increased for dogs bearing simple tubulopapillary carcinoma, intraductal papillary carcinoma, and carcinoma and malignant myoepithelioma, and an even more unfavorable prognosis was attributed to adenosquamous carcinoma, comedocarcinoma and solid carcinoma associated with a median survival time of 18, 14 and 8 months respectively. Adenosquamous carcinoma as well had the highest local recurrence rate. The worst outcome was that of anaplastic carcinoma and carcinosarcoma, both having a median survival time of only 3 months. The anaplastic and carcinosarcoma groups also had the highest metastatic rates (89% and 100%, respectively).

This study is the first to highlight that canine intraductal papillary carcinoma had a more favorable prognosis than that of other subtypes of carcinomas namely anaplastic carcinoma, carcinosarcoma, solid carcinoma, comedocarcinoma and adenosquamous carcinoma. Another interesting finding of this study is that tumor diameter (< 1 cm, between 1 and 2 cm, between 2 and 5 cm, and > 5 cm) can be a strong predictor of local recurrence/distant metastasis and an independent prognosticator of survival according to multivariate analysis. Excision margins predicted only the possibility of local

recurrence, but lymphatic invasion and histologic grade were predictive of local recurrence/distant metastasis and survival in univariate analysis (Rasotto et al., 2017).

Human inflammatory breast cancer is the most aggressive mammary cancer and its canine counterpart is represented by canine inflammatory carcinoma (IC), which has similar epidemiologic, histopathological and clinical features (Camacho et al., 2014; de Andrés et al., 2013; Marconato et al., 2009; Peña et al., 2003; Pérez Alenza et al., 2001; van Uden et al., 2015). CIC is a distinct form of mammary neoplasia and the diagnosis is achieved considering both the clinical presentation, characterized by signs of severe dermal erythema and edema, histologically associated with massive embolization of superficial dermal lymphatic vessels by neoplastic cells. It is the most aggressive and lethal type of mammary cancer in women and dogs with a fulminant clinical course. Some authors have hypothesised that this form of mammary neoplasia has a unique pathogenesis compared to other forms of canine mammary neoplasia: a distinct metastatic pattern (namely to the urinary bladder and reproductive tract) compared to mammary non-inflammatory carcinomas was pointed out (Clemente et al., 2010) and a high expression of vascular factors was seen (Camacho et al., 2014, 2013). CIC has been proposed as a valid spontaneous model to study human inflammatory breast cancer with a higher prevalence, necropsy availability and larger samples, the majority of which can be acquired prior to chemotherapy (Peña et al., 2003).

Table 1. Proposed Histologic Classification: 2010, according to Goldschmidt et al 2011.

Malignant Epithelial Neoplasms—Carcinomas	Carcinoma non-invasive (*in situ*) Carcinoma–simple a. Tubular b. Tubulopapillary c. Cystic-papillary d. Cribriform Carcinoma–micropapillary invasive Carcinoma–solid Comedocarcinoma Carcinoma–anaplastic Carcinoma arising in a complex adenoma/mixed tumor Carcinoma–complex type Carcinoma and malignant myoepithelioma Carcinoma–mixed type Ductal carcinoma Intraductal papillary carcinoma
Malignant Epithelial Neoplasms—Special Types	Squamous cell carcinoma Adenosquamous carcinoma Mucinous carcinoma Lipid-rich (secretory) carcinoma Spindle cell carcinomas a. Malignant myoepithelioma b. Squamous cell carcinoma–spindle cell variant c. Carcinoma–spindle cell variant Inflammatory carcinoma
Malignant Mesenchymal Neoplasms—Sarcomas	Osteosarcoma Chondrosarcoma Fibrosarcoma Hemangiosarcoma Other sarcomas
Carcinosarcoma—Malignant Mixed Mammary Tumor	
Benign Neoplasms	Adenoma–simple Intraductal papillary adenoma (ex duct papilloma) Ductal adenoma (ex basaloid adenoma) • With squamous differentiation (keratohyaline granules) Fibroadenoma Myoepithelioma Complex adenoma (adenomyoepithelioma) Benign mixed tumor
Hyperplasia/Dysplasia	Duct ectasia Lobular hyperplasia (adenosis) a. Regular b. With secretory activity (lactational) c. With fibrosis–interlobular fibrous connective tissue d. With atypia Epitheliosis Papillomatosis Fibroadenomatous change Gynecomastia
Neoplasms of the Nipple	Adenoma Carcinoma Carcinoma with epidermal infiltration (Paget-like disease)
Hyperplasia/Dysplasia of the Nipple	Melanosis of the skin of the nipple

Histological Grading

The most important information that can be gained from the histopathological examination of surgically excised tissue is relative to the prognosis (Matos et al., 2012; Morris, 2010). Histological grade is the most popular system for quantifying the histological malignancy of mammary carcinomas, since it is significantly associated with disease-free interval and survival and, at least for mammary cancer, is now considered essential information to be included in the histology report (Peña et al., 2013). It enhances the information already provided by the morphological-histological classification, complementing it (Karayannopoulou et al., 2005; Peña et al., 2013). Most grading systems of mammary tumors proposed for domestic animals are drawn from human medicine and are based on the application of the method by Elston and Ellis (EE) (Elston and Ellis, 2002; Goldschmidt et al., 2011; Karayannopoulou et al., 2005; Peña et al., 2013).When adapting a human histological grading system to pets, it is important to give relevance to the mentioned inter-specific differences among the histological types of mammary tumors: in bitches there is a clear prevalence of proliferation of both luminal epithelial cells and myoepithelial cells (complex tumors), while in the woman simple tumors prevail, characterized by luminal epithelial proliferation alone (Peña et al., 2013).

The most recent grading system for canine mammary tumors is that proposed in the study of Goldschmidt and colleagues (2011) and which has shown the best predictive value in the study by Peña and colleagues (2013) (Table 2).

With this system the following three parameters of the neoplasia are examined:

- glandular tubule formation;
- nuclear pleomorphism;
- mitotic count, evaluated in ten high-power fields (400x).

This last parameter is determined in the most mitotically active areas of the neoplastic tissue sample. A score is given to each parameter and the sum of the scores gives a total for the neoplasm, defining its grade of increasing malignancy: from well differentiated or grade 1, moderately differentiated or grade 2, to poorly differentiated or grade 3 (Peña et al., 2013). In the study by Karayannopoulou and colleagues, which assessed the application of the EE grading system, it was found that the 2-year post-operative survival of dogs with grade 3 carcinoma was clearly inferior to that of dogs with grade 2 or 1 tumors (Karayannopoulou et al., 2005). The system proposed by Peña and

colleagues takes into account the heterogeneity of canine mammary carcinomas, updating some aspects of the previous system, as discussed below. As far as complex and mixed neoplasms are concerned, “tubule formation” is evaluated only in epithelial areas and, in the case of tumors with a heterogeneous appearance, in the most malignant areas. The myoepithelium must not be evaluated because the neoplastic myoepithelial component, when present, is unlikely to display tubule formation, a feature which, if considered in the final score would erroneously indicate a poorly differentiated neoplasm (Peña et al., 2013). By convention, malignant myoepitheliomas are assigned an intermediate score of 2. The nuclear pleomorphism of complex and mixed neoplasms is assessed in all the malignant components. The importance of identifying the myoepithelial component is reiterated by this indication necessary to establish the histological grade of the carcinoma (Peña et al., 2014, 2013). Instead the mitotic count must be conducted at the periphery of the nodule or in the most mitotically active parts of the tumor and not only in luminal epithelial cells (Peña et al., 2013). Recently the great utility of this histological grading system was confirmed by Nguyen and colleagues who included grade 3 carcinomas as significant negative prognostic factor for overall survival (Nguyen et al., 2017).

Table 2. Criteria Histological Grading System for Canine Mammary Cancer. According to Peña et al. 2013.

TUBULES FORMATION	**NUCLEAR PLEOMORFISM**	**MITOSES PER 10 HPF** (diameter of a field= 0.55 mm)	
>75%	Uniform or regular small nucleus and occasional nucleoli	< 9 mitoses	**SCORE→ 1**
10%–75%, moderate formation of tubular arrangements admixed with areas of solid growth	Moderate degree of variation in nuclear size and shape, hyperchromatic nucleus, presence of nucleoli (some of which can be prominent)	10 - 19 mitoses i	**SCORE →2**
<10%, minimal or no tubule formation	Marked variation in nuclear size, hyperchromatic nucleus, often with more than 1 prominent nucleolus	> 20 mitoses	**SCORE → 3**
TOTAL SCORE	**GRADE OF MALIGNANCY**	Death related to mammary cancer during a follow-up period of 28 months	
3-5	I (low, well differentiated)	0%	
6-7	II (intermediate, moderately differentiated)	15.8 %	
8-9	III (high, poorly differentiated)	58.8 %	

Histological Staging

The histological stage, a term introduced by Gilbertson and colleagues in 1983 (Gilbertson et al., 1983), is the parameter through which the invasiveness of a tumor is evaluated. Histological stage 0 indicates a malignant, non-infiltrating neoplasm, i.e a tumor that does not breach the basement membrane on which the epithelium of the gland lies; at this stage the tumor is also called carcinoma *in situ*. Histological stages I, II and III represent malignant, infiltrating neoplasms that invade, respectively, local stroma (I) (Figure 2), regional lymph nodes (Figure 3) with or without the presence of neoplastic emboli (II) (Figure 4), or systemic metastases (III) (Gilbertson et al., 1983). The histological assessment, which is usually based on samples of the primary tumor and regional lymph nodes, cannot go beyond the classification of histological stage II. It is then delegated to the clinician to determine whether systemic metastases are present using further diagnostic investigations to classify the patient more correctly as having stage II (no systemic metastases) or stage III (presence of systemic metastases) (Gilbertson et al., 1983). Furthermore, it should be stressed that in order to appropriately classify a tumor as histological stage II, a careful cytological examination of the draining lymph nodes should be performed, prior to surgery, and/or a histological examination after surgical excision of the tumor. This information is essential for determining the spread of the neoplastic disease (Cassali et al., 2011). Although standard staining of the lymph node with HE enables the detection of most micrometastases, Matos and colleagues reported that staining lymph nodes with an anti-pancytokeratin antibody, which specifically identifies epithelial cells, revealed isolated malignant cells or microfoci of metastatic cells in almost 10% of the lymph nodes examined, which had been considered to be free of metastases (Matos et al., 2012). Faced with a diagnosis of a malignant, infiltrating epithelial tumor, it is therefore desirable to perform immunohistochemical studies on the lymph nodes to detect metastatic epithelial cells in samples considered negative according to the first screening with HE (Figure 5) (Carolyn, 2014; Cassali et al., 2011; Matos et al., 2012). It should be mentioned that although some studies (de Araújo et al., 2015; Szczubiał and Łopuszynski, 2011) have revealed that the presence of macrometastases (>2 mm) in regional lymph nodes is a negative prognostic factor, there are conflicting data concerning the presence of micrometastases (metastatic foci with a diameter between 0.2 and 2 mm) and isolated tumor cells (groups of cells with a diameter <0.2 mm). As for the macrometastases, the already mentioned study by Szczubiał and Łopuszynski

highlighted that the post-operative survival of bitches differed only between the group of animals without metastases and the group in which the animals had evident lymph node macrometastases (Szczubiał and Łopuszynski, 2011). On the other hand Araújo and colleagues confirmed the prognostic importance of finding macrometastases, particularly if their diameter exceeded 7 mm, and more than one lymph node was involved. However, these authors noted that the presence of isolated tumor cells, identified unequivocally by immunohistochemical studies, was associated with a shorter survival time when compared to that of animals without metastases, and probably these neoplasms had a more aggressive histological subtype. Thus, the importance that should be given to lymph node micrometastases is presently uncertain (de Araújo et al., 2015; Szczubiał and Łopuszynski, 2011).

Figure 2 - Bitch, simple tubulo-papillary mammary carcinoma. Histological stage I indicates tumors that are locally invasive; in this field it is clear that extensions of neoplastic cells (indicated by the arrows) are invading the surrounding stroma. (Haematoxylin-eosin, 40X). (From: Levi et al., 2016).

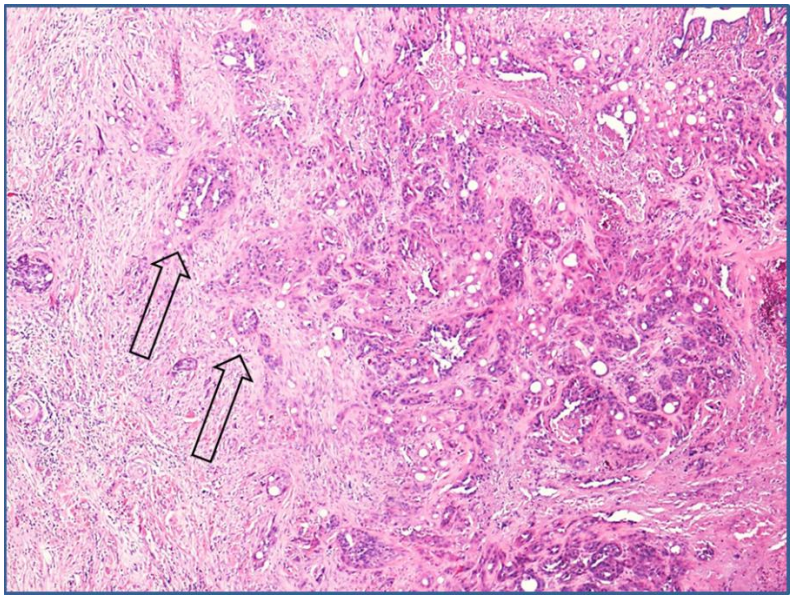


Figure 3 - Bitch, simple tubulo-papillary mammary carcinoma. Histological stage II indicates those tumors with neoplastic emboli and/or metastases in regional lymph nodes. Carcinomas metastasise predominantly via the lymphatic system: panel (a) a large embolusinside a vessel; panel (b) numerous emboli within the lymphatic system (arrows), but no embolization in blood vessels (empty arrows). (Haematoxylin-eosin, 100X). (From: Levi et al., 2016)

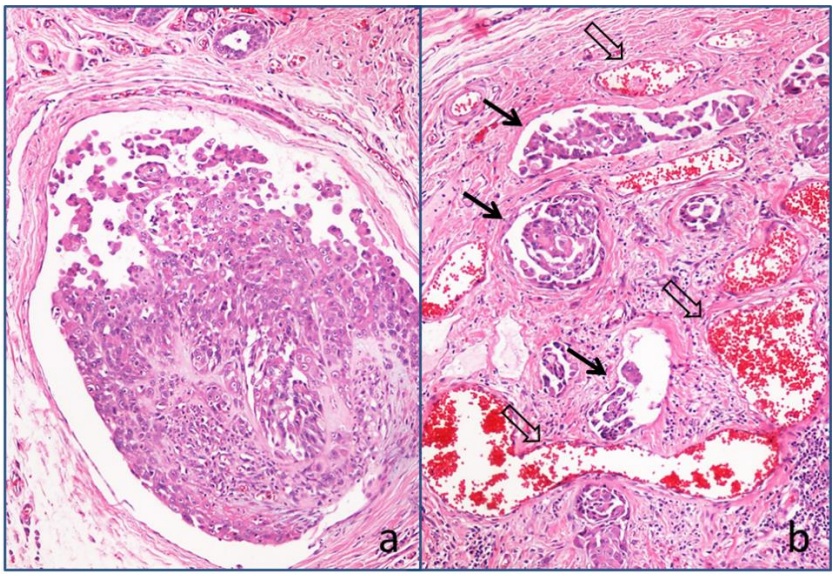


Figure 4 - Bitch, inguinal lymph node. Histological stage II refers to those tumors with neoplastic emboli and/or metastases in regional lymph nodes. In the right of the figure there is an evident metastasis (indicated by the arrow) of a mammary carcinoma expanding the draining lymph node. Two hyperplastic lymphoid follicles can be seen in the left of the figure. (Haematoxylin-eosin, 40X). (From: Levi et al., 2016)

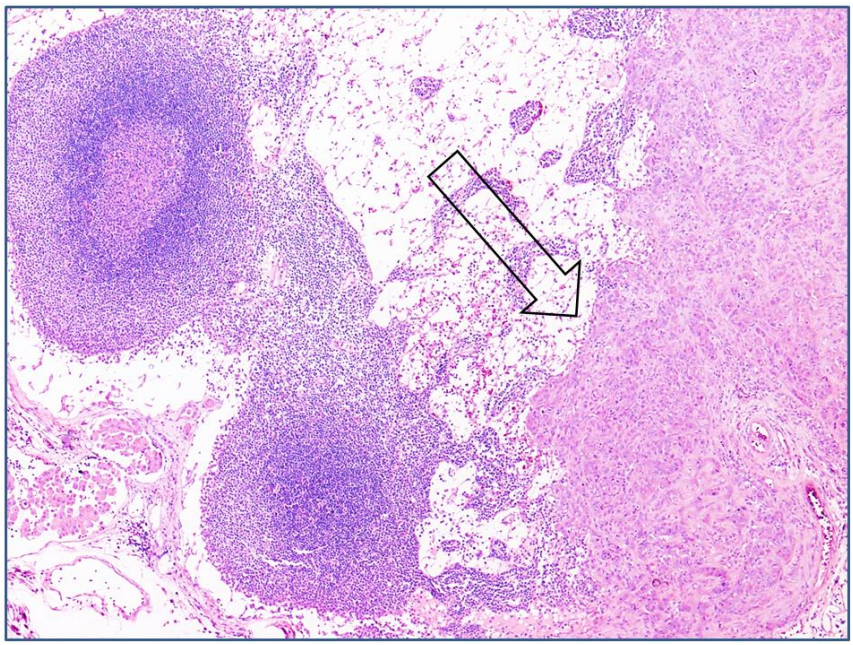
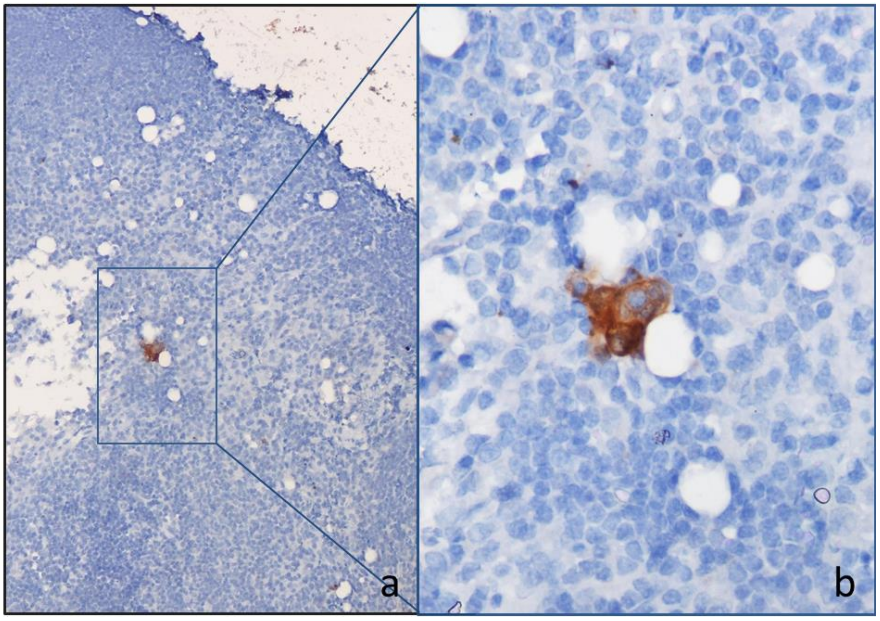


Figure 5 - Bitch, inguinal lymph node excised during mastectomy. IHC staining performed with an antibody against pancytokeratin shows a microfocus of metastatic neoplastic epithelial cells at low (a) and high magnification (b). (DAB stain, counterstaining with Papanicolaou haematoxylin; a:100X, b:400X). (From: Levi et al., 2016).



Phenotypic Classification of Canine Mammary carcinomas

Similarly to breast cancer in women, mammary carcinomas in bitches and queens constitute a heterogeneous group of tumors with regards to both morphology and biological behavior, independently of histological type (Abadie et al., 2017; Brunetti et al., 2013; Gama et al., 2008; Peña et al., 2013). Studies of the gene expression profile of breast cancer have identified molecular subtypes with different pathobiology and prognosis (Kumar et al., 2014; Perou et al., 2000; Sørlie et al., 2001a; Yang et al., 2007). Gene expression profiling is considered the gold standard for identifying subtypes of breast cancer, but it is impractical for routine clinical use and cannot be applied to tissue samples embedded in paraffin. For this reason it has been suggested to use a panel of immunohistochemical markers to divide the various carcinomas into subgroups mirroring those determined by gene expression studies. The suggested antibody panel contains antibodies targeting estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 2 (HER2), CK5/6 and CK14, enabling tumor subtypes to be defined as luminal-like if they express receptors for either estrogen or progesterone, and non-luminal-like if they do not express hormone receptors (Kumar et al., 2014). This latter category is further divided into HER2-overexpressing tumors, which overexpress the HER2 receptor (an important receptor for the corresponding growth factor, often amplified in malignant neoplasms), and basal-like tumors which have a phenotype similar to basal cells; finally, the normal-like subtype comprises the neoplasms that are negative for all three markers (Perou et al., 2000; Sørlie et al., 2001). This classification has been consistently used in human medicine: each subtype has a different prognosis, requires different treatment and has a different metastatic pattern. The non-luminal-like subtypes are those with the more aggressive clinical behavior (Kumar et al., 2014).

Being able to apply this new molecular-based system of classification to mammary tumors in veterinary medicine would be profitable: the possible role that each subtype would have as a prognostic indicator independently of the histological subtype, and more reliably, given the already described limitations of histological typing for prognostic purposes, it would open the way to a targeted treatment (Abadie et al., 2017; Peña et al., 2014; Sassi et al., 2010). In fact, in human medicine the cornerstone of treatment of breast cancers with a luminal phenotype (expressing hormone receptors) is inhibition of the ER through targeted therapy (estrogen modulators, aromatase inhibitors) (den Hollander et al., 2013). In contrast, for those tumors that show amplification of

the *HER2* gene and/or overexpression of the corresponding protein HER2, pharmacologically active molecules that selectively target this glycoprotein have been developed (for example, Trastuzumab-Herceptin™) (Tebbutt et al., 2013; Wolff et al., 2013).

The same immunohistochemical panel used for breast cancer in women can also be used for mammary tumors in bitches and queens in veterinary medicine and, overall, the results have confirmed that the known molecular phenotypes of breast cancer also exist in these species (Abadie et al., 2017; Beha et al., 2012a; Brunetti et al., 2013; Gama et al., 2008; Sassi et al., 2010). Furthermore, clinically relevant data distinguishing the various phenotypes have been obtained: Sartin and colleagues correlated the absence of receptors for estrogen and progesterone in canine mammary carcinoma with shorter survival (Sartin et al., 1992). In bitches ER has a lower expression in neoplastic mammary tissue than in the corresponding normal tissue (Beha et al., 2012a), therefore the bitch could be a good animal model and could share characteristics with hormone-dependent breast cancer in women (Abadie et al., 2017; Beha et al., 2012a; Brunetti et al., 2013). Few studies have so far provided detailed information on the prognostic significance of the different phenotypes of canine and feline mammary carcinomas; however a recent study by Abadie and colleagues has enlightened the utility of the immunophenotypes. In this study 350 invasive CMCs was classified according to two panels used in human oncology, proposed by Nielsen and by Blows that require the IHC evaluation of ER, PR, Ki-67, CK 5/6, EGFR and HER2 and already proved to be of prognostic value (Blows et al., 2010; Nielsen et al., 2004). The algorithm adapted for invasive CMCs is reported in Figure 6 and 7. Strong prognostic significance has been demonstrated for both the classification systems: luminal A or luminal 1+ showed a significantly longer disease-free interval, OS and specific survival, compared to triple-negative carcinomas (adjusted for stage). Triple-negative CMCs largely predominated (76%) and showed a more aggressive behavior than the human counterpart, while no HER2-overexpressing CMCs were observed in that caseload (Abadie et al., 2017). The presence of HER2 overexpressing mammary tumors in bitches is at present doubtful because the amplification HER2 gene in CMCs by a reliable technique (i.e. *in situ* hybridization) is missing (Abadie et al., 2017; Burrai et al., 2015; Mulas et al., 2003). Burrai and colleagues have exhaustively investigated the present issue in a detailed study using multiple molecular approaches including the detection of HER2 protein by antibody-based, transcriptomic and mass spectrometry analysis. HER2 mRNA expression was determined by

quantitative real-time (qRT) PCR, but no significant difference between benign and malignant tumors was noticed. The IHC results suggested a lack of specificity of the commonly used antibody in CMT samples and the need of further investigations to carefully assess the diagnostic and biological role of HER2 in CMTs (Abadie et al., 2017; Burrai et al., 2015).

Figure 6 – Algorithm of immunophenotypic classifications of canine mammary carcinomas adapted from Blows. (Modified from Abadie et al., 2017).
A panel of 5 immunohistochemical markers (ERα, PR, HER2, CK5/6, and EGFR) can define the immunophenotypes of CMCs.

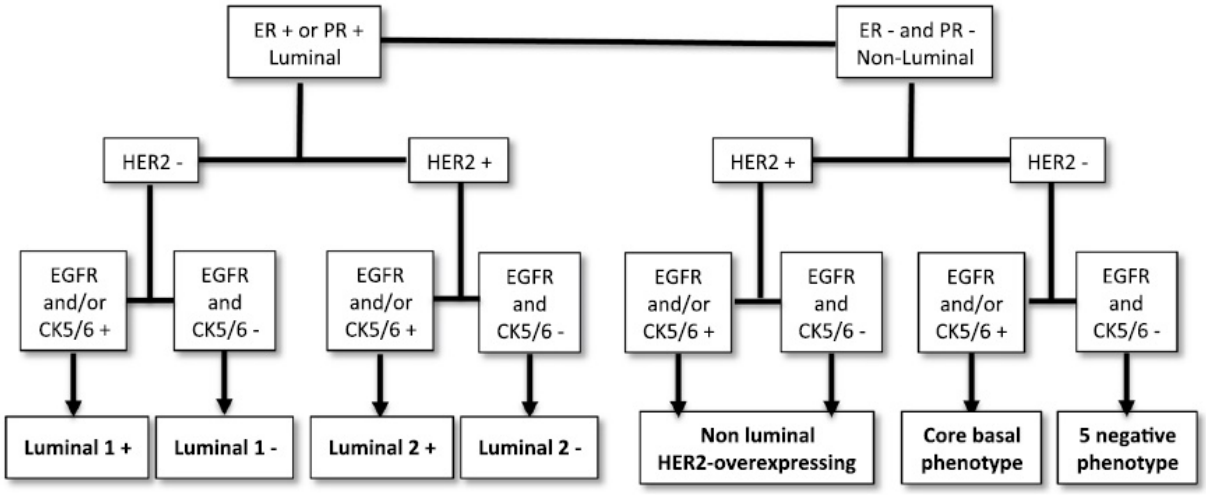
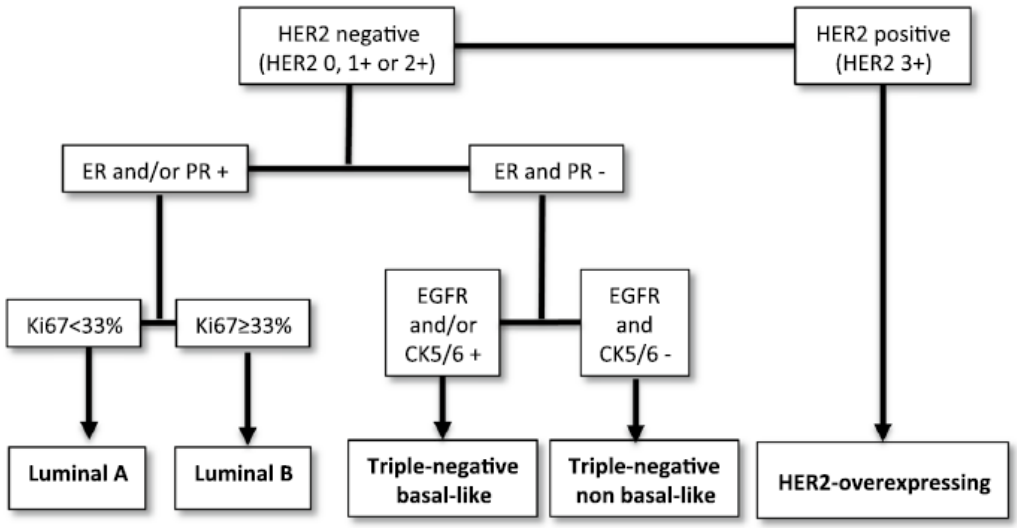


Figure 7 – Algorithm of immunophenotypic classifications of canine mammary carcinomas adapted from Nielsen (Modified from Abadie et al., 2017).
A panel of 6 immunohistochemical markers (ERα, PR, HER2, Ki-67, CK5/6, and EGFR) can define the immunophenotypes of CMCs.



As in human medicine, determining the phenotypic expression pattern of the steroid hormone and HER2 receptors in animal carcinomas could be relevant for selecting those individuals that would benefit from targeted treatment with estrogen modulators and/or aromatase inhibitors (those with luminal-type carcinomas) or with antibodies (e.g. Trastuzumab) (those with an HER2-overexpressing tumor) (Carolyn, 2014; de Las Mulas et al., 2005; Gardner et al., 2016; Novosad, 2003; Peña et al., 2014). In fact this strategy is widely used in women, whereas in veterinary medicine it is still underused, in part because of the relative difficulty and cost of performing the investigations necessary to determine receptor expression. Antibody therapy is the most used in women at present in the treatment of HER2-overexpressing breast cancer, but the limitations to translating this therapy to bitches are its cost and the fact that information on the homology of antigen expression between women and bitches is still relative scarce; further studies to clarify this issue are, therefore, necessary (Carolyn, 2014; Novosad, 2003). A study by Singer and colleagues showed a high degree of homology between the human and canine tumor-associated antigens ERBB1 and HER2, and the in vitro demonstration that the antibodies significantly inhibited the proliferation of canine mammary tumor cell lines, opened the way to useful in vivo applications (Singer et al., 2012). In their studies regarding the molecular phenotype of canine and feline mammary carcinomas, Brunetti and colleagues and Beha and colleagues have extended their investigation from the primary tumor, to regional and systemic metastases, determining whether or not the molecular phenotype was concordant in the different sites (primary tumor and metastasis) (Beha et al., 2015, 2014, 2012a; Brunetti et al., 2013). Since these studies have revealed a significant percentage of non-concordance (35% in bitches) between the phenotype of the primary tumor and metastases in the regional lymph nodes, a consequence is that the therapy based on a phenotypic analysis of only the primary tumor can be unsuccessful in discordant cases. The most frequent phenotypic change between the primary tumor and lymph node metastases in the bitch is from luminal-like to non-luminal-like, while in the queen it is from luminal-like to HER2- overexpression (Beha et al., 2015, 2014). A comparison of the phenotype of primary tumors with that of systemic metastases also revealed a certain level of discordance, although the prevailing phenotype in metastatic sites appeared to be the HER2 one, which is a particularly aggressive phenotype; this prevalence is probably the result of a selection of tumor cell clones with a greater capacity for metastatic spread (Beha et al., 2015, 2014).

Prognostic Markers

The molecular pathways associated with the development of mammary tumors have been investigated in the dog for identifying risk-associated genetic aberrations and CMTs seem to mimic breast cancer since many similarities are found at the molecular level (Gardner et al., 2016; Klopfleisch et al., 2011; Queiroga et al., 2011). In CMT, the use of prognostic markers is presently used only for research purposes (Gardner et al., 2016).
Proliferation activity has been successfully estimated and repeatedly applied in several studies on CMT evaluating PCNA, Ki67, and AgNOR (Klopfleisch et al., 2011). Quantification of AgNOR and PCNA and Ki67 IHC index has established a statistically significant association between tumor malignancy and survival of the patient affected by CMT (Bundgaard-Andersen et al., 2008; Löhr et al., 1997). Nguyen and colleagues have recently evidenced that in invasive CMSs a high Ki67 proliferation index (> 33.3%) and the absence of expression of the epidermal growth factor receptor were prognostic factors significantly associate to the OS (Nguyen et al., 2017).
It was generally assumed that steroid receptor (ER and PR) influence canine mammary carcinogenesis because of the observation that dogs spayed at a young age have a smaller prevalence of mammary tumors (Sorenmo et al., 2000). Recently a significant prognostic factor associated to shorter overall survival was ERα negativity (Nguyen et al., 2017), whereas in the past other studies had correlated the absence of ER protein expression levels with a higher grade of malignancy (de Las Mulas et al., 2005; Millanta et al., 2005).

The study by Rivera and colleagues demonstrated that germline mutations in BRCA1 and BRCA2 are significantly associated with mammary cancer in English springer spaniel, a breed that is considered predisposed to the development of CMT (Rivera et al., 2009). Human and canine gene expression data have been compared by analyzing the expression levels of about ten thousand dog/human orthologous genes (Uva et al., 2009). The authors observed a significant genes overlap (i.e. PI3K/AKT, KRAS, PTEN, WNT-beta catenin and MAPK cascade) deregulated in mammary neoplasia of the dog and human, therefore confirming the suitability of the dog as a model for breast cancer (Rivera et al., 2009).
On the other hand Klopfleisch and colleagues have pointed out that the combined expression patterns of BMP2, LTBP4, and DERL1 (Derlin-1) can

correctly distinguish benign and malignant neoplastic transformation in laser-microdissected tumor cells for each individual tumor (Klopfleisch et al., 2010). A multivariate survival study assessed the independent prognostic value of several immunohistochemical markers in CMT, in both neoplastic mammary cells and in stromal fibroblasts, being the tumor-stroma crosstalk a well-recognised important factor in the development of the neoplasia (Egeblad et al., 2010). MMP-9 and Ki-67 emerged as useful prognostic markers and the high stromal expressions of uPA and MMP-9 in aggressive tumors suggest them as a potential therapeutic target in the post-operative treatment (Santos et al., 2013).
Loss of HER2 expression in CMT, together with loss of ER expression and positivity for basal markers, namely P-cadherin, p63, CK5, has been associated with a poor prognosis (Gama et al., 2008), as already mentioned in the section regarding the molecular classification of canine mammary carcinomas (Gardner et al., 2016).
E-cadherin is a transmembrane glycoprotein with an extracellular domain promoting cellular adhesion, and an intracellular domain that interacts with α-, β- e γ- catenin (Klopfleisch et al., 2010). In mammary tumors the loss of E-cadherin, normally expressed by ductal and acinar epithelial cells, correlated with the histological grade has been shown to promote metastases (Brunetti et al., 2003; Gama and Schmitt, 2012).

Traditionally human research paves the way to investigation in veterinary medicine that reproduces studies previously performed in the human. However, there are some noteworthy exceptions: for example the investigation regarding the enzyme Cox-2 that catalyzes the synthesis of prostaglandins from arachidonic acid has been investigated for the first time in CMT, where a higher expression of Cox-2 has been found in CIC and it was correlated with the unique "inflammatory phenotype" of this neoplastic entity (Queiroga et al., 2011, 2005). The same authors studied the role of the GH/IGF-I axis and observed that local production of GH by mammary gland tissue was induced by progestin stimulation. Even if the role of progestins in triggering GH production in normal and neoplastic mammary gland remains to be clarified. This interesting study enlightens newer research themes in human disease; in fact the production of GH at the mammary gland level was discovered for the first time with this study regarding the dog, while it was still unidentified in human medicine (Queiroga et al., 2011, 2008).

VETERINARY ONCOLOGY – Chemotherapy in Dogs

The same German chemist, Paul Ehrlich, who is considered "the father of chemotherapy", was also a pioneer in understanding the usefulness of animal models in studying drug molecules with a potential anti-cancer activity (Lawrence et al., 2015). The treatment of cancer in veterinary medicine has coevolved with the treatment of cancer in people and, beyond the immediate animal cure, the greatest opportunity for veterinarians is the contribution in advancing the understanding of cancer biology, prevention, and treatment. Spontaneously developing neoplasia in pets is in fact an excellent opportunity of comparative oncology (Gardner et al., 2016; Gustafson and Page, 2013). The recent study by Nguyen and colleagues (2017) highlights how canine spontaneous cancers seem particularly relevant to human oncology for investigating the pharmacokinetics of innovative therapeutic drug *in vivo,* the pathologic response and the interactions between tumor cells and their microenvironment and the developing of metastases and survival time in the canine patient (Nguyen et al., 2017).

The prevalence of cancer in companion animals continues to rise and cancer remains a major cause of companion animal morbidity and mortality and is correlated to the longer life of our pets (Cooley et al., 2003; Dobson et al., 2008; Gustafson and Page, 2013; North and Banks, 2009).

The science of veterinary oncology is growing, with ever-increasing numbers of clients seeking treatment, and veterinarians bear the responsibility to provide the best care for their patients in respect to tumor diagnosis and treatment; in doing so veterinarians commit themselves to strengthen and honor the human-animal bond (North and Banks, 2009).

Surgery, radiation therapy and chemotherapy are conventionally the three main methods of cancer treatment (Dobson et al., 2008). Chemotherapy is the treatment of choice when facing systemic malignancies like hematopoietic tumors and metastatic cancers (Zandvliet and Teske, 2015).

The prerequisite to treatment selection is a definitive tumor diagnosis, by histopathology and the assessment, through the clinical staging, of the extent of the disease in terms of local invasion at the primary site, lymph node involvement and distant metastasis (North and Banks, 2009).

The effectiveness of the treatment is related to the type, grade and extent of the neoplastic process. It is important to identify any concurrent diseases in the oncologic patient because they could undermine the tolerance and effectiveness of the treatment (Dobson et al., 2008). The goal of an oncologic treatment is a

complete eradication of all tumor cells, including stem cells, but unfortunately even with the most effective protocols currently available this aim is not achieved in many case (Dobson et al., 2008). In veterinary medicine the response to the treatment is established on the basis of the clinical response, but there are other methods that evaluate the absence of neoplastic cells in biological samples (cytogenic response) and the disappearance of the DNA of neoplastic cells using PCR-based techniques (Zandvliet and Teske, 2015).
Frequently the oncologist has to face a discrepancy between the expected effect (efficacy) of a therapy and the observed effect (effectiveness), with the latter sadly lower than the expected efficacy. In fact while expected efficacy is based on laboratory tests or highly controlled clinic trials, the real effectiveness of the treatment is often undermined by concurrent diseases of the patient, environmental factors, iatrogenic factors (i.e. the use of the most appropriated drug and the correct dosage and administration) and decisions taken by the clinician and patient-owner. In these circumstances the failure should not be attributed to the onset of a chemoresistant tumor even if from the clinical point of view the outcome looks the same (Zandvliet and Teske, 2015).
Gustafson and Page (2013) define adjuvant therapy "the treatment with chemotherapeutic drugs following the surgical removal or radiation control of the primary tumor". The finality is to act against occult disease with a systemic exposure to the drug.
Primary or neoadjuvant therapy is defined as "the utilization of chemotherapeutic drugs prior to treatment with other modalities, primarily surgical removal of the primary tumor, with the intent of decreasing tumor size for increased control and preventing possible postoperative growth of micrometastasis."
Palliative chemotherapy consists of a therapeutic protocol "delivered to decrease clinical signs in the case of unresectable or disseminated disease that is associated with functional disturbances or pain." This cure is given to optimize symptom control, improve quality of life, and sometimes to improve survival, but not for curing the neoplasia (Gustafson and Page, 2013; Roeland and LeBlanc, 2016).
The owner must always be informed whether the intention of treatment is curative or palliative (Dobson et al., 2008).

The phenomenon of tumor resistance or selection of cells resistant to chemotherapeutic drugs is recognized in veterinary oncology and is one of the

major mechanisms of therapeutic failure during cancer drug therapy (Gustafson and Page, 2013; North and Banks, 2009; Zandvliet and Teske, 2015).
Recently two reviews, one by Klopfleisch and the other by Zandvliet and Teske have been published to highlight the most important acquisitions regarding MDR in veterinary oncology (Klopfleisch et al., 2016; Zandvliet and Teske, 2015).
Tumors that often show intrinsic resistance include melanomas, soft tissue sarcomas and gastric carcinomas, therefore these tumor entities are rarely considered good candidates for traditional chemotherapy, because there is often a poor response right from the start treatment (North and Banks, 2009).
Acquired resistance by neoplastic cells, after an initial response to the chemotherapeutic drug, is a major complication in the treatment of lymphoma (North and Banks, 2009).
However, the distinction between intrinsic and acquired MDR is fictitious because, even if the majority of neoplastic cells respond at first positively to the treatment, a niche of cancer-initiating stem cells bearing intrinsic capacities of escape the drug molecule can then proliferate resulting in recurrence of the neoplasia that clinically appear as secondary onset of a resistant neoplasia (Zandvliet and Teske, 2015).
Some authors have stated that a possible reason for treatment failures obtained with chemotherapy in dogs with multicentric lymphoma could be related by induction of ABC-transporters prior to chemotherapy when the patients were pretreated with glucocorticoids (Gavazza et al., 2008; Piek et al., 1999; Price et al., 1991). In fact in dogs as in human beings, prednisolone is a substrate of P-gp, and it could induce the upregulation of this transporter. However the induction of P-gp has been seen in lymphocytes of the lamina propria from dogs affected by inflammatory bowel disease (Allenspach et al., 2006), but a similar induction has not yet been demonstrated for cytotoxic drugs or in neoplastic cells (Zandvliet and Teske, 2015).
Treatment regimens which combine several different drugs are often more effective than single agents and may delay or avert the onset of clinical drug resistance (Dobson et al., 2008). In order to overcome both natural and acquired resistance of tumor cells and to limit high dose-associated side effects of chemotherapy with a single agent, protocols based on the combination of chemotherapeutic drugs have been established. In the human, combination chemotherapy has been shown to possess a curative effect, not present with single-agent therapy, in the case of acute lymphocytic leukemia, Hodgkin's

disease, histiocytic lymphoma, and testicular carcinoma (Gustafson and Page, 2013).
In canine oncology the curative effectiveness of combination therapy is well exemplified by lymphoma, a neoplasia for which a complete remission can be often achieved (North and Banks, 2009). Doxorubicin is the most active single-agent therapy against canine lymphoma; but combinations of doxorubicin with cyclophosphamide, vincristine, and prednisone result increase both the median remission and median survival times (Gustafson and Page, 2013; Keller et al., 1993).

A viable way to test the sensitivity of neoplastic cells to a drug and therefore its efficacy is the observation of tumor cell line panels associated with a given histotype, when treating them in the culture medium with single or multiple drug molecules. This screening model was recognized as a rich source of information about the mechanisms of growth inhibition and tumor-cell kill (Gustafson and Page, 2013). Human oncology and cancer research can draw information about drug sensitivity and genotypic characteristics from the NCI60 human tumor cell line panel and other public databases (Shoemaker, 2006; Thamm et al., 2012). In veterinary oncology the use of canine tumor cell line panels to screen drug sensitivity has become a way to identify potential drug combinations. Further studies are warranted to assess direct correlations between in vitro and in vivo efficacy (Berlow et al., 2013; Donnelly et al., 2004; Simon et al., 2001).
Simon and colleagues tested in vitro drug efficacy of chemotherapeutics in cultures of mammary gland tumors obtained from dogs. They found significant variations in susceptibility. Cultures varied in their pattern of susceptibility of tumor cells with doxorubicin vs platinum derivatives (cisplatin and carboplatin) while they did not detect differences in the in vitro susceptibility among the histologic subtypes of tumors (Simon et al., 2001).

Many issues are still debated regarding the contribution of ABC-transporters to the clinical MDR-phenotype in oncologic canine therefore further studies are warranted (Zandvliet and Teske, 2015).

Chemotherapy in Canine Mammary Tumors

At present there is a paucity of high-quality trial evidence that guide the veterinary oncologist in the cure of dogs with malignant high-risk mammary tumors (Sorenmo et al., 2013).
The efficacy of a systemic treatment of mammary tumors has still to be confirmed with studies that meet the standard of evidence based medicine. Systemic therapy has been recommended in dogs with histologically aggressive tumors, lymph node metastases and large tumors, for which surgery alone is not considered curative (Nguyen et al., 2017; Sorenmo et al., 2013).

The clinical staging is therefore of great importance for the canine patient affected by mammary neoplasia and is considered a well-established powerful prognostic factor. The original staging system was published in 1980 by Owens (Owens, 1980). Veterinary oncologists at present use the modified version of Rutteman and colleagues (Rutteman et al., 2001). This staging system should be used for dogs with mammary carcinomas (noninflammatory), but not sarcomas (Sorenmo et al., 2013)
These classifications are all based on the TNM, where: T describes the tumor size; N lymph node metastasis; and M distant metastasis. Stage advances from I to II to III as the size of the primary tumor increases from smaller than 3 cm, to between 3 and 5 cm, to larger than 5 cm (Sorenmo et al., 2013).
The survival of the dog has been strongly correlated to the tumor diameter at time of diagnosis (Rasotto et al., 2017; Sorenmo et al., 2009) and the presence of metastases (de Araújo et al., 2015). In the most recent study tumor size larger than 2 cm and positive nodal stage were reported to be significant prognostic factors for OS (Nguyen et al., 2017).

Surgical excision is still the choice treatment for all CMT except the ones already metastatic at time of diagnosis and inflammatory carcinoma (Goldschmidt et al., 2017; Sorenmo et al., 2013). Surgical approaches include nodulectomy, mammectomy, regional mastectomy, unilateral or bilateral mastectomy, as well as lymph node removal for staging. Considering the possible transformation of benign neoplastic mammary tumors into malignant ones, all the detectable masses should be excised (Sorenmo et al., 2009). In the case of canine invasive mammary carcinomas surgery alone does not assure a long survival. In the study by Nguyen and colleagues (2017), which analyses the natural history of 350 invasive CMSs for a 2-year-outcome (the most

numerous caseload available in literature), the overall survival after mastectomy was 11 months, and 41.5% of dogs died from their mammary carcinoma within 1 year post-mastectomy (Nguyen et al., 2017).

Considering that mammary tumors often exhibit steroid hormone dependence, surgical ovariohisterectomy or ovariectomy is often performed and acts as hormonal systemic therapy with the potential of significantly reduced recurrence and prolong survival, similar to the human. In human medicine the cornerstone of treatment of breast cancers with a luminal phenotype (expressing hormone receptors) is inhibition of the ER through targeted therapy (estrogen modulators, aromatase inhibitors) (den Hollander et al., 2013). In fact this strategy is widely used in women whereas in veterinary medicine it is still underused, in part because of the relative difficulty and cost of performing the investigations necessary to determine ER expression. The best known estrogen modulator is Tamoxifen which has both estrogenic and anti-estrogenic effects (Carolyn, 2014). This molecule has been the most widely used drug in women with ER-positive breast cancer (den Hollander et al., 2013). The effects of hormone therapy on canine mammary tumors are not yet completely clear: in one pilot study it was found that five of seven animals with metastases or surgically inoperable tumors had a reduction in tumor size after tamoxifen administration, but the same study highlighted the severe side effects of Tamoxifen consisting of high risk of development of pyometra and retinitis (Novosad, 2003; Tavares et al., 2010). Chang and colleagues found that ovariohysterectomy was more beneficial in dogs with complex carcinomas than in dogs with simple carcinomas; in fact dogs with aggressive and more undifferentiated mammary carcinomas are less likely to have ER-positive tumors, thus they are less likely to benefit from hormonal ablation (Chang et al., 2005; Sorenmo et al., 2013).

The real efficacy of the administration of chemotherapy in mammary tumors is still weak (Sorenmo et al., 2013).
The already mentioned study by Tran and colleagues compared 58 dogs bearing canine mammary carcinoma surgically treated with 36 dogs treated with surgery and adjunct chemotherapy (Tran et al., 2016). In dogs with stage IV or lymphatic invasion there was no statistically significant improvement of median survival time for the group treated with adjunctive chemotherapy (chemotherapy 228 days; none 194 days). Interestingly five cases with

complete surgical margins that were administered mitoxantrone and carboplatin had a mean survival of 1139 days (Tran et al., 2016).

A significant benefit from a chemotherapeutic protocol consisting of a combination of 5-fluorouracil and cyclophosphamide was seen in a prospective nonrandomized study regarding 16 dogs with high-risk mammary tumors (stage III or IV according to WHO staging system). Survival analysis indicated that the chemotherapeutic regimen had a positive influence on the disease-free interval and the survival time of the eight bitches treated with adjuvant post-operative chemotherapy compared to the eight dogs treated with surgery alone (Karayannopoulou et al., 2001).

Anthracycline or taxane combinations have shown their efficacy in the treatment of breast cancer (Nabholtz et al., 2003). Taxanes are a family of microtubule inhibitors that suppress spindle microtubule dynamics (Khanna et al., 2015). A novel formulation of paclitaxel was recently given conditional approval by the US Food and Drug Authority (FDA) Center for Veterinary Medicine for use in dogs with resectable and nonresectable squamous cell carcinoma and nonresectable stage III, IV and V mammary carcinoma. The conditional approval implies that this new drug is safe in accordance with the full FDA approval standard and that there is reasonable expectation of its effectiveness (Khanna et al., 2015).

In veterinary oncology a study regarding the use of doxorubicin and docetaxel in 31 dogs with malignant mammary gland tumors of histologic stages II and III has showed no beneficial effect (Simon et al., 2006). Another study about adjuvant gemcitabine in dogs with advanced stage (IV or V) mammary carcinoma did not establish any benefit (Marconato et al., 2008).Though additional, randomized prospective, adequately powered trials are necessary (Sorenmo et al., 2013).

Survival of dogs with inflammatory carcinoma has been successfully improved using nonsteroidal anti-inflammatory drugs (NSAIDs) combined with chemotherapy (piroxicam; piroxicam and carboplatin; piroxicam and doxorubicin; piroxicam and capecitabine; piroxicam and cisplatin) (Marconato et al., 2009). According to the results of another study, piroxicam should be considered as a single agent for the treatment of dogs with inflammatory mammary carcinoma, and should be preferred to the doxorubicin-based protocols (de M Souza et al., 2009).

Interestingly desmopressin, a vasopressin peptide analog with hemostatic properties, could minimize the spread and survival of residual mammary cancer

cells when administered during surgical excision of the primary tumor (histologic grade 2 or 3 carcinoma) (Hermo et al., 2011).

P-glycoprotein in the Dog

As already mentioned, P-gp is the best-known ABC-transporter and play a key role in efflux-mediated drug resistance (Borst and Elferink, 2002; Chen et al., 2016; Fletcher et al., 2010). Canine transcript of canine *mdr1* shows a 93% sequence homology to the human (Steingold et al., 1998). The physiologic distribution of P-gp has been described using IHC and RT-qPCR in hepatocytes, bile duct epithelial cells, pancreatic ducts, adrenal cortex, endothelial cells, especially at brain level and a milder expression was found is stomach, small intestine and colon, alveolar and bronchiolar epithelium, and both B- and T- lymphocytes (Conrad et al., 2001; Ginn, 1996). Normal canine mammary gland showed mild immunoreactivity (Badowska-Kozakiewicz and Malicka, 2010; Petterino et al., 2006), while the mammary gland epithelium of women physiologically expresses P-gp, especially the epithelium of the ducts, for the physiological activity of this excretory organ (Maliepaard et al., 2001; Pavelic et al., 1993).
In 2001 iveremectin-induced neurotoxicosis, a severe clinical concern for the Scottish collie dog, was discovered and has been attributed to a deletion of the *mdr1* gene, resulting in the production of a truncated, nonfunctional P-glycoprotein (Culmsee et al., 2004; Mealey et al., 2001). Depending on the homozygosis or heterozygosis of the mutation, Collies, but also Shetland sheepdog, Old English sheepdog, White German sheepdog, English shepherd, Long haired wippet, Australian cattle dog, Australian shepherd and Silken windhound bear the hypersensitive phenotype (Dowling, 2006; Neff et al., 2004). For many years veterinarians have treated with caution those patients that were included in this group and the common sense suggested to avoid the administration of ivermectin and similar compounds - “white feet, don’t treat”- . Nowadays an assay is available to screen dogs for the expression of P-gp (Mealey et al., 2001). From the oncologist’s point of view the usefulness of the test consists in recognizing the subjects bearing increased toxicity to chemotherapy drugs associated to absent or inferior expression of functional P-gp (Culmsee et al., 2004; Gramer et al., 2015; Mealey et al., 2001).

In veterinary medicine the role of ABC-transporters in multidrug resistance is still poorly explored (Zandvliet and Teske, 2015).
The first IHC study by Ginn and colleagues evidenced a strong P-gp immunolabeling in canine hepatomas, colorectal adenomas, colorectal carcinomas, adrenal cortical adenomas, hemangiopericytomas, apocrine gland

adenocarcinomas, transitional cell carcinomas. Differently, malignant lymphomas, malignant melanomas, leiomyosarcomas, mammary gland carcinomas, mammary gland adenomas, squamous cell carcinomas, basal cell tumors, apocrine gland adenomas, cholangiocarcinomas and thyroid gland carcinomas gave variable results (Ginn, 1996). After this, IHC detected P-gp expression in other various tumors of the dog (Badowska-Kozakiewicz and Malicka, 2010; Bergman et al., 1996a; Hifumi et al., 2010; Lee et al., 2007; Miyoshi et al., 2002; Petterino et al., 2006; Teng et al., 2012).

Few studies have been published regarding the expression of ABC-transporters in canine tumor cell lines including canine lymphoid leukemia (Page et al., 2000), osteosarcoma (Uozurmi et al., 2005), and mast cell tumor (Nakaichi et al., 2007)

For what concerns canine mammary tumor cell lines, two main studies have enlightened the importance of P-gp. Pawłowsky and colleagues were able to identify the drug specificity by siRNA-mediated gene silencing, in different ABC-transporters. They knock-down efflux pumps expression during exposure to various chemotherapeutic compounds. Interestingly they evidenced that vinblastine efflux was mediated by P-gp and MRP1, cisplatin efflux was mediated by P-gp, BCRP, MRP1 and cyclophosphamide efflux was mediated by BCRP. Small interfering RNA targeting efflux pumps could be therefore a possible way to overcame MDR in mammary tumor cells (Pawłowski et al., 2013).

Another relevant study is that by Honscha and colleagues who investigated mRNA expression of seven ABC-transporters, including P-g and BCRP, in 103 canine mammary tumor probes by RT-PCR (Honscha et al., 2009). They reported the expression of all seven different ABC-transporters in the majority of tumor probes; namely BCRP was detected in 100% of tumor probes, and P-gp in 92.2%, of tumor probes (Honscha et al., 2009).

P-gp was found to be overexpressed in two cell lines isolated from canine mammary tumors and two cell lines isolated from their lung metastases. This study pointed out that two classical P-gp inhibitors (verapamil and cyclosporin A) were ineffective in the reversion of multidrug resistance in these canine mammary cancer cells (Król et al., 2014).

A fundamental point in the evaluation of P-gp immunostaining is the individuation of a reliable cut-off value for discriminating the tumors overexpressing P-gp from the IHC negative ones. Even in human oncology the same issue has been discussed and standardized criteria have been proposed to uniform the results of research studies and the diagnostic practice (Cederbye et

al., 2016; Clarke et al., 2005). Petterino and colleagues have pointed out this issue also in IHC of CMTs and individuated the best cut-off point for P-gp expression. Receiver-operating characteristic analysis was applied to determine a threshold that concurrently optimizes sensibility and specificity and the threshold to differentiate negative from positive tissue samples has been establish at 18.40% of immunostained cells. Noteworthy this cut-off point is quite similar to that reported for human breast cancer, where 20% of positive cells in a HPF was used as a cut-off (Linn et al., 1996, 1995). With this optimal cut-off point they found that 82.4% of malignant and half of the benign tumors were Pgp-positive and a significant difference was found between histotypes of CMT overexpressing P-gp. Based on their results these authors have also suggested that routine evaluation of Pgp expression in canine mammary gland tumors may be useful for selecting cases for chemotherapy (Petterino et al., 2006).

Similar results emerged from the study of Badowska and colleagues that reported P-gp expression by IHC in 76% of CMCs, especially in complex carcinomas (Badowska-Kozakiewicz and Malicka, 2010).

Kim and colleagues investigated P-gp expression with to aim of evaluate P-gp expression by IHC, immunofluorescence and reverse transcriptase-polymerase chain reaction (RT-PCR) in malignant and benign mammary tumors. A novel finding from this study was that using the C219 antibody also myoepithelial cells in both benign and malignant tumors were P-gp-positive (Kim et al., 2012).

Most of the studies concerning P-gp in CMTs agree that P-gp expression in appear to be correlated with an increase in the grade of malignancy of the tumor and /or with a less favorable prognosis (Badowska-Kozakiewicz and Malicka, 2010; Koltai and Vajdovich, 2014; Petterino et al., 2006). Nonetheless P-gp and p53 overexpression have been correlated in determining malignancy of CMCs (Koltai and Vajdovich, 2014). In contrast to these findings, Kim and colleagues that have found that P-gp expression in malignant mammary tumors was related with favorable histopathological parameters (Kim et al., 2012).

These studies have provided a first indication that routine evaluation of P-gp expression may be useful for selecting cases for chemotherapy and may be helpful for establish the prognosis of the patient, especially in the case of canine mammary gland tumors.

Breast Cancer Resistance Protein in the Dog

BCRP was discovered in 1998, more recently than P-gp (Doyle et al., 1998), and it shares many similarity with its older cousin (Gillet and Gottesman, 2010; Gottesman et al., 2002).

If few studies are available concerning the most famous P-gp, much less is known regarding the expression of BCRP in veterinary medicine.

Most of the available studies regard BCRP expression in canine cell lines, among them in canine multicentric lymphoma higher levels of BCRP mRNA were found in in T-cell canine lymphoma compared to B-cell canine lymphoma (Zandvliet et al., 2015, 2014).

The majority of studies regarding tumors of the dog focus their attention on canine mammary tumors.

By IHC a high expression of BCRP was detected in a cohort of 54 mammary tumors, namely in 85% adenocarcinomas and almost 28% adenomas. An increased expression of BCRP was positively correlated with higher histological grade of malignancy (Nowak et al., 2009). The same authors pointed out a putative role of BCRP in contributing to a neoplastic phenotype characterized by greater malignancy and stem cell-like properties (Nowak et al., 2009).

In another comprehensive study by Honscha and colleagues BCRP mRNA was detected in all 103 canine mammary tumors. Interestingly all tumor samples expressed BCRP. The authors further investigated BCRP in a culture of stable transfected canine cells. Resistance towards doxorubicin appeared 5 times increased, at the cytotoxicity assay, while cell survival was not affected by the presence of methotrexate in the culture medium (Honscha et al., 2009). Doxorubicin has been administered in dogs with CMCs of advanced histological stage showing no beneficial effect (Simon et al., 2006), therefore on these basis the use of doxorubicin in the treatment of canine mammary neoplasia should be considered inappropriate (Honscha et al., 2009).

In the study mentioned earlier in the paragraphs describing P-gp, Pawłowski and colleagues were able to identify the drug specificity for different ABC-transporters in two canine mammary adenocarcinoma cell lines, anaplastic cancer cell line, simple carcinoma cell line and spindle-cell mammary tumor cell line. By siRNA-mediated gene silencing they found that BCRP was responsible for the resistance of neoplastic cells towards cisplatin and cyclophosphamide, while BCRP did not exert this effect towards vinblastine (Pawłowski et al., 2013). These chemotherapeutic compounds are commonly

used in breast cancer treatment and have presented some positive results also in the treatment of CMCs (Karayannopoulou et al., 2001).

These results argue for the involvement of BCRP in the biological behavior of canine mammary tumors, and pave the way to further investigations in the field of MDR mediated by BCRP, in the research of effective chemotherapeutic protocols in veterinary oncology and in elucidating the basic molecular mechanisms of carcinogenesis of animal.

Assessing ABC-Transporters Expression and Function

The presence of transmembrane ABC-transporters at cellular level can be investigated detecting the specific protein with IHC, and/or Western blot. Two main issues concerning IHC must be considered: firstly, the immunohistochemical evidence of the ABC-transporter, labeled by the antibody targeting a specific epitope, is not a proof of the functional capacity of drug transport and related MDR by the cell (Clarke et al., 2005; Leonessa and Clarke, 2003; Trock et al., 1997). Secondly, in veterinary studies most of the commercially available antibodies are made for the detection of the human or murine ATP-transporter, therefore they may not be specific enough or even not cross-reactive with other species like the canine (Ginn, 1996). However, IHC technique is still considered a good method for the detection of ABC-transporters expression, which can reflect the subcellular localization of the investigated pump (e.g. membranous, cytoplasmic diffuse, cytoplasmic perinuclear) (Zandvliet and Teske, 2015).
Among others, in the study of Bergman and colleagues has suggested IHC for the detection of P-gp expression in dogs with lymphoma, and has evidenced that P-gp expression in neoplastic cells could be a useful predictor of remission time, survival time, and the time from relapse to death (Bergman et al., 1996). Other studies investigated the presence of various MDR-associated proteins by IHC in canine tumors, for example lung tumors (Hifumi et al., 2010), mammary gland tumors (Kim et al., 2012; Koltai and Vajdovich, 2014; Nowak et al., 2009; Petterino et al., 2006) and mast cell tumors (Miyoshi et al., 2002; Teng et al., 2012).
The presence of mRNA of ABC-transporter can be evaluated by RT-qPCR. The generation of primers for different ABC-transporters and animal species is relatively easy, this technique can be performed on small tumors biopsies and a semi-quantitative value of the expression of the pump is obtained (Zandvliet

and Teske, 2015). However, the doubt regarding the functionality of the investigated ABC-transporter *in vivo* still remains (Allen et al., 2000), and the detection of mRNA levels in cell lines do not always mirror the expression of the related protein (Mao and Unadkat, 2015; Shirasaka et al., 2009).
The best way to demonstrate and quantify ABC-transporters function are dye efflux studies, but this technique is frequently practically challenging because it requires fresh, single-cell neoplastic cell suspension and adequate facilities (Clarke et al., 2005; Trock et al., 1997; Zandvliet and Teske, 2015).
There are few studies involving cell lines where the functionality of P-gp and BCRP has been investigated (Honscha et al., 2009; Page et al., 2000; Zandvliet et al., 2014)
In their study Page and colleagues characterized the chemosensitivity of wild type and multidrug resistant canine cell lines and determine the efficacy of the P-gp modulators verapamil, tamoxifen and a cyclosporin-A analog by the extent of dye retention in the drug resistant cell lines. The modulators seemed to modify the sensitivity to doxorubicin but not of cisplatin (Page et al., 2000).
The already mentioned study of Honscha and colleagues pointed out with a functional test that canine BCRP-transfected Madin-Darby canine kidney-II cells showed a 5.4-fold higher resistance doxorubicin while cell survival in the presence of methotrexate was not affected by cBCRP. they concluded that caution should considered when doxorubicin is administered for treatment of canine mammary tumors.

To conclude, in the last decade scientific research in veterinary oncology has drawn attention on the role of ABC-transporters, especially P-gp, MRP1 and BCRP, in veterinary medicine, supporting a causative role in the development of drug resistance in the dog. Further prospective studies are necessary to better understand the contribution of ABC-transporters to the clinical phenotype.

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EXPERIMENTAL SECTION

Experiment 1. IMMUNOHISTOCHEMICAL EXPRESSION OF P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN IN CANINE MAMMARY HYPERPLASIA, NEOPLASIA AND SUPPORTING STROMA

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Published in *Journal of Comparative Pathology*, Volume 155, Issue 4, November 2016, Pages 277-285, Received 7 June 2016, Accepted 13 July 2016, Available online 12 August 2016.

Introduction

The ability of cancer cells to become simultaneously resistant to different structurally unrelated drugs is known as multidrug resistance (MDR). The development of MDR in the course of chemotherapy is considered to be a major obstacle in cancer treatment. Resistance to a broad range of hydrophobic drugs can result from the expression of ATP-dependent binding cassette transporters (ABC-t). P-glycoprotein (P-GP), also known as ABCB1or MDR1, is an ABC-t efflux pump which removes cytotoxic drugs across the lipid bilayer. Breast cancer resistance protein (BCRP), also known as mitoxantrone-resistance gene (MXR), ABC-t in placenta (ABC-P) or ABCG2, is another ABC-t efflux pump consisting of a homodimer of two half-transporters (Gottesman et al., 2002; Haimeur et al., 2004; Nakanishi and Ross, 2012).

Overexpression of P-gp and BCRP reduces cellular drug accumulation and contributes to the MDR neoplastic phenotype in man and animals (Diestra et al., 2002; Haimeur et al., 2004; Leonessa and Clarke, 2003; Martinez et al., 2008; Nakanishi and Ross, 2012). Moreover, P-gp expression in breast cancer, especially when associated with P-gp expression in stromal fibroblasts, is suggestive of a more malignant phenotype (Linn et al., 1995). P-gp expression has been described in canine lymphoma, hepatic malignant and benign neoplasia, colorectal adenomas and carcinomas, adrenal cortical adenomas, perivascular wall tumors, apocrine gland adenocarcinomas, transitional cell carcinomas, melanomas, leiomyosarcomas, squamous cell carcinomas, basal

cell tumors, apocrine gland adenomas, thyroid gland carcinomas, mast cell tumors and mammary gland tumors (Bergman et al., 1996; Ginn, 1996; Koltai and Vajdovich, 2014; Lee et al., 1996; Miyoshi et al., 2002; Moore et al., 1995; Petterino et al., 2006; Tashbaeva et al., 2007). BCRP expression has been reported rarely in canine mammary gland tumors (Nowak et al., 2009; Pawłowski et al., 2013).

Chemotherapy may be indicated in dogs with aggressive or metastatic tumors such as mammary gland carcinomas, but no large-scale studies have demonstrated its benefits. The lack of effective chemotherapy for canine mammary cancer may be due to the expression of various ABC-transport proteins (Honscha et al., 2009). Studies on the effects of chemotherapeutic agents on canine mammary tumor cell cultures suggest there might be useful agents for clinical treatment (Honscha et al., 2009; Tamura et al., 2015). Dogs treated with doxorubicin or docetaxel tend to have higher long-term local control and survival rates, but there is no significant difference in the recurrence-free interval, time to metastasis or overall survival compared with untreated dogs (Simon et al., 2006). A novel formulation of paclitaxel, an important drug in human oncology, was recently given conditional approval by the US Food and Drug Authority Center for Veterinary Medicine for use in dogs with non-resectable mammary carcinoma (Khanna et al., 2015). Paclitaxel is a substrate for ABC-transporters, including P-gp and BCRP, but if and how drug efflux proteins contribute to clinical cancer resistance to paclitaxel remains unclear (Distefano et al., 1998). Pawłowski et al. (2013) reported that P-gp is responsible for vinblastine resistance in canine mammary cancer. As P-gp and BCRP are cisplatin transporters, and BCRP is also a transporter of cyclophosphamide, these authors suggested that the efflux pump expression level should be assessed before the initiation of chemotherapy (Pawłowski et al., 2013).

Aims

The aims of the present study were:

- to determine the distribution of P-gp and BCRP expression in the different components of canine hyperplastic mammary tissue and mammary tumors,
- to compare P-gp and BCRP immunoreactivity in the histological stages and histological grades of malignant progression in canine mammary carcinomas, and

- to describe P-gp and BCRP expression in the stroma associated with hyperplastic and neoplastic canine mammary tissue.

Material and Methods

Sample Selection and Histological Analysis

Formalin-fixed and paraffin wax-embedded tissues of surgical samples from 56 canine mammary glands were examined. Lymph nodes containing tumor metastases were included when available. The dogs had not received chemotherapy at the time of biopsy. Sections (3 μm) were stained with haematoxylin and eosin (HE). Lesions were classified according to the criteria of Goldschmidt et al. (2011). The histological stage of neoplastic progression of malignant tumors was defined as: stage 0, in-situ tumors; stage I, infiltration of the stroma; stage II, presence of vascular or lymphatic emboli or regional lymph node metastasis; or stage III, tumors with metastasis to distant organs (Gilbertson et al., 1983). The histological grade was established by classifying the carcinomas into three different prognostic grades of malignancy based on evaluation of the percentage of tubule formation within the carcinomatous areas, nuclear pleomorphism of the malignant components and the number of mitoses in 10 high-power fields (HPFs) in the most mitotically active areas of the tumor (Peña et al., 2013). One HPF was considered to be a field area of 2.37 mm2, in agreement with the recent proposal (Meuten et al., 2016).

Immunohistochemistry

Formalin-fixed and paraffin wax-embedded tissues were sectioned (3 μm), dewaxed with diaphane (HistoLine Laboratories, Milan, Italy) and gradually hydrated through increasing concentrations of alcohol. Endogenous peroxidase activity was blocked by incubation with H_2O_2 3% in methanol for 30 min. Antigens were unmasked by immersing the slides into 10% sodium citrate (pH 6.0) and heating in a microwave at 750 Watts for 10 min. Non-specific binding was blocked by incubation with 10% normal goat serum (NGS) in phosphate buffered saline (PBS) for 30 min at room temperature. Slides were incubated overnight at 4°C with mouse monoclonal anti-P-GP (clone C494, Signet Laboratories, Dedham, Massachusetts, USA), diluted 1 in 1,500 in 10% NGS in PBS and with mouse monoclonal anti-BCRP (clone BXP-21, Merck KGaA, Darmstadt, Germany), diluted 1 in 100 in 10% NGS in PBS. The slides were rinsed in TRIS buffer and then incubated with secondary anti-mouse antibody (biotinylated goat anti-mouse immunoglobulins; Dako, Glostrup, Denmark)

diluted 1 in 200 in 10% NGS in PBS. The reaction was amplified by the avidin-biotin method (ABC-kit elite; Vector, Burlingame, CA) and visualized after incubation with 3,3′-diaminobenzidine tetrahydrochloride 0.04% for 2 minutes in the dark, at room temperature (DAB chromogen/substrate kit; Diagnostic BioSystem, Pleasanton California, USA). Sections were counterstained with Papanicolaou hematoxylin, rinsed in tap water, dehydrated, and coverslipped. Negative controls were run in parallel, by substituting the primary antibody with an irrelevant isotype-matched antibody. Sections of normal canine liver were used as positive controls.

Samples labelled for P-gp expression were considered positive when at least 20% of the cells in 10 HPFs showed membrane or cytoplasmic labelling. This level was based on that reported by Petterino et al. (2006), of 18.40% being the best discriminating cut-off value in studies of human breast cancer (Petterino et al., 2006). BCRP expression was scored as positive if >10% of the tumor cells were labelled (Diestra et al., 2002). The 10% cut-off level is chosen for two reasons: firstly, because a small number of cells positive for MDR-related proteins may have clinical significance, and secondly, because 10% positivity is the lowest level of expression that can be most detected consistently in formalin-fixed and paraffin wax-embedded tissue sections (Diestra et al., 2002). For both P-gp and BCRP, the intensity of labelling was evaluated in comparison with that of endothelium (used as a positive internal control). Cells showing no reaction or a very faint reaction compared with endothelium were considered negative, while those showing an intensity of labelling similar to that of the endothelium were considered positive. The adjacent or supporting stroma was considered positive only if fibroblasts and/or fibrocytes showed cytoplasmic labelling of the same or greater intensity as compared with endothelium. Cases showing no reactivity of lymphatic and/or vascular endothelium to P-gp or BCRP (probably because of a deterioration of the antigen due to formalin fixation) were excluded.

Statistical Analysis

Comparison of P-gp and BCRP positivity between groups was tested by Fisher's exact method (GraphPad Software, La Jolla, California, USA; accessed January 2016). $P < 0.05$ was considered significant.

Results

The study included 47 samples of lobular hyperplasia in mammary tissue adjacent to mammary tumors, 10 benign tumors and 46 malignant tumors. Benign tumors included four simple adenomas, three complex adenomas, two benign mixed tumors and one intraductal papillary adenoma. Malignant tumors included 12 solid carcinomas, 12 simple tubulopapillary carcinomas, eight complex carcinomas, 11 mixed carcinomas and three carcinomas arising in a mixed tumor. Three carcinomas were classified as stage 0, 28 were stage I and 15 were stage II (seven with neoplastic emboli and eight with lymph node metastasis). There were 27 grade 1, 15 grade 2 and four grade 3 carcinomas (Table 1).

Table 1. Percentage and number of samples positive for P-gp and BCRP in the different cellular components, grouped according to histological diagnosis, histological stage and histological grade. H stage, histological stage; H grade, histological grade.

Cellular component		PGP expression %	BCRP expression %
Luminal epithelial cells	Hyperplasia	46.8 (22/47)	60 (27/45)
	Benign neoplasia	40 (4/10)	20 (2/10)
	Malignant neoplasia	76.8(35/46)	81.82 (36/44)
Myoepithelial cells	Benign neoplasia	40 (2/5)	20 (1/5)
	Malignant neoplasia	8.33 (2/24)	33.33 (8/24)
Metaplasia	Benign neoplasia	0 (0/2)	0 (0/2)
	Malignant neoplasia	28.75 (4/14)	25(3/12)
Carcinomatous cells	Simple carcinomas	78.26 (18/23)	100 (21/21)
	Complex and mixed carcinomas	73.91 (17/23)	60.87 (14/23)
	Emboli	83.33 (5/6)	100 (6/6)
	Lymph node metastasis	87.5 (7/8)	100 (8/8)
	Stage 0	66,66 (2/3)	66.66 (2/3)
	Stage I	75 (21/28)	77.78 (21/27)
	Stage II	86.66 (13/15)	100 (14/14)
	Grade I	59.26 (16/27)	69.23 (18/26)
	Grade II	73.33 (11/15)	78.57 (11/14)
	Grade III	75 (3/4)	100 (4/4)
Fibroblasts of adjacent or supporting stroma	Hyperplasia	12.76 (6/47)	4,44 (2/45)
	Benign neoplasia	10 (1/10)	0 (0/10)
	Malignant neoplasia	28.26 (13/46)	13.64 (6/44)
	Stage 0	0 (0/2)	0 (0/3)
	Stage I	17.86 (5/28)	3,70 (1/27)
	Stage II	53.33 (8/15)	35.71 (5/14)
	Grade I	14.81 (4/27)	0 (0/26)
	Grade II	46.67 (7/15)	21.42 (3/14)
	Grade III	50 (2/4)	75 (3/4)

Immunohistochemically, the cell membrane of hepatocytes and biliary epithelial cells and the cytoplasm of endothelial cells showed a strong reaction with both anti-P-GP and anti-BCRP reagents, while fibrocytes in the portal tracts were not labelled (Figure 1). In the case of BCRP, two samples of simple carcinoma (one of stage I and grade 1 and the other of stage II and grade 2) and the adjacent hyperplastic mammary gland were excluded from the study because the endothelium, considered as internal control, did not show the expected immunoreactivity, probably due to antigen denaturation by fixation. This was not observed for samples labelled for P-gp expression, most likely due to resistance of P-gp to fixation.

The highest expression of both proteins was found in the malignant epithelium, with 76.8% of cases (35/46 tumors) for P-gp and of 81.82% of cases (36/44 tumors) for BCRP overexpressing these markers. In hyperplasia and benign tumors, the epithelial cells expressed both proteins variably, but to a lesser extent (Table 1). There was a significant difference in the expression of both proteins in hyperplastic epithelial cells compared with malignant epithelial cells (P-GP, $P = 0.0055$; BCRP, $P = 0.0352$), and in hyperplastic epithelia and epithelium in benign tumors compared with epithelium in malignant tumors (P-GP, $P = 0.0024$; BCRP, $P = 0.0049$) (Figure. 2 A, B and 3 A, B, Graph 1).

The myoepithelial component was also evaluated in complex and mixed, benign and malignant tumors. Myoepithelium was not labelled in normal mammary tissue. P-gp and BCRP expression by myoepithelial cells was variable in both benign and malignant complex and mixed tumors (Table 1, Figure. 2A, Graph 1), but did not reach statistical significance.

P-gp and BCRP expression were compared between simple carcinomas, which are generally considered more aggressive, and complex and mixed carcinomas, characterized by a proliferation of epithelial cells and myoepithelial cells with metaplasia in mixed tumors (Chang et al., 2005; Goldschmidt et al., 2011). P-gp was overexpressed in malignant epithelial cells of 78.26% of cases (18/23 tumors) of simple carcinomas and in 73.91% of cases (17/23 tumors) of complex and mixed tumors. BCRP expression was significantly different between simple carcinomas (100% expression, 21/21 tumors) and complex and mixed carcinomas (60.87% expression, 14/23 tumors) ($P = 0.0016$).

An increase in expression between different histological stages of carcinomas for both markers was detected but did not reach statistical significance. Neoplastic cells in lymphovascular emboli and lymph node metastasis

frequently showed intense labelling (Table 1, Figures. 2C and 3C). An overall increase in both P-gp and BCRP expression was found in tumors of higher histological grades (Table 1). Grade 2 and 3 tumors, which have a more aggressive biological behaviour, had significantly higher P-gp expression compared with grade 1 tumors (P = 0.0375).

In addition, the P-gp and BCRP expression was evaluated in the stroma of the different lesions (Figures. 2D and 3D). Fibroblasts adjacent to or within areas of mammary hyperplasia or neoplasia showed variable cytoplasmic expression of P-gp and BCRP, with the lowest number of positive cases in adenomas, with 10% (1/10 tumors) and 0% (0/10 tumors), respectively, and the highest in carcinomas with 28.26% (13/46 tumors) and 13.64% (6/44 tumors), respectively (Table 1). Moreover, fibroblast expression varied between tumors with different histological stages and grades: a significant difference was found between fibroblast expression of both markers when comparing histological stage I and II carcinomas (P-GP, P = 0.0339; BCRP, P = 0.0127) and between histological grade 1 and 2 carcinomas (P-GP, P = 0.0343; BCRP, P = 0.0368) (Graph 2).

Discussion

This study examined the expression of the MDR-associated proteins P-gp and BCRP in canine mammary hyperplasia and neoplasia. Unlike in human breast cancer, mesenchymal and myoepithelial cell proliferation is common in canine mammary gland tumors (Misdorp, 2002). The present study found that there were a higher number of P-gp and BCRP positive cases in malignant tumors, with a significant higher expression in epithelial cells of carcinomas compared with hyperplastic and benign mammary epthelium (P-gp, P = 0.0024, BCRP, P = 0.0049). These findings are in accordance with previous studies showing that P-gp and BCRP expression is associated with a more malignant phenotype of tumors (Koltai and Vajdovich, 2014; Martinez et al., 2008; Nowak et al., 2009; Petterino et al., 2006). A basal level of P-gp and BCRP expression in the epithelial cells of hyperplastic mammary tissue could be explained by intrinsic expression of these proteins in mammary gland epithelium, especially the epithelium of the ducts, related to the physiological activity of an excretory organ (Maliepaard et al., 2001; Pavelic et al., 1993).

The myoepithelium in these tissues also showed a variable degree of expression of the markers. It has been reported previously that myoepithelial cells do not express P-gp and BCRP, as almost no cases in one study showed immunoreactivity with the C494 antibody, which detects *MDR1*-encoded protein (Petterino et al., 2006). In contrast, another study (Kim et al., 2012), using the antibody C219, which detects both *MDR1*- and *MDR3*-encoded P-gp, suggested that the myoepithelial compartment in mammary tumors can express P-gp and that the protein is *MDR3* encoded. This expression may be induced by growing tumors, since P-gp could be upregulated not only by drugs, but by many extracellular stimuli, and a certain degree of expression is a consistent finding.

The higher expression of P-gp and BCRP in the epithelial component of simple carcinomas, which are generally considered more aggressive (Chang et al., 2005; Goldschmidt et al., 2011), and in the epithelial component of complex and mixed carcinomas is in accordance with the findings of Nowak et al. (2009), Koltai and Vajdovich (2014) and Honscha et al. (2009). Nowak et al. (2009) reported BCRP expression in >85% of simple adenocarcinomas, and expression was correlated positively with higher grades of malignancy, while Koltai and Vajdovich (2014) found a significantly higher rate of P-gp expression in tubulopapillary carcinomas compared with complex carcinomas and benign mammary tumors. Honscha et al. (2009) stated that because BCRP was expressed in all of the carcinomas they examined, and is known to induce doxorubicin resistance, the use of doxorubicin in the treatment of canine mammary neoplasia should be considered inappropriate.

The possible variation of P-gp and BCRP expression in the malignant progression of mammary tumors was investigated by examining expression of these molecules by different histological stages and histological grades of malignancy. Stage II carcinomas, characterized by the detection of emboli or lymph node metastasis (Gilbertson et al., 1983), showed higher expression of both P-gp and BCRP compared with stage I lesions. The investigation of marker's expression in the different grades of carcinoma revealed an increase in the expression of both markers, with 73% of grade 2 or 3 carcinomas expressing P-gp or BCRP. These results support the view that in addition to potential drug resistance, carcinomas expressing these MDR markers show a more aggressive biological behaviour. Koltai and Vajdovich (2014) found that

the P-gp expression correlated with the probability of tumor recurrence after surgery and was inversely related to the survival time.

BCRP is known to confer cancer cell self-renewal capacity ('stemness'), invasiveness and aggressiveness, thereby imparting a poor prognosis (Huls et al., 2009; Nakanishi and Ross, 2012; Zhou et al., 2001). As documented by Nowak et al. (2009), high BCRP-1 expression in canine mammary tumor cells may result in a higher prevalence of less well-differentiated tumor cells and hence, potentially, greater malignancy. It is noteworthy that in five out of six stage II carcinomas, embolic neoplastic cells showed strong P-gp expression and all expressed BCRP. Likewise, lymph node metastasis always expressed BCRP (8/8 cases) and seven out of eight showed P-gp expression. This should be linked to the already mentioned association between BCRP expression and tumor aggressiveness (Nakanishi and Ross, 2012). The study of Król et al. (2010) showed that metastatic properties of canine mammary tumor cells seem to be associated with elevated P-gp expression (Król et al., 2014). However, the small number of stage 0 carcinomas (three cases) in the present study precludes any speculation about P-gp and BCRP expression in this group.

MDR development in neoplastic cells is explained by two main mechanisms. Firstly, cells natively expressing drug-efflux proteins like P-gp and BCRP retain their phenotype throughout the process of malignant transformation. Secondly, chemotherapeutics have been shown to induce genetic P-gp expression in non-expressing cells and to exert a selection pressure on resistant neoplastic cells during the course of chemotherapy (Bebawy et al., 2009; Levchenko et al., 2005; Pasquier et al., 2011; Rafii et al., 2008). Moreover, extragenetic MDR transmission has recently been reported, involving the direct transfer of P-gp protein to MDR1 non-expressing recipient cells from various donors, for instance stromal cells isolated from patients with ovarian cancer. These findings indicate a new modality of chemoresistance and possible spread in a population of tumor cells that could involve the fibrovascular stroma of the tumor. This may have important implications in the diagnostic value of P-gp expression and in the design of chemotherapy regimens (Levchenko et al., 2005; Rafii et al., 2008; Bebawy et al., 2009; Pasquier et al., 2011).

The tumor microenvironment can also promote drug resistance by altering the biological functions of certain gene clusters and preventing drug accumulation in tumor cells (Chen et al., 2014). Considering this finding, our study also

investigated P-gp and BCRP expression in tumor stroma. The highest percentage of stromal positivity was found in carcinomas, for both P-gp (28.26%, 13/46 tumors) and BCRP (13.64%, 6/44 tumors), with a significant difference between stages I and II carcinomas for both P-gp ($P = 0.0339$) and BCRP ($P = 0.0127$). This suggests an association with a malignant tumor phenotype. According to findings in women (Linn et al., 1995), P-gp expression in primary breast cancer cells, especially when associated with P-gp expression in fibroblasts of desmoplastic stroma, should have prognostic value as a marker of a more malignant phenotype.

A limit of the immunohistochemical detection of stromal expression of this marker is that when the tumor is highly vascularized, and hence there is a high small vessel density, endothelium, physiologically positive for P-gp and BCRP, could be misinterpreted as fibroblasts/fibrocytes. In addition, endothelial cells also have a complex, probably active, role in the development of MDR (Marroni et al., 2003).

Conclusions

In conclusion, this study has provided new insights into P-gp and BCRP expression in canine mammary tumors not previously exposed to chemotherapy. Both proteins were potentially expressed by the entire cellular neoplastic component (i.e. myoepithelial cells and cells associated with metaplasia), but the highest expression was found in malignant epithelial cells. The findings confirm previous reports suggesting that canine mammary tumors, especially involving epithelial cells in malignant and embolic or metastatic tumors, often express P-gp and/or BCRP, and that these proteins could be associated with a malignant and MDR phenotype. In view of this, the use of drugs that are substrates of these proteins should be carefully evaluated, and alternatives to traditional chemotherapy or administration in conjunction with MDR inhibitors might be considered in order to avoid the onset of chemoresistance. In addition, determination of MDR markers at the time of diagnosis could provide valuable information for the design of treatment protocols. Considering the expression of MDR markers in the stroma associated with these tumors, fibroblasts were found to express P-gp and BCRP and this could be associated with disease aggressiveness. Further investigations are required to confirm these findings.

Figures and Graphs

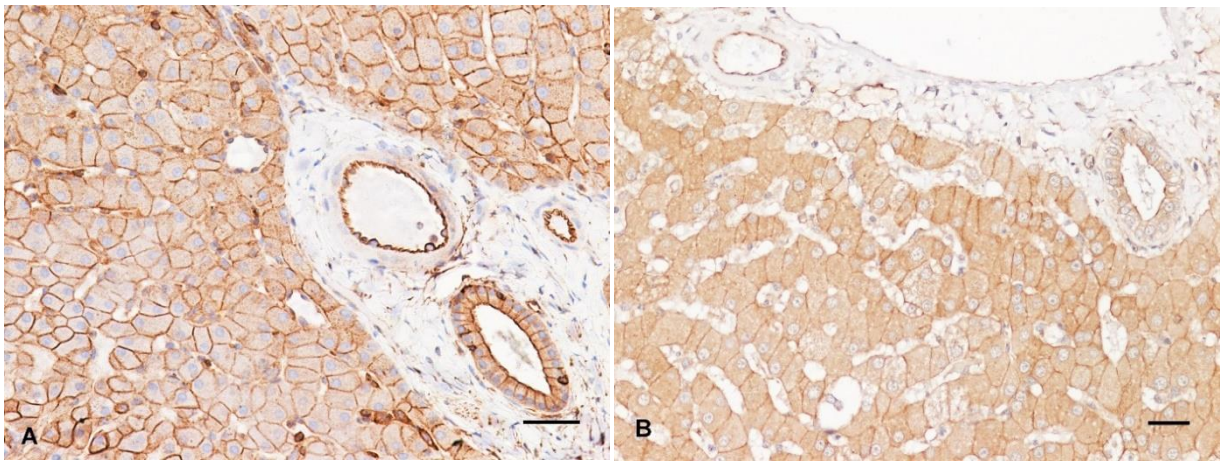


Figure. 1. Canine liver used as a positive control for (A) P-gp and (B) BCRP immunohistochemistry. The cellular membranes of hepatocytes, bile duct epithelial cells and vessel endothelium are strongly labelled. Bars, 100 microns.

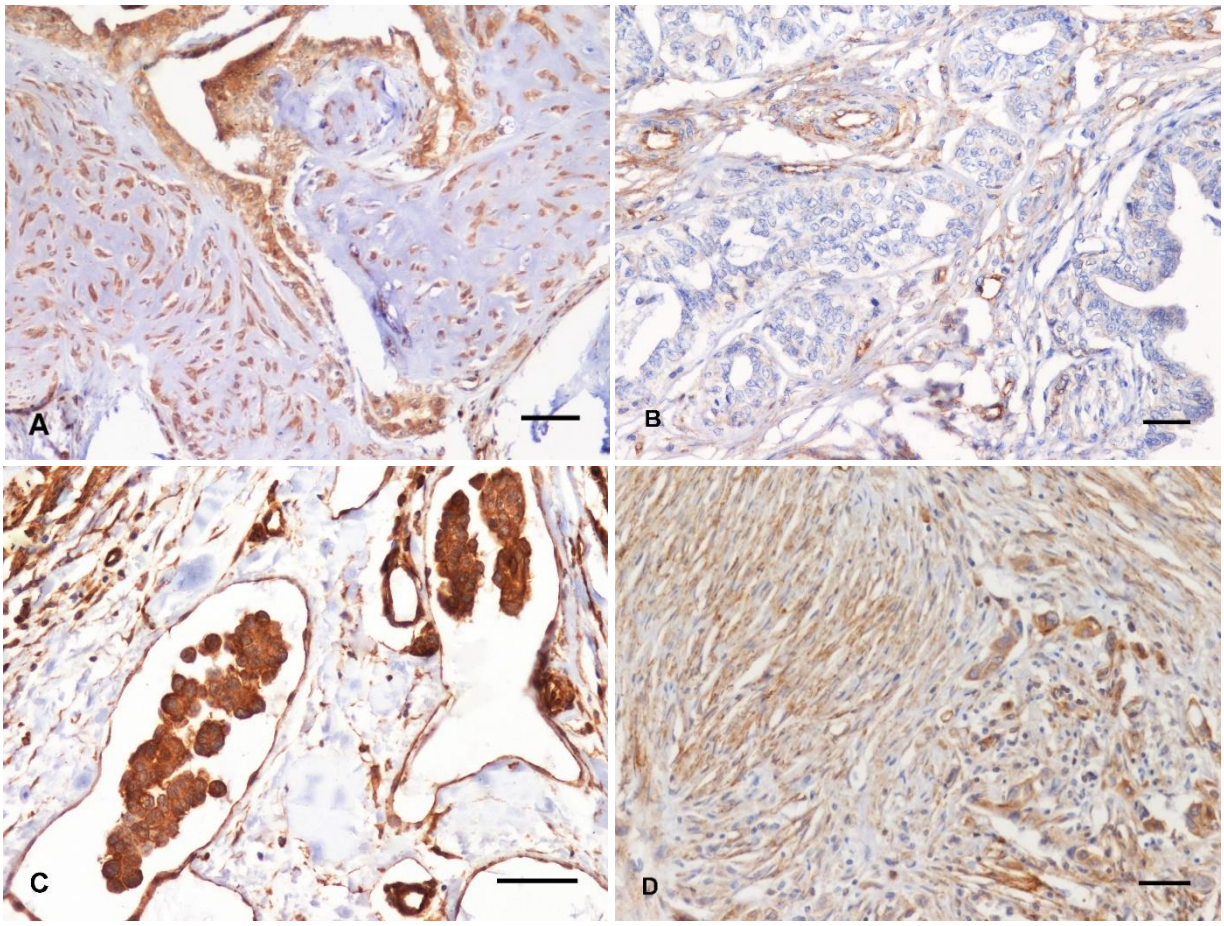


Figure. 2. IHC labelling with anti-P-GP antibody. (A) Mixed carcinoma with intense expression of P-gp in the epithelial component and in an area of cartilaginous metaplasia. (B) Simple adenoma negative for P-gp. Vascular endothelium, considered as a positive internal control, is positive. (C) A lymphovascular embolus of a tubulopapillary carcinoma with intense expression of P-gp. (D) The stroma supporting an infiltrating tubulopapillary carcinoma shows intense diffuse expression of P-gp. Bars, 100 microns.

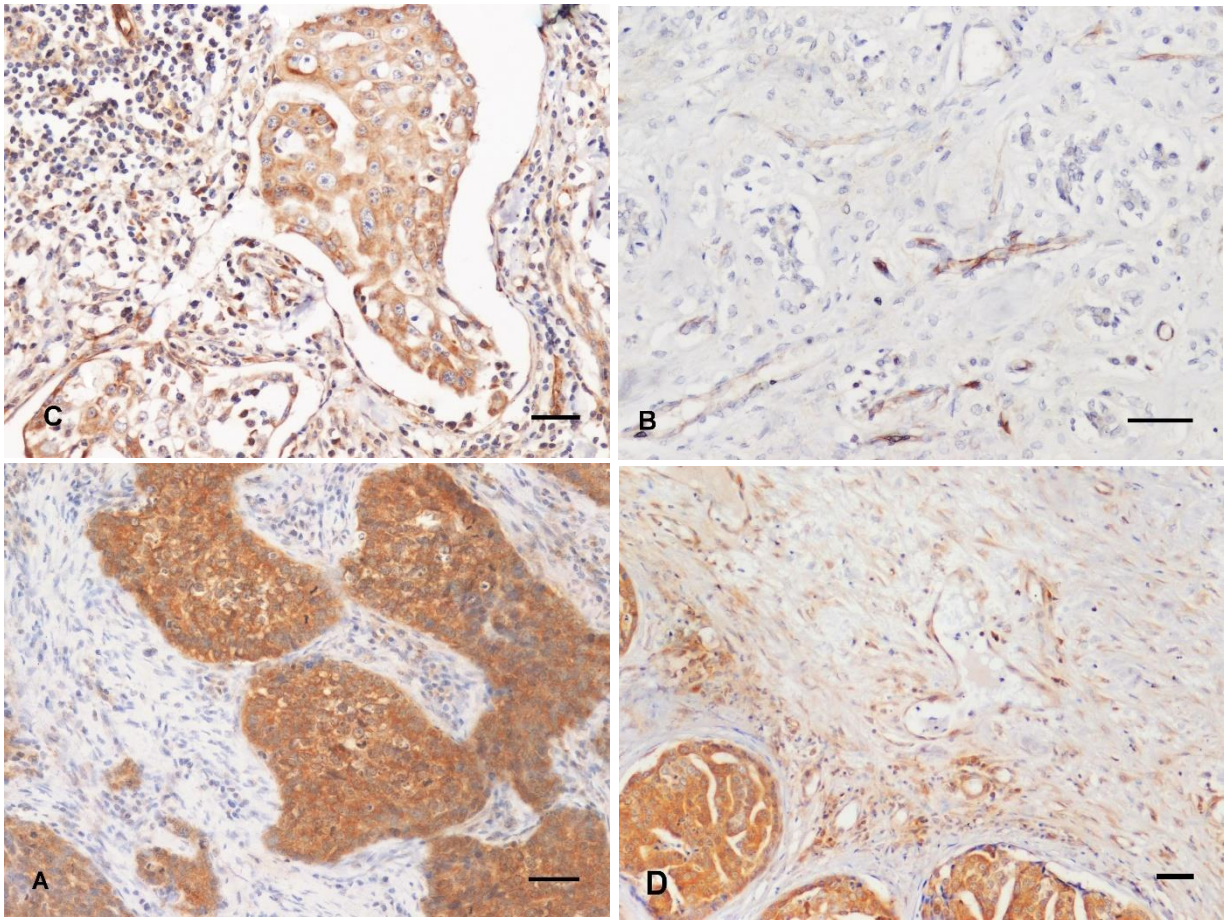
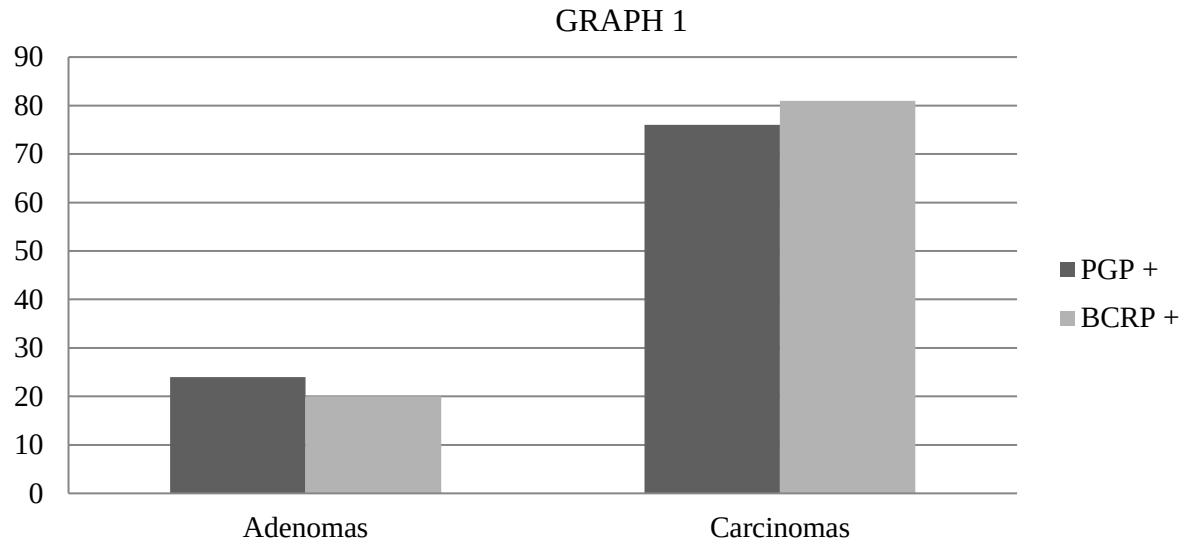
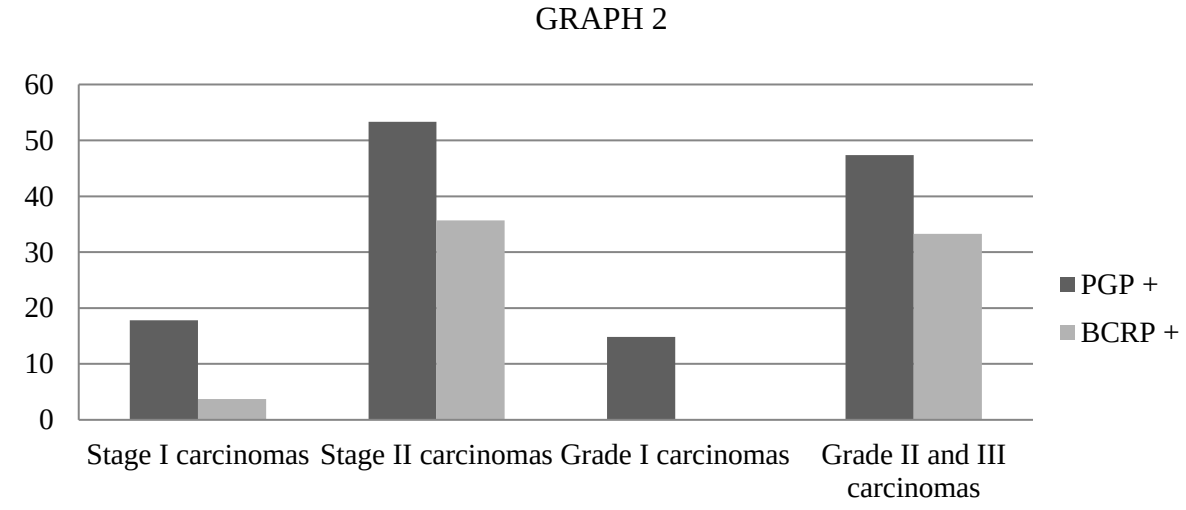


Figure 3. IHC labelling with anti-BCRP antibody. (A) Simple solid carcinoma with intense expression of BCRP by neoplastic epithelial cells. The supporting fibrous stroma is negative. (B) Hyperplastic mammary lobules negative for BCRP. Vascular endothelium, considered as a positive internal control, is positive. (C) A regional lymph node metastasis of tubulopapillary carcinoma with intense expression of BCRP. (D) The stroma supporting a simple tubulopapillary carcinoma with intense diffuse expression of BCRP. Bars, 100 microns.



Graph 1. % of canine mammary tumors expressing P-gp and BCRP in the epithelial component. The expression of P-gp and BCRP was significantly higher in malignant epithelial cells of mammary carcinomas compared to the epithelium of benign tumors.



Graph 2. % of canine mammary carcinomas expressing P-gp and BCRP in the stromal component. A significant difference in both P-gp and BCRP expression was found between fibroblast of histological stage I and II carcinomas and between fibroblasts of histological grade 1 and 2 carcinomas.

Publications and Proceedings

Poster: M. Levi, G. Sarli, B. Brunetti and C. Benazzi. "Immunohistochemical Expression of P-gp and Breast Cancer Resistance Protein in Canine Mammary Hyperplasia, Neoplasia and Supporting Stroma" 34th ESVP/ 27th ECVP Annual Meeting, Bologna, Italy, 7-10th September, 2016.

Levi, M., Brunetti, B., Sarli, G., Benazzi, C., 2016. Immunohistochemical Expression of P-gp and Breast Cancer Resistance Protein in Canine Mammary Hyperplasia, Neoplasia and Supporting Stroma. J. Comp. Pathol. 155, 277–285 (Levi et al., 2016)

Experiment 2. CHEMORESISTANCE MARKERS P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN IN CANINE INFLAMMATORY AND GRADE 3 MAMMARY CARCINOMA

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Introduction

P-gp and BCRP are well known ABC-transporter efflux pumps bearing an influential role in chemotherapy-resistance in cancers due to their capacity of exporting out from the cell a wide variety of substrates including many of the currently available chemotherapeutic compounds, preventing drug intracellular action (Gottesman et al., 2002). When ABC-transporter are overexpressed and functional in neoplastic cells before induction chemotherapy, it is defined as *de novo*/intrinsic overexpression, while secondary/acquired overexpression of ABC-transporter appears after a first treatment of chemotherapy (Chung et al., 1997). Another huge interest that promotes research regarding P-gp and BCRP is that they are considered markers of the so-called side population of cancer initiating cells; moreover regulation of stem cell biology by ABC-transporters has emerged as an important new field of investigation (Bunting, 2002; Hirschmann-Jax et al., 2004; Holohan et al., 2013; Moitra, 2015).

Information regarding the complex role P-gp and BCRP are reported in human breast cancer research (Faneyte et al., 2002; Leonessa and Clarke, 2003), while at present, data on the role of ABC-transporters in drug resistance in veterinary oncology are still limited (Klopfleisch et al., 2016; Zandvliet and Teske, 2015)

Canine mammary tumors are a heterogeneous group of tumors, from their pathological aspects to their clinical behavior (Sorenmo, 2003). To provide more information regarding the prognosis of the examined tumor, carcinomas of the bitch are routinely classified in three different histological grades of malignancy according to the most recent proposed system (Peña et al., 2013).

This system has proved to be a useful prognostic tool, facilitating histological interpretation, and offering uniform criteria for veterinary pathologists (Peña et al., 2013). Carcinomas of histological grade 3 are the rarest, and have the worst prognosis with a 58.8% of cancer related death and recurrence/metastasis in a follow up period of 28 months, as evidenced in the study of Peña and colleagues (2013).
Treatment guidelines for malignant canine mammary gland tumors have been proposed (Sorenmo, 2003), however the administration of a chemotherapeutical regiment to dog with aggressive canine mammary carcinomas (CMCs) have been documented with conflicting results (Gustafson and Page, 2013; Karayannopoulou et al., 2001; Karayannopoulou and Lafioniatis, 2016; Marconato et al., 2008; Simon et al., 2006). Based on the limited clinical information available in veterinary medicine, the drugs that are effective in human breast cancer, such as cyclophosphamide, 5-fluorouracil, and doxorubicin, have been translated into veterinary oncology in attempt to treat CMCs (Goldschmidt et al., 2017; Karayannopoulou and Lafioniatis, 2016; Sorenmo, 2003).

Human inflammatory breast cancer (IBC) and its counterpart in the dog, canine inflammatory mammary cancer (IC), are considered the most malignant types of mammary cancer (Giordano and Hortobagyi, 2003; Marconato et al., 2009; Peña et al., 2003; Pérez Alenza et al., 2001; van Uden et al., 2015). IBC and IC are clinic-pathological entities, diagnosed on the basis of a rapid progression of signs, the presence of dermal erythema, induration and edema of the mammary gland, and the histological feature of massive embolization of neoplastic cells in superficial dermal lymphatic vessels (Pérez Alenza et al., 2001; van Uden et al., 2015). This neoplasia bears a unique rapid-growing, angioinvasive, and angiogenic phenotype, which is far from being completely understood (Giordano and Hortobagyi, 2003; Kleer et al., 2000; Clemente et al., 2010b).
The therapeutic approach in women with IBC usually involves neoadjuvant anthracycline- and taxane-based chemotherapy treatment associated, when feasible, to ablative surgery, and locoregional radiotherapy (van Uden et al., 2015). Despite recent advances in multimodality treatments, the prognosis of women with IBC is still poor, with a median disease-free survival of less than 2.5 years (Kleer et al., 2000; Woodward, 2015). In veterinary oncology there are few studies that describe medical treatment for patients with canine IC, suggesting a beneficial effect with the administration of nonsteroidal anti-inflammatory drugs like piroxicam, as single-agent (de M Souza et al., 2009),

or piroxicam combined with carboplatin, or doxorubicin, or capecitabine or cisplatin (Marconato et al., 2009). The search for new therapeutic strategies to improve survival is needed in both species.
The aforementioned chemotherapeutic molecules (i.e. anthracycline) are substrates of many ABC-transporters, and in human oncology the onset of chemoresistance towards these compounds has been attributed to the overexpression of P-gp and BCRP (Faneyte et al., 2002; Nakanishi and Ross, 2012; Nieth and Lage, 2005). Few studies are available regarding the expression of this important modulators of drug resistance in canine mammary tumors (Badowska-Kozakiewicz and Malicka, 2010; Honscha et al., 2009; Kim et al., 2012; Koltai and Vajdovich, 2014; Król et al., 2014; Pawłowski et al., 2013; Petterino et al., 2006).
IMCs and CMCs have been proposed as useful spontaneous models for the study of their human counterparts (Abadie et al., 2017; Nguyen et al., 2017; Peña et al., 2003), therefore a better understanding of their biological behavior and chemoresistant phenotype would be helpful in both human and veterinary oncology.

Aims

Considering that, to the best of our knowledge, there are no studies describing the expression of MDR-associated markers in IMC, and few information are available regarding CMC's response to chemotherapy, the aims of this study were:

- to describe the expression of chemoresistance-markers P-gp and BCRP in ICs, in the neoplastic emboli and primary tumor;
- to describe the expression of chemoresistance-markers P-gp and BCRP in histological grade 3 non-inflammatory carcinomas (C3s);
- to compare P-gp and BCRP expression between canine IC and comparable highly malignant non-inflammatory carcinomas, namely C3.

Material and Methods

Sample Selection and Histological Analysis
Samples were retrieved from the archive of the Department of Animal Medicine, Surgery and Pathology of the Complutense University of Madrid, Spain. Thirty-eight formalin-fixed and paraffin-embedded tissue samples of mammary gland from 38 dogs were selected at under light microscopes based

on the clinical and histopathological diagnoses of IC and C3. In cases of ICs the primary mammary carcinoma was included in the study when available.
Non-IMC were classified according to the current histologic classification proposed by Goldschmidt and colleagues (Goldschmidt et al., 2011). Only grade 3 neoplasms were included in the study. Canine mammary carcinomas were classified into three grades of malignancy based on evaluation of the percentage of tubule formation within the carcinomatous areas, nuclear pleomorphism of the malignant components and the number of mitoses in 10 high-power fields (HPFs) in the most mitotically active areas of the tumor (Peña et al., 2013).

Immunohistochemistry

Formalin-fixed and paraffin wax-embedded tissues were sectioned (3 μm) and placed in a PT module (Lab Vision) containing EDTA buffer solution (pH 8.0) (Master Diagnostica, MAD-004072R/D), heated for 20 minutes at 95°C and cooled down to 60°C (dewaxing and antigen retrieval). Slides were rinsed in warm tap water and placed in an automated Immunostainer device (Lab Vision Corporation, Fremont, CA) for immunohistochemistry.
Slides were incubated for 120 minutes at room temperature with the following primary antibodies:

- Mouse monoclonal anti-P-gp/CD243 (C494, GTX23365) Manufacturer: Gene Tex International - dilution 1:1500
- Mouse monoclonal anti-BCRP (BXP-21, Millipore-MAB4146) Manufacturer: Merk - dilution 1:200.

Immunolabeling was detected with a peroxidase detection system Master Polymer Plus Detection System, (Master Diagnostica, MAD-000237QK). After immunostaining the slides were counterstained with Mayer's hematoxylin and permanently mounted with Depex.
Sections of normal canine liver were used as positive controls, and endothelium of blood and lymphatic vessels as positive control internal to the examined tissue. Corresponding negative control slides were processed in parallel by replacing the primary antibody with non-reactive antibody. The same criteria illustrated in the previous experiment of this thesis were applied for evaluating the immunolabeling. P-gp and BCRP was considered positive when >20% and >10% of cells were labelled for P-gp (Petterino et al., 2006) and BCRP (Diestra et al., 2002), respectively. Cases showing no reactivity of lymphatic and/or vascular endothelium to P-gp or BCRP (probably because of a deterioration of the antigen due to formalin fixation) were excluded.

Statistical Analysis

Comparison of P-gp and BCRP expression between groups was analyzed by Fisher's exact method (GraphPad Software, La Jolla, California, USA; accessed May, 2017). $P < 0.05$ was considered significant.

Results

The selected samples included 18 C3s and 20 ICs. Regarding ICs the diagnosis was based on the typical clinical presentation (with dermal erythema and edema in the ventral abdominal dermis) associated, at histology, with neoplastic emboli in superficial lymphatic dermal vessels. Emboli of IC were detectable in every case (20), while primary carcinoma was available in 15 IC cases. According to the anamnesis attached to the reports, dogs had not received chemotherapy at the time of biopsy.

Histotypes of primary ICs included 6 micropapillary invasive carcinomas, 3 comedocarcinomas, 2 lipid-rich carcinomas, 2 solid carcinomas, 1 adenosquamous and 1 anaplastic carcinoma. In 8 cases of IC the embolized lymphatic vessels were surrounded by desmoplasia, often with severe dermal edema, while in only 2 cases there were multifocal areas of intratumor colliquative necrosis and in only 2 cases an abundant inflammatory infiltrate composed by neutrophils, lymphocytes and plasma cells was associated to the neoplasia. (Figure 1 A and B).

Histotypes of C3 included 7 solid carcinomas, 5 micropapillary invasive carcinomas, 2 complex carcinomas, 1 tubulopapillary carcinoma, 1 comedocarcinoma, 1 anaplastic carcinoma and 1 adenosquamous carcinoma.

In 8 cases of C3 there were multifocal areas of colliquative necrosis in more than 50% of the tumor (Figure 1 C and D).

The grading of C3s was revised and corresponded to the highest histological grade proposed by Peña and colleagues (Peña et al., 2013). In 15 cases of IC for which the primary mammary tumor, which presumably gave origin to the IC was present and the evaluation of the histological grade of malignancy, according to Peña and colleagues (2013), corresponded to histological grade 3 in every case.

At IHC, the cell membrane of hepatocytes and biliary epithelial cells and endothelial cells showed a strong reaction with both anti-P-GP and anti-BCRP antibodies. One case of IC with no available primary tumor, was excluded from the evaluation of the immunostaining with anti-BCRP antibody, because the endothelium, considered as internal control, did not show the expected immunoreactivity with BCRP, probably due to antigen denaturation by fixation.

In ICs, emboli of neoplastic cells were evaluated separately from the primary tumor and compared to each other.
Immunolabeling pattern was mainly membranous for P-gp showing a particularly strong reaction in neoplastic emboli of IC (Figure 2).
Membranous and cytoplasmic stain was seen for BCRP (Figure 3).

Emboli of ICs were often characterized by the presence of cells with peculiar morphological features, severe anisokaryosis and anisocytosis. Some cells stood out for a morphology reminiscent of anomalous endothelial cells, characterized by a rim of elongated cytoplasm containing elongated eccentric nuclei encircling an empty round space (endothelial-like cells, vasculogenic mimicry phenomenon). These cells were intensely stained by P-gp. (Figure 4).

Considering ICs and C3s together, P-gp was expressed in 63.13% (24/38), and BCRP was expressed in 72.97% (27/37).

P-gp was highly expressed in the different tumors, namely 85% of the embolic component of ICs overexpressed P-gp (17/20), 80% of the primary ICs (12/15), while fewer cases of C3s overexpressed P-gp (7/18, 38.89%).
Interestingly P-gp was significantly higher in emboli of IC vs C3 (P-GP P=0.006) and in primary IC vs C3 (P-gp P=0.032) (Graph 1).

There was no significant difference in BCRP expression among groups of IC and C3, being BCRP overexpressed in 78.95% of emboli of IC (15/19), 80% of primary IC (12/15) and 66.67% of C3 (12/18) (Graph 2).
These results are synthetized in Graph 1 for P-gp and Graph 2 for BCRP.

The groups of ICs and C3s were compared for evaluating in how many cases per group, P-gp and BCRP were both expressed or not, in the same case.
In the group of ICs

- 63.15 % of the cases (12/19) overexpressed both the markers (P-gp+/ BCRP+) (Figure 5),
- 36.84 % of the cases (7/19) overexpressed at least one marker (P-gp +/ BCRP - or P-gp -/ BCRP +)
- none (0/19) resulted negative for both the markers (P-gp -/ BCRP -).

In the group of C3s
- 38.8 % of the cases (7/18) overexpressed both the markers (P-gp+/ BCRP+),
- 27.77% of the cases (5/18) overexpressed at least one marker (P-gp +/ BCRP - or P-gp -/ BCRP +)
- 33.33% of the cases (6/18) resulted negative for both the markers (P-gp -/ BCRP -).

An example of IC expressing both P-gp and BCRP (P-gp+/ BCRP+) is shown in Figure 5.

Discussion

With this study the intrinsic overexpression of chemoresistance-associated markers P-gp and BCRP have been described in canine ICs and C3s, two groups of rare and biologically aggressive canine mammary tumors, suitable for a chemotherapeutic approach.
The most numerous histotypes of primary IC were micropapillary invasive carcinomas (6/20 cases) and comedocarcinomas (3/20 cases). Similarly in C3 there were 7 solid carcinomas and 5 micropapillary invasive carcinomas out of 18 cases. Comedocarcinoma and solid carcinoma have been recently recognized as histoypes indicating a poor prognosis (Rasotto et al., 2012). The high number of these histotypes in our caseload is therefore due to the aggressiveness of ICs and C3.
Overall, an elevated number of cases overexpressing P-gp (63%) and BCRP (72%) were found. Chemoresistance associated to the expression of P-gp and BCRP in this cohort of canine mammary tumors, which have not received chemotherapy at time of biopsy, is presumably an intrinsic phenomenon, meaning that ABC-transporter, overexpressed by neoplastic cells, may lead to the failure of induction chemotherapy from the start (Chung et al., 1997; Petterino et al., 2006).

In this study ICs overexpressed P-gp in 85% of the emboli and in 80% of the primary carcinomas, while in the group of C3s P-gp was overexpressed in 38% of the cases. BCRP expression was elevated in all the groups, and will be discussed later. To the best of our knowledge, there are no study documenting P-gp and BCRP expression in canine ICs.

In human oncology a significant heterogeneity in ATP-transporters overexpression is seen across the studies, and a valid summary measure is difficult to substantiate (Clarke et al., 2005). In human breast cancer P-gp has been identified as a marker of poor prognosis and a high number of P-gp positive cases (64%) have been reported prior to chemotherapy (Linn et al., 1996, 1995). P-gp positive tumors are three times less likely to respond to chemotherapy (Clarke et al., 2005).

Our results in the dog can be compared to the ones of the other studies available on P-gp expression in canine mammary tumors. The percentage of P-gp positive carcinomas fluctuates: Badowska-Kozakiewicz and Malicka (2010) reported 76% of P-gp positive cases, Koltai and Vajdovic (2014) found 66.94% of P-gp positive cases, Ginn and colleagues (1996) found a 63.2% of P-gp expression in mammary carcinomas and of Petterino et al. (2006) referred 49.75% of P-gp-positive tubulopapillar carcinomas in their caseload. Most of the authors are in agreement that an increase in P-gp expression seems to be related with the degree of malignancy of the tumor (Badowska-Kozakiewicz and Malicka, 2010; Koltai and Vajdovich, 2014; Petterino et al., 2006).

In breast cancer BCRP has been reported to be overexpressed in around 33% of malignancies and has been correlated with resistance to 5-fluorouralic (Mao and Unadkat, 2015, 2005; Yuan et al., 2008).

In veterinary oncology few studies regarding BCRP overexpression are available, however the results are coherent in reporting that BCRP is highly overexpressed. In our study BCRP was overexpressed in 78.95% of emboli of IC, 80% of primary IC and 66.67% of C3. These results slightly differ from the ones of Nowak and colleagues (2009) that reported BCRP expression in >85% of simple adenocarcinomas and a positive correlation with the degree of malignancy was found. Honscha et al. (2009) stated that because BCRP was expressed in all of the canine mammary cancer cell lines they examined, and BCRP is known to induce doxorubicin resistance, the use of doxorubicin in the treatment of canine mammary neoplasia should be considered carefully (Honscha et al., 2009).

The results of our previous study (Experiment 1) are coherent with these last findings. In fact we found that P-gp and BCRP expression were significantly higher in malignant epithelial cells of CMCs than in benign tumors (Levi et al., 2016).

Another interesting finding in the present study is that all ICs overexpressed at least one of the two pumps. Furthermore we found that a high number of ICs and C3 overexpressed both BCRP and P-gp (in 63.1% of ICs and 38.8% of C3 respectively), thus presumably this tumor is capable of developing resistance towards drug molecules that are both P-gp and BCRP substrates, bearing a more complex multidrug resistant phenotype. The expression by the same tumor of more than one ABC-transporter has been reported in the study of Honscha and colleagues (2009), where 103 canine mammary tumor probes were investigated for mRNA expression of seven ABC-transporters, including P-gp and BCRP. More than half of tumor samples (56.1%) expressed all of the examined ABC-transport proteins, 92.2% overexpressed P-gp and 100% overexpressed BCRP (Honscha et al., 2009).
These findings suggest that chemoresistance associated to overexpression of P-gp and BCRP pumps, is a phenomenon present in dogs with C3 and IC, therefore caution should be employed when drugs that are substrates of P-gp and BCRP (i.e. doxorubicin, vincristin, mitoxantrone) are prescribed in the treatment of IMC and C3 and canine mammary gland tumors. Potentially, routine evaluation of the expression MDR markers P-gp and BCRP, may be useful for the selection of patients for chemotherapy. In human medicine some patients appear to have a naturally more aggressive phenotype associated to high levels of baseline P-gp expression (Clarke et al., 2005). However, further studies are necessary to define the actual role of P-gp in mammary gland tumors as a marker for MDR, and its utility as a prognostic factor in human and veterinary medicine (Gottesman et al., 2002; Zandvliet and Teske, 2015).

A striking feature in both normal and cancer stem cells is their high level of ABC-transporter expression compared with their more differentiated progeny (Fletcher et al., 2010). Normal stem cells have multiple mechanisms to protect them from cytotoxic insults, which include highly active drug-efflux pumps (Klopfleisch et al., 2016; Moitra, 2015). These quiescent constitutively drug resistant cancer stem cells remain present in low frequency within the heterogeneous tumor and serve as a reservoir for drug resistant tumor relapses (Zandvliet and Teske, 2015). Particularly BCRP is able to evidence the 'side-

population' of stem cells from different human tissue (Zhou et al., 2001). For several tumor types, the tumor grade, a low degree of differentiation and poor patient outcome correlate with the presence of transcriptional programs closely resembling those seen in embryonic stem cells (Fletcher et al., 2010). As already discussed BCRP was highly expressed between both ICs and C3s in this study, therefore this subpopulation of neoplastic cells could belong to the cohort of cancer initiating cells/cancer stem cells and could play a role in the unique biologically aggressive, invasive, and chemoresistant phenotype of IC and C3.

Finally in this study a statistically significantly higher expression of P-gp was found in inflammatory carcinomas compared to non-inflammatory carcinomas. Furthermore when examined under a light microscope, it was evident that neoplastic emboli of ICs had often a peculiar morphologic appearance, characterized by the presence of cells with cytoplasmic empty spaces covered by the plasma-membrane, intensely stained by P-gp, forming channel-like structures. This aspect seems to recapitulate vasculogenic mimicry, a phenomenon that takes place in cancer and consists of microvascular channels composed of genetically deregulated, aggressive tumor cells that support the neoplasia, without participation by endothelial cells and independent of angiogenesis (Folberg et al., 2000; Shirakawa et al., 2002). Vasculogenic mimicry has been described in IBC (Folberg et al., 2000; Shirakawa et al., 2002) and in canine ICs (Clemente et al., 2013, 2010a).
Nevertheless some authors have described that P-gp is highly expressed in endothelial cells at specialized sites (Allen et al., 2000; Huls et al., 2009; Marroni et al., 2003; Schinkel, 1997) and in intratumoral blood vessels invasive breast cancer with metastasis to lymph nodes (Badowska-Kozakiewicz et al., 2017), and up-regulation of P-gp gene mdr1 has been associated with increased metastatic capacities by canine mammary cancer cells (Król et al., 2014). Some neoplastic cells of canine inflammatory mammary carcinomas have been hypothesized to have endothelial-like morphological features (Clemente el al 2010a); therefore the elevated expression of P-gp in this cells could suggest that P-gp plays a role in the peculiar phenotype of IC characterized by special migration ability, angiogenesis, and drug resistance.

Conclusions

Overexpression of P-gp and BCRP seems to be a diffuse phenomenon in dogs with C3 and IC, therefore caution should be employed in administering drugs that are substrates of P-gp and BCRP, since an associated chemoresistance might be present. Routine evaluation of the expression MDR markers may be useful for selection of patients for chemotherapy.

BCRP and P-gp could contribute to the biologically aggressive nature and the peculiar phenotype of C3 and ICs because they are implicated in the regulation of cancer stem cell biology. Since both markers were overexpressed in IC, we could hypothesize that P-gp could play a specific pathogenic role in the characteristic “inflammatory phenotype” of this particular disease.

Future prospective studies are needed, to demonstrate the association between chemoresistance and overexpression of these markers in canine mammary cancer, to better understand the complexity of its pathogenic mechanisms in order to evaluate and choose adequate chemotherapy protocols in each patient with highly malignant mammary cancer.

Spontaneously occurring canine mammary cancer represents an excellent model of human breast cancer that can provide powerful information helpful in understanding its human counterpart, so far still greatly understudied.

Figures and Graphs

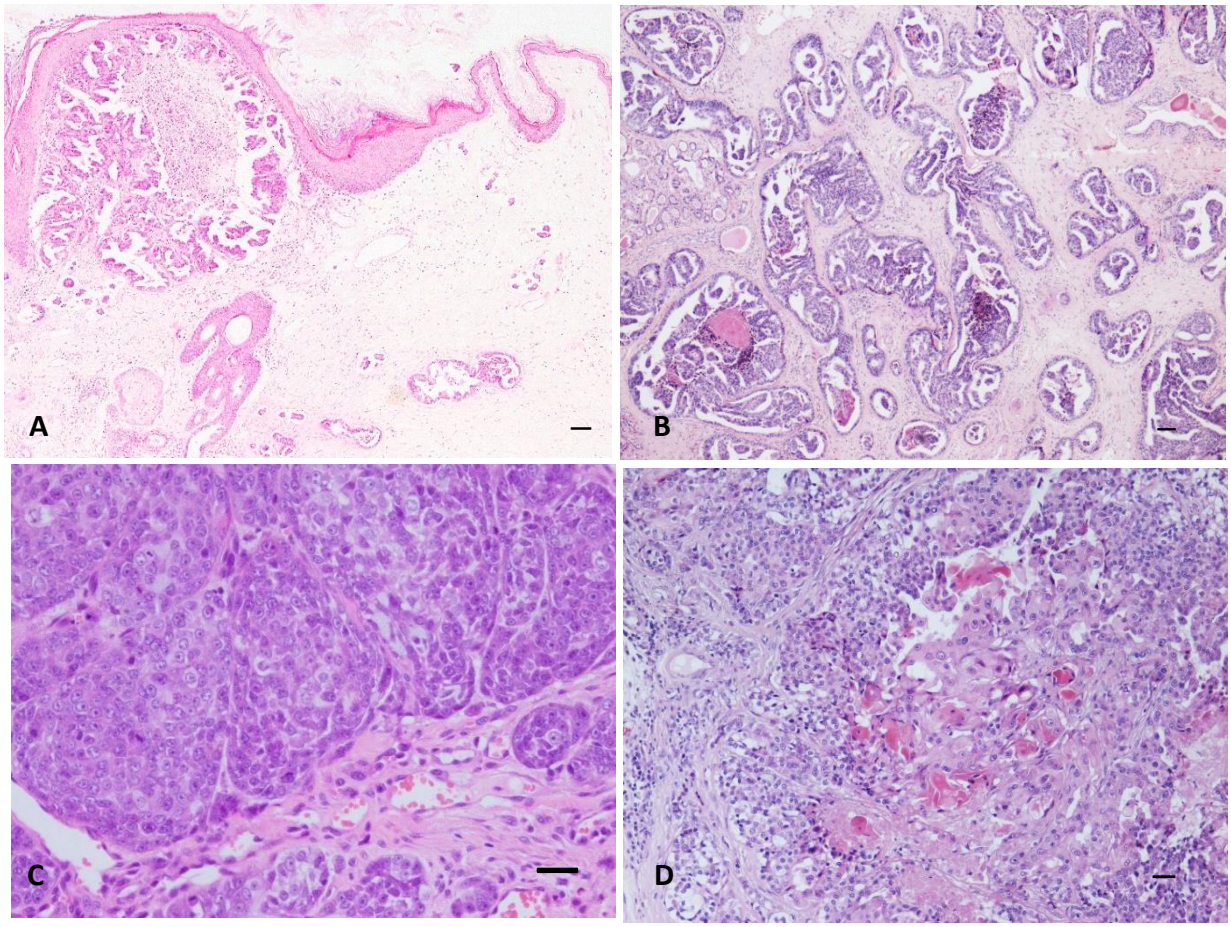


Figure 1. HE. (A) Canine mammary inflammatory carcinoma with massive embolization of neoplastic cells in superficial dermal lymphatic vessels, associated to interstitial dermal edema and moderate inflammatory infiltrate. (B) Canine mammary inflammatory carcinoma, primary mammary tumor of micropapillary invasive histotype. (C) Canine grade 3 mammary carcinoma, solid histotype. (D) Canine grade 3 mammary carcinoma, adenosquamous histotype. Bars, 100 microns.

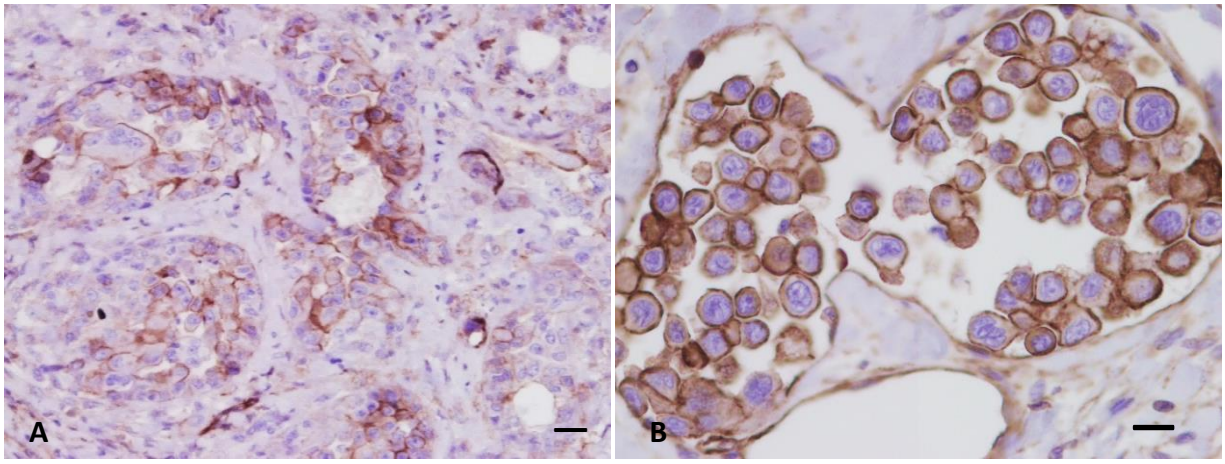


Figure 2. IHC labelling with anti-P-gp antibody. (A) Primary tumor of inflammatory carcinoma with intense membranous expression of P-gp in malignant epithelial cells. (B) Emboli of inflammatory carcinoma with intense membranous expression of P-gp in malignant epithelial cells. The endothelium of lymphatic vessel, considered as a positive internal control, is positive. Bars, 100 microns.

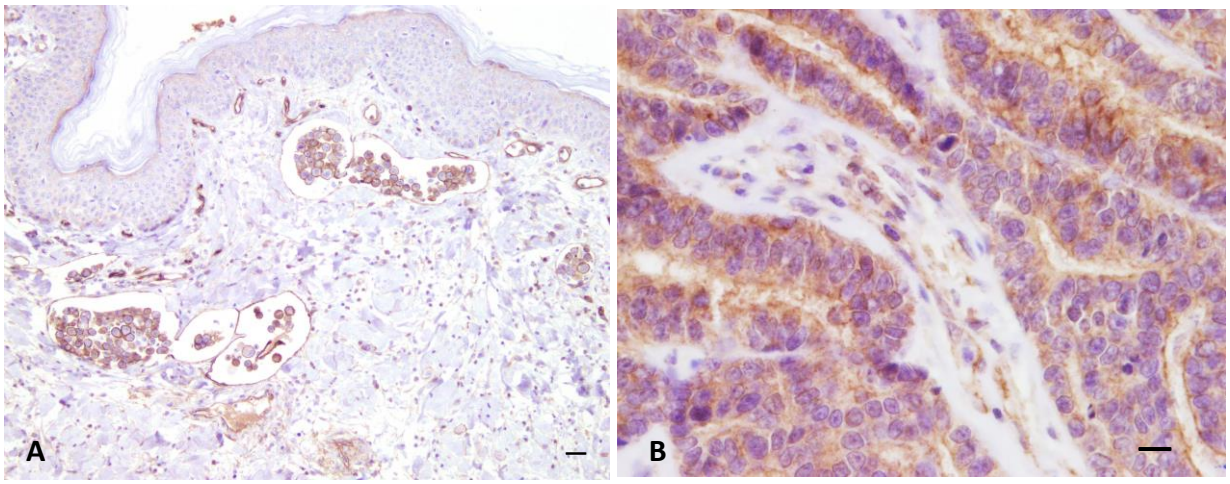


Figure 3. IHC labelling with anti-BCRP antibody. (A) Emboli of inflammatory carcinoma in superficial dermal lymphatic vessels with intense membranous expression of P-gp in malignant epithelial cells. The endothelium of lymphatic vessel, considered as a positive internal control, is positive. (B) Canine grade 3 mammary carcinoma, tubulopapillary histotype, with intense cytoplasmic and membranous expression of P-gp in malignant epithelial cells. Bars, 100 microns.

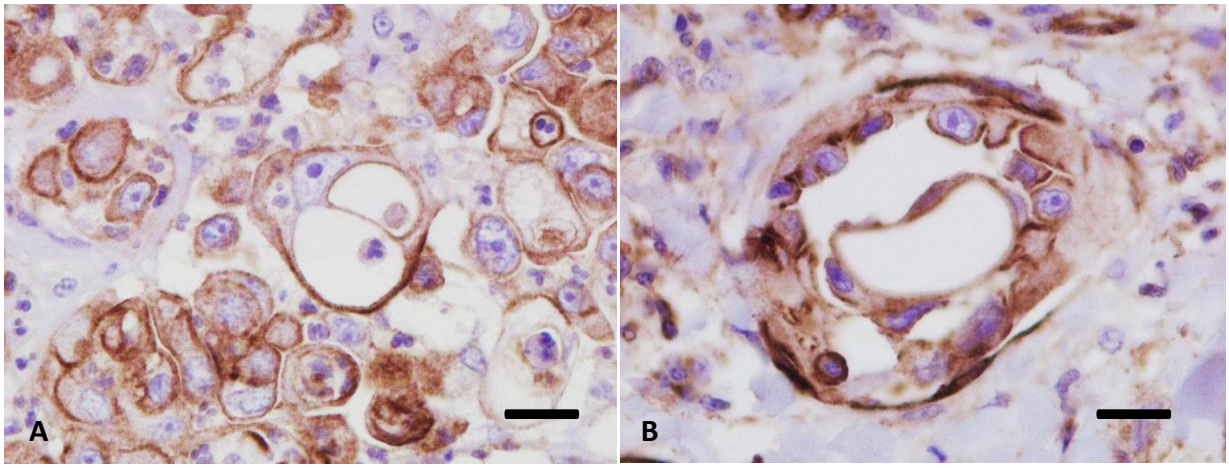


Figure 4. IHC labelling with anti-P-gp antibody. (A) Neoplastic cells of mammary inflammatory carcinoma with severe anisokaryosis and anysocytosis, characterized by a rim of elongated cytoplasm containing eccentric nuclei, encircling an empty round, intensely stained with P-gp. (B) Embolus of mammary inflammatory carcinoma showing an atypical neoplastic cell, characterized by a rim of elongated cytoplasm containing elongated eccentric nuclei, encircling an empty round space, intensely stained with P-gp. Both neoplastic cells (A and B) are suggestive of vasculogenic mimicry. Bars, 100 microns.

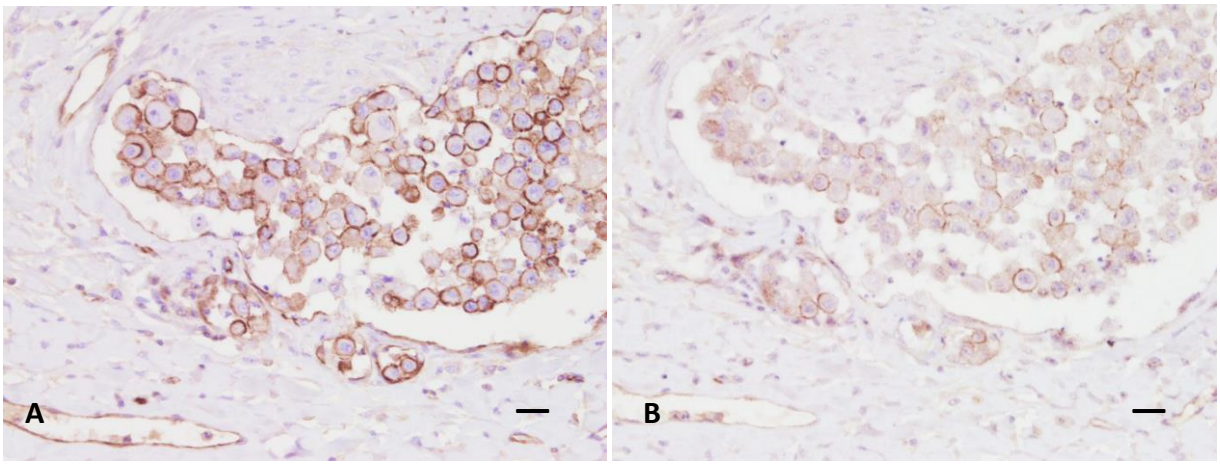
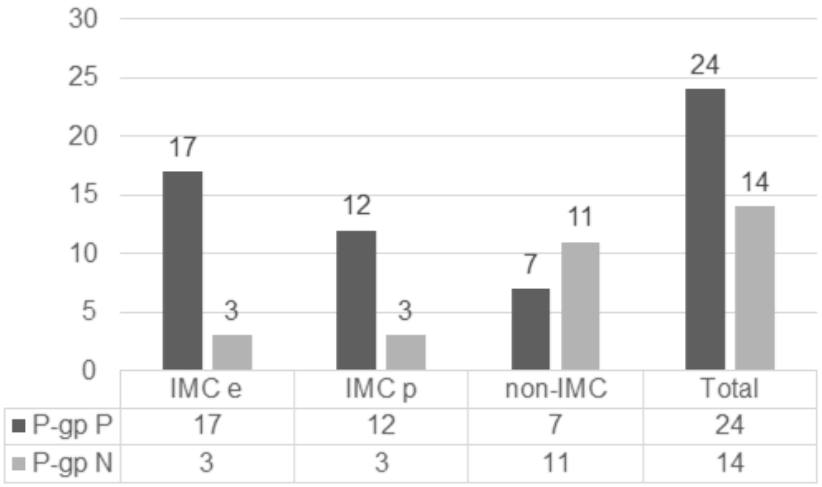
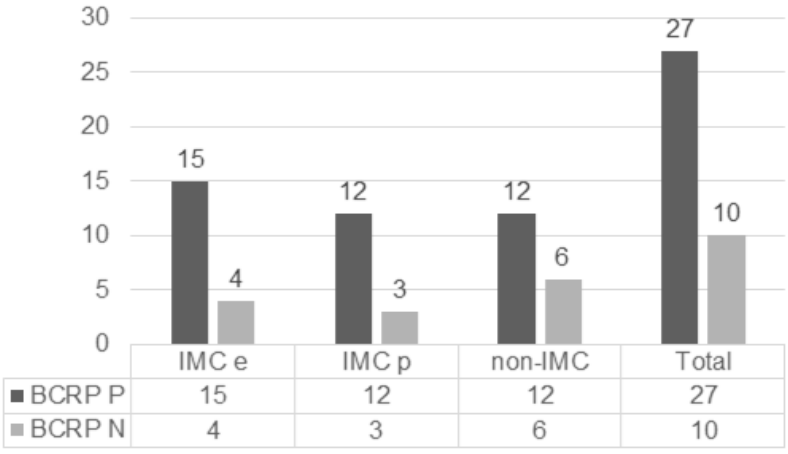


Figure 5. IHC labelling with anti-P-gp antibody (A) and anti-BCRP (B). Embolus of mammary inflammatory carcinoma. Neoplastic cells, in the same area, express both P-gp and BCRP, with intense membranous staining and mild cytoplasmic staining. Bars, 100 microns.



Graph 1). P-gp expression in neoplastic emboli and primary tumor of canine inflammatory mammary cancer (IMC), and canine non-inflammatory histologic grade III mammary carcinoma (non-IMC). Emboli of IMC expressed P-gp in 85% of the cases (17/20), and primary IMC overexpressed P-gp in 80% of the primary IMCs (12/15). Fewer cases of non-IMCs expressed P-gp (7/18, 38.89%).



Graph 2). BCRP expression in neoplastic emboli and primary tumor of canine inflammatory mammary cancer (IMC), and canine non-inflammatory histologic grade III mammary carcinoma (non-IMC). BCRP was expressed in 78.95% of emboli of IMC (15/19), 80% of primary IMC (12/15) and 66.67% of non-IMC (12/18).

Publications and Proceedings

Oral presentation: M. Levi, L. Peña, B. Brunetti, A. Alonso-Diez, L.V. Muscatello, M.D. Pérez-Alenza, C. Benazzi e G. Sarli. "Chemoresistance Markers P-gp and Bcrp in Canine Inflammatory and Grade 3 Mammary Carcinoma". 3rd Joint European Congress of the ESVP, ECVP and ESTP-Lyon, France, August 30 – September 02, 2017

Experiment 3. ORIGINAL PURPOSE AND TECHNICAL PITFALLS

This part of my PhD research project was initially born with the aim of collecting a numerous group of canine mammary carcinomas to investigate the expression of P-gp and BCRP in association to the molecular phenotypical classification of mammary carcinomas translated from human breast cancer (Perou et al., 2000) to canine mammary carcinomas (Gama et al., 2008; Sassi et al., 2010).
The classification of the canine mammary into molecular phenotypes requires the assessment of the IHC expression of ER, -PR, -HER2, -CK 5/6 and -CK14 (Sassi et al., 2010) or ER, -PR, -HER2, -CK 5/6 and -CK14 (Gama et al., 2008) or, according to the most recent proposal, of ERα, PR, Ki-67, HER2, CK 5/6, and EGFR (Abadie et al., 2017; Nguyen et al., 2017). Some authors have recently proposed guidelines in order to standardize and uniform the criteria for the evaluation of the molecular phenotype of CMCs (Peña et al., 2014).

IHC technical pitfalls with Estrogen Receptor

In this molecular classification, it is fundamental to assess the expression of the hormonal receptors ER for a first subdivision of carcinomas into luminal-like and non-luminal like (Peña et al., 2014).
In the course of my research activity the attempt to develop a reliable IHC protocol for the use of the antibody anti-ER (Polyclonal, Invitrogen, Product:18-0174z) was not fruitful. The standardization has included, as positive control, FF-PE samples of canine uterus, as suggested in the literature (Peña et al., 2014). An optimal immunostaining of the nuclei of the endometrium and myometrium was achieved with the IHC performed in our laboratory (Figure 1); however, in the case of mammary gland tumors adjacent to normal mammary glands, the staining pattern was unreliable, with a faint immunostaining impossible to evaluate. The hyperplastic mammary gland often available in the section, and considered as the positive internal control, was unstained, despite many attempts in performing variations of IHC procedures (variations in time and temperature of heat- and enzymatic antigen retrieval, dilution and incubation of the primary antibody, incubation with the DAB chromogen).
A possible explication of the inability to obtain a reliable staining of the mammary gland tissue could be related to the peculiar location of the gland,

surrounded by abundant subcutaneous adipose tissue. This coat of lipids may cause a delay in the penetration of formaldehyde into the sample and consequently tissue fixation.
In human oncology this pitfall has been reported, among all, in the study of Khoury and colleagues who investigated the effect of a delay in formalin fixation on breast biomarkers, namely ER PR and HER2 (Khoury et al., 2009), and in the study of Masood and colleagues that evaluated the effect of fixation on ER and PR analyses in three cases (Masood et al., 1998). Briefly the authors concord on the finding that when there was a delay in the onset of fixation immunoreactivity was severely affected. A delay of formalin fixation for 12 h determined a decrease in the expression of ER and PR from 100% each to 61.2 and 40.4%, respectively (Khoury et al., 2009). In the consensus recommendations on ER testing in breast cancer by IHC the importance of a correct fixation procedure was highlighted: "Breast resection specimens must be sectioned, placed in fixative as quickly as possible and the time recorded. Tissue sections must be immersed in an adequate volume of fixative (ratio of tissue/fixative = 1:20) within a maximum of 1 hour from removal" (Yaziji et al., 2008). Similar recommendations have been proposed for IHC of canine mammary tissue species (Peña et al., 2014). Many times in the diagnostic practice mammary sample are fixed during surgery, and, depending on the patient state of nutrition a thick coat of adipose tissue can often invalidate the penetration of the fixative, undermining the correct fixation of the mammary nodule. This issue related to inadequate fixation did not happen in our cases when the positive control was a uterine sample, collected during an ovariohysterectomy in our structure, readily fixed in formalin, and, in general, with samples where adipose tissue was not surrounding the nodule.
These technical pitfalls open new future perspective for research regarding how technical procedures can affect IHC results in veterinary medicine following the human model.

IHC technical pitfalls with Human Epidermal Growth Factor Receptor 2

In the last years new findings regarding HER2 amplification have called into question its relevance in the pathogenesis and prognosis of CMTs and the possibility to detect the supposed overexpression of the receptor HER2 by IHC. Briefly, the antibody HER2 (polyclonal, Dako A0485) has been used in many studies on canine mammary carcinomas (Beha et al., 2015, 2014, 2012; Gama et al., 2008; Kim et al., 2011; Sassi et al., 2010). Doubts regarding the

possibility to detect HER2 amplification in mammary tumors of dogs have arisen for methodological issues present in many studies and related to the selection of antibodies, dilution, scoring criteria, and absence of appropriate controls (Abadie et al., 2017). The amplification HER2 gene was never found in CMCs by chromogenic *in situ* hybridization (de Las Mulas et al., 2005), and the existence of HER2-positive mammary carcinomas in dogs is still under debate. In their recent and complete study, Burrai and colleagues (2015) have investigated HER2 expression in canine mammary tumors by antibody-based, transcriptomic and mass spectrometry analysis. Their findings suggest that IHC bear a lack of specificity of the FDA-approved antibody in CMT samples. Moreover, in the same study, HER2 was not detected by mass spectrometry in a protein-expressing carcinoma at the IHC investigation. (Burrai et al., 2015).
In the present research project, therefore, the IHC evaluation of HER2 expression in the collection of mammary carcinoma has been delayed with the intention to find a more reliable antibody and a more validated technique.

The following part of this thesis regarding experiment 3 is therefore, a first step of a wider, still ongoing project regarding the evaluation of multidrug resistance markers P-gp and BCRP in association with the molecular phenotypes CMCs. We are present, for now, the investigation of P-gp and BCRP expression by IHC in a cohort of CMCs, and the findings regarding this MDR markers.

Experiment 3. IMMUNOHISTOCHEMICAL EXPRESSION OF P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN IN CANINE MAMMARY CARCINOMAS

Introduction

Overexpression of the ATP-dependent drug efflux pump is a major molecular mechanism of multidrug resistance in human cancer (Gottesman et al., 2002) and for their complex role P-gp and BCRP have been widely studied in human breast cancer research (Faneyte et al., 2002; Leonessa and Clarke, 2003),
Overexpression of P-gp and BCRP reduces cellular drug accumulation and contributes to the MDR neoplastic phenotype in man and animals (Diestra et al., 2002; Haimeur et al., 2004; Leonessa and Clarke, 2003; Martinez et al., 2008; Nakanishi and Ross, 2012). *De novo*/intrinsic overexpression of P-gp and

BCRP is responsible for the failure of the chemotherapy at the first treatment (Chung et al., 1997). At present, data on the role of ABC-transporters in drug resistance in veterinary oncology are still limited (Klopfleisch et al., 2016; Zandvliet and Teske, 2015)

Limited information is available in veterinary medicine regarding chemotherapy in canine mammary carcinomas.The drugs that are effective in human breast cancer have been translated into veterinary oncology in attempt to treat CMCs and treatment guidelines have been proposed, however conflicting results are still obtained regarding their effectiveness (Gustafson and Page, 2013; Karayannopoulou et al., 2001; Karayannopoulou and Lafioniatis, 2016; Marconato et al., 2008; Simon et al., 2006; Sorenmo, 2003). The onset of chemoresistance towards many of the commonly used compounds has been attributed to the overexpression of P-gp and BCRP (Faneyte et al., 2002; Nakanishi and Ross, 2012; Nieth and Lage, 2005). Few studies are available regarding the expression of this important modulators of drug resistance in mammary tumors (Badowska-Kozakiewicz and Malicka, 2010; Honscha et al., 2009; Kim et al., 2012; Koltai and Vajdovich, 2014; Król et al., 2014; Pawłowski et al., 2013; Petterino et al., 2006).

Mammary tumors in the dog have been proposed as useful spontaneous models for the study of breast cancer (Abadie et al., 2017; Caceres et al., 2015; Nguyen et al., 2017; Peña et al., 2003), therefore a better understanding of their biological behavior and chemoresistant phenotype would be helpful in both human and veterinary oncology.

Aims

The aims of the present study were:

- to determine the distribution of P-gp and BCRP expression in a group of canine mammary carcinomas;
- to compare P-gp and BCRP immunoreactivity in the histological stages and histological grades of malignant progression in canine mammary carcinomas.

Material and Methods

Sample Selection and Histological Analysis

Formalin-fixed and paraffin wax-embedded tissues of surgical samples from 54 canine mammary carcinomas were collected from the archive of the service of Pathology of the DIMEVET, University of Bologna. According to the

anamnestic reports dogs had not received chemotherapy at the time of biopsy. HE stained section were examined under a light microscopes and carcinomas were classified according to the morphologic criteria of Goldschmidt et al. (2011). The histological stage of neoplastic progression of malignant tumors was defined by the observation of HE sections under a light microscope, as: stage 0, in-situ tumors; stage I, infiltration of the stroma by carcinomatous cells that cross the myoepithelial layer surrounding luminal cells; stage II, presence of vascular or lymphatic emboli or regional lymph node metastasis; or stage III, tumors with metastasis to distant organs (Gilbertson et al., 1983). The histological grade was established by classifying the carcinomas into three different prognostic grades of malignancy based on evaluation of the percentage of tubule formation within the carcinomatous areas, nuclear pleomorphism of the malignant components and the number of mitoses in 10 high-power fields (HPFs) in the most mitotically active areas of the tumor (Peña et al., 2013). One HPF was considered to be a field area of 2.37 mm2, in agreement with a recent proposal (Meuten et al., 2016).

Immunohistochemistry

The following procedure is the same that has been performed in experiment 1. FF-PE samples were sectioned with a microtome (3 μm), dewaxed with diaphane (HistoLine Laboratories, Milan, Italy) and gradually rehydrated through increasing concentrations of alcohol. Endogenous peroxidase activity was blocked by incubation with H2O2 3% in methanol for 30 min. Antigens were unmasked by immersing the slides into 10% sodium citrate (pH 6.0) and heating in a microwave at 750 Watts for 10 min. Non-specific binding was blocked by incubation with 10% normal goat serum (NGS) in phosphate buffered saline (PBS) for 30 min at room temperature. Slides were incubated overnight at 4°C with mouse monoclonal anti-P-GP (clone C494, Signet Laboratories, Dedham, Massachusetts, USA), diluted 1 in 1,500 in 10% NGS in PBS and with mouse monoclonal anti-BCRP (clone BXP-21, Merck KGaA, Darmstadt, Germany), diluted 1 in 100 in 10% NGS in PBS. The slides were rinsed in TRIS buffer and then incubated with secondary anti-mouse antibody (biotinylated goat anti-mouse immunoglobulins; Dako, Glostrup, Denmark) diluted 1 in 200 in 10% NGS in PBS. The reaction was amplified by the avidin-biotin method (ABC-kit elite; Vector, Burlingame, CA) and visualized after incubation with 3,3′-diaminobenzidine tetrahydrochloride 0.04% for 2 minutes in the dark, at room temperature (DAB chromogen/substrate kit; Diagnostic BioSystem, Pleasanton California, USA). Sections were counterstained with

Papanicolaou hematoxylin, rinsed in tap water, dehydrated, and coverslipped. Negative controls were run in parallel, by substituting the primary antibody with an irrelevant isotype-matched antibody. Sections of normal canine liver were used as positive controls.

As a consequence of the low dilution of the antibody in this standardized protocol, IHC with BCRP was evaluated in only 26 carcinomas because of the total use of the reagent available at that time.

Samples labelled for P-gp expression were considered positive when at least 20% of the cells in 10 HPFs showed membrane or cytoplasmic labelling. This cut-off level was based on what reported by Petterino and colleagues (2006), with 18.40% being the best discriminating cut-off value in studies of human breast cancer (Petterino et al., 2006). BCRP expression was scored as positive if >10% of the tumor cells were labelled (Diestra et al., 2002). The 10% cut-off level was chosen for two reasons: firstly, because a small number of cells positive for MDR-related proteins may have clinical significance, and secondly, because 10% positivity is the lowest level of expression that can be most consistently detected in formalin-fixed and paraffin wax-embedded tissue sections (Diestra et al., 2002). For both P-gp and BCRP, the intensity of labelling was evaluated in comparison with that of endothelium (used as a positive internal control). Cells showing no reaction or a very faint reaction compared with endothelium were considered negative, while those showing an intensity of labelling similar to that of the endothelium were considered positive. The adjacent or supporting stroma was considered positive only if fibroblasts and/or fibrocytes showed cytoplasmic labelling of the same or greater intensity as compared with endothelium. Cases showing no reactivity of lymphatic and/or vascular endothelium to P-gp or BCRP (probably because of a deterioration of the antigen due to formalin fixation) were excluded.

Statistical Analysis

Comparison of P-gp and BCRP positivity between groups was tested by Fisher's exact method (GraphPad Software, La Jolla, California, USA; accessed October 2017). $P < 0.05$ was considered significant.

Results

The study included 54 samples of CMC.

According to the histomorphological classification the group included:

- 12 complex carcinomas,

- 12 simple tubulopapillary carcinomas,
- 10 carcinomas arising in a mixed tumor,
- 5 tubular carcinomas,
- 3 solid carcinomas,
- 2 adenosquamous carcinomas,
- 2 micropapillary intraductal carcinomas,
- 2 mixed carcinomas,
- 2 comedocarcinomas,
- 1 cribriform carcinoma,
- 1 lipid-rich carcinoma
- 1 inflammatory carcinoma.

According to the histological staging system proposed by Gilbertson et al. (1983) the caseload was composed of:

- 3 stage 0/ *in situ* carcinomas,
- 46 stage I carcinomas
- 5 stage II carcinomas (1 with lymphovascular invasion and 4 with lymph node metastases).

According to the histological grading proposed by Peña et al. (2011) the caseload was composed by:

- 40 grade 1 carcinomas,
- 9 grade 2 carcinomas,
- 4 grade 3 carcinomas.

In the case of the 1 inflammatory carcinoma this grading system was not applied: for their peculiar aggressiveness and elevated angioinvasive properties, per definition, inflammatory carcinomas cannot be graded (Goldschmidt et al., 2011).

At immunohistochemistry the cell membrane of hepatocytes and biliary epithelial cells and the cytoplasm of endothelial cells showed a strong reaction with both anti-P-GP and anti-BCRP reagents.

IHC with P-gp showed that 63% of the cases (34/54) overexpressed P-gp in carcinomatous cells, and IHC with BCRP showed that 76.9% of the cases (20/26) cases overexpressed BCRP in carcinomatous cells 1).

Both P-gp (Figure 2) and BCRP (Figure 3) showed a membranous and occasionally cytoplasmic staining pattern.

The immunostaining with both P-gp and BCRP was available in 26 cases.
In 50% of the cases (13/26) the same carcinoma overexpressed both P-gp and BCRP (Figure 4), in 34.6% of the cases (9/26) just one of the chemoresistance-markers was expressed by the carcinoma, and in 15.4% of the cases (4/26) no expression of P-gp and BCRP was found (Graph 2).
The inflammatory carcinoma was found to express both P-gp and BCRP.
A statistical analysis of the distribution of the expression of P-gp and BCRP was performed comparing the different histological subtypes, histological staging and grading. No statistically significant difference was found in the expression of P-gp and BCRP, as both the markers had a variable % of positive cases among the different groups.

Discussion

This study examined the expression of the MDR-associated proteins P-gp in 54 CMCs and BCRP in 26 in CMCs by IHC.
The major finding of this experiment was that, in CMCs, epithelial carcinomatous cells often overexpressed P-gp and BCRP, in 63% and 76.9% of the cases, respectively, and that half of carcinomas co-expressed both P-gp and BCRP, before being subjected to a chemotherapeutic regimen (intrinsic chemoresistance).

Such an elevated percentage of P-gp- and BCRP-positive carcinomas is consistent with that reported in the literature. In human breast cancer high percentages of P-gp positive cases at IHC have been reported in various studies in pre-treatment tumor samples (Cordon-Cardo et al., 1990; Linn et al., 1996, 1995). The proportion of breast tumors expressing P-gp considering many human studies was 41.2%, but substantial heterogeneity has been found in this value across individual studies (Trock et al., 1997).
In veterinary oncology most of the authors have evidenced that an increased in P-gp expression seems to be related with the degree of malignancy of the tumor, and the number of P-gp-positive cases found by these author is consistent with the findings of our experiments, i.e. around 60% (Badowska-Kozakiewicz and Malicka, 2010; Koltai and Vajdovich, 2014; Petterino et al., 2006).

Studies investigating BCRP expression in breast cancer reported lower percentages of positivity, with 22 to 33% of their caseloads overexpressing BCRP (Lemos et al., 2009; Mao and Unadkat, 2015, 2005; WANG et al., 2013; Yuan et al., 2008). However, studies in veterinary oncology have reported higher levels of BCRP expression compared to the ones in breast cancer. In fact all the canine mammary cell lines examined in the study of Honscha et al. (2009) were found to express BCRP. In an IHC study >85% of simple adenocarcinomas were BCRP-positive and correlation between BCRP increased expression and degree of malignancy was found (Nowak et al., 2009). According to these findings the overexpression of BCRP is a more common phenomenon in CMCs than in human breast cancer.

In human oncology is frequent to find a co-expression of P-gp and BCRP in tumor cancers (Chen et al., 2016). In this study we found that half of the carcinomas co-overexpressed P-gp and BCRP, thus presumably having a more complex and chemoresistant phenotype. Nevertheless these pumps have an overlapped specificity for a broad range of substrates, so selective inhibition of one ABC-efflux transporters could be compensated by the remaining transporters (Chen et al., 2016).

The expression of P-gp and BCRP has been reported to be associated with peculiar features of malignancy, 'stemness', invasiveness and aggressiveness, thereby imparting a poor prognosis (Dean et al., 2005; Huls et al., 2009; Nakanishi and Ross, 2012; Zhou et al., 2001).
The present study has investigated the expression of P-gp and BCRP in a cohort of malignant tumors, classified according to the histological grade, a parameter by which the malignancy of the carcinoma is assessed and correlates with prognosis (Goldschmidt et al., 2011; Peña et al., 2013), and according to the histological stage proposed by which the invasiveness of the carcinomas is assessed (Gilbertson et al., 1983)
In this study no statistical significant difference of P-gp and BCRP expression have been found among groups and histological grades and stages. However, in our caseload the group of stage I tumor was over-represented compared to the other stages, with 46 out of 54 cases for P-gp, and 22 out of 26 for BCRP invalidating a statistical comparison. The same happened with the comparison of P-gp and BCRP expression based on the histological grade of malignancy with grade 1 over-represented (40 out of 54 cases for P-gp, and 17 out of 26 for BCRP).

This mirrors the fact that stage I and grade 1 tumors are the most common subtypes of mammary carcinoma in the dog, a species with a lower percentage of aggressive tumor compared to the woman and cat (Sorenmo et al., 2013).
The differentiation between histological stage 0 and stage I has been recently revised suggesting the use of IHC with p63 for differentiating between the in situ carcinomas and the invasive ones (Nguyen et al., 2017; Peña et al., 2014). To better recognize stage 0-*in situ* mammary carcinomas, Nguyen and colleagues have performed IHC with p63 to evidence the continuity of the myoepithelial layer surrounding the luminal epithelial component in stage 0 carcinomas. IHC with p63 is of paramount importance in human breast diagnostic but it is rarely, if ever, performed in veterinary studies (Nguyen et al., 2017). In those studies, including ours, where the invasive nature of the CMC has not been consistently confirmed by p63 immunohistochemistry, a significant part of stage 0 carcinomas may have been diagnosed as invasive (Stage I) causing an impairment of the comparison between groups. Recently in the study of Nguyen and colleagues (2017), *in situ* mammary carcinomas have been carefully excluded from analysis, using p63 immunohistochemistry when necessary, which is rarely performed in veterinary studies, but of paramount importance in human breast oncology.
In previous studies, where the invasive nature of the CMC has not been consistently confirmed by p63 immunohistochemistry, the higher incidence of *in situ* carcinomas may explain the high level of hormone receptor-positive (luminal) neoplasms (Abadie et al., 2017).

Heterogeneity of results is a common feature even in human studies evaluating the expression and prognostic role of MDR associated markers, due to both methodological and biological factors (Cederbye et al., 2016). Nonetheless, a high expression P-gp was often found even if without a consistent association with cancer stage (Clarke et al., 2005). However, comparing different techniques it has been suggested that the correlations with prognosis appear more evident in studies using immunohistochemistry, in adjuvant and neoadjuvant settings, but care must be taken in interpreting staining results when only one or two monoclonal antibodies are used. (Pavelic et al., 1993). The best way to demonstrate and quantify ABC-transporters functionality is using dye efflux studies, but this technique is frequently practically challenging because it requires fresh, single-cell neoplastic cell suspension and adequate facilities (Zandvliet and Teske, 2015). Clinical trials of drug resistance represent the ultimate test of a protein's functional role in clinical drug

resistance and would be the best way to investigate chemoresistance associated with P-gp and BCRP expression (Leonessa and Clarke, 2003).

Conclusions

In conclusion, this study has provided insights into P-gp and BCRP IHC expression in canine mammary tumors not previously exposed to chemotherapy. Both these chemoresistance markers were overexpressed in canine mammary carcinomas in high percentage, and in the half of the cases the same carcinoma overexpressed both the proteins thus presumably bearing multiple mechanisms of chemoresistance and a complex MDR stem cell-like phenotype. The findings confirm previous reports suggesting that canine mammary tumors often express P-gp and/or BCRP, and these proteins could be associated with a malignant and MDR phenotype. In veterinary oncology caution should be used in administering classical chemotherapeutic regimens involving P-gp and BCRP substrates. Screening for the presence of these MDR makers, as suggested in human medicine, may be beneficial. Further studies testing in vivo the functionality of P-gp and BCRP, and perspective clinical trials evaluating dog responses to chemotherapy are warranted.

Figures and Graphs

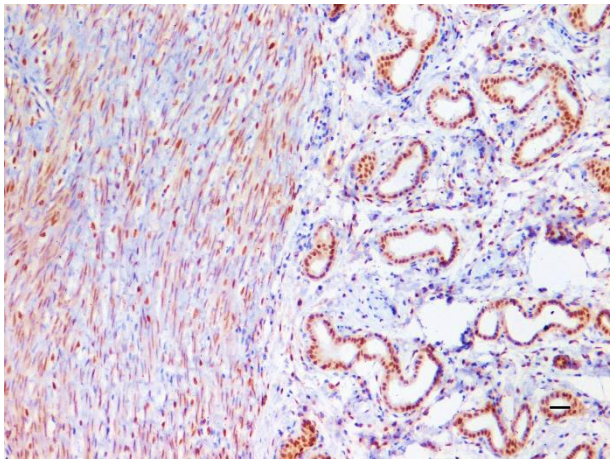


Figure 1. Canine normal uterus. IHC labelling with anti-ER antibody, positive control. Endometrial epithelial cells and myocytes of the myometrium show an intense nuclear immunostaining. Bars, 100 microns.

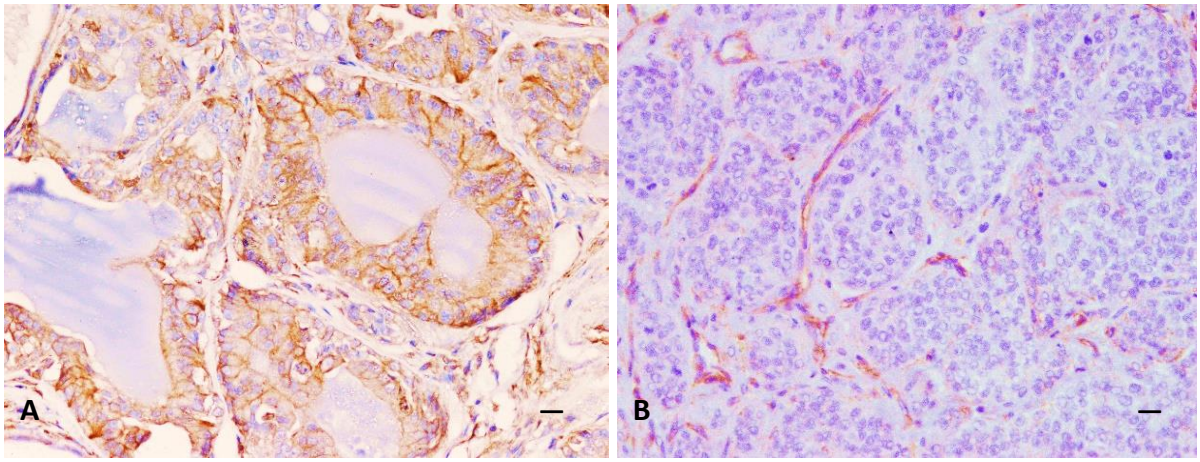


Figure 2. IHC labelling with anti-P-GP antibody. (A) Tubulopapillary carcinoma with intense membranous and weak cytoplasmic expression of P-gp in the epithelial component. (B) Solid carcinoma negative for P-gp. Vascular endothelium, considered as a positive internal control, shows intense staining with P-gp. Bars, 100 microns.

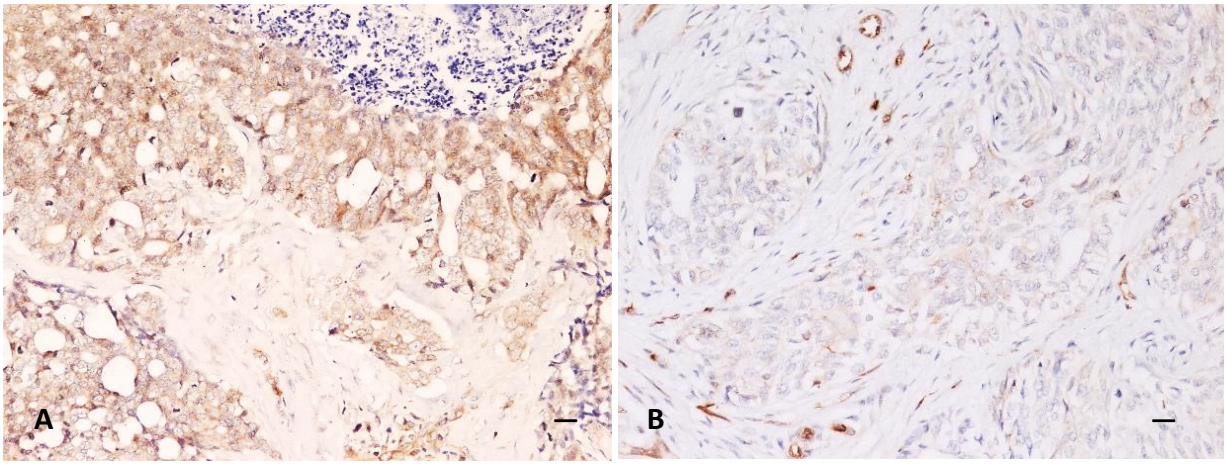


Figure 3. Figure 2. IHC labelling with anti-BCRP antibody. (A) Comedocarcinoma with intense membranous and cytoplasmic expression of BCRP in the epithelial component. (B) Tubulopapillary carcinoma negative for BCRP. Vascular endothelium, considered as a positive internal control, shows intense staining with BCRP. Bars, 100 microns.

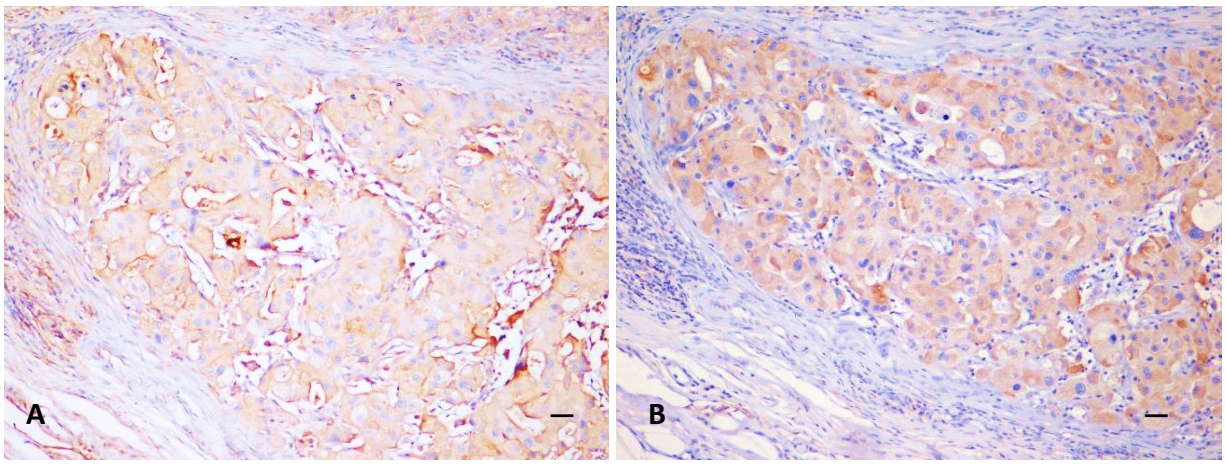
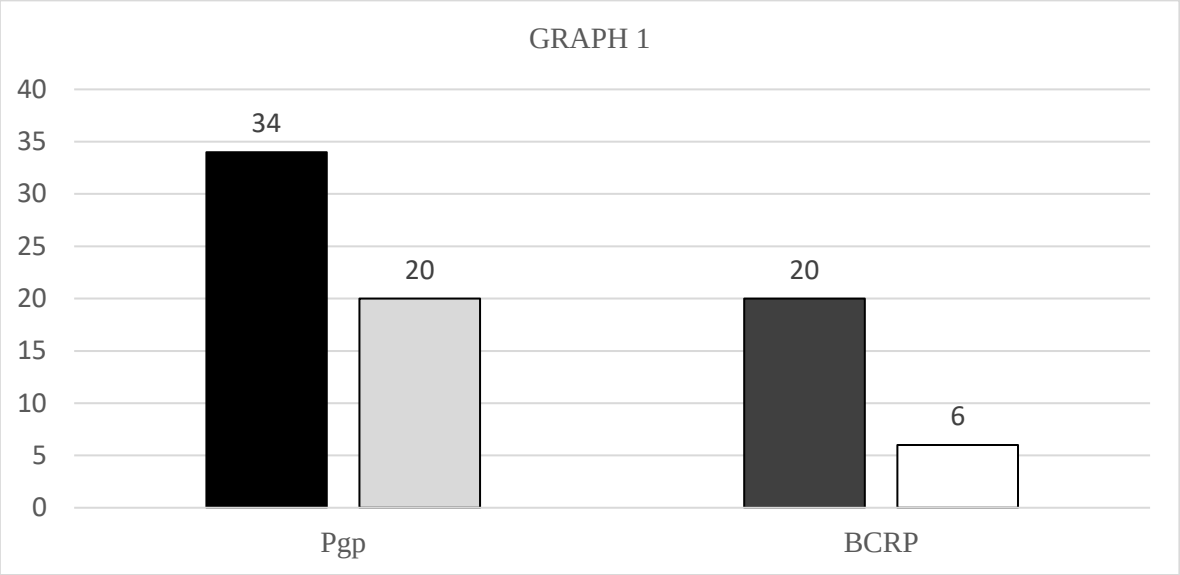
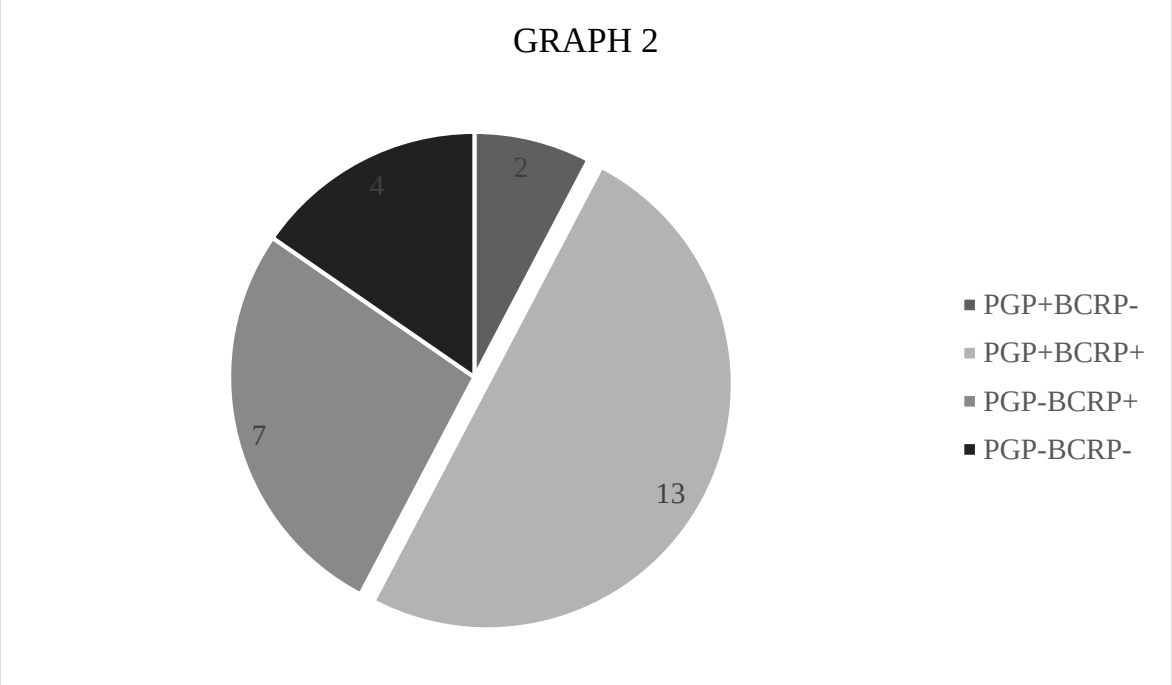


Figure 4. IHC labelling with anti-P-gp antibody (A) and anti-BCRP (B). Solid carcinoma. Neoplastic cells, in the same area, express both P-gp and BCRP, with intense membranous staining and cytoplasmic staining. Bars, 100 microns.



Graph 1. IHC expression of P-gp and BCRP in canine mammary carcinomas. P-gp was overexpressed in epithelial malignant cells of 63% carcinomas (34/54), while BCRP was overexpressed in epithelial malignant cells of 76,9% of carcinomas (20/26). (Black column: P-gp positive cases, light gray column: P-gp negative cases; dark gray column: BCRP positive cases, white column: BCRP negative cases).



Graph 2. IHC expression of both P-gp and BCRP in the same carcinoma. In 50% of the cases (13/26) the same carcinoma overexpressed both P-gp and BCRP, in 34.6% of the cases (9/26) just one of the chemoresistance-markers was expressed by the carcinoma (7/26 P-gp-/BCRP+ and 2/26 P-gp+/BCRP-), and in 15.4% of the cases (4/26) no expression of P-gp and BCRP was present in the same carcinoma.

Publications and Proceedings

A substantial part of this work regarding the classification into molecular phenotypes of canine mammary carcinomas is still ongoing (standardization of IHC protocols for ER and HER2 evaluation), and will be developed in the following months collecting a wider caseload and hopefully solving some of the technical issues we encountered.

Case Report. INCREASED EXPRESSION OF THE CHEMORESISTANCE MARKERS P-GLYCOPROTEIN AND BREAST CANCER RESISTANCE PROTEIN IN A CANINE CUTANEOUS MAST CELL TUMOR TREATED WITH CHEMOTHERAPY AND TYROSINE KINASE INHIBITOR

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Introduction

The onset of multidrug resistance can be related to P-gp and BCRP expression, already described in canine neoplasia (Zandvliet and Teske, 2015). Medical therapeutic approach for canine mast cell tumors (MCT) includes conventional chemotherapy and tyrosine kinase inhibitors (TKIs) (Hahn, 2014).
The protoconcogene *c-Kit* encodes the KIT protein, a tyrosine kinase receptor whose IHC expression pattern has been associated to different biological behavior in MCTs (Kiupel et al., 2004).
TKIs are drug molecules able to block dysregulated KIT activity (Hahn, 2014).
A case of a non-responsive to treatment MCT is described below and IHC expression of P-gp, BCRP and KIT were retrospectively, examined, before and after treatment with Vinblastine + Prednisone.

Description of the Case

A 7-year-old, female spayed, mixed-breed, dog was referred for a cutaneous carpal mass of 3 cm of diameter, and ipsilateral prescapular lymphadenomegaly.
Lesions were surgically excised and the histologic diagnosis was cutaneous MCT (Patnaik's Grade 2, Kiupel's low grade) with lymph node metastases. The owner refused adjuvant chemotherapy. Six months later, 3 new large masses consisting of MCT were diagnosed by cytology, in the surgical scar region.

A neoadjuvant protocol with Vinblastine (2 mg/m^2 every week IV) + Prednisone (1 mg/kg SID PO) was initiated.
After three doses, the disease progressed and the tumors were excised: histologic diagnosis was cutaneous MCT (Patnaik's Grade 2, Kiupel's high grade), infiltrating subcutaneous MCT and MCT lymph node metastasis.
A new protocol consisting of Vinblastine (1.6 mg/m^2 every week IV) + the TKI Masitinib (12.5 mg/kg SID).
After 60 days, the dog developed new MCTs and a rescue protocol with Lomustine (70 mg/m^2 every 3 weeks PO) was initiated. Due to the disease progression, the dog was not necropsied, and therefore samples from the last stage of this MCT were not available. Overall survival was 285 days.
A retrospective IHC exam with P-gp (C494), BCRP (BXP-21), KIT (CD117) was performed and evaluated with already described methods (Diestra et al., 2002; Kiupel et al., 2004; Petterino et al., 2004).

Results

Samples collected before chemotherapy with Vinblastine did not show P-gp (0%cell/HPF) and BCRP (<10%cell/HPF) expression; KIT staining expression was pattern I in the cutaneous mass, and pattern III in the node metastasis (Figure 1).
After chemotherapy with Vinblastine, an increased expression of P-gp from null to low (<10%cell/HPF) in the cutaneous metastasis, and from null to intermediate (10-50% cell/HPF) in the subcutaneous and nodal metastasis were detected. BCRP expression was increased and positive in all the samples (>10%cell/HPF); KIT staining was pattern III (diffuse cytoplasmic) in all the samples (Figure 2).

Discussion and Conclusions

The overexpression of P-gp in canine mast cell tumor have seldom been investigated in the literature. Petterino and colleagues have reported 76% of P-gp positive cases in their study (Petterino et al., 2004), Miyoshi and colleagues have indicated 15% of P-gp-positive MCT (Miyoshi et al., 2002) and Teng and colleagues have revealed 87.5% of P-gp-positve MCT (Teng et al., 2012), with a staining pattern (cytoplasmic and membranous) similar to what we found in our case. The expression of P-gp in canine MCT cell lines have been correlated with drug resistance (Nakaichi et al., 2007).

The increase in P-gp expression, seen in case of MCT after the first treatment with Vinblastine, could be related with the onset of P-glycoprotein-mediated chemoresistance. Indeed Vinblastine is a well-known substrate of P-gp known to induce a functional overexpression of this protein (Zandvliet and Teske, 2015). However, it cannot be excluded that the increased expression of P-gp could be related to the administration of prednisolone, that is a substrate of this pump. The significance of interaction between P-gp and prednisolone has been investigated in the study of Teng and colleagues who found a high degree of P-gp expression in both the pre-treatment and post-treatment MCT specimens (Teng et al., 2012).
Another possible interaction, investigated in canine lymphomas, is the one between P-gp and Masitinib. The potential of Masitinib to revert MDR in canine malignant lymphoma using an in vitro model with canine lymphoid cell lines has been investigated by Zandvliet and colleagues (2013). Masitinib had a mild antiproliferative effect on lymphoid cells and inhibited P-gp function, and has been proposed to have effective inverse doxorubicin resistance onset in canine lymphoma (Zandvliet et al., 2013). However, in this case treatment with Masitinib was not effective in reversing drug resistance, probably emerged towards Vinblastine.

Vinblastine, instead, is not a substrate of BCRP (Nakanishi and Ross, 2012) (Zandvliet and Teske, 2015), but TKIs (namely imatinib, gefitinib, lapatinib) have been reported to be substrates of BCRP (Ozvegy-Laczka et al., 2005) and this transporter has a complex, not yet fully understood, role in the modulation of TKIs. Therefore, BCRP could have interact with Masitinib action leading to the failure of the second protocol.
BCRP is also known to be a stem cell marker, and its expression in cancer cells could be a manifestation of metabolic and signaling pathways that confer multiple mechanisms of drug resistance, self-renewal (stemness), invasiveness and aggressiveness, thereby impart a poor prognosis (Nakanishi and Ross, 2012); all these latter features were present in this case.

The rescue protocol, based on Lomustine, has been proposed as an effective regimen to face chemoresistance in veterinary oncology, partly because Lomustine is not a substrate of P-gp and BCRP (Flory et al., 2008). However, the failure of response in this case can be related to other complex mechanisms of drug resistance (Zandvliet and Teske, 2015).

Pattern III (cytoplasmic) of KIT, seen in all our metastases, is reported to be related to the worst prognosis (Kiupel et al., 2004).

This case of aggressive MCT in a dog suggests something about the complex mechanism that can lead to the failure of a chemotherapeutic treatment. The increased expression of P-gp, BCRP and KIT could all play a role in the chemoresistant and malignant phenotype of this case of MCT.

Figures

Figure 1

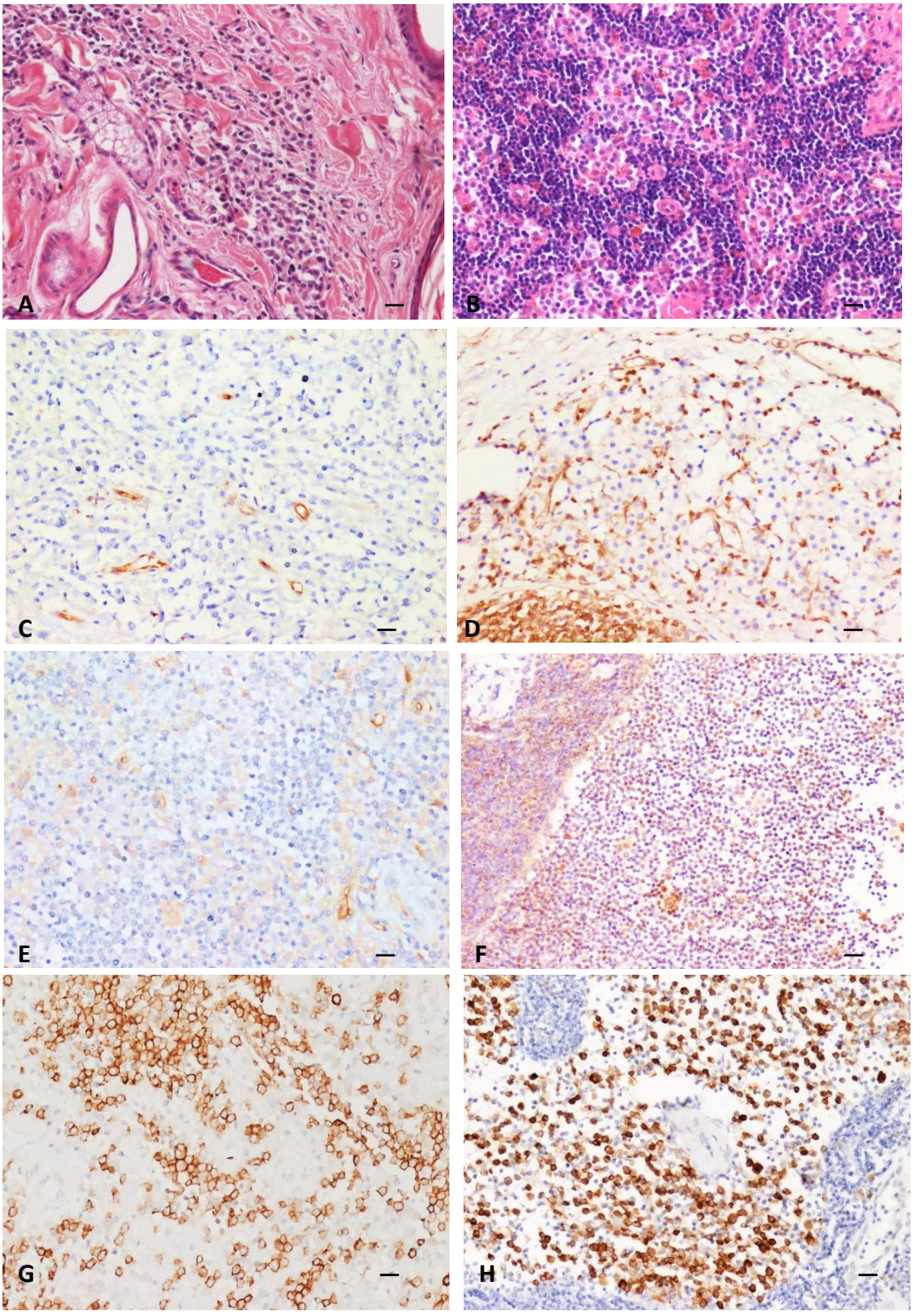

Figure 1. Pre-treatment samples.
HE: Histology of the primary cutaneous MCT (A) and the lymph node metastasis (B).
IHC P-gp: The primary cutaneous MCT (C) and the lymph node metastasis (D) did not show P-gp expression (0%cell/HPF), vascular endothelium was the positive internal CTR.
IHC BCRP: The primary cutaneous MCT (E) and the lymph node metastasis (F) did not show BCRP expression (<10%cell/HPF), vascular endothelium was the positive internal CTR.
IHC CD117: KIT staining expression was pattern I/membranous in the cutaneous mass (G), and pattern III/diffuse cytoplasmic in the node metastasis (H).

Figure 2

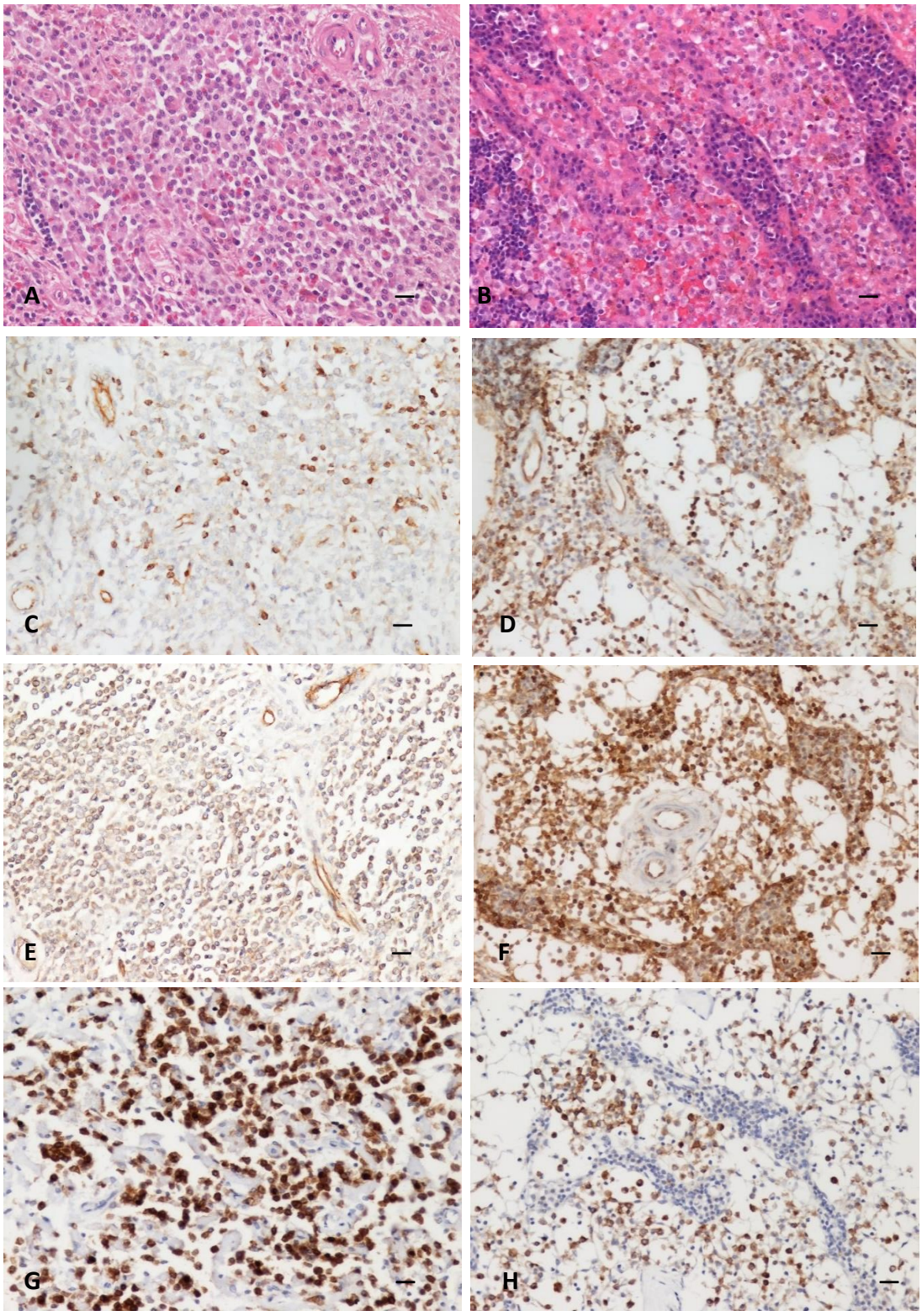

Figure 2: Post-chemotherapy (Vinblastine + Prednisone) samples.
HE: Histology of the cutaneous metastasis of MCT (A) and the MCT lymph node metastasis (B).
IHC P-gp: In the cutaneous metastasis the expression of P-gp was low (<10%cell/HPF) (C). in the lymph node metastasis the expression of P-gp was intermediate (10-50% cell/HPF) (D).
IHC BCRP: In both the cutaneous and the lymph node metastases BCRP expression was positive (>10%cell/HPF) (E and F).
IHC CD117: In both the cutaneous and the lymph node metastases KIT staining was pattern III (diffuse cytoplasmic) (G and H).

Publications and Proceedings

Levi M., Valenti P., Benazzi C., Brunetti B. "Increased expression of the chemoresistance markers P-gp (P-GP) and breast cancer resistance protein (BCRP) in a canine cutaneous mast cell tumor treated with chemotherapy and tyrosine kinase inhibitor". XIII AIPVET Congress - lXX SISVET Congress, Palermo, Italy, 13-16th June, 2016.

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