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# GENETIC POLYMORPHISMS
## An Approach To Canine Mammary Tumors

Ana Canadas Sousa

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Ana Maria Canadas Pereira De Sousa

# GENETIC POLYMORPHISMS
## An Approach To Canine Mammary Tumors

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

**Orientador** Professora Doutora Patrícia Dias-Pereira
Categoria – Professor Auxiliar
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP).

**Co-orientador** Professora Doutora Marta Amaro dos Santos
Categoria – Professora Auxiliar
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP).

**Co-orientador** Professor Doutor Rui Manuel Medeiros
Categoria - Professor Afiliado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP). Coordenador do Grupo de Investigação Oncologia Molecular e Patologia Viral do IPO Porto

***Aim for the moon. If you miss, you may hit a star.***

W. Clement Stone

## DECLARATION

In accordance to the disposed in “nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009”, the author of this thesis declares to have actively participated in the elaboration and execution of experimental work that led to the results presented, as well as in its interpretation and in the writing of the respective manuscripts. The following original manuscripts are integral parts of this PhD thesis:

Canadas, A., M. Santos, A. Nogueira, J. Assis, M. Gomes, C. Lemos, R. Medeiros and P. Dias-Pereira (2018). "Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes." J Vet Diagn Invest **30**(5): 733-738.

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# THESIS OUTLINE

The present thesis has been divided into **ten chapters**.

In **Chapter 1** the state of the art in canine mammary tumors and in genetic polymorphisms is presented.

**Chapter 2** summarizes the aims of the thesis.

**Chapters 3**, **4**, **5**, **6**, **7** and **8** enclose original published results and data presently in revision, submitted and in preparation for submission.

**Chapter 3** emphasizes the importance of the genetic polymorphisms in canine mammary tumors susceptibility. In this work multiple genes known to influence human breast cancer risk are studied. This study, entitled “Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes”, is published in J Vet Diagn Invest, 2018, **30**(5): 733-738.

**Chapter 4** reports the influence of single nucleotide polymorphisms in CDH1 gene on the risk, clinicopathological features and outcome of canine mammary tumors. This study, entitled “Influence of E-cadherin genetic variation in canine mammary tumor risk, clinicopathological features and prognosis” was submitted to Vet Comp Oncol and is currently under revision.

**Chapter 5** focuses the relationship between single nucleotide polymorphisms in ESR1 gene and the clinicopathological features and outcome of canine mammary tumors. This study, entitled “Estrogen Receptors genotypes and canine mammary neoplasia” was submitted for publication to the BMC Veterinary Research and is currently under revision.

**Chapter 6** reports the association between single nucleotide polymorphisms in COMT gene and clinicopathological characteristics and overall survival of canine mammary tumors. This study, entitled “Catechol-o-methyltransferase genotypes are associated with progression and biological behaviour of canine mammary tumours." is published in Vet Comp Oncol **16**(4): 664-669.

**Chapter 7** included a survival study, comprising the two most common histological classification methods, as well as the two most used histological grading systems for canine mammary tumors. This study, entitled "Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study." is published in Vet Pathol **56**(2): 208-219.

**Chapter 8** addresses the association between individual genetic profiles and histological type and grade of canine mammary malignant tumors. This study, entitled “Single Nucleotide Polymorphisms influence histological type and grade of canine malignant mammary tumors” is currently under preparation to be submitted in soon.

In **Chapter 9** a general discussion is presented, combining data obtained in all the previous studies/chapters.

Finally, future perspectives are described in **Chapter 10**.

# RESUMO

Os tumores mamários constituem a neoplasia mais frequente na cadela, sendo caracterizados por uma apresentação clínica, comportamento biológico e características morfológicas e moleculares variáveis. A investigação do cancro da mama na mulher demonstrou a influência de variações genéticas, tais como os polimorfismos de um único nucleótido (SNPs), no risco, progressão, resposta ao tratamento e na sobrevivência.

Atualmente dado o avanço e os crescente número de recursos tecnológicos disponíveis, o patologista veterinário deverá incluir na sua avaliação não só a parte morfológica mas também as caraterísticas genéticas e moleculares das neoplasias mamárias da cadela. Deste modo, contribuirá para a aquisição de informações relevantes acerca do risco, resposta ao tratamento e prognóstico da doença.

Nesta tese foi avaliada a influência de variações genéticas no risco, nas características clínico-patológicas e prognóstico dos tumores mamários nas cadelas. Diferentes SNPs em proto-oncogenes (*HER2*, *EGFR*), genes supressores tumorais (*TP53*, *STK11, CDH1*), genes envolvidos na identificação de danos e reparação do DNA (*BRCA1*, *BRCA2*, *RAD51*, *CHEK2*, *PTEN*, *BRIP1*) e no metabolismo hormonal (*ESR1*, *PGR*, *PLRL*, *COMT*) foram incluídos nos estudos.

O SNP no gene STK11 (rs22928814) foi associado a aumento do risco de desenvolvimento de tumores mamários, enquanto que os SNPs nos genes RAD51 (rs23623251 e rs23642734) e CDH1 (rs850805755 e rs852280880) foram associados a uma menor suscetibilidade para o desenvolvimento de neoplasias mamárias.

Identificou-se ainda associações significativas entre as variações genéticas nos genes STK11 (rs22928814), RAD51 (rs23623251 e rs23642734), CHEK2 (rs397511718), HER2 (rs24537329) e COMT (rs23350589, rs23322686, rs23336579 e rs852564758) e parâmetros clinico-patológicos relacionados com um prognóstico menos favorável, nomeadamente início precoce da doença, presença de múltiplos tumores, maior tamanho tumoral, subtipos histológicos de comportamento biológico agressivo, grau histológico elevado e existência de invasão vascular. Pelo contrário, foram observadas associações significativas entre os SNPs nos genes CDH1 (rs850805755, rs852280880 e rs852639930), BRCA2 (rs23255542), HER2 (rs24537331), ESR1 (rs397512133, rs397510462, rs851327560 e rs397510612) e COMT (rs851328636, rs853133060 e rs853046495) e um início tardio da doença, tumores de tamanho reduzido,

crescimento tumoral de tipo expansivo, subtipos histológicos menos agressivos, baixo grau histológico, reduzido pleomorfismo nuclear e baixo índice de prognóstico.

A reconhecida heterogeneidade histológica dos tumores mamários caninos tem contribuído para o desenvolvimento de diferentes sistemas de classificação histológica e métodos de gradação. Nesta tese foi efetuado um estudo comparativo pormenorizado entre as classificações histológicas mais utilizadas (classificação da Organização Mundial de Saúde e a classificação de 2011) e entre os métodos de gradação mais frequentemente aplicados (grau histológico de Nottingham e grau histológico de Nottingham adaptado à cadela) aos tumores mamários caninos. Ambos os sistemas de classificação relevaram relevância prognóstica dado terem sido identificados subtipos histológicos associados a bom prognóstico e subtipos de índole agressiva relacionados a um prognóstico desfavorável, por sua vez, associado a risco de morte relacionada à neoplasia. Adicionalmente, o grau histológico foi associado à sobrevivência, independentemente do sistema de classificação empregue, observando-se apenas discretas diferenças entre os dois métodos.

Na generalidade os dados reportados reforçam a importância das características genéticas e fenotípicas no desenvolvimento, progressão e comportamento biológico dos tumores mamários das cadelas. Apesar de preliminares, estes resultados colocam em evidência o papel importante do perfil genético no aprofundamento do conhecimento dos tumores mamários e ressaltam a importância das principais características histopatológicas relacionadas com o prognóstico da doença, incluindo a sua classificação histológica. Com base nestas evidências, compreende-se que a interação entre o perfil genético do animal e as características do tumor são determinantes para obter um retrato mais detalhado da neoplasia mamária na espécie canina, sendo ambos reconhecidos como ferramentas valiosas para o maneio clínico da doença, auxiliando na implementação de medidas preventivas e terapêuticas, adaptadas a cada animal de acordo com os critérios da chamada medicina veterinária de precisão.

# ABSTRACT

Canine mammary tumors are a common neoplasia characterized by a considerable variation in clinical, morphologic and molecular attributes, as well as in biological behaviour. Genetic research in human breast cancer has demonstrated a relationship between genetic variations (such as single nucleotide polymorphisms - SNPs) and the risk, progression, treatment response and outcome of the disease.

Nowadays different technological resources are available and this forces to review the role of the veterinary pathologist in mammary oncology. By using that technology, the pathologist has the opportunity to search for genetic, molecular and morphological features that may provide relevant information regarding the progression, response to treatment and outcome of the disease.

Herein the influence of genetic variation on the risk, clinicopathological features and outcome of canine mammary tumors was evaluated. Canine SNPs in proto-oncogenes (*HER2*, *EGFR*), tumor suppressor genes (*TP53*, *STK11, CDH1*), genes involved in DNA damage recognition and repair (*BRCA1*, *BRCA2*, *RAD51*, *CHEK2*, *PTEN*, *BRIP1*) and in hormonal metabolism (*ESR1*, *PGR*, *PRLR*, *COMT*) were assessed.

The SNP in STK11 gene (rs22928814) was associated with a high risk for developing canine mammary tumors, while the SNPs in RAD51 (rs23623251 and rs23642734) and CDH1 (rs850805755 and rs852280880) genes were related to a decreased susceptibility to the disease.

Furthermore, a significant relationship between genetic variations in STK11 (rs22928814), RAD51 (rs23623251 and rs23642734), CHEK2 (rs397511718), HER2 (rs24537329) and COMT (rs23350589, rs23322686, rs23336579 and rs852564758) genes and clinicopathological parameters related to a guarded prognosis, namely early onset of the disease, multiple tumors, larger tumor size, aggressive histotypes, high histological grade carcinomas and vascular invasion was detected. On the other hand, significant associations were reported between SNPs in genes CDH1 (rs850805755, rs852280880 and rs852639930), BRCA2 (rs23255542), HER2 (rs24537331), ESR1 (rs397512133, rs397510462, rs851327560 and rs397510612) and COMT (rs851328636, rs853133060 and rs853046495) and a later onset of the disease, small tumor size, expansive tumoral growth, less aggressive histotypes, low histological grade, reduced nuclear pleomorphism and low prognostic index.

The marked histological heterogeneity of canine mammary tumors had contributed to the development of different histological classification schemes and grading methods. In this thesis a comprehensive comparison between the most commonly used histological classifications (World Health Organization classification and the 2011 classification) and grading systems (Nottingham histological grade and the canine-adapted Nottingham histological grade) of canine mammary tumors was performed. Both the classification schemes had prognostic relevance by identifying histotypes associated with good prognosis, as well as aggressive histological subtypes related to poor outcome and high risk of cancer related death. Furthermore, the histological grade was associated with survival, irrespective of the grading system employed and only slight different were detected between the two grading methods.

In general, this data reinforce the importance of both genetic and phenotypic features in the development, progression and biological behavior of canine mammary tumors. Allthough preliminary, these results suggest that the genetic background constitutes an important clue for a comprehensive knowledge of canine mammary tumors and underline the importance of key histopathological features related to the outcome of the disease, including the histological classification. Based on the present data, the interaction between the animal's genetic profile and the tumor features are determinants to get the full portrait of the canine neoplastic mammary disease, and both are valuable tools for the clinical management of the disease, assisting in the implementation of animal-tailored preventive and therapeutic measures in the line of the precision veterinary medicine.

# ABBREVIATION LIST

AgNOR – Argyrophilic nucleolar organizer region

BRCA1 - Breast cancer type 1 susceptibility protein

BRCA2 – Breast cancer type 2 susceptibility protein

BRIP1 - BRCA1-interacting protein 1

ca-NHG – Canine-adapted Nottingham histological grade

CDH1 – Cadherin-1

CHEK2 – Checkpoint kinase 2

CMTs – Canine mammary tumors

COMT – Catechol-O-methyltransferase

COX-2 – Prostaglandin-endoperoxide synthase 2

DFS – Disease-free survival

DNA – Deoxyribonucleic acid

E2 – Estradiol

EGFR – Epidermal growth factor receptor

ER – Estrogen receptor

ESR1 – Estrogen receptor 1

HER2 – Human epidermal growth factor 2

LSP1 – Lymphocyte specific protein 1

MAP3K1 – Mitogen-activated kinase kinase kinase 1

MMP-9 – Matrix metalloproteinase-9

NHG – Nottingham histological grade

NPI – Nottingham prognostic index

OHE - Ovariohysterectomy

OS – Overall survival

PCNA – Proliferating cell nuclear antigen

PECAM – Platelet-endothelial cell adhesion molecule-1

PGR – Progesterone receptor

PTEN – Phosphatase and tensin homolog

PRLR – Prolactin receptor

RAD51 – RAD51 recombinase

RNA – Ribonucleic acid

ROC – Receiver operating curve

ROS – Reactive oxygen species

SNPs – Single nucleotide polymorphisms

STK11 – Serine/threonine kinase 11

TP53 – Tumor protein p53

uPA - Urokinase plasminogen activator

VEGF – Vascular endothelial growth factor

vet-NPI – Veterinary adapted Nottingham prognostic index

WHO – World Health Organization

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# CHAPTER 01

## General Introduction

## Genetic and Cancer

Cancer, the so-called “disease of the genome” is defined as an uncontrolled “new growth” (Yates *et al.* 2012, Garraway *et al.* 2013, Kumar *et al.* 2015, Cullen *et al.* 2017). In this process normal cells undergo different and irreversible genetic changes making them less responsive to growth control mechanisms, allowing them to expand beyond their normal anatomic boundaries, and thus creating a microscopically or macroscopically detectable neoplasm (Nowell 1976, Kusewitt *et al.* 2016).

Three recognized steps are essential in the multistage process of cell transformation: initiation, promotion and progression (Pitot 1993, Kumar *et al.* 2015, Cullen *et al.* 2017). The initiation step comprises an irreversible DNA damage caused by a mutagenic agent (initiator). Many intrinsic and extrinsic agents can be responsible to trigger the neoplastic process. Intrinsic factors include endogenous substances, reactive oxygen species (ROS), polymerase errors, decreased telomere length, altered telomere activity, mistakes in chromosome segregation, among others. Extrinsic factors include chemicals, physical and infectious agents (Kusewitt *et al.* 2016, Wu *et al.* 2016). The promotion phase reflects the proliferation of initiated genetically unstable cells, which may be favoured by growth factors and external stimuli (promoters). The progression phase represents an irreversible state characterized by genetic instability and increased tumor cell heterogeneity (Barrett *et al.* 1987, Cullen *et al.* 2017). In the course of the neoplastic transformation process cells acquire several traits, collectively recognized as the “hallmarks of cancer”, that allow them to survive in different environments (Cullen *et al.* 2017). These capabilities include unlimited replicative activity, insensitivity to anti-growth signals, evasion of apoptosis, self-sufficiency in growth signals, promotion of angiogenesis and increased invasive properties (Hanahan *et al.* 2000, Hanahan 2011). More recently, inflammation has been suggested to represent the seventh hallmark of cancer (Coussens *et al.* 2002, Mantovani 2009, Fouad *et al.* 2017).

Cancer is a sequential and stepwise process, involving a diversity of genetic and epigenetic changes, which ultimately affect gene expression, driving to the development of a neoplastic phenotype (Baylin *et al.* 2000, You *et al.* 2012, Cullen *et al.* 2017). Genetic injuries or DNA damage are regular events occurring in a cell population, however each organism holds complex mechanisms of repair, like

checkpoints and tumor suppressors proteins, that can minimize such damage (Motoyama *et al.* 2004, Hakem 2008). The persistence of the damage and the resultant synthesis of altered DNA in the daughter strand lead to the “fixation of a mutation” (Uecker *et al.* 2011, Kumar *et al.* 2015). A fixed mutation has the potential to alter gene expression or gene copy numbers and consequently modify protein structure, function and/or amount of protein production (Camps *et al.* 2007, Kumar *et al.* 2015, Kusewitt *et al.* 2016). Gene expression can also be enhanced or suppressed by epigenetic events. In this case, despite alterations in the DNA primary sequence do not occur, changes in DNA methylation, histone modifications and nucleosome remodelling may interfere with gene expression (Jones *et al.* 2007, Sharma *et al.* 2010, Kanwal *et al.* 2012, Deans *et al.* 2015, Kumar *et al.* 2015, Kusewitt *et al.* 2016, Cullen *et al.* 2017). Several researchers have underlined the importance of epigenetic changes in the carcinogenic process (Baylin *et al.* 2011, Sandoval *et al.* 2012).

There are various types of genetic alterations, some of them involving changes in the number (aneuploidy) or configuration of chromosome, while others comprise subtle changes in DNA strands. Slight alterations in DNA strands such as insertions, deletions, inversions, mutations, and single nucleotide polymorphisms (SNPs) comprise the majority of the genetic changes. SNPs are by far the most common type of genetic variation in the human genome. They involve a variation in a single nucleotide at a specific region of the genome, which is common in a determined population, occurring in a frequency of at least 1% of the individuals (Weiss 1998, Brookes 1999, Sachidanandam *et al.* 2001, Wright 2001, Goldstein *et al.* 2005). SNPs can occur in proto-oncogenes and tumor suppressor genes, in genes responsible for DNA repair, cell cycle regulation, metabolism and immunity, among others. Accordingly, SNPs may influence several organic functions and participate in different mechanisms of complex diseases, namely carcinogenesis (Angele *et al.* 2003, Povel *et al.* 2012, Broustas *et al.* 2014, Karrman *et al.* 2015).

SNPs can be present in different locations of the gene: promoters, exons, introns, 5´- and 3´UTRs regions (Tatarinova *et al.* 2016, Deng *et al.* 2017). Exonal SNPs are classified as non-synonymous and synonymous, based in the capability to replace the encoded amino acid (Hunt *et al.* 2009, Deng *et al.* 2017). Non-synonymous SNPs involve amino acids changes resulting in modifications in the protein structure (Yates *et al.* 2013, Deng *et al.* 2017). In synonymous SNPs there

is no amino acids change, so the primary structure of the protein is not altered (Hunt *et al.* 2009). Despite the fact that they do not involve amino acid substitutions, synonymous SNPs may interfere with the accuracy and speed of transcription and/or translation, induce alternative splicing and produce structural changes in messenger RNA or in its stability. These attributes may result in important changes in the conformation, stability and functional activity of the protein produced (Hunt *et al.* 2009, Deng *et al.* 2017) and, thus become biologically relevant. Intronic SNPs have been neglected for a long period of time since they are not locate in coding regions (Cooper 2010). They are, however, involved in multiple tasks including gene expression modification by cis-acting regulatory elements, regulation of protein synthesis by mRNA splicing, influence in genomic imprinting, regulation of gene expression via long non-coding RNA and interference in chromatin looping (Cooper 2010, Deng *et al.* 2017). Recently, a significant number of investigations showed that intronic SNPs could be associated with disease susceptibility, including human breast cancer (Marian *et al.* 2011, Robbez-Masson *et al.* 2013, Ng *et al.* 2014, Deng *et al.* 2017, Esmaeili *et al.* 2018). The influence of intronic SNPs was also demonstrated in other diseases like several canine metabolic disorders (Udagawa *et al.* 2014, Plassais *et al.* 2017), ophthalmologic and (Dekomien *et al.* 2003, Kanemaki *et al.* 2013) parasitic diseases (Sanchez-Robert *et al.* 2008, Mani *et al.* 2016). In mammary carcinogenesis of canine species, different authors also investigated intronic SNPs as having a potential role in risk or in clinicopathological features of CMTs (Borge *et al.* 2013, Enginler *et al.* 2014). SNPs involved in promoter and in 5´- and 3´UTRs regions have also been described as being related with canine diseases such as diabetes (Short *et al.* 2010) or with the increased risk of mammary tumors (Sun *et al.* 2015).

Besides the recognized effect of individual SNPs in cancer, the combined effect of functionally relevant SNPs may additively contribute to alter cancer risk or disease progression (Onay *et al.* 2006, Chuang *et al.* 2012, Sapkota *et al.* 2014). This event, called epistasis, involves a gene-to-gene interdependency phenomenon by which the effect of one gene is influenced by the presence of other(s) gene(s) (de Visser *et al.* 2011, Yates *et al.* 2012). This SNP-SNP interaction represents an important target in oncological investigation, namely in breast cancer (Yang *et al.* 2013, Jamshidi *et al.* 2015).

The investment in genetic research made in the last decades allowed the recognition of a huge inter-individual genetic variability, largely attributed to SNPs. This individual genetic diversity may be responsible for significant biochemical and functional effects, thus interfering with the susceptibility, development, clinical course and response to treatment of several diseases, including human breast cancer (Erichsen *et al.* 2004, Wang *et al.* 2017). Genetic signatures are nowadays considered a useful instrument for the development of prevention, screening and early detection strategies in human mammary cancer. They can also represent a powerful tool for personalized medicine and theranostics, assisting in the selection of the most effective and safe therapeutic protocols in cancer management, according to the patient genetic profile (Erichsen *et al.* 2004, Offit 2011, Stover *et al.* 2015, Wang *et al.* 2017).

Despite the fact that SNPs were studied in different fields of veterinary research, their assessment in the context of canine mammary carcinogenesis is still scarce. Notwithstanding, there is a growing body of evidence underlining the importance of the genetic background in the susceptibility, clinical and pathological features and outcome of canine mammary tumors (CMTs). In 2007, Bhattacharya and colleagues evaluated the influence of the polymorphism on the ZuBeCa3 microsatellite (highly polymorphic repetitive DNA sequences) in CMTs. The authors found that this SNP was more frequent in cancer cases than in control animals, associating it with an increased susceptibility of mammary tumors development, as well as with the histological subtypes of mammary tumors, namely benign and malignant tumors including solid and papillary adenocarcinoma (Bhattacharya *et al.* 2007). Later, Dias Pereira and colleagues demonstrated a relation between canine COMT (catechol-O-methyltransferase) SNPs G216A (rs853133060) and G482A (rs853046495) and important clinicopathological features of CMTs. They showed a protective effect of the COMT G482A genetic variant on the age at onset of mammary tumors, with carriers of the variant allele presenting a threefold likelihood of developing mammary tumors after 9 years, comparing with wild-type animals (Dias Pereira *et al.* 2008). These authors also demonstrated the influence of COMT genotypes on the outcome of CMTs, with carriers of genetic variations presenting an higher risk of local recurrences (Dias Pereira *et al.* 2008, Dias Pereira *et al.* 2009a). This finding provides useful clinical information regarding the selection of the most suitable surgical approach for each dog, based on genetic profile. Other

authors described an association between SNPs in BRCA 1 and BRCA2 genes and an increased susceptibility for CMTs development (specifically for malignant tumors and when considering BRCA1 in particular) (Rivera *et al.* 2009). However, these results are not consensual and were not confirmed by other authors in a different cohort (Borge *et al.* 2011). The relationship between other SNPs in genes known to be involved in human breast cancer (namely BRIP1, CDH1, CHEK2, EGFR, ESR1, FGFR2, HER2, LSP1, MAP3K1, PTEN, RCAS1, STK11, TOX3 and TP53 genes) and CMTs susceptibility has been assessed by some investigators, but no significant associations were found (Rivera *et al.* 2009, Borge *et al.* 2011). More recently, a significant difference in allele frequencies for several SNPs in canine ESR1 gene was described when comparing high-risk (English Springer Spaniel, Boxer, Cocker Spaniel and Dachshund) and low-risk (Beagle, Bernese Mountain Dog, Collie and Shetland Sheepdog) canine breeds for developing mammary tumors (Borge *et al.* 2013).

In spite of the scarce information in the field of veterinary genetic oncology available to date, the identification of genetic profiles associated with the susceptibility and progression of CMTs arises as a potential tool in the conception of preventive/surveillance protocols for high-risk animals and in an individualized clinical management of the disease based on the animal's genetic profile (Blomme *et al.* 2010, Klopfleisch 2016, Lloyd *et al.* 2016, Pang *et al.* 2016).

**Canine mammary tumors**

Canine mammary tumors are characterized by a remarkably heterogeneous morphology and biological behavior, constituting therefore a focus of intense research by veterinary oncologists and pathologists (Sorenmo 2003, Rivera *et al.* 2009, Salas *et al.* 2015). The development of recent molecular tools such as complete coverage of the canine genome (Lindblad-Toh *et al.* 2005), cDNA microarrays, and proteomic analyses have been crucial for unravelling several features of CMTs complex pathogenesis (Klopfleisch *et al.* 2011, Abdelmegeed *et al.* 2018).

Epidemiologic data of CMTs varies according to geographic location and medical practices of ovariohysterectomy (OHE), however it is consensual that CMTs represent about 30-70% of all canine cancers (Dorn *et al.* 1968, Merlo *et al.* 2008,

Sorenmo *et al.* 2011, Vascellari *et al.* 2016), being more frequent in females than male dogs, especially in intact ones (Dorn *et al.* 1968, Moulton 1990, Sorenmo 2003, Salas *et al.* 2015, Klopfleisch 2016, Goldschmidt *et al.* 2017).

Fifty to almost 70% of dogs with CMTs have multiple, synchronous or metachronous tumors (Fowler *et al.* 1974, Benjamin *et al.* 1999, Misdorp 2002, Sorenmo 2003, Sorenmo *et al.* 2009) of the same or different histological type (Misdorp 2002, Sorenmo 2003, Sorenmo *et al.* 2009).

The peak incidence of CMTs occurs around 8-9 years old, with oldest animals being affected mainly by malignant tumors (Sorenmo *et al.* 2009, Sleeckx *et al.* 2011, Vascellari *et al.* 2016). The development of mammary tumors in dogs younger than five years old is rare and, in most cases, only benign tumors are diagnosed at that age (Kurzman *et al.* 1986, Perez Alenza *et al.* 2000, Sleeckx *et al.* 2011). Also large-breed dogs seems to develop mammary neoplasms at a younger age than small-breed ones (Goldschmidt *et al.* 2017).

Malignant tumors account for 50-80% of the CMTs (Dorn *et al.* 1968, Sorenmo *et al.* 2000, Rivera *et al.* 2009, Benavente *et al.* 2016a, Melin *et al.* 2016, Goldschmidt *et al.* 2017). It has been suggested that malignant mammary tumors can develop within pre-existing benign tumors and that dogs with pre-malignant lesions, or with history of a previous malignant mammary tumors, show an increased risk of developing a new mammary malignant lesion (Sorenmo *et al.* 2009).

Several studies referred a significantly higher incidence of CMTs in pure breed dogs than in mixed-breed ones (Dorn *et al.* 1968, Schneider *et al.* 1969, Merlo *et al.* 2008, Goldschmidt *et al.* 2017), which can be related to an inbreeding effect (Dorn *et al.* 1976), emphasizing a potential genetic basis in canine mammary carcinogenesis (Kurzman *et al.* 1986, Boldizsar *et al.* 1992, Sorenmo 2003, Egenvall *et al.* 2005, Sorenmo *et al.* 2011). According to some authors, English Springer Spaniels, Cocker Spaniels, German Shepherds, Maltese, Yorkshire Terriers, Dachshunds, English Setter, Labrador Retriever, Pointer, Doberman and Schnauzer dog breeds are at increased risk of developing CMTs, however this evidence varied according to the geographic location and breed popularity (Mitchell *et al.* 1974, Priester 1979, Kurzman *et al.* 1986, Yamagami *et al.* 1996, Egenvall *et al.* 2005, Jitpean *et al.* 2012, Vascellari *et al.* 2016).

The mammary gland is found specifically in mammals being nutrition and immunity the primordial functions to provide the young born (Cowie 1974, Sorenmo

*et al.* 2011, Schlafer *et al.* 2016, Goldschmidt *et al.* 2017). In canine species, mammary glands extend to the ventral thorax and abdomen, parallel along the midline of both thoracic and abdominal walls (Cowie 1974, Sleeckx *et al.* 2011). Commonly, females have five pairs of glands: cranial (M1) and caudal (M2) thoracic mammary glands; cranial (M3) and caudal (M4) abdominal mammary glands; and inguinal (M5) mammary gland pairs (Sleeckx *et al.* 2011, Sorenmo *et al.* 2011).

Histologically, the mammary gland is a modified apocrine sweat gland, with a tubuloalveolar structure, divided into lobules by a well-vascularized stromal connective tissue (Santos *et al.* 2010). The fibro-vascular and adipocyte-rich stroma placed around ductal or glandular elements form the intra-lobular stroma (Sorenmo *et al.* 2011). The branching system begins in the secretory alveoli which drains to the intra-lobular ducts, then to interlobular ducts, and finally to the large lactiferous ducts that ends in a lactiferous sinus. The sinus continues into the nipple sinus, and opens in the nipple surface via the papillary duct (Sleeckx *et al.* 2011, Sorenmo *et al.* 2011, Schlafer *et al.* 2016). Papillary duct exits via an ostium and is characterized by the presence of a sphincter. The nipple is covered by epidermis that exhibits a keratinized stratified squamous epithelium (Goldschmidt *et al.* 2017). Secretory alveoli are lined by simple cuboidal to columnar epithelium, with variable numbers of intra-cytoplasmatic lipid droplets, and by star-shaped myoepithelial cells (responsible for milk let-down) placed on a continuous basement membrane (Goldschmidt *et al.* 2017). Smaller intra-lobular ducts are lined by a single layer of cuboidal epithelium while larger ducts are lined by a bi-layered cuboidal epithelium. All the ducts are surrounded by fusiform contractile myoepithelial cells (Sleeckx *et al.* 2011, Goldschmidt *et al.* 2017). Along with the mature adipose tissue in the interstitial tissue, a variable amount of histiocytic cells, plasma cells and small lymphocytes can be identified (Schlafer *et al.* 2016).

The intense remodelation under hormonal stimulation since puberty and during the entire reproductive life of the dog is an important feature of this organ. During each estrous cycle, the mammary gland experience successive proliferative and regressive changes, more pronounced in the most caudal (M4 and M5) gland pairs (Mulligan 1942, Rehm *et al.* 2007, Santos *et al.* 2010). This complex process, controlled by ovarian steroid hormones and growth factors, results in cyclic coordinated functional and morphological changes (Mulligan 1942, Moulton 1990, Santos *et al.* 2010).

Considering vascularization and lymphatic drainage, branches of the internal thoracic artery, intercostal arteries, superficial epigastric artery, cranial abdominal artery and branches of the external pudendal artery, comprise the net of mammary glands vascularization. Veins anatomy is parallel to the course of arteries, and the most important includes the cranial superficial epigastric and external pudendal veins. Lymphatic drainage is made usually by 1-3 branches in each gland to the nearest superficial lymph node. Lymphatic drainage can interconnect along the same side but not connect across midline (Patsikas *et al.* 1996, Pereira *et al.* 2003, Sorenmo *et al.* 2011, Goldschmidt *et al.* 2017).

Notwithstanding this organization, vascular anatomy can greatly change in the presence of a neoplastic disease. It is reported that neoplastic mammary glands exhibit diverse vascular anastomosis and more changes in the lymphatic drainage pattern compared with the healthy ones (Pereira *et al.* 2003, Pereira *et al.* 2008).

Besides the risk of CMTs development associated with the dog's genetic profile, age and breed previously mentioned, there are other factors, like hormonal exposition (mainly estrogens and progesterone), growth factors (growth hormone, vascular endothelial growth factor, insulin growth factor) and obesity, that seem to influence the susceptibility to CMTs. (Schneider *et al.* 1969, Sonnenschein *et al.* 1991, Perez Alenza *et al.* 1998, Sorenmo 2003, Queiroga *et al.* 2005, Rivera *et al.* 2009).

In humans, the increased or prolonged exposure of the mammary tissue to estrogens constitutes a recognized risk factor for breast carcinogenesis. This is also widely recognized in CMTs development (Frye *et al.* 1967, Schneider *et al.* 1969, Misdorp 1991, Rutteman 1992, Sorenmo *et al.* 2011). In fact, mammary tumors occur almost exclusively in females and most of the cases described in males were associated with testicular neoplasms with concomitant hormonal disturbances, like estrogens-secreting Sertoli cell tumors (Hamilton 1974, Misdorp 2002). Furthermore, iatrogenic sources such the administration of hormonal contraceptives, have been associated with an increased risk of developing mammary neoplasia in bitches (Misdorp 1988, Misdorp 1991, Rutteman 1992), particularly malignant tumors (Misdorp 1991, Stovring *et al.* 1997).

According to some authors, bitches spayed before the first estrous have only 0.5% risk of developing mammary tumors compared with sexually intact dogs. A risk of 8% and 26% was reported for bitches spayed before the second estrous and

any estrous thereafter, respectively (Schneider *et al.* 1969). Until now, spaying recommendations follow Schneider indications but contradictory opinions have emerged in the last years, aiming attention at the disadvantages of early spaying, such as urinary incontinence, splenic disorders, cardiac and musculoskeletal disorders or the development of different types of neoplasia such as osteosarcoma or lymphoma (Beauvais *et al.* 2012a, Beauvais *et al.* 2012b, Torres de la Riva *et al.* 2013, Hart *et al.* 2014).

Obesity also seems to play an important role in the development of CMTs (Perez Alenza *et al.* 1998, Sorenmo 2003, Sleeckx *et al.* 2011, Sorenmo *et al.* 2011), with some studies reporting a correlation between obesity and high fat diets and the risk of CMTs (Shofer *et al.* 1989, Sonnenschein *et al.* 1991, Perez Alenza *et al.* 1998). Recently, Lim and colleagues (2015) demonstrated that the proportion of poorly differentiated mammary tumors was significantly higher in overweight or obese dogs (Lim *et al.* 2015). These findings can be related to the increased levels of free bioavailable estrogens resulting from the impaired function of sex hormone-binding globulin in obese bitches (Sorenmo 2003, Sorenmo *et al.* 2011). Besides, it is possible that leptin promotes cell proliferation of the canine mammary gland, as previously reported in human breast cancer (Niu *et al.* 2013, Schmidt *et al.* 2015).

Histopathology remains the gold standard for diagnosis and classification of CMTs (Goldschmidt *et al.* 2011). Histological examination allows the identification of the cellular population involved (epithelial, myoepithelial, or mesenchymal tissue) and a detailed morphological description of every cell population in order to assign the tumor's histological subtype (Misdorp *et al.* 1999, Goldschmidt *et al.* 2011). Each neoplastic cell population is scrutinized, by evaluating the degree of cell differentiation, the presence of atypical features (such as those concerning the nuclear-cytoplasmatic ratio, the nucleoli number and morphology) and mitotic activity, including the presence of abnormal mitotic figures (Misdorp *et al.* 1999, Santos *et al.* 2015b). The presence of vascular invasion or lymphatic permeabilization is also pivotal information that should be routinely assessed and included in pathological reports (Kamstock *et al.* 2011).

CMTs are characterized by a highly variable morphology, being classified as simple (involving only luminal epithelial or myoepithelial cells), complex (comprising luminal epithelial and myoepithelial cells), mixed (including luminal epithelial and/or myoepithelial cells, and osseous/cartilaginous metaplastic tissue) or as

mesenchymal tumors (Misdorp *et al.* 1999, Goldschmidt *et al.* 2011). This typical high heterogeneity of CMTs and the subjectivity inherent to some tumor categories, can pose a challenge to the pathologist in an accurate classification of the lesions. In an attempt to apply well-defined criteria to categorize mammary neoplasms, different classification schemes for CMTs have been developed during the last forty years, based on the available categorization for human breast cancer.

The first classification system dates from 1974 and was endorsed by the World Health Organization (WHO) (Hampe *et al.* 1974). Three major groups of malignant tumors were described - carcinoma, sarcoma and carcinosarcoma. In the carcinoma group the authors considered the adenocarcinoma (tubular, papillary and papillary cystic – simple or complex), solid carcinoma (simple or complex), spindle cell carcinoma (simple or complex), anaplastic carcinoma, squamous cell carcinoma and mucinous carcinoma as the main six groups (Table 1). The sarcoma group included four categories: osteosarcoma, fibrosarcoma, combined sarcoma and other sarcomas. Benign tumors were classified as adenoma, papilloma, fibroadenoma and benign soft tissue tumors. This classification system also contemplated several dysplastic lesions, as shown in Table 1.

Twenty-five years later, the WHO published a revised and updated classification system for CMTs (Misdorp *et al.* 1999). Four main groups were defined: malignant tumors, benign tumors, unclassified tumors and mammary hyperplasia and dysplasias (Table 2 and 3).

Table 1. Canine mammary tumors classification according to Hampe et al (1974).

| 1974 Histologic Classification (malignant tumors) | 1974 Histologic Classification (benign tumors) |
|---|---|
| **I. Carcinoma** | **IV. Benign** |
| A. Adenocarcinoma | A. Adenoma |
| 1. Tubular | B. Papilloma |
| (a) Simple type | 1. Duct papilloma |
| (b) Complex type | 2. Duct papillomatosis |
| 2. Papillary | C. Fibroadenoma |
| (a) simple type | 1. Pericanilicular |
| (b) Complex type | 2. Intracanilicular |
| 3. Papillary cystic | (a) noncellular type |
| (a) simple type | (b) cellular type |
| (b) complex type | 3. Benign mixed tumor |
| B. Solid carcinoma | 4. Total fibroadenomatous change |
| (a) simple type | D. Benign soft tissue tumor |
| (b) complex type | **V. Unclassified Tumors** |
| C. Spindle cell carcinoma | **VI. Dysplasias** |
| (a) simple type | A. Cyst |
| (b) complex type | 1. Nonpapillary |
| D. Anaplastic carcinoma | 2. Papillary |
| E. Squamous cell carcinoma | B. Adenosis |
| F. Mucinous carcinoma | C. Epitheliosis |
| **II. Sarcoma** | D. Duct ectasia |
| A. Osteosarcoma | E. Fibrosclerosis |
| B. Fibrosarcoma | F. Gynecomastia |
| C. Combined sarcoma | G. Other nonneoplastic proliferative lesions |
| D. Other sarcomas | 1. Noninflammatory lobular hyperplasia |
| **III. Carcinosarcoma (malignant mixed tumor)** | 2. Inflammatory lobular hyperplasia |

Malignant subtypes of tumors were reorganised into seven categories: non-infiltrating carcinoma, complex carcinoma, simple carcinoma (tubulopapillary, solid and anaplastic carcinoma), special type carcinoma (spindle cell, squamous cell, mucinous and lipid-rich carcinoma), sarcoma (fibrosarcoma, osteosarcoma, others sarcoma), carcinosarcoma and carcinoma or sarcoma in benign tumors (Table 2). A simplification regarding the categorization of benign tumors and hyperplastic/dysplastic lesions was achieved with this histological classification system, comparing to the previous one (Table 3).

Table 2. Classification of canine mammary malignant tumors according to the WHO (Misdorp *et al*, 1999) and Goldschmidt *et al* (2011).

| **WHO Histologic Classification** | **2011 Histologic Classification** |
|---|---|
| **Malignant tumors** | **Malignant Epithelial Neoplasms** |
| Non-infiltrating (in situ) carcinoma | Carcinoma–in situ |
| Complex carcinoma | Carcinoma–simple: |
| Simple carcinoma: | Tubular |
| Tubulopapillary carcinoma | Tubulopapillary |
| Solid carcinoma | Cystic-papillary |
| Anaplastic carcinoma | Cribriform |
| Special type of carcinomas | Carcinoma–micropapillary invasive |
| Spindle cell carcinoma | Carcinoma–solid |
| Squamous cell carcinoma | Comedocarcinoma |
| Mucinous carcinoma | Carcinoma–anaplastic |
| Lipid-rich carcinoma | Carcinoma in a complex adenoma/mixed tumors |
| Sarcoma | Carcinoma–complex type |
| Fibrosarcoma | Carcinoma and malignant myoepithelioma |
| Osteosarcoma | Carcinoma–mixed type |
| Other sarcomas | Ductal carcinoma |
| Carcinosarcoma | Intraductal papillary carcinoma |
| Carcinoma or sarcoma in benign tumors | **Malignant Epithelial Neoplasms—Special Types** |
| | Squamous cell carcinoma |
| | Adenosquamous carcinoma |
| | Mucinous carcinoma |
| | Lipid-rich (secretory) carcinoma |
| | Spindle cell carcinomas |
| | Malignant myoepithelioma |
| | Squamous cell carcinoma–spindle cell variant |
| | Carcinoma–spindle cell variant |
| | Inflammatory carcinoma |
| | **Malignant Mesenchymal Neoplasms—Sarcomas** |
| | Osteosarcoma |
| | Chondrosarcoma |
| | Fibrosarcoma |
| | Hemangiosarcoma |
| | Other sarcomas |
| | **Carcinosarcoma** |

In addition, the system aimed to ensure uniformity in the criteria used to establish categories and classification of the lesions (Sleeckx *et al.* 2011, Matos *et al.* 2012), and provide reliable prognostic information for surgeons and oncologists. In fact the prognostic value of the histological subtypes was focused by that WHO classification, according to which complex carcinomas have a better prognosis than simple carcinomas; moreover, within the group of simple carcinomas, the anaplastic carcinomas seem to have a worse prognosis compared to that of solid type and the latest to that of tubulopapillary type (Misdorp *et al.* 1999).

Table 3. Classification of canine mammary benign tumors, hyperplasia and dysplasia according to the WHO (Misdorp *et al*, 1999) and Goldschmidt *et al* (2011).

| **WHO Histologic Classification** | **2011 Histologic Classification** |
|---|---|
| **Benign tumors** | **Benign Neoplasms** |
| Adenoma | Adenoma–simple |
| Simple adenoma | Duct papilloma |
| Complex adenoma | Ductal adenoma (basaloid adenoma) |
| Basaloid adenoma | With squamous differentiation |
| Fibroadenoma: | Fibroadenoma |
| Low-cellularity fibroadenoma | Myoepithelioma |
| High-cellularity fibroadenoma | Complex adenoma |
| Benign mixed tumor | Benign mixed tumor |
| Duct papilloma | **Hyperplasia/Dysplasia** |
| **Unclassified Tumors** | Duct ectasia |
| **Mammary hyperplasia and dysplasia** | Lobular hyperplasia |
| Ductal hyperplasia | Regular |
| Lobular hyperplasia: | With secretory activity |
| Epithelial hyperplasia | With fibrosis |
| Adenosis | With atypia |
| Cysts | Epitheliosis |
| Duct ectasia | Papillomatosis |
| Focal fibrosis | Fibroadenomatous change |
| Gynecomastia | Gynecomastia |
| | **Neoplasms of the Nipple** |
| | Adenoma |
| | Carcinoma |
| | Carcinoma with epidermal infiltration |
| | **Hyperplasia/Dysplasia of the Nipple** |
| | Melanosis of the skin of the nipple |

The prognostic value of WHO classification method was confirmed by several subsequent studies (Karayannopoulou *et al.* 2005, Santos *et al.* 2013, Carvalho *et al.* 2016), while other authors did not found significant associations between the histological type and survival (de Las Mulas *et al.* 2005, Santos *et al.* 2015b).

More recently a new classification scheme for CMTs has been proposed by Goldschmidt and colleagues (Goldschmidt *et al.* 2011). In this new proposed classification system, a special emphasis was given to several cellular details, namely to the architectural arrangements adopted by the neoplastic cells, as well as to other particular histological features, such as the presence of comedo-like necrotic areas or areas with squamous differentiation. The involvement and characterization of all the different cell populations involved in the neoplastic process, namely the myoepithelium, was also stressed. The high level of detail of the histological picture and the characteristic heterogeneity of CMTs resulted in the elaboration of an exhaustive and complete classification scheme, reflected by an increased number of tumor types and subtypes, especially regarding malignant

neoplasia (Table 2 and 3). New tumor subtypes emerged, namely comedocarcinoma, adenosquamous carcinoma, mixed carcinomas, ductal carcinomas, intraductal papillary carcinomas, carcinoma and malignant myoepithelioma or inflammatory carcinomas (Table 2). Moreover, the adoption of this classification system frequently implies the use of immunohistochemistry techniques including smooth muscle actin, calponin, vimentin and p63 markers, which are essential to identify myoepithelial cells, for example in the carcinoma and malignant myoepithelioma subtype (Goldschmidt *et al.* 2011, Sorenmo *et al.* 2011). Despite being adopted by some researchers in the latest years (de Andres *et al.* 2013, Im *et al.* 2014, Mainenti *et al.* 2014), the prognostic value of this classification system was only confirmed, so far, by two investigations (Pena *et al.* 2013, Rasotto *et al.* 2017), being also considered an independent prognostic factor by multivariable analysis of a prospective cohort of CMTs (Rasotto *et al.* 2017). According to these authors, tubulopapillary carcinomas and intraductal papillary carcinomas exhibited worse prognosis than simple tubular carcinomas or complex carcinomas. Furthermore, based on survival times, carcinosarcomas and anaplastic carcinomas were considered the most aggressive tumors followed by comedocarcinomas, adenosquamous carcinomas and solid carcinomas. Besides, this classification scheme allowed the identification of different subtypes of carcinomas composed of both luminal epithelial and myoepithelial cells, which have different survival times. For instance, complex or mixed carcinomas seem to have a better prognosis than carcinoma and malignant myoepithelioma, underlining the importance of the myoepithelial component in malignant CMTs.

Nowadays, pathologists face some incertitude about the system that should employ in the diagnosis and histological classification of CMTs, since there is no data regarding a rational comparison between the prognostic relevance of the WHO system and that proposed by Goldschmidt and colleagues.

The histological grade of CMTs represents another important task for veterinary pathologists. The assignment of a grade to a malignant tumor is crucial in order to provide additional prognostic information (Misdorp 2002, Goldschmidt *et al.* 2011, Santos *et al.* 2014, Santos *et al.* 2015b). Traditionally, histological grading systems adopted for CMTs followed the human methods and since the early 70´s, Misdorp and Hampe (Hampe *et al.* 1974) and later Misdorp and Hart (Misdorp *et al.* 1976) started the journey in this field, based on Bloom and Richardson method,

documenting characteristics like the degree of differentiation, considering tubule formation, hyperchromatism and mitosis; and anaplasia including size irregularity, shape and staining of nuclei (Misdorp *et al.* 1976). Subsequently adjustments were made, emphasizing for example the importance of lymphoid cells (Gilbertson *et al.* 1983). Moreover, two studies used the Scarff-Bloom-Richardson system, which included cell differentiation, nuclear pleomorphism, and mitotic index with the addiction of vascular invasion (Perez Alenza *et al.* 1997, Pena *et al.* 1998). More recently, the so called Nottingham histological grade method (NHG) described by Elston and Ellis in 1991 for human breast cancer (Elston *et al.* 1991) has been the method more frequently used by veterinary pathologists and research groups devoted to the study of CMTs (Karayannopoulou *et al.* 2001, Nieto *et al.* 2003, Karayannopoulou *et al.* 2005, Clemente *et al.* 2010, Carvalho *et al.* 2011, Kim *et al.* 2013). The Elston and Ellis grading method include a semi-quantitative assessment of three histological parameters: tubular formation, nuclear pleomorphism and mitotic figures count. For all these parameters a numerical score is given (1, 2 and 3) and the total score is used to assign the grade: grade I (total score 3-5), II (total score 6-7) or III (total score 8-9) (Elston *et al.* 1991) (Table 4). NHG has been associated with disease progression and cancer-related death, demonstrating the prognostic value of histological grade in CMTs (Karayannopoulou *et al.* 2005, Santos *et al.* 2013, Santos *et al.* 2015b).

Besides the widely use of NHG, it is recognized that the application of a human classification method in CMTs unleash some questions, so adjustments are needed to provide an adequate method for canine species (Goldschmidt *et al.* 2011, Matos *et al.* 2012).

Table 4. Histological grading methods used in CMTs (NHG method and ca-NHG method).

| | Score | | |
|---|---|---|---|
| **Grading Parameter** | **1** | **2** | **3** |
| *Tubule formation* | | | |
| NHG (epithelial cells) | > 75% | 10-75% | < 10% |
| ca-NHG (epithelial cells) | > 75% | 10-75%<br>Malignant myoepitheliomas* | < 10% |
| *Nuclear pleomorphism* | | | |
| NHG (epithelial cells) | Small, uniform | Moderate increase in size and variability; visible nucleoli | Marked variation in size and shape; prominent or multiple nucleoli |
| ca-NHG (all malignant cells) | Similar to NHG | Similar to NHG, but hyperchromatic nuclei; presence of nucleoli | Similar to NHG, but hyperchromatic nuclei; ≥1 prominent nucleoli |
| *Mitotic count* (in 10 HPF) | | | |
| NHG (in epithelial cells; cut-offs adjusted to field area; presenting those for a field diameter of 0.55mm) | ≤8 | 9-17 | ≥18 |
| ca-NHG (in epithelial and myoepithelial cells) | 0-9 | 10-19 | ≥20 |
| **Sum of scores** | **3 - 5** | **6 - 7** | **8 - 9** |
| **Histological Grade** | **I** | **II** | **III** |

Histological Grade: I (low, well differenced); II (intermediate, moderately differentiated); III (high, poorly differentiated). *According to the ca-NHG method, all malignant myoepithelioma are scored 2 for tubule formation parameter (Pena *et al.* 2013).

In 2013 Peña and co-workers proposed a “canine adapted-NHG” (ca-NHG, also known in the literature as “Peña system”). This system contemplated new criteria for assessing the parameters nuclear pleomorphism and mitotic count (Pena *et al.* 2013). For instance, for the subtype “malignant myoepithelioma”, the tubular formation parameter is always scored with the value 2 (Table 4); moreover, in this grade system, the mitotic count is made in both luminal and myoepithelial component, and not only in the luminal component as in the NHG method. This new method presents some modifications still object of discussion, namely the attribution of a score 2 to the myoepithelial cells in tubular formation parameter, knowing that

these cell population is not able to form tubules; the absence of a suggestion to grading carcinosarcomas; the unclear criteria to analyse nuclear pleomorphism in each malignant component and also the inexistence of comparative studies in terms of survival which would allow the validation for its standardization and application worldwide. Recent studies demonstrated that ca-NHG provided independent prognostic information (Pena *et al.* 2013, Mainenti *et al.* 2014, Carvalho *et al.* 2016), while others did not confirm this observation in a multivariable regression model (Rasotto *et al.* 2017). Besides the low number of prospective survival studies, it is known that interobserver variability in grading systems is one of the main pitfalls in the evaluation of malignant tumors in canine species (Santos *et al.* 2015c). To minimize this bias, a grading method should be validated in various prospective cohorts in order to be universally accepted and adopted (Santos *et al.* 2015c).

In the search for reliable prognostic tools for CMTs, Santos and co-authors (2015) demonstrated the advantageous application of an index formula, adapted from the well-known human Nottingham Prognostic Index (NPI) (Haybittle *et al.* 1982, Lee *et al.* 2008, Santos *et al.* 2015b). Originally created for women breast cancer, this formula includes three prognostic factors: tumor size; lymph node stage and histologic grade (Haybittle *et al.* 1982, Blamey 1996). Santos et al (2015) adapted this formula to CMTs and computed as: NPI= [tumor size (cm) ×0.2] +NHG (1, 2 or 3 respectively for grades I, II and III) +evidence of vascular invasion/regional lymph node metastases (1 or 2 if absent or present, respectively). The authors found in this study an optimal veterinary adapted NPI cut-off of 4.25, based on the maximal pair of sensitivity and specificity values; cases with a vet-NPI ≥ 4.25 were associated with poorer outcomes (Santos *et al.* 2015b). This work triggered a novel approach in prognostic factors in CMTs offering new prognostic information to include in the histopathological report for oncologists and surgeons.

For these last decades, researchers had dedicated their efforts to improve knowledge about CMTs, by analysing several clinicopathological factors and their role in the prognosis of the disease, aiming to support the implementation of better and effective treatments (Matos *et al.* 2012, Santos *et al.* 2013, Benavente *et al.* 2016a).

Multiple factors seem to influence the course of the disease and age is considered one of them. Increased age is related to shorter overall survivals (OS) and shorter disease-free survivals (DFS) (Hellmen *et al.* 1993, Perez Alenza *et al.*

1997, Pena *et al.* 1998). In some studies, however, age was not associated with survival variables (Philibert *et al.* 2003, Chang *et al.* 2005).

Another important prognostic factor is tumor size. Various studies described that tumors larger than 3.0 cm diameter were associated with a decreased OS (Yamagami *et al.* 1996, Philibert *et al.* 2003, Sorenmo 2003, Ferreira *et al.* 2009, Sorenmo *et al.* 2011, Santos *et al.* 2013). Moreover, malignant tumors larger than 3.0 cm diameter were also characterized by loss of progesterone receptor or higher proliferation index, which contributed to a worst prognosis (Ferreira *et al.* 2009, Sorenmo *et al.* 2009). In another study, tumors larger than 5.0 cm diameter were associated with the presence of lymph node metastasis (Chang *et al.* 2005).

Rapid and invasive growth skin ulceration, and the status of the completeness of the surgical margins are all related to a poor prognosis of dogs with the disease (Hellmen *et al.* 1993, Yamagami *et al.* 1996, Perez Alenza *et al.* 1997, Pena *et al.* 1998, Sarli *et al.* 2002, Ferreira *et al.* 2009, Tran *et al.* 2016).

Histological classification (Hellmen *et al.* 1993, Misdorp *et al.* 1999, Goldschmidt *et al.* 2011, Rasotto *et al.* 2017), histological grade (Karayannopoulou *et al.* 2005, Pena *et al.* 2013, Santos *et al.* 2015b) as well as vet-NPI showed to be of great value in terms of the definition of the disease outcome (Santos *et al.* 2015b).

Furthermore, vascular invasion/lymph node and distant metastasis (mainly in the lungs) are well recognized as decisive prognostic factors in CMTs (Sorenmo 2003, Sleeckx *et al.* 2011, Sorenmo *et al.* 2011, Santos *et al.* 2013, Klopfleisch 2016).

There are several immunohistochemical markers that, besides of assisting in the diagnosis of CMTs, have also been suggested to constitute valuable prognosticators. The expression of hormone receptors is considered to be important for prognostic definition, with lack of both progesterone and estrogens expression being found in more aggressive tumors (Nieto *et al.* 2000, de Las Mulas *et al.* 2005, Chang *et al.* 2009). Moreover, some studies showed HER2 overexpression in mammary malignant tumors. This overexpression was related to indicators of poor prognosis like marked nuclear pleomorphism, high histological grade and elevated mitotic count (Martin de las Mulas *et al.* 2003, Dutra *et al.* 2004). However, Ressel et al (2013) did not found a relation between the expression of HER2 and prognosis in malignant neoplasia's (Ressel *et al.* 2013). Proliferation markers, such as AgNOR, PCNA and Ki67 have also been mentioned as relevant prognostic factors

for CMTs (Pena *et al.* 1998, Sarli *et al.* 2002, Santos *et al.* 2013, Queiroga *et al*. 2016). In a study by Peña and co-workers, a significant relation was found between lower proliferative indices and non-malignant tumors and dysplastic lesions, comparing to malignant tumors. Furthermore, multivariable analyses concerning metastasis, disease-free survival, and overall survival revealed that the Ki-67 index had prognostic value (Pena *et al.* 1998). Also in Santos et al (2013) Ki-67 marker was identified as an independent prognostic marker of CMTs. In this study, Ki-67 was associated with the development of recurrences/distant metastases as well as with shorter DFS and OS intervals, both in univariate and multivariable analysis (Santos *et al.* 2013).

Cox-2 overexpression in CMTs was related to tumor dedifferentiation, lymph node metastasis at the time of surgery, development of distant metastasis during the follow-up, and poor disease-free and overall survival (Millanta *et al.* 2006a, Dias Pereira *et al.* 2009b, Lavalle *et al.* 2009, Queiroga *et al.* 2010, Queiroga *et al.* 2011a).

Interactions between cancer cells and stroma are known to be critical for tumor growth and invasion in human breast cancer (Egeblad *et al.* 2010). CMTs are characterized by an abundant and very rich stroma (Moulton 1990, Misdorp *et al.* 1999, Misdorp 2002). The stromal cells includes fibroblasts, myofibroblasts, endothelial cells, pericytes, lipoblasts and inflammatory cells, all immersed in an extracellular matrix (Orimo *et al.* 2006). During the last years, stroma became a valuable model for the study of the tumoral microenvironment with various investigations exploring its role in the neoplasm's development (Hu *et al.* 2008b, Hu *et al.* 2008a, Le Bitoux *et al.* 2008, Mao *et al.* 2013). As for epithelial neoplastic cells, the application of advanced pathological and genetic molecular techniques on stromal cells, seems to be very useful in the molecular characterization of the different cell types that constitute the tumor microenvironment, getting possible valuable information for CMTs prevention and treatment (Mao *et al.* 2013).

Matrix-associated proteases have been related to CMTs prognosis. In fact these proteases facilitate proliferation, invasion and angiogenesis by cancer cells (Brooks *et al.* 2010, Santos *et al.* 2013). MMP-9 was considered an independent prognostic marker and the high expression of both uPA and MMP-9 proteases were associated with poor outcomes, suggesting that these molecules can be of great utility in the post-operative treatment of CMTs (Santos *et al.* 2013).

Besides matrix-associated proteases, other stromal components, such as collagen signatures, have been assessed in the context of CMTs microenvironment. In human breast cancer various studies highlighted the importance of collagen organization in determining whether collagen plays a tumor-permissive or tumor restrictive role in breast cancer progression or recurrence (Boyd *et al.* 2011, Conklin *et al.* 2011, Conklin *et al.* 2018). Information about this issue in the veterinary field is scarce and not universally recognized as a prognostic factor, however, one study demonstrated that collagen density, as well as fiber width, length and straightness, were inversely correlated with the overall survival time reflecting a possible role of these elements as prognostic biomarkers (Case *et al.* 2017).

It is widely accepted that increased micro-vessel density and angiogenesis is a characteristic of malignant tumors (Folkman 2006, Hanahan 2011). In CMTs the increased expression of angiogenic factors (vascular endothelial growth factor - VEGF, factor VIII-related antigen, platelet endothelial cell adhesion molecule – PECAM, among others) has been described in more aggressive tumors (Graham *et al.* 1999, Restucci *et al.* 2000, Clemente *et al.* 2013, Santos *et al.* 2013, Carvalho *et al.* 2015). However, in survival studies the results are still controversial. For some authors there is an association between angiogenic factors, like VEGF, and OS (Carvalho *et al.* 2015), while for others no correlation was found between those variables (Millanta *et al.* 2006b, Santos *et al.* 2013).

Inflammation can contribute to the neoplastic transformation of cancer cells (Coussens *et al.* 2002, Colotta *et al.* 2009). Lymphocytic aggregates are documented in CMTs and T-lymphocytes function has been highlighted as being associated with lymphatic invasion and high histological grade carcinomas (Estrela-Lima *et al.* 2010, Carvalho *et al.* 2011, Saeki *et al.* 2012, Kim *et al.* 2013). Recently the presence of T-lymphocytes was related to an increase of angiogenesis, tumor aggressiveness and poor prognosis, emerging as a possible prognostic factor in CMTs (Carvalho *et al.* 2015).

Despite the fact that several different molecular markers were assessed as prognosticators in CMTs, they are still not routinely used in ordinary histological practice. The low number of prospective multivariable survival analyses, the lack of reproducibility regarding technical aspects and evaluation systems, as well as the contradictory results published to date constrains their application in histological routine (Matos *et al.* 2012, Santos *et al.* 2013, Peña *et al.* 2014).

There is a marked diversity in CMTs inter individual biological behavior, which hamper the identification of reliable and universal prognostic factors, and that may be partially explained by the dog's genetic profile. In humans, exhaustive research in molecular genetic and oncology displayed a relation between the host genetic profile, namely SNPs, and the response to treatment and clinical outcome of breast cancer disease (Hirshfield 2008, Buonaguro *et al.* 2014). Several SNPs in proto-oncogenes, tumor suppressor genes, genes involved in DNA damage recognition/repair and in hormonal metabolism, among others, have been considered relevant prognostic factors for breast cancer (Wang *et al.* 2017, Yu *et al.* 2019). Despite of the scarcity of data available regarding CMTs, Dias Pereira and colleagues reported an association between SNPs in canine COMT gene and the outcome of CMTs, identifying a genetic profile related to a higher risk of recurrences (Dias Pereira *et al.* 2009a). In CMTs this is an issue still underexplored, but highly promising, that deserves further investigation.

Considering CMTs treatment, surgical excision with clean histologic margins, remains the gold-standard treatment for CMTs, with the exception of inoperable metastatic disease and most of the inflammatory carcinoma (MacEwen *et al.* 1985, Karayannopoulou *et al.* 2001, Pena *et al.* 2003, Sorenmo 2003, Clemente *et al.* 2009, Marconato *et al.* 2009). Surgical techniques are usually adapted to the size, anatomical location and include lumpectomy, simple mastectomy, regional mastectomy, and unilateral or bilateral mastectomy (Novosad 2003).

The benefit of OHE at the time of mammary tumors excision remain ambiguous. Various studies advocate no significant advantages for dogs treated by mastectomy and ovariectomized at the same time than those submitted only to mastectomy (Schneider *et al.* 1969, Yamagami *et al.* 1996, Morris *et al.* 1998). Curiously, others shown improved survival time in dogs treated by mastectomy and OHE at the same time (Sorenmo *et al.* 2000). Recent studies demonstrated that OHE performed at the time of mammary tumor excision reduces the risk of new tumors by about 50% among dogs with benign tumors (Kristiansen *et al.* 2013) and that dogs with grade II, ER-positive mammary tumors, or increased E2 seric levels are likely to benefit with the OHE procedure at the time of tumors removal (Kristiansen *et al.* 2016).

The use of chemotherapy is considered an adjuvant treatment for mammary tumors when surgical excision alone yields unsatisfactory results, namely for tumors

exhibiting lymphatic or vascular invasion, or in cases of inoperable tumors (Karayannopoulou *et al.* 2001, Marconato *et al.* 2008, Clemente *et al.* 2009, Lavalle *et al.* 2012, Arenas *et al.* 2016, de Campos *et al.* 2016, Tran *et al.* 2016). Nevertheless, contradictory results have been published and most of the studies included low number of dogs and different drug protocols, which can probably explain the discrepancies in the results. For example, a positive influence of chemotherapy using a 5-fuorouracil and cyclophosphamide combination on DFS interval and OS time was described in Karayannopoulou and co-workers (Karayannopoulou *et al.* 2001). On the contrary, no benefit in OS rates was observed in Simon´s work with the use of chemotherapy including doxorubicin or docetaxel in a postoperative protocol (Simon *et al.* 2006).

Adjuvant radiation and endocrine therapy with antiestrogens, such as tamoxifen, have not shown any benefits compared to surgery alone in dogs (Karayannopoulou *et al.* 2001, Novosad 2003). Tamoxifen is an estrogen receptor antagonist that binds to estrogen receptors, preventing estrogen stimulation of existing cancer cells. It has shown, however, both anti-estrogenic and estrogenic effects causing pyometra in dogs (Tavares *et al.* 2010, Sleeckx *et al.* 2011). Also the use of PR antagonists as post-surgery adjuvant therapy yielded controversial results (Benavente *et al.* 2016a). A recent study showed that aglepristone, demonstrated inhibitory effects on proliferation of progesterone receptor positive canine mammary carcinoma cells (Guil-Luna *et al.* 2011).

The administration of anti-Cox-2 agents (Lavalle *et al.* 2009, Arenas *et al.* 2016), desmopressin (Hermo *et al.* 2008, Hermo *et al.* 2011, Benavente *et al.* 2016b) and anti-angiogenic drugs, such as interleukin 12 (Cicchelero *et al.* 2017) or toceranib (Rossi *et al.* 2018), have been applied in CMTs clinical trials, but data available is very limited and further investigations are mandatory (Benavente *et al.* 2016b).

The use of canine interferon-β and HSV-thymidine kinase/ganciclovir after surgery, was recently described by Finocchiaro et al (2018). A correlation was found between the administration of these drugs and better survival times, maintaining the animal's quality of life (Finocchiaro *et al.* 2018).

Novel therapeutic approaches are emerging like the use of a p62 anti-cancer DNA vaccine (sequestosome-1) (Gabai *et al.* 2014). In this pilot study, locally advanced malignant mammary lesions decreased or stabilized their growth, while

no overall toxic effects were detected. According to the authors, the observed anti-tumor activity was associated with lymphocyte infiltration and tumor encapsulation via fibrotic reaction (Gabai *et al.* 2014).

Therapies emphasizing oncolytic virotherapy have been studied and are a promising treatment strategy for cancer in humans (Fukuhara *et al.* 2016, Marelli *et al.* 2018), including breast cancer (Cody *et al.* 2015, Suryawanshi *et al.* 2017). In the veterinary field, several viruses have been reported as oncolytic agents like adenovirus, canine distemper virus or measles virus (Sánchez *et al.* 2018). Preliminary results reported that oncolytic agents colonize canine mammary cancer cells, resulting in inhibition of tumor growth and in a marked reduction of the tumor size, however further studies are needed to confirm these findings (Gentschev *et al.* 2009, Shoji *et al.* 2016).

In spite of all the studies, continuous investigation is required covering a larger number of dogs. Furthermore, research regarding neoadjuvant and/or adjuvant procedures is important in order to provide treatment options for dogs with aggressive forms of CMTs, aiming to prolong their survival time maintaining their quality of life.

## REFERENCES

Abdelmegeed, S. M. and S. Mohammed (2018). "Canine mammary tumors as a model for human disease." Oncology Letters **15**(6): 8195-8205.

Angele, S., P. Romestaing, N. Moullan, M. Vuillaume, B. Chapot, M. Friesen*, et al.* (2003). "ATM haplotypes and cellular response to DNA damage: association with breast cancer risk and clinical radiosensitivity." Cancer Res **63**(24): 8717-8725.

Arenas, C., L. Pena, J. L. Granados-Soler and M. D. Perez-Alenza (2016). "Adjuvant therapy for highly malignant canine mammary tumours: Cox-2 inhibitor versus chemotherapy: a case-control prospective study." Vet Rec **179**(5): 125.

Barrett, J. C. and R. W. Wiseman (1987). "Cellular and Molecular Mechanisms of Multistep Carcinogenesis: Relevance to Carcinogen Risk Assessment." Environmental Health Perspectives **76**: 65-70.

Baylin, S. B. and J. G. Herman (2000). "DNA hypermethylation in tumorigenesis: epigenetics joins genetics." Trends Genet **16**(4): 168-174.

Baylin, S. B. and P. A. Jones (2011). "A decade of exploring the cancer epigenome - biological and translational implications." Nat Rev Cancer **11**(10): 726-734.

Beauvais, W., J. M. Cardwell and D. C. Brodbelt (2012a). "The effect of neutering on the risk of mammary tumours in dogs-a systematic review." J Small Anim Pract **53**(6): 314-322.

Beauvais, W., J. M. Cardwell and D. C. Brodbelt (2012b). "The effect of neutering on the risk of urinary incontinence in bitches - a systematic review." J Small Anim Pract **53**(4): 198-204.

Benavente, M., C. Bianchi and M. Aba (2016a). "Canine Mammary Tumors: Risk Factors, Prognosis and Treatments." Journal of Veterinary Advances **6**: 1291-1300.

Benavente, M. A., C. P. Bianchi, F. Imperiale and M. A. Aba (2016b). "Antiproliferative Effects of Oxytocin and Desmopressin on Canine Mammary Cancer Cells." Front Vet Sci **3**: 119.

Benjamin, S. A., A. C. Lee and W. J. Saunders (1999). "Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles." Vet Pathol **36**(5): 423-436.

Bhattacharya, T. K., S. Rani, S. K. Maiti, S. Dayal, P. Kumar and A. Sharma (2007). "Polymorphism of ZuBeCa3 microsatellite and its association with mammary tumor in dogs." Int J Immunogenet **34**(3): 161-165.

Blamey, R. W. (1996). "4. The design and clinical use of the nottingham prognostic index in breast cancer." The Breast **5**(3): 156-157.

Blomme, E. A. and B. B. Spear (2010). "Theranostics in veterinary medicine: where are we heading?" Vet J **185**(3): 237-238.

Boldizsar, H., O. Szenci, T. Muray and J. Csenki (1992). "Studies on canine mammary tumours. I. Age, seasonal and breed distribution." Acta Vet Hung **40**(1-2): 75-87.

Borge, K. S., A. L. Borresen-Dale and F. Lingaas (2011). "Identification of genetic variation in 11 candidate genes of canine mammary tumour." Vet Comp Oncol **9**(4): 241-250.

Borge, K. S., M. Melin, P. Rivera, S. I. Thoresen, M. T. Webster, H. von Euler*, et al.* (2013). "The ESR1 gene is associated with risk for canine mammary tumours." BMC Veterinary Research **9**: 69-69.

Boyd, N. F., L. J. Martin, M. J. Yaffe and S. Minkin (2011). "Mammographic density and breast cancer risk: current understanding and future prospects." Breast Cancer Research **13**(6): 223.

Brookes, A. J. (1999). "The essence of SNPs." Gene **234**(2): 177-186.

Brooks, S. A., H. J. Lomax-Browne, T. M. Carter, C. E. Kinch and D. M. Hall (2010). "Molecular interactions in cancer cell metastasis." Acta Histochem **112**(1): 3-25.

Broustas, C. G. and H. B. Lieberman (2014). "DNA Damage Response Genes and the Development of Cancer Metastasis." Radiation research **181**(2): 111-130.

Buonaguro, F. M., D. Pauza, M. L. Tornesello, P. Hainaut, R. Franco and F. M. Marincola (2014). "Cancer Diagnostic and Predictive Biomarkers." BioMed Research International **2014**: 3.

Camps, M., A. Herman, E. Loh and L. A. Loeb (2007). "Genetic constraints on protein evolution." Critical reviews in biochemistry and molecular biology **42**(5): 313-326.

Carvalho, M. I., I. Pires, M. Dias, J. Prada, H. Gregorio, L. Lobo*, et al.* (2015). "Intratumoral CD3+ T-lymphocytes immunoexpression and its association with c-Kit, angiogenesis, and overall survival in malignant canine mammary tumors." Anal Cell Pathol (Amst) **2015**: 920409.

Carvalho, M. I., I. Pires, J. Prada, L. Lobo and F. L. Queiroga (2016). "Ki-67 and PCNA Expression in canine mammary tumors and adjacent nonneoplastic mammary glands: prognostic impact by a multivariate survival analysis." Vet Pathol **53**(6): 1138-1146.

Carvalho, M. I., I. Pires, J. Prada and F. L. Queiroga (2011). "T-lymphocytic infiltrate in canine mammary tumours: clinic and prognostic implications." In Vivo **25**(6): 963-969.

Case, A., B. K. Brisson, A. C. Durham, S. Rosen, J. Monslow, E. Buza*, et al.* (2017). "Identification of prognostic collagen signatures and potential therapeutic stromal targets in canine mammary gland carcinoma." PloS one **12**(7): e0180448-e0180448.

Chang, C. C., M. H. Tsai, J. W. Liao, J. P. Chan, M. L. Wong and S. C. Chang (2009). "Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors." J Am Vet Med Assoc **235**(4): 391-396.

Chang, S. C., C. C. Chang, T. J. Chang and M. L. Wong (2005). "Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002)." J Am Vet Med Assoc **227**(10): 1625-1629.

Chuang, L.-Y., Y.-D. Lin, H.-W. Chang and C.-H. Yang (2012). "An Improved PSO Algorithm for Generating Protective SNP Barcodes in Breast Cancer." PLoS ONE **7**(5): e37018.

Cicchelero, L., S. Denies, H. Haers, K. Vanderperren, E. Stock, L. Van Brantegem*, et al.* (2017). "Intratumoural interleukin 12 gene therapy stimulates the immune system and decreases angiogenesis in dogs with spontaneous cancer." Vet Comp Oncol **15**(4): 1187-1205.

Clemente, M., P. J. De Andres, L. Pena and M. D. Perez-Alenza (2009). "Survival time of dogs with inflammatory mammary cancer treated with palliative therapy alone or palliative therapy plus chemotherapy." Vet Rec **165**(3): 78-81.

Clemente, M., M. D. Perez-Alenza, J. C. Illera and L. Pena (2010). "Histological, immunohistological, and ultrastructural description of vasculogenic mimicry in canine mammary cancer." Vet Pathol **47**(2): 265-274.

Clemente, M., A. R. Sanchez-Archidona, D. Sardon, L. Diez, A. Martin-Ruiz, S. Caceres*, et al.* (2013). "Different role of COX-2 and angiogenesis in canine inflammatory and non-inflammatory mammary cancer." Vet J **197**(2): 427-432.

Cody, J. J. and D. R. Hurst (2015). "Promising oncolytic agents for metastatic breast cancer treatment." Oncolytic Virother **4**: 63-73.

Colotta, F., P. Allavena, A. Sica, C. Garlanda and A. Mantovani (2009). "Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability." Carcinogenesis **30**(7): 1073-1081.

Conklin, M. W., J. C. Eickhoff, K. M. Riching, C. A. Pehlke, K. W. Eliceiri, P. P. Provenzano*, et al.* (2011). "Aligned collagen is a prognostic signature for survival in human breast carcinoma." Am J Pathol **178**(3): 1221-1232.

Conklin, M. W., R. E. Gangnon, B. L. Sprague, L. Van Gemert, J. M. Hampton, K. W. Eliceiri*, et al.* (2018). "Collagen Alignment as a Predictor of Recurrence after Ductal Carcinoma In Situ." Cancer Epidemiol Biomarkers Prev **27**(2): 138-145.

Cooper, D. N. (2010). "Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes." Hum Genomics **4**(5): 284-288.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-867.

Cowie, A. T. (1974). "Proceedings: Overview of the mammary gland." J Invest Dermatol **63**(1): 2-9.

Cullen, J. M. and M. Breen (2017). An Overview of Molecular Cancer Pathogenesis, Prognosis, and Diagnosis. Tumors in Domestic Animals. D. J. Meuten. Raleigh, NC, USA, Wiley Blackwell**:** 1-25.

de Andres, P. J., J. C. Illera, S. Caceres, L. Diez, M. D. Perez-Alenza and L. Pena (2013). "Increased levels of interleukins 8 and 10 as findings of canine inflammatory mammary cancer." Vet Immunol Immunopathol **152**(3-4): 245-251.

de Campos, C. B., G. E. Lavalle, S. Fialho Ligorio, F. Camargo Nunes, R. A. Carneiro, R. L. Amorim*, et al.* (2016). "Absence of significant adverse events following thalidomide administration in bitches diagnosed with mammary gland carcinomas." Vet Rec **179**(20): 514.

de Las Mulas, J. M., Y. Millan and R. Dios (2005). "A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog." Vet Pathol **42**(2): 200-212.

de Visser, J. A., T. F. Cooper and S. F. Elena (2011). "The causes of epistasis." Proc Biol Sci **278**(1725): 3617-3624.

Deans, C. and K. A. Maggert (2015). "What do you mean, "epigenetic"?" Genetics **199**(4): 887-896.

Dekomien, G. and J. T. Epplen (2003). "Evaluation of the canine RPE65 gene in affected dogs with generalized progressive retinal atrophy." Mol Vis **9**: 601-605.

Deng, N., H. Zhou, H. Fan and Y. Yuan (2017). "Single nucleotide polymorphisms and cancer susceptibility." Oncotarget **8**(66): 110635-110649.

Dias Pereira, P., C. C. Lopes, A. J. Matos, D. Pinto, F. Gartner, C. Lopes*, et al.* (2008). "Estrogens metabolism associated with polymorphisms: influence of COMT G482a genotype on age at onset of canine mammary tumors." Vet Pathol **45**(2): 124-130.

Dias Pereira, P., C. C. Lopes, A. J. Matos, D. Pinto, F. Gartner, C. Lopes*, et al.* (2009a). "Influence of catechol-O-methyltransferase (COMT) genotypes on the prognosis of canine mammary tumors." Vet Pathol **46**(6): 1270-1274.

Dias Pereira, P., C. C. Lopes, A. J. Matos, M. Santos, F. Gartner, R. Medeiros*, et al.* (2009b). "COX-2 expression in canine normal and neoplastic mammary gland." J Comp Pathol **140**(4): 247-253.

Dorn and R. Shneider (1976). "Inbreeding and canine mammary cancer." Journal National Cancer Institution **57**(3).

Dorn, C. R., D. O. N. Taylor, R. Shneider, H. H. Hibbard and M. R. Klauber (1968). "Survey of Animal Neoplasms in Alameda and Contra Costa Counties, California. II. Cancer Morbidity in Dogs and Cats From Alameda County." Journal of The National Cancer Institute **40**(2): 307-318.

Dutra, A. P., N. V. Granja, F. C. Schmitt and G. D. Cassali (2004). "c-erbB-2 expression and nuclear pleomorphism in canine mammary tumors." Braz J Med Biol Res **37**(11): 1673-1681.

Egeblad, M., E. S. Nakasone and Z. Werb (2010). "Tumors as organs: complex tissues that interface with the entire organism." Dev Cell **18**(6): 884-901.

Egenvall, A., B. N. Bonnett, P. Ohagen, P. Olson, A. Hedhammar and H. von Euler (2005). "Incidence of and survival after mammary tumors in a population of over 80,000 insured female dogs in Sweden from 1995 to 2002." Prev Vet Med **69**(1-2): 109-127.

Elston, C. W. and I. O. Ellis (1991). "Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up." Histopathology **19**(5): 403-410.

Enginler, S. O., I. Akis, T. S. Toydemir, K. Oztabak, D. Haktanir, M. C. Gunduz*, et al.* (2014). "Genetic variations of BRCA1 and BRCA2 genes in dogs with mammary tumours." Vet Res Commun **38**(1): 21-27.

Erichsen, H. C. and S. J. Chanock (2004). "SNPs in cancer research and treatment." British Journal of Cancer **90**(4): 747-751.

Esmaeili, R., N. Abdoli, F. Yadegari, M. Neishaboury, L. Farahmand, A. Kaviani*, et al.* (2018). "Unique CD44 intronic SNP is associated with tumor grade in breast cancer: a case control study and in silico analysis." Cancer Cell International **18**(1): 28.

Estrela-Lima, A., M. S. Araujo, J. M. Costa-Neto, A. Teixeira-Carvalho, S. M. Barrouin-Melo, S. V. Cardoso*, et al.* (2010). "Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates." BMC Cancer **10**: 256.

Ferreira, E., A. C. Bertagnolli, M. F. Cavalcanti, F. C. Schmitt and G. D. Cassali (2009). "The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours." Vet Comp Oncol **7**(4): 230-235.

Finocchiaro, L. M. E., A. I. M. Spector, L. Agnetti, M. F. Arbe and G. C. Glikin (2018). "Combination of Suicide and Cytokine Gene Therapies as Surgery Adjuvant for Canine Mammary Carcinoma." Vet Sci **5**(3).

Folkman, J. (2006). "Angiogenesis." Annu Rev Med **57**: 1-18.

Fouad, Y. A. and C. Aanei (2017). "Revisiting the hallmarks of cancer." American journal of cancer research **7**(5): 1016-1036.

Fowler, E. H., G. P. Wilson and A. Koestner (1974). "Biologic behavior of canine mammary neoplasms based on a histogenetic classification." Vet Pathol **11**(3): 212-229.

Frye, F., C. Dorn, D. Taylor, H. Hibbard and M. Klauber (1967). "Characteristics of canine mammary gland tumor cases." Journal of the American Animal Hospital Association **3**: 1-12.

Fukuhara, H., Y. Ino and T. Todo (2016). "Oncolytic virus therapy: A new era of cancer treatment at dawn." Cancer science **107**(10): 1373-1379.

Gabai, V., F. M. Venanzi, E. Bagashova, O. Rud, F. Mariotti, C. Vullo*, et al.* (2014). "Pilot study of p62 DNA vaccine in dogs with mammary tumors." Oncotarget **5**(24): 12803-12810.

Garraway, L. A. and E. S. Lander (2013). "Lessons from the cancer genome." Cell **153**(1): 17-37.

Gentschev, I., J. Stritzker, E. Hofmann, S. Weibel, Y. A. Yu, N. Chen*, et al.* (2009). "Use of an oncolytic vaccinia virus for the treatment of canine breast cancer in nude mice: preclinical development of a therapeutic agent." Cancer Gene Ther **16**(4): 320-328.

Gilbertson, S. R., I. D. Kurzman, R. E. Zachrau, A. I. Hurvitz and M. M. Black (1983). "Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs." Vet Pathol **20**(2): 127-142.

Goldschmidt, H., L. Pena and V. Zappulli (2017). Tumors of the mammary gland. Tumors in Domestic Animals. D. J. Meuten. Ames, IA, John Wiley & Sons Inc.**:** 723-765.

Goldschmidt, M., L. Pena, R. Rasotto and V. Zappulli (2011). "Classification and grading of canine mammary tumors." Vet Pathol **48**(1): 117-131.

Goldstein, D. B. and G. L. Cavalleri (2005). "Genomics: understanding human diversity." Nature **437**(7063): 1241-1242.

Graham, J. C. and R. K. Myers (1999). "The prognostic significance of angiogenesis in canine mammary tumors." J Vet Intern Med **13**(5): 416-418.

Guil-Luna, S., R. Sanchez-Cespedes, Y. Millan, F. J. De Andres, E. Rollon, V. Domingo*, et al.* (2011). "Aglepristone decreases proliferation in progesterone receptor-positive canine mammary carcinomas." J Vet Intern Med **25**(3): 518-523.

Hakem, R. (2008). "DNA-damage repair; the good, the bad, and the ugly." The EMBO journal **27**(4): 589-605.

Hamilton, J. M. (1974). "Comparative aspects of mammary tumors." Adv Cancer Res **19**(0): 1-45.

Hampe, J. F. and W. Misdorp (1974). "Tumours and dysplasias of the mammary gland." Bull World Health Organ **50**(1-2): 111-133.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. a. W., R. A. (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hart, B. L., L. A. Hart, A. P. Thigpen and N. H. Willits (2014). "Long-term health effects of neutering dogs: comparison of Labrador Retrievers with Golden Retrievers." PLoS One **9**(7): e102241.

Haybittle, J. L., R. W. Blamey, C. W. Elston, J. Johnson, P. J. Doyle, F. C. Campbell*, et al.* (1982). "A prognostic index in primary breast cancer." Br J Cancer **45**(3): 361-366.

Hellmen, E., R. Bergstrom, L. Holmberg, I. B. Spangberg, K. Hansson and A. Lindgren (1993). "Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases." Vet Pathol **30**(1): 20-27.

Hermo, G. A., P. Torres, G. V. Ripoll, A. M. Scursoni, D. E. Gomez, D. F. Alonso*, et al.* (2008). "Perioperative desmopressin prolongs survival in surgically treated bitches with mammary gland tumours: a pilot study." Vet J **178**(1): 103-108.

Hermo, G. A., E. Turic, D. Angelico, A. M. Scursoni, D. E. Gomez, C. Gobello*, et al.* (2011). "Effect of adjuvant perioperative desmopressin in locally advanced canine

mammary carcinoma and its relation to histologic grade." J Am Anim Hosp Assoc **47**(1): 21-27.

Hirshfield, K. (2008). "SNPs as prognostic indicators in cancer outcomes." Clinical Cancer Research **14**(15 Supplement): ED04-01-ED04-01.

Hu, M. and K. Polyak (2008a). "Microenvironmental regulation of cancer development." Curr Opin Genet Dev **18**(1): 27-34.

Hu, M. and K. Polyak (2008b). "Molecular characterisation of the tumour microenvironment in breast cancer." Eur J Cancer **44**(18): 2760-2765.

Hunt, R., Z. E. Sauna, S. V. Ambudkar, M. M. Gottesman and C. Kimchi-Sarfaty (2009). "Silent (synonymous) SNPs: should we care about them?" Methods Mol Biol **578**: 23-39.

Im, K. S., N. H. Kim, H. Y. Lim, H. W. Kim, J. I. Shin and J. H. Sur (2014). "Analysis of a new histological and molecular-based classification of canine mammary neoplasia." Vet Pathol **51**(3): 549-559.

Jamshidi, M., R. Fagerholm, S. Khan, K. Aittomaki, K. Czene, H. Darabi*, et al.* (2015). "SNP-SNP interaction analysis of NF-kappaB signaling pathway on breast cancer survival." Oncotarget **6**(35): 37979-37994.

Jitpean, S., R. Hagman, B. Strom Holst, O. V. Hoglund, A. Pettersson and A. Egenvall (2012). "Breed variations in the incidence of pyometra and mammary tumours in Swedish dogs." Reprod Domest Anim **47 Suppl 6**: 347-350.

Jones, P. A. and S. B. Baylin (2007). "The epigenomics of cancer." Cell **128**(4): 683-692.

Kamstock, D. A., E. J. Ehrhart, D. M. Getzy, N. J. Bacon, K. M. Rassnick, S. D. Moroff*, et al.* (2011). "Recommended guidelines for submission, trimming, margin evaluation, and reporting of tumor biopsy specimens in veterinary surgical pathology." Vet Pathol **48**(1): 19-31.

Kanemaki, N., K. T. Tchedre, M. Imayasu, S. Kawarai, M. Sakaguchi, A. Yoshino*, et al.* (2013). "Dogs and humans share a common susceptibility gene SRBD1 for glaucoma risk." PLoS One **8**(9): e74372.

Kanwal, R. and S. Gupta (2012). "Epigenetic modifications in cancer." Clinical Genetics **81**(4): 303-311.

Karayannopoulou, M., E. Kaldrymidou, T. C. Constantinidis and A. Dessiris (2001). "Adjuvant post-operative chemotherapy in bitches with mammary cancer." J Vet Med A Physiol Pathol Clin Med **48**(2): 85-96.

Karayannopoulou, M., E. Kaldrymidou, T. C. Constantinidis and A. Dessiris (2005). "Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method." J Comp Pathol **133**(4): 246-252.

Karrman, K., A. Castor, M. Behrendtz, E. Forestier, L. Olsson, M. Ehinger*, et al.* (2015). "Deep sequencing and SNP array analyses of pediatric T-cell acute lymphoblastic leukemia reveal NOTCH1 mutations in minor subclones and a high incidence of uniparental isodisomies affecting CDKN2A." J Hematol Oncol **8**: 42.

Kim, J. H., S. K. Chon, K. S. Im, N. H. Kim and J. H. Sur (2013). "Correlation of tumor-infiltrating lymphocytes to histopathological features and molecular phenotypes in canine mammary carcinoma: A morphologic and immunohistochemical morphometric study." Can J Vet Res **77**(2): 142-149.

Klopfleisch, R. (2016). Mammary Tumors. Veterinary Oncology - a short textbook. K. R. Cham Switzerland, Springer International Publishing**:** 100-104.

Klopfleisch, R., H. von Euler, G. Sarli, S. S. Pinho, F. Gartner and A. D. Gruber (2011). "Molecular carcinogenesis of canine mammary tumors: news from an old disease." Vet Pathol **48**(1): 98-116.

Kristiansen, V. M., A. Nodtvedt, A. M. Breen, M. Langeland, J. Teige, M. Goldschmidt*, et al.* (2013). "Effect of ovariohysterectomy at the time of tumor removal in dogs with benign mammary tumors and hyperplastic lesions: a randomized controlled clinical trial." J Vet Intern Med **27**(4): 935-942.

Kristiansen, V. M., L. Pena, L. Diez Cordova, J. C. Illera, E. Skjerve, A. M. Breen*, et al.* (2016). "Effect of Ovariohysterectomy at the Time of Tumor Removal in Dogs with Mammary Carcinomas: A Randomized Controlled Trial." J Vet Intern Med **30**(1): 230-241.

Kumar, V., A. K. Abbas and J. C. Aster (2015). Neoplasia. Robbins and Cotran pathologic basis of disease. Philadelphia, PA, Elsevier/Saunders.

Kurzman, I. and S. R. Gilbertson (1986). "Prognostic Factors in Canine Mammary Tumors." Seminars in veterinary medicine and surgery **1**(1): 25-32.

Kusewitt, D. F. and L. J. Rush (2016). Neoplasia and Tumor Biology. Pathologic Basis of Veterinary Disease J. F. Zachary and M. D. McGavin, Elsevier Health Sciences.

Lavalle, G. E., A. C. Bertagnolli, W. L. Tavares and G. D. Cassali (2009). "Cox-2 expression in canine mammary carcinomas: correlation with angiogenesis and overall survival." Vet Pathol **46**(6): 1275-1280.

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

Le Bitoux, M. A. and I. Stamenkovic (2008). "Tumor-host interactions: the role of inflammation." Histochem Cell Biol **130**(6): 1079-1090.

Lee, A. H. and I. O. Ellis (2008). "The Nottingham prognostic index for invasive carcinoma of the breast." Pathol Oncol Res **14**(2): 113-115.

Lim, H. Y., K. S. Im, N. H. Kim, H. W. Kim, J. I. Shin and J. H. Sur (2015). "Obesity, expression of adipocytokines, and macrophage infiltration in canine mammary tumors." Vet J **203**(3): 326-331.

Lindblad-Toh, K., C. M. Wade, T. S. Mikkelsen, E. K. Karlsson, D. B. Jaffe, M. Kamal*, et al.* (2005). "Genome sequence, comparative analysis and haplotype structure of the domestic dog." Nature **438**(7069): 803-819.

Lloyd, K. C., C. Khanna, W. Hendricks, J. Trent and M. Kotlikoff (2016). "Precision medicine: an opportunity for a paradigm shift in veterinary medicine." J Am Vet Med Assoc **248**(1): 45-48.

MacEwen, E. G., H. J. Harvey, A. K. Patnaik, S. Mooney, A. Hayes, I. Kurzman*, et al.* (1985). "Evaluation of effects of levamisole and surgery on canine mammary cancer." J Biol Response Mod **4**(4): 418-426.

Mainenti, M., R. Rasotto, P. Carnier and V. Zappulli (2014). "Oestrogen and progesterone receptor expression in subtypes of canine mammary tumours in intact and ovariectomised dogs." Vet J **202**(1): 62-68.

Mani, T., C. Bourguinat, K. Keller, E. Carreton, A. Peregrine and R. K. Prichard (2016). "Polymorphism in ion channel genes of Dirofilaria immitis: Relevant knowledge for future anthelmintic drug design." Int J Parasitol Drugs Drug Resist **6**(3): 343-355.

Mantovani, A. (2009). "Cancer: Inflaming metastasis." Nature **457**(7225): 36-37.

Mao, Y., E. T. Keller, D. H. Garfield, K. Shen and J. Wang (2013). "Stromal cells in tumor microenvironment and breast cancer." Cancer Metastasis Rev **32**(1-2): 303-315.

Marconato, L., R. M. Lorenzo, F. Abramo, A. Ratto and E. Zini (2008). "Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumours in dogs." Vet Comp Oncol **6**(2): 90-101.

Marconato, L., G. Romanelli, D. Stefanello, C. Giacoboni, U. Bonfanti, G. Bettini*, et al.* (2009). "Prognostic factors for dogs with mammary inflammatory carcinoma: 43 cases (2003-2008)." J Am Vet Med Assoc **235**(8): 967-972.

Marelli, G., A. Howells, N. R. Lemoine and Y. Wang (2018). "Oncolytic Viral Therapy and the Immune System: A Double-Edged Sword Against Cancer." Frontiers in immunology **9**: 866-866.

Marian, C., H. M. Ochs-Balcom, J. Nie, B. V. Kallakury, C. B. Ambrosone, M. Trevisan*, et al.* (2011). "FGFR2 intronic SNPs and breast cancer risk: associations

with tumor characteristics and interactions with exogenous exposures and other known breast cancer risk factors." International journal of cancer **129**(3): 702-712.

Martin de las Mulas, J., J. Ordas, Y. Millan, V. Fernandez-Soria and S. Ramon y Cajal (2003). "Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study." Breast Cancer Res Treat **80**(3): 363-367.

Matos, A. J., C. S. Baptista, M. F. Gartner and G. R. Rutteman (2012). "Prognostic studies of canine and feline mammary tumours: the need for standardized procedures." Vet J **193**(1): 24-31.

Melin, M., P. Rivera, M. Arendt, I. Elvers, E. Muren, U. Gustafson*, et al.* (2016). "Genome-Wide Analysis Identifies Germ-Line Risk Factors Associated with Canine Mammary Tumours." PLoS Genet **12**(5): e1006029.

Merlo, D. F., L. Rossi, C. Pellegrino, M. Ceppi, U. Cardellino, C. Capurro*, et al.* (2008). "Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy." J Vet Intern Med **22**(4): 976-984.

Millanta, F., S. Citi, D. Della Santa, M. Porciani and A. Poli (2006a). "COX-2 expression in canine and feline invasive mammary carcinomas: correlation with clinicopathological features and prognostic molecular markers." Breast Cancer Res Treat **98**(1): 115-120.

Millanta, F., G. Silvestri, C. Vaselli, S. Citi, G. Pisani, D. Lorenzi*, et al.* (2006b). "The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: a preliminary study of autocrine and paracrine loops." Res Vet Sci **81**(3): 350-357.

Misdorp, W. (1988). "Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins." Vet Q **10**(1): 26-33.

Misdorp, W. (1991). "Progestagens and mammary tumours in dogs and cats." Acta Endocrinol (Copenh) **125 Suppl 1**: 27-31.

Misdorp, W. (2002). Tumors of the Mammary Gland. Tumors in Domestic Animals. D. J. Meuten. Ames: IA, Iowa State Press**:** 575-606.

Misdorp, W., R. W. Else, E. Hellmen and L. TP. (1999). World Health Organization international histological classification of tumors of domestic animals Washington, D.C. : Armed Forces Institute of Pathology.

Misdorp, W. and A. A. Hart (1976). "Prognostic factors in canine mammary cancer." J Natl Cancer Inst **56**(4): 779-786.

Mitchell, L., F. A. De la Iglesia, M. S. Wenkoff, A. A. Van Dreumel and G. Lumb (1974). "Mammary tumors in dogs: survey of clinical and pathological characteristics." The Canadian Veterinary Journal **15**(5): 131-138.

Morris, J. S., J. M. Dobson, D. E. Bostock and E. O'Farrell (1998). "Effect of ovariohysterectomy in bitches with mammary neoplasms." Vet Rec **142**(24): 656-658.

Motoyama, N. and K. Naka (2004). "DNA damage tumor suppressor genes and genomic instability." Current Opinion in Genetics & Development **14**(1): 11-16.

Moulton, J. (1990). Tumors of the mammary gland. . Tumors in Domestic Animals. M. JE. Berkeley, University of California Press. **3:** 518-546.

Mulligan, R. M. (1942). "Histological studies on the canine female genital tract." Journal of Morphology **71**(3): 431-448.

Ng, T., M. Chan, C. C. Khor, H. K. Ho and A. Chan (2014). "The genetic variants underlying breast cancer treatment-induced chronic and late toxicities: a systematic review." Cancer Treat Rev **40**(10): 1199-1214.

Nieto, A., L. Pena, M. D. Perez-Alenza, M. A. Sanchez, J. M. Flores and M. Castano (2000). "Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance." Vet Pathol **37**(3): 239-247.

Nieto, A., M. D. Perez-Alenza, N. Del Castillo, E. Tabanera, M. Castano and L. Pena (2003). "BRCA1 expression in canine mammary dysplasias and tumours: relationship with prognostic variables." J Comp Pathol **128**(4): 260-268.

Niu, J., L. Jiang, W. Guo, L. Shao, Y. Liu and L. Wang (2013). "The Association between Leptin Level and Breast Cancer: A Meta-Analysis." PloS one **8**(6): e67349-e67349.

Novosad, C. A. (2003). "Principles of treatment for mammary gland tumors." Clin Tech Small Anim Pract **18**(2): 107-109.

Nowell, P. C. (1976). "The clonal evolution of tumor cell populations." Science **194**(4260): 23-28.

Offit, K. (2011). "Personalized medicine: new genomics, old lessons." Hum Genet **130**(1): 3-14.

Onay, V. U., L. Briollais, J. A. Knight, E. Shi, Y. Wang, S. Wells*, et al.* (2006). "SNP-SNP interactions in breast cancer susceptibility." BMC Cancer **6**: 114.

Orimo, A. and R. A. Weinberg (2006). "Stromal fibroblasts in cancer: a novel tumor-promoting cell type." Cell Cycle **5**(15): 1597-1601.

Pang, L. Y. and D. J. Argyle (2016). "Veterinary oncology: Biology, big data and precision medicine." Vet J **213**: 38-45.

Patsikas, M. N. and A. Dessiris (1996). "The Lymph Drainage of the Mammary Glands in the Bitch: a Lymphographic

Study. Part 11: the 3rd Mammary Gland." Anatomy, Histology, Embryology **25**: 139-143.

Pena, L., P. J. De Andres, M. Clemente, P. Cuesta and M. D. Perez-Alenza (2013). "Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics." Vet Pathol **50**(1): 94-105.

Peña, L., A. Gama, M. H. Goldschmidt, J. Abadie, C. Benazzi, M. Castagnaro*, et al.* (2014). "Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry." Vet Pathol **51**(1): 127-145.

Pena, L., M. D. Perez-Alenza, A. Rodriguez-Bertos and A. Nieto (2003). "Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases." Breast Cancer Res Treat **78**(2): 141-148.

Pena, L. L., A. I. Nieto, D. Perez-Alenza, P. Cuesta and M. Castano (1998). "Immunohistochemical detection of Ki-67 and PCNA in canine mammary tumors: relationship to clinical and pathologic variables." J Vet Diagn Invest **10**(3): 237-246.

Pereira, C. T., F. L. N. Marques, J. Williams, B. W. D. Martin and P. P. Bombonato (2008). "99mTc-Labeled Dextran For Mammary Lymphoscintigraphy In Dogs." Veterinary Radiology & Ultrasound **49**(5): 487-491.

Pereira, C. T., S. C. Rahal, J. C. de Carvalho Balieiro and A. A. Ribeiro (2003). "Lymphatic drainage on healthy and neoplasic mammary glands in female dogs: can it really be altered?" Anat Histol Embryol **32**(5): 282-290.

Perez Alenza, D., G. R. Rutteman, L. Pena, A. C. Beynen and P. Cuesta (1998). "Relation between habitual diet and canine mammary tumors in a case-control study." J Vet Intern Med **12**(3): 132-139.

Perez Alenza, M. D., L. Pena, N. del Castillo and A. I. Nieto (2000). "Factors influencing the incidence and prognosis of canine mammary tumours." J Small Anim Pract **41**(7): 287-291.

Perez Alenza, M. D., L. Pena, A. I. Nieto and M. Castano (1997). "Clinical and pathological prognostic factors in canine mammary tumors." Ann Ist Super Sanita **33**(4): 581-585.

Philibert, J. C., P. W. Snyder, N. Glickman, L. T. Glickman, D. W. Knapp and D. J. Waters (2003). "Influence of host factors on survival in dogs with malignant mammary gland tumors." J Vet Intern Med **17**(1): 102-106.

Pitot, H. C. (1993). "The molecular biology of carcinogenesis." Cancer **72**(3 Suppl): 962-970.

Plassais, J., M. Rimbault, F. J. Williams, B. W. Davis, J. J. Schoenebeck and E. A. Ostrander (2017). "Analysis of large versus small dogs reveals three genes on the canine X chromosome associated with body weight, muscling and back fat thickness." PLoS genetics **13**(3): e1006661-e1006661.

Povel, C. M., J. M. Boer, N. C. Onland-Moret, M. E. Dolle, E. J. Feskens and Y. T. van der Schouw (2012). "Single nucleotide polymorphisms (SNPs) involved in insulin resistance, weight regulation, lipid metabolism and inflammation in relation to metabolic syndrome: an epidemiological study." Cardiovasc Diabetol **11**: 133.

Priester, W. A. (1979). "Occurrence of mammary neoplasms in bitches in relation to breed, age, tumour type, and geographical region from which reported." Journal Small Animal Practice **20**: 1-11.

Queiroga, F. L., M. D. Perez-Alenza, G. Silvan, L. Pena, C. Lopes and J. C. Illera (2005). "Role of steroid hormones and prolactin in canine mammary cancer." J Steroid Biochem Mol Biol **94**(1-3): 181-187.

Queiroga, F. L., I. Pires, L. Lobo and C. S. Lopes (2010). "The role of Cox-2 expression in the prognosis of dogs with malignant mammary tumours." Res Vet Sci **88**(3): 441-445.

Queiroga, F. L., I. Pires, M. Parente, H. Gregorio and C. S. Lopes (2011). "COX-2 over-expression correlates with VEGF and tumour angiogenesis in canine mammary cancer." Vet J **189**(1): 77-82.

Rasotto, R., D. Berlato, M. H. Goldschmidt and V. Zappulli (2017). "Prognostic Significance of Canine Mammary Tumor Histologic Subtypes: An Observational Cohort Study of 229 Cases." Vet Pathol **54**(4): 571-578.

Rehm, S., D. J. Stanislaus and A. M. Williams (2007). "Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels." Birth Defects Res B Dev Reprod Toxicol **80**(3): 233-245.

Ressel, L., R. Puleio, G. R. Loria, I. Vannozzi, F. Millanta, S. Caracappa*, et al.* (2013). "HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome." Res Vet Sci **94**(2): 299-305.

Restucci, B., G. De Vico and P. Maiolino (2000). "Evaluation of angiogenesis in canine mammary tumors by quantitative platelet endothelial cell adhesion molecule immunohistochemistry." Vet Pathol **37**(4): 297-301.

Rivera, P., M. Melin, T. Biagi, T. Fall, J. Haggstrom, K. Lindblad-Toh*, et al.* (2009). "Mammary tumor development in dogs is associated with BRCA1 and BRCA2." Cancer Res **69**(22): 8770-8774.

Robbez-Masson, L. J., C. Bodor, J. L. Jones, H. C. Hurst, J. Fitzgibbon, I. R. Hart*, et al.* (2013). "Functional analysis of a breast cancer-associated FGFR2 single nucleotide polymorphism using zinc finger mediated genome editing." PLoS One **8**(11): e78839.

Rossi, F., S. Sabattini, M. Vascellari and L. Marconato (2018). "The impact of toceranib, piroxicam and thalidomide with or without hypofractionated radiation therapy on clinical outcome in dogs with inflammatory mammary carcinoma." Vet Comp Oncol **16**(4): 497-504.

Rutteman, G. R. (1992). "Contraceptive steroids and the mammary gland: is there a hazard?--Insights from animal studies." Breast Cancer Res Treat **23**(1-2): 29-41.

Sachidanandam, R., D. Weissman, S. C. Schmidt, J. M. Kakol, L. D. Stein, G. Marth*, et al.* (2001). "A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms." Nature **409**(6822): 928-933.

Saeki, K., Y. Endo, K. Uchida, R. Nishimura, N. Sasaki and T. Nakagawa (2012). "Significance of tumor-infiltrating immune cells in spontaneous canine mammary gland tumor: 140 cases." J Vet Med Sci **74**(2): 227-230.

Salas, Y., A. Marquez, D. Diaz and L. Romero (2015). "Epidemiological Study of Mammary Tumors in Female Dogs Diagnosed during the Period 2002-2012: A Growing Animal Health Problem." PLoS One **10**(5): e0127381.

Sanchez-Robert, E., L. Altet, M. Utzet-Sadurni, U. Giger, A. Sanchez and O. Francino (2008). "Slc11a1 (formerly Nramp1) and susceptibility to canine visceral leishmaniasis." Vet Res **39**(3): 36.

Sánchez, D., G. Cesarman-Maus, A. Amador-Molina and M. Lizano (2018). "Oncolytic Viruses for Canine Cancer Treatment." Cancers **10**(11): 404.

Sandoval, J. and M. Esteller (2012). "Cancer epigenomics: beyond genomics." Curr Opin Genet Dev **22**(1): 50-55.

Santos, A. A., C. C. Lopes, J. R. Ribeiro, L. R. Martins, J. C. Santos, I. F. Amorim*, et al.* (2013). "Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study." BMC Vet Res **9**: 1-11.

Santos, M., C. Correia-Gomes, R. Marcos, A. Santos, A. De Matos, C. Lopes*, et al.* (2015a). "Value of the Nottingham Histological Grading Parameters and Nottingham Prognostic Index in Canine Mammary Carcinoma." Anticancer Res **35**(7): 4219-4227.

Santos, M., C. Correia-Gomes, A. Santos, A. de Matos, P. Dias-Pereira and C. Lopes (2015b). "Interobserver Reproducibility of Histological Grading of Canine Simple Mammary Carcinomas." J Comp Pathol **153**(1): 22-27.

Santos, M., C. Correia-Gomes, A. Santos, A. de Matos, E. Rocha, C. Lopes*, et al.* (2014). "Nuclear pleomorphism: role in grading and prognosis of canine mammary carcinomas." Vet J **200**(3): 426-433.

Santos, M., R. Marcos and A. M. Faustino (2010). "Histological study of canine mammary gland during the oestrous cycle." Reprod Domest Anim **45**(5): e146-154.

Sapkota, Y., J. R. Mackey, R. Lai, C. Franco-Villalobos, S. Lupichuk, P. J. Robson*, et al.* (2014). "Assessing SNP-SNP interactions among DNA repair, modification and metabolism related pathway genes in breast cancer susceptibility." PLoS One **8**(6): e64896.

Sarli, G., R. Preziosi, C. Benazzi, G. Castellani and P. S. Marcato (2002). "Prognostic value of histologic stage and proliferative activity in canine malignant mammary tumors." J Vet Diagn Invest **14**(1): 25-34.

Schlafer, D. H. and R. A. Foster (2016). Female Genital System, Elsevier.

Schmidt, S., J. M. Monk, L. E. Robinson and M. Mourtzakis (2015). "The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: potential effects of exercise." Obes Rev **16**(6): 473-487.

Schneider, R., C. R. Dorn and D. O. Taylor (1969). "Factors influencing canine mammary cancer development and postsurgical survival." J Natl Cancer Inst **43**(6): 1249-1261.

Sharma, S., T. K. Kelly and P. A. Jones (2010). "Epigenetics in cancer." Carcinogenesis **31**(1): 27-36.

Shofer, F. S., E. G. Sonnenschein, M. H. Goldschmidt, L. L. Laster and L. T. Glickman (1989). "Histopathologic and dietary prognostic factors for canine mammary carcinoma." Breast Cancer Res Treat **13**(1): 49-60.

Shoji, K., M. Yoneda, T. Fujiyuki, Y. Amagai, A. Tanaka, A. Matsuda*, et al.* (2016). "Development of new therapy for canine mammary cancer with recombinant measles virus." Mol Ther Oncolytics **3**: 15022.

Short, A. D., N. M. Saleh, B. Catchpole, L. J. Kennedy, A. Barnes, C. A. Jones*, et al.* (2010). "CTLA4 promoter polymorphisms are associated with canine diabetes mellitus." Tissue Antigens **75**(3): 242-252.

Simon, D., D. Schoenrock, W. Baumgartner and I. Nolte (2006). "Postoperative adjuvant treatment of invasive malignant mammary gland tumors in dogs with doxorubicin and docetaxel." J Vet Intern Med **20**(5): 1184-1190.

Sleeckx, N., H. de Rooster, E. J. Veldhuis Kroeze, C. Van Ginneken and L. Van Brantegem (2011). "Canine mammary tumours, an overview." Reprod Domest Anim **46**(6): 1112-1131.

Sonnenschein, E. G., L. T. Glickman, M. H. Goldschmidt and L. J. McKee (1991). "Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study." Am J Epidemiol **133**(7): 694-703.

Sorenmo, K. (2003). "Canine mammary gland tumors." Veterinary Clinics of North America: Small Animal Practice **33**(3): 573-596.

Sorenmo, K. U., V. M. Kristiansen, M. A. Cofone, F. S. Shofer, A. M. Breen, M. Langeland*, et al.* (2009). "Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence." Vet Comp Oncol **7**(3): 162-172.

Sorenmo, K. U., R. Rasotto, V. Zappulli and M. H. Goldschmidt (2011). "Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms." Vet Pathol **48**(1): 85-97.

Sorenmo, K. U., F. S. Shofer and M. H. Goldschmidt (2000). "Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma." J Vet Intern Med **14**(3): 266-270.

Stover, D. G. and N. Wagle (2015). "Precision medicine in breast cancer: genes, genomes, and the future of genomically driven treatments." Curr Oncol Rep **17**(4): 15.

Stovring, M., L. Moe and E. Glattre (1997). "A population-based case-control study of canine mammary tumours and clinical use of medroxyprogesterone acetate." APMIS **105**(8): 590-596.

Sun, W., X. Yang, H. Qiu, D. Zhang, H. Wang, J. Huang*, et al.* (2015). "Relationship between three novel SNPs of BRCA1 and canine mammary tumors." The Journal of Veterinary Medical Science **77**(11): 1541-1543.

Suryawanshi, Y. R., T. Zhang and K. Essani (2017). "Oncolytic viruses: emerging options for the treatment of breast cancer." Med Oncol **34**(3): 43.

Tatarinova, T. V., E. Chekalin, Y. Nikolsky, S. Bruskin, D. Chebotarov, K. L. McNally*, et al.* (2016). "Nucleotide diversity analysis highlights functionally important genomic regions." Scientific reports **6**: 35730-35730.

Tavares, W. L., G. E. Lavalle, M. S. Figueiredo, A. G. Souza, A. C. Bertagnolli, F. A. Viana*, et al.* (2010). "Evaluation of adverse effects in tamoxifen exposed healthy female dogs." Acta Vet Scand **52**: 67.

Torres de la Riva, G., B. L. Hart, T. B. Farver, A. M. Oberbauer, L. L. Messam, N. Willits*, et al.* (2013). "Neutering dogs: effects on joint disorders and cancers in golden retrievers." PLoS One **8**(2): e55937.

Tran, C. M., A. S. Moore and A. E. Frimberger (2016). "Surgical treatment of mammary carcinomas in dogs with or without postoperative chemotherapy." Vet Comp Oncol **14**(3): 252-262.

Udagawa, C., N. Tada, J. Asano, K. Ishioka, K. Ochiai, M. Bonkobara*, et al.* (2014). "The genetic association study between polymorphisms in uncoupling protein 2 and uncoupling protein 3 and metabolic data in dogs." BMC Res Notes **7**: 904.

Uecker, H. and J. Hermisson (2011). "On the Fixation Process of a Beneficial Mutation in a Variable Environment." Genetics **188**(4): 915-930.

Vascellari, M., K. Capello, A. Carminato, C. Zanardello, E. Baioni and F. Mutinelli (2016). "Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer." Prev Vet Med **126**: 183-189.

Wang, C.-Q., C.-H. Tang, Y. Wang, L. Jin, Q. Wang, X. Li*, et al.* (2017). "FSCN1 gene polymorphisms: biomarkers for the development and progression of breast cancer." Scientific Reports **7**(1): 15887.

Weiss, K. M. (1998). "In search of human variation." Genome Res **8**(7): 691-697.

Wright, A. F. (2001). Genetic Variation: Polymorphisms and Mutations. eLS, John Wiley & Sons, Ltd.

Wu, S., S. Powers, W. Zhu and Y. A. Hannun (2016). "Substantial contribution of extrinsic risk factors to cancer development." Nature **529**(7584): 43-47.

Yamagami, T., T. Kobayashi, K. Takahashi and M. Sugiyama (1996). "Prognosis for canine malignant mammary tumors based on TNM and histologic classification." J Vet Med Sci **58**(11): 1079-1083.

Yang, C. H., Y. D. Lin, L. Y. Chuang and H. W. Chang (2013). "Evaluation of breast cancer susceptibility using improved genetic algorithms to generate genotype SNP barcodes." IEEE/ACM Trans Comput Biol Bioinform **10**(2): 361-371.

Yates, C. M. and M. J. Sternberg (2013). "The effects of non-synonymous single nucleotide polymorphisms (nsSNPs) on protein-protein interactions." J Mol Biol **425**(21): 3949-3963.

Yates, L. R. and P. J. Campbell (2012). "Evolution of the cancer genome." Nature reviews. Genetics **13**(11): 795-806.

You, J. S. and P. A. Jones (2012). "Cancer genetics and epigenetics: two sides of the same coin?" Cancer Cell **22**(1): 9-20.

## CHAPTER 02

# Aims and Objectives

Despite of the well-known influence of genetic variation in human breast cancer development and progression, the impact of genetic SNPs in CMTs is scarcely studied. Considering that the knowledge of the animal genetic profile and its role on the risk, development and prognosis of the disease represents an important step for the application of precision medicine strategies in veterinary oncology, herein the influence of SNPs into the development, outcome and into the phenotypical features of CMTs will be studied. For this purpose, SNPs in genes known to be involved in mammary carcinogenesis, including oncogenes, tumor suppressor genes, genes participating in DNA repair and genes implicated in hormonal metabolism, will be assessed. To accomplish this goal we defined the following specific aims:

- To evaluate the relationship between canine SNPs and the risk of CMTs;
- To evaluate the association between canine SNPs and relevant clinicopathological features of CMTs;
- To evaluate the relationship between canine SNPs and the clinical outcome of the disease.

Histopathology remains the gold standard method for classifying and grading CMTs. Recently, new classification and grading system for CMTs have been proposed; nowadays, pathologists face uncertainty regarding which method is most suitable for the evaluation of mammary tumors in canine species. Considering this, we intend to compare the two most widely used histological classification systems [the WHO (Misdorp et al 1999) and the 2011 proposal (Goldschmidt et al 2011)] and two grading methods [the NHG (Elston and Ellis 1991) and the ca-NHG (Peña et al 2013)]. To fulfil this goal we defined the following specific aims:

- To apply the classification and grading systems to a prospective series of CMTs;
- To compare the classification and grading systems, identifying advantages and limitations of each system when applied to routine diagnostic practice;
- To evaluate the association of the histotype (defined by each histological classification system) and the histological grade (defined by the NHG and the ca-NHG methods) with the overall survival;

- To evaluate the association of the histotype (defined by each histological classification system) and histological grade (defined by the NHG and the ca-NHG systems) with other relevant clinicopathological features of CMTs;
- To correlate the histotype (defined by each classification system) and the histological grade (defined by the NHG and the ca-NHG systems) with the genetic profile of the animal.

## CHAPTER 03

# Canine Mammary Tumor Risk is Associated with Polymorphisms in RAD51 and STK11 Genes

Check for updates

*Brief Communication*

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# Canine mammary tumor risk is associated with polymorphisms in *RAD51* and *STK11* genes

**Ana Canadas, Marta Santos, Augusto Nogueira, Joana Assis, Mónica Gomes, Carolina Lemos, Rui Medeiros, Patrícia Dias-Pereira**[1]

Abstract. Cancer is a complex disease involving genetic and phenotypic changes. Several single nucleotide polymorphisms (SNPs) have been associated with the risk of breast cancer development in women; however, little is known regarding their influence on canine mammary tumor risk. We assessed the influence of SNPs in genes related to human breast cancer susceptibility, with respect to the risk of development of mammary tumors in dogs. Sixty-seven canine SNPs in proto-oncogenes, tumor suppressor genes, genes involved in DNA repair, and in hormonal metabolism were evaluated in 212 bitches with mammary tumors and in 161 bitches free of mammary neoplasia. A significant association with mammary neoplasia risk was identified for 2 SNPs in *RAD51* (*rs23623251* and *rs23642734*) and one SNP in the *STK11* gene (*rs22928814*). None of the other SNPs were related to the risk of mammary tumor development. The identification of genetic profiles associated with risk of mammary neoplasia is of great importance, supporting the implementation of specific clinical management strategies in high-risk animals.

Key words: Canine mammary tumor; polymorphisms; *RAD51*; risk; *STK11*.

Advances in molecular, genetic, and clinical research have markedly increased our knowledge of the basic biology of cancer. Cancer is clearly recognized as a complex disease involving a sequence of genetic, epigenetic, and phenotypic changes. The disease is characterized by aberrant gene structure or function that drive the progressive switch of normal cells into neoplastic ones.[7,14] Exhaustive research in molecular genetics applied to oncology has led to the identification of genetic profiles associated with clinical and pathologic features of neoplasms, with the biological behavior of the tumors, and with their response to treatment.[3,19] In humans, several single nucleotide polymorphisms (SNPs), mostly in proto-oncogenes, tumor suppressor genes, genes coding for proteins involved in DNA repair, and in sexual steroid hormone metabolism have been associated with an increased risk of cancer development, including breast cancer.[13]

To date, only a few studies have reported an association between SNP and canine mammary tumor (CMT) risk. Some authors described significantly different allele frequencies for several SNPs when comparing high- and low-risk breeds for mammary tumors, underlining the importance of the genetic background in the susceptibility to mammary tumor development in dogs.[2] Other authors have recently associated SNPs in *BRCA1* and *BRCA2* genes with mammary neoplasm development in dogs.[5,20] Furthermore, an association between SNP in the *ESR1* gene and the risk for mammary tumors in English Springer Spaniels has also been described.[2]

We assessed the influence of SNPs in genes known to be associated with human breast cancer susceptibility, in the risk of development of CMT. For that purpose, 67 canine SNPs in proto-oncogenes (*HER-2, EGFR*), tumor suppressor genes (*TP53, STK11*), and genes involved in DNA damage recognition and repair (*BRCA1, BRCA2, RAD51, CHECK2, PTEN, BRIP1*) and in hormonal metabolism (*ESR1, PGR, PRLR, COMT*) were selected (Table 1).

A case-control study was conducted involving 373 dogs: 212 bitches with histologically confirmed mammary tumors (mean age: 10.0 y); and 161 bitches >7-y-old, without

Instituto de Ciências Biomédicas de Abel Salazar–Universidade do Porto (ICBAS-UP), Porto, Portugal (Canadas, Santos, Lemos, Dias-Pereira); Molecular Oncology and Viral Pathology Group, Portuguese Institute of Oncology of Porto (IPO Porto) Research Center (CI-IPOP), Porto, Portugal (Nogueira, Assis, Gomes, Medeiros); i3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal (Lemos); UnIGENe, Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal (Lemos); CEBIMED, Faculty of Health Sciences of Fernando Pessoa University, Porto, Portugal (Medeiros); FMUP, Faculty of Medicine of Porto, University of Porto, Porto, Portugal (Medeiros); Research Department, Portuguese League Against Cancer (LPCC–NRNorte), Porto, Portugal (Medeiros).

[1]Corresponding author: Patrícia Dias-Pereira, Instituto de Ciências Biomédicas de Abel Salazar–Universidade do Porto (ICBAS-UP), Rua Jorge Viterbo Ferreira nº228, 4050-313 Porto, Portugal.
pdiaspereira@yahoo.com.br

Table 1. Genes and single nucleotide polymorphisms (SNPs) assessed for association to canine mammary tumor risk.

| Gene | CFA | SNP | Minor allele | Minor allele frequency | | Major allele |
|---|---|---|---|---|---|---|
| | | | | Controls | Cases | |
| Proto-oncogene | | | | | | |
| *EGFR* | 18 | *rs22662624* | – | 0 | 0 | T |
| | | *rs397513721* | C | 0.363 | 0.349 | T |
| | | *rs851560790* | – | 0 | 0 | C |
| | | *rs8581001* | T | 0.065 | 0.035 | C |
| *HER-2* | 9 | *rs24537331* | A | 0.348 | 0.334 | G |
| | | *rs397510212* | – | 0 | 0 | T |
| | | *rs397509770* | A | 0.065 | 0.036 | G |
| | | *rs397512599* | G | 0.171 | 0.153 | A |
| | | *rs397511049* | C | 0.397 | 0.379 | T |
| | | *rs397512289* | T | 0.169 | 0.143 | C |
| | | *rs24616607* | C | 0.010 | 0.007 | G |
| | | *rs24537329* | T | 0.481 | 0.488 | C |
| | | *rs397513043* | C | 0.081 | 0.076 | T |
| Tumor suppressor genes | | | | | | |
| *STK11* | 20 | *rs22928814* | T | 0.149 | 0.257 | C |
| *TP53* | 5 | *rs852701327* | – | 0 | 0 | C |
| | | *rs852416666* | – | 0 | 0 | G |
| | | *rs851620436* | – | 0 | 0 | C |
| DNA damage repair | | | | | | |
| *BRCA1* | 9 | *rs397509570* | G | 0.472 | 0.495 | A |
| | | *rs397511319* | G | 0.351 | 0.370 | C |
| | | *rs397512112* | – | 0 | 0 | T |
| *BRCA2* | 25 | *rs23250374* | C | 0.394 | 0.422 | T |
| | | *rs397511123* | DEL | 0.404 | 0.425 | AAC |
| | | *rs23255542* | G | 0.442 | 0.434 | A |
| | | *rs397510884* | – | 0 | 0 | C |
| | | *rs397512126* | – | 0 | 0 | T |
| *BRIP* | 9 | *rs397511271* | G | 0.103 | 0.109 | A |
| | | *rs397512960* | C | 0.360 | 0.351 | T |
| | | *rs24602743* | A | 0.009 | 0.012 | G |
| | | *rs397511741* | G | 0.463 | 0.479 | A |
| *CHEK2* | 26 | *rs23299237* | C | 0.041 | 0.032 | T |
| | | *rs397511718* | G | 0.170 | 0.213 | T |
| *PTEN* | 26 | *rs397513087* | T | 0.044 | 0.049 | C |
| | | *rs397510459** | – | – | – | – |
| | | *rs397510595* | A | 0.130 | 0.094 | G |
| *RAD51* | 30 | *rs23623225* | T | 0.034 | 0 | C |
| | | *rs851723852* | – | 0 | 0 | C |
| | | *rs23623251* | C | 0.376 | 0.325 | T |
| | | *rs23642734* | C | 0.419 | 0.401 | T |
| | | *rs523631166* | – | 0 | 0 | G |
| Hormonal metabolism | | | | | | |
| *ESR1* | 1 | *rs397512133* | A | 0.126 | 0.090 | G |
| | | *rs21970417* | – | 0 | 0 | G |
| | | *rs851327560* | C | 0.244 | 0.197 | T |
| | | *rs397510462* | A | 0.128 | 0.090 | G |
| | | *rs21960513* | C | 0.475 | 0.439 | T |
| | | *rs397512038* | G | 0.183 | 0.118 | A |
| | | *rs397510612* | C | 0.134 | 0.095 | T |
| | | *rs21953930* | – | 0 | 0 | T |
| | | *rs9176904* | – | 0 | 0 | T |

*(continued)*

Table 1. (continued)

| Gene | CFA | SNP | Minor allele | Minor allele frequency | | Major allele |
|---|---|---|---|---|---|---|
| | | | | Controls | Cases | |
| | | *rs852887655* | A | 0.116 | 0.096 | G |
| | | *rs852398698* | DEL | 0.289 | 0.267 | TTC |
| | | *rs852684753* | DEL | 0.278 | 0.265 | TTTC |
| *COMT* | 26 | *rs853046495* | T | 0.156 | 0.122 | C |
| | | *rs23350589* | T | 0.447 | 0.459 | C |
| | | *rs9008012* | – | 0 | 0 | G |
| | | *rs853133060* | T | 0.146 | 0.146 | C |
| | | *rs851328636* | G | 0.147 | 0.141 | C |
| | | *rs23322686* | T | 0.432 | 0.444 | C |
| | | *rs2336579* | C | 0.465 | 0.458 | T |
| | | *rs23367171* | – | 0 | 0 | G |
| | | *rs852549365* | A | 0.294 | 0.317 | G |
| | | *rs852564758* | T | 0.432 | 0.444 | C |
| | | *rs9008011* | – | 0 | 0 | DEL |
| *PGR* | 21 | *rs8875007* | T | 0.245 | 0.259 | DEL |
| | | *rs22996864* | – | 0 | 0 | C |
| | | *rs397512502* | A | 0.025 | 0.044 | T |
| *PRLR* | 35 | *rs23932236* | C | 0.373 | 0.420 | G |
| | | *rs852622584* | – | 0 | 0 | G |

CFA = canine chromosome number; dash (–) = allele undetected in the population.
* SNP undetected.

evidence of mammary neoplasia (mean age: 9.0 y). The breeds represented in both groups were very similar, with 45% and 46% of mixed-breed dogs included in case and control population, respectively. Furthermore, 32% and 22.4% of the bitches from the case and control group, respectively, belonged to a series of 5 more representative breeds: Poodle, Boxer, Labrador Retriever, Cocker Spaniel, and German Shepherd. All of the owners gave informed consent regarding the use of the material for research purposes. Our study was performed under an institutionally approved protocol (Approval P151/2016).

Genomic DNA was extracted from peripheral blood samples obtained by standard venipuncture (High Pure PCR template preparation kit, Roche, Mannheim, Germany). The DNA quality was evaluated by measuring the optical density, and the quantity was assessed (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, Waltham, MA). SNP genotyping was performed (MassARRAY iPLEX Gold technology, Agena Bioscience, San Diego, CA) in the Genomics Unit of the genotyping service of the Gulbenkian Institute of Science (Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência, Portugal).

Statistical analysis of data was performed (SPSS for Windows v.18, SPSS, Chicago, IL). Chi-square analysis was used to compare categorical variables. A 5% level was considered to define statistical significance. The observed number of each genotype was compared with that expected for a population in the Hardy–Weinberg equilibrium by using a goodness-of-fit chi-square test. Linkage disequilibrium (LD) analysis was performed for SNPs in genes in the same chromosome. Data were calculated and LD maps were constructed (Haploview v.4.2, https://www.broadinstitute.org/haploview/haploview).

A significant association with CMT risk was identified for 3 SNPs: *rs23623251* and *rs23642734* in *RAD51*, and *rs22928814* in the *STK11* gene ($p$ = 0.004; $p$ = 0.037; $p$ = 0.008, respectively). For *RAD51* SNP *rs23623251*, TT animals are more likely to develop mammary neoplasia than C allele carriers (odds ratio [OR] = 1.86; confidence interval [CI] = 1.22–2.83). In fact, TT animals correspond to 50.9% of all bitches with mammary tumors and only up to 35.4% of the animals from the control group. Furthermore, for *RAD51* SNP *rs23642734*, the TT genotype represents 42.9% of animals with mammary tumors and 31.9% of the animals from the control population. Our data demonstrate that TT animals have a higher risk of developing mammary tumors than C allele carriers (OR = 1.58; CI = 1.03–2.43). LD analysis was performed for SNPs *rs23623251* and *rs23642734*, and demonstrated that, in our population, these loci are in LD ($r^2$ = 0.75; Fig. 1). For *STK11* SNP *rs22928814*, T allele carriers have a higher probability of developing CMT than CC animals (OR = 1.82; CI = 1.17–2.84). Indeed, T carriers represent 40.3% of bitches with mammary neoplasia and only 26.7% of the animals from the control group. No significant association was found between the other SNPs included in our study and the risk of CMT development. The LD plots of

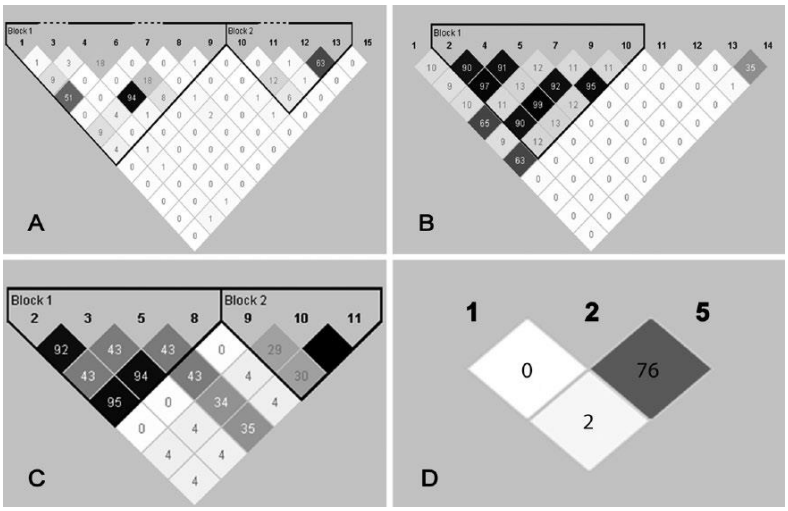


Figure 1. Linkage disequilibrium (LD) maps of genotyped single nucleotide polymorphisms (SNPs), including controls and canine mammary tumor cases. The dark gray-to-white gradient reflects higher to lower LD values. Dark gray diamonds without a number indicate complete LD ($r^2 = 1$). A. Chr9 (*HER-2*, *BRCA1*, and *BRIP*) SNPs included: *rs24537331, rs397509770, rs397512599, rs2453732, rs24616607, rs397512289, rs397513043, rs397511271, rs24602743, rs397511741, rs397512960, rs397509570*. Fixed SNPs: *rs397510212, rs397512112*. B. Chr26 (*COMT*, *CHEK2*, *PTEN*) SNPs included: *rs853046495, rs23350589, rs23322686, rs23336579, rs851328636, rs852564758, rs853133060, rs23299237, rs397511718, rs397513087, rs397510595*. Fixed SNPs: *rs9008012, rs23367171, rs852549365*. C. Chr1 (*ESR1*) SNPs included: *rs397510462, rs397512133, rs851327560, rs397510612, rs852887655, rs852684753, rs852398698.* Fixed SNPs: *rs21970417, rs9176904, rs21953930*. D. Chr30 (*RAD51*) SNPs included: *rs23623225, rs23623251, rs23642734*. Fixed SNPs: *rs23631166, rs851723852* .

all studied SNPs, including SNPs found to be associated with CMT in the different populations (control and disease groups), are presented as supporting information (Fig. 1). In 21 SNPs, LD could not be estimated because most of them were fixed SNPs (a homozygous SNP that has spread throughout an entire population) and some were not in agreement with Hardy–Weinberg equilibrium.

In a subsequent analysis, the association between *RAD51 rs23623251* and *rs23642734* and *STK11 rs22928814* and the tumor type (benign or malignant) was assessed. No statistically significant association was found for any of the SNPs evaluated. However, for *STK11* SNP *rs22928814*, CC carriers revealed a tendency to be associated with the development of benign tumors ($p = 0.067$). In fact, 40% of CC carriers developed benign neoplasms, whereas only 27.5% of the other genotypes developed this type of lesion. The association between *RAD51 rs23623251* and *rs23642734* and *STK11 rs22928814* and the histologic classification of malignant tumors was also evaluated. For that purpose, malignant neoplasms were divided into 3 groups: A = complex carcinomas + carcinoma in benign tumors; B = simple carcinomas; C = carcinosarcomas + special type carcinomas + sarcomas. No significant relationship was observed between the animals' genetic profile and the histologic subtype of the malignant mammary tumors for any of the SNPs assessed.

RAD51 is a protein involved in DNA repair pathways by homologous recombination.[1] Proper function of DNA repair enzymes is crucial for maintaining genome integrity. Failure of these proteins can lead to an exponential increase in DNA mutations, resulting in genomic instability and, eventually, in higher cancer susceptibility.[14] *RAD51* also interacts with *BRCA2*, a well-recognized high-penetrance breast cancer susceptibility gene.[18] As in humans, changes in *RAD51* messenger RNA and protein expression were described in CMTs, and an interaction between both *BRCA2* and *RAD51* canine genes has also been documented.[10,11,15,16] STK11 is a serine/threonine protein kinase that plays several functions as a tumoral suppressor through control of cell growth and polarity, regulation of AMPK and other kinases, and cell cycle governance.[8] In humans, *STK11* mutations are associated with predisposition to Peutz–Jeghers syndrome, a condition characterized by gastrointestinal polyposis, mucocutaneous hyperpigmentation, and increased cancer susceptibility, namely breast cancer.[12] There are also some analogies in the *STK* expression pattern between human breast cancer and feline mammary tumors.[4] Some genetic variations in *RAD51* and *STK11* genes were found in several types of human neoplasia, including breast cancer, hence they are both considered breast cancer susceptibility genes.[9] Comparative oncology is a rapidly growing and promising field of research.

Merging scientific data from human and veterinary oncologic investigation clearly benefits knowledge of cancer development, progression, and management.[22] Canine and feline mammary tumors share several epidemiologic, clinical, and pathologic features with breast cancer, which makes them appropriate animal models for the translational study of this disease.[17] Our findings suggest that *RAD51* and *STK11* may play a similar role in mammary carcinogenesis in both humans and companion animals, thus constituting a valuable target for comparative oncology research.

Despite the fact that these particular SNPs in canine *RAD51* (*rs23623251* and *rs23642734*) and *STK11* (*rs22928814*) genes are located in introns (non-coding DNA regions), they may have a functional significance in mammary tumor risk through several pathways, namely by coding for small regulatory elements that control gene expression or by affecting alternative splicing. Additionally, they may be linked to other functional SNPs from the same gene that indeed have impact on gene expression and/or function.[14,21]

Data from our investigation do not entirely match previous studies, which associated SNPs in *ESR1*, *BRCA1*, and *BRCA2* genes with increased risk of CMT.[2,5,20] This dissimilarity may be explained by differences in the canine population studied (such as sample size and composition), by the inclusion criteria, or by the laboratory methodologies employed.

Costs associated with genetic testing make inclusion of genetic tests difficult in routine veterinary oncology protocols. However, in an era characterized by growing concerns about animal health and welfare, it is reasonable to assume that an increasing number of pet owners will pursue genetic tests. This type of methodology allows for the identification of genetic profiles associated with a higher risk of mammary neoplasia, which may be reflected in long-term savings. The development of specific surveillance and clinical monitoring protocols in a group of high-risk animals could significantly reduce costs associated with the clinical management of the disease and also improve the animals' quality of life. Furthermore, such development supports the implementation of preventive strategies aiming to reduce the exposure to other known risk factors (e.g., early spaying recommendation and avoidance of hormonal anti-contraceptive therapy).[6,23]

It is noteworthy that although most of the SNPs assessed in our study are not significantly associated with CMT risk, they may indeed influence the clinicopathologic features and biological behavior of the neoplastic disease, which are areas that deserve evaluation in future investigations.

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## References

1. Baumann P, West SC. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. Trends Biochem Sci 1998;23:247–251.
2. Borge KS, et al. The ESR1 gene is associated with risk for canine mammary tumours. BMC Vet Res 2013;9:69.
3. Cardoso F, et al. 70-gene signature as an aid to treatment decisions in early-stage breast cancer. N Engl J Med 2016;375:717–729.
4. De Maria R, et al. Feline STK gene expression in mammary carcinomas. Oncogene 2002;21:1785–1790.
5. Enginler SO, et al. Genetic variations of BRCA1 and BRCA2 genes in dogs with mammary tumours. Vet Res Commun 2014;38:21–27.
6. Gagnon J, et al. Recommendations on breast cancer screening and prevention in the context of implementing risk stratification: impending changes to current policies. Curr Oncol 2016;23:e615–e625.
7. Garraway LA, Lander ES. Lessons from the cancer genome. Cell 2013;153:17–37.
8. Hezel AF, Bardeesy N. LKB1; linking cell structure and tumor suppression. Oncogene 2008;27:6908–6919.
9. Kleibl Z, Kristensen VN. Women at high risk of breast cancer: molecular characteristics, clinical presentation and management. Breast 2016;28:136–144.
10. Klopfleisch R, Gruber AD. Increased expression of BRCA2 and RAD51 in lymph node metastases of canine mammary adenocarcinomas. Vet Pathol 2009;46:416–422.
11. Klopfleisch R, et al. RAD51 protein expression is increased in canine mammary carcinomas. Vet Pathol 2010;47:98–101.
12. Korsse SE, et al. Targeting LKB1 signaling in cancer. Biochim Biophys Acta 2013;1835:194–210.
13. Mavaddat N, et al. Genetic susceptibility to breast cancer. Mol Oncol 2010;4:174–191.
14. Newkirk K, et al. Neoplasia and tumor biology. In: Zachary J, ed. Pathologic Basis of Veterinary Disease. 6th ed. St. Louis, MO: Elsevier, 2016:286–321.
15. Ochiai K, et al. Polymorphisms of canine BRCA2 BRC repeats affecting interaction with RAD51. Biomed Res 2015;36:155–158.

16. Ozmen O, et al. Somatic SNPs of the BRCA2 gene at the fragments encoding RAD51 binding sites of canine mammary tumors. Vet Comp Oncol 2017;15:1479–1486.
17. Pinho SS, et al. Canine tumors: a spontaneous animal model of human carcinogenesis. Transl Res 2012;159:165–172.
18. Prakash R, et al. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. Cold Spring Harb Perspect Biol 2015;7:a016600.
19. Ripperger T, et al. Breast cancer susceptibility: current knowledge and implications for genetic counseling. Eur J Hum Genet 2009;17:722–731.
20. Rivera P, et al. Mammary tumor development in dogs is associated with BRCA1 and BRCA2. Cancer Res 2009;69:8770–8774.
21. Samuels DC, et al. Finding the lost treasures in exome sequencing data. Trends Genet 2013;29:593–599.
22. Schiffman JD, Breen M. Comparative oncology: what dogs and other species can teach us about humans with cancer. Philos Trans R Soc Lond B Biol Sci 2015;370:pii:20140231.
23. Walsh MF, et al. Genomic biomarkers for breast cancer risk. Adv Exp Med Biol 2016;882:1–32.

# CHAPTER 04

## Influence of E-Cadherin Genetic Variation in Canine Mammary Tumor Risk, Clinicopathological Features and Prognosis

## Influence of E-Cadherin Genetic Variation in Canine Mammary Tumor Risk, Clinicopathological Features and Prognosis

**Abstract**

E-cadherin is a cell adhesion molecule that participates in several cellular processes that guarantee the maintenance of structural and functional integrity of epithelial tissues. E-cadherin plays an important role in mammary carcinogenesis and various studies have demonstrated the effect of *CDH1* genetic variation in risk, progression and biological behavior of human breast cancer. Although there are some recognized genetic variations in canine *CDH1* gene, their influence in canine mammary tumor development and progression has not been previously evaluated. In this study, we aim to assess the influence of *CDH1* SNPs rs850805755, rs852280880 and rs852639930 in the risk, clinicopathological features and clinical outcome of canine mammary tumors. A case-control study was conducted involving 206 bitches with mammary tumors and 161 bitches free of mammary neoplasia. *CDH1* SNPs rs850805755 and rs852280880 were associated with a decreased risk and a later onset of mammary tumor development. Furthermore, these SNPs were related to the development of small size carcinomas, of low histological grade and low nuclear pleomorphism. SNP rs852639930 was associated with the development of small size tumors with a non-infiltrative, non-invasive growth pattern. Data from the present investigation demonstrate that these *CDH1* genetic variants could have a protective role in canine mammary tumors, by being associated with low risk of tumor development, delayed onset of the disease and less aggressive clinicopathological features.

**Keywords:** canine mammary tumor, CDH1 gene, E-cadherin, risk, SNP

## Introduction

Tissues' architectural arrangements are maintained by several cell adhesion molecules, including those of the cadherin's family. Cadherins are calcium-dependent transmembrane glycoproteins responsible for cell-to-cell adhesion [1,2]. They participate in tissue morphogenesis and differentiation and also play an important role in several processes such as cell shape, polarity, proliferation and motility. E-cadherin/CDH1 (epithelial) is present in most epithelial tissues, namely the mammary gland, guaranteeing its structural and functional integrity [3].

As the major component of epithelial adherent junctions, structural or functional alterations in E-cadherin can compromise cell-to-cell adhesion [4-6], favoring tumor development and facilitating neoplastic cells motility, thus increasing the tumoral metastatic potential [7]. In fact, loss/alterations in E-cadherin expression and genetic changes in *CDH1* gene have been described in several types of human cancer, including breast cancer. Furthermore, E-cadherin downregulation has been associated to aggressive clinicopathological features of breast cancer, metastasis, decreased disease-free and overall survival [8-11], underlining the importance of this cell adhesion molecule as a prognostic marker.

Several immunohistochemical studies reported decreased E-cadherin membrane expression, as well as E-cadherin cytoplasmic internalization, in canine mammary carcinomas [12-16]. These features have been linked to aggressive clinicopathological characteristics of mammary carcinomas, such as high histological grade and mitotic index, larger tumor size, infiltrative or invasive growth and lymph node metastasis [4,12-14]. Besides, some authors found a significant association between E-cadherin down expression and shorter disease-free survival [14] and overall survival [4,14,15]. Recently, gene expression profiling research documented significant differences between metastatic and non-metastatic canine mammary tumors, with some authors linking the loss of cell adhesion molecules to the metastatic subgroup [17-20]. Although genetic variability in canine *CDH1* gene is well recognized, namely single nucleotide polymorphisms (SNPs), its influence in canine mammary tumors (CMT) development and progression is largely unknown. In this study we aim to investigate the association between SNPs rs850805755, rs852280880 and rs852639930 in *CDH1* gene and risk, clinicopathological features and clinical outcome of CMT.

**Materials and Methods**

A case-control study was conducted including 206 bitches with histologically confirmed mammary tumor and a control group with 161 bitches (≥7 years old), with no clinical evidence of mammary neoplasia nor history of other neoplasia, and with no relevant diseases. Dogs with mammary tumors were treated with radical unilateral mastectomy (removal of an entire unilateral mammary chain) and/or partial mastectomy (removal of at least one mammary gland, always including the affected one), according to the best surgical practice criteria. Owners provided consent for surgery with curative intents as well as for the use of the material for research purposes. This protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences Abel Salazar, University of Porto (P151/2016).

Mammary specimens were collected after surgery, fixed immediately in a 10% buffered formalin solution and routinely processed for histological examination. For each case, the age at the time of the diagnosis, the number of tumors (one or multiple tumors) and the tumor size -corresponding to the largest diameter measured by the same pathologist (AC) during trimming- were recorded. Histological diagnosis was established by consensus of 3 observers (AC, MS and PDP) using the criteria of the World Health Organization for the classification of mammary tumors of dogs and cats [21]. The presence of vascular invasion and of regional lymph node metastases at the time of diagnosis was assessed. Immunohistochemistry was used to assist in the recognition of biphasic tumors, namely for confirming the involvement of myoepithelium in the neoplasia, and to ascertain the presence of lymph node metastasis. Antibodies against pan-cytokeratin, cytokeratin 14 and p63 were used for that purpose, following current guidelines [22]. Each tumor was evaluated for the mode of growth and classified as either expansive, when it was delimited with a capsule; infiltrative, when not delimited by a capsule, but without signs of vascular invasion or lymph node metastasis; and invasive in the presence of vascular invasion or lymph node metastases. In cases of dogs with multiple malignant tumors, a reference lesion was assigned for the statistical study, as previously reported [23].

Histological grading was performed according to the Nottingham histologic grading method (NHG) [24] based on the assessment of three parameters: tubule formation, nuclear pleomorphism and mitotic counts. A veterinary-adaptation of the human Nottingham Prognostic Index (vet-NPI) was also computed, as previously reported [25]. The cut-off of 4 was considered for vet-NPI, *i.e*. vet-NPI>4 was interpreted as a marker of worse prognosis and decreased survival.

The animals were followed for a 2 year post-operative period. Follow-up data was obtained by consulting the medical records and the referring veterinarian. Disease-specific overall survival (OS) was calculated from the time of diagnosis to the date of the animal's death/euthanasia due to the neoplastic disease. Animals that died or were euthanized for unrelated causes and those that were lost to follow-up were censored, respectively, at the time of death and at the data of their last clinical examination. Euthanasia was performed only in terminal stage of the disease. Necropsy examination was performed upon the owner consent.

Blood samples (obtained by standard venipuncture) were collected from both cases and controls for genomic DNA extraction using High Pure PCR Template preparation kit (Roche). The DNA quality was evaluated by measuring the optical density and the quantity was assessed employing the NanoDrop 1000 Spectrophotometer. SNP genotyping was performed using MassARRAY iPLEX Gold Technology at the *Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência.*

Three canine *CDH1* (chromosome 5) synonymous SNP were studied (Figure 1). The rs850805755 corresponds to a change G-> A: GT**C**/GT**T** (Valine). SNP rs852280880 is responsible for the change C->T: AC**G**/AC**A** (Threonine). SNP rs852639930 corresponds to the change C->T: AA**G**/AA**A** (Lysine).

Statistical analysis of data was performed using the computer software SPSS for Windows (version 18). Chi-square analysis and multivariate logistic binary regression analysis using backward stepwise (Wald) were used to investigate the significance of the relationship between *CDH1*1 SNPs and the clinicopathological variables. A 5% level was considered to define statistical significance. Linkage disequilibrium (LD) analysis was performed using Haploview 4.2 software.

**Results**

Three hundred and sixty-seven bitches were enrolled in this study. The mean age of dogs with mammary tumors was 10.2 years old (7-18 years old) and in the control group was 9.5 years old (7-18 years old). In this series, 65.5% of the cases exhibited multiple mammary tumors. Seventy-three bitches (35.4%) had only benign tumors and 133 (64.6%) presented at least one malignant tumor. In this series, the mean tumor size for benign tumors was 2.1 cm ± 1.9 cm (range 0.3 to 8.5 cm diameter) and for malignant tumors 3.7 cm ± 3.1 cm (range 0.3 to 16.5 cm diameter). According to the Nottingham histological grading method 36 (34.0%), 47 (44.0%) and 23 (21.7%) out of 106 carcinomas, were classified as

grade I, II and III carcinomas, respectively. Vascular invasion and lymph nodes metastases were identified in 16.9% and 26.7% of the cases, respectively. Two-year follow-up data was available for 116 dogs with malignant mammary tumors, 31 of which (26.7%) died due to progression of the neoplastic disease. At the end of the follow-up period, 57 animals (49.1%) were alive. Animals lost to follow-up (n=18; 15.5%) and animals that died from other causes (n=10; 8.6%) were censored.

Genotype distributions were in concordance with the Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) analysis revealed that SNPs rs850805755 and rs852280880 were in complete LD ($r^2$=1), demonstrating that alleles at these loci were non-randomly associated (Figure 2).

Twenty-nine animals (18.0%) from the control population and 21 (10.2%) of the animals with mammary tumor were carriers of the variant allele for SNPs rs850805755 and rs852280880. For SNP rs852639930, 18 animals (11.2%) from the control group and 12 (5.9%) from the group with mammary tumor were carriers of the variant allele. Variant allele carriers for both SNPs rs850805755 and rs852280880 were nearly 2 fold less susceptible to develop mammary tumors than wild type animals (p= 0.03; OR=1.9; CI= 1.06-3.54), while SNP rs852639930 did not influence significantly mammary tumor risk (Table 1).

Table 2 display the results regarding the association between SNPs considered and several clinicopathological parameters: tumor number and size, histological classification, mode of growth, NHG grade, NHG grading parameters (tubule formation, nuclear pleomorphism and mitotic index), vet-NPI, vascular invasion and lymph node metastases.

A correlation between the animals' age at the time of the tumor diagnosis and SNPs rs850805755 and rs852280880 was found. Indeed, carriers of the genetic variant tended to develop mammary tumors later in life, compared with the wildtype genotype (p= 0.043) (Figure 3).

When evaluating other clinicopathological variables, we found that carriers of the variant allele for SNPs rs850805755 and rs852280880 presented smaller malignant tumors (≤ 3cm) than wildtype animals (p= 0.038). An association was also observed between rs850805755 and rs852280880 and the histological grade of mammary carcinomas (p=0.025): only 7.1% of grade II/III carcinomas were found in variant allele carriers subgroup. These SNPs were also associated with nuclear pleomorphism (p= 0.041). In fact, none of the variant allele carriers exhibited carcinomas scored 3 for nuclear pleomorphism, while 32.3% of the wildtype animals did. Furthermore, SNPs rs850805755 and rs852280880

were also related to the vet-NPI, with 84.6% of the variant allele carries showing vet-NPI≤ 4 (p=0.035).

Regarding the SNP rs852639930, variant allele carriers presented a predisposition to develop smaller tumors (≤3cm), especially when considering only the subgroup of malignant ones (p=0.004 and p= 0.003, respectively). This SNP was also related to the mode of growth of the tumoral lesion, with variant allele carriers exhibiting mainly an expansive growth pattern (p= 0.012).

No significant associations could be established between any of the studied SNPs and the number of tumors, histological classification, tubule formation, mitotic index, vascular invasion and lymph node metastasis. None of the SNPs was related to disease-specific overall survival. In the final step of the multivariate logistic regression analysis, and after exclusion of non-significant variables, vet-NPI emerged as an independent variable associated with the SNPs rs850805755 and rs852280880, while the variable mode of growth pattern was independently associated with the SNP rs852639930.

**Discussion**

As a major cell adhesion molecule, E-cadherin plays a pivotal role in formation and differentiation of epithelial tissues. E-cadherin regulates several cellular processes (including cell shape, polarity, intercellular cohesion and motility), thus assuring the maintenance of structural and functional integrity of epithelia [1,26]. Over the last decade, various studies have underlined the role of human *CDH1* genetic variation in the risk, clinicopathological findings, progression and biological behavior of breast cancer [27-30]. However, and although there are some recognized genetic variations in canine *CDH1* gene, their influence in CMT development and progression is largely unknown. In this vein, this study aimed to evaluate the influence of canine *CDH1* genetic variations in the risk, clinicopathological features and prognosis of CMT.

Our data suggest that *CDH1* SNP rs850805755 and rs852280880 could play a protective effect against the development of CMT, with variant allele carriers presenting a nearly twofold decreased risk of developing mammary tumors when compared to wildtype dogs. Besides, SNPs rs850805755 and rs852280880 are also significantly associated with the age of the animal at the time of the tumor's diagnosis, with variant allele carriers developing mammary tumors later in life than wildtype dogs. Taken together, our findings suggest a likely dual protective role of SNPs rs850805755 and rs852280880 against the development of CMT, being associated with a low risk and a later onset of mammary tumor

development. Considering the role E-cadherin plays in the architectural organization of epithelial tissues, it can be hypothesized that SNPs rs850805755 and rs852280880 codify a form of E-cadherin that may be more effective in the maintenance of mammary gland structural integrity during physiological growth, differentiation and regression cycles, an important feature in guaranteeing its resistance to carcinogenic events. Indeed, recent research identified loss of cell polarity and collapse of luminal arrangements (processes associated to E-cadherin down-regulation) as early events in carcinogenesis [31], reinforcing the role of this cell adhesion molecule in neoplastic transformation. Besides, it is also conceivable that the structure of the E-cadherin molecule codified by these genetic variants can be more resistant to spontaneous or carcinogen-induced mutations.

Our results also point for a possible beneficial effect of SNPs rs850805755 and rs852280880 within the group of animals with mammary tumors, as these SNPs are related to clinicopathological features classically associated to a good prognosis, such as small size carcinomas (≤ 3cm), low histological grade and low nuclear pleomorphism [32-37]. Similarly, SNP rs852639930 seems to display a favorable role in the group of animals bearing mammary tumors, being associated to the development of small size (≤ 3cm) tumors, with a non-infiltrative, non-invasive growth pattern. As a cell adhesion molecule, E-cadherin favors intercellular adherence, contributing to maintain the tissue's architectural arrangements and preventing cell detachment and migration. E-cadherin is, in fact, considered an important invasion suppressor. Changes in E-cadherin expression and/or function have been implicated in mammary tumor progression and spread. E-cadherin takes part in the epithelial-mesenchymal transition (EMT), a phenotype modulation process characterized by loss of epithelial traits and acquisition of mesenchymal-like features. One of the hallmarks of EMT is a progressive down-regulation of cell-cell adhesion epithelial molecules (such as E-cadherin) and *de novo* expression of mesenchymal markers, driving to remarkable phenotypic changes. This program constitutes an adaptive advantage for neoplastic cells, disrupting intercellular contact and increasing their ability to invade neighbouring tissues [38-40]. Indeed, according to previous investigations in CMT, E-cadherin low-expression carcinomas are usually moderate/high grade tumors, characterized by disruption of tubular arrangements and high nuclear pleomorphism, with an infiltrative/invasive growth pattern and an unfavorable prognosis, reflecting loss of cell adhesive properties [5,12,14,41]. Our data, demonstrating an association between SNPs rs850805755, rs852280880 and rs852639930 in canine *CDH1*gene and clinicopathological attributes related to good prognosis, suggest that these genetic variants may be associated with an increase in E-cadherin cohesive

properties. It is reasonable to assume that, through changes in the transcription/translation process, these genetic variants may codify more efficient forms of the molecule, thus resulting in stronger intercellular cohesion, or may favor a stronger sustained linkage between E-cadherin and the other components of the complex cadherin-catenin-cytoplasmic actin, contributing to a highly resistant cell-to-cell adhesion system.

In this study, the frequency of the assessed *CDH1* SNPs is low, both in the control population and in the group of animals with mammary tumors. Curiously, these genetic variants are associated with a decreased risk of the disease and with clinicopathological parameters related to good prognosis. In general, it is expected that due to a natural selection effect the protective genetic variant becomes predominant over the time in a population [42,43]. In this vein, it is conceivable that these SNPs may constitute recent events, which have not yet reach equilibrium among descendants. On the other hand, it cannot be excluded that some of the animals from the control group had subclinical internal/visceral tumors. This hypothesis seems unlike, and the number of animals affected would represent a minority, because the possibility of internal neoplasia without systemic illness is always reduced and the most frequent tumors in canine species, such as mammary and skin tumors, were not reported by the clinician neither detected by the owner[44,45]. Although most of the research performed to date focus on disease-causing genetic variants and on genetic profiles related to a guarded prognosis, the recognition of the full spectrum of susceptibility genotypes offers a broader understanding of the complex pathways that drive to the neoplastic transformation. In fact, several recent reports emphasize the importance of cancer-preventive genetic variants in guiding the clinical decision process and as a valuable therapeutic target [46-48].

Although being synonymous, these SNPs can be functionally relevant, interfering with mRNA splicing, affecting mRNA structure, stability or interaction with specific ligands, or modulating translation kinetics, thus resulting in changes in the structure, stability, function and/or expression levels of the protein [49,50].

A possible source of pitfall in this study may be related to the acquisition of follow-up data from different clinicians. For the statistical analysis we choose the “disease-specific overall survival” as the primary endpoint, instead of the “overall survival” (time of death irrespective of the cause). We believe that the latter could introduce an unwanted bias in the study, since we confirmed that a consistent number of dogs from the original cohort died from causes not related to the mammary neoplasia (such as metabolic disorders, cardiac failure, trauma and neurodegenerative diseases). In this study, 84% of the animals whose death was related to progression of the mammary disease had evidences of vascular invasion

and/or lymph node metastasis. We could not confirm metastatic spread of the neoplastic disease in the remaining 16% of cases (n=5) since the owners did not allow necropsy examination. Nevertheless, those animals were clinically monitored during the follow-up period, presented anorexia, progressive weight loss and respiratory distress (similar to that observed in dogs with confirmed metastatic spread of the disease), and other potential causes of illness were excluded by the clinicians.

Taken together, our data demonstrate an association between canine *CDH1* SNPs rs850805755, rs852280880 and rs852639930 and low risk and less aggressive clinicopathological features of CMT. The identification of genetic profiles related to the susceptibility, progression and prognosis of mammary neoplasia is of paramount importance in a clinical context. Recognizing high-risk genetic profiles could allow the establishment of preventive and surveillance protocols, as well as the implementation of individualized therapeutic measures appropriated for a specific patient at a defined time, which constitutes the basis of personalized cancer medicine [51-54].Further research, considering a higher number of bitches and focusing on other molecules that participate in the complex axis cadherin-catenin-cytoplasmic actin, as well as in the EMT process, is required to confirm and to expand the present data.

**Conflict of interest statement**

The authors declare no conflicts of interest.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## References


1. Takeichi M. Cadherins: A Molecular Family Important in Selective Cell-Cell Adhesion. *Annual Review of Biochemistry.* 1990;59(1):237-252.
2. Moll R, Mitze M, Frixen UH, Birchmeier W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *The American journal of pathology.* 1993;143(6):1731-1742.
3. Andrews JL, Kim AC, Hens JR. The role and function of cadherins in the mammary gland. *Breast cancer research : BCR.* 2012;14(1):203-203.
4. Brunetti B, Sarli G, Preziosi R, Leprotti S, Benazzi C. E-cadherin expression in canine mammary carcinomas with regional lymph node metastases. *Journal of veterinary medicine A, Physiology, pathology, clinical medicine.* 2003;50(10):496-500.
5. Sarli G, Preziosi R, De Tolla L, Brunetti B, Benazzi C. E-cadherin immunoreactivity in canine mammary tumors. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.* 2004;16(6):542-547.
6. Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. *Molecular cancer research : MCR.* 2010;8(5):629-642.
7. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. *Cellular and molecular life sciences : CMLS.* 2008;65(23):3756-3788.
8. Liu T, Zhang X, Shang M, et al. Dysregulated expression of Slug, vimentin, and E-cadherin correlates with poor clinical outcome in patients with basal-like breast cancer. *Journal of surgical oncology.* 2013;107(2):188-194.
9. Adamo B, Ricciardi GRR, Ieni A, et al. The prognostic significance of combined androgen receptor, E-Cadherin, Ki67 and CK5/6 expression in patients with triple negative breast cancer. *Oncotarget.* 2017;8(44):76974-76986.
10. Kovac B, Makela TP, Vallenius T. Increased alpha-actinin-1 destabilizes E-cadherin-based adhesions and associates with poor prognosis in basal-like breast cancer. *PloS one.* 2018;13(5):e0196986.
11. Luo CW, Wu CC, Chang SJ, et al. CHD4-mediated loss of E-cadherin determines metastatic ability in triple-negative breast cancer cells. *Experimental cell research.* 2018;363(1):65-72.

12. Matos AJ, Lopes C, Carvalheira J, Santos M, Rutteman GR, Gartner F. E-cadherin expression in canine malignant mammary tumours: relationship to other clinico-pathological variables. *Journal of comparative pathology.* 2006;134(2-3):182-189.
13. Matos AJ, Lopes CC, Faustino AM, Carvalheira JG, Rutteman GR, Gartner Mde F. E-cadherin, beta-catenin, invasion and lymph node metastases in canine malignant mammary tumours. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica.* 2007;115(4):327-334.
14. Gama A, Paredes J, Gartner F, Alves A, Schmitt F. Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. *Veterinary journal (London, England : 1997).* 2008;177(1):45-53.
15. Yoshida K, Yoshida S, Choisunirachon N, et al. The relationship between clinicopathological features and expression of epithelial and mesenchymal markers in spontaneous canine mammary gland tumors. *The Journal of veterinary medical science.* 2014;76(10):1321-1327.
16. Asproni P, Ressel L, Millanta F, Vannozzi I, Poli A. Co-localization of PTEN and E-cadherin in canine mammary hyperplasias and benign and malignant mammary tumors. *Research in veterinary science.* 2015;103:113-118.
17. Boggs RM, Wright ZM, Stickney MJ, Porter WW, Murphy KE. MicroRNA expression in canine mammary cancer. *Mammalian genome : official journal of the International Mammalian Genome Society.* 2008;19(7-8):561-569.
18. Klopfleisch R, von Euler H, Sarli G, Pinho SS, Gartner F, Gruber AD. Molecular carcinogenesis of canine mammary tumors: news from an old disease. *Veterinary pathology.* 2011;48(1):98-116.
19. von Deetzen M-C, Schmeck BT, Gruber AD, Klopfleisch R. Malignancy Associated MicroRNA Expression Changes in Canine Mammary Cancer of Different Malignancies. *ISRN Veterinary Science.* 2014;2014:5.
20. Bulkowska M, Rybicka A, Senses KM, et al. MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. *BMC cancer.* 2017;17(1):728.
21. Misdorp W, Else RW, Hellmen E, TP. L. *World Health Organization international histological classification of tumors of domestic animals* Vol VII. 2nd ed: Washington, D.C. : Armed Forces Institute of Pathology; 1999.

22. Peña L, Gama A, Goldschmidt MH, et al. Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. *Veterinary pathology.* 2014;51(1):127-145.
23. Canadas A, Santos M, Nogueira A, et al. Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.* 2018;30(5):733-738.
24. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology.* 1991;19(5):403-410.
25. Santos M, Correia-Gomes C, Marcos R, et al. Value of the Nottingham Histological Grading Parameters and Nottingham Prognostic Index in Canine Mammary Carcinoma. *Anticancer research.* 2015;35(7):4219-4227.
26. Ladoux B, Mège R-M. Mechanobiology of collective cell behaviours. *Nature Reviews Molecular Cell Biology.* 2017;18:743.
27. Tipirisetti NR, Govatati S, Govatati S, et al. Association of E-cadherin single-nucleotide polymorphisms with the increased risk of breast cancer: a study in South Indian women. *Genetic testing and molecular biomarkers.* 2013;17(6):494-500.
28. Ma YY, Wu WQ, Liu ZC, et al. The CDH1 -160C/A polymorphism is associated with breast cancer: evidence from a meta-analysis. *World J Surg Oncol.* 2016;14(1):169.
29. Memni H, Macherki Y, Klayech Z, et al. E-cadherin genetic variants predict survival outcome in breast cancer patients. *Journal of translational medicine.* 2016;14(1):320.
30. Zarei F, Menbari MN, Ghaderi B, Abdi M, Vahabzadeh Z. Higher risk of progressing breast cancer in Kurdish population associated to CDH1 -160 C/A polymorphism. *EXCLI journal.* 2017;16:1198-1205.
31. Halaoui R, Rejon C, Chatterjee SJ, et al. Progressive polarity loss and luminal collapse disrupt tissue organization in carcinoma. *Genes & development.* 2017;31(15):1573-1587.

32. Hellmen E, Bergstrom R, Holmberg L, Spangberg IB, Hansson K, Lindgren A. Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases. *Veterinary pathology.* 1993;30(1):20-27.
33. Perez Alenza MD, Pena L, Nieto AI, Castano M. Clinical and pathological prognostic factors in canine mammary tumors. *Annali dell'Istituto superiore di sanita.* 1997;33(4):581-585.
34. Chang SC, Chang CC, Chang TJ, Wong ML. Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002). *Journal of the American Veterinary Medical Association.* 2005;227(10):1625-1629.
35. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, Dessiris A. Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *Journal of comparative pathology.* 2005;133(4):246-252.
36. Sorenmo KU, Kristiansen VM, Cofone MA, et al. Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. *Vet Comp Oncol.* 2009;7(3):162-172.
37. Santos M, Correia-Gomes C, Santos A, et al. Nuclear pleomorphism: role in grading and prognosis of canine mammary carcinomas. *Veterinary journal (London, England : 1997).* 2014;200(3):426-433.
38. Ashaie MA, Chowdhury EH. Cadherins: The Superfamily Critically Involved in Breast Cancer. *Current pharmaceutical design.* 2016;22(5):616-638.
39. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology. *Pathology.* 2007;39(3):305-318.
40. Das V, Bhattacharya S, Chikkaputtaiah C, Hazra S, Pal M. The basics of epithelial-mesenchymal transition (EMT): A study from a structure, dynamics, and functional perspective. *Journal of cellular physiology.* 2019.
41. Restucci B, Papparella S, De Vico G, Maiolino P. E cadherin expression in normal and neoplastic canine mammary gland. *Journal of comparative pathology.* 1997;116(2):191-202.
42. Quintana-Murci L. Understanding rare and common diseases in the context of human evolution. *Genome biology.* 2016;17(1):225-225.
43. Vicens A, Posada D. Selective Pressures on Human Cancer Genes along the Evolution of Mammals. *Genes.* 2018;9(12):582.

44. Sorenmo KU, Worley DR, Goldschmidt MH. Tumors of the Mammary Gland. In: Withrow SJ, Vail DM, Page RL, eds. *Withrow and MacEwen's Small Animal Clinical Oncology (Fifth Edition).* Saint Louis: W.B. Saunders; 2013:538-556.
45. Hauck ML. Tumors of the Skin and Subcutaneous Tissues. In: Withrow SJ, Vail DM, Page RL, eds. *Withrow and MacEwen's Small Animal Clinical Oncology (Fifth Edition).* Saint Louis: W.B. Saunders; 2013:305-320.
46. Butler JM, Hall N, Narendran N, Yang YC, Paraoan L. Identification of candidate protective variants for common diseases and evaluation of their protective potential. *BMC genomics.* 2017;18(1):575.
47. Tan H. On the Protective Effects of Gene SNPs Against Human Cancer. *EBioMedicine.* 2018;33:4-5.
48. Schwartz MLB, Williams MS, Murray MF. Adding Protective Genetic Variants to Clinical Reporting of Genomic Screening Results: Restoring Balance. *Jama.* 2017;317(15):1527-1528.
49. Hunt R, Sauna ZE, Ambudkar SV, Gottesman MM, Kimchi-Sarfaty C. Silent (synonymous) SNPs: should we care about them? *Methods in molecular biology (Clifton, NJ).* 2009;578:23-39.
50. Fernandez-Calero T, Cabrera-Cabrera F, Ehrlich R, Marin M. Silent Polymorphisms: Can the tRNA Population Explain Changes in Protein Properties? *Life (Basel, Switzerland).* 2016;6(1).
51. Sabatier R, Goncalves A, Bertucci F. Personalized medicine: present and future of breast cancer management. *Critical reviews in oncology/hematology.* 2014;91(3):223-233.
52. Stover DG, Wagle N. Precision medicine in breast cancer: genes, genomes, and the future of genomically driven treatments. *Current oncology reports.* 2015;17(4):15.
53. Aboulkheyr Es H, Montazeri L, Aref AR, Vosough M, Baharvand H. Personalized Cancer Medicine: An Organoid Approach. *Trends in Biotechnology.* 2018;36(4):358-371.
54. Low SK, Zembutsu H, Nakamura Y. Breast cancer: The translation of big genomic data to cancer precision medicine. *Cancer science.* 2018;109(3):497-506.

## Figures

**Figure 1.** Schematic illustration of the selected SNP of canine *CDH1* gene. SNPs are named by their cluster ID numbers in the public SNP database.

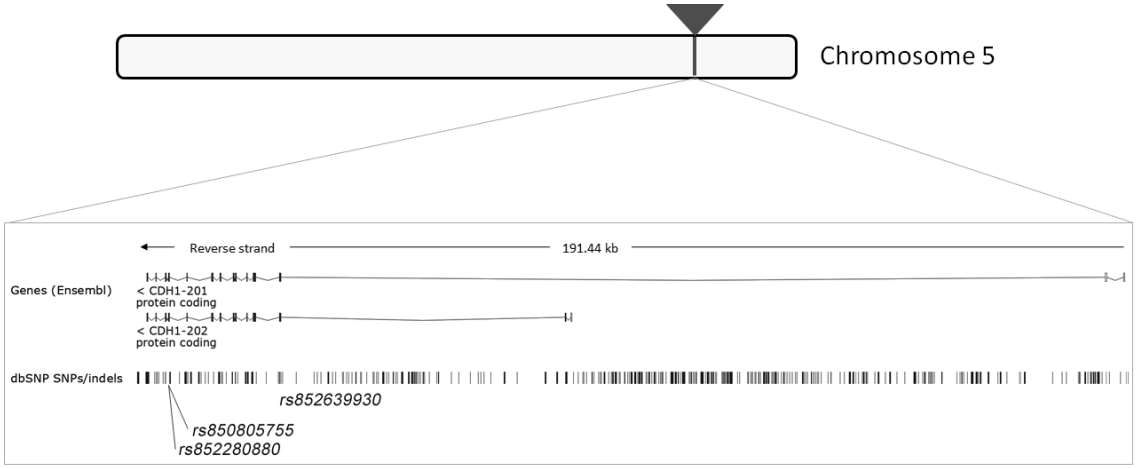



**Figure 2.** Linkage disequilibrium (LD) map of *CDH1*1 SNPs rs850805755 (5), rs852280880 (6) and rs852639930 (7), including controls and canine mammary tumor cases. The dark grey-to-white gradient reflects higher to lower LD values. Dark diamonds without a number indicate complete LD ($r^2$=1).

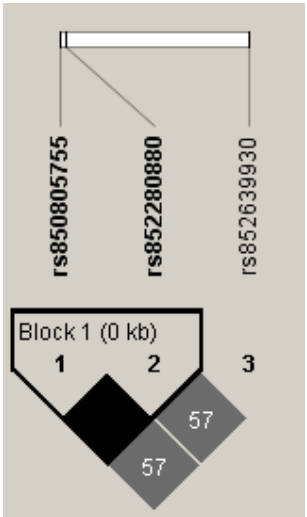
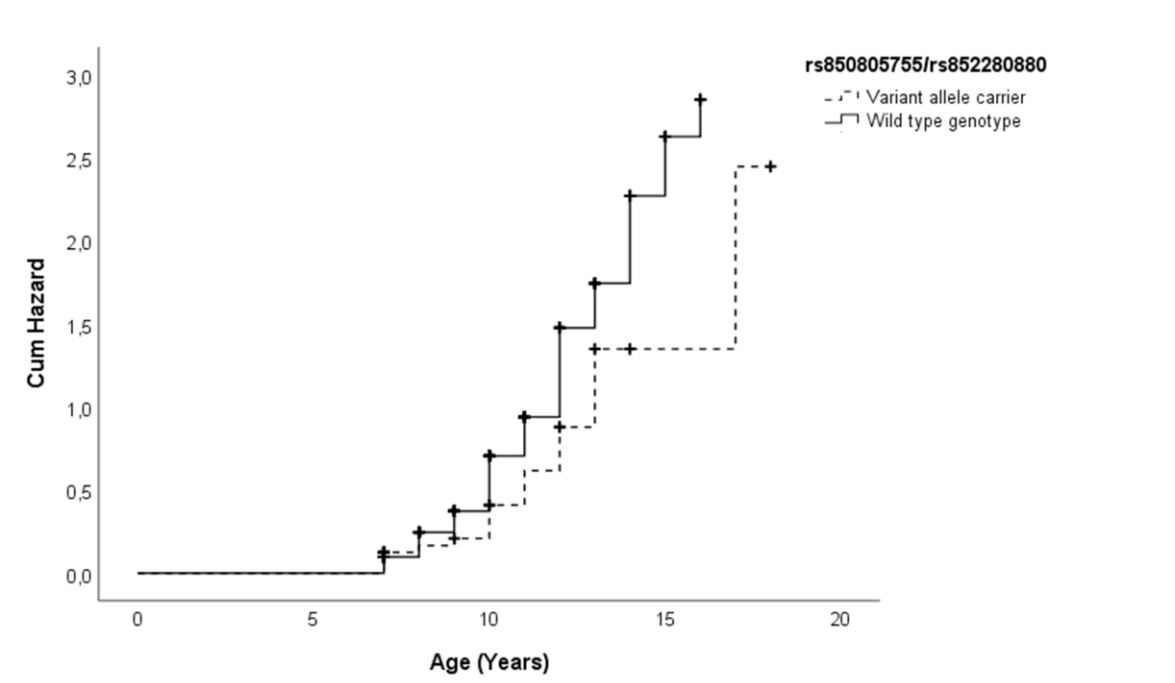


**Figure 3.** Association of *CDH1* SNP rs850805755, rs852280880 and the animal's age at the time of the tumor diagnosis (*p*=0.043).




## Tables

**Table 1.** Distribution of *CDH1* SNPs genotypes in the control and in the mammary tumor groups.

| **Single nucleotide polymorphism** | N (%) dogs Control (N=161) | Mammary Tumor (N=206) | ***p*** |
|---|---|---|---|
| ***rs850805755 / rs852280880*** | | | |
| GG / CC | 132 (82.0) | 185 (89.8) | |
| A carrier / T carrier | 29 (18.0) | 21 (10.2) | ***0.030*** |
| ***rs852639930*** | | | |
| CC | 143 (88.8) | 194 (94.2) | |
| T carrier | 18 (11.2) | 12 (5.9) | 0.063 |

**Table 2.** Association between canine *CDH1* genotypes and clinicopathological features of canine mammary tumors.

| ***Independent variables*** | **Presence of genetic variation (n= number of cases)** | | | | | |
|---|---|---|---|---|---|---|
| | rs850805755/ rs852280880 | | ***p*** | rs852639930 | | ***p*** |
| | **GG/CC (n/%)** | **A/T Allele (n/%)** | | **CC (n/%)** | **T Allele (n/%)** | |
| ***Number of tumors*** | | | | | | |
| Single | 63 (88.7) | 8 (11.3) | | 67 (94.4) | 4 (5.6) | |
| Multiple | 122 (90.4) | 13 (9.6) | NS | 127 (94.1) | 8 (5.9) | NS |
| ***Tumor size*** | | | | | | |
| ≤ 3cm | 99 (51.3) | 15 (7.8) | | 103 (90.4) | 11 (9.6) | |
| > 3cm | 75 (38.9) | 4 (2.1) | 0.063 | 79 (100.0) | 0 (0.0) | ***0.004*** |
| ***Tumor size (only malignant)*** | | | | | | |
| ≤ 3cm | 54 (84.4) | 10 (15.6) | | 56 (87.5) | 8 (12.5) | |
| > 3cm | 62 (95.4) | 3 (4.6) | ***0.038*** | 65 (100) | 0 (0.0) | ***0.003*** |
| ***Histological classification*** | | | | | | |
| *Benign* | 66 (90.4) | 7 (9.6) | | 69 (94.5) | 4 (5.5) | |
| *Malignant* | 119 (89.5) | 14 (10.5) | NS | 125 (94.0) | 8 (6.0) | NS |
| ***Mode of growth pattern*** | | | | | | |
| *Expansive* | 43 (84.3) | 8 (15.7) | | 44 (86.3) | 7 (13.7) | |
| *Infiltrative* | 42 (89.4) | 5 (10.6) | | 46 (97.9) | 1 (2.1) | |
| *Invasive* | 34 (97.1) | 1 (2.9) | NS | 35 (100.0) | 0 (0.0) | ***0.012*** |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***NHG parameters*** | | | | | | |
| ***Tubule formation*** | | | | | | |
| *Score 1* | 20 (80.0) | 5 (20.0) | | 23 (92.0) | 2 (8.0) | |
| *Score 2* | 42 (91.3) | 4 (8.7) | | 43 (93.5) | 3 (6.5) | |
| *Score 3* | 31 (88.6) | 4 (11.4) | NS | 32 (91.4) | 3 (8.6) | NS |
| ***Nuclear pleomorphism*** | | | | | | |
| *Score 1* | 9 (90.0) | 1 (10.0) | | 9 (90.0) | 1 (10.0) | |
| *Score 2* | 54 (81.8) | 12 (18.2) | | 59 (89.4) | 7 (10.6) | |
| *Score 3* | 30 (100.0) | 0 (0.0) | ***0.041*** | 30 (100.0) | 0 (0.0) | NS |
| ***Mitotic index*** | | | | | | |
| *Score 1* | 35 (81.4) | 8 (18.6) | | 39 (90.7) | 4 (9.3) | |
| *Score 2* | 26 (92.9) | 2 (7.1) | | 27 (96.4) | 1 (3.6) | |
| *Score 3* | 32 (91.4) | 3 (8.6) | NS | 32 (91.4) | 3 (8.6) | NS |
| ***NHG*** | | | | | | |
| *Grade I* | 28 (77.8) | 8 (22.2) | | 32 (89.9) | 4 (11.1) | |
| *Grade II* | 43 (91.5) | 4 (8.5) | | 44 (93.6) | 3 (6.4) | |
| *Grade III* | 22 (95.7) | 1 (4.3) | NS | 22 (95.7) | 1 (4.3) | NS |
| ***NHG groups*** | | | | | | |
| *Grade I* | 28 (77.8) | 8 (22.2) | | 32 (89.9) | 4 (11.1) | |
| *Grade II+III* | 65 (92.9) | 5 (7.1) | ***0.025*** | 66 (94.3) | 4 (5.7) | NS |
| ***Vet-NPI*** | | | | | | |
| *≤4.0* | 50 (82.0) | 11 (18.0) | | 54 (88.5) | 7 (11.5) | |
| *>4.0* | 43 (95.6) | 2 (4.4) | ***0.035*** | 44 (97.8) | 1 (2.2) | NS |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Vascular invasion*** | | | | | | |
| *No* | 95 (88.0) | 13 (12.0) | | 100 (92.6) | 8 (7.4) | |
| *Yes* | 21 (95.5) | 1 (4.5) | NS | 22 (100.0) | 0 (0.0) | NS |
| ***Lymph node metastases*** | | | | | | |
| *No* | 74 (87.1) | 11 (12.9) | | 79 (92.9) | 6 (7.1) | |
| *Yes* | 30 (96.8) | 1 (3.2) | NS | 31 (100.0) | 0 (0.0) | NS |

Legend: SNP: single nucleotide polymorphism; NHG: Nottingham grade; vet-NPI: veterinary adapted Nottingham prognostic index.

# CHAPTER 05

## Estrogen Receptors Genotypes and Canine Mammary Neoplasia

**Estrogen Receptors genotypes and canine mammary neoplasia**

Ana Canadas-Sousa[a⊥] (canadas.ana@gmail.com), Marta Santos[b] (mssantos@icbas.up.pt), Bárbara Leal[a] (baleal@icbas.up.pt), Rui Medeiros[d] (ruimedeiros@ipoporto.min-saude.pt), Patrícia Dias-Pereira[a] (pdiaspereira@yahoo.com.br)

[a]*Department of Pathology and Molecular Immunology, Instituto Ciências Biomédicas Abel Salazar, ICBAS – UPorto, University of Porto, Portugal.*

[b]*Department of Microscopy, Instituto Ciências Biomédicas Abel Salazar, ICBAS – UPorto, University of Porto, Portugal*

[d]*Molecular Oncology and Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), Portuguese Oncology Institute of Porto (IPO-Porto), Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal*

⊥Corresponding author:

Rua de Jorge Viterbo Ferreira, 228

4050-313 Porto, Portugal

Tel.: +351220428271

E-mail address: canadas.ana@gmail.com

**Abstract**

**Background:**

Estrogens are essential for the development and proper function of several hormone-dependent organs. There are, however, several lines of evidence associating estrogens with mammary carcinogenesis. A marked individual genetic variability concerning estrogens biosynthesis, metabolism and mechanism of action was recognized and associated with human breast cancer susceptibility, clinical features and progression. Although some genetic variations in canine *ESR1 gene* were reported, their influence in clinicopathological features and progression of canine mammary tumors has not been fully evaluated. This study aims to assess the influence of SNPs in *ESR1 gene* (rs397512133, rs397510462, rs851327560, rs397510612, rs852887655, rs852684753 and rs852398698) in canine mammary tumors characteristics and progression. A group of 155 non neutered bitches with mammary tumors was enrolled and submitted to follow-up for 24 months after surgery.

**Results:**

Genetic profiles associated with a later onset of mammary tumors and less aggressive clinicopathological features, namely smaller tumor size (≤ 3cm) with extensive tubular differentiation and low canine-adapted prognostic index (vet-NPI), were identified in this study.

**Conclusions:**

Our data suggest that the *ESR1* genetic profile may help on the decision regarding the selection of individual tailored preventive measures against canine mammary tumors development, such as early neutering.

**Keywords:** Canine mammary tumors, *ESR1* gene, SNP, genetic profile

**Background**

Estrogens are crucial for normal development and function of several organs and systems, namely the mammary gland. In women, estrogens play a pivotal role in the development of the mammary branching ductal-alveolar system in puberty, throughout the menstrual cycle and also during pregnancy (Sternlicht *et al.* 2006, Stingl 2011, Macias *et al.* 2012, Arendt *et al.* 2015, Hilton *et al.* 2018).

Due to their pro-proliferative and anti-apoptotic affects, estrogens have also been implicated in human breast cancer development and progression (Parl *et al.* 2009, Santen *et al.* 2009, Chen *et al.* 2013). Furthermore, some of the intermediate compounds derived from estrogens metabolism have a well-recognized genotoxic action (Cavalieri *et al.* 2000, Liehr 2000, Santen *et al.* 2009). Indeed, several conditions related to increased or prolonged exposure of the mammary tissue to estrogens, such as early menarche, late menopause, oral contraception or hormone replacement therapy, constitute well-known risk factors for human breast cancer (Dall *et al.* 2017).

There is a large body of evidence linking estrogens to mammary carcinogenesis, also in canine species. Most mammary tumors are reported in females and the few cases documented in males are related to estrogen-secretor testicular neoplasms (Saba *et al.* 2007, Kwon *et al.* 2017). Besides, canine contraceptive hormonal therapy has long been associated with an increased risk of mammary tumors (Rutteman 1992). On the other hand, the protective effect of ovariectomy against the development of mammary neoplasia has been advocated for decades (Schneider *et al.* 1969, Sorenmo *et al.* 2000, Kristiansen *et al.* 2016). Moreover, levels of serum steroid hormones were reported to be higher in dogs with mammary carcinomas than in normal ones (Queiroga *et al.* 2005, Illera *et al.* 2006).

Estrogens bind to estrogen receptors (ER) found in the normal canine mammary gland. Several studies reported changes in the expression pattern of ER in canine mammary gland in the course of neoplastic transformation and progression. An underexpression of ER has been documented in canine malignant mammary tumors, compared to benign neoplasms and to the normal mammary tissue (Rutteman *et al.* 1988, Nieto *et al.* 2000, de Las Mulas *et al.* 2005, Queiroga *et al.* 2011b). This feature reinforces the importance of estrogens in canine mammary carcinogenesis. Furthermore, the decreased ER expression has been related to larger tumor size and lymph node metastasis, suggesting that ER status can be regarded as a marker with predictive and prognostic value in canine mammary tumors (Nieto *et al.* 2000, de Las Mulas *et al.* 2005).

Over the last decades, a considerable individual genetic variability concerning estrogens biosynthesis, metabolism and mechanism of action was recognized in humans. This individual genetic background is considered a significant contributor to breast cancer susceptibility, allowing the identification of subpopulations of women with higher breast cancer risk (Martin *et al.* 2000, Bugano *et al.* 2008, Sun *et al.* 2015a, Evans *et al.* 2018). It has also been related to specific breast cancer clinical features, as well as to the clinical course of the disease (van 't Veer *et al.* 2002, Veronesi *et al.* 2005, Larsen *et al.* 2014). In canine species data regarding the genetic profile related to estrogens and mammary tumor risk is not consensual. Some *ESR1* genetic differences were described between breed dogs known to be at high and at low risk of mammary tumor development; furthermore, an association between *ESR1* variation and the susceptibility to mammary tumors was described in a cohort of English Springer Spaniels (Borge *et al.* 2011, Borge *et al.* 2013). However, in a recent investigation, our group could not confirm a

relationship between *ESR1* genetic profile and the risk of development of mammary tumors (Canadas *et al.* 2018a). On the other hand, genetic variations in canine *COMT* gene (which encodes catechol-O-methyltransferase, an enzyme involved in estrogens metabolism through inactivation of carcinogenic catechol estrogens) has not been proved to influence susceptibility to canine mammary tumors. Nevertheless, *COMT* genetic variation has been linked to the age of onset of canine mammary carcinomas, to the development of high grade mammary carcinomas, vascular invasion and recurrences (Dias Pereira *et al.* 2008, Dias Pereira *et al.* 2009a, Canadas *et al.* 2018b). To date, and to the best of the author's knowledge, the influence of *ESR1* genetic profile in clinicopathological features and progression of canine mammary tumors has not been fully assessed.

The aim of this study is to investigate the association between seven single nucleotide polymorphisms (SNPs) in canine *ESR1* gene (rs397512133, rs397510462, rs851327560, rs397510612, rs852887655, rs852684753 and rs852398698) and clinicopathological features of canine mammary tumors and the clinical outcome of the disease.

**Results**

One hundred and fifty five non neutered bitches were included in this study. The mean age of the whole population was 10.1 years-old (7-18 years-old): 9.8 years for dogs with benign tumors and 10.3 years for dogs with malignant tumors. Multiple tumours were diagnosed in 69.0% of the cases. Fifty-six (36.1%) animals had only benign tumors and 99 (63.9%) presented at least one malignant tumor. Based on the Nottingham histological grading method 25/77 (32.5%) carcinomas were graded I, 36/77 (46.8%) were graded II and 16/77 (20.8%) were graded III.

Vascular invasion and lymph node metastasis were observed in 14.6% (14/96) and in 23.5% (20/85) of cases, respectively. Two-year follow-up data was available for 88 dogs with malignant tumors. Of those, 44 (50%) were alive at the end of the follow-up period, while 21 (23.9%) died due to progression of the disease. Animals lost to follow-up (n=16; 18.2%) and animals that died from causes not related to the mammary neoplasia (n=7; 8%) were censored.

A significant association was found between the animal's age at the time of the tumor diagnosis and SNPs rs397512133, rs397510462, rs851327560 and rs397510612. In fact, carriers of the variant allele for these SNPs developed mammary tumors later than wildtype dogs (p= 0.014; p= 0.005; p= 0.007 and p= 0.008, respectively) (Figure2).

Table 2 presents results regarding the association between the studied SNPs and clinicopathological parameters, namely tumor number and size, histological classification (benign/malignant), mode of growth, NHG histological grade, NHG grading parameters (tubule formation, nuclear pleomorphism and mitotic index), NHG total score, vet-NPI, vascular invasion and lymph node metastases.

Carriers of variant allele for SNPs rs397512133, rs397510462, rs851327560 and rs397510612 developed smaller size carcinomas (≤ 3cm) than wildtype animals (p= 0.014; p= 0.020; p= 0.016; p= 0.020, respectively). The NHG grading parameters (tubule formation, nuclear pleomorphism and mitotic counts) were evaluated and a statistically significant relationship was observed between SNPs rs852887655, rs852684753 and rs852398698 and tubule formation (p= 0.039; p= 0.014; p= 0.026, respectively). The majority of carcinomas scored 3 for tubule formation were found in wildtype animals. In fact, only 7.1% (rs852887655), 22.2% (rs852684753) and 25.0% (rs852398698) of the carcinomas scored 3 for this

parameter corresponded to variant allele carriers. Furthermore, a statistically significant association was found between rs851327560 and the vet-NPI (p= 0.023). Most cases of vet-NPI>4 (80.6%) corresponded to wild type animals, while only 19.4% of those cases were observed in variant allele carriers.

No significant associations could be established between any of the SNPs considered and the number of tumors, histological classification (benign/malignant tumors) and pattern of tumor growth, NGH histological grade, nuclear pleomorphism, mitotic counts, vascular invasion and lymph node metastasis. Furthermore, none of the SNPs considered were related to OS.

**Discussion**

In this study genetic variations of the canine *ESR1* were associated with the development of less aggressive canine mammary tumors.

Our results demonstrated that carriers of the variant allele for SNPs rs397512133, rs397510462, rs851327560 and rs397510612 tended to develop mammary neoplasia later in life than wildtype dogs. This result finds parallel in data from a previous investigation of our group demonstrating that SNP in canine *COMT* gene were associated with the age of onset of mammary tumors (Dias Pereira *et al.* 2008). According to that study, variant allele carriers for SNP rs853046495 (also known as SNP *COMT* G482A) presented a threefold likelihood of developing mammary tumors after 9 years of age, when compared to wildtype animals. Furthermore, a significantly longer waiting time of onset of malignant disease was observed in variant allele carriers than in wildtype animals.

The presence of the variant allele for SNPs rs397512133, rs397510462, rs851327560 and rs397510612 was also associated with the development of small size malignant tumors (≤ 3cm). Tumor size has long been considered an important

prognostic factor in canine mammary neoplasia, with tumors larger than 3cm being associated with short survival times (Yamagami *et al.* 1996, Perez Alenza *et al.* 1997, Ferreira *et al.* 2009, Sorenmo *et al.* 2009).

Our data also demonstrated that variant allele genotypes for SNPs rs852887655, rs852684753 and rs852398698 were associated to carcinomas with a high percentage of tubular arrangements, a feature related to well differentiated and low-malignancy neoplasias, and usually associated with good prognosis (Fisher *et al.* 1987).

Despite the fact that none of the SNPs studied was related to NHG histological grade, carriers of the variant allele for rs851327560 were significantly associated with low vet-NPI (≤4). This recently described index combines well-recognized prognostic factors for canine mammary tumors, namely tumor size, NHG histological grade and vascular/lymph node invasion. Previous studies from our group demonstrated that this canine-adapted index is associated with disease free-interval and OS in bitches (Santos *et al.* 2015b, Canadas *et al.* 2019).

Taken together, our results allow the identification of a subgroup of dogs that tend to develop mammary tumors at older ages and with less aggressive clinicopathological features (small size tumors, with extensive tubular differentiation and low vet-NPI). Based on these findings, we hypothesize that carriers of the variant allele for *ESR1* SNPs (rs397512133, rs397510462, rs851327560, rs397510612, rs852887655, rs852684753 and rs852398698) may possess receptors less sensitive to estrogen binding, resulting in a mammary tissue less responsive to the hormone, thus being more protected from its carcinogenic action. In those dogs, a longer period of exposition to estrogens would be required to achieve carcinogenic levels, which explains the development of tumors in older age.

Despite the fact that SNPs assessed in this study are synonymous or intronic, and do not involve amino acid changes, they can induce alternative splicing, promote changes in RNA stability or structure, interfere with the speed and accuracy of transcription and/or translation and disturb protein folding (Hunt *et al.* 2009, Cooper 2010, Fernandez-Calero *et al.* 2016, Ramirez-Bello *et al.* 2017). These apparently slight features often explain the diversity of individual reaction to hormonal stimulus exhibited by different animals.

There are several epidemiological, biochemical and toxicological lines of evidence associating estrogens with mammary carcinogenesis (Yager *et al.* 1996, Cavalieri *et al.* 2000, Liehr 2000, Gail 2015). However, estrogens are also essential for the development and proper function of several hormone-dependent organs (Hughes *et al.* 2009, Swedenborg *et al.* 2009, Xiao *et al.* 2010). In fact, there are various detrimental effects related to neutering, such as urinary incontinence, musculoskeletal disorders and development of different types of neoplasia (Prymak *et al.* 1988, Ru *et al.* 1998, Ware *et al.* 1999, Veronesi *et al.* 2009, Villamil *et al.* 2009, Beauvais *et al.* 2012b, Hart *et al.* 2014, Arlt *et al.* 2017). Besides, several sources of bias were identified in previous studies that link neutering to a decreased risk of mammary neoplasia. A more recent revision of the data previously available was performed, demonstrating that the scientific support for this evidence is weak (Beauvais *et al.* 2012a). In this vein, a growing debate on whether and when spaying should be recommended has emerged. Several researchers have questioned the value of early spaying, taking into account the secondary effects of this procedure in the animal's health and quality of life in the medium and long term (Beauvais *et al.* 2012b, Torres de la Riva *et al.* 2013, Hart *et al.* 2014). It is important to identify subsets of animals with increased risk or more susceptible to the development of

aggressive mammary tumors that, in spite of the medium-long term secondary effects resulting from neutering, could benefit from that intervention. Similarly, recognition of the animals in which the deleterious consequences from neutering overlap their protective effect against the development of mammary neoplasia is of uttermost importance, avoiding unnecessary surgical interventions.

## Conclusions

Our data demonstrate that canine *ESR1* genetic profile may constitute a rational basis for the evaluation of the cost-benefit ratio related to early spaying, assisting in the selection of the animals that could benefit from neutering as an individualized preventive strategy against the development of canine mammary neoplasia.

## Methods

The study was conducted involving 155 non neutered bitches with histologically confirmed mammary tumors collected from the Veterinary Pathology Laboratory (ICBAS-University of Porto). All animals were treated with radical unilateral and/or partial mastectomy. Owners provided consent for surgery with curative intents as well as for the use of the material for research purposes. This protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences Abel Salazar, University of Porto (P151/2016).

After surgery, mammary specimens were immediately fixed in a 10% buffered formalin solution and routinely processed for histopathological examination. For each case, clinicopathological features including age at the time of the diagnosis, tumor number and size (corresponding to the largest diameter

measured by a pathologist (ACS) during trimming) were recorded. The histological diagnosis was established by consensus of 3 pathologists (ACS, MS and PDP) in a multi-head microscope, using the criteria of the World Health Organization for the classification of mammary tumors of dogs and cats (Misdorp *et al.* 1999). Each malignant tumor was assessed for the mode of growth and the presence of vascular invasion and regional lymph node metastasis, as previously described (Canadas *et al.* 2019). In the subgroup of animals with multiple malignant tumors, a reference lesion was assigned for the statistical study, according to criteria previously reported (Canadas *et al.* 2019). Histological grading was performed based on the Nottingham histological grading method -NHG (Elston *et al.* 1991). A veterinary adaptation of the human Nottingham Prognostic Index (vet-NPI) was also computed, as previously reported (Santos *et al.* 2015b).

Follow-up data was obtained by consulting the medical records and by contact with the referring veterinarian. Disease-specific overall survival (OS) was calculated from the time of diagnosis to the date of the animal's death/euthanasia due to the neoplastic disease. Animals that died or were euthanized for unrelated causes and those that were lost to follow-up were censored, respectively, at the time of death and at the data of their last clinical examination. Euthanasia was performed only in terminal stage of the disease. Necropsy examination was performed upon the owner consent.

Genomic DNA was extracted from peripheral blood samples (obtained by standard venipuncture) using High Pure PCR Template preparation kit (Roche). The DNA quality was evaluated by measuring the optical density and the quantity was assessed employing the NanoDrop 1000 Spectrophotometer. SNP genotyping was

performed using MassARRAY iPLEX Gold Technology at the *Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência.*

Seven canine *ESR1* (chromosome 1) SNPs were assessed as referred in Table 1 and Figure 1. Statistical analysis of data was performed using the computer software SPSS for Windows (version 25). Chi-square analysis (or Fisher's exact test, when appropriated) was used to evaluate the significance of the relationship between *ESR1* SNPs and the categorical variables. Cumulative risk curves were computed using Kaplan-Meier product-limit estimates method, with Log-rank (Mantel-Cox) tests being used to estimate the differences in risk of canine mammary tumors development according to each genetic profile. A 5% level was considered to define statistical significance.

## Abbreviations

COMT: Catechol-O-methyltransferase; ESR1: Estrogen receptor 1; Estrogen receptors (ER); NHG: Nottingham histologic grade; OS: Overall survival; SNP: Single nucleotide polymorphism; vet-NPI: veterinary adapted Nottingham Prognostic Index.

## Declarations

## Ethics approval and consent to participate

This protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences Abel Salazar, University of Porto (P151/2016).

Informed consent was obtained from all owners of animals that participate in this study. Euthanasia, required by the owners or proposed by the veterinary physician, was performed only in terminal stage of the disease using pentobarbital.

**Availability of data and materials**

The datasets used and analysed for this study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

**Funding**

No funding was used in this study.

**Authors' contributions**

ACS helped conceive the study and its design, collected the control and cases blood samples, carried out the DNA isolation, performed CMT analysis and drafted the manuscript. MS contributed to conceive the study and its design, performed CMT analysis and revised the manuscript. BL and RM contributed to the SNP and haplotype association analysis, statistical analysis and revised the manuscript. PDP conceived the study and its design, performed CMT analysis and drafted the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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**References**

1. Hilton N, Clarke L, Graham D: **Estrogen and progesterone signalling in the normal breast and its implications for cancer development**. *Molecular and cellular endocrinology* 2018, **466**:2-14.

2. Macias H, Hinck L: **Mammary Gland Development**. *Wiley interdisciplinary reviews Developmental biology* 2012, **1**(4):533-557.

3. Arendt LM, Kuperwasser C: **Form and function: how estrogen and progesterone regulate the mammary epithelial hierarchy**. *Journal of mammary gland biology and neoplasia* 2015, **20**(1-2):9-25.

4. Stingl J: **Estrogen and progesterone in normal mammary gland development and in cancer**. *Hormones & cancer* 2011, **2**(2):85-90.

5. Sternlicht MD, Kouros-Mehr H, Lu P, Werb Z: **Hormonal and local control of mammary branching morphogenesis**. *Differentiation; research in biological diversity* 2006, **74**(7):365-381.

6. Parl FF, Dawling S, Roodi N, Crooke PS: **Estrogen metabolism and breast cancer: a risk model**. *Annals of the New York Academy of Sciences* 2009, **1155**:68-75.

7. Santen R, Cavalieri E, Rogan E, Russo J, Guttenplan J, Ingle J, Yue W: **Estrogen mediation of breast tumor formation involves estrogen receptor-dependent, as well as independent, genotoxic effects**. *Annals of the New York Academy of Sciences* 2009, **1155**:132-140.

8. Chen F-P, Chien M-H, Chen H-Y, Huang T-S, Ng Y-T: **Effects of estradiol and progestogens on human breast cells: Regulation of sex steroid receptors**. *Taiwanese Journal of Obstetrics and Gynecology* 2013, **52**(3):365-373.

9. Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D: **Chapter 4: Estrogens as Endogenous Genotoxic Agents—DNA Adducts and Mutations**. *JNCI Monographs* 2000, **2000**(27):75-94.

10. Liehr JG: **Is estradiol a genotoxic mutagenic carcinogen?** *Endocrine reviews* 2000, **21**(1):40-54.

11. Dall GV, Britt KL: **Estrogen Effects on the Mammary Gland in Early and Late Life and Breast Cancer Risk**. *Frontiers in oncology* 2017, **7**:110.

12. Saba, Rogers, Newman, Mauldin, Vail: **Mammary Gland Tumors in Male Dogs**. *Journal of veterinary internal medicine* 2007, **21**:1056-1059.

13. Kwon S-C, Yoo D-Y, Ko M, Lee K-Y, Kwak H-H, Park I-C, Hwang I-K, Choi J-H, Chung J-Y: **Mammary gland tumors in a male Cocker Spaniel**. *Acta veterinaria Scandinavica* 2017, **59**:20.

14. Rutteman GR: **Contraceptive steroids and the mammary gland: is there a hazard?--Insights from animal studies**. *Breast cancer research and treatment* 1992, **23**(1-2):29-41.

15. Schneider R, Dorn CR, Taylor DO: **Factors influencing canine mammary cancer development and postsurgical survival**. *Journal of the National Cancer Institute* 1969, **43**(6):1249-1261.

16. Sorenmo KU, Shofer FS, Goldschmidt MH: **Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma**. *Journal of veterinary internal medicine* 2000, **14**(3):266-270.

17. Kristiansen VM, Pena L, Diez Cordova L, Illera JC, Skjerve E, Breen AM, Cofone MA, Langeland M, Teige J, Goldschmidt M *et al*: **Effect of Ovariohysterectomy at the Time of Tumor Removal in Dogs with Mammary Carcinomas: A Randomized Controlled Trial**. *Journal of veterinary internal medicine* 2016, **30**(1):230-241.

18. Queiroga FL, Perez-Alenza MD, Silvan G, Pena L, Lopes C, Illera JC: **Role of steroid hormones and prolactin in canine mammary cancer**. *The Journal of steroid biochemistry and molecular biology* 2005, **94**(1-3):181-187.

19. Illera JC, Perez-Alenza MD, Nieto A, Jimenez MA, Silvan G, Dunner S, Pena L: **Steroids and receptors in canine mammary cancer**. *Steroids* 2006, **71**(7):541-548.

20. Rutteman GR, Misdorp W, Blankenstein MA, van den Brom WE: **Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: different receptor profile in non-malignant and malignant states**. *British journal of cancer* 1988, **58**(5):594-599.

21. Nieto A, Pena L, Perez-Alenza MD, Sanchez MA, Flores JM, Castano M: **Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance**. *Veterinary pathology* 2000, **37**(3):239-247.

22. de Las Mulas JM, Millan Y, Dios R: **A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog**. *Veterinary pathology* 2005, **42**(2):200-212.

23. Queiroga FL, Raposo T, Carvalho MI, Prada J, Pires I: **Canine mammary tumours as a model to study human breast cancer: most recent findings**. *In vivo (Athens, Greece)* 2011, **25**(3):455-465.

24. Martin A-M, Weber BL: **Genetic and Hormonal Risk Factors in Breast Cancer**. *JNCI: Journal of the National Cancer Institute* 2000, **92**(14):1126-1135.

25. Bugano DD, Conforti-Froes N, Yamaguchi NH, Baracat EC: **Genetic polymorphisms, the metabolism of estrogens and breast cancer: a review**. *European journal of gynaecological oncology* 2008, **29**(4):313-320.

26. Evans DG, Howell SJ, Howell A: **Personalized prevention in high risk individuals: Managing hormones and beyond**. *Breast (Edinburgh, Scotland)* 2018, **39**:139-147.

27. Sun MY, Du HY, Zhu AN, Liang HY, de Garibay GR, Li FX, Li M, Yang XX: **Genetic polymorphisms in estrogen-related genes and the risk of breast cancer among Han Chinese women**. *International journal of molecular sciences* 2015, **16**(2):4121-4135.

28. Larsen MJ, Thomassen M, Gerdes A-M, Kruse TA: **Hereditary Breast Cancer: Clinical, Pathological and Molecular Characteristics**. *Breast Cancer : Basic and Clinical Research* 2014, **8**:145-155.

29. Veronesi A, Giacomi Cd, Magri MD, Lombardi D, Zanetti M, Scuderi C, Dolcetti R, Viel A, Crivellari D, Bidoli E *et al*: **Familial breast cancer: characteristics and outcome of BRCA 1–2 positive and negative cases**. *BMC cancer* 2005, **5**:70-70.

30. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT *et al*: **Gene expression profiling predicts clinical outcome of breast cancer**. *Nature* 2002, **415**:530.

31. Borge KS, Borresen-Dale AL, Lingaas F: **Identification of genetic variation in 11 candidate genes of canine mammary tumour**. *Vet Comp Oncol* 2011, **9**(4):241-250.

32. Borge KS, Melin M, Rivera P, Thoresen SI, Webster MT, von Euler H, Lindblad-Toh K, Lingaas F: **The ESR1 gene is associated with risk for canine mammary tumours**. *BMC veterinary research* 2013, **9**:69-69.

33. Canadas A, Santos M, Nogueira A, Assis J, Gomes M, Lemos C, Medeiros R, Dias-Pereira P: **Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes**. *Journal of veterinary diagnostic*

*investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2018, **30**(5):733-738.

34. Canadas A, Santos M, Pinto R, Medeiros R, Dias-Pereira P: **Catechol-o-methyltransferase genotypes are associated with progression and biological behaviour of canine mammary tumours**. *Vet Comp Oncol* 2018.

35. Dias Pereira P, Lopes CC, Matos AJ, Pinto D, Gartner F, Lopes C, Medeiros R: **Influence of catechol-O-methyltransferase (COMT) genotypes on the prognosis of canine mammary tumors**. *Veterinary pathology* 2009, **46**(6):1270-1274.

36. Dias Pereira P, Lopes CC, Matos AJ, Pinto D, Gartner F, Lopes C, Medeiros R: **Estrogens metabolism associated with polymorphisms: influence of COMT G482a genotype on age at onset of canine mammary tumors**. *Veterinary pathology* 2008, **45**(2):124-130.

37. Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, Mongil CM, Grondahl AM, Teige J, Goldschmidt MH: **Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence**. *Vet Comp Oncol* 2009, **7**(3):162-172.

38. Yamagami T, Kobayashi T, Takahashi K, Sugiyama M: **Prognosis for canine malignant mammary tumors based on TNM and histologic classification**. *The Journal of veterinary medical science* 1996, **58**(11):1079-1083.

39. Perez Alenza MD, Pena L, Nieto AI, Castano M: **Clinical and pathological prognostic factors in canine mammary tumors**. *Annali dell'Istituto superiore di sanita* 1997, **33**(4):581-585.

40. Ferreira E, Bertagnolli AC, Cavalcanti MF, Schmitt FC, Cassali GD: **The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours**. *Vet Comp Oncol* 2009, **7**(4):230-235.

41. Fisher ER, Sass R, Fisher B: **Pathologic findings from the national surgical adjuvant breast project. Correlations with concordant and discordant estrogen and progesterone receptors**. *Cancer* 1987, **59**(9):1554-1559.

42. Santos M, Correia-Gomes C, Marcos R, Santos A, De Matos A, Lopes C, Dias-Pereira P: **Value of the Nottingham Histological Grading Parameters and Nottingham Prognostic Index in Canine Mammary Carcinoma**. *Anticancer research* 2015, **35**(7):4219-4227.

43. Canadas A, Franca M, Pereira C, Vilaca R, Vilhena H, Tinoco F, Silva MJ, Ribeiro J, Medeiros R, Oliveira P *et al*: **Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study**. *Veterinary pathology* 2018:300985818806968.

44. Cooper DN: **Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes**. *Human genomics* 2010, **4**(5):284-288.

45. Ramirez-Bello J, Jimenez-Morales M: **[Functional implications of single nucleotide polymorphisms (SNPs) in protein-coding and non-coding RNA genes in multifactorial diseases]**. *Gaceta medica de Mexico* 2017, **153**(2):238-250.

46. Hunt R, Sauna ZE, Ambudkar SV, Gottesman MM, Kimchi-Sarfaty C: **Silent (synonymous) SNPs: should we care about them?** *Methods in molecular biology (Clifton, NJ)* 2009, **578**:23-39.

47. Fernandez-Calero T, Cabrera-Cabrera F, Ehrlich R, Marin M: **Silent Polymorphisms: Can the tRNA Population Explain Changes in Protein Properties?** *Life (Basel, Switzerland)* 2016, **6**(1).

48. Yager JD, Liehr JG: **Molecular mechanisms of estrogen carcinogenesis**. *Annual review of pharmacology and toxicology* 1996, **36**:203-232.

49. Gail MH: **Twenty-five years of breast cancer risk models and their applications**. *Journal of the National Cancer Institute* 2015, **107**(5).

50. Hughes ZA, Liu F, Marquis K, Muniz L, Pangalos MN, Ring RH, Whiteside GT, Brandon NJ: **Estrogen receptor neurobiology and its potential for translation into broad spectrum therapeutics for CNS disorders**. *Current molecular pharmacology* 2009, **2**(3):215-236.

51. Xiao J, Wang NL, Sun B, Cai GP: **Estrogen receptor mediates the effects of pseudoprotodiocsin on adipogenesis in 3T3-L1 cells**. *American journal of physiology Cell physiology* 2010, **299**(1):C128-138.

52. Swedenborg E, Power KA, Cai W, Pongratz I, Ruegg J: **Regulation of estrogen receptor beta activity and implications in health and disease**. *Cellular and molecular life sciences : CMLS* 2009, **66**(24):3873-3894.

53. Arlt S, Wehrend A, Reichler IM: **[Neutering of female dogs - old and new insights into Pros and Cons]**. *Tierarztliche Praxis Ausgabe K, Kleintiere/Heimtiere* 2017, **45**(4):253-263.

54. Hart BL, Hart LA, Thigpen AP, Willits NH: **Long-term health effects of neutering dogs: comparison of Labrador Retrievers with Golden Retrievers**. *PloS one* 2014, **9**(7):e102241.

55. Prymak C, McKee LJ, Goldschmidt MH, Glickman LT: **Epidemiologic, clinical, pathologic, and prognostic characteristics of splenic hemangiosarcoma and splenic hematoma in dogs: 217 cases (1985)**. *Journal of the American Veterinary Medical Association* 1988, **193**(6):706-712.

56. Ru G, Terracini B, Glickman LT: **Host related risk factors for canine osteosarcoma**. *Veterinary journal (London, England : 1997)* 1998, **156**(1):31-39.

57. Veronesi MC, Rota A, Battocchio M, Faustini M, Mollo A: **Spaying-related urinary incontinence and oestrogen therapy in the bitch**. *Acta veterinaria Hungarica* 2009, **57**(1):171-182.

58. Villamil JA, Henry CJ, Hahn AW, Bryan JN, Tyler JW, Caldwell CW: **Hormonal and sex impact on the epidemiology of canine lymphoma**. *Journal of cancer epidemiology* 2009, **2009**:591753.

59. Ware WA, Hopper DL: **Cardiac tumors in dogs: 1982-1995**. *Journal of veterinary internal medicine* 1999, **13**(2):95-103.

60. Beauvais W, Cardwell JM, Brodbelt DC: **The effect of neutering on the risk of urinary incontinence in bitches - a systematic review**. *The Journal of small animal practice* 2012, **53**(4):198-204.

61. Beauvais W, Cardwell JM, Brodbelt DC: **The effect of neutering on the risk of mammary tumours in dogs-a systematic review**. *The Journal of small animal practice* 2012, **53**(6):314-322.

62. Torres de la Riva G, Hart BL, Farver TB, Oberbauer AM, Messam LL, Willits N, Hart LA: **Neutering dogs: effects on joint disorders and cancers in golden retrievers**. *PloS one* 2013, **8**(2):e55937.

63. Misdorp W, Else RW, Hellmen E, TP. L: **World Health Organization international histological classification of tumors of domestic animals** vol. VII, 2nd edn: Washington, D.C. : Armed Forces Institute of Pathology; 1999.

64. Elston CW, Ellis IO: **Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up**. *Histopathology* 1991, **19**(5):403-410.

Figures

Figure 1

Schematic illustration of the selected SNPs of *ESR1* gene located in the chromosome 1. The selected SNPs are represented on the illustration by their cluster ID numbers in the public SNP database. Coding regions are shown as a box on a horizontal bar whereas the horizontal bar represents the noncoding region of the illustrated fragment. Green: synonymous SNP; blue: intronic SNPs.

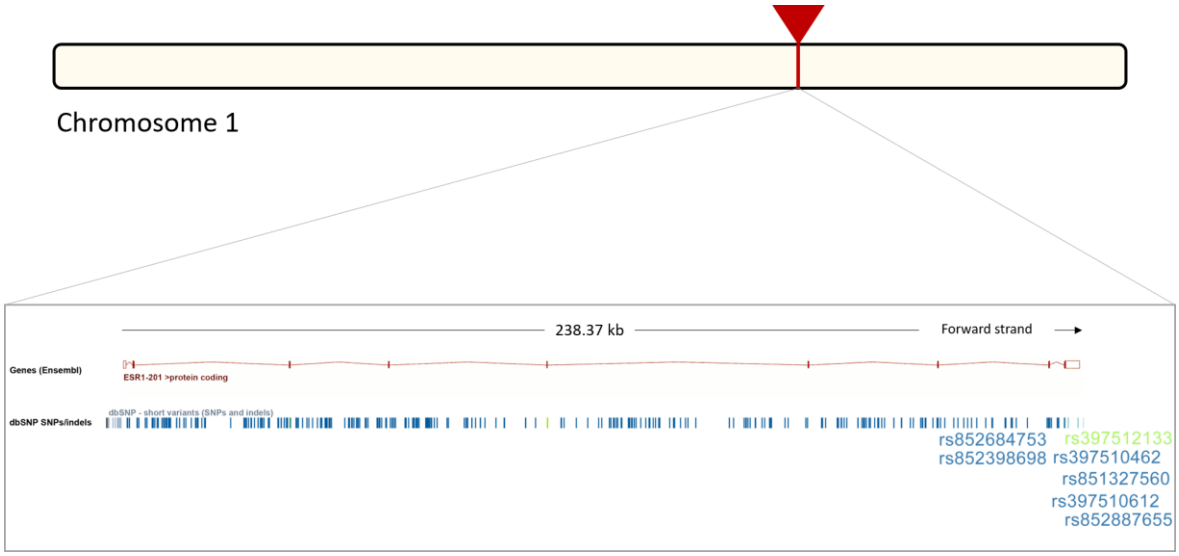
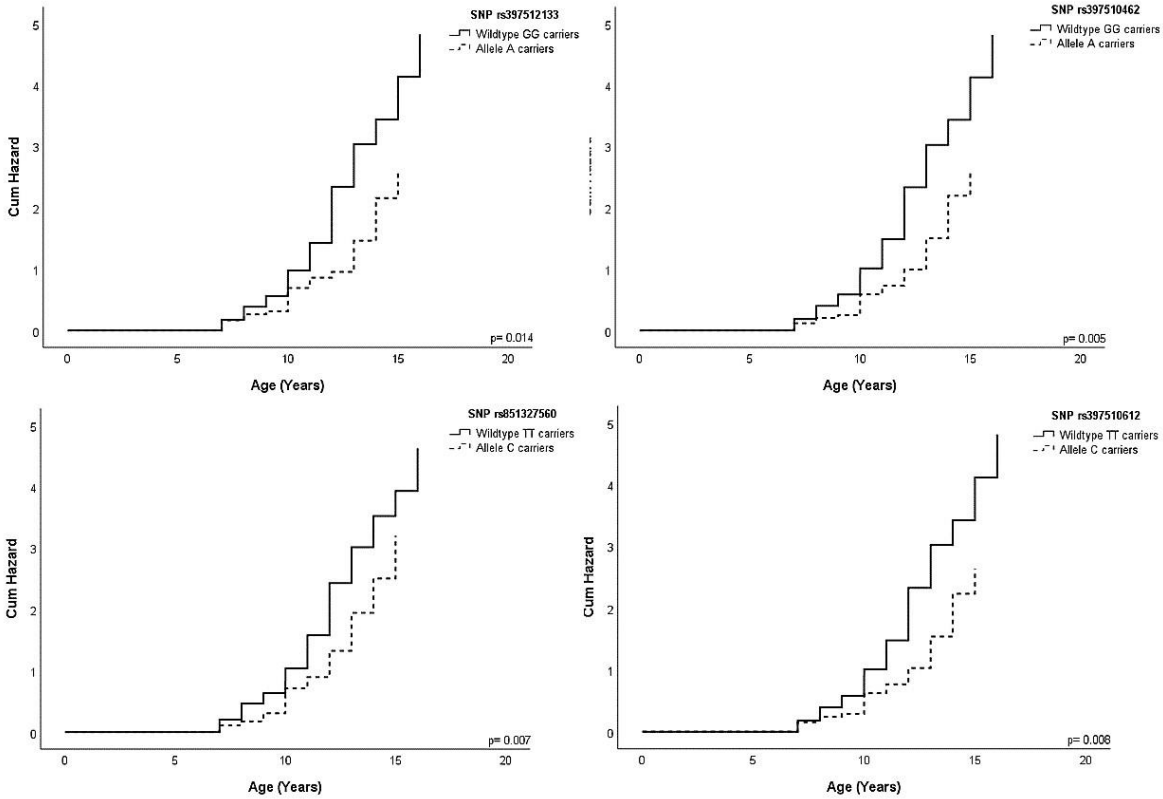


Figure 2

Association between the animal's age at the time of the tumor diagnosis and SNPs of *ESR1* gene. A: SNP rs397512133; B: SNP rs397510462; C: SNP rs851327560 and D: SNPrs397510612.




Tables

Table 1. SNPs assessed in this study.

| SNP | Type | Location | Change | Amino acid |
|---|---|---|---|---|
| rs397512133 | Synonymous | Exon 8 | G->A: CT**G**-CT**A** | Leucine |
| rs397510462 | Intronic | 42.32 Mb to 42.37 Mb | G->A | - |
| rs851327560 | Intronic | 42.32 Mb to 42.37 Mb | T->C | - |
| rs397510612 | Intronic | 42.32 Mb to 42.37 Mb | T->C | - |
| rs852887655 | Intronic | 42.32 Mb to 42.37 Mb | G->A | - |
| rs852684753 | Intronic | 42.32 Mb to 42.37 Mb | TTTTC/- | - |
| rs852398698 | Intronic | 42.32 Mb to 42.37 Mb | TTC/- | - |

**Table 2.** Association between canine *ESR1* SNPs and clinicopathological features of canine mammary tumors.

| ***Clinicopathological Variables*** | **Single Nucleotide Polymorphisms (n= number of cases)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | rs397512133 | | ***p*** | rs397510462 | | ***p*** | rs851327560 | | ***P*** | rs397510612 | | ***P*** |
| | **GG (n/%)** | **A Allele (n/%)** | | **GG (n/%)** | **A allele (n/%)** | | **TT (n/%)** | **C allele (n/%)** | | **TT (n/%)** | **C allele (n/%)** | |
| ***Age*** | | | | | | | | | | | | |
| <10 | 79 (85.9 | 13 (14.1) | | 80 (87.0) | 12 (13.0) | | 67 (72.8) | 25 (27.2) | | 79 (85.9) | 13 (14.1) | |
| ≥10 | 46 (78.0) | 13 (22.0) | NS | 44 (74.6) | 15 (25.4) | NS | 35 (59.3) | 24 (40.7) | NS | 44 (74.6) | 15 (25.4) | NS |
| ***Number of Tumors*** | | | | | | | | | | | | |
| Single | 40 (83.3) | 8 (16.7) | | 41 (85.4) | 7 (14.6) | | 36 (75.0) | 12 (25.0) | | 40 (83.3) | 8 (16.7) | |
| Multiple | 88 (82.2) | 19 (17.8) | NS | 86 (80.4) | 21 (19.6) | NS | 69 (64.5) | 38 (35.5) | NS | 86 (80.4) | 21 (19.6) | NS |
| ***Tumor Size[a]*** | | | | | | | | | | | | |
| ≤ 3cm | 66 (78.6) | 18 (21.4) | | 65 (77.4) | 19 (22.6) | | 54 (64.3) | 30 (35.7) | | 65 (77.4) | 19 (22.6) | |
| > 3cm | 55 (91.7) | 5 (8.3) | ***0.034*** | 54 (90.0) | 6 (10.0) | ***0.049*** | 45 (75.0) | 15 (25.0) | NS | 54 (90.0) | 6 (10.0) | ***0.049*** |
| ***Tumor Size (malignant)*** | | | | | | | | | | | | |
| ≤ 3cm | 35 (76.1) | 11 (23.9) | | 34 (73.9) | 12 (26.1) | | 26 (56.5) | 20 (43.5) | | 34 (73.9) | 12 (26.1) | |
| > 3cm | 46 (93.9) | 3 (6.1) | ***0.014*** | 45 (91.8) | 4 (8.2) | ***0.020*** | 39 (79.6) | 10 (20.4) | ***0.016*** | 45 (91.8) | 4 (8.2) | ***0.020*** |
| ***Histological type*** | | | | | | | | | | | | |
| *Benign* | 43 (76.8) | 13 (23.2) | | 44 (78.6) | 12 (21.4) | | 37 (66.1) | 19 (33.9) | | 43 (76.8) | 13 (23.2) | |
| *Malignant* | 85 (85.9) | 14 (14.1) | NS | 83 (83.8) | 16 (16.2) | NS | 68 (68.7) | 31 (31.3) | NS | 83 (83.8) | 16 (16.2) | NS |
| ***Mode of growth*** | | | | | | | | | | | | |
| *Expansive* | 31 (81.6) | 7 (18.4) | | 29 (76.3) | 9 (23.7) | | 23 (60.5) | 15 (39.5) | | 29 (76.3) | 9 (23.7) | |
| *Infiltrative plus Invasive* | 54 (88.5) | 7 (11.5) | NS | 54 (88.5) | 7 (11.5) | NS | 45 (73.8) | 16 (26.2) | NS | 54 (88.5) | 7 (11.5) | NS |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***NHG Parameters*** | | | | | | | | | | | | |
| ***Tubule formation*** | | | | | | | | | | | | |
| *Score 1 plus Score 2* | 12 (70.6) | 5 (29.4) | | 12 (70.6) | 5 (29.4) | | 8 (47.1) | 9 (52.9) | | 12 (70.6) | 5 (29.4) | |
| *Score 2* | 29 (90.6) | 3 (9.4) | NS | 29 (90.6) | 3 (9.4) | NS | 23 (71.9) | 9 (28.1) | NS | 29 (90.6) | 3 (9.4) | NS |
| ***Nuclear pleomorphism*** | | | | | | | | | | | | |
| *Score 1 plus Score 2* | 52 (85.2) | 9 (14.8) | | 50 (82.0) | 11 (18.0) | | 40 (65.6) | 21 (34.4) | | 50 (82.0) | 11 (18.0) | |
| *Score 3* | 13 (81.3) | 3 (18.8) | NS | 13 (81.3) | 3 (18.8) | NS | 11 (68.8) | 5 (31.3) | NS | 13 (81.3) | 3 (18.8) | NS |
| ***Mitotic count*** | | | | | | | | | | | | |
| *Score 1 plus Score 2* | 44 (83.0) | 9 (17.0) | | 43 (81.1) | 10 (18.9) | | 34 (64.2) | 19 (35.8) | | 43 (81.1) | 10 (18.9) | |
| *Score 3* | 21 (87.5) | 3 (12.5) | NS | 20 (83.3) | 4 (16.7) | NS | 17 (70.8) | 7 (29.2) | NS | 20 (83.3) | 4 (16.7) | NS |
| ***Total score*** | | | | | | | | | | | | |
| *Score <7* | 40 (87.0) | 6 (13.0) | | 39 (84.8) | 7 (15.2) | | 31 (67.4) | 15 (32.6) | | 39 (84.8) | 7 (15.2) | |
| *Score ≥7* | 25 (80.6) | 6 (19.4) | NS | 24 (77.4) | 7 (22.6) | NS | 20 (64.5) | 11 (35.5) | NS | 24 (77.4) | 7 (22.6) | NS |
| ***NHG*** | | | | | | | | | | | | |
| *Grade I plus Grade II* | 50 (82.0) | 11 (18.0) | | 49 (80.3) | 12 (19.7) | | 38 (62.3) | 23 (37.7) | | 49 (80.3) | 12 (19.7) | |
| *Grade III* | 15 (93.8) | 1 (6.3) | NS | 14 (87.5) | 2 (12.5) | NS | 13 (81.3) | 3 (18.8) | NS | 14 (87.5) | 2 (12.5) | NS |
| ***Vet-NPI*** | | | | | | | | | | | | |
| *≤4.0* | 35 (77.8) | 10 (22.2) | | 34 (75.6) | 11 (24.4) | | 25 (55.6) | 20 (44.4) | | 34 (75.6) | 11 (24.4) | |
| *>4.0* | 29 (93.5) | 2 (6.5) | NS | 28 (90.3) | 3 (9.7) | NS | 25 (80.6) | 6 (19.4) | ***0.023*** | 28 (90.3) | 3 (9.7) | NS |
| ***Vascular invasion*** | | | | | | | | | | | | |
| *No* | 68 (82.9) | 14 (17.1) | | 66 (80.5) | 16 (19.5) | | 53 (64.6) | 29 (35.4) | | 66 (80.5) | 16 (19.5) | |
| *Yes* | 14 (100.0) | 0 (0.0) | NS | 14 (100.0) | 0 (0.0) | NS | 12 (85.7) | 2 (14.3) | NS | 14 (100.0) | 0 (0.0) | NS |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Lymph node metastases*** | | | | | | | | | | | | |
| *No* | 54 (83.1) | 11 (16.9) | | 52 (80.0) | 13 (20.0) | | 41 (63.1) | 24 (36.9) | | 52 (80.0) | 13 (20.0) | |
| *Yes* | 19 (95.0) | 1 (5.0) | NS | 19 (95.0) | 1 (5.0) | NS | 17 (85.0) | 3 (15.0) | NS | 19 (95.0) | 1 (5.0) | NS |

[α] Including benign and malignant tumors.

Legend: NHG – Nottingham histological grade; WHO – World Health Organization; vet-NPI – veterinary-adapted Nottingham Prognostic Index.

**Table 2. (Continuation)** Association between canine *ESR1* SNPs and clinicopathological features of canine mammary tumours.

| *Clinicopathological Variables* | Single Nucleotide Polymorphisms (n= number of cases) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | rs852887655 | | *p* | rs852684753 | | *p* | rs852398698 | | *P* |
| | **GG (n/%)** | **A Allele (n/%)** | | **Non DEL (n/%)** | **Del (n/%)** | | **TTC (n/%)** | **Del/TTC (n/%)** | |
| ***Age*** | | | | | | | | | |
| <10 | 78 (84.8) | 14 (15.2) | | 53 (58.2) | 38 (41.8) | | 53 (57.6) | 39 (42.4) | |
| ≥10 | 49 (83.1) | 10 (16.9) | NS | 31 (53.4) | 27 (46.6) | NS | 31 (52.5) | 28 (47.5) | NS |
| ***Number of Tumors*** | | | | | | | | | |
| Single | 43 (91.5) | 4 (8.5) | | 26 (55.3) | 21 (44.7) | | 26 (54.2) | 22 (45.8) | |
| Multiple | 87 (81.3) | 20 (18.7) | NS | 61 (57.5) | 45 (42.5) | NS | 61 (57.0) | 46 (43.0) | NS |
| ***Tumor Size[α]*** | | | | | | | | | |
| ≤ 3cm | 69 (83.1) | 14 (16.9) | | 45 (53.6) | 39 (46.4) | | 45 (53.6) | 39 (46.4) | |
| > 3cm | 51 (85.0) | 9 (15.0) | NS | 36 (62.1) | 22 (37.9) | NS | 36 (60.0) | 24 (40.0) | NS |
| ***Tumor Size (malignant)*** | | | | | | | | | |
| ≤ 3cm | 35 (76.1) | 11 (23.9) | | 26 (56.5) | 20 (43.5) | | 26 (56.5) | 20 (43.5) | |
| > 3cm | 43 (87.8) | 6 (12.2) | NS | 31 (64.6) | 17 (35.4) | NS | 31 (63.3) | 18 (36.7) | NS |
| ***Histological type*** | | | | | | | | | |
| *Benign* | 49 (89.1) | 6 (10.9) | | 28 (50.9) | 27 (49.1) | | 28 (50.0) | 28 (50.0) | |
| *Malignant* | 81 (81.8) | 18 (18.2) | NS | 59 (60.2) | 39 (39.8) | NS | 59 (59.6) | 40 (40.4) | NS |
| ***Mode of growth*** | | | | | | | | | |
| *Expansive* | 32 (84.2) | 6 (15.8) | | 22 (59.5) | 15 (40.5) | | 22 (57.9) | 16 (42.1) | |
| *Infiltrative plus Invasive* | 49 (80.3) | 12 (19.7) | NS | 37 (60.7) | 24 (39.3) | NS | 37 (60.7) | 24 (39.3) | NS |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***NHG Parameters*** | | | | | | | | | |
| ***Tubule formation*** | | | | | | | | | |
| *Score 1 plus Score 2* | 36 (73.5) | 13 (26.5) | | 24 (49.0) | 25 (51.0) | | 24 (49.0) | 25 (51.0) | |
| *Score 3* | 26 (92.9) | 2 (7.1) | ***0.039*** | 21 (77.8) | 6 (22.2) | ***0.014*** | 21 (75.0) | 7 (25.0) | ***0.026*** |
| ***Nuclear pleomorphism*** | | | | | | | | | |
| *Score 1 plus Score 2* | 48 (78.7) | 13 (21.3) | | 35 (58.3) | 25 (41.7) | | 35 (57.4) | 26 (42.6) | |
| *Score 3* | 14 (87.5) | 2 (12.5) | NS | 10 (62.5) | 6 (37.5) | NS | 10 (62.5) | 6 (37.5) | NS |
| ***Mitotic count*** | | | | | | | | | |
| *Score 1 plus Score 2* | 41 (77.4) | 12 (22.6) | | 29 (55.8) | 23 (44.2) | | 29 (54.7) | 24 (45.3) | |
| *Score 3* | 21 (87.5) | 3 (12.5) | NS | 16 (66.7) | 8 (33.3) | NS | 16 (66.7) | 8 (33.3) | NS |
| ***Total score*** | | | | | | | | | |
| *Score <7* | 35 (76.1) | 11 (23.9) | | 25 (55.6) | 20 (44.4) | | 25 (54.3) | 21 (45.7) | |
| *Score ≥7* | 27 (87.1) | 4 (12.9) | NS | 20 (64.5) | 11 (35.3) | NS | 20 (64.5) | 11 (35.5) | NS |
| ***NHG*** | | | | | | | | | |
| *Grade I plus Grade II* | 47 (77.0) | 14 (23.0) | | 33 (55.0) | 27 (45.0) | | 33 (54.1) | 28 (45.9) | |
| *Grade III* | 15 (93.8) | 1 (6.3) | NS | 12 (75.0) | 4 (25.0) | NS | 12 (75.0) | 4 (25.0) | NS |
| ***vet-NPI*** | | | | | | | | | |
| *≤4.0* | 33 (73.3) | 12 (26.7) | | 24 (54.5) | 20 (45.5) | | 24 (53.3) | 21 (46.7) | |
| *>4.0* | 28 (90.3) | 3 (9.7) | NS | 20 (64.5) | 11 (35.5) | NS | 20 (64.5) | 11 (35.5) | NS |
| ***Vascular invasion*** | | | | | | | | | |
| *No* | 66 (80.5) | 16 (19,5) | | 48 (59.3) | 33 (40.7) | | 48 (58.5) | 34 (41.5) | |
| *Yes* | 12 (85.7) | 2 (14.3) | NS | 9 (64.3) | 5 (35.7) | NS | 9 (64.3) | 5 (35.7) | NS |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***Lymph node metastases*** | | | | | | | | | |
| *No* | 51 (78.5) | 14 (21.5) | | 38 (59.4) | 26 (40.6) | | 38 (58.5) | 27 (41.5) | |
| *Yes* | 18 (90.0) | 2 (10.0) | NS | 13 (65.0) | 7 (35.0) | NS | 13 (65.0) | 7 (35.0) | NS |

[α] Including benign and malignant tumors.

Legend: NHG – Nottingham histological grade; WHO – World Health Organization; vet-NPI – veterinary-adapted Nottingham Prognostic Inde

# CHAPTER 06

## Catechol-O-Methyltransferase Genotypes are Associated with Progression and Biological Behavior of Canine Mammary Tumors

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ORIGINAL ARTICLE

# Catechol-o-methyltransferase genotypes are associated with progression and biological behaviour of canine mammary tumours

Ana Canadas[1] | Marta Santos[2] | Ricardo Pinto[3] | Rui Medeiros[3,4,5,6] | Patrícia Dias-Pereira[1]

[1]Department of Pathology and Molecular Immunology, Instituto Ciências Biomédicas Abel Salazar, ICBAS–UPorto, University of Porto, Porto, Portugal

[2]Department of Microscopy, Instituto Ciências Biomédicas Abel Salazar, ICBAS–UPorto, University of Porto, Porto, Portugal

[3]Molecular Oncology and Viral Pathology Group, IPO-Porto Research Centre, Portuguese Institute of Oncology of Porto (IPO-Porto), Porto, Portugal

[4]Faculty of Medicine, University of Porto, Porto, Portugal

[5]FP-ENAS Research Unit, UFP Energy, Environment and Health Research Unit, CEBIMED, Biomedical Research Centre, University Fernando Pessoa, Porto, Portugal

[6]LPCC, Research Department, Portuguese League Against Cancer (LPPC-NRN), Porto, Portugal

Correspondence
Patrícia Dias-Pereira, Department of Pathology and Molecular Immunology, Instituto Ciências Biomédicas Abel Salazar, ICBAS–UPorto, University of Porto, Rua Jorge Viterbo Ferreira, no. 228, 4050-313 Porto, Portugal.
Email: pdiaspereira@yahoo.com.br

The enzyme catechol-o-methyltransferase (COMT) is responsible for inactivation of catechol estrogens, which are well-recognized carcinogenic metabolites. Two single-nucleotide polymorphisms (SNPs) in canine COMT gene were previously associated with the age of onset of mammary tumours and with the clinical progression of the disease. However, no information is available regarding the impact of other known SNPs in COMT gene in canine mammary tumours. The aim of the present study is to evaluate the influence of COMT SNP in clinicopathological features and outcome of canine mammary tumours. A case series study was conducted involving 155 non-neutered bitches with mammary tumours submitted to follow-up for 24 months after surgery. Three genotypes were considered: Genotype 1 (rs853046495); Genotype 2 (rs23350589, rs23322686, rs23336579, and rs852564758); Genotype 3 (rs851328636 and rs853133060). Genotype 1 was associated to low degree of tubular differentiation. Genotype 2 was related to the development of intermediate/high-histological-grade carcinomas and to vascular invasion. Genotype 3 was associated to reduced nuclear pleomorphism and well-differentiated carcinomas. Data from the present investigation allowed the identification of COMT genetic profiles associated with pathological features of mammary tumours that constitute relevant prognostic factors. The assessment of the COMT genotypes may represent a helpful tool in the clinical management of canine mammary tumours, assisting in the selection of individualized preventive and therapeutic strategies, according to the animals' genetic profile.

KEYWORDS

canine mammary tumour, COMT, histological grade, SNP, vascular invasion

## 1 | INTRODUCTION

Canine mammary tumours (CMTs) are considered the most common neoplasms in female dogs,[1–4] representing 30% to 70% of all canine tumours.[2,5] It is widely recognized that reproductive and hormonal factors play a determinant role in both breast cancer and CMT development. In fact, CMTs are reported to occur almost exclusively in females, especially those submitted to hormonal contraceptive therapy.[6,7] Besides, some authors demonstrated that ovariectomy has a protective effect against the development of mammary tumours, and the earlier this intervention is performed the more noticeable this sparring effect becomes.[6,8] In addition, dogs with mammary neoplasia exhibit higher levels of serum estrogens than normal ones.[9] These features strongly implicate sexual steroid hormones, namely estrogens, in CMT development.

Estrogens are known to participate in the carcinogenic process by promoting proliferative activity of the mammary epithelium and through the accumulation of mutagenic metabolites, namely catechol estrogens. Catechol estrogens are considered carcinogenic metabolites that can bind to DNA producing depurinating adducts, and are oxidized to semi-quinones and quinones, in a process that generates reactive oxygen species able to induce DNA damage.[10,11]

The enzyme catechol-o-methyltransferase (COMT) plays a pivotal role in estrogens metabolism, being responsible for the methylation

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and inactivation of catechol estrogens.[12] In this sense, COMT is regarded as a caretaker enzyme, being considered a protective tool against estrogens dependent cancers.[13,14] Several single-nucleotide polymorphisms (SNPs) in human *COMT* gene have been described and extensively analysed as breast cancer risk factors leading, however, to contradictory results.[15] Similarly, there are various recognized SNPs in canine *COMT* gene, which is located at chromosome 26. Dias Pereira and colleagues demonstrated the influence of *COMT* SNP G216A (also known as *rs853133060*) and G482A (also known as *rs853046495*) in some clinicopathological features (namely the waiting time of onset of malignant tumours) and in the outcome of the neoplastic disease.[16,17] However, no information is available regarding the impact of other known SNPs in canine *COMT* gene in CMT. The aim of the present study is to evaluate the association of seven canine *COMT* gene SNPs with the clinicopathological features and outcome of CMT.

## 2 | MATERIALS AND METHODS

A case series study was conducted involving 155 non-neutered bitches with histologically confirmed mammary tumours. All the cases were collected from the Veterinary Pathology Laboratory (Instituto Ciências Biomédicas Abel Salazar [ICBAS]-University of Porto). Owners provided consent for surgery with curative intents (partial or radical mastectomy), as well as for the use of the material for research purposes. This protocol was approved by the Ethics Committee of the ICBAS-University of Porto (P151/2016).

Samples were collected immediately after surgery, fixed in a 10% buffered formalin solution and routinely processed for histopathologic examination. For each dog, the age at the time of the diagnosis, the number of tumours (one or multiple tumours), and the tumour size (corresponding to the largest diameter measured during trimming) were recorded. Histological diagnosis was established by consensus of three observers in a multi-head microscope, using the criteria proposed by the World Health Organization for the classification of mammary tumours of dogs and cats.[18] Each malignant tumour was assessed for the mode of growth and classified as either expansive, when it was delimited by a fibrous capsule; infiltrative, when not delimited by a capsule, but without signs of vascular invasion or lymph node metastasis; and invasive, when vascular invasion or lymph node metastases were detected. Histological grading of mammary carcinoma was performed according to the Nottingham method (Nottingham histological grade [NHG]) originally designed to grade human breast cancer.[19] A veterinary adaptation of the human Nottingham prognostic index (vet-NPI) was also computed, as previously reported.[20] Additionally, the presence of vascular invasion and regional lymph node metastases was assessed. In cases of dogs with multiple malignant tumours, a reference lesion was assigned for the statistical study. The reference lesion was considered as the tumour presenting vascular invasion (primary criterion), higher nuclear pleomorphism (secondary criterion) or the one with the largest diameter (tertiary criterion). Follow-up data were obtained by consulting the medical records and by contact to the reference veterinarian. Disease-specific overall survival was calculated from the time of diagnosis to the date of the animal's death/euthanasia because of the neoplastic disease. Animals that died or were euthanized for unrelated causes and those that were lost to follow-up were censored, respectively, at the time of death and at the data of their last clinical examination. Euthanasia was performed only in terminal stage of the disease. Necropsy examination was performed upon the owner consent.

For the genetic study, genomic DNA was extracted from peripheral blood samples (obtained by standard venipuncture) using high pure polymerase chain reaction Template preparation kit (Roche, Mannheim, Germany). The DNA quality was evaluated by measuring the optical density, and the quantity was assessed employing the NanoDrop 1000 Spectrophotometer. SNP genotyping was performed using MassARRAY iPLEX Gold Technology (Agena Bioscience, San Diego, CA) at the Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência.

Seven *COMT* SNP were studied: one non-synonymous SNP *rs853046495*; two synonymous SNP: *rs23350589* and *rs853133060*; and four intronic SNP: *rs23322686*, *rs23336579*, *rs851328636*, and *rs852564758* (Figure 1). The SNP *rs853046495* is located in exon 4 and is responsible for a missense variant C → T: CGG/CAG

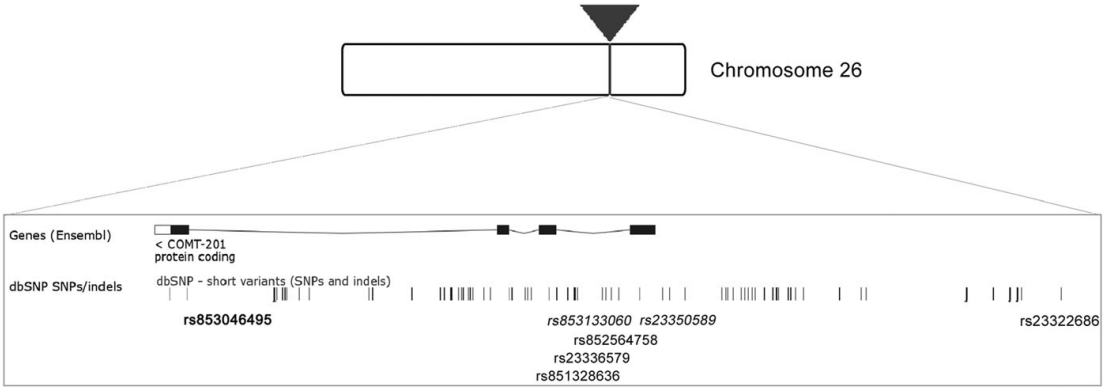


**FIGURE 1** Schematic illustration of the selected single-nucleotide polymorphism (SNP) of canine catechol-o-methyltransferase (*COMT*) gene. SNPs are named by their cluster ID numbers in the public SNP database. Coding regions are depicted as a dark box, and the horizontal bar represents the non-coding region of the illustrated fragment. Bold rs: non-synonymous SNP; italics rs: synonymous SNP; normal rs: intronic SNP

(Arginine → Glutamine). The *rs23350589* corresponds to a synonymous variant located in exon 1, responsible for the change T → C: GT**A**/GT**G** (Valine). The synonymous SNP *rs853133060* is located in exon 3, being responsible for the change C → T: TC**G**/TC**A** (Serine). All intronic SNPs are located between 29.36 and 29.38 Mb in chromosome 26. SNP *rs23322686* is responsible for the change T → C; *rs23336579* is responsible for the change C → T; *rs851328636* is responsible for change C → G and *rs852564758* for the change T → C.

In a recent report, we performed a linkage disequilibrium analysis and demonstrated that canine *COMT* SNP *rs23350589*, *rs23322686*, *rs23336579*, and *rs852564758* were in linkage. The same was observed for SNP *rs851328636* and *rs853133060*. [21] As alleles at these loci are non-randomly associated, we opted to analyse these SNP as blocks. Accordingly, for statistical analysis of data, three genotypes were considered: Genotype 1 represented carriers of SNP *rs853046495*; Genotype 2 was identified as carriers of at least one of the SNP *rs23350589*, *rs23322686*, *rs23336579* and *rs852564758*; Genotype 3 included carriers of at least one of the SNP *rs851328636* and *rs853133060*. Statistical analysis of data was performed using the computer software SPSS for Windows (version 18, Chicago, IL). Chi-square analysis was used to investigate the significance of the relationship between SNPs and the categorical variables. A 5% level was considered to define statistical significance.

## 3 | RESULTS

One hundred and fifty five non-neutered female dogs were included in the study. The median age of the study population was 10.1 years. On histological examination, 56 tumours were classified as benign (36.1%) and 99 as malignant (63.9%). Thirty-six animals were assigned to Genotype 1, 103 animals to Genotype 2 and 44 animals to Genotype 3.

Results regarding the association between the COMT genotypes and clinicopathological parameters of the neoplastic disease, including age at the tumour diagnosis, tumour number and size, tumour growth pattern, histological classification, NHG parameters (tubule formation, nuclear pleomorphism and mitotic index), NHG total score, NHG, vet-NPI, vascular invasion and lymph node metastases are shown in Table 1.

When evaluating NHG parameters, a statistically significant relationship was observed between SNP *rs853046495* (Genotype 1) and the percentage of tubule formation ($P = 0.022$); indeed, 86.6% of carriers of the variant allele were scored ≥2 for this parameter. Furthermore, a statistically significant relationship was found between Genotype 3 and low degree of nuclear pleomorphism ($P = 0.036$) and NHG total score < 7 ($P = 0.049$). None of the Genotype 3 animals were scored 3 for nuclear pleomorphism. On the other hand, Genotype 3 animals represented 79% of the cases with NHG total score < 7. Furthermore, only 17.3% of grade II/III carcinomas corresponded to Genotype 3 animals, demonstrating an association between this genotype and low-histological-grade carcinomas ($P = 0.031$). On the contrary, Genotype 2 was related to grade II/III carcinomas ($P = 0.038$). Additionally, a statistically significant association was found between Genotype 2 and vascular invasion ($P = 0.024$); almost all the cases (92.9%) with neoplastic emboli were found in Genotype 2 animals.

No significant associations could be established between any of the three genotypes considered and age of the animal, tumour number, size or type of growth, histological classification, mitotic index, vet-NPI and lymph node metastases. Furthermore, none of the genotypes studied was related to OS.

## 4 | DISCUSSION

In recent years, several investigations have highlighted the importance of the individual genetic profile in the definition of various clinical and pathological features of mammary neoplasms, as well as in the biological behaviour and prognosis of breast cancer disease.[11,22–26] Most of these studies have focused on non-synonymous SNP, which are able to induce changes in the amino acid sequence and may thus interfere directly with the protein functional activity. However, recent research has shown that, although not altering the protein primary structure, synonymous SNP can affect the speed and accuracy of transcription and/or translation, induce alternative splicing and changes in messenger RNA (mRNA) structure/stability. These apparently subtle features can be responsible for significant changes in the amount of protein produced, as well as in its kinetics parameters, conformation (secondary and tertiary structure), stability and functional activity.[27,28] Specifically regarding COMT enzyme, a marked decrease in the levels of protein expression and in its enzymatic activity have been attributed to changes in the secondary structure of mRNA because of synonymous SNP.[29] Furthermore, intronic SNP also seems to have influence on several human multi-factorial diseases, including breast cancer.[30] Introns are non-coding sequences accounting for 24% of the entire human genome, removed by splicing when exons are bound. These usually long sequences contain microRNA genes and several sequences known to play an important role in gene expression regulation, affecting[31,32] splicing, alternative splicing and splicing efficiency.[30,31]

Data from the present study allowed the identification of *COMT* genetic profiles associated with important pathological features of CMT, such as histological grade, grading parameters and vascular invasion, which are relevant factors for prognostication of the neoplastic disease.

Genotype 2, a haplotype based on a block of one synonymous and three intronic SNP, was significantly related to the development of intermediate/high-histological-grade mammary carcinomas and to vascular invasion. It is conceivable that this haplotype may interfere with COMT enzymatic activity, coding a variant form of the enzyme less efficient in catechol estrogens inactivation. The accumulation of these genotoxic metabolites, known to have a carcinogenic effect, drives to the development of more aggressive neoplastic cell clones, thus explaining the higher histological grade and the invasive potential of this group of carcinomas.

On the contrary, Genotype 3 (a haplotype involving a block of one synonymous and one intronic SNP) was significantly associated to well-differentiated carcinomas with lower nuclear pleomorphism. Our results suggest that this haplotype may play a protective effect, contributing to the development of less aggressive mammary carcinomas.

**TABLE 1** Association between canine *COMT* genotypes and clinicopathological features of canine mammary tumours

| | Genotype 1[b] | | | Genotype 2[c] | | | Genotype 3[d] | | |
|---|---|---|---|---|---|---|---|---|---|
| **Independent variables** | **Yes (*n* [%])** | **No (*n* [%])** | **p** | **Yes (*n* [%])** | **No (*n* [%])** | **p** | **Yes (*n* [%])** | **No (*n* [%])** | **p** |
| Age | | | | | | | | | |
| ≤10 | 19 (20.7) | 73 (79.3) | | 58 (63.0) | 34 (37.0) | | 23 (25.0) | 69 (75.0) | |
| >10 | 15 (25.9) | 43 (74.1) | 0.458 | 44 (74.6) | 15 (25.4) | 0.140 | 19 (32.2) | 40 (67.8) | 0.335 |
| Number of tumours | | | | | | | | | |
| Single | 12 (25.0) | 36 (75.0) | | 28 (58.3) | 20 (41.7) | | 15 (31.3) | 33 (68.8) | |
| Multiple | 24 (22.6) | 82 (77.4) | 0.749 | 75 (70.1) | 32 (29.9) | 0.152 | 29 (27.1) | 78 (72.9) | 0.596 |
| Tumour size[a] | | | | | | | | | |
| ≤3 cm | 20 (24.1) | 63 (75.9) | | 57 (67.9) | 27 (32.1) | | 23 (27.4) | 61 (72.6) | |
| >3 cm | 13 (21.7) | 47 (78.3) | 0.734 | 41 (68.3) | 19 (31.7) | 0.952 | 17 (28.3) | 43 (71.7) | 0.900 |
| Tumour size (malignant tumours) | | | | | | | | | |
| ≤3 cm | 12 (26.7) | 33 (73.3) | | 29 (63.0) | 17 (37.0) | | 14 (30.4) | 32 (69.6) | |
| >3 cm | 12 (24.5) | 37 (75.5) | 0.809 | 34 (69.4) | 15 (30.6) | 0.513 | 14 (28.6) | 35 (71.4) | 0.842 |
| Histological classification | | | | | | | | | |
| Benign | 11 (19.6) | 45 (80.4) | | 37 (66.1) | 19 (33.9) | | 15 (26.8) | 41 (73.2) | |
| Malignant | 25 (25.5) | 73 (74.5) | 0.408 | 66 (66.7) | 33 (33.3) | 0.940 | 29 (29.3) | 70 (70.7) | 0.739 |
| Mode of growth | | | | | | | | | |
| Expansive | 11 (27.9) | 27 (71.1) | | 26 (68.4) | 12 (31.6) | | 9 (23.7) | 29 (76.3) | |
| Infiltrative | 11 (29.7) | 26 (70.3) | | 23 (62.2) | 14 (37.8) | | 15 (40.5) | 22 (59.5) | |
| Invasive | 3 (13.0) | 20 (87.0) | 0.292 | 17 (70.8) | 7 (29.2) | 0.749 | 5 (20.8) | 19 (79.2) | 0.160 |
| NHG parameters | | | | | | | | | |
| Tubule formation | | | | | | | | | |
| Score 1 | 2 (12.5) | 14 (87.5) | | 12 (70.6) | 5 (29.4) | | 5 (29.4) | 12 (70.6) | |
| Score 2 | 11 (34.4) | 21 (65.6) | | 22 (68.7) | 10 (31.3) | | 11 (34.4) | 21 (65.6) | |
| Score 3 | 2 (7.1) | 26 (92.9) | **0.022** | 21 (75.0) | 7 (25.0) | 0.864 | 3 (10.7) | 25 (89.3) | 0.092 |
| Nuclear pleomorphism | | | | | | | | | |
| Score 1 | 1 (14.3) | 6 (85.7) | | 7 (100.0) | 0 (0) | | 2 (28.6) | 5 (71.4) | |
| Score 2 | 14 (26.4) | 39 (73.6) | | 35 (64.8) | 19 (35.2) | | 17 (31.5) | 37 (61.5) | |
| Score 3 | 0 (0.0) | 16 (100.0) | 0.062 | 13 (81.3) | 3 (18.8) | 0.095 | 0 (0.0) | 16 (100.0) | **0.036** |
| Mitotic index | | | | | | | | | |
| Score 1 | 8 (25.0) | 24 (75.0) | | 22 (68.8) | 10 (31.3) | | 10 (31.3) | 22 (68.8) | |
| Score 2 | 2 (10.0) | 18 (90.0) | | 16 (76.2) | 5 (23.8) | | 4 (19.0) | 17 (81.0) | |
| Score 3 | 5 (20.8) | 19 (79.2) | 0.412 | 17 (70.8) | 7 (29.2) | 0.839 | 5 (20.8) | 19 (79.2) | 0.524 |
| Total score | | | | | | | | | |
| Score < 7 | 11 (24.4) | 34 (75.6) | | 31 (67.4) | 15 (32.6) | | 15 (32.6) | 31 (67.4) | |
| Score ≥ 7 | 4 (12.9) | 27 (87.1) | 0.214 | 24 (77.4) | 7 (22.6) | 0.339 | 4 (12.9) | 27 (87.1) | **0.049** |
| NHG | | | | | | | | | |
| Grade I | 7 (28.0) | 18 (72.0) | | 14 (56.0) | 11 (44.0) | | 10 (40.0) | 15 (60.0) | |
| Grade II | 7 (20.0) | 28 (80.0) | | 31 (86.1) | 5 (13.9) | | 7 (19.4) | 29 (80.6) | |
| Grade III | 1 (6.3) | 15 (93.8) | 0.233 | 10 (62.5) | 6 (37.5) | **0.025** | 2 (12.5) | 14 (87.5) | 0.084 |
| Vet-NPI | | | | | | | | | |
| ≤4.0 | 11 (24.4) | 34 (75.6) | | 31 (68.9) | 14 (31.1) | | 13 (28.9) | 32 (71.1) | |
| >4.0 | 4 (12.9) | 27 (87.1) | 0.214 | 24 (75.0) | 8 (25.0) | 0.559 | 6 (18.8) | 26 (81.3) | 0.309 |
| Vascular invasion | | | | | | | | | |
| No | 32 (24.4) | 99 (75.6) | | 51 (62.2) | 31 (37.8) | | 25 (30.5) | 57 (69.5) | |
| Yes | 1 (7.7) | 12 (92.3) | 0.171 | 13 (92.9) | 1 (7.1) | **0.024** | 3 (21.4) | 11 (78.6) | 0.491 |
| Lymph node metastases | | | | | | | | | |
| No | 23 (22.1) | 81 (77.9) | | 45 (69.2) | 20 (30.8) | | 18 (27.7) | 47 (72.3) | |
| Yes | 3 (15.8) | 16 (84.2) | 0.535 | 13 (65.0) | 7 (35.0) | 0.722 | 5 (25.0) | 15 (75.0) | 0.813 |

Abbreviations: *COMT*, catechol-o-methyltransferase; NHG, Nottingham histological grade; vet-NPI, veterinary adaptation of the Nottingham prognostic index.
*P* values < 0.05 are written in bold.
[a] Includes benign and malignant tumours.
[b] Genotype 1 (*rs853046495*).
[c] Genotype 2 (*rs23350589*, *rs23322686*, *rs23336579*, and *rs852564758*).
[d] Genotype 3 (*rs851328636* and *rs853133060*).

On the other hand, the single non-synonymous SNP included in this study (Genotype 1), did not prove to be determinant in the definition of clinicopathological features and progression of CMTs, when assessed individually. Only the proportion of tubular arrangements within the tumour was related to this specific SNP (being associated with lower degree of tubular differentiation), however with no significant changes in the score or histological grade of carcinomas. Our results are in accordance with data published in a previous study,[16] demonstrating that no significant relationship can be established between SNP *rs853046495* and the number and histological type of mammary tumours, nor with lymph node metastasis. On the other hand, we cannot confirm the previously reported association between this SNP and the age of onset of mammary tumours, which may be related to differences in the cohort populations and to the small representativeness of this SNP, accounting for only 23.7% of the animals included in the present study.

Our results emphasize the importance of genetic variation in estrogens metabolic pathways as prognosticators in both human and canine mammary neoplasia. Recently, some investigators described an association between genetic SNP in Cytochrome P450 (CYP) enzymes (involved in estrogens biosynthesis) and the patients' age, tumour size, histological grade and OS of breast cancer.[33,34] Data from the present investigation identified *COMT* haplotypes with apparently opposite effects regarding CMTs progression, reinforcing the role of hormonal factors as prognosticators in this type of neoplasm.

Genetic signatures emerged as a useful tool assisting in the development of prevention, screening and early detection protocols in human breast cancer. Furthermore, they also play a relevant role helping in the selection of most appropriated therapeutic strategies, as well as in predicting individual response to treatment and clinical outcome of the neoplastic disease.[35,36] In veterinary medicine, the identification of genetic profiles related to the progression of the neoplastic disease also holds great promise in the clinical management of CMT, assisting in the implementation of individualized preventive and therapeutic approaches, according to the animal's genetic profile. Based on our findings, it is conceivable that *COMT* encoded by Genotype 2 corresponds to a low-activity form of the enzyme, less efficient in catechol estrogens metabolization. In this sense, Genotype 2 animals could benefit from early sterilization (instead of the use of oestrogen-based contraceptives), in order to reduce the exposure to endogenous estrogens and catechol estrogens, thus ensuring that the low-activity form of COMT would be sufficient to guarantee their inactivation, minimizing their carcinogenic potential.

Additional studies, assessing COMT functional activity, as well as the enzyme structure, stability and kinetic parameters, are warranted in order to disclose the mechanisms by which these SNPs influence clinicopathological features and progression of CMT. Additionally, comprehensive investigations are required to evaluate the interaction/interdependence between SNP from different genes involved in estrogens biosynthesis and metabolism, and to understand the role epistasis play in the development and progression of CMT.

In conclusion, this study allowed the identification of canine *COMT* genetic profiles associated to relevant pathological features of mammary tumours (namely histological grade, grading parameters and vascular invasion) which may be critical in the progression and biological behaviour of the neoplastic disease. These findings open new perspectives in the clinical management of CMT, supporting the implementation of individualized preventive and therapeutic procedures, based on the animal's genetic profile.

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## Conflict of interest

The authors declare no conflicts of interest.

## ORCID

*Patrícia Dias-Pereira* http://orcid.org/0000-0002-8845-794X

## REFERENCES

1. Moulton J. Tumors of the mammary gland. In: Je M, ed. *Tumors in Domestic Animals*. Vol 3. 3rd ed. Berkeley, CA: University of California Press; 1990:518-546.
2. Sorenmo KU, Rasotto R, Zappulli V, Goldschmidt MH. Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. *Vet Pathol*. 2011;48(1):85-97.
3. Enginler SO, Akis I, Toydemir TS, et al. Genetic variations of BRCA1 and BRCA2 genes in dogs with mammary tumours. *Vet Res Commun*. 2014;38(1):21-27.
4. Klopfleisch R. Mammary tumors. In: Klopfleisch R, ed. *Veterinary Oncology—A Short Textbook*. 1st ed. Cham, Switzerland: Springer International Publishing; 2016:100-104.
5. Klopfleisch R, von Euler H, Sarli G, Pinho SS, Gartner F, Gruber AD. Molecular carcinogenesis of canine mammary tumors: news from an old disease. *Vet Pathol*. 2011;48(1):98-116.
6. Misdorp W. Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins. *Vet Q*. 1988;10(1):26-33.
7. Sorenmo KU, Shofer FS, Goldschmidt MH. Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *J Vet Intern Med*. 2000;14(3):266-270.
8. Schneider R, Dorn CR, Taylor DO. Factors influencing canine mammary cancer development and postsurgical survival. *J Natl Cancer Inst*. 1969;43(6):1249-1261.
9. Queiroga FL, Perez-Alenza MD, Silvan G, Pena L, Lopes C, Illera JC. Role of steroid hormones and prolactin in canine mammary cancer. *J Steroid Biochem Mol Biol*. 2005;94(1–3):181-187.
10. Xie T, Ho S-L, Ramsden D. Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol Pharmacol*. 1999;56(1):31-38.
11. Cheng T-C, Chen S-T, Huang C-S, et al. Breast cancer risk associated with genotype polymorphism of the catechol estrogen-metabolizing genes: a multigenic study on cancer susceptibility. *Int J Cancer*. 2005;113(3):345-353.
12. Yager JD, Liehr JG. Molecular mechanisms of estrogen carcinogenesis. *Annu Rev Pharmacol Toxicol*. 1996;36:203-232.
13. Lavigne JA, Helzlsouer KJ, Huang H-Y, et al. An association between the allele coding for a low activity variant of catechol-

O-methyltransferase and the risk for breast cancer. *Cancer Res*. 1997; 57(24):5493-5497.
14. Cavalieri EL, Stack DE, Devanesan PD, et al. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc Natl Acad Sci U S A*. 1997;94(20):10937-10942.
15. Qin X, Peng Q, Qin A, et al. Association of COMT Val158Met polymorphism and breast cancer risk: an updated meta-analysis. *Diagn Pathol*. 2012;7:136.
16. Dias Pereira P, Lopes CC, Matos AJ, et al. Estrogens metabolism associated with polymorphisms: influence of COMT G482a genotype on age at onset of canine mammary tumors. *Vet Pathol*. 2008;45(2):124-130.
17. Dias Pereira P, Lopes CC, Matos AJ, et al. Influence of catechol-O-methyltransferase (COMT) genotypes on the prognosis of canine mammary tumors. *Vet Pathol*. 2009;46(6):1270-1274.
18. Misdorp W, Else RW, Hellmen E, Lipscomb TP. *World Health Organization International Histological Classification of Tumors of Domestic Animals*. Vol VII. 2nd ed. Washington, DC: Armed Forces Institute of Pathology; 1999.
19. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. 1991;19(5): 403-410.
20. Santos M, Correia-Gomes C, Marcos R, et al. Value of the Nottingham histological grading parameters and Nottingham prognostic index in canine mammary carcinoma. *Anticancer Res*. 2015;35(7):4219-4227.
21. Canadas A, Santos M, Nogueira A, et al. Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes. *J Vet Diagn Invest*. 2018. https://doi.org/10.1177/1040638718789231. Accessed July 20, 2018.
22. Desmedt C, Sotiriou C, Piccart-Gebhart MJ. Development and validation of gene expression profile signatures in early-stage breast cancer. *Cancer Invest*. 2009;27(1):1-10.
23. Garraway LA, Lander ES. Lessons from the cancer genome. *Cell*. 2013; 153(1):17-37.
24. Stover DG, Wagle N. Precision medicine in breast cancer: genes, genomes, and the future of genomically driven treatments. *Curr Oncol Rep*. 2015;17(4):15.
25. Kuo SH, Yang SY, You SL, et al. Polymorphisms of ESR1, UGT1A1, HCN1, MAP3K1 and CYP2B6 are associated with the prognosis of hormone receptor-positive early breast cancer. *Oncotarget*. 2017; 8(13):20925-20938.
26. Yersal O, Barutca S. Biological subtypes of breast cancer: prognostic and therapeutic implications. *World J Clin Oncol*. 2014;5(3):412-424.
27. Hunt R, Sauna ZE, Ambudkar SV, Gottesman MM, Kimchi-Sarfaty C. Silent (synonymous) SNPs: should we care about them? *Methods Mol Biol*. 2009;578:23-39.
28. Deng N, Zhou H, Fan H, Yuan Y. Single nucleotide polymorphisms and cancer susceptibility. *Oncotarget*. 2017;8(66):110635-110649.
29. Nackley AG, Shabalina SA, Tchivileva IE, et al. Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science*. 2006;314(5807): 1930-1933.
30. Cooper DN. Functional intronic polymorphisms: buried treasure awaiting discovery within our genes. *Hum Genomics*. 2010;4(5): 284-288.
31. Ramirez-Bello J, Jimenez-Morales M. Functional implications of single nucleotide polymorphisms (SNPs) in protein-coding and non-coding RNA genes in multifactorial diseases. *Gaceta Medica de Mexico*. 2017; 153(2):238-250.
32. Ul Hussain M. Micro-RNAs (miRNAs): genomic organisation, biogenesis and mode of action. *Cell Tissue Res*. 2012;349(2):405-413.
33. Darabi H, Czene K, Wedren S, et al. Genetic variation in the androgen estrogen conversion pathway in relation to breast cancer prognosticators. *Breast Cancer Res Treat*. 2011;127(2):503-509.
34. Bai X, Xie J, Sun S, Zhang X, Jiang Y, Pang D. The associations of genetic polymorphisms in CYP1A2 and CYP3A4 with clinical outcomes of breast cancer patients in northern China. *Oncotarget*. 2017; 8(24):38367-38377.
35. Feigelson HS, Henderson BE. Future possibilities in the prevention of breast cancer: role of genetic variation in breast cancer prevention. *Breast Cancer Res*. 2000;2(4):277-282.
36. Stemke-Hale K, Hennessy B, Mills GB, Mitra R. Molecular screening for breast cancer prevention, early detection, and treatment planning: combining biomarkers from DNA, RNA, and protein. *Curr Oncol Rep*. 2006;8(6):484-491.

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## CHAPTER 07

# Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study

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# Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study

Ana Canadas[1], Miguel França[2], Cristina Pereira[3], Raquel Vilaça[4], Hugo Vilhena[5,6,7], Flora Tinoco[8], Maria João Silva[9], Jorge Ribeiro[2], Rui Medeiros[10], Pedro Oliveira[11], Patrícia Dias-Pereira[1], and Marta Santos[12]

## Abstract

Histopathology remains the cornerstone for diagnosing canine mammary tumors (CMTs). Recently, 2 classification systems (the World Health Organization [WHO] classification of 1999 and the proposal of 2011) and 2 grading methods based on the human Nottingham grade have been used by pathologists. Despite some evidence that the histological subtype and grade are prognostic factors, there is no comprehensive comparative study of these classification and grading systems in the same series of CMTs. In this study, the 2 classifications and the 2 grading methods were simultaneously applied to a cohort of 134 female dogs with CMTs. In 85 animals with malignant tumors, univariable and multivariable survival analyses were performed. Using the 2 systems, the proportion of benign (161/305, 53%) and malignant (144/305, 47%) tumors was similar and no significant differences existed in categorization of benign tumors. However, the 2011 classification subdivided malignant tumors in more categories—namely, those classified as complex, solid, and tubulopapillary carcinomas by the WHO system. Histological subtype according to both systems was significantly associated with survival. Carcinomas arising in benign tumors, complex carcinomas, and mixed carcinomas were associated with a better prognosis. In contrast, carcinosarcomas and comedocarcinomas had a high risk of tumor-related death. Slight differences existed between the 2 grading methods, and grade was related to survival only in univariable analysis. In this cohort, age, completeness of surgical margins, and 2 index formulas adapted from human breast cancer studies (including tumor size, grade, and vascular/lymph node invasion) were independent prognostic factors.

## Keywords

canine mammary tumors, classification, grade, prognosis, survival

[1]Department of Pathology and Molecular Immunology, Instituto Ciências Biomédicas Abel Salazar, ICBAS, UPorto, University of Porto, Porto, Portugal
[2]UPVet—Veterinary Hospital, Instituto Ciências Biomédicas Abel Salazar, ICBAS, UPorto, University of Porto, Porto, Portugal
[3]PetzAnimal Veterinary Clinic, Matosinhos, Portugal
[4]Custóias Veterinary Clinic, Matosinhos, Portugal
[5]Baixo Vouga Veterinary Hospital, Águeda, Portugal
[6]Department of Veterinary Sciences, Vasco da Gama University School, Coimbra, Portugal
[7]Animal and Veterinary Research Center (CECAV), University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[8]Dra. Flora Tinoco Veterinary Clinic, Maia, Portugal
[9]Matosinhos Veterinary Clinic, Matosinhos, Portugal
[10]Molecular Oncology and Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal
[11]Department of Populations Studies, Instituto Ciências Biomédicas Abel Salazar, ICBAS, UPorto, University of Porto, Porto, Portugal
[12]Department of Microscopy, Instituto Ciências Biomédicas Abel Salazar, ICBAS, UPorto, University of Porto, Porto, Portugal

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Corresponding Author:
Marta Santos, Department of Microscopy, Instituto Ciências Biomédicas Abel Salazar, ICBAS, UPorto, University of Porto, Rua de Jorge Viterbo Ferreira, 228, Porto 4050, Portugal.
Email: mssantos@icbas.up.pt

Canine mammary tumors (CMTs) are the most frequent neoplasm in intact female dogs. Despite the efforts made on CMT research, which have contributed to a remarkable increase in knowledge of their biology, histological evaluation remains the cornerstone for classifying and grading CMTs. The intrinsic morphological heterogeneity of these tumors, with a frequent involvement of different cell populations, challenges the pathologist's task of providing an accurate classification. For nearly 2 decades, most veterinary pathologists used the official histological classification for CMTs endorsed by the World Health Organization (WHO),[18]which included 6 benign tumor categories and 14 malignant subtypes. Several studies have underlined the prognostic value of this classification system,[3,14,18,21] while other authors have not found significant associations between histological type and survival variables.[5,22] More recently, Goldschmidt and coworkers[9] presented an alternative histological classification scheme for CMTs, recognized as the 2011 classification, which emphasizes architectural arrangements of neoplastic cells and involvement of myoepithelial cells in the neoplastic process. The 2011 classification is a complete and exhaustive classification scheme,[13] with 7 benign and 23 malignant subtypes,[9] and its prognostic value was recently demonstrated,[19,20] emerging also as an independent prognostic factor by multivariate analysis of a prospective cohort of CMTs.[20] Currently, veterinary pathologists face uncertainty regarding the histological classification of CMTs as it is not possible to compare the prognostic relevance of these histological classification systems based on published data, since they do not refer to the same cohorts of animals. Moreover, to our knowledge, there are no published reports comparing the prognostic value of the WHO classification and of the 2011 classification in the same series of female dogs with CMTs.

Traditionally, the grading of malignant CMTs has followed that of the human counterparts. In a reference book, Misdorp[17] adopted the numerical human grading method of Bloom and Richardson (1957),[2] which included 3 parameters: tubule formation, nuclear pleomorphism, and number of mitoses/hyperchromatic nuclei per high-power field. Each parameter is scored from 1 to 3 and the overall grade determined by the final combined score. However, during the past decade, the most used grading system for CMTs has been the so-called Nottingham histological grade (NHG) described by Elston and Ellis[8] in 1991. This method is a refinement of the Bloom and Richardson method, also including the assessment of tubule formation, nuclear pleomorphism, and mitotic counts. In NHG, the evaluation of tubule formation is semiquantitative (with percentages) and mitotic counts are scored considering the field area of the microscope used (Suppl. Table S1).[8] In CMTs, the NHG has been associated in univariate analysis with disease progression and cancer-related death, especially when grade I plus grade II were compared to grade III.[14,21,22] Recently, our group demonstrated that the computation of an index formula, adapted from the well-known human Nottingham Prognostic Index[11,15] and that included NHG, tumor size, and a variable related to the spread of the disease (vascular/lymph node invasion), had prognostic value in CMTs.[22] The diversity of cellular populations involved in CMTs, which often includes luminal epithelium, myoepithelium and mesenchymal components, and the great heterogeneity of their histological patterns, also challenged veterinary pathologists when grading the tumors. In a recent proposed grading scheme, Peña and colleagues[19] attempted to circumvent this by including all cell types in the assessment of the histological grade of CMTs. These authors described a canine adaptation of the human Nottingham histological grade (ca-NHG) system, which included modifications in the assignment of the parameters nuclear pleomorphism and mitotic count[19] (Suppl. Table S1). Recent studies demonstrated that ca-NHG provided independent prognostic information,[3,16,19] while others did not confirm that observation.[20] At this point, the number of CMTs series addressing the prognostic value of grading systems is relatively small, and to our knowledge, a comparative prognostic study of the NGH and ca-NHG is still missing. The aims of the present study were (1) to compare the WHO and 2011 histological classification systems in a prospective series of CMTs to identify advantages and limitations of each system when applied to diagnostic practice, (2) to evaluate the association of the histological diagnosis (performed according to each classification system) and histological grade (based on NHG and ca-NHG methods) with the overall survival, and (3) to evaluate the association between other relevant clinicopathological variables and survival.

## Materials and Methods

### *Study Population and Histological Analysis*

A cohort of female dogs with spontaneous CMTs diagnosed between January 2015 and July 2016 in the Veterinary Pathology Laboratory—ICBAS-UP at the University of Porto was prospectively included in this study. Dogs included in this study underwent surgery as the only treatment. All animals were treated with radical unilateral mastectomy (removal of an entire unilateral mammary chain) and/or partial mastectomy (removal of 1 to 3 glands, including the affected ones). Owners provided consent for surgery with curative intent, as well as the use of the material for research purposes. This protocol was approved by the institutional Ethics Committee (P151/2016).

Samples were immediately fixed in 10% buffered formalin after the surgery and routinely processed for histopathology. All the slides resulting from at least the largest transverse section of each tumor were screened (mean of 2 slides [range, 1–7] per benign tumor and mean of 4 slides [range, 1–18] per malignant tumor). All the regional and/or intramammary lymph nodes identified in the mastectomy specimens were sectioned (2 to 3 longitudinal sections), embedded, and submitted to histopathological examination. The presence of a malignant myoepithelial component was confirmed by immunohistochemistry using a panel of mouse monoclonal antibodies, including pancytokeratin (clone AE1/AE3 [Invitrogen, Camarillo, CA], 1:300 dilution, antigen retrieval in water bath with

Target Retrieval Solution [Dako, Glostrup, Denmark]), vimentin (clone V9, Dako, 1:500 dilution, water bath with Target Retrieval Solution [Dako]), smooth muscle actin (clone HHF35, Dako, 1:300 dilution, water bath with Target Retrieval Solution [Dako]), and p63 (clone 4A4, Ventana, Tucson, AZ, ready-to-use, antigen retrieval in pressure cooker with sodium citrate buffer). The histological classifications and grades were assigned at the same time, blinded to survival outcomes. The histological diagnosis was established by consensus in a joint review by 3 pathologists (A.C., M.S., and P.D.-P.) using a multihead microscope, according to the WHO[18] and to the 2011 classification[9] systems. Histological grading was performed by consensus of the same 3 pathologists who classified the tumors, based on the criteria defined by the NHG[8] and by the ca-NHG method.[19] In the former, grading parameters were evaluated only in epithelial cells and sarcomas were excluded. Following the original publication,[19] inflammatory carcinomas, carcinosarcomas, and sarcomas were not graded by the ca-NHG method. The cutoffs for establishing the score of the mitotic counts of the NHG were adjusted to the high-power field (HPF) of the microscope (Nikon E600 Eclipse with a field diameter of 0.55 mm, HPF area of 0.237 $mm^2$; Nikon, Tokyo, Japan), and the counting was performed in 10 HPFs that the observers judged to be the most mitotically active parts of the tumor (avoiding areas with necrosis and large amounts of stroma). This approach followed the current guidelines for human breast carcinomas.[7] Vascular invasion was defined as the presence of tumor emboli within endothelial-lined spaces without distinguishing between lymphatic and blood vessels. The lymph node was considered positive for metastases when 1 or more groups of 2 or more atypical, nonlymphoid and nonhematopoietic cells were seen in the subcapsular sinus or elsewhere within the node. In addition, the Nottingham Prognostic Index was adapted for use in CMTs from a human breast cancer study[11] and computed as veterinary-adapted Nottingham prognostic index, vet-NPI = (tumor size [cm] × 0.2) + NHG (1, 2, or 3, respectively, for grades I, II, and III) + evidence of vascular invasion and/or regional lymph node metastases (1 or 2, respectively, if absent or present), as previously described.[22] A similar index formula was computed but excluding the information about lymph node metastasis (veterinary-adapted, vascular-based Nottingham prognostic index vet-NPI-V).

### Follow-up Study and Survival Data

The follow-up data were obtained by contacting the referring veterinarian and from the medical records. Disease-specific overall survival (OS) was calculated from the date of diagnosis to the date of the animal's death/euthanasia due to the neoplastic disease. Animals that died or were euthanized for causes unrelated to the mammary tumor and those that were lost to follow-up were censored, respectively, at the time of death or at the time of their last clinical examination. In the subgroup of cases with multiple malignant tumors, a reference lesion was assigned for the survival study. Based on previous studies and evidence, the reference lesion was considered the tumor presenting peritumoral vascular invasion (primary criterion) or the one with the highest nuclear pleomorphism (secondary criterion) or, if necessary, the one with the largest diameter (tertiary criterion).[22] Necropsy was performed upon the owner's consent.

### Statistical Analysis

The concordance of the histological diagnosis in benign tumors according to the 2 classification systems was tested using the McNemar-Bowker test. Fisher exact method and $\chi^2$ test were used to assess the association between clinicopathological variables and number of disease-related deaths. Receiver operating characteristic (ROC) curves were used to assess the sensitivity and specificity of the vet-NPI and vet-NPI-V for predicting OS. Disease-specific survival curves were computed using the Kaplan-Meier product-limit estimates method, with log-rank (Mantel-Cox) tests being used to estimate the differences in survival fractions according to several clinicopathological parameters. Cox proportional hazard regression models were used to evaluate the prognostic role of the clinicopathological variables in univariable and multivariable analyses. Supplemental Table S2 describes the clinicopathological variables included in the regression analyses. Only the variables that proved to be significant in the univariable analysis were included in the multivariable regression models. Backward stepwise (Wald) selection was performed to arrive at the most parsimonious model. Cox regression coefficients were used to estimate the hazard of each independent variable of the model. The 95% confidence interval (CI) is given for all variables that figured in the final Cox regression model. The survival analyses were performed in the sample of cases with at least 1 malignant tumor for which follow-up data were available. All the analyses were performed using SPSS Statistics, version 24 (SPSS, Inc, an IBM Company, Chicago, IL). Statistical significance was set at 5%.

## Results

### Histopathologic Descriptive Findings

This study included 134 female dogs aged 5 to 18 years (mean, 9.9 years). In this cohort, 23 of 134 (17%) were spayed, 84 of 134 (63%) were nulliparous, and only 4 of 134 (3%) female dogs had been under contraceptive medication.

The number of cases assigned to each histological subtype, according to each classification system, is presented in Tables 1 and 2. In this cohort, multiple tumors were diagnosed in 80 of 134 (60%) of the cases. The proportion of benign and malignant tumors (161/305 [53%] vs 144/305 [47%], respectively) in this series was similar, irrespective of the classification system employed. According to both classification methods, the most frequent benign and malignant tumors were complex adenoma (55/161 [34%] in both classification systems) and complex carcinoma (49/144 [34%] and 23/144 [16%] according to the WHO and to the 2011 classification, respectively). Within the

**Table 1.** Histological Subtypes of 161 Benign Canine Mammary Tumors Classified According to the World Health Organization (WHO) Classification[18] and the 2011 Classification.[9]

| WHO Classification | 2011 Classification |
|---|---|
| Complex adenoma ($n = 55$) | Complex adenoma (adenomyoepithelioma) ($n = 55$) |
| Benign mixed tumor ($n = 40$) | Benign mixed tumor ($n = 40$) |
| Simple adenoma ($n = 37$) | Adenoma, simple ($n = 35$) |
| Basaloid adenoma ($n = 13$) | Ductal (basaloid) adenoma ($n = 14$) |
| Fibroadenoma ($n = 2$) | Fibroadenoma ($n = 2$) |
| Duct papilloma ($n = 14$) | Intraductal papillary adenoma (duct papilloma) ($n = 15$) |

**Table 2.** Histological Subtypes and Grades of 144 Malignant Canine Mammary Tumors.[a]

| | | NHG | | | ca-NHG | | |
|---|---|---|---|---|---|---|---|
| WHO Classification | 2011 Classification | I | II | III | I | II | III |
| Complex carcinoma ($n = 49$) | Carcinoma, complex type ($n = 23$)[b] | 12 | 10 | 1 | 11 | 10 | 1 |
| | Carcinoma, mixed type ($n = 18$) | 12 | 4 | 2 | 12 | 4 | 2 |
| | Carcinoma and malignant myoepithelioma ($n = 6$) | 4 | 2 | — | 3 | 2 | 1 |
| | Adenosquamous carcinoma ($n = 1$) | 1 | — | — | 1 | — | — |
| | Intraductal papillary carcinoma ($n = 1$) | — | 1 | — | 1 | — | — |
| Tubulopapillary carcinoma ($n = 31$) | Carcinoma, tubulopapillary ($n = 10$) | 3 | 6 | 1 | 3 | 6 | 1 |
| | Intraductal papillary carcinoma ($n = 6$) | 2 | 4 | — | 3 | 3 | — |
| | Carcinoma, cystic-papillary ($n = 4$) | 2 | 2 | — | 2 | 2 | — |
| | Carcinoma, tubular ($n = 4$) | 3 | 1 | — | 3 | 1 | — |
| | Adenosquamous carcinoma ($n = 3$) | — | 3 | — | — | 3 | — |
| | Comedocarcinoma ($n = 2$) | — | 2 | — | — | 2 | — |
| | Micropapillary carcinoma ($n = 1$)[b] | — | 1 | — | — | — | — |
| | Carcinoma of the nipple ($n = 1$) | 1 | — | — | 1 | — | — |
| Solid carcinoma ($n = 20$) | Carcinoma, solid ($n = 8$)[b] | 1 | 1 | 6 | 1 | 1 | 5 |
| | Adenosquamous carcinoma ($n = 6$) | — | 1 | 5 | — | 1 | 5 |
| | Comedocarcinoma ($n = 4$) | — | — | 4 | — | — | 4 |
| | Carcinoma, cribriform ($n = 2$) | — | 1 | 1 | — | 1 | 1 |
| Carcinoma in benign tumor ($n = 16$) | Carcinoma arising in a complex adenoma ($n = 11$) | 9 | 2 | — | 9 | 2 | — |
| | Carcinoma arising in a mixed tumor ($n = 5$) | 5 | — | — | 5 | — | — |
| Carcinosarcoma ($n = 8$) | Carcinosarcoma ($n = 8$) | 4 | 3 | 1 | — | — | — |
| Sarcoma ($n = 7$) | Sarcoma ($n = 7$) | — | — | — | — | — | — |
| Squamous cell carcinoma ($n = 3$) | Squamous cell carcinoma ($n = 1$) | — | 1 | — | — | 1 | — |
| | Adenosquamous carcinoma ($n = 2$) | — | — | 2 | — | — | 2 |
| Sarcoma in benign tumor ($n = 3$) | Sarcoma ($n = 3$) | — | — | — | — | — | — |
| Carcinoma in situ ($n = 3$) | Carcinoma in situ ($n = 3$) | — | — | — | — | — | — |
| Spindle cell carcinoma ($n = 2$) | Spindle cell carcinoma (malignant myoepithelioma) ($n = 2$) | 1 | 1 | — | — | 2 | — |
| Mucinous carcinoma ($n = 1$) | Adenosquamous carcinoma ($n = 1$) | — | 1 | — | — | 1 | — |
| Anaplastic carcinoma ($n = 1$) | Anaplastic carcinoma ($n = 1$) | — | 1 | — | — | 1 | — |

Abbreviations: ca-NHG, canine-adapted Nottingham Histological Grading; NHG, Nottingham Histological Grading; WHO, World Health Organization; —, no cases.

[a] Histologic subtypes were classified according to the WHO classification[18] and the 2011 classification.[9] Histologic grades were assigned according to the NHG method[8] and the ca-NHG method.[19]

[b] One case with clinicopathological features of inflammatory carcinoma was not graded by the ca-NHG system.

group of benign tumors, there were no significant differences between the 2 systems (Table 1). Only 2 WHO simple adenomas were reclassified by the 2011 classification, one as ductal (basaloid) adenoma and the other as intraductal papillary adenoma. One simple adenoma (WHO classification) was reclassified as ductal (basaloid) adenoma by the 2011 classification, as it presented slit-like lumina and bilayered cords with columnar luminal cells. Another simple adenoma was reclassified as intraductal papillary adenoma according to the 2011 classification, as multifocal intraductal growth with peripheral sclerosis and hyalinization was observed (features not described for duct papilloma in the WHO classification system).

In the group of malignant neoplasms, several differences in the histological diagnoses were found between the classification schemes (Table 2). Tumors diagnosed as complex carcinomas by the WHO system were divided by the criteria of the

2011 classification mainly into complex carcinoma, mixed carcinoma, and carcinoma and malignant myoepithelioma (Fig. 1a,b). Moreover, the 2011 classification subdivided the simple carcinomas into several subtypes: WHO tubulopapillary carcinomas were subdivided in 8 categories by the 2011 classification (Suppl. Figs. S1 and S2), and the majority of WHO solid carcinomas were divided into other categories (mainly adenosquamous carcinoma and comedocarcinoma) (Table 2, Fig. 2). The number of carcinomas in situ, carcinosarcomas, carcinomas arising in benign tumors, and anaplastic carcinomas was the same for both classifications. As the 2011 classification did not consider the category sarcoma arising in benign tumors (as the WHO classification did), these tumors were included in the sarcoma group. Another novel category of the 2011 classification was carcinoma of the nipple, and 1 case in this series was consistent with this entity. In addition, in 3 carcinomas (1 micropapillary, 1 solid, and 1 complex carcinoma), for which the clinician reported inflammatory signs, the histological hallmark of inflammatory carcinomas defined in the 2011 classification (ie, neoplastic emboli obliterating the dermal lymphatic vessels) was present.

At the time of diagnosis, 14.6% (21/144) of malignant tumors showed peritumoral vascular invasion. One or more lymph nodes (inguinal and/or intramammary) were identified during the macroscopic pathological examination in 75 of 98 cases with at least 1 malignant tumor; of these, lymph node metastases were detected in 20 of 75 cases (26.7%).

### *Histologic Classification—Relationship to Survival*

Two-year follow-up data were available for 85 bitches with at least 1 malignant tumor (53 of 85 had a single malignant tumor, with or without additional benign tumors). Cases fulfilling the criteria of inflammatory carcinoma of the 2011 classification were excluded from survival analysis regarding the histotype defined by the 2011 classification. Of the 85 cases, 26 of 85 (30.6%) died due to disease progression during the follow-up period (necropsy was performed in 4 cases and disseminated metastatic disease was confirmed histologically), 31 of 85 (36.4%) were alive 2 years after the surgery, 10 of 85 (11.8%) died of causes not related to the mammary disease, and 18 of 85 (21.2%) were lost to follow-up.

Both the WHO and the 2011 classification systems were significantly associated with tumor-specific OS ($P < .001$; Figs. 3, 4). According to the WHO classification, cases with complex carcinoma or with carcinoma or sarcoma arising in benign tumor had significantly better prognosis compared to all other subtypes ($P = .002$ and $P = .05$, respectively) (Fig. 5). None of the animals with a carcinoma or sarcoma arising in benign tumor died of neoplastic disease. On the contrary, all 5 animals with carcinosarcoma died of causes related to the disease (Fig. 6), and this subtype had a shorter OS (mean, 12.6 months; 95% CI, 5.6–19.6 months) compared to all other subtypes (mean, 20.0 months; 95% CI, 18.4–21.6 months; $P < .001$). When simple carcinomas (tubulopapillary, solid, and anaplastic) as a single group were compared to all other subtypes, no

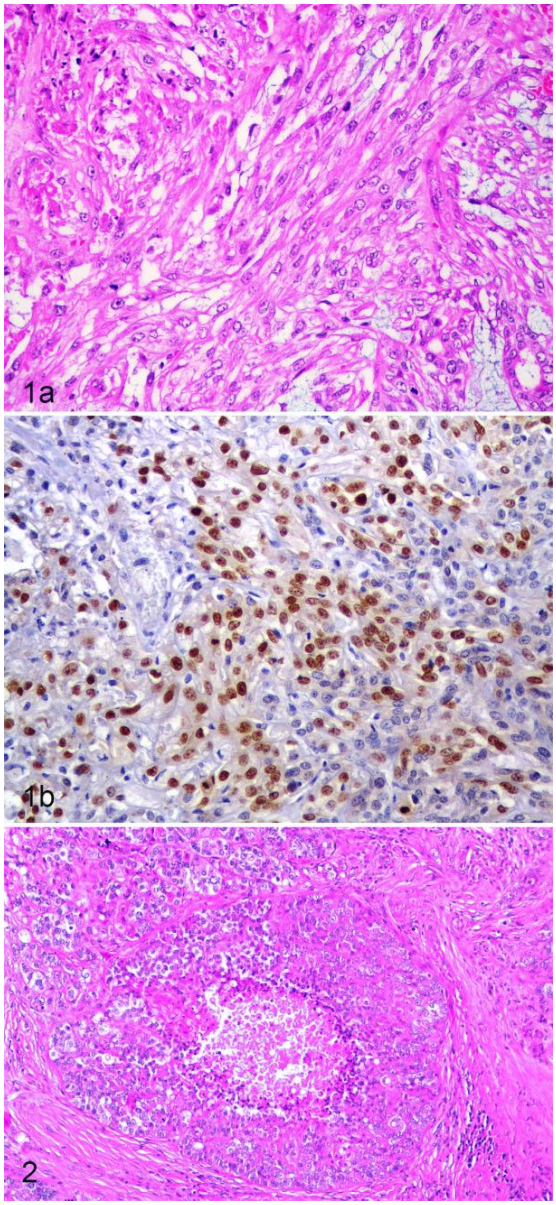



**Figure 1.** Carcinoma and malignant myoepithelioma, mammary gland, dog. (a) This malignant tumor, composed of epithelial cells arranged in tubules and fusiform cells, was classified as complex carcinoma by the World Health Organization classification. Hematoxylin and eosin (HE). (b) The fusiform cells display anisokaryosis, mitoses, and immunoreactivity for p63. Thus, the tumor was reclassified as carcinoma and malignant myoepithelioma by the 2011 classification criteria. **Figure 2.** Mammary carcinoma, dog. Solid growth of cells with central necrosis ("comedo") separated by septa of hyaline fibrous stroma. This tumor was classified as solid carcinoma by the World Health Organization classification and was reclassified as comedocarcinoma by the 2011 classification criteria. HE.

statistical association with OS was observed. Moreover, the subdivision of simple carcinomas into solid and tubulopapillary subtypes did not provide prognostic information. No difference was observed between the survival curves of cases with special types of carcinomas (squamous cell, mucinous, and spindle cell carcinomas) and cases with other types of malignant tumors.

Regarding the 2011 classification system, the histological subtypes associated with better outcome ($P < .001$) were complex carcinoma, mixed carcinoma, and carcinoma arising in benign tumor; none of the animals with these tumors died of causes related to the neoplastic disease. On the other hand, carcinosarcoma, comedocarcinoma, and adenosquamous carcinoma had shorter survival ($P < .001$, $P = .005$, and $P = .049$, respectively) (Figs. 7, 8). As with the WHO classification, the OS of cases with simple carcinomas (including tubular, tubulopapillary, cystic-papillary, cribriform, and solid) and intraductal papillary carcinomas or cases with sarcomas (including osteosarcomas) was not significantly different from the survival of cases with other subtypes of malignant tumors.

The association between the histological diagnosis and OS was also evaluated by univariable regression analysis. For that purpose, tumors were regrouped according to the established criteria of the original classification system and/or considering the results of the previously described survival analysis. The defined groups for each histological classification system and the results of the univariable regression analysis are presented in Table 3. In the WHO method, the complex carcinomas were assigned as reference for the computation of the hazard ratio, while in the 2011 classification, the reference lesion was the group of simple carcinomas plus intraductal papillary carcinomas (no event was registered for cases with complex carcinomas, mixed carcinomas, and carcinomas arising in complex adenoma/benign mixed tumor, so these cases were excluded from the regression analysis). The regression analysis confirmed the significant relationship between the WHO classification and OS, and carcinosarcoma was the subtype with the highest risk of disease-specific death. All other subtypes (except carcinomas or sarcomas arising in benign tumors) had a significantly higher risk of cancer-related death compared to complex carcinomas. For the 2011 classification, cases diagnosed with a carcinosarcoma or a carcinoma and malignant myoepithelioma had 4 times higher risk of disease-related death than cases with simple or intraductal papillary carcinomas. No difference was detected in the risk for dogs with other subtypes compared to simple or intraductal papillary carcinomas (Table 3).

Because of the low number of cases included in the present series, it was not possible to perform a meaningful analysis of the subtype-specific outcome of the following histotypes: anaplastic carcinomas, each type within the group of special-type carcinomas (either by the WHO or by the 2011 classification), as well as micropapillary invasive, ductal, and intraductal papillary carcinomas; carcinoma and malignant myoepithelioma; and each of the types of simple carcinomas defined by the 2011 classification.

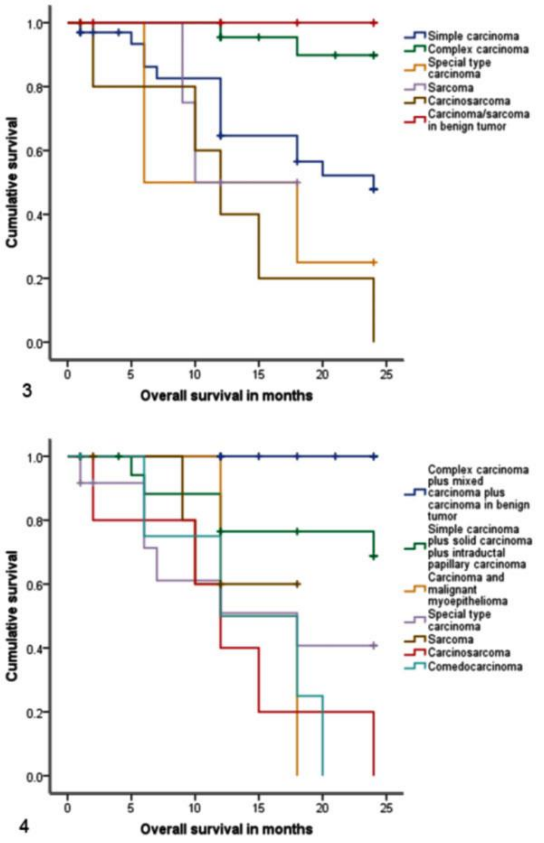
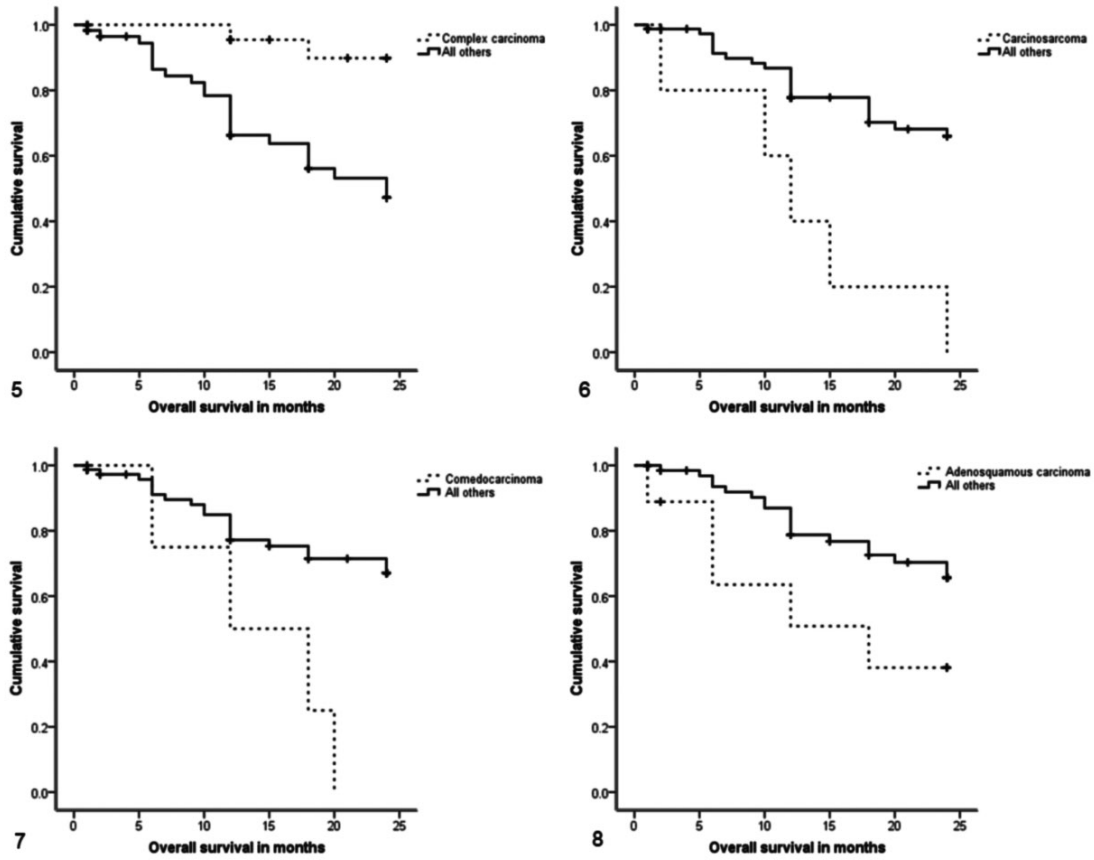



**Figure 3.** Kaplan-Meier overall survival curves according to histological classifications in 85 female dogs with malignant mammary tumors. Diagnoses of tumor type made in accordance with the World Health Organization classification were significantly associated with differences in survival. Compared to other categories, cases of carcinoma or sarcoma arising in benign tumors had significantly longer survival, and cases of carcinosarcoma had significantly shorter survival ($P < .001$). Vertical marks represent censored cases. **Figure 4.** Kaplan-Meier overall survival curves according to histological classifications in 85 female dogs with malignant mammary tumors. The 2011 classification was also significantly associated with differences in survival. Compared to other categories, cases of carcinoma arising in benign tumors, complex carcinoma, and mixed carcinoma had longer survival and cases of carcinosarcoma had shorter survival ($P < .001$). Vertical marks represent censored cases.

## *Histologic Grade and Relationship to Survival*

When the NHG and ca-NHG grading systems were applied to this series of 144 malignant tumors, there were no differences in the final grade or in the scores of each grading parameter in most cases. The few differences observed between the 2 grading systems were related mainly to mitotic count scores, less




**Figures 5–8.** Kaplan-Meier survival curves for canine malignant mammary tumor subtypes. **Figure 5.** Cases diagnosed as complex carcinomas by the World Health Organization (WHO) classification had a significantly better survival time than cases diagnosed with any other histological subtype ($P = .002$). **Figure 6.** Cases diagnosed with carcinosarcoma by the WHO criteria had a shorter overall survival time ($P < .001$). **Figure 7.** Cases diagnosed with comedocarcinoma by the 2011 classification had a poor overall survival compared to cases with other types of mammary carcinoma ($P = .005$). **Figure 8.** Cases of adenosquamous carcinoma tended to have a shorter survival time ($P = .049$). Vertical marks represent censored cases in each group.

frequently to the nuclear pleomorphism scores, and also to the fact that, according to ca-NHG, malignant myoepitheliomas should always be assigned with score 2 in tubule formation. The nuclear pleomorphism scores by NHG and by ca-NHG were different in 2 complex carcinomas (reclassified as carcinoma and malignant myoepithelioma by the 2011 classification). The mitotic counts assigned by ca-NHG were lower in 6 cases compared to the score assigned by NHG, and in 2 cases, the scores assigned by ca-NHG were higher. After the sum of the scores, grade discrepancies were observed in 7 of 144 malignant tumors (4 complex carcinomas according to the WHO system, which were reclassified as carcinoma and malignant myoepithelioma by the 2011 classification, 1 complex carcinoma reclassified as intraductal papillary carcinoma, 1 tubulopapillary carcinoma reclassified as intraductal papillary carcinoma, and 1 spindle cell carcinoma reclassified as malignant myoepithelioma). Considering only the cases with follow-up data, the distribution of the grades according to the criteria of the NHG and ca-NHG methods is shown in Tables 4 and 5.

The histological grade was significantly associated with OS for both grading methods; OS was longer for cases with grade I tumors compared to cases with grade II or grade III tumors ($P = .019$ for NHG and $P = .005$ for ca-NHG) (Figs. 9, 10). The grading parameters of tubule formation and nuclear pleomorphism were related to the survival outcome, irrespective of the grading method employed ($P = .036$ for tubule formation by NHG and ca-NHG, respectively; $P = .006$ and $P = .02$ for nuclear pleomorphism by NHG and ca-NHG, respectively).

**Table 3.** Univariable Cox Regression Analysis of the Association Between Histological Subtypes (According to the WHO[18] and the 2011 Classification[9] Systems) and Overall Survival in 85 Female Dogs With Malignant Mammary Tumors.

| | Univariable Analysis | | |
|---|---|---|---|
| Variable | HR | 95% CI | *P* Value |
| WHO classification subtypes | | | |
| Complex carcinoma | Reference | — | .017 |
| Solid carcinoma | 6.75 | 1.36–33.47 | .019 |
| Tubulopapillary carcinoma | 6.28 | 1.30–30.25 | .022 |
| Carcinosarcoma | 20.01 | 3.85–104.04 | <.001 |
| Sarcoma (including osteosarcoma) | 10.45 | 1.44–75.63 | .020 |
| Carcinoma or sarcoma in benign tumor | — | — | NS |
| Anaplastic, spindle cell, mucinous, squamous cell carcinomas | 16.66 | 3.03–91.72 | .001 |
| 2011 classification subtypes[a] | | | |
| Tubular, tubulopapillary, cystic-papillary, intraductal papillary, and cribriform carcinoma | Reference | — | NS |
| Solid, anaplastic, adenosquamous carcinoma, SCC, malignant myoepithelioma | — | — | NS |
| Comedocarcinoma | — | — | NS |
| Sarcoma (including osteosarcoma) | — | — | NS |
| Carcinosarcoma, carcinoma and malignant myoepithelioma | 4.02 | 1.25–12.86 | .019 |

Abbreviations: CI, confidence interval; HR, hazard ratio; NS, not significant; SCC, squamous cell carcinoma; WHO, World Health Organization; —, not applicable.

[a]As no event was registered for female dogs with complex, mixed carcinomas and carcinomas arising in complex adenoma/benign mixed tumor, these cases were excluded from this analysis; inflammatory carcinomas were also excluded.

Carcinomas with tubule formation scores of 1 or 2 had longer OS compared to those of score 3 ($P = .026$ for NHG and $P = .012$ for ca-NHG). Similar results were obtained for nuclear pleomorphism ($P = .001$ for NHG and $P = .005$ for ca-NHG). No association between mitotic count scores and OS was found for either grading method. The distribution of mitotic count scores was also analyzed within each subtype of tumors: the subgroup of simple, solid, and intraductal papillary carcinomas by the 2011 classification with mitotic activity scores of $\geq 2$ was associated with a short OS. For all other histological subtypes, mitotic counts had no significant relation with OS.

Peritumoral vascular invasion was observed in 19 cases included in the survival study. Lymph nodes were available in 66 of 85 cases, and metastases were detected in 19 cases (16 of these also had vascular invasion, and 3 had metastases with no evidence of vascular invasion). Vascular invasion and lymph node metastases were both negatively associated with the OS in univariable regression analysis (Suppl. Table S2).

**Table 4.** Relationship of Histologic Subtype According to the WHO Classification[18] and Histologic Grade According to the NHG and ca-NHG Methods, in Female Dogs With Malignant Mammary Tumors Included in the Survival Study.[a]

| | NHG, No. | | | ca-NHG, No. | | |
|---|---|---|---|---|---|---|
| WHO Classification | I | II | III | I | II | III |
| Complex carcinoma | 11 | 14 | 1 | 11 | 13 | 2 |
| Tubulopapillary carcinoma | 5 | 13 | 1 | 6 | 12 | 1 |
| Solid carcinoma | — | 2 | 11 | — | 2 | 11 |
| Anaplastic carcinoma | — | 1 | — | — | 1 | — |
| Spindle cell carcinoma | — | 1 | — | — | 1 | — |
| Squamous cell carcinoma | — | 1 | 2 | — | 1 | 2 |
| Mucinous carcinoma | — | 1 | — | — | 1 | — |
| Carcinosarcoma | 2 | 2 | 1 | — | — | — |
| Carcinoma in benign tumor | 7 | — | — | 7 | — | — |

Abbreviations: ca-NHG, canine-adapted Nottingham Histological Grading; NHG, Nottingham Histological Grading; WHO, World Health Organization; —, no cases.

[a]Carcinoma in situ, sarcoma arising in benign tumors, and sarcomas were not graded by NHG, while carcinoma in situ, carcinosarcomas, and sarcomas were not graded by ca-NHG.

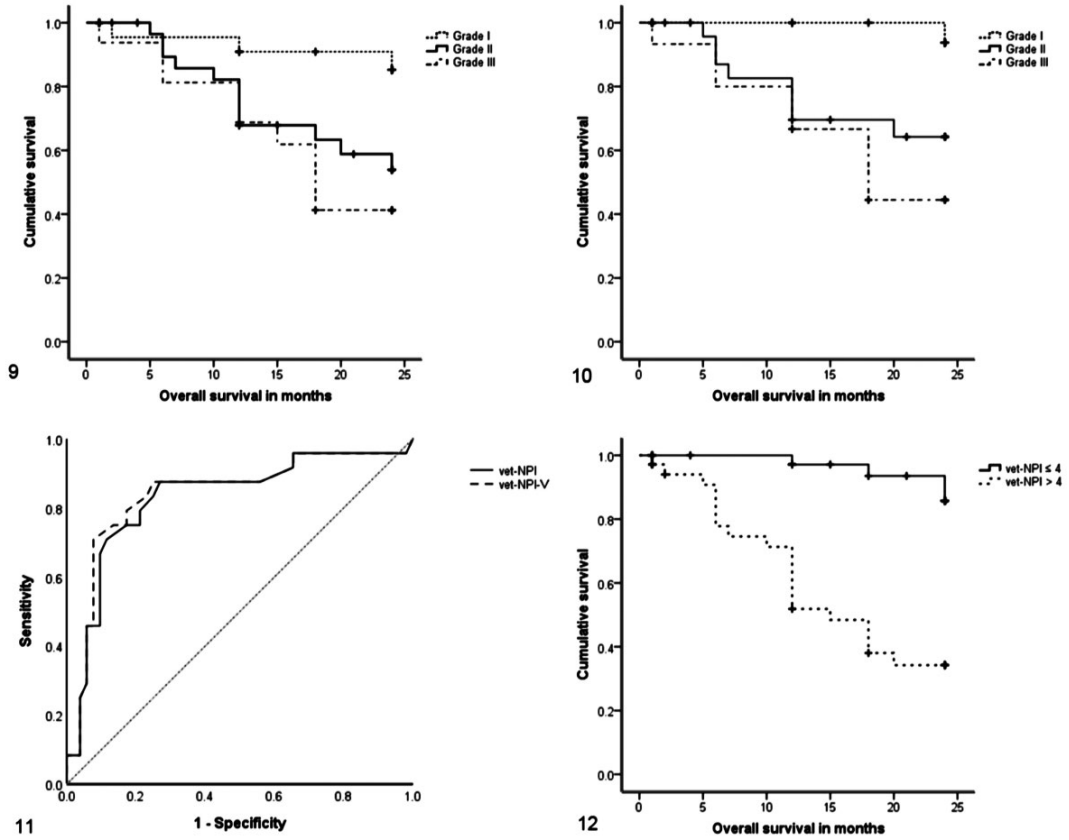
**Table 5.** Relationship of Histologic Subtype According to the 2011 Classification Criteria[9] and Histologic Grade According to the NHG and ca-NHG Methods, in Female Dogs With Malignant Mammary Tumors Included in the Survival Study.[a]

| | NHG, No. | | | ca-NHG, No. | | |
|---|---|---|---|---|---|---|
| 2011 Classification | I | II | III | I | II | III |
| Adenosquamous carcinoma | 1 | 3 | 5 | 1 | 3 | 5 |
| Complex carcinoma | 5 | 9 | 1 | 5 | 9 | 1 |
| Carcinoma and malignant myoepithelioma | — | 2 | — | — | 1 | 1 |
| Carcinoma arising in benign tumor | 7 | — | — | 7 | — | — |
| Mixed carcinoma | 5 | 3 | — | 5 | 3 | — |
| Intraductal papillary carcinoma | — | 3 | — | 1 | 2 | — |
| Tubulopapillary carcinoma | 2 | 3 | 1 | 2 | 3 | 1 |
| Tubular carcinoma | 3 | 1 | — | 3 | 1 | — |
| Anaplastic carcinoma | — | 1 | — | — | 1 | — |
| Cribriform carcinoma | — | 1 | 1 | — | 1 | 1 |
| Squamous cell carcinoma | — | 1 | — | — | 1 | — |
| Cystic-papillary carcinoma | — | 2 | — | — | 2 | — |
| Comedocarcinoma | — | 2 | 3 | — | 2 | 3 |
| Malignant myoepithelioma | — | 1 | — | — | 1 | — |
| Solid carcinoma | — | — | 3 | — | — | 3 |
| Carcinosarcoma | 2 | 2 | 1 | — | — | — |

Abbreviations: ca-NHG, canine-adapted Nottingham Histological Grading; NHG, Nottingham Histological Grading; WHO, World Health Organization; —, no cases.

[a]Carcinoma in situ, sarcoma arising in benign tumors, and sarcomas were not graded by NHG, while carcinoma in situ, carcinosarcomas, and sarcomas were not graded by ca-NHG.

In the descriptive ROC curve analysis, the discriminative power of vet-NPI and vet-NPI-V for identifying tumor-associated death was high (area under the curve [AUC] = 0.83; 95% CI, 0.72–0.94, $P < .001$ for vet-NPI and AUC =




**Figures 9–10.** Overall survival (OS) curves of female dogs with malignant mammary tumors. **Figure 9.** Animals with grade I or II tumors had significantly longer OS compared to animals with grade III tumors according to the Nottingham histological grade (NHG) method ($P = .019$). **Figure 10.** Animals with grade I or II tumors had a better OS compared to animals with grade III tumors according to the canine adapted-NHG ($P = .005$). **Figure 11.** Receiver operating characteristic (ROC) curve for tumor-specific OS in cases based on veterinary-adapted indexes modified from the human Nottingham Prognostic Index (veterinary-adapted Nottingham Prognostic Index [vet-NPI] and veterinary-adapted, vascular-based Nottingham Prognostic Index [vet-NPI-V]). Area under the curve (AUC) = 0.83; 95% confidence interval (CI), 0.72–0.94, $P < .001$ for vet-NPI and AUC = 0.84; 95% CI, 0.73–0.95, $P < .001$ for vet-NPI-V. **Figure 12.** Kaplan-Meier survival curves. Animals with a veterinary-adapted, vascular-based Nottingham Prognostic Index (vet-NPI-V) ≤4 had significantly longer OS compared to animals with vet-NPI-V >4 ($P < .001$). Censoring is indicated by vertical marks.

0.84; 95% CI, 0.73–0.95, $P < .001$ for vet-NPI-V) (Fig. 11). A cutoff of 4.0 for both indexes was selected for statistical purposes. This threshold allowed stratification of cases with significant differences in survival: cases with vet-NPI ≤4.0 had a mean OS of 23.4 months (95% CI, 22.5–24.3 months), whereas vet-NPI >4.0 had a mean and a median OS of 15.3 months (95% CI, 12.6–18.0 months) and 15.0 months (95% CI, 10.8–18.8) ($P < .001$), respectively; cases with vet-NPI-V ≤4.0 had a mean OS of 22.8 months (95% CI, 22.8–24.5 months), whereas vet-NPI-V >4.0 had a mean and a median OS of 15.1 months (95% CI, 12.4–17.7 months) and 15.0 months (95% CI, 11.2–18.8), respectively ($P < .001$) (Fig. 12).

In the final multivariable Cox models (Suppl. Table S2) after exclusion of nonsignificant variables, only age (cut-point of 12 years), surgical margins, vet-NPI, vet-NPI-V, and vascular invasion emerged as independent prognostic factors.

## Discussion

This work represents the first comprehensive comparison of the WHO[18] and the 2011 classification[9] systems, as well as the NHG[8] and the ca-NHG[19] grading methods, performed in the same series of CMTs. Using both classification systems, the proportion of benign and malignant tumors was 4:4.5,

which is in accordance with the existing literature.[10,17,23] The most frequent benign and malignant tumors were complex adenoma and complex carcinoma, respectively, independently of the classification scheme used, thus corroborating findings in other CMTs series.[19,20,24] Within the benign tumors, no significant differences were found between the 2 classification systems. Only 2 simple adenomas categorized by the WHO classification were reclassified differently by 2011 classification. This difference resulted from the fact that in the 2011 classification, the description of some benign tumor subtypes (including basaloid and intraductal papillary adenomas) was more detailed than that found in WHO classification, allowing the observer to appraise other subtle histological features presented in the tumor. As expected, the 2011 classification system subdivided malignant tumors in more categories compared to the WHO classification. This was particularly evident for simple carcinomas; for instance, in our series, the WHO tubulopapillary carcinomas and solid carcinomas were subdivided in 8 and 5 subcategories, respectively, according to the 2011 classification scheme. In our opinion, some of the simple carcinoma categories appeared subjective (such as tubular, tubulopapillary, or ductal carcinomas), while the diagnosis for others seemed relatively straightforward (such as comedocarcinoma). Despite this, in the 2011 classification system, there was no quantitative description of the proportion of specific morphological features, such as squamous or comedo areas, required for the diagnosis of adenosquamous carcinoma or comedocarcinoma. This limitation was also present in the WHO classification regarding other histological subtypes, such as mucinous carcinoma.

Herein, we confirmed the prognostic value of the WHO classification—namely, the better survival time in dogs with complex carcinomas and the worst outcome for those with carcinosarcoma, which is in accordance with findings from other authors.[3,18,21] However, in this series, it was not possible to support the association of mammary sarcomas with a worse prognosis[4,12] or the increased clinical aggressiveness of solid carcinomas described in other studies.[18] It is noteworthy that the carcinomas arising in benign tumors, diagnosed either according to the WHO or to the 2011 classification criteria, were not associated with disease-related deaths in this 2-year follow-up study. This is in line with a previous study[20] and reinforces the evidence that they resemble benign tumors in their biological behavior. However, it should be stressed that in both systems, the criteria for classification of a tumor as a carcinoma arising in benign tumor did not include a description of the number of foci and/or proportion of cells with malignant phenotype. Although further studies are needed to clarify the clinical value of this subtype of CMTs, we recommended that a comment about the expected benign clinical course of these tumors should be included in pathology reports.

Regarding the 2011 classification, the number of cases included in this series in some of the categories was too small to allow separate survival analysis. Considering the tumor categories with at least 5 cases, the 2011 classification clearly identified histological subtypes associated with a good prognosis (such as complex- and mixed-type carcinomas) and tumors associated with a poor survival (such as comedocarcinomas and carcinosarcomas), thus corroborating the findings of a recent study.[20] The 2011 classification scheme was more accurate than the WHO classification scheme, because it identified 2 subgroups (comedocarcinomas and adenosquamous carcinomas) within the WHO solid carcinoma category with a short survival time. Similarly, the 2011 classification scheme identified carcinoma and malignant myoepithelioma as distinctive from other WHO complex carcinomas, and this tumor type has been associated with a more guarded prognosis.[20] The present series included only 2 cases of carcinoma and malignant myoepithelioma with survival data, and both died of tumor-related causes during the follow-up period. This finding, suggests that the simultaneous proliferation of malignant epithelial and malignant myoepithelial cells increases the clinical aggressiveness of CMTs.

The histological grade (according to either the NHG or ca-NHG methods) was associated with OS, but the association was not independent, similar to the findings of some studies[20–22] but contradicting others.[3,19] Variation in the interpretation of grading criteria (including interobserver variability) and different field diameters of the microscopes used in the different studies could have contributed to these discrepancies. To our knowledge, this is the first study where the 2 grading methods were applied to the same cohort of CMTs, and according to our findings, only slight differences existed between them. It is important to note that we excluded hyperchromatic nuclei for the assignment of the grading scores (although they were included in the evaluation of the nuclear pleomorphism of the ca-NHG method) because robust evidence has been built in human breast pathology that hyperchromatic nuclei represent apoptosis rather than mitosis.[7] In addition, the individual prognostic value of hyperchromatic/apoptotic nuclei in CMTs has never been identified, and a recent report failed to detect an association between the expression of apoptosis-related proteins and survival.[6] This exclusion might have contributed to underestimating the grade of some tumors, and this deserves clarification in further studies.

Interestingly, when histological grade, tumor size, and vascular/lymph node invasion were considered together in an index formula adapted from the validated human Nottingham Prognostic Index,[15] they showed an independent prognostic value. This finding confirmed a previous study of our group, where the index formula was adapted to CMTs for the first time.[22] Similar results were obtained when an alternative index formula including vascular invasion but not the lymph node status was computed. Based on these findings, we believe that the results of these prognostic index formulas could be routinely included in pathology reports, thus providing additional relevant prognostic data to clinicians. These index formulas should be validated in other prospective cohorts.

We also investigated the individual value of each of the grading parameters, a subject frequently neglected in prognostic studies of CMTs. In this series, tubule formation and nuclear pleomorphism were associated with OS for both grading methods—namely, when each parameter was classified as a 2-tiered system (score 1 or 2 vs score 3). As in the study by Carvalho and coworkers,[3] none of these parameters retained their association with survival in multivariable models. In addition, in this cohort, the mitotic counts had no prognostic value when all subtypes of tumors were considered. This observation confirmed previous findings from our group[21,22] but contradicted results of others.[3] Interestingly, mitotic activity seemed to be valuable for prognostication only for simple and for intraductal papillary carcinomas defined by the 2011 classification. Thus, the association of the grading parameters and prognosis indicates that they should be included in routine pathology reports of malignant CMTs, in addition to the overall histological grade that is more commonly reported.

In this study, age (<12 years) and the completeness of the surgical margins emerged as independent protective factors in CMTs, as previously reported.[1,12,23] This evidence reinforces the need for early diagnosis and a prompt and adequate surgical treatment.[1] As biologically expected, vascular invasion and the presence of lymph node metastases at the time of the diagnosis were relevant for prognostication in CMTs. However, only vascular invasion retained the prognostic value in the multivariable analyses, as previously reported.[22] The lower statistical significance of the association between lymph node metastasis and OS in Cox multivariable models may have resulted from missing data for this parameter in 19 cases. A similar trend was reported in other CMTs studies.[5,12,21]

A limitation of this study was the relatively small number of cases, which precluded analyzing the association of some histological subtypes (especially those concerning the 2011 classification) with survival. Moreover, bias could have been introduced by obtaining the follow-up data from different clinicians. In addition, some cases enrolled in the survival study presented more than 1 malignant tumor, and only 1 tumor per animal was selected for the statistical analysis.

In conclusion, our findings demonstrated that the CMT classification systems were very similar in terms of the categorization of benign tumors, and both were prognostically relevant by identifying malignant tumors with a good prognosis and others with a high risk of cancer-related death. Large multi-institutional studies are warranted for evaluating the prognostic value of some categories in the 2011 histopathological classification system. Finally, it should be recommended that pathologists include the histotype defined by both classification systems in their routine reports of malignant CMTs. This will support clinicians and oncologists in their clinical and therapeutic decisions, as well as allow large, multi-institutional survival studies necessary for full standardization of the histological assessment of the CMTs.

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## ORCID iD

Marta Santos http://orcid.org/0000-0002-1350-4254

## References

1. Betz D, Schoenrock D, Mischke R, et al. Nolte I: postoperative treatment outcome in canine mammary tumors. Multivariate analysis of the prognostic value of pre- and postoperatively available information. *Tierarztl Prax Ausg K Kleintiere Heimtiere*. 2012;**40**(4):235–242.
2. Bloom HJG, Richardson WW. Histological Grading and Prognosis in Breast Cancer: A Study of 1409 Cases of which 359 have been Followed for 15 Years. *Br J Cancer*. 1957; **11**(3): 359–377.
3. Carvalho MI, Pires I, Prada J, et al. Queiroga FL: Ki-67 and PCNA expression in canine mammary tumors and adjacent nonneoplastic mammary glands: prognostic impact by a multivariate survival analysis. *Vet Pathol*. 2016;**53**(6): 1138–1146.
4. Chang SC, Chang CC, Chang TJ, et al. Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998–2002). *J Am Vet Med Assoc*. 2005;**227**(10):1625–1629.
5. de Las Mulas JM, Millan Y, Dios R. A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Vet Pathol*. 2005;**42**(2):200–212.
6. Dolka I, Krol M, Sapierzynski R. Evaluation of apoptosis-associated protein (Bcl-2, Bax, cleaved caspase-3 and p53) expression in canine mammary tumors: an immunohistochemical and prognostic study. *Res Vet Sci*. 2016; **105**:124–133.
7. Ellis IO, Simpson JF, Reis-Filho JS, et al. Invasive breast carcinoma: introduction and general features: grading. In: Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van der Vijver MJ, eds. *WHO Classification of Tumors of the Breast*. Lyon, France: IARC; 2012:19–20.
8. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer, I: the value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. 1991;**19**(5):403–410.
9. Goldschmidt M, Peña L, Rasotto R, et al. Classification and grading of canine mammary tumors. *Vet Pathol*. 2011;**48**(1):117–131.
10. Goldschmidt H, Peña L, Zappulli V. Tumors of the mammary gland. In: Meuten DJ, ed. *Tumors in Domestic Animals*. 5th ed. Ames, IA: John Wiley; 2017:723–765.
11. Haybittle JL, Blamey RW, Elston CW, et al. A prognostic index in primary breast cancer. *Br J Cancer*. 1982;**45**(3):361–366.
12. Hellmen E, Bergstrom R, Holmberg L, et al. Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases. *Vet Pathol*. 1993; **30**(1):20–27.
13. Im KS, Kim NH, Lim HY, et al. Analysis of a new histological and molecular-based classification of canine mammary neoplasia. *Vet Pathol*. 2014;**51**(3): 549–559.
14. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, et al. Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *J Comp Pathol*. 2005;**133**(4):246–252.

15. Lee AH, Ellis IO. The Nottingham Prognostic Index for invasive carcinoma of the breast. *Pathol Oncol Res*. 2008;**14**(2):113–115.
16. Mainenti M, Rasotto R, Carnier P, et al. Oestrogen and progesterone receptor expression in subtypes of canine mammary tumours in intact and ovariectomised dogs. *Vet J*. 2014;**202**(1):62–68.
17. Misdorp W. Tumors of the mammary gland. In: Meuten DJ, ed. *Tumors in Domestic Animals*. 4th ed. Ames, IA: Iowa State Press; 2002:575–606.
18. Misdorp W, Else RW, Hellmen E, et al. *World Health Organization International Histological Classification of Tumors of Domestic Animals*. Washington, DC: Armed Forces Institute of Pathology; 1999.
19. Peña L, De Andres PJ, Clemente M, et al. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. *Vet Pathol*. 2013;**50**(1):94–105.
20. Rasotto R, Berlato D, Goldschmidt MH, et al. Prognostic significance of canine mammary tumor histologic subtypes: an observational cohort study of 229 Cases. *Vet Pathol*. 2017;**54**(4):571–578.
21. Santos AA, Lopes CC, Ribeiro JR, et al. Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study. *BMC Vet Res*. 2013;**9**:1.
22. Santos M, Correia-Gomes C, Marcos R, et al. Value of the Nottingham histological grading parameters and Nottingham Prognostic Index in canine mammary carcinoma. *Anticancer Res*. 2015;**35**(7):4219–4227.
23. Sorenmo K. Canine mammary gland tumors. *Vet Clin North Am Small Animal Pract*. 2003;**33**(3):573–596.
24. Sorenmo KU, Kristiansen VM, Cofone MA, et al. Canine mammary gland tumours: a histological continuum from benign to malignant; clinical and histopathological evidence. *Vet Comp Oncol*. 2009;**7**(3):162–172.

## CHAPTER 08

# Single Nucleotide Polymorphisms Influence Histological Type and Grade of Canine Malignant Mammary Tumors

**Single Nucleotide Polymorphisms influence histological type and grade of canine malignant mammary tumors**

Ana Canadas-Sousa[a], Marta Santos[b], Rui Medeiros[c], Patrícia Dias-Pereira[a⊥]

[a]*Department of Pathology and Molecular Immunology, Instituto Ciências Biomédicas Abel Salazar, ICBAS – UPorto, University of Porto, Portugal.*

[b]*Department of Microscopy, Instituto Ciências Biomédicas Abel Salazar, ICBAS – UPorto, University of Porto, Portugal*

[c]*Molecular Oncology and Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), Portuguese Oncology Institute of Porto (IPO-Porto), Rua Dr António Bernardino de Almeida, Porto, Portugal*

⊥Corresponding author:
Rua de Jorge Viterbo Ferreira, 228
4050-313 Porto, Portugal
Tel.: +351220428271
E-mail address: pdiaspereira@yahoo.com.br

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## Abstract

Histological examination of canine mammary tumors is mandatory for determining the histological type and grade of the lesions, features that provide relevant information regarding the biological behaviour of the disease. Different histological classification systems and grading methods have been used in canine mammary tumors characterization. In this study, the relationship between the genetic profile of 87 female dogs, considering SNPs in genes known to be involved in mammary carcinogenesis, and the histological type and grade of malignant mammary tumors was assessed. Our results demonstrated a significant relation between genetic variation in genes RAD51, BRCA2, CHEK2, HER2, CDH1, COMT and PGR and these morphological features of the mammary lesions. Specifically, SNPs in RAD51 (rs23623251 and rs23642734), CHEK2 (rs397511718), HER2 (rs24537329) and PGR (rs8875007) were related to aggressive histotypes, with moderate to high histological grade. On the other hand, SNPs in BRCA2 (rs23255542), HER2 (rs24537331), CDH1 (rs852280880 and rs850805755) and COMT (rs851328636, rs853133060 and rs85346495) were associated with tumor histotypes of good prognosis and of low histological grade. Our data provides preliminary evidence for a genotypic-phenotypic correlation in mammary tumors in canine species, highlighting the mechanisms of their genesis, development and progression.


## Introduction

Histological examination of canine mammary lesions is still the gold standard for diagnosis and classification. It constitutes an important source of information concerning the clinical management of the patient, namely regarding therapeutic approach and follow-up planning. The recognized high microscopic heterogeneity of canine mammary tumors (CMTs) led to the development of different histological classification systems, a subject that is far from consensus (Hampe *et al.* 1974, Misdorp *et al.* 1999, Goldschmidt *et al.* 2011). For nearly 20 years, the World Health Organization (WHO) histological classification system was widely used by veterinary pathologists to categorize CMTs (Misdorp *et al.* 1999). The prognostic value of this method was underlined by several investigations (Karayannopoulou *et*

*al.* 2005, Santos *et al.* 2013, Carvalho *et al.* 2016), while other authors did not confirm this finding (de Las Mulas *et al.* 2005, Santos *et al.* 2015). A recent new classification scheme for CMTs, highlighting the architectural arrangements adopted by the neoplastic cells and the participation of the myoepithelium, was proposed (Goldschmidt *et al.* 2011). The prognostic value of this classification system was underlined by Peña et al (2013) and Rasotto et al (2017). More recently, our group performed a comprehensive comparison between the WHO and the 2011 histological classification systems in a prospective series of CMTs, confirming the prognostic relevance of both methods, by identifying mammary tumors associated with better prognosis, as well as histological subtypes related to poor survival and high risk of cancer related death (Canadas *et al.* 2019a).

To date, the most used grading system in CMTs is based on the Elston and Ellis method (Nottingham histological grade - NHG) adopted from women breast cancer, which involves the assessment of 3 parameters: tubule formation, nuclear pleomorphism and mitotic counts (Elston *et al.* 1991). Recently, the canine-adapted Nottingham histological grading method (ca-NHG) was described, encompassing slight differences compared to the original NHG method. The main difference between both methods relies in the assessment of all neoplastic cell types (including myoepithelium) to score the grading parameters by the ca-NHG method (Pena *et al.* 2013).

Breast cancer classification has evolved from the traditional classification systems based in morphology and histological grade, into a new era encompassing the molecular stratification of the tumor (Perou *et al.* 2000, Eliyatkın *et al.* 2015, Ahn *et al.* 2016). The categorization of mammary tumors should rely on their subjacent pathogenesis that is known to involve genetic determinants (Song *et al.* 2015, Bettaieb *et al.* 2017, Rakha *et al.* 2017, Russnes *et al.* 2017). In human breast cancer, numerous genetic variants seem to be involved in tumor susceptibility, histological subtype, as well as in the progression and outcome of the disease (Easton *et al.* 2007, Michailidou *et al.* 2017, Milne *et al.* 2017, Wang *et al.* 2017, Lilyquist *et al.* 2018). There is a growing body of evidence associating genetic variation with CMTs risk and with some clinicopathological features of the tumors (Dias Pereira *et al.* 2008, Borge *et al.* 2013, Canadas *et al.* 2018a, Canadas *et al.* 2018b), including the histological grade and grading parameters (Canadas *et al.*

2019a). However, the influence of the genetic profile in CMTs histotype is largely unknown.

Our aim is to investigate the relationship between SNPs in genes known to be involved in mammary carcinogenesis and the histotype and grade of malignant CMTs.

## Materials and Methods

Eighty-seven female dogs with malignant mammary tumors were included in this study. All animals were treated with radical unilateral and/or partial mastectomy. Owners provided consent for surgery with curative intents, as well as for the use of the material for research purposes, being the protocol approved by the Ethics Committee of the Institute of Biomedical Sciences Abel Salazar, University of Porto (P151/2016). After surgery, tissue samples were immediately fixed in a 10% buffered formalin solution and routinely processed for histopathological examination. The histological diagnosis was established by consensus of 3 pathologists (ACS, MS and PDP) in a multi-head microscope, according to the World Health Organization histological classification of mammary tumors of dogs and cats (Misdorp *et al.* 1999) and to the 2011 classification system (Goldschmidt *et al.* 2011). In cases of dogs with multiple malignant tumors, a reference lesion was assigned for the statistical study. The reference lesion was considered as the tumor presenting peritumoral vascular invasion (primary criterion), or the one with the highest nuclear pleomorphism (secondary criterion), or the one with the largest diameter (tertiary criterion). Whenever necessary, immunohistochemistry was performed as previously reported, in order to confirm the histogenesis of the neoplastic cells (Goldschmidt *et al.* 2011, Canadas *et al.* 2019a). Based on the data from previous survival studies (Rasotto *et al.* 2017, Canadas *et al.* 2019a) malignant mammary tumors were divided into 3 groups (G1, G2 and G3) corresponding to different levels of aggressiveness (Table 1). Histological grading was performed based on the Nottingham histological grading method - NHG (Elston *et al.* 1991) and on the canine-adapted Nottingham histological grading method – ca-NHG (Pena *et al.* 2013). For statistical purposes, the 3 histological grades were considered independently - Grade I (low, well-differentiated tumor) vs Grade II (intermediate, moderately-differentiated tumor) vs Grade III (high, poorly

differentiated tumor) - as well as grouped (Grades I + II vs Grade III; Grade I vs Grades II + III).

Genomic DNA was extracted from peripheral blood samples (obtained by standard venipuncture) using High Pure PCR Template preparation kit (Roche). The DNA quality was evaluated by measuring the optical density and the quantity was assessed employing the NanoDrop 1000 Spectrophotometer. SNP genotyping was performed using MassARRAY iPLEX Gold Technology at the *Unidade de Genómica/Serviço de Genotipagem do Instituto Gulbenkian de Ciência.*

Based on the high complexity of the mammary neoplastic transformation, SNPs in different gene families, namely proto-oncogenes (HER2), tumor suppressor genes (STK11, CDH1), genes involved in DNA damage recognition and repair (BRCA1, BRCA2, BRIP, CHEK2, PTEN, RAD51) and in hormonal metabolism (ESR1, COMT, PGR, PRLR) were included in this study (Table 2).

Statistical analysis of data was performed (SPSS for Windows v.18, SPSS, Chicago, IL). Chi-square analysis was used to compare categorical variables. A 5% level was considered to define statistical significance.

## Results

A significant association between SNPs in RAD51 gene (rs23623251 and rs23642734) and tumor WHO histotypes related to worse prognosis was observed (p= 0.005 and p= 0.016 for rs23623251 and rs23642734, respectively) (Table 3). Nearly 91% of G3 tumors were found in variant allele carriers. None of the other SNPs assessed in this study were associated with the tumor histological subtype as defined by the WHO system (Table 3).

When tumors were classified according to the 2011 proposal, an association was found between SNPs in genes RAD51 (rs23642734), HER2 (rs24537329) and PGR (rs8875007) and tumor histotypes related to a worse prognosis (p= 0.011, p= 0.046 and p= 0.029, respectively). Indeed, 77%, 92% and nearly 62% of the G3 tumors, respectively, were found in variant allele carriers for these genetic variants (Table 3). On the other hand, SNPs in COMT (rs851328636 and rs853133060), HER2 (rs24537331) and BRCA2 (rs23255542) genes were related to less aggressive tumor histotypes (p= 0.027, p= 0.049, p= 0.049 and p= 0.019, respectively). Only 15% of the G3 tumors were observed in variant allele carriers for

these SNPs in COMT gene. Furthermore, 69% and 83% of the G1 tumors were diagnosed in carriers of genetic variations in genes HER2 (rs24537331) and BRCA2 (rs23255542), respectively (Table 3). None of the other SNPs evaluated were significantly associated with tumor histotypes based on the 2011 classification.

No statistically significant association was observed between any of the SNPs included in this study and histological grading of CMTs (irrespective of the method employed), when the 3 histological grades were considered independently. When tumors were graded based on the NHG method, a significant association was observed between SNP in RAD51 gene (rs23642734) and intermediate/high grade neoplasms (p= 0.029). Nearly 62% of grade II and II tumors were found in variant allele carriers for this SNP (Table 4). On the contrary, SNPs in genes COMT (rs85346495), CDH1 (rs852280880 and rs850805755) and BRCA2 (rs23255542) were significantly associated with low-grade mammary carcinomas (p= 0.048, p= 0.045, p= 0.045 and p= 0.038, respectively). In fact, only 11% and 5% of Grade II/III tumors were observed in carriers of genetic variations in COMT and CDH1 genes, respectively. Besides, 81% of the well-differentiated tumors were diagnosed in variant allele carriers for BRCA2 gene. The association between rs85346495 in COMT gene and histological grade was confirmed when Grade I and II tumors were considered together (p= 0.031); indeed, none of the variant allele carriers for this SNP developed high histological grade neoplasms (Table 4).

Additionally, a significant association between SNPs in genes RAD51 (rs23642734; p= 0.048) and CHEK2 (rs397511718; p= 0.035) and intermediate/high grade mammary carcinomas (assessed according to the ca-NHG method) was documented (Table 4). Nearly 78% and 81% of variant allele carriers for these SNPs in RAD51 and in CHEK2 genes, respectively, developed Grade II or Grade III carcinomas. On the other hand, the SNP rs85346495 in COMT gene was significantly associated (p= 0.030) with low grade carcinomas, as all variant allele carriers exhibiting grade I carcinomas (Table 4).

## Discussion

CMTs represent a heterogeneous disease, comprising diverse molecular changes, histological features, clinical expressions and biological behaviors (Sorenmo 2003). Histopathological examination of CMTs play a determinant role in the phenotypic characterization of the lesions, allowing proper histological classification and grading (Elston *et al.* 1991, Misdorp *et al.* 1999, Goldschmidt *et al.* 2011, Pena *et al.* 2013). Mammary tumor phenotypes can be further defined on the basis of the molecular characteristics of the lesion. The appropriated categorization of mammary neoplasms provides relevant prognostic information that can assist in the clinical management of the disease. Recently, the application of molecular techniques to human breast cancer research has underlined a correlation between genetic changes and histopatological features of the lesions. Indeed, several genetic alterations, mainly in tumor suppressor genes and oncogenes, were reported and linked to specific characteristics of breast cancer, providing insights into the molecular basis of breast cancer morphological phenotypes (Tsuda 2009, Heng *et al.* 2017).

In this study we assessed the relationship between the animal genetic profile and the histological type and grade of CMTs. The two most widely used systems for histological classification (Misdorp *et al.* 1999, Goldschmidt *et al.* 2011) and for grading (Elston *et al.* 1991, Pena *et al.* 2013) of CMTs were employed. Our results demonstrated a significant relation between genetic variation in genes known to be involved in mammary carcinogenesis (namely RAD51, BRCA2, CHEK2, HER2, CDH1, COMT and PGR) and these morphological features of the mammary lesions. Specifically, we found that SNPs in genes RAD51 (rs23623251 and rs23642734), CHEK2 (rs397511718), HER2 (rs24537329) and PGR (rs8875007) were associated with the development of aggressive CMTs histotypes, with moderate to high histological grade. On the contrary, SNPs in BRCA2 (rs23255542), HER2 (rs24537331), CDH1 (rs852280880 and rs850805755) and COMT (rs851328636, rs853133060 and rs85346495) were related to tumor histotypes of good prognosis and of low histological grade.

In a previous report we did not find a significant association between most of the SNPs included in the present study and the risk of CMTs (Canadas *et al.* 2018a). Although carriers of genetic variants in genes CHEK2 (rs397511718), HER2

(rs24537329) and PGR (rs8875007) do not exhibit an increased risk of develop CMTs, when the disease occurs it tends to be clinically aggressive, with the development of tumors of high histological grade and of histotypes related to poor prognosis. These animals could thus benefit from the implementation of rigorous clinical monitoring protocols, in order to achieve an early detection of the mammary lesions and a timely surgical approach performed in early stages of the neoplastic disease. It could also be reasonable to consider prophylactic mastectomy and the reduction of risk factors (namely suppression of hormonal therapy) in female dogs presenting genetic variations related to the development of aggressive breast tumors. On the other hand, despite the fact that RAD51 SNPs rs23623251 and rs23642734 were related to a decreased risk of CMTs (Canadas *et al.* 2018a), within the group of animals with mammary neoplasia, these genetic variants seem to be associated with unfavorable clinicopathological characteristics, namely aggressive histotypes and moderate to less differentiated mammary carcinomas.

In a previous study we also demonstrated that carriers of the variant allele for CDH1 SNPs rs850805755 and rs852280880 had an almost two-fold lower risk for mammary tumors development compared with wild-type animals (Canadas *et al.* 2019b). Data from the present investigation underline and expand the “protective effect” of these genetic variants in canine mammary carcinogenesis. In fact, when the disease occurs, variant allele carriers for these SNPs tend to develop CMTs of low histological grade and with a better prognosis.

Taken together, our data documented an association between specific genetic variations in genes involved in mammary carcinogenesis and two of the most relevant histopathological features of CMTs, namely histological type and grade, providing preliminary evidence for a genotypic-phenotypic correlation in mammary tumors in this animal species. This issue highlights the mechanisms of genesis, development and progression of CMTs, which is essential for the improvement of effective diagnostic and treatment approaches. Knowledge of the individual genetic profile has been considered a useful tool in the clinical approach of mammary neoplastic disease, allowing the application of specific screening and early detection protocols, as well as assisting the clinician in the selection of the most suitable therapeutic procedure (Lloyd *et al.* 2016, Pang *et al.* 2016).

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**Conflict of interest statement**

The authors declare no conflicts of interest.

**References**

Ahn, S. G., S. J. Kim, C. Kim and J. Jeong (2016). "Molecular Classification of Triple-Negative Breast Cancer." Journal of breast cancer **19**(3): 223-230.

Bettaieb, A., C. Paul, S. Plenchette, J. Shan, L. Chouchane and F. Ghiringhelli (2017). "Precision medicine in breast cancer: reality or utopia?" J Transl Med **15**(1): 139.

Borge, K. S., M. Melin, P. Rivera, S. I. Thoresen, M. T. Webster, H. von Euler*, et al.* (2013). "The ESR1 gene is associated with risk for canine mammary tumours." BMC Veterinary Research **9**: 69-69.

Canadas, A., M. Franca, C. Pereira, R. Vilaca, H. Vilhena, F. Tinoco*, et al.* (2019a). "Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study." Vet Pathol **56**(2): 208-219.

Canadas, A., M. Santos, R. Medeiros and P. Dias Pereira (2019b). "Influence of E-cadherin genetic variation in canine mammary tumor risk, clinicopathological features and prognosis." Veterinary and Comparative Oncology: *In Revision*.

Canadas, A., M. Santos, A. Nogueira, J. Assis, M. Gomes, C. Lemos*, et al.* (2018a). "Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes." J Vet Diagn Invest **30**(5): 733-738.

Canadas, A., M. Santos, R. Pinto, R. Medeiros and P. Dias-Pereira (2018b). "Catechol-o-methyltransferase genotypes are associated with progression and biological behaviour of canine mammary tumours." Vet Comp Oncol **16**(4): 664-669.

Carvalho, M. I., R. Silva-Carvalho, I. Pires, J. Prada, R. Bianchini, E. Jensen-Jarolim*, et al.* (2016). "A Comparative Approach of Tumor-Associated Inflammation in Mammary Cancer between Humans and Dogs." Biomed Res Int **2016**: 4917387.

de Las Mulas, J. M., Y. Millan and R. Dios (2005). "A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog." Vet Pathol **42**(2): 200-212.

Dias Pereira, P., C. C. Lopes, A. J. Matos, D. Pinto, F. Gartner, C. Lopes*, et al.* (2008). "Estrogens metabolism associated with polymorphisms: influence of COMT G482a genotype on age at onset of canine mammary tumors." Vet Pathol **45**(2): 124-130.

Easton, D. F., K. A. Pooley, A. M. Dunning, P. D. P. Pharoah, D. Thompson, D. G. Ballinger*, et al.* (2007). "Genome-wide association study identifies novel breast cancer susceptibility loci." Nature **447**(7148): 1087-1093.

Eliyatkın, N., E. Yalçın, B. Zengel, S. Aktaş and E. Vardar (2015). "Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way." The journal of breast health **11**(2): 59-66.

Elston, C. W. and I. O. Ellis (1991). "Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up." Histopathology **19**(5): 403-410.

Goldschmidt, M., L. Pena, R. Rasotto and V. Zappulli (2011). "Classification and grading of canine mammary tumors." Vet Pathol **48**(1): 117-131.

Hampe, J. F. and W. Misdorp (1974). "Tumours and dysplasias of the mammary gland." Bull World Health Organ **50**(1-2): 111-133.

Heng, Y. J., S. C. Lester, G. M. Tse, R. E. Factor, K. H. Allison, L. C. Collins*, et al.* (2017). "The molecular basis of breast cancer pathological phenotypes." J Pathol **241**(3): 375-391.

Karayannopoulou, M., E. Kaldrymidou, T. C. Constantinidis and A. Dessiris (2005). "Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method." J Comp Pathol **133**(4): 246-252.

Lilyquist, J., K. J. Ruddy, C. M. Vachon and F. J. Couch (2018). "Common Genetic Variation and Breast Cancer Risk—Past, Present, and Future." Cancer Epidemiology Biomarkers & Prevention **27**(4): 380-394.

Lloyd, K. C., C. Khanna, W. Hendricks, J. Trent and M. Kotlikoff (2016). "Precision medicine: an opportunity for a paradigm shift in veterinary medicine." J Am Vet Med Assoc **248**(1): 45-48.

Michailidou, K., S. Lindström, J. Dennis, J. Beesley, S. Hui, S. Kar*, et al.* (2017). "Association analysis identifies 65 new breast cancer risk loci." Nature **551**: 92.

Milne, R. L., K. B. Kuchenbaecker, K. Michailidou, J. Beesley, S. Kar, S. Lindström*, et al.* (2017). "Identification of ten variants associated with risk of estrogen-receptor-negative breast cancer." Nature Genetics **49**: 1767.

Misdorp, W., R. W. Else, E. Hellmen and L. TP. (1999). World Health Organization international histological classification of tumors of domestic animals Washington, D.C. : Armed Forces Institute of Pathology.

Pang, L. Y. and D. J. Argyle (2016). "Veterinary oncology: Biology, big data and precision medicine." Vet J **213**: 38-45.

Pena, L., P. J. De Andres, M. Clemente, P. Cuesta and M. D. Perez-Alenza (2013). "Prognostic value of histological grading in noninflammatory canine mammary

carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics." Vet Pathol **50**(1): 94-105.

Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees*, et al.* (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-752.

Rakha, E. A. and A. R. Green (2017). "Molecular classification of breast cancer: what the pathologist needs to know." Pathology **49**(2): 111-119.

Rasotto, R., D. Berlato, M. H. Goldschmidt and V. Zappulli (2017). "Prognostic Significance of Canine Mammary Tumor Histologic Subtypes: An Observational Cohort Study of 229 Cases." Vet Pathol **54**(4): 571-578.

Russnes, H. G., O. C. Lingjaerde, A. L. Borresen-Dale and C. Caldas (2017). "Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters." Am J Pathol **187**(10): 2152-2162.

Santos, A. A., C. C. Lopes, J. R. Ribeiro, L. R. Martins, J. C. Santos, I. F. Amorim*, et al.* (2013). "Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study." BMC Vet Res **9**: 1-11.

Santos, M., C. Correia-Gomes, R. Marcos, A. Santos, A. De Matos, C. Lopes*, et al.* (2015). "Value of the Nottingham Histological Grading Parameters and Nottingham Prognostic Index in Canine Mammary Carcinoma." Anticancer Res **35**(7): 4219-4227.

Song, Q., S. D. Merajver and J. Z. Li (2015). "Cancer classification in the genomic era: five contemporary problems." Hum Genomics **9**: 27.

Sorenmo, K. (2003). "Canine mammary gland tumors." Veterinary Clinics of North America: Small Animal Practice **33**(3): 573-596.

Tsuda, H. (2009). "Gene and chromosomal alterations in sporadic breast cancer: correlation with histopathological features and implications for genesis and progression." Breast Cancer **16**(3): 186-201.

Wang, C.-Q., C.-H. Tang, Y. Wang, L. Jin, Q. Wang, X. Li*, et al.* (2017). "FSCN1 gene polymorphisms: biomarkers for the development and progression of breast cancer." Scientific Reports **7**(1): 15887.

Table 1. Canine mammary malignant tumors categories defined according to their aggressiveness potential.

| Tumor Groups | | Aggressiveness | WHO Classification | 2011 Classification |
|---|---|---|---|---|
| Group (G1) | 1 | Low | Complex carcinoma<br>Carcinoma in benign tumor | Carcinoma – complex type<br>Carcinoma - mixed type<br>Carcinoma arising in a complex adenoma<br>Carcinoma arising in a benign mixed tumor |
| Group (G2) | 2 | Moderate | Simple carcinoma (except anaplastic carcinoma) | Carcinoma - solid<br>Carcinoma - simple<br>Intraductal papillary carcinoma<br>Carcinoma and malignant myoepithelioma<br>Sarcoma |
| Group (G3) | 3 | High | Anaplastic carcinoma<br>Special type carcinomas (squamous cell carcinoma, spindle cell carcinoma, mucinous carcinoma)<br>Carcinosarcoma | Carcinoma - anaplastic<br>Comedocarcinoma<br>Special type carcinomas (squamous cell carcinoma, adenosquamous carcinoma, malignant myoepitheloma, inflammatory carcinoma)<br>Carcinosarcoma |

Table 2. Genes and single nucleotide polymorphisms (SNPs) assessed for association to histological type and grade of canine mammary tumors.

| Gene | Canine chromosome number | SNP |
|---|---|---|
| *Proto-oncogene* | | |
| *HER2* | 9 | *rs24537331*<br>*rs397509770*<br>*rs397512599*<br>*rs397511049*<br>*rs397512289*<br>*rs24537329*<br>*rs397513043* |
| *Tumor suppressor genes* | | |
| *STK11* | 20 | *rs22928814* |
| *CDH1* | 5 | *rs850805755*<br>*rs852639930*<br>*rs852280880* |
| *DNA damage repair genes* | | |
| *BRCA1* | 9 | *rs397509570*<br>*rs397511319* |
| *BRCA2* | 25 | *rs23250374*<br>*rs23255542* |
| *BRIP* | 9 | *rs397511271*<br>*rs397512960*<br>*rs397511741* |
| *CHEK2* | 26 | *rs397511718* |
| *PTEN* | 26 | *rs397510595* |
| *RAD51* | 30 | *rs23623251*<br>*rs23642734* |
| *Hormonal metabolism genes* | | |
| *ESR1* | 1 | *rs397512133*<br>*rs851327560*<br>*rs397510462*<br>*rs397510612*<br>*rs852887655*<br>*rs852398698*<br>*rs852684753* |
| *COMT* | 26 | *rs853046495*<br>*rs23350589*<br>*rs853133060*<br>*rs851328636*<br>*rs23322686*<br>*rs2336579*<br>*rs852564758* |
| *PGR* | 21 | *rs8875007*<br>*rs397512502* |
| *PRLR* | 35 | *rs23932236* |

Table 3. Statistically significant associations between SNPs and histological type of CMTs based on the WHO and the 2011 classification systems.

| | *WHO classification* | | | *2011 classification* | | |
|---|---|---|---|---|---|---|
| ***SNPs*** | Groups (n/%) | | | Groups (n/%) | | |
| | G1 | G2 | *G3* | *G1* | *G2* | *G3* |
| RAD51_ rs23623251 | | | | | | |
| VA | 12 (35.3) | 19 (45.2) | 10 (90.0) | - | - | - |
| W | 22 (64.7) | 23 (54.8) | 1 (1.9) | - | - | - |
| p | | | 0.005 | - | - | - |
| RAD51_rs23642734 | | | | | | |
| VA | 14 (41.2) | 22 (52.4) | 10 (90.9) | 11 (37.9) | 15 (46.9) | 20 (76.9) |
| W | 20 (58.8) | 20 (47.6) | 1 (9.1) | 18 (62.1) | 17 (53.1) | 6 (23.1) |
| p | | | 0.016 | | | 0.011 |
| COMT_rs851328636 | | | | | | |
| VA | - | - | - | 3 (10.7) | 12 (37.5) | 4 (15.4) |
| W | - | - | - | 25 (89.3) | 20 (62.5) | 22 (84.6) |
| p | | | - | | | 0.027 |
| COMT_rs85133060 | | | | | | |
| VA | - | - | - | 4 (13.8) | 12 (37.5) | 4 (15.4) |
| W | - | - | - | 25 (86.2) | 20 (62.5) | 22 (84.6) |
| p | | | - | | | 0.049 |
| HER2_rs24537331 | | | | | | |
| VA | - | - | - | 20 (69.0) | 14 (43.8) | 10 (38.5) |
| W | - | - | - | 9 (31.0) | 18 (56.3) | 16 (61.5) |
| p | | | - | | | 0.049 |
| HER2_rs24537329 | | | | | | |
| VA | - | - | - | 19 (65.5) | 22 (68.8) | 24 (92.3) |
| W | - | - | - | 10 (34.5) | 10 (31.3) | 2 (7.7) |
| p | | | | | | 0.046 |
| BRCA2_rs23255542 | | | | | | |
| VA | - | - | - | 20 (83.3) | 17 (54.8) | 12 (46.2) |
| W | - | - | - | 4 (16.7) | 14 (45.2) | 14 (53.8) |
| p | | | | | | 0.019 |
| PGR_rs8875007 | | | | | | |
| VA | - | - | - | 10 (35.7) | 9 (28.1) | 16 (61.5) |
| W | - | - | - | 18 (64.3) | 23 (71.9) | 10 (38.5) |
| P | | | - | | | 0.029 |

Legend: VA – variant allele; W- wildtype genotype; - no statistical significance.

Table 4. Statistically significant associations between SNPs and histological grading of CMTs based on the NHG and on the ca-NHG methods.

| | ***NHG method*** | | | | ***Ca-NHG method*** | | | |
|---|---|---|---|---|---|---|---|---|
| ***SNPs*** | *Grade I Vs Grade II plus III (n/%)* | | *Grade I plus II Vs Grade III (n/%)* | | *Grade I Vs Grade II plus III (n/%)* | | *Grade I plus II Vs Grade III (n/%)* | |
| RAD51_rs23642734 | | | | | | | | |
| VA | 8 (34.8) | 34 (61.8) | - | - | 9 (13.8) | 31 (62.0) | - | - |
| W | 15 (65.2) | 21 (38.2) | - | - | 15 (86.2) | 19 (38.0) | - | - |
| p | | 0.029 | | - | | 0.048 | | - |
| *COMT*_rs85346495 | | | | | | | | |
| VA | 7 (34.4) | 6 (10.9) | 13 (22.0) | 0 (0.0) | - | - | 13 (23.3) | 0 (0.0) |
| W | 16 (69.6) | 49 (89.1) | 46 (78.0) | 19 (100.0) | - | - | 43 (76.8) | 18 (100.0) |
| p | | 0.048 | | 0.031 | | - | | 0.030 |
| *CDH1*_rs852280880 | | | | | | | | |
| VA | 5 (21.7) | 3 (5.5) | - | - | - | - | - | - |
| W | 18 (78.3) | 52 (94.5) | - | - | - | - | - | - |
| p | | 0.045 | | - | | - | | - |
| *CDH1*_rs850805755 | | | | | | | | |
| VA | 5 (21.7) | 3 (5.5) | - | - | - | - | - | - |
| W | 18 (78.3) | 52 (94.5) | - | - | - | - | - | - |
| p | | 0.045 | | - | | - | | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BRCA2_rs23255542 | | | | | | | | |
| VA | 17 (81.0) | 28 (54.9) | - | - | - | - | - | - |
| W | 4 (19.0) | 23 (45.1) | - | - | - | - | - | - |
| p | | 0.038 | | | | - | | - |
| *CHEK2*_rs39751171 | | | | | | | | |
| 8 | | | | | | | | |
| VA | - | - | - | - | 6 (25.0) | 25 (51.0) | - | - |
| W | - | - | - | - | 18 (75.0) | 24 (49.0) | - | - |
| p | | - | | - | | 0.035 | | |

Legend: VA – variant allele; W- wildtype genotype; - no statistical significance.

CHAPTER 09

# General Discussion

Cancer is recognized as a complex and multifactorial disease, being characterized by a sequence of genetic, epigenetic and phenotypic alterations (Stratton *et al.* 2009, Sharma *et al.* 2010, Klopfleisch *et al.* 2011). In the progressive process of the neoplastic transformation, cells acquire special biological features that confer them the ability to be independent and self-sustained. Those capabilities include growing beyond boundaries and limits, unlimited replicability, resistance to apoptosis, insensitivity to anti-growth signals and growth signals autonomy, sustained angiogenesis and invasive properties in stromal microenvironment (Hanahan *et al.* 2011).

Genetic research applied to oncology has brought information about the interaction between genetic profiles, clinical aspects of the disease (biological behavior and response to treatments) and pathological or morphological features of the neoplasia (Desmedt *et al.* 2009, Roukos *et al.* 2014, Bertucci *et al.* 2018). Considering the long list of the multiple factors involved in cancer susceptibility, the genetic inter-individual variability is considered crucial for patient-tailored approach strategies that can be followed by oncologists and surgeons. This new approach is called precision or personalized medicine (Low *et al.* 2018, Chen *et al.* 2019). With respect to human breast cancer there is a strong body of evidence suggesting that multiple factors, including genetic ones, lead to an increased susceptibility to develop this neoplasia (Rojas *et al.* 2016, Akram *et al.* 2017). It has been suggested that genetic variations, such as SNPs (either isolated or in combination with others SNP - epistasis), can play an important role on the risk, progression, treatment response and outcome of human breast cancer (Onay *et al.* 2006, Sapkota *et al.* 2014, Shiovitz *et al.* 2015).

In the present era, the massive technological development and availability of different genetic tools opens up an opportunity of reviewing the role of the veterinary pathologist in mammary oncology. The pathologist has now the chance of go beyond the *mere* histopathological diagnosis, assessing also the relationship between genetics and morphology, and being able to provide relevant information to the clinician considering the different stages of the neoplastic transformation and progression.

New challenges are currently presented to the clinicians and to the pathologists, namely those regarding oncologic disease prevention. The identification of groups of female dogs with greater susceptibility to develop

mammary neoplasia or those that tend to develop more aggressive tumors should be a goal. These achievements would support the definition of preventive strategies and the establishment of protocols and guidelines for surveillance and early detection of the disease, especially designed for the at-risk groups of dogs.

On the other hand, in cases of malignant mammary tumors, the combined efforts of the clinician and the pathologist should be driven to the identification of robust prognostic factors that can assist in the management of the animals with the disease. Once the disease is clinically installed, it is important to identify, in light of the personalized medicine for oncologic animals, patient characteristics (such as those related to the animal genetic profile) and tumor histopathological features, which can be decisive to define the prognosis. It is also essential to investigate the relationship between the animal´s genetic profile and the phenotypic manifestation of the neoplastic disease. Based on this information the clinician can, for example, adjust the extent of the surgical intervention to the animal's profile or evaluate the need to implement adjuvant therapeutic, according to each individual case.

In this thesis the impact of individual genetic profiles in mammary tumors susceptibility in canine species was assessed. Furthermore, the association between SNPs in different genes and the clinicopathological features of CMTs, including age, tumor number and size, growth pattern, histological type, histological grade and corresponding grading parameters (tubule formation, nuclear pleomorphism and mitotic index), prognostic index formula, vascular invasion, lymph node metastases and outcome was evaluated. Furthermore, the two most widely used systems for histological classification (Misdorp *et al.* 1999, Goldschmidt *et al.* 2011) and grading (Elston *et al.* 1991, Pena *et al.* 2013) of CMTs were compared, related to clinicopathological features and overall survival of CMTs, as well as to the genetic profile of the animal.

**Genetic profile and risk of CMTs**

Based on the high complexity of the mammary neoplastic transformation and progression, which involves gene families participating in different steps of the carcinogenic process, SNPs in several gene groups known to be involved in human breast cancer were assessed in our study. In this vein, proto-oncogenes (HER2, EGFR), tumor suppressor genes (TP53, STK11, CDH1), genes involved in DNA damage recognition and repair (BRCA1, BRCA2, RAD51, CHEK2, PTEN, BRIP1)

and in hormonal metabolism (ESR1, PGR, PRLR, COMT) were included (Canadas *et al.* 2018a).

Our data demonstrated an association between the genetic profiles related to different gene families (namely tumor suppressor genes -STK11 and CDH1- and genes involved in DNA repair -RAD51) and the risk of CMTs development (Canadas *et al.* 2018a). Genetic variation in human STK11, CDH1 and RAD51 genes have also been found to be related to different types of neoplasia, including breast cancer (Beeghly-Fadiel *et al.* 2010, Korsse *et al.* 2013, Kleibl *et al.* 2016).

In this investigation, dogs with STK11 SNP rs22928814 had an almost two-fold higher risk to develop mammary tumors than wild-type carriers (Canadas *et al.* 2018a). This finding contradicts previous studies reporting no association between the same SNP and mammary tumors susceptibility (Borge *et al.* 2011, Borge *et al.* 2013). STK11 gene is responsible for the codification of a serine/threonine kinase that acts as a tumoral suppressor, controlling cell proliferation and apoptosis, as well as cell growth and polarity (Dupuy *et al.* 2013, Korsse *et al.* 2013, Li *et al.* 2014). Disturbances in these cellular processes are involved in the genesis of the neoplastic transformation, being actually recognized as the hallmarks of cancer (Hanahan *et al.* 2011). STK11 is a gene associated with human Peutz-Jeghers syndrome, a condition characterized by an elevated risk of multiple cancer types, including breast cancer (van Lier *et al.* 2010, Kleibl *et al.* 2016, Antov *et al.* 2017). It is conceivable that genetic variation in canine STK11 gene may be related to a reduced production of the enzyme or to a synthesis of an altered and less effective form of the enzyme, which impairs its anti-tumoral action.

On the contrary, our data demonstrated that genetic variation on RAD51 (rs23623251 and rs23642734) was related to a decreased susceptibility to the development of CMTs (Canadas *et al.* 2018a). RAD51 gene is associated with BRCA2, and plays a pivotal role in the maintenance of the genome integrity and stability, being involved in recombination repair of double-stranded damaged DNA. RAD51 participates in a complex network of interactions that includes DNA damage sensors, tumor suppressors, and cell cycle and apoptotic regulators (Haaf *et al.* 1995, Sullivan *et al.* 2018), features known to be involved in tumor initiation and progression (Richardson 2005). Based on our data, it is reasonable to suppose hypothesize that the RAD51 SNPs assessed in this study may enhance RAD 51 protein function or improve its interaction with BRCA2, guaranteeing a highly

efficient DNA repair process, thus protecting the mammary gland tissue from carcinogenic insults.

Our results also reported an association between mammary tumor susceptibility and SNPs rs850805755 and rs852280880 (which were found to be in complete linkage) in canine CDH1 gene. Carriers of the variant allele for these SNPs had an almost two-fold lower risk for mammary tumors development compared with wild-type animals (*Canadas et al, in revision*). CDH1 gene codifies E-cadherin, a cell adhesion molecule that participates in several processes such as cell shape, polarity, proliferation and motility, and can thus be implicated in the progression and metastatic ability of neoplasia (Takeichi 1990, van Roy *et al.* 2008, Yilmaz *et al.* 2010). Based on E-cadherin function in maintaining the architectural arrangements of epithelial tissues, we hypothesize that these SNPs may codify a more stable E-cadherin protein, assuring mammary gland structural integrity during physiological growth, differentiation and regression cycles. This constitutes an important feature in guaranteeing mammary tissue resistance to carcinogenic events (Halaoui *et al.* 2017). In fact, loss of cell polarity and collapse of luminal arrangements (processes associated with E-cadherin down-regulation) are recognized as early events in carcinogenesis, underlining the role of this cell adhesion molecule in neoplastic transformation.

Although most of the research performed to date has focused on the identification of disease-causing genetic variants, recognition of the full spectrum of susceptibility genotypes offers a broader understanding of the complex pathways that are in the basis of the neoplastic transformation. The characterization of both cancer-related and cancer-preventive genetic variants assures a better categorization of animals' genetic subgroups. Recognition of disease-protective SNPs constitutes a useful opportunity for biomedical research, since they are regarded as health-maintaining variants. The existence of cancer-protective variants, which confer a selective advantage and are not associated with adverse effects in a population, is important to guide clinical decision making process, in the development of novel drugs and represents a valuable therapeutic target (Harper *et al.* 2015, Butler *et al.* 2017, Schwartz *et al.* 2017, Tan 2018).

Overall, our data underline the importance of tumor suppressor genes and of genes involved in the recognition and in the repair of DNA damage in canine mammary carcinogenesis, as previously reported for human breast cancer

(Ripperger *et al.* 2009, Mavaddat *et al.* 2010, Smolarz *et al.* 2019) strengthening the intricate multistep nature of the neoplastic transformation process.

**Genetic profile and clinicopathological features of CMTs**

Besides of their influence on CMTs risk, SNPs in STK11, RAD51 and CDH1 genes were also related to clinicopathological prognostic factors of the disease.

This investigation demonstrated that SNP rs22928814 in STK11 gene was associated with the development of mammary tumors before 10 years of age (*P*=0.008). Variant allele carriers for this SNP also exhibited a tendency to develop malignant rather than benign tumors (*P*=0.052). Furthermore, within the group of malignant lesions, this genetic variant was associated with the occurrence of larger tumors (>3cm) (*P*=0.004) (Data not published). Altogether, our results suggest this genetic variant represents a risk factor regarding not only the susceptibility to CMTs, but also the development of mammary lesions earlier in life, as well as the occurrence of larger malignant tumors. Our data finds parallel in that reported for human breast cancer, in which STK11 down-expression has been associated to large tumor size, high proliferative activity and high histological grade, lymph node metastasis, as well as lymph node metastasis, shorter relapse free interval and worse overall survival (Shen *et al.* 2002, Fenton *et al.* 2006, Zhuang *et al.* 2006, Syed *et al*, 2019). Our findings underline the importance of STK11 genetic profile as a risk and prognostic marker for CMTs, as previously demonstrated for human breast cancer. As previously explained, it is feasible to assume that rs22928814 in canine STK11gene may be responsible for a decreased synthesis of the serine/threonine kinase or for less efficient forms of the enzyme that compromises its tumor suppressor activity. The assessment of this SNP may be very relevant in order to apply preventive measures and to propose surveillance protocols, both aiming to reduce CMTs incidence and their aggressiveness.

Interestingly, despite the fact that RAD51 SNPs rs23623251 and rs23642734 were related to a decreased risk of CMTs, within the group of animals with mammary neoplasia, these genetic variants were associated with aggressive clinicopathological characteristics. Our data revealed that genetic variation in RAD51 gene was associated with the development of multiple mammary tumors, rather than a single neoplastic lesion. In fact, 72% of the carriers of the variant allele

for SNP rs23623251 in this gene develop multiple tumors, comparing with 59% of the wild-type animals (*P*=0.046) (Data not published). Fifty to nearly 70% of dogs have multiple mammary lesions (Fowler *et al.* 1974, Benjamin *et al.* 1999, Misdorp 2002, Sorenmo 2003, Sorenmo *et al.* 2009) but so far little is known about the genetic relationship between the tumors. Recently, Santos and colleagues performed a high-resolution oligonucleotide array comparative genomic hybridisation and presented preliminary evidences of distinct genetic profiles in independent tumors of the same dog, suggesting an independent pathogenesis for multiple CMTs (Santos *et al.* 2017). A significant relationship was also found between RAD51 SNPs rs23623251 and rs23642734 and tumor histotypes (defined by the WHO and the 2011 histological classification systems) related to poor prognosis, such as anaplastic carcinoma, comedocarcinoma, special type carcinoma and carcinosarcoma (Canadas-Sousa et al, *in preparation*). Furthermore, our results demonstrated an association between rs23642734 in RAD51 gene and the development of moderate to less differentiated malignant mammary tumors (histological grade II and III), irrespective of the grading method employed (NHG or ca-NHG). This finding is in accordance with data regarding human breast cancer, in which RAD51 low-expression has been considered a marker of poor prognosis, being correlated to several clinicopathological factors, such as high histological grade and lymph node metastasis (Soderlund *et al.* 2007, Hallajian *et al.* 2017). Furthermore, genetic variability in RAD51 human gene was associated with highly aggressive histotypes namely triple-negative breast cancer (Smolarz *et al.* 2013, Michalska *et al.* 2015). Our data suggest that, although carriers of genetic variants in RAD51 gene are less susceptible to develop mammary tumor, in cases were the disease occurs it tends to be more aggressive, with the development of multiple lesions, with a histotype related to poor prognosis and higher histological grade. Despite more data in other cohorts of animals is needed, we can anticipate that this evidence may support a recommendation for an early wide and more radical surgical treatment of the mammary lesions in dogs with such genetic profiles.

Our data demonstrated that SNPs in CDH1 gene (rs850805755 and rs852280880) were associated with the age of the animal at the time of the tumor's diagnosis, with variant allele carriers developing mammary tumors later in life than wild type dogs (Canadas et al, *in revision*). Based on the well-known role E-cadherin

plays in the architectural organization of epithelial tissues, it can be hypothesized that SNPs rs850805755 and rs852280880 codify a form of E-cadherin that may be more efficient in the maintenance of mammary gland structural integrity in the course of physiological growth, differentiation and regression cycles. This is an important issue regarding the resistance of the mammary gland to carcinogenic insults and may, therefore, influence the age at which the animal develops mammary tumors. Besides, one cannot exclude the hypothesis that the structure of the E-cadherin molecule codified by these genetic variants can be more resistant to spontaneous or carcinogen-induced mutations, thus explaining the development of mammary tumors later in life. These SNPs were also related to clinicopathological features traditionally associated with a good prognosis, such as small size carcinomas (≤ 3cm), low histological grade (defined by the NHG method), low vet-NPI and reduced nuclear pleomorphism. Similarly, CDH1 SNP rs852639930 was related to the development of small size (≤ 3cm) tumors, with a non-infiltrative, non-invasive growth pattern (Canadas et al, *in revision*). E-cadherin is responsible for cell-to-cell adhesion thus maintaining architectural arrangements and hampering cell detachment and migration. In fact, E-cadherin low-expression carcinomas are characterized by a series of aggressive histological features such as loss of tubular organization, high nuclear pleomorphism moderate/high histological grade and an infiltrative/invasive growth pattern, revealing decreased adhesive attributes of the neoplastic cells (Sarli *et al.* 2004, Matos *et al.* 2006, Gama *et al.* 2008). Our results suggest that these CDH1 genetic variants may contribute to increase E-cadherin cohesive properties, codifying more efficient forms of the molecule, or may promote a stable interconnection between E-cadherin and the other elements of the axis cadherin-catenin-cytoplasmic actin, favoring a highly resistant cell-to-cell adhesion system. It should be stressed that our data regarding CDH1 genetic variation is not in accordance with that from Borge et al (2011), in which SNPs in CDH1 gene were assessed but no results were achieved concerning their influence on the clinicopathological features of CMTs.

This investigation also demonstrated a significant relationship between genetic variants in BRCA2 (rs23255542) and HER2 (rs24537331) genes and the development of tumor histotypes associated with a better prognosis and/or of low histologic grade. On the contrary, SNPs in CHEK2 (rs397511718) and HER2 (rs24537329) genes were found to be related to tumor histotypes characterized by

worse prognosis or of higher histologic grade (Canadas-Sousa et al, *in preparation*). Being a highly complex disease, it is conceivable that mammary neoplasia susceptibility and clinicopathological features are determined by the interaction of several genetic variants (that conforms a specific pattern of SNPs) and environmental factors, rather than by an individual genetic variant (Ripperger *et al.* 2009, Sapkota *et al.* 2013). Accordingly, further studies are needed in order to accurately disclose the intricate framework that reflects the interplay between these SNPs (epistasis), and ascertain the involvement of environmental determinants, namely the animal's hormonal background.

There is a strong body of evidence recognizing estrogens as a crucial factor for the neoplastic transformation of the mammary tissue, considering their proliferative effect and their metabolization into genotoxic metabolites that can form adducts with the DNA, driving to genetic changes (Yager *et al.* 2006, Parl *et al.* 2009, Yager 2012). Catechol-O-methyltransferase (COMT) is an important enzyme responsible for inactivation of specific genotoxic metabolites, the cathecol-estrogens (Yager *et al.* 1996, Cavalieri *et al.* 1997). Genetic variation in genes coding for estrogen receptors or enzymes involved in estrogen signaling and in metabolic pathways have been associated with human breast cancer susceptibility, as well as with specific clinicopathological features and the clinical course of the disease (Martin *et al.* 2000, Bugano *et al.* 2008, Sun *et al.* 2015, Evans *et al.* 2018).

Based on the role of the complex metabolic pathways of estrogens in breast tissue and considering previous studies demonstrating that SNPs in genes involved in estrogens metabolism can influence the age of onset and recurrences rate of CMTs (Dias Pereira *et al.* 2008, Dias Pereira *et al.* 2009), we included in this study genes related to the such pathways, namely ESR1 (estrogen receptor) and COMT.

In this study it was not possible to demonstrate an association between SNPs in ESR1 or COMT genes and CMTs risk (Canadas *et al.* 2018b, Canadas-Sousa *et al*, s*ubmitted*). As previously referred, it should be stressed that these genetic variations may not represent individual decisive factors for mammary tumor risk in canine species, but may be relevant when considered in conjunction with other genetic and/or environmental elements. Indeed, the significance of SNPs (especially in low penetrance genes) on human breast cancer risk may rely upon gene-gene (epistasis) or gene-environmental cooperation (Moore 2003, Houlston *et*

*al.* 2004, Wunsch Filho *et al.* 2005, Shen 2009, Lin *et al.* 2015, Rudolph *et al.* 2016, Cole *et al.* 2017, DeWan 2018, Gonzales *et al.* 2018).

However, our data revealed a significant relationship between genetic variations in ESR1 gene (rs397512133, rs397510462, rs851327560 and rs397510612) and less aggressive clinicopathological characteristics of CMTs, namely later onset of the disease, small tumor size and low vet-NPI. Moreover, ERS1 SNPs rs852887655, rs852684753 and rs852398698 were associated with a high percentage of tubular arrangements, a feature related to well differentiated and low-malignancy neoplasia, and also usually associated with good prognosis. An increased lifetime exposure to estrogens has been associated with a high breast cancer risk in humans, due to their proliferative action and to the harmful effect of estrogens metabolites on the DNA (Clemons *et al.* 2001, Travis *et al.* 2003). Animals with a regulated production of estrogens, hormonal receptors and enzymes involved in their metabolic pathways may be more exposed to estrogens and thus more prone to DNA damage and to genetic changes related to the development of aggressive neoplastic phenotypes. It is thus conceivable that carriers of the variant allele for these SNPs may have receptors less sensitive to estrogens binding and a mammary tissue less responsive to the hormone, being more protected from its carcinogenic activity. These dogs would need longer periods of estrogens exposition to reach carcinogenic levels, which explains the development of mammary tumors later in life and their less aggressive clinicopathological characteristics (small tumor size, high percentage of tubular arrangements and low vet-NPI).

Besides, this investigation demonstrated an association between COMT genetic profiles and prognostic factors of malignant CMTs, namely tumor histotype, histological grade, grading parameters nuclear pleomorphism and tubular formation, and vascular invasion (Canadas *et al.* 2018b). In fact, carriers of SNPs rs851328636 and/or rs853133060 (Genotype 3) tended to develop well-differentiated carcinomas with lower nuclear pleomorphism. A significant association was also found between these SNPs and the development of tumor histoypes, defined by the 2011 classification system (Goldschmidt *et al.* 2011), related to a better prognosis, namely complex carcinoma and carcinoma in benign tumor. Our investigation also demonstrated that SNP rs853046495 (Genotype 1) was related to the development of low to moderate grade mammary carcinomas (assessed on the basis of both the

NHG and ca-NHG methods). According to our data, this genotype may be responsible for the codification of a variant form of COMT enzyme with higher catalytic activity, being thus more efficient in the metabolization of the catechol-estrogens, resulting in a decreased exposure of the mammary tissue to their carcinogenic action. On the contrary, genetic variants of at least one of the SNP rs23350589, rs23322686, rs23336579 and rs852564758 (Genotype 2) were related to the development of aggressive mammary tumors of intermediate/high histological grade and to vascular invasion. It is reasonable to assume that this variant genotype may codify an unstable or less competent form of the COMT enzyme, rendering the inactivation of genotoxic metabolites less effective. The accumulation of these carcinogenic compounds would culminate into the development of aggressive neoplastic phenotypes, reflected by the high histological grade and invasive capability of this group of mammary carcinomas.

Taken together, our data demonstrate that although the studied SNPs in ESR1 and COMT genes do not play a decisive role in CMTs risk, these genetic variants may be important in the growth and development of CMTs, influencing several relevant clinicopathological features of the neoplastic disease. Depending on the position the genetic variations occupy in the gene and on their consequences regarding the structure and function of the protein codified, SNPs within the same gene can play a protective or a deleterious effect on mammary neoplastic disease in canine species. Furthermore, it cannot be excluded that the association with other genetic or environmental factors may also influence the role these genetic variants play in the carcinogenic process.

Interestingly, in this investigation, some of the SNPs found to be related with the risk and clinicopathological characteristics of CMTs (namely in RAD51, STK11, ESR1 and COMT genes) are intronic, i.e., located in non-coding regions of the genome. Introns account for nearly 24% of the human genome, being removed by splicing when exons are bound. As non-coding sequences, introns have been neglected for many years; lately they have gained much attention, being currently associated with several human diseases, including breast cancer (Santarpia *et al.* 2016, Gyorffy *et al.* 2018). Introns contain microRNA genes and various sequences that participate in gene regulation through several mechanisms: altering exon skipping, RNA splicing and inducing alternative splicing; interfering with splicing efficiency; coding for regulatory elements that govern gene expression; or they may

be linked to other functional SNPs in the same gene (Cooper 2010, Samuels *et al.* 2013, Deng *et al.* 2017). Likewise, some synonymous SNPs (located in CDH1, ESR1 and COMT genes) were also associated with the susceptibility and clinicopathological features of CMT. Although located in coding regions, synonymous SNPs do not alter the amino acid sequence of the encoded protein, and were thus considered for years as not relevant in the functional activity of the protein. Nevertheless, it is currently known that the protein structure, stability, kinetic parameters and function can be modulated indirectly by different genetic mechanisms, including synonymous SNPs. Indeed, a large body of evidence has pointed to the importance of these genetic variants in the speed and accuracy of transcription and/or translation, in promoting alternative splicing and inducing changes in mRNA structure/stability (Hunt *et al.* 2009, Deng *et al.* 2017). These apparently subtle features may explain the diversity of particular reaction to neoplastic stimulus exhibited by different individuals. Our data reinforces the importance of the whole genome, beyond the coding regions responsible for changes in the primary structure of the proteins, in the process of neoplastic transformation and progression. Despite most of the research performed to date has been centered on the analysis of non-synonymous coding SNPs, other slight (and apparently less functionally relevant) genetic changes should be assessed as putative intervenient in the multifactorial process of mammary carcinogenesis.

**Phenotype profile of CMTs - classification and grade**

Histopathology remains the gold standard for diagnosis and classification of canine mammary tumors (Misdorp 2002, Rasotto *et al.* 2017). However, the typically high histological heterogeneity of CMTs poses a challenge to the pathologist in an accurate classification of the lesions (Misdorp *et al.* 1999, Goldschmidt *et al.* 2011, Sorenmo *et al.* 2011). In an attempt to apply well-defined criteria to categorize mammary neoplasms, different classification schemes for CMTs have been developed. For more than a decade the classification system developed by the WHO presided over the diagnosis of canine mammary neoplasia (Misdorp *et al.* 1999). More recently, a new classification scheme emphasizing architectural arrangements of neoplastic cells and the involvement of myoepithelial cells in the neoplastic process was proposed (Goldschmidt *et al.* 2011). Nowadays pathologists

face an undeniable degree of uncertainty concerning the histological classification of CMTs, since there is no data regarding a rational comparison between the prognostic relevance of the WHO system and of the new proposal by Goldschmidt and colleagues. The same is happening regarding histological grading of canine mammary tumors. The histological grade represents a very important task for veterinary pathologists, crucial to provide additional prognostic information (Karayannopoulou *et al.* 2005, Matos *et al.* 2012, Rasotto *et al.* 2012, Santos *et al.* 2013, Santos *et al.* 2015, Canadas *et al.* 2019). The most widely used grading method in CMTs is the Nottingham histological grade method (NHG) described by Elston and Ellis for human breast cancer (Elston *et al.* 1991). Recently, a "canine adapted-NHG" (ca-NHG), which involves the assessment of all different neoplastic cell types, was proposed (Pena *et al.* 2013). To date, a comparative prognostic study of both grading methods is missing, thus lacking rationale criteria that allow a substantiated choice of the ideal histological grading method for canine mammary carcinomas.

In this investigation we performed a comprehensive comparison of the WHO and the 2011 classification systems in a series of CMTs and evaluated their relationship with the overall survival (Canadas *et al.* 2019). No significant differences were observed in categorization of benign tumors, however within the group of malignant tumors, several differences in histological diagnosis were found between the two methods, with the 2011 classification system subdividing the tumors into more categories compared to the WHO classification. For example, the 2011 classification system subdivided WHO tubulopapillary, solid and complex carcinomas into 17 distinct histological entities. Our results confirmed the prognostic relevance of both classification schemes by identifying mammary tumors associated with better prognosis (complex carcinomas, carcinoma arising in benign tumor and mixed carcinoma) as well as histological subtypes related to poor survival and high risk of cancer related death (comedocarcinoma, adenosquamous carcinoma and carcinosarcoma) (Canadas *et al.* 2019).

We also compared histological grade of canine mammary carcinomas based on NHG and ca-NHG methods and evaluated its association with the OS (Canadas *et al.* 2019). Our data demonstrated high similitude in the histological grade and in the scores of each grading parameters evaluated according to both methods in most cases, with discrepancies found in only 7/144 (4.9%) cases. Furthermore, our

results revealed that histological grade is significantly associated with OS for the 2 grading systems. The grading parameters tubule formation and nuclear pleomorphism, evaluated according to the criteria of the two grading methods, were significantly related to OS, and only mitotic count was not associated with prognosis, irrespective of the grading system employed (Canadas *et al.* 2019).

Data from this investigation emphasize the complexity of mammary carcinogenesis in canine species, highlighting the importance of both genetic and phenotypic features in the development, progression and biological behavior of the disease. Knowing the intricate mechanisms and the several intervening elements in this process constitutes a very useful tool in the clinical management of the neoplastic disease, as well as in the definition of prognosis. Genetic signatures arise as a foundation for the development of prevention, screening and early detection protocols for CMTs. They are also critical for select individual therapeutic schemes, predict the response to treatment and anticipate the outcome of the neoplastic disease. On the other hand, a rationale description and classification of the tumoral lesions and the recognition of key histopathological features related to the outcome of the disease (such as the histological grade) continues to be a relevant task of each veterinary pathologist.

## REFERENCES

Akram, M., M. Iqbal, M. Daniyal and A. U. Khan (2017). "Awareness and current knowledge of breast cancer." Biol Res **50**(1): 33.

Antov, G., G. Antov, M. Krasteva, S. Andonova, A. Savov, S. Angelova*, et al.* (2017). STK11 gene mutations among patients with sporadic breast cancer.

Beeghly-Fadiel, A., W. Lu, Y. T. Gao, J. Long, S. L. Deming, Q. Cai*, et al.* (2010). "E-cadherin polymorphisms and breast cancer susceptibility: a report from the Shanghai Breast Cancer Study." Breast Cancer Res Treat **121**(2): 445-452.

Benjamin, S. A., A. C. Lee and W. J. Saunders (1999). "Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles." Vet Pathol **36**(5): 423-436.

Bertucci, F., A. De Nonneville, P. Finetti, D. Perrot, M. Nilbert, A. Italiano*, et al.* (2018). "The Genomic Grade Index predicts postoperative clinical outcome in patients with soft-tissue sarcoma." Ann Oncol **29**(2): 459-465.

Borge, K. S., A. L. Borresen-Dale and F. Lingaas (2011). "Identification of genetic variation in 11 candidate genes of canine mammary tumour." Vet Comp Oncol **9**(4): 241-250.

Borge, K. S., M. Melin, P. Rivera, S. I. Thoresen, M. T. Webster, H. von Euler*, et al.* (2013). "The ESR1 gene is associated with risk for canine mammary tumours." BMC Veterinary Research **9**: 69-69.

Bugano, D. D., N. Conforti-Froes, N. H. Yamaguchi and E. C. Baracat (2008). "Genetic polymorphisms, the metabolism of estrogens and breast cancer: a review." Eur J Gynaecol Oncol **29**(4): 313-320.

Butler, J. M., N. Hall, N. Narendran, Y. C. Yang and L. Paraoan (2017). "Identification of candidate protective variants for common diseases and evaluation of their protective potential." BMC Genomics **18**(1): 575.

Canadas, A., M. Franca, C. Pereira, R. Vilaca, H. Vilhena, F. Tinoco*, et al.* (2019). "Canine Mammary Tumors: Comparison of Classification and Grading Methods in a Survival Study." Vet Pathol **56**(2): 208-219.

Canadas, A., M. Santos, A. Nogueira, J. Assis, M. Gomes, C. Lemos*, et al.* (2018a). "Canine mammary tumor risk is associated with polymorphisms in RAD51 and STK11 genes." J Vet Diagn Invest **30**(5): 733-738.

Canadas, A., M. Santos, R. Pinto, R. Medeiros and P. Dias-Pereira (2018b). "Catechol-o-methyltransferase genotypes are associated with progression and biological behaviour of canine mammary tumours." Vet Comp Oncol **16**(4): 664-669.

Canadas, A., M. Santos, R. Medeiros and P. Dias Pereira. "Influence of E-cadherin genetic variation in canine mammary tumor risk, clinicopathological features and prognosis."Veterinary and Comparative Oncology: *In Revision*.

Canadas-Sousa, A., M. Santos, B. Leal, R. Medeiros and P. Dias-Pereira "Estrogen Receptors genotypes and canine mammary neoplasia." BMC Veterinary Research *Submitted*.

Canadas-Sousa, A., M. Santos, R. Medeiros and P. Dias-Pereira "Single Nucleotide Polymorphisms influence histological type and grade of canine malignant mammary tumors". *In preparation*.

Cavalieri, E. L., D. E. Stack, P. D. Devanesan, R. Todorovic, I. Dwivedy, S. Higginbotham*, et al.* (1997). "Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators." Proceedings of the National Academy of Sciences **94**(20): 10937-10942.

Chen, L., J. E. Manautou, T. P. Rasmussen and X.-b. Zhong (2019). "Development of precision medicine approaches based on inter-individual variability of BCRP/ABCG2." Acta Pharmaceutica Sinica B.

Clemons, M. and P. Goss (2001). "Estrogen and the Risk of Breast Cancer." New England Journal of Medicine **344**(4): 276-285.

Cole, B. S., M. A. Hall, R. J. Urbanowicz, D. Gilbert-Diamond and J. H. Moore (2017). "Analysis of Gene-Gene Interactions." Curr Protoc Hum Genet **95**: 1.14.11-11.14.10.

Cooper, D. N. (2010). "Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes." Hum Genomics **4**(5): 284-288.

Deng, N., H. Zhou, H. Fan and Y. Yuan (2017). "Single nucleotide polymorphisms and cancer susceptibility." Oncotarget **8**(66): 110635-110649.

Desmedt, C., C. Sotiriou and M. J. Piccart-Gebhart (2009). "Development and Validation of Gene Expression Profile Signatures in Early-Stage Breast Cancer." Cancer Investigation **27**(1): 1-10.

DeWan, A. T. (2018). "Gene-Gene and Gene-Environment Interactions." Methods Mol Biol **1793**: 89-110.

Dias Pereira, P., C. C. Lopes, A. J. Matos, D. Pinto, F. Gartner, C. Lopes*, et al.* (2008). "Estrogens metabolism associated with polymorphisms: influence of COMT G482a genotype on age at onset of canine mammary tumors." Vet Pathol **45**(2): 124-130.

Dias Pereira, P., C. C. Lopes, A. J. Matos, D. Pinto, F. Gartner, C. Lopes*, et al.* (2009). "Influence of catechol-O-methyltransferase (COMT) genotypes on the prognosis of canine mammary tumors." Vet Pathol **46**(6): 1270-1274.

Dupuy, F., T. Griss, J. Blagih, G. Bridon, D. Avizonis, C. Ling*, et al.* (2013). "LKB1 is a central regulator of tumor initiation and pro-growth metabolism in ErbB2-mediated breast cancer." Cancer Metab **1**(1): 18.

Elston, C. W. and I. O. Ellis (1991). "Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up." Histopathology **19**(5): 403-410.

Evans, D. G., S. J. Howell and A. Howell (2018). "Personalized prevention in high risk individuals: Managing hormones and beyond." Breast **39**: 139-147.

Fenton, H., B. Carlile, E. A. Montgomery, H. Carraway, J. Herman, F. Sahin*, et al.* (2006). "LKB1 protein expression in human breast cancer." Appl Immunohistochem Mol Morphol **14**(2): 146-153.

Fowler, E. H., G. P. Wilson and A. Koestner (1974). "Biologic behavior of canine mammary neoplasms based on a histogenetic classification." Vet Pathol **11**(3): 212-229.

Gama, A., J. Paredes, F. Gartner, A. Alves and F. Schmitt (2008). "Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival." Vet J **177**(1): 45-53.

Goldschmidt, M., L. Pena, R. Rasotto and V. Zappulli (2011). "Classification and grading of canine mammary tumors." Vet Pathol **48**(1): 117-131.

Gonzales, M. C., J. Grayson, A. Lie, C. H. Yu and S. P. K. Shiao (2018). "Gene-environment interactions and predictors of breast cancer in family-based multi-ethnic groups." Oncotarget **9**(49): 29019-29035.

Gyorffy, B., L. Pongor, G. Bottai, X. Li, J. Budczies, A. Szabo*, et al.* (2018). "An integrative bioinformatics approach reveals coding and non-coding gene variants associated with gene expression profiles and outcome in breast cancer molecular subtypes." Br J Cancer **118**(8): 1107-1114.

Haaf, T., E. I. Golub, G. Reddy, C. M. Radding and D. C. Ward (1995). "Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes." Proceedings of the National Academy of Sciences **92**(6): 2298-2302.

Halaoui, R., C. Rejon, S. J. Chatterjee, J. Szymborski, S. Meterissian, W. J. Muller*, et al.* (2017). "Progressive polarity loss and luminal collapse disrupt tissue organization in carcinoma." Genes Dev **31**(15): 1573-1587.

Hallajian, Z., F. Mahjoubi and N. Nafissi (2017). "Simultaneous ATM/BRCA1/RAD51 expression variations associated with prognostic factors in Iranian sporadic breast cancer patients." Breast Cancer **24**(4): 624-634.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Harper, A. R., S. Nayee and E. J. Topol (2015). "Protective alleles and modifier variants in human health and disease." Nat Rev Genet **16**(12): 689-701.

Houlston, R. S. and J. Peto (2004). "The search for low-penetrance cancer susceptibility alleles." Oncogene **23**(38): 6471-6476.

Hunt, R., Z. E. Sauna, S. V. Ambudkar, M. M. Gottesman and C. Kimchi-Sarfaty (2009). "Silent (synonymous) SNPs: should we care about them?" Methods Mol Biol **578**: 23-39.

Karayannopoulou, M., E. Kaldrymidou, T. C. Constantinidis and A. Dessiris (2005). "Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method." J Comp Pathol **133**(4): 246-252.

Kleibl, Z. and V. N. Kristensen (2016). "Women at high risk of breast cancer: Molecular characteristics, clinical presentation and management." Breast **28**: 136-144.

Klopfleisch, R., H. von Euler, G. Sarli, S. S. Pinho, F. Gartner and A. D. Gruber (2011). "Molecular carcinogenesis of canine mammary tumors: news from an old disease." Vet Pathol **48**(1): 98-116.

Korsse, S. E., M. P. Peppelenbosch and W. van Veelen (2013). "Targeting LKB1 signaling in cancer." Biochim Biophys Acta **1835**(2): 194-210.

Li, J., J. Liu, P. Li, X. Mao, W. Li, J. Yang*, et al.* (2014). "Loss of LKB1 disrupts breast epithelial cell polarity and promotes breast cancer metastasis and invasion." Journal of experimental & clinical cancer research : CR **33**(1): 70-70.

Lin, C., C. M. Chu, J. Lin, H. Y. Yang and S. L. Su (2015). "Gene-gene and gene-environment interactions in meta-analysis of genetic association studies." PLoS One **10**(4): e0124967.

Low, S. K., H. Zembutsu and Y. Nakamura (2018). "Breast cancer: The translation of big genomic data to cancer precision medicine." Cancer Sci **109**(3): 497-506.

Martin, A.-M. and B. L. Weber (2000). "Genetic and Hormonal Risk Factors in Breast Cancer." JNCI: Journal of the National Cancer Institute **92**(14): 1126-1135.

Matos, A. J., C. S. Baptista, M. F. Gartner and G. R. Rutteman (2012). "Prognostic studies of canine and feline mammary tumours: the need for standardized procedures." Vet J **193**(1): 24-31.

Matos, A. J., C. Lopes, J. Carvalheira, M. Santos, G. R. Rutteman and F. Gartner (2006). "E-cadherin expression in canine malignant mammary tumours: relationship to other clinico-pathological variables." J Comp Pathol **134**(2-3): 182-189.

Mavaddat, N., A. C. Antoniou, D. F. Easton and M. Garcia-Closas (2010). "Genetic susceptibility to breast cancer." Mol Oncol **4**(3): 174-191.

Michalska, M. M., D. Samulak, H. Romanowicz and B. Smolarz (2015). "Single Nucleotide Polymorphisms (SNPs) of RAD51-G172T and XRCC2-41657C/T Homologous Recombination Repair Genes and the Risk of Triple- Negative Breast Cancer in Polish Women." Pathol Oncol Res **21**(4): 935-940.

Misdorp, W. (2002). Tumors of the Mammary Gland. Tumors in Domestic Animals. D. J. Meuten. Ames: IA, Iowa State Press**:** 575-606.

Misdorp, W., R. W. Else, E. Hellmen and L. TP. (1999). World Health Organization international histological classification of tumors of domestic animals Washington, D.C. : Armed Forces Institute of Pathology.

Moore, J. H. (2003). "The ubiquitous nature of epistasis in determining susceptibility to common human diseases." Hum Hered **56**(1-3): 73-82.

Onay, V. U., L. Briollais, J. A. Knight, E. Shi, Y. Wang, S. Wells*, et al.* (2006). "SNP-SNP interactions in breast cancer susceptibility." BMC Cancer **6**: 114.

Parl, F. F., S. Dawling, N. Roodi and P. S. Crooke (2009). "Estrogen metabolism and breast cancer: a risk model." Ann N Y Acad Sci **1155**: 68-75.

Pena, L., P. J. De Andres, M. Clemente, P. Cuesta and M. D. Perez-Alenza (2013). "Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics." Vet Pathol **50**(1): 94-105.

Rasotto, R., D. Berlato, M. H. Goldschmidt and V. Zappulli (2017). "Prognostic Significance of Canine Mammary Tumor Histologic Subtypes: An Observational Cohort Study of 229 Cases." Vet Pathol **54**(4): 571-578.

Rasotto, R., V. Zappulli, M. Castagnaro and M. H. Goldschmidt (2012). "A retrospective study of those histopathologic parameters predictive of invasion of the lymphatic system by canine mammary carcinomas." Vet Pathol **49**(2): 330-340.

Richardson, C. (2005). "RAD51, genomic stability, and tumorigenesis." Cancer Lett **218**(2): 127-139.

Ripperger, T., D. Gadzicki, A. Meindl and B. Schlegelberger (2009). "Breast cancer susceptibility: current knowledge and implications for genetic counselling." European journal of human genetics : EJHG **17**(6): 722-731.

Rojas, K. and A. Stuckey (2016). "Breast Cancer Epidemiology and Risk Factors." Clin Obstet Gynecol **59**(4): 651-672.

Roukos, D. H., G. G. Baltogiannis, C. S. Katsouras, A. Bechlioulis, K. K. Naka, C. Batsis*, et al.* (2014). "Novel next-generation sequencing and networks-based

therapeutic targets: realistic and more effective drug design and discovery." Curr Pharm Des **20**(1): 11-22.

Rudolph, A., J. Chang-Claude and M. K. Schmidt (2016). "Gene-environment interaction and risk of breast cancer." Br J Cancer **114**(2): 125-133.

Samuels, D. C., L. Han, J. Li, S. Quanghu, T. A. Clark, Y. Shyr*, et al.* (2013). "Finding the lost treasures in exome sequencing data." Trends Genet **29**(10): 593-599.

Santarpia, L., G. Bottai, C. M. Kelly, B. Gyorffy, B. Szekely and L. Pusztai (2016). "Deciphering and Targeting Oncogenic Mutations and Pathways in Breast Cancer." Oncologist **21**(9): 1063-1078.

Santos, A. A., C. C. Lopes, J. R. Ribeiro, L. R. Martins, J. C. Santos, I. F. Amorim*, et al.* (2013). "Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study." BMC Vet Res **9**: 1-11.

Santos, M., C. Correia-Gomes, R. Marcos, A. Santos, A. De Matos, C. Lopes*, et al.* (2015). "Value of the Nottingham Histological Grading Parameters and Nottingham Prognostic Index in Canine Mammary Carcinoma." Anticancer Res **35**(7): 4219-4227.

Santos, M., P. Dias-Pereira, C. Williams, C. Lopes and M. Breen (2017). "Malignant canine mammary tumours: Preliminary genomic insights using oligonucleotide array comparative genomic hybridisation analysis." Vet J **222**: 68-71.

Sapkota, Y., J. R. Mackey, R. Lai, C. Franco-Villalobos, S. Lupichuk, P. J. Robson*, et al.* (2013). "Assessing SNP-SNP Interactions among DNA Repair, Modification and Metabolism Related Pathway Genes in Breast Cancer Susceptibility." PLoS ONE **8**(6): e64896.

Sapkota, Y., J. R. Mackey, R. Lai, C. Franco-Villalobos, S. Lupichuk, P. J. Robson*, et al.* (2014). "Assessing SNP-SNP interactions among DNA repair, modification and metabolism related pathway genes in breast cancer susceptibility." PLoS One **8**(6): e64896.

Sarli, G., R. Preziosi, L. De Tolla, B. Brunetti and C. Benazzi (2004). "E-cadherin immunoreactivity in canine mammary tumors." J Vet Diagn Invest **16**(6): 542-547.

Schwartz, M. L. B., M. S. Williams and M. F. Murray (2017). "Adding Protective Genetic Variants to Clinical Reporting of Genomic Screening Results: Restoring Balance." Jama **317**(15): 1527-1528.

Sharma, S., T. K. Kelly and P. A. Jones (2010). "Epigenetics in cancer." Carcinogenesis **31**(1): 27-36.

Shen, J. (2009). "Evaluation of environmental and personal susceptibility characteristics that modify genetic risks." Methods Mol Biol **471**: 163-177.

Shen, Z., X. F. Wen, F. Lan, Z. Z. Shen and Z. M. Shao (2002). "The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma." Clin Cancer Res **8**(7): 2085-2090.

Shiovitz, S. and L. A. Korde (2015). "Genetics of breast cancer: a topic in evolution." Ann Oncol **26**(7): 1291-1299.

Smolarz, B., M. M. Michalska, D. Samulak, H. Romanowicz and L. Wojcik (2019). "Polymorphism of DNA repair genes in breast cancer." Oncotarget **10**(4): 527-535.

Smolarz, B., M. Zadrozny, J. Duda-Szymanska, M. Makowska, D. Samulak, M. M. Michalska*, et al.* (2013). "RAD51 genotype and triple-negative breast cancer (TNBC) risk in Polish women." Pol J Pathol **64**(1): 39-43.

Soderlund, K., L. Skoog, T. Fornander and M. S. Askmalm (2007). "The BRCA1/BRCA2/Rad51 complex is a prognostic and predictive factor in early breast cancer." Radiother Oncol **84**(3): 242-251.

Sorenmo, K. (2003). "Canine mammary gland tumors." Veterinary Clinics of North America: Small Animal Practice **33**(3): 573-596.

Sorenmo, K. U., V. M. Kristiansen, M. A. Cofone, F. S. Shofer, A. M. Breen, M. Langeland*, et al.* (2009). "Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence." Vet Comp Oncol **7**(3): 162-172.

Sorenmo, K. U., R. Rasotto, V. Zappulli and M. H. Goldschmidt (2011). "Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms." Vet Pathol **48**(1): 85-97.

Stratton, M. R., P. J. Campbell and P. A. Futreal (2009). "The cancer genome." Nature **458**(7239): 719-724.

Sullivan, M. R. and K. A. Bernstein (2018). "RAD-ical New Insights into RAD51 Regulation." Genes (Basel) **9**(12).

Sun, M. Y., H. Y. Du, A. N. Zhu, H. Y. Liang, G. R. de Garibay, F. X. Li*, et al.* (2015). "Genetic polymorphisms in estrogen-related genes and the risk of breast cancer among Han Chinese women." Int J Mol Sci **16**(2): 4121-4135.

Syed, B. M., A. R. Green, D. A. L. Morgan, I. O. Ellis and K. L. Cheung (2019). "Liver Kinase B1-A Potential Therapeutic Target in Hormone-Sensitive Breast Cancer in Older Women." Cancers (Basel) **11**(2).

Takeichi, M. (1990). "Cadherins: A Molecular Family Important in Selective Cell-Cell Adhesion." Annual Review of Biochemistry **59**(1): 237-252.

Tan, H. (2018). "On the Protective Effects of Gene SNPs Against Human Cancer." EBioMedicine **33**: 4-5.

Travis, R. C. and T. J. Key (2003). "Oestrogen exposure and breast cancer risk." Breast cancer research : BCR **5**(5): 239-247.

van Lier, M. G., A. Wagner, E. M. Mathus-Vliegen, E. J. Kuipers, E. W. Steyerberg and M. E. van Leerdam (2010). "High cancer risk in Peutz-Jeghers syndrome: a systematic review and surveillance recommendations." Am J Gastroenterol **105**(6): 1258-1264; author reply 1265.

van Roy, F. and G. Berx (2008). "The cell-cell adhesion molecule E-cadherin." Cell Mol Life Sci **65**(23): 3756-3788.

Wunsch Filho, V. and M. A. Zago (2005). "Modern cancer epidemiological research: genetic polymorphisms and environment." Rev Saude Publica **39**(3): 490-497.

Yager, J. D. (2012). "Catechol-O-methyltransferase: characteristics, polymorphisms and role in breast cancer." Drug Discov Today Dis Mech **9**(1-2): e41-e46.

Yager, J. D. and N. E. Davidson (2006). "Estrogen carcinogenesis in breast cancer." N Engl J Med **354**(3): 270-282.

Yager, J. D. and J. G. Liehr (1996). "Molecular mechanisms of estrogen carcinogenesis." Annu Rev Pharmacol Toxicol **36**: 203-232.

Yilmaz, M. and G. Christofori (2010). "Mechanisms of motility in metastasizing cells." Mol Cancer Res **8**(5): 629-642.

Zhuang, Z. G., G. H. Di, Z. Z. Shen, J. Ding and Z. M. Shao (2006). "Enhanced expression of LKB1 in breast cancer cells attenuates angiogenesis, invasion, and metastatic potential." Mol Cancer Res **4**(11): 843-849.

# CHAPTER 10

## Future Perspectives

Although CMTs are among the most common tumors affecting female dogs, and are thus widely studied, it is also recognized that the inherent carcinogenic process is still underexplored, especially concerning to its genetic basis. The identification of individual's genetic profile, specifically regarding the influence of SNPs, is recognised as relevant for clinical decisions in human breast cancer. Even though preliminary, our results extend this perspective to the context of CMTs. Indubitably a larger number of cases must be included in future studies, emphasizing the animal´s characteristics and features of the neoplastic lesion characteristics for granting generalization of the present results in incoming years. Certainly, the identification of low and high-risk groups for developing mammary neoplastic disease and the accurate identification of clinicopathological features, especially those related with aggressive malignant tumors, will allow the establishment of preventive measures before the disease development and of suitable therapeutic approaches after the detection of the neoplasia. Medical examination and follow-up strategies could be tailored for each case and surgery protocols could be applied with a bidirectional scope: preventive and curative intents.

Future studies comprising an integrated analysis involving other tumor suppressor genes, genes included in the hormonal regulation and oncogenes are being planned. The genes BRCA1 and BRCA2 are involved in ensuring genome stability and are pointed as a pivotal cause of the majority of the hereditary human breast cancer cases (Kuchenbaecker *et al.* 2014, Pfeffer *et al.* 2017, Sun *et al.* 2017). Previous studies in canine species involving these genes have been reported focusing mainly SNPs identification and exploring mammary tumors susceptibility, however with no reference to clinicopathological features of CMTs (Tsuchida *et al.* 2001, Rivera et al. 2009, Borge *et al.* 2011, Enginler *et al.* 2014, Ochiai *et al.* 2015). As far as we know only two reports revealed association between SNPs in BRCA1 and BRCA2 genes and mammary tumors susceptibility (Rivera et al. 2009, Enginler et al. 2014). A future aim is to investigate the associations between SNPs in BRCA1 and BRCA2 genes and the mammary neoplasia features in our dog population. Also HER2 gene, whose overexpression is related to poor outcome in human breast cancer (Sun *et al.* 2017, Harbeck 2018), will be evaluated concerning the influence of its genetic variants on the clinicopathological variables, including survival of CMTs. Along with ESR1 and COMT genes, Progesterone Receptor gene (PGR

gene) and Prolactin Receptor gene (PRLR gene), are also known to be relevant in women breast cancer in terms of susceptibility and treatment protocols (Samson *et al.* 2016, Nagini 2017, Tian *et al.* 2018). We aim to assess the correlation between the PGR and PRL genotypes and the phenotypical features of the disease in canine species. Genetic variations in other genes, namely PTEN, CHEK2 and BRIP genes, which have also been studied in human breast cancer (van der Groep *et al.* 2011, Lalloo *et al.* 2012), are being considered as targets of our research. It is important to state that the role of the genetic variation in all these genes on phenotype of CMTs has never been comprehensively assessed concerning clinicopathological features of CMTs, as far as we known.

Comprehensive studies are also required to evaluate the interaction/interdependence between different SNPs, which can help to understand the role epistasis play in the development of such a complex disease, as mammary neoplasia (Niel *et al.* 2015, Wang *et al.* 2017).

It is also a goal of our research to fully characterize our series of CMTs in order to establish a comparison with the individual genetic prolife. For this purpose, we intend to perform immunohistochemistry techniques to evaluate tissue expression patterns of the proteins codified by the studied genes. The relationship of this data with the other clinicopathological variables, including animal outcome, will be evaluated.

Additional studies, assessing the functional activity of enzymes and other proteins codified by the genetic variants, are needed to disclose the mechanisms by which these SNPs influence clinicopathological features and progression of CMTs. Besides, further biochemical studies are crucial to confirm the variation in the structure, stability and kinetic parameters of those proteins that can indeed be attributed to the SNPs.

One of the major phenotypical features of CMTs is their clinical and morphological heterogeneity. Another peculiar morphological feature of CMTs is the common presence of more than one cell population within a single tumor and the abundance of stroma (Misdorp 2002). In breast cancer different reports showed that stroma has an important influence in the development of neoplasia (Mao *et al.* 2013, Soysal *et al.* 2015, McCuaig *et al.* 2017, Polonia *et al.* 2017). On the contrary, few studies have been dedicated to this issue in veterinary research (Santos *et al.* 2015, Case *et al.* 2017, Ettlin *et al.* 2017). It would be interesting to evaluate the

genotypic and phenotypic changes of all the neoplastic components of the mammary lesion, including epithelial and mesenchymal tumors cells, as well as the surrounding stroma, using laser capture microdissection. With this approach, an interesting comparison between constitutional and somatic genetic changes in dogs and in their mammary tumors could be performed.

One factor that definitely contributes to the variability of CMTs is the high prevalence of multiple tumors along the mammary gland on the first animal´s examination (Sorenmo 2003, Sorenmo *et al.* 2013, Goldschmidt *et al.* 2017). A recent preliminary study pointed to the genetic clonal independence of multiple synchronous tumors and showed that within each tumor genetic heterogeneity can be detected (Santos *et al.* 2017). Given the clinical relevance of multiple tumors and of the decision regarding their surgical approach, it is our intention to pursuit with this line of investigation.

Additionally, large multi-institutional studies are warranted for evaluating the prognostic value of some histological subtypes of CMTs, especially those concerning the 2011 classification. Also supplementary efforts are needed in order to minimize bias related to the obtainment of follow-up data from different clinicians.

Given the high number of dog breeds future analyses should be addressed using stratification systems (Parker *et al.* 2004, American Kennel Club 2019, FCI 2019).

We are aware that the present data is a small piece of the large puzzle that is the carcinogenic process in canine mammary tissue. Hopefully the present data and the results of further studies could be used to design specific surveillance and clinical monitoring protocols in female dogs. Eventually similar genetic studies will be performed involving other types of neoplasia. The establishment of genetic tests for application on the routine practice and the definition of potential targets for specific therapeutic protocols in dogs with mammary tumors are definitely relevant goals for all the veterinary medical community and for owners, who are increasingly concerned with the welfare of their pet animals.

## REFERENCES

American Kennel Club. (2019). *Dog Breeds - Types Of Dogs - American Kennel Club*. [online] Available at: https://www.akc.org/dog-breeds/ [Accessed 2 May 2019].

Borge, K. S., A. L. Borresen-Dale and F. Lingaas (2011). "Identification of genetic variation in 11 candidate genes of canine mammary tumour." Vet Comp Oncol **9**(4): 241-250.

Case, A., B. K. Brisson, A. C. Durham, S. Rosen, J. Monslow, E. Buza*, et al.* (2017). "Identification of prognostic collagen signatures and potential therapeutic stromal targets in canine mammary gland carcinoma." PloS one **12**(7): e0180448-e0180448.

Enginler, S. O., I. Akis, T. S. Toydemir, K. Oztabak, D. Haktanir, M. C. Gunduz*, et al.* (2014). "Genetic variations of BRCA1 and BRCA2 genes in dogs with mammary tumours." Vet Res Commun **38**(1): 21-27.

Ettlin, J., E. Clementi, P. Amini, A. Malbon and E. Markkanen (2017). "Analysis of Gene Expression Signatures in Cancer-Associated Stroma from Canine Mammary Tumours Reveals Molecular Homology to Human Breast Carcinomas." Int J Mol Sci **18**(5).

Fci.be. (2019). *Fédération Cynologique Internationale*. [online] Available at: http://www.fci.be/en/ [Accessed 2 May 2019].

Goldschmidt, H., L. Pena and V. Zappulli (2017). Tumors of the mammary gland. Tumors in Domestic Animals. D. J. Meuten. Ames, IA, John Wiley & Sons Inc.**:** 723-765.

Harbeck, N. (2018). "Advances in targeting HER2-positive breast cancer." Curr Opin Obstet Gynecol **30**(1): 55-59.

Kuchenbaecker, K. B., S. L. Neuhausen, M. Robson, D. Barrowdale, L. McGuffog, A. M. Mulligan*, et al.* (2014). "Associations of common breast cancer susceptibility alleles with risk of breast cancer subtypes in BRCA1 and BRCA2 mutation carriers." Breast cancer research : BCR **16**(6): 3416-3416.

Lalloo, F. and D. G. Evans (2012). "Familial breast cancer." Clin Genet **82**(2): 105-114.

Mao, Y., E. T. Keller, D. H. Garfield, K. Shen and J. Wang (2013). "Stromal cells in tumor microenvironment and breast cancer." Cancer Metastasis Rev **32**(1-2): 303-315.

McCuaig, R., F. Wu, J. Dunn, S. Rao and J. E. Dahlstrom (2017). "The biological and clinical significance of stromal-epithelial interactions in breast cancer." Pathology **49**(2): 133-140.

Misdorp, W. (2002). Tumors of the Mammary Gland. Tumors in Domestic Animals. D. J. Meuten. Ames: IA, Iowa State Press**:** 575-606.

Nagini, S. (2017). "Breast Cancer: Current Molecular Therapeutic Targets and New Players." Anticancer Agents Med Chem **17**(2): 152-163.

Niel, C., Sinoquet, C., Dina, C., & Rocheleau, G. (2015). "A survey about methods dedicated to epistasis detection." Frontiers in genetics **6**: 285.

Ochiai, K., T. Ishiguro-Oonuma, Y. Yoshikawa, C. Udagawa, Y. Kato, M. Watanabe*, et al.* (2015). "Polymorphisms of canine BRCA2 BRC repeats affecting interaction with RAD51." Biomed Res **36**(2): 155-158.

Parker, H. G., L. V. Kim, N. B. Sutter, S. Carlson, T. D. Lorentzen, T. B. Malek, et al. (2004). "Genetic Structure of the Purebred Domestic Dog." Science **304**(5674): 1160-1164.

Pfeffer, C. M., B. N. Ho and A. T. K. Singh (2017). "The Evolution, Functions and Applications of the Breast Cancer Genes BRCA1 and BRCA2." Cancer genomics & proteomics **14**(5): 293-298.

Polonia, A., R. Pinto, J. F. Cameselle-Teijeiro, F. C. Schmitt and J. Paredes (2017). "Prognostic value of stromal tumour infiltrating lymphocytes and programmed cell death-ligand 1 expression in breast cancer." J Clin Pathol **70**(10): 860-867.

Samson, M., N. Porter, O. Orekoya, J. R. Hebert, S. A. Adams, C. L. Bennett*, et al.* (2016). "Progestin and breast cancer risk: a systematic review." Breast Cancer Res Treat **155**(1): 3-12.

Santos, A. A. and A. J. Matos (2015). "Advances in the understanding of the clinically relevant genetic pathways and molecular aspects of canine mammary tumours. Part 2: invasion, angiogenesis, metastasis and therapy." Vet J **205**(2): 144-153.

Santos, M., P. Dias-Pereira, C. Williams, C. Lopes and M. Breen (2017). "Malignant canine mammary tumours: Preliminary genomic insights using oligonucleotide array comparative genomic hybridisation analysis." Vet J **222**: 68-71.

Sorenmo, K. (2003). "Canine mammary gland tumors." Veterinary Clinics of North America: Small Animal Practice **33**(3): 573-596.

Sorenmo, K. U., D. R. Worley and M. H. Goldschmidt (2013). Tumors of the Mammary Gland. Withrow and MacEwen's Small Animal Clinical Oncology (Fifth Edition). S. J. Withrow, D. M. Vail and R. L. Page. Saint Louis, W.B. Saunders**:** 538-556.

Soysal, S. D., A. Tzankov and S. E. Muenst (2015). "Role of the Tumor Microenvironment in Breast Cancer." Pathobiology **82**(3-4): 142-152.

Sun, Y.-S., Z. Zhao, Z.-N. Yang, F. Xu, H.-J. Lu, Z.-Y. Zhu*, et al.* (2017). "Risk Factors and Preventions of Breast Cancer." International journal of biological sciences **13**(11): 1387-1397.

Tian, J. M., B. Ran, C. L. Zhang, D. M. Yan and X. H. Li (2018). "Estrogen and progesterone promote breast cancer cell proliferation by inducing cyclin G1 expression." Braz J Med Biol Res **51**(3): 1-7.

Tsuchida, S., S. Ikemoto and M. Tagawa (2001). "Microsatellite polymorphism in intron 14 of the canine BRCA1 gene." J Vet Med Sci **63**(4): 479-481.

van der Groep, P., E. van der Wall and P. J. van Diest (2011). "Pathology of hereditary breast cancer." Cellular oncology (Dordrecht) **34**(2): 71-88.

Wang W, Xu ZZ, Costanzo M, Boone C, Lange CA, Myers CL (2017). "Pathway-based discovery of genetic interactions in breast cancer." PLoS Genet **13**(9): e1006973.