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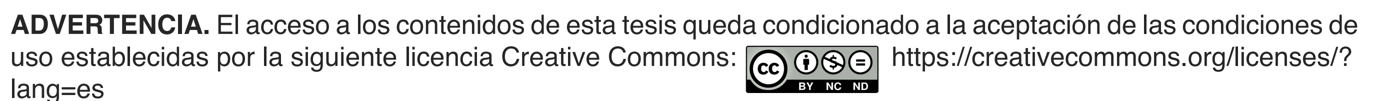
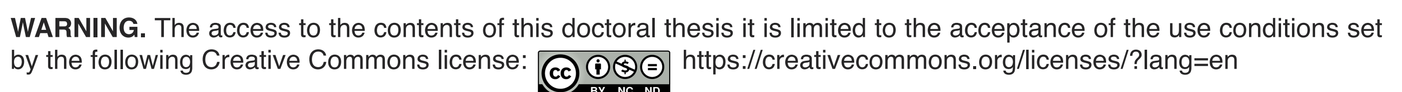
ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  https://creativecommons.org/licenses/?lang=es

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

SERUM AND TISSUE BIOMARKERS FOR CLINICAL DIAGNOSIS OF CANINE MAMMARY TUMOURS

PhD Thesis

Animal Medicine and Health

Makchit Galadima

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Director and Tutor:

Maria Montserrat Rivera del Alamo

Directors:

Juan Enrique Rodriguez Gil

Mariana Teles Pereira

Universitat Autònoma de Barcelona
Departament de Medicina i Cirurgia Animals

La Dra Maria Montserrat Rivera del Alamo, profesora agregada del Departament de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, el Dr. Joan Enric Rodríguez-Gil, profesor titular del Departament de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, y la Dra. Mariana Teles Pereira, investigadora Ramón y Cajal del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autonòma de Barcelona

Certifican:

Que la tesis titulada **"Serum and tissue biomarkers for clinical diagnosis of canine mammary tumours"**, presentada por la doctoranda Makchit Galadima Saleh Fale para optar al grado de Doctor en Veterinaria se ha realizado bajo nuestra dirección y, considerándola acabada, autorizamos su presentación para que sea juzgada por la correspondiente comisión.

Bellaterra, Noviembre de 2023

Mª Montserrat Rivera del Alamo   Mariana Teles Pereira   Joan Enric Rodríguez-Gil

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SUMMARY

Canine mammary tumours (CMTs) are naturally occurring cancer that it is frequent in intact female dogs, and rare in male dogs. The annual reported incidence is approximately 198 cases of every 100,000 female dogs, with this incidence tending to be higher in regions of the world where early spaying is seldom practised. The exact cause of CMTs is still unknown, even though myriads of risk factors have been attributed. The milestone for prognosis in CMTs is an early diagnosis, with late diagnosis being usually associated with low survival rates after the surgical treatment. Unfortunately, CMTs may be associated with non-specific clinical signs, which makes early detection by the owner, or during routine physical examinations difficult.

Like in human oncology, recent advances made in the diagnosis of CMTs include the discovery of biomarkers in both mammary gland tissues by immunohistochemistry techniques and blood/plasma/serum by other molecular methods. So far in veterinary oncology, only a few biomarkers have been discovered and standardized for use. Therefore, we hypothesized that biomarkers in the serum and mammary tissue can be used in the diagnosis of CMTs.

The objectives of the present study were two-fold. Firstly, the serum of female dogs was assessed in the determination of suitable biomarkers for CMTs. To this end, blood was sampled from 30 female dogs and subsequently centrifuged. The collected serum was analysed through CD antibody microarrays targeting 90 CD surface markers and 56 cytokines/chemokines. A total of five CD proteins, namely CD20, CD45RA, CD53, CD59, and CD99, were selected due to their differential protein abundance in relation to either log FC values or p-values. These were further analysed utilizing immunoblotting techniques to validate the microarray results. CD45RA showed a significantly lower ($P<0.05$) abundance in the serum obtained from the individuals presenting mammary neoplasia in comparison to the control group. In contrast, CD99 resulted significantly more abundant in neoplastic female dogs than in healthy patients. Finally, CD20 showed a significantly higher abundance in individuals carrying a malignant mammary tumour in comparison to healthy patients, although no differential expression between malignant and benign tumours was observed. It was concluded that both CD99 and CD45RA are indicators of mammary tumour presence, although without differentiating between malignant and benign canine mammary neoplasia.

Secondly, the present study aimed to assess the gene expression mRNA in mammary tissue of both healthy and diagnosed CMT female dogs through quantitative PCR (RT-qPCR). The target genes chosen for this study included vascular endothelial growth factor A (VEGFα), CD20, progesterone receptor (PGR), hyaluronidase 1 (HYAL1), programmed death ligand 1 (PDL1), epidermal growth factor (EGF), relaxin 2 (RLN2), and matrix metallopeptidase 3 (MMP3). Fifty-eight mammary tissue samples from female dogs, both healthy (n=3) and presenting mammary tumours (n-58), were analysed for this purpose. Transcription levels of HYAL1, VEGFα, CD20 and *PGR* genes showed similar higher expressions in both benign and malignant mammary tumours in comparison to healthy individuals, whereas MMP3*,* EGF, PDL-1, and RLN2 genes were found at higher expression levels in malignant tumours than in the benign tumours and the control group. Therefore, it was concluded that HYAL1, VEGFα, CD20 and PGR genes are markers for tumourigenesis, whereas MMP3, EGF, PDL-1, and RLN2 genes are markers for malignancy. Gene expressions for *CD20*, *PGR*, *EGF*, *RLN2* and *MMP3* are significantly correlated, suggesting that the combination of these molecules may have potential as malignancy biomarkers in CMTs.

In conclusion, this study demonstrated the involvement of specific serum CD proteins as well as specific genes expressed in mammary tissue in the detection of tumoural anomalies.

ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immune Deficiency Syndrome
Akt	Protein kinase B
APC	Adenomatous Polyposis Coli Tumour-Suppressor Protein
AP-1	Activator Protein 1
ARG 1	Arginase-1
ATP	Adenosine Triphosphate
AXIN	Axis Inhibition
B cell	B Lymphocyte
BCLXL	B Cell Lymphoma-Extra Large
BCLXS	B Cell Lymphoma-2 isoform
BCL2	B Cell Lymphoma 2
B. fragilis	*Bacteroides Fragilis*
BMP	Basic Metabolic Panel
BMI-1	B Lymphoma Mo-MLV insertion region 1 Homolog
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
CSF-1	Colony Stimulating Factor-1

CA 15-3	Cancer Antigen 15-3
CCL5	C-C motif Chemokine Ligand 5
CEA	Carcinoembryonic Antigen
CDH1	Cadherin-1
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin Dependent Kinase 4
CDK6	Cyclin Dependent Kinase 6
CD4+	Cluster of Differentiation 4 positive
CD8	Cluster of Differentiation 8
CEUS	Contrast-Enhanced Ultrasound
cfDNA	Cell Free Deoxyribonucleic Acid
cfRNA	Cell Free Ribonucleic Acid
CHEK1	Checkpoint Kinase 1
CHEK2	Checkpoint Kinase 2
C. koseri	*Citrobacter koseri*
Class III PI3K	Class 3 Phospoinositide 3-Kinase
CMA	Chaperone-Mediated Autophagy
cMYC	Cellular Myelocytomatosis

COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CMT	Canine Mammary Tumour
CREB	cAMP Response Element-Binding Protein
CRD	Cystein-Rich Domain
CTC	Circulating Tumour Cells
ctDNA	Circulating Deoxyribonucleic Acid
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTC	Disseminated Tumour Cells
E. aerogenes	*Enterobacter aerogenes*
E cadherin	Epithelial Cadherin
E. Coli	*Escherichia coli*
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cellular Adhesion Molecule
ERBB2	Erythroblastic Oncogene B
ESR1	Estrogen Receptor 1

ER	Oestrogen Receptor
ERα	Oestrogen Receptor Alpha
Erβ	Oestrogen Receptor Beta
ER-	Oestrogen Receptor Negative
ER+	Oestrogen Receptor Positive
EVs	Extracellular Vesicles
E_2	Oestrogen
FADD	Fas Associated Death Domain
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FGFR2	Fibroblast Growth Factor Receptor 2
FLT1	Fms-related Tyrosine Kinase
FOXA1	Forkhead Box Protein 1
FOXO	Forkhead Box O
FNAC	Fine Needle Aspirate Cytology
GH	Growth Hormone
GHR+	Growth Hormone Receptor-Positive
GMDSC	Granulocytic Myeloid-Derived Suppressor Cells

GPCR	G Protein-Coupled Receptor
GRB2	Growth Factor Receptor Bound 2
GSK-3β	Glycogen Synthase Kinase-3β
GSTP1	Glutathione S Transferase Pi
HYAL-1	Hyaluronidase-1
H. B. virus	Hepatitis B Virus
H. C. virus	Hepatitis C Virus
H. Pylori	*Helicobacter pylori*
HBC	Human Breast Cancer
HER-2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HH	Hedgehog
HIF 1α	Hypoxia Inducible Factor 1 Alpha
HIF 1β	Hypoxia Inducible Factor 1 Beta
HIF 2	Hypoxia Inducible Factor 2
HIF 3	Hypoxia Inducible Factor 3
HLA-II	Human Leukocyte Antigen-II
HOXD10	Homeobox D10

HYAL-1	Hyaluronidase-1
ICE	Interleukin-1-Converting Enzyme
IGF-2	Insulin-like Growth Factor-2
IGF 1R	Insulin-like Growth Factor 1 Receptor
IGFBPS	Insulin-like Growth Factor Binding Proteins
IκB	IkappaB
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1Ra	Interleukin 1 Receptor Antagonist
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-16	Interleukin-16

IL-18	Interleukin-18
IL-23	Interleukin-23
IL-33	Interleukin-33
IL-36α	Interleukin-36α
IL-36β	Interleukin-36β
IL-36γ	Interleukin-36γ
IL-37	Interleukin-37
IL-38	Interleukin-38
INF-γ	Interferon-γ
iNOS	Inducible Nitric Oxide Synthase
INSR-A	Insulin Receptor-A
INSR-B	Insulin Receptor-B
JAK2	Janus Kinase 2
kDa	Kilo Dalton
Ki-67	Kiel-67
K. pneumoniae	*Klebsiella Pneumoniae*
LAMP-2A	Lysosomal Associated Membrane Protein-2A
LDH	Lactate Dehydrogenase

LEF-1	Lymphoid Enhancer Binding Factor-1
LSP1	Lymphocyte Specific Protein-1
MAGI3	Membrane Associated Guanylate Kinase
MAPK	Mitogen-Activate Protein Kinase
MAP3K1	Mitogen-Activated Protein Kinase 1
MaSC	Mammary Stem Cell
MDSCs	Myeloid-Derived Suppressor cells
MIF	Migration Inhibition Factor
MKI-67	Marker of Proliferation Ki-67
miRNA	Micro Ribonucleic Acid
MLH1	MutL Homolog 1
MMPs	Matrix Metalloproteinases
MMP-1	Matrix Metalloprotease 1
MMP-2	Matrix Metalloprotease 2
MMP-3	Matrix Metalloprotease 3
MMP-9	Matrix Metalloprotease 9
MMTV	Mouse Mammary Tumour Virus
mRNA	Messenger Ribonucleic Acid

MUC-1	Mucin-1
MSA4	Membrane Spanning 4-domain Family A
mTOR	Mammalian Target Of Rapamycin
NAD	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor-Kappa light chain enhancer activated B cells
N cadherin	Neural Cadherin
NK	Natural Killer cell
NLRP3	NACHT, LRR, and PYD domains-containing protein 3
P cadherin	Placental Cadherin
PCNA	Proliferating Cell Nuclear Antigen
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
PD-I	Programmed Death 1
PGE2	Prostaglandin E2
PGF	Prostaglandin F
PGRMC1	Progesterone Receptor Membrane Component 1
PI	Proliferative Index
PR	Progesterone Receptor

PR-	Progesterone Receptor Negative
PR+	Progesterone Receptor Positive
PTCH1	Protein Patched Homolog 1
PTEN	Phosphatase and Tensin homolog
P4	Progesterone
P53	Protein 53
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
RAS	Renin Angiotensin System
RCAS1	Receptor binding Cancer Antigen expressed on SISO Cells
RhoC	Ras Homolog Family Member C
RLN2	Relaxin
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
TNF-mRNA	Tumour Necrosis factor- Messenger Ribonucleic Acid
SF	Serum Ferritin
SFRP-1	Secreted Frizzled Related Protein 1

SFRP-2	Secreted Frizzled Related Protein 2
SFRP-3	Secreted Frizzled Related Protein 3
SFRP-4	Secreted Frizzled Related Protein 4
SFRP-5	Secreted Frizzled Related Protein 5
SHH	Sonic Hedgehog
siRNA	Small Interfering Ribonucleic Acid
SIRT3	Sirtuin 3
S. japonicum	*Schistosoma japonicum*
S. mekongi	*Schistosoma mekongi*
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducer and Activator of Transcription 3
STAT5	Signal Transducer and Activator of Transcription 5
TAN	Tumour Associated Neutrophil
TAM	Tumour Associated Macrophage
T cell	T Lymphocyte
TCR	T cell Receptor
TGF-β	Transforming Growth Factor-Beta
T. Gondii	*Toxoplasma Gondii*

Th1	T Helper 1 cell
Th17	T Helper 17 cell
TME	Tumour Microenvironment
TNF-α	Tumour Necrosis Factor α
TNM system	Tumour Size, Number of lymph nodes involved, Metastasis
TNF-mRNA	Tumour Necrosis Factor- Messenger Ribonucleic Acid
TOX3	TOX High Mobility Group Box Family Member 3
TP53	Tumour Protein 53
TRAIL	TNF-Related Apoptosis-Inducing Ligand
tRNA	Transfer Ribonucleic Acid
Treg	T Regulatory Cell
TRF2	Telomeric Repeat-Binding Factor 2
T. vaginalis	*Toxoplasma vaginalis*
TWIST1	Twist family BHLH Transcription Factor 1
TWIST 2	Twist Homolog 2
VEGF	Vascular Endothelial Growth Factor
VEGF-A	Vascular Endothelial Growth Factor A
VEGF-B	Vascular Endothelial Growth Factor B

VEGF-C	Vascular Endothelial Growth Factor C
VEGF-D	Vascular Endothelial Growth Factor D
WHO	World Health Organization
WNT	Wingless-related Integration Site
XCL1	Chemokine (C motif) Ligand
XIAP	X-linked Inhibitor of Apoptosis Protein
ZEB1	Zinc finger E-Box-Binding Homeobox 1
ZEB2	Zinc finger E-Box-Binding Homeobox 2

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1.BASIC CONCEPTS OF THE CANINE MAMMARY TUMOUR

Mammary gland tumours are the most frequent cancers in women and intact female dogs (Kabir et al., 2016; Carvalho et al., 2016), appearing extremely sporadically in ruminants, horses and swine (Baba & Catoi, 2007). The dog-intact female is, among the monitored domestic species so far, the most frequently affected (Gelaleti et al., 2012; Gupta et al., 2012; Saito et al., 2013; Giacomo et al., 2022), showing a prevalence of about three times higher than in women (Yoshikawa et al., 2005; Micheal et al., 2012; Hussain et al., 2018; Kuppusamy et al., 2019; Valdivia et al., 2021). Thus, the annual incidence has been reported to be approximately 198 cases in every 100,000 female dogs, whereas human breast cancer has been reported to be 85 cases per 100,000 women (Chocteau et al., 2019; Gray et al., 2020).

Canine mammary tumours (CMTs) are highly heterogeneous spontaneous naturally occurring neoplasias of mammary gland affecting older non-spayed bitches (Sorenmo, 2003; Sleeckx et al., 2011; Kabir et al., 2016; Diao et al., 2019; dos Anjos et al.,2019; Varallo et al., 2019; Valdivia et al., 2021). It is rare in male dogs, reported to be 62 times less likely to develop the condition than female dogs (Saba et al., 2007; Kivrak & Aydin, 2017). Analysing the percentage of each canine tumour type, CMTs rank second to skin cancers (Gupta et al., 2012; Kabir et al., 2016; Kivrak & Aydin, 2017; Sharma et al., 2018; Irac et al., 2019), and constitute about 50 to 70% of all cancers (Sorenmo, 2003; Sleeckx et al., 2011; Manuali et al., 2012; Kabir et al., 2016; Diao et al., 2019; Varallo et al., 2019; Valdivia et al., 2021).

1.1.- Prevalence of canine mammary tumours

CMTs have been reported worldwide with regional differences in occurrence. The highest incidences are in countries that do not routinely practice early spaying (Sleeckx et al., 2011; Valdivia et al., 2021), such as Scandinavia and Spain, whereas North America and some Western European countries report the lowest incidences (Sleeckx et al., 2011). CMTs can originate from the epithelial, glandular, mesenchymal, or connective tissues in the mammary gland (Sorenmo,

2003; Valdivia et al., 2021). Human breast cancers (HBC) are from epithelial tissue, while CMTs frequently contain myoepithelial and mesenchymal components (Borge et al., 2013). Single or multiple mammary glands can be affected simultaneously, with 60% of tumours located within the caudal mammary glands. These tumours might vary histologically within a single specific tumour and between different tumours in the same dog (Visan et al., 2016; Gray et al., 2020). In addition, CMTs are either benign or malignant (Kivrzak & Aydin, 2017; Hussain et al., 2018; Lee et al., 2021), although both types may be found within a single dog (Sorenmo, 2003; Sleeckx et al., 2011). Younger dogs are more likely to present benign tumours than older dogs (Sleeckx et al., 2011; Torres et al., 2021). Close to 50% of CMTs are malignant (Sorenmo, 2003; Sleeckx et al., 2011; Valdivia et al., 2021) and have the potential to metastasize to the lung, and regional lymph nodes and, less frequently, to other organs such as the liver, kidneys and adrenal glands, brain, eyes and bones (Peres-Alenza et al., 2000; Sorenmo, 2003), skin, uterus, heart, pancreas, spleen and muscle (Sleeckz et al., 2011; Petrov et al., 2014). Metastasis is the main cause of cancer-related deaths in dogs (dos Anjos et al., 2019).

1.2. - Aetiology of canine mammary tumour

Currently, the exact cause or causes for the appearance of mammary tumours is unknown (Sharma et al., 2018), except for certain strains of mice where the Mouse Mammary Tumour Virus (MMTV) is causative (Abdelmegeed & Mohammed, 2018; Lawson & Glenn, 2019; Afzal et al., 2022). However, myriads of risk factors are implicated in the development of CMTs such as age, breed, genetic predisposition, hormones and growth factors, biomarkers like cyclooxygenase-2 (COX-2), diet, obesity, and environmental pollutants (Sleeckz et al., 2011; Thumser-Henner et al., 2020; Torres et al., 2021).

- Age: The incidence of CMTs increases with age but may vary depending on the natural life span of the breed of dog. Most publications report an average age range of 8–11 years, indicating that they are malignancies of middle-aged or older dogs. A retrospective study, performed by Salas et al. (2015) to investigate the epidemiological characteristics of CMT revealed that adult female dogs (9 to 12 years old) showed the highest incidences, followed by 5- to 8-year-old females.

Mammary tumours are rare in dogs under 5 years of age, unless they have been treated with progestins (Sleeckx et al., 2011; Torres et al., 2021).

- ***Hormones***: Historically, it has been stated that bitches that are spayed before the first oestrous cycle have a 0.5% risk of developing mammary tumours compared with intact ones (Schneider et al., 1969). On the other hand, bitches spayed before the second oestrous were said to have an 8% risk, while those spayed after the second oestrous and before two and a half years of age have a 26% risk (Schneider et al., 1969). This idea has become an overall accepted dogma. However, a later study (Beauvais et al., 2012) performed a systematic review of more than 11147 publications focused on the link between neutering/not-neutering practice and the incidence of CMTs. This study demonstrated that nearly all the evaluated studies showed a high to moderate risk of bias, concluding that there is no robust evidence that early neutering completely avoids the appearance of mammary tumours in the bitch. Most recent studies showed that spaying is an effective adjunct to mastectomy in dogs with mammary gland carcinoma and that the timing of spaying is important in influencing survival (Kristiansen et al., 2013; Kristiansen et al., 2016; Banchi et al., 2022). According to them, performing an ovariohysterectomy concurrently with tumour removal reduced the risk for subsequent mammary tumours by 47% in bitches with non-malignant mammary tumours (Kristiansen et al., 2013; Kristiansen et al., 2016; Banchi et al., 2022).

In women and female dogs, the risk of developing mammary tumours depends on the duration of exposure of mammary tissue to bioavailable oestrogens (E_2) and the associated cumulative mitotic activity (Sorenmo, et al., 2019; Vendramini et. al., 2020). Physiologically, mammary gland tissue comes under ovarian steroids every oestrous cycle; therefore, removing the proliferating stimuli (E_2) through early spaying is of greatest benefit to CMTs prevention. Similarly, the few reported cases of mammary tumours in males are related to oestrogen secretory-testicular tumours (Saba et al., 2007; Kwon et al., 2017). Although ovarian steroids are incriminated in mammary carcinogenesis, the mechanisms are still inconclusive. It is worth noting though that these steroids are crucial for mammary gland development and functioning from puberty, pregnancy, and throughout the reproductive life of animals. Oestrogens promote mammary epithelial cell proliferation causing elongation of the ductal tree. Progesterone (P_4) stimulates side branching and development of the alveolar buds which differentiates into alveoli. Oestrogen and P_4 mediate their actions on mammary gland epithelial through oestrogen receptor (ER) and progesterone receptors

(PR) respectively. Oestrogen binds to ERα and ERβ, which are nuclear ligand-activated transcription factors, to modulate the transcription of target genes. Transcription factors need nuclear receptor co-regulators to mediate their action on target DNA sequences. In this case, ER signalling is dependent on forkhead box A1 (FOXA1) expression, which promotes local DNA unwinding, facilitating the access of ER to DNA (Alferez et al., 2018). Hormones are known to be a cause of DNA damage, which always leads to gene mutation (Marchi et al., 2022).

The involvement of E_2 in human breast cancer development and progression is linked to their pro-proliferative and anti-apoptotic effects, and the exposure period of the mammary gland to E_2 (Queiroga et al., 2009; Sleeckz et al., 2011; Canadas et al., 2018b; Canadas-Sousa et al., 2019; Torres et al., 2021). Similarly, some of the intermediate compounds from oestrogen metabolism have a well-documented genotoxic action (Santen et al., 2009; Canadas-Sousa, et al., 2019). Studies in mice revealed that the mechanism includes the generation of mutagenic/genotoxic metabolites from E_2 oxidative metabolism. These mutagenic metabolites of A-ring E_2, such as 2- and 4- catechol oestrogen and derivatives and oestrogen-3,4-quinone derivative, accumulate in the mammary tissue. The 2- and 4-catechol oestrogen and derivatives can be carcinogenic in mammary tissue, while the oestrogen-3,4-quinone derivative may form unstable adducts that interact with adenine and guanine bases which bind to DNA, producing depurinating adducts, being then oxidized to semi-quinones and quinones in a process that generates reactive oxygen species (ROS) able to induce DNA damage and gene mutation (Timmermans-Sprang et al., 2017; Canadas et al., 2018b; Canadas-Sousa et al., 2019; Torres et al., 2021).

While there is no information demonstrating the contribution of E_2 metabolites in canine mammary carcinogenesis, there is evidence of high serum and mammary E_2 in dogs with mammary tumours relative to those without tumours. Likewise, there are higher 17β-oestradiol serum levels in dogs with benign and malignant tumours than in those with normal mammary tissues (Torres et al., 2021). This is because E_2 is synthesized also by normal and tumour mammary tissues besides the ovary (Torres et al., 2021). The local mammary gland E_2 production is carried out by steroidogenic enzymes such as CYP19-aromatase, 17-β- hydroxysteroid dehydrogenase type I, and steroid sulfatase (Torres et al., 2021). Results thus suggest, on one hand, that local steroid synthesis is more relevant in mammary carcinogenesis than circulating E_2 and there is a maintenance of a high concentration of these hormones in mammary tissues (Torres et al., 2021). Additionally, changes

in tissue E_2 concentration are positively correlated with the expression of mammary gland epidermal growth factor receptor (EGFR) (Torres et al., 2021).

Several studies on CMTs have reported expressions of both PR and ER in normal and neoplastic mammary tissues. While ER are highly expressed in benign tumours compared to malignant tumours, PR are under-expressed in hyperplastic/dysplastic over benign to malignant tumours (Sleeckx et al., 2011; Abdelmegeed & Mohammed, 2018). Tumours expressing low ER and high PR are associated with a guarded prognosis, although not all studies agree (Sleeckz et al., 2011). Malignant tumours tend to lose hormonal dependency as the disease progresses towards metastasis, in other words, they are negative for both ER and PR. In human breast cancer, the negative status of ER and PR is in fact associated with a poorer prognosis (Queiroga et al., 2011; Abdelmegeed & Mohammed, 2018). In addition to the differences in the steroid hormone receptors in normal and neoplastic mammary tissue, serum levels of steroid hormones in bitches with malignant tumours were significantly higher compared with healthy dogs or bitches with benign tumours (Sorenmo, 2003; Sleeckz et al., 2011). Another circumstance that is thought to affect CMTs development is pseudopregnancy, but it is still debated, although, most likely, there is no effect (Sorenmo, 2003; Sleeckz et al., 2011; Benavente et al., 2018). Likewise, contraception with progestins may increase the incidence of CMTs (Sorenmo, 2003; Sleeckz et al., 2011).

Like E_2, P_4 is crucial in mammary gland development. It controls the proliferation and morphogenesis of the luminal epithelium at puberty, pregnancy, parturition, lactation, and involution; it also initiates the expansion of stem cells to generate progenitor cells for alveologenesis. These roles are conducted through paracrine pathways (wingless integrated (WNT), receptor activator of nuclear factor kappa B ligand (RANKL), insulin-like growth factor-2 (IGF-2)) (Rajaram & Brisken, 2011; Obr & Edwards, 2012; Rosen & Roarty, 2014; Alferez et al., 2017; Timmermans-Sprang et al., 2018). In addition, at mid to late gestation, P_4 suppress secretory activation until parturition. This role is mediated partly by PR and prolactin/STAT5 signalling to inhibit the induction of milk protein gene expression, thereby preventing milk protein production and inhibiting the closure of tight junctions (Obr & Edwards, 2012).

Growth hormone (GH) also participates in mammary development and carcinogenesis. It is primarily produced in the differentiated pituitary or placental cells but can also be produced locally within the mammary gland under the influence of P_4. GH is responsible for the expansion of

mammary stem cells (MaSC) and progenitor cells when the mammary gland tissue grows (Timmermans-Sprang et al., 2017; Canadas-Sousa et al., 2019). Both stem and progenitor cells are necessary to ensure the proper long-term maintenance of mammary tissue structure and function during puberty, pregnancy, and lactation (Timmermans-Sprang et al., 2017). Additionally, progenitor cells are more proliferative and prone to oncogenic transformation (Timmermans-Sprang et al., 2017). This undesirable consequence of GH is mediated through two mechanisms. The first one is a direct effect on growth hormone receptor-positive (GHR+) cells, which are in the mammary stem cell (MaSC) compartment. The second mechanism takes place in the stromal compartment, where indirect GH effects are mediated by the synthesis and release of insulin-like growth factor-1 (IGF-1) by stromal cells (Timmermans-Sprang et al., 2017). This involvement of GH is launched through the activation of several components linked to the cell gene regulatory system such as the signal transducer of activator of transcription 3 (STAT3), Janus kinase 2 (JAK2)/ signal transducer of activator of transcription 5 (STAT5), wingless integrated (WNT) and receptor activator of nuclear factor kappa B ligand (RANKL) (Timmermans-Sprang et al., 2017; Alferez et al., 2018). However, not all mammary tumours have reactivity with hormonal receptor antibodies, with only approximately 50–77% of epithelial mammary gland tumours expressing hormonal receptors (Nieto et al., 2000).

- *Obesity and nutrition:* Obesity is a common nutritional disease in humans as well as dogs (Sleeckx et al., 2011; Gary et al., 2020; Tesi et al., 2020). Like in humans, several countries have demonstrated a prevalence of obesity among dogs (Marchi et al., 2022). For instance, in the United States and Australia, studies have shown that between 29 and 33.5% of dogs were overweight and between 5.1 and 7.6 %, were obese. In Brazil and Japan, between 25.9 and 39.8% were overweight and the rate of obesity was between 14.6 and 15.1%. Furthermore, in Spain and China, obesity rates of 40.9 and 44.4% in dogs were respectively reported (Marchi et al., 2022). Obesity has been associated with the risk of developing specific types of cancers in both women and bitches (Sleeckx et al., 2011). It has been estimated that 4-8% of all cancers in humans including breast, colorectal, oesophageal, kidney, gall bladder, uterine, pancreatic, and liver cancer are attributed to obesity, although the underlying mechanism is complex, yet inconclusive (Pati et al., 2023). They have consistently been shown to increase rates of breast cancer in postmenopausal women (Calle et al., 2004; Pischon et al., 2008). Obesity in humans is not only associated with an increased risk of cancer but also with a worse prognosis (recurrence and death) (Calle et al., 2004; Pishon et al.,

2008; Aune et al., 2016; Petrelli et al., 2021). In addition, overweight and obesity have been reported to increase the risk of a wide range of chronic diseases, including cardiovascular diseases, type 2 diabetes, several types of cancer, gallbladder disease, gout, osteoarthritis, and several other conditions, as well as mortality for any cause in humans (Aune et al., 2016). A study by Tesi et al. (2020) illustrated the relatedness between body condition scores and the prognosis of CMTs. The results revealed that a higher body condition is directly proportional to the aggressiveness of the tumour and negatively affects the survival time in bitches with these mammary tumours. It has been noted that a thin body conformation at 9-12 months of age reduced the risk of CMTs by 40 and 99% in non-spayed and spayed bitches respectively (Queiroga et al., 2011; Sleeckx et al., 2011; Magalhaes et al., 2020). Also, the consumption of a homemade diet (rich in beef and pork but low in chicken) as opposed to commercial food, is related to the risk of developing mammary tumour and dysplasia (Parez-Alenza et al., 1998; Alenza et al., 2000; Magalhaes et al., 2020). All these data highlight the importance of diet in the development of CMTs, although the exact mechanism/s by which this influence is established is not known.

- ***Breed***: Canine mammary tumours like other diseases can occur in any breed of dog, but certain breeds have higher susceptibility than others (Borge et al., 2013). These breed-related differences would indicate differential number and/or expression of risk alleles within these specific breeds, although the inherited risk factors of CMTs are unknown (Borge et al., 2013). An important factor involved with these breed-related differences could be the rigorous in-breeding applied to produce modern breeds of dogs from a few ancestors. This practice results in extreme phenotypic variation between breeds but with limited genetic variation from their ancestors. The breeds of dogs that have shown higher susceptibility to CMTs are miniature and toy Poodles, English Springer Spaniels, Brittany, German Shepherds, Cocker Spaniels, Dachshund Yorkshire Terriers, Maltese, West Highland White Terriers, Chihuahua, Beagle, English Setter, Bichon Fries, Terrier, Boxer, Pointer, and Afghan hound (Baba & Catoi, 2007; Sorenmo et al., 2010; Sleeckx et al., 2011; Borge et al., 2013; Tavasoly et al., 2013; Vahedi et al., 2019; Giovanni et al., 2020). However, considerable discrepancies exist between studies regarding the breed's susceptibility to CMTs.

It is noteworthy that the analysis of human *BRCA1* and *BRCA2* genes has established those specific mutations, either familial or inherited through the germline related to an increased lifetime risk of having breast tumours. However, the gene pack that is linked with the launching of human breast

tumours seems not to be the same as that related to canine ones. In this respect, a study by Rivera et al. (2009) that analysed the association of 10 selected human breast cancer genes (namely *BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1*, *RCAS1, TOX3*, and *TP53*) with CMTs in female English Springer Spaniels dogs showed that only two genes, *BRCA1* and *BRCA2*, were significantly associated with CMTs. Furthermore, Borge et al. (2013) genotyped 52 single nucleotide polymorphisms (*SNPs*) in 10 human cancer-associated genes in 2 different datasets to identify genes/alleles associated with the development of CMTs. The identification of CMT-associated *SNPs* in the oestrogen receptor 1 (*ESR1*) gene in two independent datasets suggests that this gene might be involved in CMT development. Vahedi et al. (2019) evaluated *DLA-DRB1* allele genetic polymorphism in a dog population. The result revealed the association between *DLA-DRB1* genes and the development of CMTs.

- ***Pesticides***: The build-up of pesticide residues in adipose tissues has been implicated in cancers, birth defects, endocrine disruption, and immunological, behavioural, neurological, and reproductive discrepancies in human and animal species (Gautam et al., 2020). Several studies on the role of pesticides in mammary tumours have given ambiguous results (Gautam et al., 2020). In this sense, a study performed by Andrade et al. (2010) detected and further identified the presence of pyrethroid insecticides in adipose tissue adjacent to malignant mammary tumours in bitches, thus suggesting the involvement of pyrethroids in CMTs carcinogenesis. The role of pesticides has also been described in mammary carcinoma in the male dog (Figueiroa et al., 2012). In another study performed by Colodel et al. (2012), which investigated the relationship between pyrethroid residues and the aggressiveness of mammary carcinoma in female dogs, the findings showed no statistical correlation between the pyrethroids and the aggressiveness of mammary carcinoma in bitches. However, a separate study performed by Gautam et al. (2020) to detect pesticides in serum, mammary tissue and mammary adipose tissue of canines suffering from spontaneous malignant mammary neoplasia, yielded differential accumulation of pesticides in serum and mammary tissue/adipose tissue. Nevertheless, no statistical association existed between the total pesticide concentrations among serum, mammary tissue and mammary adipose tissue in malignant CMT cases.

1.3.- Clinical signs

Usually, dog owners notice tumours when the tumour has grown visibly or they are found during a routine physical examination (Sorenmo, 2003; Thumser-Henner et al., 2020). Most of these dogs are clinically healthy when they are initially presented and, if metastases are present, dogs may show non-specific symptoms such as fatigue, lethargy, weight loss, dyspnoea, cough, lymphoedema or lameness (Sleeckz et al., 2011; Petrov et al., 2014). Nonetheless, the severity of clinical signs depends on the extent and location of the metastases (Sleeckz et al., 2011; Petrov et al., 2014).

1.4.- Diagnosis and prognosis

Mammary gland tumours are diagnosed using several diagnostic techniques. Some of which are non-invasive offering early diagnosis, while others are invasive hence late diagnosis. These techniques include:

- *Cytological examination*: The milestone of mammary neoplasia is an early diagnosis, which will allow the application of a precocious treatment and, in turn, provide a better prognosis for the patient. In women, early diagnosis of cancer lesions is performed through cytological examination. This technique of diagnosis is usually employed on palpable lesions such as the salivary gland, lymph node, thyroid, and mammary gland. Samples from lesions are obtained through fine needle aspiration cytology (FNAC) (Shafiee et al., 2013). This method is inexpensive, non-invasive, and yield rapid result; but only a small amount of tumour mass is available to analyse (Haziroglu et al., 2010; Sontas et al., 2012; Shafiee et al., 2013). In breast cancers, FNAC is done after clinical exploration and mammography. FNAC is an indispensable diagnostic method in breast cancer showing 65-96% sensitivity and 83-100% specificity (Sontas et al., 2012). Unlike, its accuracy is poorly reported in CMTs (Cassali et al., 2007) and its use is restricted probably due to the lower frequency of diagnosis of mammary tumours in dogs compared to women (Haziroglu et al., 2010; Sontas et al., 2012). However, some studies suggest that cytology is a valuable diagnostic tool for CMT despite its low accuracy when the number of samples is inadequate (Simon et al., 2009; Haziroglu et al., 2010; Sontas et al., 2012; Dolka et al., 2018).

- *Histopathological examination*: Currently, this is the mainstay diagnostic method for CMTs, despite the morphological heterogeneity of these tumours, with frequent involvement of different cell populations (Canadas et al., 2018a). It is an invasive method, time-consuming, and delays the application of specific treatments for malignant cases (Sontas et al., 2012). In addition, the initial stages of CMTs may not be detectable through histopathological assessment (Bergman, 2003; Gama et al., 2003; Novosad, 2003).

- *Ultrasonographical examination*: Another non-invasive method of diagnosing CMTs and human breast cancers is ultrasonographical examination. Various ultrasound techniques have been explored in human and veterinary medicines, such as B-mode ultrasound, Contrast-enhanced ultrasound (CEUS), and Real-time elastography (Soler et al., 2016; Sahoo et al., 2020). These techniques increase the sensitivity and accuracy of diagnosis, but the definitive diagnosis of mammary tumours is based on histopathology examination (Soler et al., 2016). Several studies have employed ultrasound techniques in CMTs, yet the results were dissimilar (Soler et al., 2016; Feliciano et al., 2017; Sahoo et al., 2020).

- *Liquid biopsy*: Liquid biopsy is a tumour diagnostic technique that investigates the content of biomarkers in body fluids. These body fluids include blood, urine, pleural fluid, ascites fluid, saliva, sputum, synovial fluids, milk, amniotic fluid, seminal fluid, and cerebrospinal fluid. The biomarkers include circulating tumour cells (CTC) from tumours or pieces of circulating tumour DNA (ctDNA), cell-free RNA (cfRNA), and exosomes released from tumour cells into the body fluids. Liquid biopsy is currently being used as a complementary or alternative diagnostic technique to surgical biopsy (Michela, 2021). The advantages accrue to this method of diagnosis are that it involves minimal invasion to obtain samples, and measure the markers diagnostically when tumours are at the pre-symptomatic stage (Xue et al., 2019; Agashe & Kurzrock, 2020; Adashek et al., 2021; Michela, 2021). In addition, it improves personalized cancer treatment regimes, monitors response to treatment, detects resistance to therapy and detects tumour relapse after potentially curative treatment (Crowley et al., 2013). Therefore, this technique provides enhanced sensitivity and ease of repeated sampling during the treatment period. In human oncology, several applications of liquid biopsy have been used to detect tumours, such as the circulating tumour cell (CTC) test and circulating tumour DNA (ctDNA), also known as cell-free DNA (cfDNA) test (Crowley et al., 2013). Krugiyak et al. (2021) used blood-based liquid biopsy

on multiple blood and cancer tissues from dogs with several types of cancer diagnoses. Flory et al. (2023) retrospectively audited medical records of dogs diagnosed with cancer. In addition, prospectively subject blood samples from dogs to detect preclinical disease using blood-based liquid biopsy.

Tumours are prognostically examined by pathologists who use the World Health Organization (WHO) TNM system (T: the size of the tumour; N; affectation of lymph nodes; M: distant metastasis) to clinically stage CMTs post-surgically for doctors to determine the size of the tumour, the spread, and the treatment option (Gray et al., 2020; Valdivia et al., 2021). Numerical values are assigned to the letters in the acronym. To get values for T; when there is no tumour, T0 is assigned, T1 is assigned when the diameter is < 3cm, T2 involves tumours of 3–5 cm as maximum diameter and T3 is assigned to tumours >5 cm in diameter. In cases of multiple tumours, the largest one will be used as the basis for classification (Karayannopoulou et al., 2005).

The involvement of lymph nodes is assessed by N0 (where no lymph node is involved), N1-N3 (indicating some degree of nodal spread, with a progressively distal spread from N1 to N3). N-values are assessed differently for specific tumours and their regional lymph node drainage. For instance, in colorectal cancer, N1 indicates the involvement of 1-3 regional nodes. N2 can be 4-6 regional nodes, while N3 indicates >7 regional nodes involved. Nx is used when lymph nodes are unable to be assessed.

Finally, M denotes distant metastases of the primary tumour, where M0 is used if no distant metastasis is present and M1 when there is evidence of distant metastasis (Rosen & Sapra, 2022).

In addition to tumour staging, pathologists grade tumours histologically (Gundim et al., 2016). The clinical grade was assigned as I, II, III, or IV according to the extent of the tumour and the prognosis (Martins et al., 2016). A tumour that is graded 0 is carcinoma *in situ*, not considered cancerous but may become cancer in the future, grade I means a localized tumour, grade II is assigned to a locally advanced tumour in early stage, grade III is a locally advanced tumour in late stage, and grade IV is applied to metastatic tumours (Rosen & Sapra, 2022). One characteristic of cancer that provides worse clinical staging is metastasis formation, besides being linked directly to a poor prognosis (Lopes-Neto 2017).

1.5. - Treatment

- ***Surgery***: As in human breast cancer, disease staging for CMTs is mandatory before beginning definitive treatment (Gray et al., 2020). Surgery is the primary treatment option for CMTs in the absence of metastases and inflammation (Baba & Catoi, 2007), intending to remove the tumour mass with clean margins to prevent developing new tumours in the remaining glands. Surgical intervention could either be a simple lumpectomy, local mastectomy, regional mastectomy, total chain mastectomy, or bilateral total mastectomy (Gray et al., 2020; Valdivia et al., 2021). It is recommended that, if a single tumour (<1cm) is present, lumpectomy is opted for. If the tumour is larger and centrally located within the gland, a simple mastectomy is performed. If a single tumour mass is located between two mammary glands, or multiple masses in consecutive glands, regional mastectomy is indicated (Valdivia et al., 2021). Stratmann et al. (2018) conducted a study to investigate the recurrence of new mammary tumours in the remaining mammary chain tissue after regional mastectomy. The results revealed that 58% of dogs develop new mammary growth in the ipsilateral mammary chain after regional mastectomy of a single tumour. Though surgery is regarded as the mainstay treatment of mammary tumours, there is a consistently high mortality rate of bitches after mastectomy. This demonstrates that surgical resection is unsatisfactory (Stratmann et al., 2018). In cases of inflammatory mammary carcinoma and distant (organ) metastasis, surgery is contraindicated.

- ***Adjuvant treatment***: To lower the risk of tumour recurrences and metastases after surgery, adjuvant therapy such as chemotherapy, cyclooxygenase (COX) inhibitors, hormone therapy, and virotherapy could be considered as adjuvant treatments (Kivrak & Aydin, 2017; Valdivia et al., 2021).

2. BIOMARKERS

An evolving diagnostic option is the combination of ultrasound examination with the evaluation of certain biomarkers to increase the effectiveness of non-invasive clinical diagnostics (Kaszak et al., 2022). There are ongoing studies on specific biomarkers that have been detected in both mammary gland tissues by immunohistochemistry technique and blood/plasma/serum by other molecular methods, both in human and veterinary oncology. Unlike in human oncology, where several prognostic markers have been discovered and standardized for use, in veterinary oncology, the search for possible markers is underway (Neumann et al., 2017; Jain et al., 2019; Senhorello et al., 2019; Estaller et al., 2022). However, increasing evidence suggests that these human-derived markers can be useful in CMTs evaluation (Gray et al., 2020). So far, only a few biomarkers have been used in veterinary clinical practice due to low sensitivity and specificity (Neumann et al., 2017; Jain et al., 2019; Senhorello et al., 2019; Estaller et al., 2022).

Focusing on the important criteria to label a marker as ideal, it must (1) be produced by the tumour, (2) disseminate through the body fluids, and (3) be detected by non-invasive methods. In addition, marker levels should increase proportionally to the tumour malignancy or progression (Jain et al., 2019; Senhorello et al., 2019). Despite worldwide constant efforts, and after so many years of their discovery, there is no such biomarker which exists in the strict sense, i.e., marker having a specificity of 100% (undetectable in healthy and benign patients) and with 100% sensitivity (always detectable in malignancy and early stages of a tumour) (Jain et al., 2019). In CMTs, as in HBC, evaluating a single biomarker cannot accurately provide insight into the diagnosis and prognosis due to the heterogeneity of mammary carcinomas and the large number of cellular events involved in cell growth, differentiation, proliferation, invasion and metastasis. In fact, the investigation of multiple molecular alterations in conjunction has great relevance for the understanding of mammary carcinoma progression (Araujo et al., 2016). Some of these biomarkers are discussed below.

Biomarkers of cell proliferation

- *Ki-67 (Kiel-67)*: Ki-67 is a non-histone nuclear cortex regulatory protein expressed in various types of cancers. It is encoded by the MKI67 gene (Nashimura et al., 2010; Penult–Llorca et al., 2017). It is under-expressed during the G1, S, and G2 phases and peaks in the mitotic phase. In

non-cancer mammary tissue, Ki-67 is expressed at a very low level, while highly expressed in cancer tissue. This indicates its involvement in cell proliferation (Urruticoechea, 2005; Nashimura et al., 2010; Carvalho et al., 2016; Neumann et al., 2017; Menon et al., 2019). In veterinary oncology, undetected Ki-67 expression in serum has been reported in healthy bitches, low levels in bitches carrying benign mammary tumours, and high levels of Ki-67 in malignant tumour patients (Neumann et al., 2017). This suggests that there is a relatedness of malignancy with elevated Ki-67 serum concentrations in dogs. In another study, Estaller et al. (2022) suggest that Ki-67 may be useful as a potential serum tumour marker. These results are in contraction with those by Araujo et al. (2016), who hypothesized that studying single molecular markers in CMTs probably cannot accurately account for the heterogeneity of this disease.

- *Proliferation cell nuclear antigen (PCNA)*: PCNA is a protein of DNA polymerase δ, expressed on the nucleus during the DNA synthesis phase of the cell cycle. It increases after the G1 phase, peaks at the S phase and then falls after G2. Finally, it shows extremely low levels in the M phase and G0 phase in quiescent cells (Carvalho et al., 2016). PCNA is involved in cell cycle continuity by maintaining the DNA repair process, cell cycle control, chromatin assembly and RNA transcription. In human oncology, it is considered a marker of proliferation when evaluated alongside ER, PR, Ki-67 or HER-2, but it is disqualified as a marker because of its non-specificity. In veterinary oncology, PCNA expression is greater in large malignant tumours, skin ulceration, histological grade II or III, and the presence of regional lymph node metastasis (Kaszak et al., 2018). Carvalho et al. (2016) investigated the proliferation index (PI) of Ki-67 and PCNA in neoplastic and adjacent non-neoplastic mammary gland tissue, together with several clinicopathological features (tumour size, tumour histological type, differentiation grade, nuclear grade, histological grade of malignancy and lymph node metastasis) to clarify their potential value as predictors of worse prognosis. From the data obtained, it can be stated that a positive and statistically significant correlation exists between the PI of Ki-67, and PCNA in neoplastic and non-neoplastic tissues of benign and malignant tumours, suggesting that growth factors produced by the tumour might act on the adjacent non-neoplastic mammary gland in a paracrine manner, contributing to a high proliferative tumoural microenvironment. In addition, in malignant tumours, the high Ki-67 and PCNA in the adjacent non-neoplastic mammary tissue revealed a statistical association with clinicopathological features of tumour aggressiveness. This might be due to the participation of PCNA in DNA synthesis and repair, which might explain the association between

the high PCNA index in adjacent non-neoplastic mammary tissues with the more aggressive tumour. Although, it has been reported that PCNA expression may be stimulated by cytokines without inducing DNA synthesis, or as a result of its association with DNA repair. Therefore, its expression may not be restricted to cancer, and it is recommended to evaluate the expression of PCNA in combination with other biomarkers of cell proliferation such as Ki-67 (Pena et al., 1998).

- *Human epidermal growth factor receptor 2 (HER-2)*: HER-2, also known as erbB-2, is a membrane tyrosine kinase and oncogene belonging to the ErbB2 family (Dutra et al., 2004; Ressel et al., 2013; Campos et al., 2015; Sakai et al., 2021). Under normal circumstances, it functions in regulating cell proliferation, survival, angiogenesis, and migration. In human breast cancer, over-expression of HER-2 is routinely used as a prognostic marker on its own or in correlation with other proteins (p53, Ki-67, ER, PR). In addition, it is a predictive marker, in the sense that analysis of HER-2 status is necessary for the selection of patients with mammary carcinomas who could benefit from trastuzumab treatment (Ressel et al., 2013; Sakai et al., 2021). In veterinary oncology, the role of HER-2 has been investigated in feline and canine mammary tumours by *in situ* hybridization. As in women, HER-2 over-expression in CMTs appears to be correlated with morphological parameters suggestive of malignancy and poor prognosis (Dutra et al., 2004; Ressal et al., 2013).

Biomarkers of cell cycle process

- *Cyclins*: There are three types of cyclins, namely A, B, and G1 type. They function as regulatory proteins. The G1-type consist of cyclins C, D, and E (Murakami et al., 2000). In addition, Cyclin D has three subtypes including D1, D2, and D3 cyclins. Cyclin D regulates cell cycle activity by forming complexes with two types of cyclin-dependent kinases (CDK4 and CDK6) during the G1 phase of the cell cycle (Murakami et al., 2000; Sfacteria et al., 2003; Kaszak et al., 2022). CDK6 is poorly represented in canine cells but its regulatory function is evident when it complexes with CDK4 to be activated to catalyse (Sfacteria et al., 2003). It has been reported that Cyclin D can transactivate oestrogen receptor response (enabling mammary cells to grow without oestrogen stimulation) and, hence, contribute to oestrogen-independent breast tumour development (Sfacteria et al., 2003). In humans, Cyclin D1 overexpression has been detected in breast, neck, head, and oesophageal cancer (Murakami et al., 2000). Overexpression of cyclin D1 correlated to

metastasis and shorter life expectancy in breast cancer individuals in addition to overall survival, the small size of primary tumours, low histological grade, and oestrogen receptor-positive (ER+) status (Hwang et al., 2003; Roy et al., 2006). In veterinary oncology, Murakami et al. (2000) analysed the expression of cyclin A, cyclin D1 and p53 in mammary tumours, squamous cell carcinomas, and basal cell tumours in dogs and cats. The results suggest that cyclin A may play a role in the proliferation of canine malignant mammary tumours, feline mammary carcinomas, and squamous cell carcinomas of dogs and cats, whereas p53 may be associated with tumour development in mammary carcinomas and squamous cell carcinomas of dogs and cats. The expression of cyclin D1 in precancerous and cancerous lesions of the canine mammary gland has been suggested to be related to mitotic activity (Sfacteria et al., 2003). Mammary lesions that expressed cyclin D1 display a high proliferative ratio, whereas lesions without cyclin D1 expression show a low proliferative ratio. E-type cyclins (cyclin E1 and cyclin E2) are expressed during the late G1 phase until the end of S-phase of the cell cycle. E cyclin expression is regulated at the level of gene transcription by the E2F transcription factor family members and by its degradation through the proteasome pathway (Moroy & Geisen, 2004). E cyclins complexes with CDK2 to regulate the cell cycle at the S-phase, initiate DNA replication, control genomic stability, and cancer transformation. Deregulation of the complex causes replication stress in the S-phase and chromosome segregation errors in the M-phase leading to genomic instability and carcinogenesis (Moroy & Geisen, 2004; Kaszak et al., 2022). Overexpression of E cyclins has been associated with many tumours such as breast cancer, leukaemia, lymphomas, and others (Moroy & Geisen, 2004). In human breast cancer, cyclin E is suggested as a prognostic factor, while cyclin D1 and E are used as cellular markers (Keyomarsi et al., 2002; Hunt et al., 2017)

Biomarkers of cell apoptosis

- ***Programmed cell death protein-1(PD-1)***: PD-1 is also called CD279 and belongs to the B7-CD28 family. It is a 50−55 kDa type I transmembrane glycoprotein expressed on various innate and adaptive immune cells (activated T cells, B cells, monocytes, dendritic cells, regulatory T cells, and natural killer T cells), but not on resting naive T cells (Jiang et al., 2019; Qin et al., 2019; Salmaninejad et al., 2019; Laba et al., 2022). PD-1 exerts its inhibitory role by binding to its ligands (PD-L1 and PD-L2). Though they bind to the same receptor, they do not have different role, but

their expression pattern is different (Salmaninejad et al., 2019). PD-L1 has a much broader tissue distribution in the sense that it is constitutively expressed on T and B cells, dendritic cells, macrophages and non-haematopoietic cells (keratinocytes and non-parenchymal cells including vascular endothelial cells, pancreas, islets cells, cells in the placenta and testicles) (Salmaninejad et al., 2019; Jubel et al., 2020; Laba et al., 2022). Some studies have demonstrated the PD-L1 expression on the surface of several types of tumour cells, such as non-small-cell lung cancer, hematologic malignancies, and virus-infected cells (Salmaninejad et al., 2019). PD-L2 expression is limited to antigen-presenting cells (dendritic cells, macrophages and cultured bone marrow-derived mast cells), and non-hematopoietic tissues such as respiratory tract epithelial cells (Salmaninejad et al., 2019; Laba et al., 2022). PD-1 binds to PD-L1 and inhibits T-cell activation and cytokine production (Huang et al., 2018; Jiang et al., 2019; Han et al., 2020; Jubel et al., 2020). PD-L1 has been demonstrated to be expressed in various canine malignant tumours such as canine oral melanoma, osteosarcoma, hemangiosarcoma, mast cell tumour, mammary adenocarcinoma and prostate adenocarcinoma (Maekawa et al., 2016). Likewise, PD-1 and PDL-1 have a high potential as biomarkers in canine tumours (Song et al., 2021).

- *Protein 53 (p53)*: P53 is a tumour suppressor protein and is the 'guardian of the genome' because it detects DNA damage and triggers the apoptosis of damaged cells (Munday et al., 2019). It is responsible for cell cycle control, apoptosis, and suppression of tumour development (Shimada et al., 2003). The p53 gene mutation is common in breast cancers and CMTs (Sorenmo, 2003). In human and veterinary oncology, the expression of p53 is associated with a worse prognosis and shorter survival time (Klopfleisch & Gruber, 2009; Pan et al., 2017). Klopfleisch & Gruber (2009), investigated the mRNA expression of p21, p27 and p53 in highly defined laser microdissected tissue samples of canine mammary adenomas, adenocarcinomas, and their lymph node metastases, compared to non-neoplastic mammary tissues of the same dog. The results show that p21 is over-expressed in primary adenocarcinomas but reduced in lymph node metastases independently of p53 expression. Munday et al. (2019) evaluated p53 and p16 in CMTs through immunostaining techniques to predict the prognostic behaviour of neoplasia. The results suggest that there was no significant alteration in either p53 or p16 that altered the behaviour of the tumour nor predicted prognosis. Another study (Brunetti et al., 2020) suggested that the combined use of p53, ER, and Ki-67 may help predict the biological behaviour of CMTs.

Biomarkers of Metastatic Potential

- *Cadherins*: Cadherins are calcium-dependent transmembrane glycoproteins that mediate cell adhesion, thus playing a vital role in maintaining tissue architectural arrangement. They consist of at least five subfamilies, including classical cadherins of type I, closely related cadherins of type II, desmosomal cadherins, proto-cadherins, and a variety of cadherin-related molecules (Roy & Berx, 2008). The most identified cadherins are the E-, N-, and P-cadherins, but the most evaluated is the E-cadherin (Epithelial cadherin) which is found in most epithelial tissues including mammary tissues (Knudsen & Wheelock, 2005; Canadas et al., 2019). E-cadherin is a type I transmembrane glycoprotein that is encoded by the E-cadherin gene (CDH1) (Lui et al., 2016). E-cadherin binds to β-catenin to form a complex to mediate cell–cell adhesion and prevent cells in a primary tumour from metastasizing. In many systems, cadherin adhesion promotes cell differentiation while suppressing growth. These properties suggest that the loss or alteration in E-cadherin by mammary epithelial cells will favour tumour development and will facilitate the motility of neoplastic cells and increase metastases (Matos et al., 2006; Nowak et al., 2007). It has been reported that epithelial tumours lose E-cadherin partially or totally as they progress towards malignancy. This inactivation of E-cadherin could be due to mutations, epigenetic silencing, endocytosis, and increased expression of non-epithelial cadherins (Roy & Berx, 2008). In human breast cancer, low expression of E-cadherin correlates with tumour histological grade, tumour size, lymph node status and poor prognosis while the total loss of expression indicates epithelial-mesenchyma transition (EMT), which induces cell dissemination through increased cell migration and invasion. In most CMTs, low expression is concomitant to increased tumour development and progression, tumour malignancy, aggressiveness of metastases and overall survival. In both species, E-cadherin should be evaluated together with other biomarkers, like Ki-67. Even though, E-cadherin is not the most evaluated biomarker of CMTs (Kaszak et al., 2018). A case-control study performed by Canadas et al. (2019) revealed that CDH1 genetic variants could have a protective role in CMTs, by being associated with minimal risk of tumour development, delayed onset of the disease and less aggressive clinicopathological features.

- *Carcinoembryonic antigen (CEA)*: CEA is a 200kDa glycoprotein involved in intracellular adhesion. It is produced by gastrointestinal mucosa cells, localized in epithelial cell membranes in lesser amounts. It is over-expressed by the cancer cells of the colon, breast, and lungs. It was

among the first identified tumour biomarkers in breast cancer and has been associated with tumour progression (Campos et al., 2012; Senhorello et al., 2019; Fan et al., 2021). Generally, CEA is regarded as a non-specific tumour marker due to its low sensitivity and specificity in early diagnosis. Hence, it should be combined with other tumour markers in auxiliary diagnosis, evaluation of effectiveness, and prognosis of the tumour (Valencakova-Agyagosova et al., 2012; Fan et al., 2021). Serum levels of CEA in CMTs have yielded contradictory results. Some studies have reported high levels of CEA in bitches with mammary tumour dogs compared to healthy dogs (Senhorello et al., 2019), whereas another study evaluating the serum levels of CA 15-3, CEA, and lactate dehydrogenase (LDH) in female dogs (Campos et al., 2012) observed measurable values in bitches with various stages of mammary cancer. However, there were no significant differences in CEA among groups. Similarly, Jain et al. (2021) evaluated CEA, CA 15- 3, and miRNA expression in healthy, benign, and malignant mammary tumour bitches. The results showed that CA 15-3 was more sensitive than CEA but the detection of both will increase sensitivity.

- *Carbohydrate antigen 15-3(CA 15-3)*: CA 15–3 also known as mucin 1/muc-1, is a mucinous large glycoprotein expressed on the apical side of normal epithelial and some other cell types. In normal cells, it can act as a lubricant, moisturizer, and physical barrier against degradative enzymes. However, in cancer cells, it often undergoes aberrant glycosylation and over-expression (Chen et al., 2021). CA 15-3 is involved in cancer invasion, metastasis, angiogenesis, and apoptosis by its participation in intracellular signalling processes and the regulation of related biomolecules (Chen et al., 2021). It is over-expressed in epithelial cancers and is associated with large tumours, lymph node metastases, and poor prognosis in many cancers. It has been extensively studied in breast cancer and is widely used as a serum biomarker (Manuali et al., 2012; Zhao et al., 2014). In addition, it is used as a monitoring tool for chemotherapy (Vafaei et al., 2022). There is a paucity of information regarding its application as a serum biomarker in CMTs (Manuali et al., 2012). De Oliveira et al. (2009) investigated the expression of MUC-1 on normal mammary tissue cells and tumour tissue cells and correlated it to distant metastases. The results show that MUC-1 is expressed apically on the cell membrane in normal tissue, whereas in the cytoplasm of CMTs tissues. Campos et al. (2015) compare serum and tissue levels of HER-2 and MUC-1 in CMTs and correlate with regional lymph node metastasis. The results revealed that while serum and tissue levels of HER-2 do not correlate with the presence of regional metastasis, increased serum levels of MUC-1 are associated with the presence of metastasis to regional lymph nodes.

Finally, a study performed by Fan et al. (2021) suggests the combining of CA 15-3, CEA and SF (serum ferritin) as markers to diagnose CMTs.

- *Vascular endothelial growth factor (VEGF)*: VEGF is a protein encoded by four genes (VEGF-A, B, C, and D), and expressed in lung, gastric, ovarian, endothelial, and breast cancers. VEGF-A and VEGF-B are responsible for angiogenesis, while VEGF-C and VEGF-D, are for lymphangiogenesis (Ferrara, 2004; Milanta et al., 2010). In human oncology, over-expression of VEGF leads to tumour growth and metastasis, while its under-expression results in suppression of tumour development (Lawicki et al., 2016; Zajkowska et al., 2016). In CMT, it has been shown that increased VEGF serum levels correlate with worse clinical stages, poor prognosis, and lower survival rates. Since it is usually reported in malignant tumours with infiltrative growth, hence, it is considered a marker of tumour growth and metastasis. Generally, it is a good marker for early diagnosis of HBC and CMT. In addition, its sensitivity increases when combined with CA 15-3 (Lawicki et al., 2016; Zajkowska et al., 2016). On the other hand, VEGF-C has been suggested as a candidate marker for predicting metastasis of CMTs, since its expression did not correlate with tumour size or the patient's age but was significantly higher in malignant mammary tumours and related to lymph node metastasis (Qui et al., 2008). Increased levels of iNOS, COX-2 and VEGF gene mRNA levels have been also correlated with the histological grade of malignancy in dogs with mammary tumours (Anadol et al., 2017). A study by Sakalauskaite et al. (2021) reported that VEGF-B and EGFR genes were over-expressed in mammary gland carcinomas compared to adjacent tissue. Furthermore, differences in VEGF-B, FLT1, ERBB2, GRB2, RAC1, CDH1 and HYAL-1 gene expression have been found in different breed dogs (German Shepherd, Yorkshire Terrier) and mixed-breed dogs indicating that a dog's breed could determine a molecular difference, outcome of cancer and should be accounted as a confounding factor in the future gene expression research.

Gene Expression

- *Breast cancer susceptibility gene (BRCA1)*: BRCA1 and BRCA2 are tumour suppressor genes, and a mutation in any of the genes predisposes one to breast, pancreatic, prostate, and ovarian cancers. They contribute to DNA repair in response to DNA damage and regulate some genes involved in the cell cycle and apoptosis (Yoshikawa et al., 2015; Varol et al., 2018). These genes have been incriminated in HBCs and CMTs (Yoshikawa et al., 2015; Varol et al., 2018; Thumser-

Henner et al., 2020; Giacomo et al., 2022). Several studies in humans have reported over 400 mutations in BRCA genes, most of the mutations can be considered unique, and each family may present a specific mutation. Also, certain mutations have been frequently observed at some geographical locations and in some ethnicities. Women carrying a BRCA2 mutation have a substantial risk of about 81-88% risk of developing breast cancer (Varol et al., 2018). However, little is known about BRCA2 expression in CMTs. Chromosomal aneuploidy has been reported in CMTs, hence repair proteins (BRCA2 and Rad51) may be involved in the aetiology (Yoshikawa et al., 2005). In this sense, the mutation of the BRCA1 and BRCA2 genes has been reported to be associated with CMTs in English Springer Spaniels (Rivera et al., 2009). However, other studies (Yoshikawa et al., 2015) showed reduced BRCA2 expression in tumour tissues as compared to normal mammary tissues.

- *<u>Relaxin (RLX)</u>*: Relaxin is a peptide hormone belonging to the insulin/insulin-like growth factor family (Binder et al., 2004). It is produced in the ovary and the placenta of the bitch (Lamp et al., 2009). However, it is also assumed to be produced by healthy mammary gland tissue, but this is yet to be proven (Silvertown et al 2003). It plays a critical role by inducing cervical ripening and lengthening of the interpubic ligament before parturition and promotes uterine growth and the development of mammary glands and the teats in the female dog. Furthermore, it is involved in the reorganization of non-reproductive tissues by decreasing the synthesis and deposition of interstitial collagens thereby antagonizing the development of interstitial fibrosis of the kidney and the liver of rats and stimulating wound healing through upregulation of neovascularization (Binder et al., 2004).

Normal, benign, and malignant tumour breast tissues in women have been shown to express relaxin (Tashima et al., 1994; Hombach-Klonisch et al., 2000). Elevated levels of relaxin are reported in human cardiovascular insufficiency and degenerative ligament disease (Lamp et al., 2009). In the dog, high levels of relaxin are observed in pregnant bitches, and in dogs with hip dysplasia. Matrix metalloproteases (MMPs) are essential for relaxin-induced modification of stroma tissue such as the reorganization of benign tissues and progression of malignant tumours. Relaxin has been established as a marker for poor prognosis in human breast cancer (Binder et al., 2004). A preliminary study performed by Pawlowski et al. (2013) suggested that increased expression of relaxin and MAGI3 can be used as prognostic markers for mammary cancer in dogs.

- *Secreted frizzled-related protein-2 (SFRP-2)*: Secreted frizzled-related proteins are a family of glycoproteins, composed of SFRP-1, SFRP-2, SFRP-3 (FRZB), SFRP-4, and SFRP-5 in humans, mice, and chicken. They are characterized by a frizzled-related cysteine-rich domain (CRD) at the N-terminus and a netrin module at the C-terminus (Saito et al., 2014; Liang et al., 2019; Wu et al., 2020). SFRPs modulate and bind directly to Wnt ligands to block the interaction between Wnt and frizzled receptors, thus regulating the Wnt/β-catenin signalling pathway. In addition, they can bind with frizzled receptors to form a non-functional complex that prevents Wnt/β- catenin signalling activation (Saito et al., 2014; Liu et al., 2017; Liang et al., 2019). The Wnt/β-catenin pathway is constitutively active in multiple cancers, and closely associated with breast cancer initiation and metastasis (Saito et al., 2014; Lui et al., 2017; Wu et al., 2020). Studies show a diverse functional profile of the SFRPs in breast cancer concerning WNT signalling. For instance, SFRP-1 acts as a WNT antagonist in breast carcinoma cells and blocks thrombospondin-induced adhesion and migration, whereas SFRP-4 is a WNT antagonist that mediates apoptosis in breast cancer stem cells, and inhibits proliferation, metastasis, and epithelial-mesenchymal transition. Finally, SFRP-2 has a pro-oncogenic tendency (Liang et al., 2019; Wu et al., 2020).

In human cancers, SFRP-2 is often methylated and its down-regulation is closely related to Wnt signalling activity and tumour progression. While emerging evidence showed that SFRP-2 is recognized as an antagonist to the Wnt signalling and a novel molecule of tumour suppressors, a steady flow of research has indicated that it may also have tumour promotion effects in some cancer types (Liu et al., 2017). SFRP-2 expression is decreased in some tumour types where it correlates with increased Wnt signalling. Wu et al. (2020) studied the expression levels of various SFRPs in breast cancer patients. The results revealed that SFRP1/3/4/5 were under-expressed, while SFRP-2 was over-expressed in breast cancer compared to healthy control. On the other hand, SFRP-2 was reported to be abundantly expressed in CMT cells but not in normal canine mammary gland cells (Lee et al., 2003, 2004). The marked differential expression of SFRP-2 reveals that this protein may be a potential candidate marker for CMTs.

Pro-inflammatory cytokines

Cytokines are secretory proteins that mediate intercellular communication in the immune system. Some of them and their receptors are produced in the organisms under physiological and pathological conditions (Esquivel-Velazquez et al., 2014). Pro-inflammatory cytokines are secreted by Th1 cells, CD4+ cells, dendritic cells (DC) and macrophages (Turner et al., 2014). The key pro-inflammatory cytokines are IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) (Zhang et al., 2007; Turner et al., 2014). They are involved in inflammatory responses to stimulate or suppress the immune system against infection, cancer, and other diseases (Turner et al., 2014).

- *Interleukins-1 (IL-1)*: Members of this family are IL-1α, IL-1β, the IL-1 receptor antagonist [IL-1Ra], IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-38 (Kaneko et al., 2019; Boraschi, 2022). IL-1α and IL-1β are synthesised and expressed by numerous cell types such as monocytes, macrophages, neutrophils, hepatocytes, and tissue macrophages. While IL-1α is active in both its precursor and mature form, IL-1β becomes active after cleaving by caspase-1 or IL-1-converting enzyme (ICE) (Turner et al., 2014). IL-1 can act as a leucocytic pyrogen, leucocytic endogenous mediator, and an inducer of several components of the acute-phase response and the recruitment of TAMs and MDSCs, which promote tumour development in breast cancer (Kaneko et al., 2019). IL-1β plays a similar role, except for its association with breast cancer. The immunoreactive IL-1β is detected in approximately 90% of invasive breast carcinomas. In addition, its levels are significantly higher in invasive carcinomas compared to benign lesions (Jin et al., 1997). In veterinary oncology, Machado et al. (2015) evaluated the levels of cytokines and nitric oxide oxidative and antioxidant status, as well as the activity of adenosine deaminase and butyrylcholinesterase in the serum of healthy dogs as well as those diagnosed with mammary carcinoma. The results showed high levels of cytokines (TNF-α, INF-γ, IL-1, and IL-6), NOx (nitrite/nitrate), protein oxidation, and antioxidant power in dogs with mammary carcinoma when compared with healthy dogs. In another study, Monkong et al. (2020) evaluated serum IL-8 levels and correlated them with clinicopathological parameters in healthy and mammary tumour-bearing dogs. The results showed higher levels of IL-8 in the mammary tumour group compared to the healthy group.

- *Interleukin-6 (IL-6)*: IL-6 is a 26kDa glycoprotein secreted by monocytes and macrophages during acute inflammation, and T cells during chronic inflammation (, Chen et al., 2022). It is

involved in the acute immune response and haematopoiesis through the stimulation of antibody production and effector T-cell development, promoting differentiation or proliferation of several non-immune cells (Tanaka et al., 2014; Chen et al., 2022). Uncontrolled synthesis of IL-6 plays a pathological role in chronic inflammation and autoimmunity (Tanaka et al., 2014). As a key tumour promoter, its overexpression may facilitate tumour growth by suppressing apoptosis and promoting angiogenesis. Over-expression in breast cancer tissues stimulates Jagged-1 expression to promote cell growth and maintain the aggressive phenotype (Naugler & Karin, 2008). Higher circulating IL-6 levels are correlated with shorter survival in patients with metastatic breast cancer. Response to IL-6 in breast cancer is closely dependent on ER and PR expression implying that hormone-sensitive cancer cells are more responsive than hormone-insensitive cells. On the other hand, IL-6 has been shown to stimulate oestrogen levels in breast cancer. Co-expression and elevation of Shh and IL-6 in serum could be co-regulated, share common mechanisms, and may serve as a biomarker panel (Noman et al., 2018). A study performed by Szczubial et al. (2018) determined the concentrations of IL-6 and IL-10 in the plasma of healthy, benign and malignant mammary gland tumours bearing bitches. The results showed higher levels of IL-6 and IL-10 in benign and malignant mammary tumours compared to healthy bitches. It suggests that circulating IL-6 and IL-10 concentrations could help identify malignant forms of mammary gland tumours in dogs. In another study performed by Andalauz et al. (2016), sixty proinflammatory cytokines were evaluated in the serum of canine mammary tumour bitches and the data suggest that a combination of inflammatory cytokines can be a potential diagnostic biomarker. Ren et al. (2023), in a study which investigated the expression of serum/tissue IL-6, IL-8 and IL-10 in canine mammary gland tumours, observed that serum levels of these cytokines are higher in the malignant tumour group as compared to benign and control groups. Likewise, IL-6 and IL-8 were also highly expressed in malignant tumour tissues. Hence, it suggests that IL-6 and IL-8 may be used as potential biomarkers in canine mammary gland tumours.

- *Tumour necrosis factor-α (TNF-α)*: TNF-α is a cytokine, identified as a major regulator of inflammatory responses. It is secreted by activated macrophages, T lymphocytes, and natural killer cells. Abnormal or excessive production is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, psoriasis, and non-infectious uveitis (Jang et al., 2021). It is over-expressed in a variety of cancers, especially breast cancer, and it correlates with augmented tumour

cell proliferation, higher malignancy grade, increased occurrence of metastasis and generally poor prognosis (Mercogliano et al., 2020). A study performed by Castanheira et al. (2013) reports increased levels of TNF-mRNA expression in dogs with mammary gland tumours compared with healthy dogs. Furthermore, Kim et al. (2022) examined the expression of endogenous tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in canine mammary tumours and analysed its correlation with downstream molecules Fas-associated protein with death domain (FADD) and caspase-3, and to the apoptotic index. The TRAIL protein expression was significantly decreased in mammary carcinoma compared to healthy, and benign mammary adenoma.

Hormone receptors

Oestrogen receptor/Progesterone receptor (ER/PR): Oestrogen and progesterone play a role in mammary gland and reproductive tract development (Kim et al., 2014). Studies have shown that HBCs that are ER+ and PR+ correlate to favourable prognosis, while ER- and PR- relate to more aggressive tumours and worse outcomes (Timmermans-Sprang et al., 2017). Contrary, CMTs that are ER+ or PR+ are usually non-malignant and relate to favourable prognosis (Millanta et al., 2005; Mainenti et al., 2014) They are the most studied biomarkers of mammary neoplasias and have remained good prognostic markers for HBCs and CMTs (Tavares et al., 2010).

Progesterone receptor membrane component 1 (PGRMC1): PGRMC1 is expressed in various tissues particularly those high in P450 activity such as the liver, kidney and adrenals (Ahmad et al., 2010; Kaluka et al., 2015). It is expressed in males and females alike (Rohe et al., 2009), and it is overexpressed in a wide variety of cancers in mammalian species. These cancers include lung, thyroid, colon, cervix, sebaceous glands, oral, head and neck, bladder, ovary, and breast (Rohe et al., 2009; Ahmed et al., 2010; Hampton et al., 2015; Terzaghi et al., 2020), and in Alzheimer disease (Kim et al., 2018). It is detected at high levels in the plasma of patients with lung and renal cancer patients but lower in healthy individuals (Terzaghi et al., 2020). Reports on PGRMC1 expression in veterinary oncology have been very scarce. In this sense, Terzaghi et al., (2020) demonstrated the expression of PGRMC1 in healthy and neoplastic canine mammary gland tissues. The expression of PGRMC1 is high in healthy tissues and adenomas, while it decreases in

carcinomas, and is slightly expressed in less differentiated tumours or the less differentiated compartment in the same tumour. PGRMC1 is expressed in the blood and bladder tissue of healthy and bladder cancer cattle (Russo et al., 2017). It is involved in carcinogenesis and is considered to be a putative biomarker for diagnosis and prognosis in many human cancers (Terzaghi et al., 2020). It promotes tumour growth through the induction of cell proliferation, chemotherapy resistance and invasion (Crudden et al., 2005; Ahmad et al., 2010; Hampton et al., 2015; Furuhata et al., 2020). PGRMC1 may mediate the proliferation and progression of breast cancer cells potentially by altering lipid metabolism, activation of oncogenic signalling pathways, such as ERα expression and activation, as well as EGFR signalling (Asperger et al., 2020). According to Cai et al. (2020), higher PGRMC1 concentrations in blood were associated with greater tumour growth in breast cancer tissue. In lung cancer cells it is associated with epidermal growth factor receptor (EGFR), and the formed complex (PGRMC1-EGFR) enhances tumour invasion by partly activating matrix metalloproteinases (Hampton et al., 2015). A study by Zhang et al. (2015) reports that PGRMC1 interaction with ERα may increase the risk of developing breast cancer, especially when treated with hormone therapy. Similarly, the oestradiol/norethisterone combination increases proliferation and PGRMC1 over-expressing breast cancer cells, suggesting that undetected tumour cells over-expressing pgrmc1 may likely develop into frank tumour cells in women undergoing hormonal therapy (Neubauer et al., 2013). The expression of PGRMC1 correlates with metastasis to lymph nodes, large tumour size and poor overall- and tumour-free survival (Asperger et al., 2020). Clark et al. (2016) report that PGRMC1 promotes the survival of human breast cancer cells and the growth of xenograft tumours. Human PGRMC1 promotes cell death in cancer cells after oxidative damage, probably due to its activation of P450 proteins, activates the pro-survival protein kinase Akt and inactivates the cell death-associated protein IκB, suggesting multiple avenues for regulating cell survival (Rohe et al., 2009).

Cluster of differentiation (CD) proteins

CD proteins are cell surface molecules expressed on leukocytes and other cells associated with the immune system. They often act as receptors or ligands of immune cells. Some function in cell signalling, while others mediate cell adhesion (Xiong & Xu, 2014).

Cluster of Differentiation 20(CD20): CD20 is a 35-37kDa non-glycosylated transmembrane protein, expressed on the surface of almost all normal and malignant B cells except plasma cells (Cragg et al., 2005; Ruuls et al., 2008; Singh et al., 2014; Miligy et al., 2017; Kozlova et al., 2020; Li et al., 2020; Pavlasova & Mraz, 2020; Zhang et al., 2021). It exists on the cell surface as homodimeric and homo-tetrameric oligomers; and is close to other proteins of the same family (CD53, CD81, and CD82), forming supramolecular complexes (Pavlasova & Mraz, 2020). It belongs to the MS4A (membrane spanning 4-domain family A) protein family (Pavlasova & Mraz, 2020), and is encoded by the MS4A1 gene (Mudd et al., 2021). CD20 has four membrane-spanning hydrophobic domains, single intracellular and double extracellular loop with both N-and C-terminal regions within the cytoplasmic matrix (Singh et al., 2014; Pavlasova & Mraz, 2020). It resides in the lipid raft domain of the plasma membrane where it probably functions as a store-operated calcium channel following ligation of the B cell receptor (Cragg et al., 2005; Pavlasova & Mraz, 2020). CD20 is also expressed at low levels in a subset of T cells largely those bearing γδ T-cell receptors due to its ubiquitous nature (Jubala et al., 2005; Singh et al., 2014). The T cell response is inhibited by resting B cells and facilitated by activated B cells (Miligy et al., 2017). CD20 has no known physiological ligand nor the exact biological function of CD20 despite the success of CD20 monoclonal antibodies (Cragg et al. 2005; Ruuls et al., 2008; Boross & Leusen, 2012; Klasener et al., 2021), but it is known to be involved in cell differentiation, cell cycle regulation and signal transduction (Mudd et al., 2021; Zhang et al., 2021). CD20 is a target for diagnostic and therapeutic monoclonal antibodies (Jubala et al., 2015; Zhang et al., 2021), and this therapeutic effect of CD20 antibodies may be partly due to the targeting of a subpopulation of T cells that selectively expresses CD20 lower than B cells (Vlaming et al., 2021).

Most human B-cell malignancies show strong CD20 expression, but it is inconsistent on T-cell acute lymphoblastic leukaemia, chronic lymphocytic leukaemia, non-Hodgkin's lymphoma and multiple myeloma (Jubala et al., 2005; Mudd et al., 2021; Zhang et al., 2021). Studies have reported the expression of CD20 in CMTs. Rismanchi et al. (2015) conducted the first pathological study of canine primary breast lymphoma and the description of its clinicopathological characteristics as an animal model for human primary breast lymphoma. A previous study by Andaluz et al. (2016) identified biomarkers in healthy and mammary tumour-bearing bitches. CD20 and CD45RA were selected as potential prognostic markers that can help to elucidate the role of T- and B-cells during tumorigenesis. Tricarico et al. (2022) investigated the relationship

between the inflammatory cell response, cancer grade, and progression in canine breast cancer using biopsies collected for diagnostic purposes and in the unselected population under anti-HER2 treatments. The finding that trastuzumab i.v-s.c. and trastuzumab emtansine showed, respectively, high neutrophil and platelet count decrease with no cancer progression in the absence of reports of lymphocyte decrease, and that a high density of CD20+ lymphocytes was observed in the less severe canine breast cancer samples along with high neutrophil and platelet count.

Cluster of Differentiation 45: The CD45 receptor is a cell surface glycoprotein with tyrosine phosphatase activity. It is expressed on all nucleated haematopoietic cells, except for mature erythrocytes and platelets. CD45 has been identified as a rheostat of T and B cell activation, by regulating Src family protein tyrosine kinases and thereby, the signalling cascade in leukocytes. CD45 plays a role in their differentiation and autoimmunity (Martinez et al., 2018). The loss of CD45 results in hyperphosphorylation of the Lck C-terminal tail (Y505), thereby reducing the amount of active Lck, which invariably impairs T-cell development when TCR signalling is required (Raiter et al., 2017). CD45 along with epithelial markers, such as epithelial cell adhesion molecule (EpCAM) and keratin, are expressed by circulating cancer cells (CTCs) from carcinomas therefore regarded as biomarkers (Babayan et al., 2013). Hillebrand et al. (2016), isolated CD24+CD90+CD45− cells from primary MMTV-PyMT breast tumours.

Cluster of Differentiation 53(CD53): CD53 is a 20-25kDa tetraspanin protein and has no known ligand (Hernandez-Torres et al., 2000; Marchetti et al., 2021). It is expressed on the surface of haematopoietic cells (including B and T cells, dendritic cells, natural killer cells, granulocytes, and monocytes/macrophages) (Schaper & van Spriel, 2018; Dunlock, 2020; Marchetti et al., 2021). Members of this family regulate protein trafficking and signal transduction, cell adhesion and migration, T and B cell proliferation, as well as leukocyte migration into tumour microenvironment function, tumour growth, and metastasis (Schaper & van Spriel, 2018; Lombardo et al., 2019; Dunlock, 2020; Ding et al., 2021). CD53 is considered a negative regulator of IL-6, TNFα and IL-1β, and a promoter of B cell receptor-dependent protein kinase C signalling (Schaper & van Spriel, 2018; Marchetti et al., 2021). Ligation of the CD53 antigen with monoclonal antibodies modulates several biological processes such as intracellular calcium mobilization in human B-cells and monocytes, and rat macrophages, induction of homotypic adhesion, also induces the expression of nitric oxide synthase (van Winde et al., 2015).

Cluster of Differentiation 59(CD59): CD59 is an 18-20-kDa membrane glycosylated protein present on the surface of a large number of different cells, including heart, liver, spleen, kidney, erythrocytes, monocytes, granular cells and vena umbilicalis endothelial cells (Li et al., 2011; Weinstock et al., 2015; Blom, 2017). It is firmly inserted into the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Failure of this anchor causes the loss of this protein from the cell membrane, which leads to an enhanced sensitivity towards complement attack. CD59 binds to complement components CS and C9 to prevent binding of C9 to C5b-8 by competing for binding to a neoepitope on C8. This prevents the polymerization of C9, which is required for the formation of the membrane attack complex (MSC) (Babiker et al., 2002; Xu et al., 2005; Li et al., 2011; Weinstock et al., 2015). It is encoded by the CD59 gene (Xiong et al., 2018) and its known ligand is CD2 (Li et al., 2011, Ouyang et al., 2016). CD59 is regarded as a complement inhibitor which prevents injury or lysis of erythrocytes and other vascular cells (Babiker et al., 2002; Li et al., 2011; Weinstock et al., 2015; Ouyang et al., 2016; Blom, 2017; Xiong et al., 2018). It complexes with CD2 to function as a signal stimulant to induce T cell activation (Li et al., 2011; Weinstock et al., 2015), which consequently forms adhesion with other T cells or tissue cells to further regulate the growth of tissue cells (Li et al., 2011; Li et al., 2022). CD59 in tumour cells can be hijacked to escape complement-based immune surveillance (Li et al., 2022). Overexpression of CD59 has been demonstrated in most solid tumours including breast cancers (Ouyang et al., 2016; Xiong et al., 2018), Alzheimer's disease, ulcerative colitis and rheumatoid arthritis (Li et al 2011; Ouyang et al., 2016). In breast cancer patients, loss of CD59 expression has been described as an independent prognostic marker (Xu et al., 2005). The absence or low-level expression of CD59 may be partly responsible for the pathogenesis of diabetes, multiple sclerosis, haemolytic anaemia, acquired immune deficiency disease (AIDS) and resistance to rituximab treatment in B cell cancer patients (Madjd et al., 2003; Li et al., 2011; Xiong et al., 2018). It has been reported that down downregulation of intracellular CD59 causes a marked reduction in the ability of β cells to secrete insulin but removal of CD59 from the cell surface using phospholipase C has no effect on insulin secretion (Li et al., 2022).

Cluster of Differentiation 99 (CD99): CD99 is a 32kDa transmembrane glycoprotein expressed on leucocytes and activated endothelium (Huijbers et al., 2019), mature plasma cells, granulosa cells of the ovary, Sertoli cells, pancreatic islet cells (Milanezi et al., 2001; Manara et al., 2018; Pasello et al., 2018; Vaikari et al., 2020), cortical thymocytes, endothelial cells, bone marrow CD34

positive cells, stromal lymphocytes and a broad range of haematopoietic cells. Its highest expression is in the immature lymphocytes, granulocytes (such as immature thymic T-lineage cells and tonsillar lymphoid progenitor cells), and immature basal keratinocytes (Pasello et al., 2018). It is encoded by the MIC2 gene located in the pseudoautosomal region in the distal short arms of the X and Y chromosomes (Buccar et al., 2013; Manara et al., 2018; Pasello et al., 2018). It does not share structural homology with any known family of proteins, except the Xga protein and CD99 antigen-like 2 (CD99L2) (Alberti et al 2002; Pasello et al. 2018). Two isoforms of CD99 exist; CD99 type I (CD99wt) and CD99 type II (CD99sh) (Manara et al., 2018; Huijbers et al., 2019), and have distinct expression and functional profiles in both normal and malignant tissues (Scotlandi et al., 2007; Seol et al., 2012; Huijbers et al., 2019). Although the role of CD99 is still obscured, it has been known to trigger CD99-mediated signalling events with agonistic CD99 monoclonal antibodies in haematopoietic cells to function in cellular processes, such as cell adhesion and migration, homotypic aggregation, apoptosis, expression upregulation, and the transport of various membrane proteins (T cell receptor complex (TCR) and HLA-II proteins) in normal cells (Byun et al., 2006; Soel et al., 2012; Buccar et al., 2013; Manara et al., 2018; Pasello et al., 2018; Cardoso et al., 2019; Huijbers et al., 2019; Takheaw et al., 2019; Vaikari et al., 2020). It is also involved in inflammation, signal transduction and cytokine production (Takheaw et al., 2019). In tumour cells, it promotes tumour migration, invasion and metastasis. High expression of CD99 has been reported in acute lymphoblastic leukaemia, acute myeloid leukaemia, myelodysplastic syndromes, Ewing's sarcoma and stem cells (Pasello et al., 2018; Huijbers et al., 2019). Sporadic expression was reported in synovial sarcoma, mesenchymal chondrosarcoma, rhabdomyosarcoma, thymic tumours, hemangiopericytoma, gastrointestinal and pulmonary neuroendocrine tumours, sex-cord stromal tumours, melanoma, and breast carcinoma, where it acts as a tumour suppressor. Diffuse expression was seen in benign or early-stage pancreatic endocrine neoplasms, gastric adenocarcinoma, gallbladder carcinoma, and osteosarcomas, while it is absent or lower in the malignant or advanced-stage of these tumours (Manara et al., 2018; Pasello et al., 2018). In inflammatory cells, CD99 has been related to Th1 cell differentiation and the activation and proliferation of mature T cells (Cardoso et al., 2019).

Circulating tumour cells (CTC): Circulating tumour cells are cells which break from the primary tumour or secondary lesion into the peripheral circulation. They do not attach to the extracellular matrix because they resist anoikis (Sundling & Iowe, 2019; Michela, 2021). In addition, they occur

as single (individual CTC) and in cluster (cluster CTC) comprising of 2-50 cells consisting of tumour cells and probably normal cells. Although, clusters of CTC are less prevalent, but possess greater metastatic potential than the former. (Yu et al., 2011; Alix-Panabieres & Pantel, 2013). Larger clusters have been detected in several cancers. The presence of clusters correlated with poor prognosis in lung, breast, and prostate cancer patients (Hou et al., 2012; Aceto et al., 2014). The presence of CTCs in blood negatively correlates with progression-free and overall survival of early stages of lung, gastric and pancreatic cancers, and melanoma (Rhim et al., 2014). Some studies have associated CTC number with primary tumour size, lymph node status, presence of metastasis and prognosis. CTCs are seeds of tumour metastasis and, therefore, are regarded as potential biomarkers for diagnosis and prognosis as the number of CTCs of over 5 cells per 7.5 ml of blood detected by the cellSearch system in metastatic breast cancer signifies poor diagnosis (Hiraiwa et al., 2008; Rack et al., 2014). Moreover, CTCs have been incorporated into the 7th edition of the American Joint Committee on Cancer staging system for breast cancer as an index of distant metastases (Sundling & Lowe, 2019). A high CTC number predicts liver metastases from peri-ampullary or pancreatic adenocarcinoma (Tien et al., 2016). Similarly, a lower CTC number during treatment predicts the success of therapy (Hayes et al., 2006; Bidard et al., 2016). Marconato et al. (2019) evaluated CTCs and DTCs (disseminated tumour cells) in metastatic mammary tumours and the result showed similarity to those of metastatic breast cancers, thus being a predictor of shorter overall survival.

Circulating tumour DNA (ctDNA): They account for only 0.1-10% of total circulating cell-free DNA (cfDNA) from tumour cells. They are found in healthy, cancer, pregnancy and organ transplant individuals (Gai & Sun, 2019). They are used for early cancer detection, offering information about the genetic profile of the primary tumour and metastases. The methods of detecting ctDNA are often based on sequencing of somatic mutation in cfDNA but the diagnostic sensitivity of these methods is low among patients with early-stage cancer due to low somatic mutations. However, the method that is based on DNA methylation is preferred because aberrant DNA methylation occurs early in tumour development and can be tissue and cancer-type specific. Some cfDNA-based methylation epigenetic biomarkers in clinical use are VIM and SEPT9 for colorectal cancer, PTGER9/SHOX2 in lung cancer, and GSTP1 in prostate cancer (Shen et al., 2018). A study performed by Lee et al. (2019) reported methylation of LINE-1 in cell-free DNA as a biomarker for human breast cancers and dog mammary tumours. Another study performed

by Stover et al. (2018) characterized metastatic triple-negative breast cancers using cell-free DNA. Likewise, Leal et al. (2020) used circulating tumour DNA analysis to detect residual disease of gastric cancer in the cat.

Extracellular vesicles (EVs): EVs are 30-100nm membrane-bound, saucer-shaped vesicles secreted by cells and found in body fluids (Kalluri, 2016). The three types of EVs are microvesicles, exosomes, and apoptotic bodies. EVs contain lipids, nucleic acids, and proteins from donor cells. EVs in body fluid can reach distal tissues and transfer their content to other cells thereby contributing to biological processes (Zaborowski et al., 2015). EVs released by tumour cells modify host cell phenotype and promote tumour growth. Ovarian cancer cells release EVs that contain CD147, which promotes the expression of MMP1, MMP2, and MMP9 in endothelial cells (Millimaggi et al., 2007). EVs analyzed for their proteins or nucleic acids are useful as markers for a variety of cancers. For instance, human EVs proteins such as CD9, CD63, CD81, TSG101, Alix, and heat-shock chaperones are used as markers (Abels & Breakefields, 2016). Sammarco et al. (2018) identified and characterised EVs from canine and feline mammary tumour cells. The results of a study by Logozzi et al. (2009) showed that high levels of tumour exosomes in the plasma of tumour-bearing animals and humans express CD63 and caveolin, thus being useful as potential markers for melanoma.

MicroRNA (miRNA): These are small noncoding RNA molecules secreted in body fluids. In HBCs, a number of miRNAs have displayed diagnostic and prognostic potentials, such as miR-9, miR-10b, miR-17-5p, miR-148a and miR-335 (Wang et al., 2019), although miR-21 is regarded the most promising biomarker (Jeong et al., 2019). However, there is limited information on miRNA in CMTs, but it has been reported that cfa-miR-96 and cfa-miR-149 are oncogenic and tumour-suppressive (Bulkowska et al., 2017). Similarly, a study by Heishima et al. (2017) reports that miR-214 and miR-126 are potential candidate biomarkers for canine neoplasias.

Metabolomic markers

Modified nucleosides have been proposed to be potential biomarkers for early diagnosis of cancers (Seidel et al., 2006, Henneges et al., 2009). Modified nucleosides are end products of transfer RNA (tRNA) metabolism which are excreted when they are not reutilized in the salvage pathway.

Cancer-bearing humans and animals excrete high levels of these metabolites in their urine due to high turnover of tRNA in tumour tissues (Fischbein et al., 1983; Seidel et al., 2006), while non-tumour individuals excrete low to normal levels. After effective surgery or chemotherapy, high levels of modified nucleosides return to normal (Fischbein et al., 1983; Vreken & Tavenier, 1987). Approximately 100 modified nucleosides are known (Bullinger et al., 2007), and 1-methyladenosine, 1-methylguanosine, 2-methylguanosine, and pseudouridine may serve as biomarkers lung, liver, breast, bladder, colon, urogenital tract, thyroid, ovarian cancers, leukaemia, and Hodgkin disease (Seidel et al., 2015). In HBC, 5-hydroxymethyl-2-deoxyuridine and 8-hydroxy-2-deoxyguanosine were observed (Woo et al., 2009). A study by Budczies et al. (2012) reported that the cytidine-5-monophosphate/pentadecanoic acid metabolic ratio can be used to differentiate invasive breast cancer from normal breast tissues. In canine bladder cancer, urea, choline, citrate acetone, methyl guanidine, and β-hydroxybutyrate are potential biomarkers (Zhang et al., 2012). In CMTs, 5-hydroxyindolacetic acid, serotonin, indoxyl sulphate, 3,4-dihydroxy-L-phenyalanine, and epinephrine were detected in the urine samples in high concentrations, thus being suggested as useful biomarkers (Valko-Rokytovska et al., 2020).

3. TUMOUR PROLIFERATION AND IMMUNITY

In 1863, Rudolph Virchow observed the presence of leukocytes in human cancers and proposed that "inflammation occurs before tumour development''. This statement has remained globally accepted, hence, the connection between inflammation and cancer was established (Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Grivennikov et al., 2010; Morisson, 2012). Unlike in humans, there is a dearth of precise data to support this relationship in domestic animals. However, probably, such relatedness is so (Heidland et al., 2006; Grivennikov et al., 2010; Morisson, 2012). Epidemiological studies suggest the involvement of chronic inflammation in the development of various human cancers (Mantovani et al., 2008). Approximately 25% of human cancers are caused by either chronic inflammation, chronic infection, or both. Chronic inflammatory conditions increase the risk of developing several kinds of cancer including breast, liver, bowel, urinary bladder, prostate, gastric mucosa, ovary, and skin cancers (Morisson, 2012). Later in 1928, Karl-

Heinrich Bauer observed mutations in plants and animals and proposed that cancers probably originated from somatic mutations (Brucher & Jamall, 2014). Balkwill and Mantovani (2001) suggested that if genetic damage is the "match that lights the fire" of cancer, some types of inflammation may provide the "fuel that feeds the flames". Almost 90% of all cancers are attributed to somatic mutations and environmental causes, while very few are related to germline mutations. Often, a single mutation is not sufficient to initiate cancer but requires at least 4-5 mutations. In addition, each mutation will be transmitted to successive cell offspring (Grivennikov et al., 2010). Any cell presenting these damages may become the loci of cancer development. Cells that have minimal mutation can survive without any meaningful change after repairs, whereas those with major irreversible mutations are signalled for altered development and death. For neoplastic processes to start, the induced mutations must persist and resist cell repair processes as well as be readable to DNA polymerase, which will maintain the mutation (Morisson, 2012; Xia et al., 2014). In this way, mutations will accumulate, eventually leading to a precancerous lesion (Morisson, 2012; Xia et al., 2014). Molecular studies in genetically modified mice report that oncogenic changes can induce an inflammatory response which ultimately results in the development of various cancer types (Mantovani et al., 2008).

3.1.- Inflammation

Inflammation is the set of physiological responses by the body to repair itself in the presence of any disturbance to normal tissue homeostasis (Lu et al., 2006; Morisson, 2012). These disturbances could range from acute tissue damage to physical injury, ischemia, or lesions induced by toxins, infection, or parasites (Coussens & Werb, 2002; Morisson, 2012; Murata, 2018; Khandia & Munjal, 2020). The inflammatory response could either be acute or chronic (Aggarwal et al., 2014). During acute inflammation, the innate immune system is activated and the immune cells (macrophages, mast cells, dendritic cells, and natural killer cells) initiate the release of cytokines, chemokines, matrix-remodelling proteases (MMPs), and reactive oxygen and nitrogen species to mediate inflammation, thus leading to the destruction of pathogens and, finally, the repair of tissue damage (Morisson, 2012; Turner et al., 2014). If inflammation persists for a long time, it becomes chronic, where the inflammatory microenvironment is dominated by lymphocytes, macrophages, and plasma cells (Lu et al., 2006; Morrison, 2012).

Various tumours develop from different types of inflammation. For instance, 20% of cancers are initiated by chronic microbial infections, 30% by tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35% by dietary factors (Grivennikov et al., 2010). During microbial infections, the host body attempts to eliminate the pathogen through an inflammatory response which may eventually contribute to cancer development (Grivennikov et al., 2010; Khandia & Munjal, 2020). Some of these infections are discussed here.

- Bacteria infection: Infection with certain bacteria including *Escherichia coli, Helicobacter pylori, Bacteroides fragilis, Streptococcus gallolyticus, Enterococcus spp, Enterobacter aerogenes, Citrobacter koseri* and *Klebsiella pneumonia* may initiate inflammation. When these microbes attach to the intestinal epithelium, they directly cause the proliferation of epithelial cells. Chronic infection of the gastric mucosa by *H. pylori* infection results in the production of nitric oxide, which also damages the host nucleotide DNA and alters the transcriptional regulation through DNA methyl-transferase activity (Mantovani et al., 2008). In addition, *H.pylori* produce toxins which damage the epithelial barrier causing inflammation (Khandia & Munjal, 2020). A few strains of *E. coli, Enterobacter aerogenes, C. koseri* and *K. pneumoniae* produce a toxin called colibactin. Colibactin can induce the DNA double-strand break, chromosome aberrations, and cell cycle arrest in the G2/M phase of cell division. Infection by colibactin *E. coli* could promote the development of human colorectal cancer. It also induces the inhibition of the mutL homologue 1 (MLH1) mismatch repair protein, which could lead to the genomic instability associated with pks *E. coli* infection (Fais et al., 2018; Khandia & Munjal, 2020). Enterotoxigenic *B. fragilis* produces a toxin that triggers the expression of cyclooxygenase (COX-2) which in turn releases prostaglandin E2 (PGE2). PGE2 induces inflammation and cell proliferation by binding to the cell receptor and activating oncogenic signalling pathways resulting in intestinal tract tissue injury and colorectal cancer (Cheng et al., 2020; Khandia & Munjal, 2020).

- Fungal infection: Infection with *Candida albicans* can cause inflammation and increases the host's susceptibility to cancer such as oral, gastric, and colorectal cancer (Yu & Liu, 2022). Its adherence with endothelial cells may trigger the release of proinflammatory cytokines (IL-1β, IL-8, and TNF-α) and induce chronic inflammation and T helper 17 (Th17) cells immune response, and production of carcinogens (Khandia & Munjal, 2020; Yu & Liu, 2022).

- Protozoan infection: Infections by *Trichomonas vaginalis* and *Toxoplasma gondii* can trigger inflammation (Khandia & Munjal, 2020). *T. gondii* infection can alter the host immune response and produce polarization toward T helper1 (Th1) cells response as such causing an increase in IFN-γ and IL-12 production which activates anti-tumour response (Khandia & Munjal, 2020). During asexual reproduction of *T. gondii,* the resultant bradyzoite cysts persist in the host becoming a lifelong chronic infection due to the inability of drugs to eliminate the cysts. These cysts persist in the brain, heart, lung, and skeletal muscle tissues. The cysts provoke the immune system (e.g., lymphocytes, plasma cells and macrophages), stimulating mild inflammation, and inhibiting programmed cell death. *T. gondii* infection has a 1.8-fold increase in the risk of brain cancers and causes glioma in experimental animals (Thomas et al., 2012). Infection with *T. vaginalis* has been associated with cervical and prostate cancers (Stark et al., 2009).

- Helminth infection: Helminth parasites modulate the immune responses of their hosts to avoid immune effector cells and molecules. These immunomodulatory activities can impact the outcomes of several inflammatory diseases, such as multiple sclerosis, arthritis, type 1 diabetes, and inflammatory bowel disease, like ulcerative colitis and Crohn's disease (Ledesma-Soto et al., 2015). The larval stage of *Taenia crassiceps* down-regulates the pro-inflammatory cytokines while upregulating IL-4 and IL-10 and ameliorate Dextran Sodium Sulphate mediated colitis (Ledesma-Soto et al., 2015). The eggs of *Schistosoma mansoni, S. japonicum* and *S. mekong,* in the bladder can trigger the production of IL-4, IL-5, and IL-13, which further activate the macrophages, and a granulomatous host-immune response and finally induces chronic inflammation (Colley et al., 2014).

- Viral infection: Infections with *Hepatitis B virus, H. C. virus, Epsteine-Barr virus, Human T-lymphotropic virus-I, Kaposi's sarcoma herpesvirus,* and *Merkel cell polyomavirus* predisposes the body to inflammation and eventually cancer initiation (Read & Douglas, 2013; Khandia & Munjal, 2020). During chronic viral infection, the infected cells induce the production of cytokines (IL-1β, tumour necrosis factor-α (TNF-α) and IL-6) and create an inflammatory microenvironment. Both the viral infection and virus-mediated inflammation activate NF-κB, STAT3 and the MAPK signalling pathways to promote tumour development (Fan et al., 2013; Read & Douglas, 2013). Chronic *Hepatitis B virus* and *H. C virus* infections can induce liver cancer due to chronic inflammation, liver damage and endoplasmic reticulum stress (Read &

Douglas, 2014). *Epstein-Barr Virus* is associated with various cancers including Hodgkin's lymphoma, Burkitt's lymphoma, lymphomas in immunosuppressed individuals, and some carcinomas (Read & Douglas, 2014). *Kaposi's sarcoma herpesvirus* infection is usually oncogenic in immunocompromised individuals such as AIDS patients who have undergone organ transplants. It is associated with Kaposi's sarcoma primary effusion lymphoma, and multicentric Castleman's disease (Gonclaves et al., 2017; Read & Douglas, 2014). *Human Papilloma Virus* is implicated in cervical cancers and oropharyngeal cancers (Read & Douglas, 2014; Berman & Schiller, 2017; Okunade, 2020). Other viruses have also been implicated in human cancers including the *Human mammary tumour virus*, *Human endogenous retrovirus*, and *Merkel cell polyomavirus* (Read & Douglas, 2014).

3.2.- Relationship between inflammation and cancer

Although the role of chronic inflammation in cancer development has been globally accepted (Mantovani et al., 2008), the detailed mechanisms of how it promotes tumour growth and how cancerous cells suppress anti-tumour immunity remain a significant challenge (Lu et al., 2006; Fan et al., 2013). However, it is known that cancer development is triggered by a completely different type of inflammation, though having similar attributes to inflammation such as the presence of inflammatory cells and inflammatory mediators in tumour tissues, tissue remodelling and angiogenesis similar to that in chronic inflammatory responses and tissue repair (Mantovani et al., 2008).

Inflammation and cancer are linked through two pathways, namely intrinsic and extrinsic inflammatory pathways (Mantovani et al., 2008). The intrinsic pathway is triggered by genetic mutations in various oncogenes including renin-angiotensin system (RAS) and c-myelocytomatosis (c-MYC) family members. On the other hand, the extrinsic pathway is activated by inflammation due to microbial infections, autoimmune diseases, obesity, tobacco smoking, asbestos exposure and excessive alcohol consumption, all of which increase cancer risk and stimulate malignant progression (Mantovani et al., 2008; Todoric et al., 2016). Cells that have undergone genetic mutations, generate inflammatory mediators, thus creating an inflammatory microenvironment in the absence of preexisting inflammation (Mantovani et al., 2008).

Conversely, the extrinsic pathway predisposes to cancers (including colon, prostate, and pancreas cancer) at sites of inflammation or infection (Mantovani et al., 2006). Both pathways activate transcription factors (NF-κB, STAT3, and HIF1α) in tumour cells (Todoric et al., 2016).

Intrinsic inflammatory response builds up a pro-tumorigenic microenvironment through the recruitment of leucocytes and lymphocytes, cytokines, chemokines, and induction of angiogenic switch. Besides independent cell proliferation, oncogenes induce transcription pathways that cause remodelling of the tumour microenvironment (Givennikov et al., 2010). During the pro-tumorigenic response by tumour cells, STAT3, which is one of the critical transcription pathways, is activated in numerous types of cancer. The activity of STAT3 is activated by IL-6, IL-11, other cytokines, and growth factors (Todoric et al., 2016). It plays an important role in the tumour microenvironment by promoting the expression of IL-23, a pro-tumorigenic immune response, while inhibiting IL-12 expression, an antitumorigenic immune response. Hence, inhibition of STAT3 blocks tumour development at an early stage and regression of established tumours at a late stage (Todoric et al., 2016). The activation of NF-κB, another critical transcription pathway that is activated, results in different responses depending on the site. For instance, in malignant cells, the resultant response is the increased expression of tumour-promoting genes, whereas, when in the TME, it increases the expression of inflammatory cytokines and growth factors. It also promotes EMT and angiogenesis (Todoric et al., 2016). Prolonged inhibition of NF-κB can lead to profound inflammation due to activation of NLRP3 inflammasome leading to enhanced production of IL-1β and subsequent neutrophilia (Todoric et al., 2016). These transcription factors initiate the production of inflammatory mediators (cytokines, growth factors, reactive oxygen or nitrogen species, prostaglandins, and proteolytic enzymes) by macrophages, neutrophils, lymphocytes, dendritic cells, natural killer cells, fibroblasts, adipocytes, endothelial cells and, sometimes, by the cancer cells (Morisson, 2012; Todoric et al., 2016). Macrophages and other inflammatory cells also generate cytokines, growth factors, and reactive oxygen and nitrogen species which may cause DNA damage and resultant permanent genomic alterations (Lu et al., 2006; Morrison, 2012; Aggarwal et al., 2014; Todoric et al., 2016). Reactive oxygen and nitrogen species cause tissue damage, which may produce peroxynitrite which reacts with DNA causing mutations in proliferating epithelial and stroma cells (Lu et al., 2006). In addition, macrophages and T lymphocytes may release tumour necrosis factor-α (TNF-α) and macrophage migration inhibitory factors to further aggravate the DNA damage. Migration inhibitory factor impairs p53-

dependent protective responses, thus causing the accumulation of oncogenic mutations. It also interferes with the Rb-E2F pathway in regulating apoptosis (Lu et al., 2006). Cytokines also activate transcription factors in inflammatory cells stroma cells and tumour cells resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated (Grivennikov et al., 2010; Capettini et al., 2012; Liu et al., 2015). Some mediators stimulate the proliferation of cancer cells and inhibit apoptosis at the same time, hence promoting the accumulation of oncogenic mutations, while others influence other components of the tumour microenvironment (Todoric et al., 2026).

The proliferation of tumour cells is crucial for cancer development, survival, and metastasis. It is an overly complex process, which is characterized by the altered expression and/or activity of cell cycle regulatory proteins and the activation of signal transduction pathways (Feitelson et al., 2015). Three mechanisms are involved in the early steps of cancer development that favour the proliferation and survival of cancer stem cells, namely epithelial-mesenchymal transition, autophagy, and altered metabolism (Warburg effect) (Feitelson et al., 2015). These steps are interdependent with one to another, thus creating a complex environment to facilitate tumour growth (Feitelson et al., 2015).

- Epithelial-mesenchymal transition (EMT): EMT is a dynamic multistep process that involves loss of intercellular adhesion, destruction of the cancer basement membrane and extracellular matrix, reconstruction of cytoskeleton and enhancement of cell motility, which also increases the resistance to cancer treatment (Kalluri & Weinberg, 2009; Chen et al., 2019). The completion of an EMT is signalled by the degradation of the underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated (Kalluri & Weinberg, 2009). Epithelial cells are held together by various cell adhesion molecules, such as claudins and E-cadherin, to form polarized sheets (Gheldof & Berx, 2013). In addition, the basement membrane anchors epithelial cells to the matrix surface and maintains apical-basal polarity through connections between intermediate filaments and hemidesmosomes (Gheldof & Berx, 2013). Cell adhesion to both the basement membrane and adjacent cells is critical for maintaining the epithelial phenotype (Gheldof & Berx, 2013). Three types of EMTs have been described (Figure 1). Type 1 EMT is a non-pathological process that occurs at distinct stages of embryonic development, including gastrulation and neural crest cell delamination (Kalluri &

Weinberg, 2009; Gheldof & Berx, 2013; Marconi et al., 2021). This EMT is required to produce mesenchymal cells (primary mesenchyme) that are capable of successively undergoing a mesenchymal-epithelial transition to create secondary epithelial (Marconi et al., 2021). Type 2 EMT is associated with the wound-healing process following trauma and inflammatory damage, where the epithelial cells differentiate into fibroblast-like cells to rebuild tissues (Marconi et al., 2021). It is a necessary feature for the correct development processes such as tissue regeneration, organ fibrosis, and wound healing (Roche, 2018). Finally, type 3 EMT occurs in cancer processes, where metastatic cancer cells have undergone genetic and epigenetic modifications to escape apoptosis and induce an invasive phenotypic transformation (Marconi et al., 2021). In other words, EMT is a physiological mechanism used by cancer cells with pathological consequences (Roche, 2018). During this process, epithelial cells lose their main characteristics, gaining instead an invasive and migratory mesenchymal phenotype that allows them to leave the tissue parenchyma and enter the systemic circulations during metastasis (Gonzalez & Medici, 2014; Kaufhold & Bonavida, 2014).

EMT is initiated and maintained by transcription factors which repress the expression of E-cadherin, which is a critical molecule in the EMT process. Other molecules involved in EMT include claudins and desmosomes (Gheldof & Berx, 2013; Gonzalez & Medici, 2014; Chen et al., 2019; Casas et al., 2022). Transcription factors involved in the repression of E-cadherin include ZEB protein, Snail factor, Twist, and lymphoid enhancer-binding factor-1 (LEF-1) (Gheldof and Berx, 2013; Gonzalez & Medici, 2014; Chen et al., 2019; Casas et al., 2022).

In this sense, the Snail family of transcription factors is composed of three proteins, namely Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc). These proteins are expressed in neoplastic epithelial and mesenchymal cells, as well as in non-neoplastic ones such as fibroblasts and macrophages in wounded and inflamed tissues (Wu & Zhou, 2010). Snail1 and Snail2 are critical in regulating EMT by binding to CDH1 (Cadherin1) promoter, resulting in E-cadherin repression. This repression will lead to the dissemination of cancer cells and, consequently, the appearance of metastasis (Gonzalez & Medici, 2014). Aside from E-cadherin, claudins are also down-regulated with concomitant up-regulation of vimentin and fibronectin among other biomarkers (Kaufhold & Bonavida, 2014). Expression of Snail in mammary tumours is associated with metastasis, tumour recurrence and poor prognosis. In addition, it is involved in immune responses such as induction

of immunosuppression and immunoresistance through immunosuppressive cytokines, regulatory T cells, impaired dendritic cells, and cytotoxic T lymphocyte resistance. It also increases the resistance to chemo and radiation therapy (Wu & Zhou, 2010; Kaufhold & Bonavida, 2014; Feitelson et al., 2015).

The ZEB family transcription factors (ZEB1 and ZEB2) are also critical in EMT through binding the CDH1 promoter site, invariably repressing E-cadherin activity (Gonzalez & Medici, 2015; Chen et al., 2019; Scott & Omilusik, 2019). In addition to their role in EMT, ZEBs are widely expressed by various immune cells, particularly in mice, where they regulate important transcription networks necessary for cell differentiation, maintenance, and function (Gonzalez & Medici, 2014; Scott & Omilusik, 2019). The abundance of ZEB proteins on the CDH1 promoter is inhibited by the miR-200 family. In turn, ZEB1 and ZEB2 bind to the E-box promoters of miR-200, creating a reciprocal feedback loop controlling EMT. Interestingly, the expression of miR-200 family members enhances the recolonization of metastatic cells, highlighting the role of this regulatory loop in maintaining the mesenchymal phenotype and demonstrating the reversibility of EMT (Gonzalez & Medici, 2014). It is worth noting that Snail1 and ZEB1 work together to repress E cadherin and to achieve complete EMT (Kaufhold & Bonavida, 2014).

Twist (Twist1 and Twist2) also initiate EMT. In human mammary cells, Twist1 binds the Snail2 promoter and stimulates its expression to induce EMT (Qin et al., 2012; Gonzalez & Medici, 2014). It mediates the regulation of transcription by acting in a concerted fashion with BMI1 (a polycomb-group repressor complex protein) to repress E-cadherin and the cell cycle inhibitor p16INK4α (Casas et al., 2011). In addition, Twist1 is responsible for the expression of several microRNAs which mediate the inhibition of HOXD10 (Homeobox protein) expression and RhoC gene. Twist1 plays a vital role in cancer invasion, metastasis, apoptosis, and acquired resistance by cancer cells to chemotherapy (Qin et al., 2012; Gonzalez & Medici, 2014). Suppression of Twist expression in highly metastatic mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung. The ectopic expression of Twist results in the loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility, suggesting that Twist contributes to metastasis by promoting EMT (Gonzalez & Medici, 2014).

LEF-1 is a T-cell factor detected in tumours and is considered a mesenchymal marker. It can directly induce EMT through its repression of E-cadherin by forming complexes with β-catenin, which is degraded due to GSK-3β mediated phosphorylation. It also interacts with Snail 1 through Wnt, phosphatidylinositol-3 kinase (P13K) and transforming growth factor-beta 1 (TGF-β1) signalling pathways. Inhibiting LEF-1 activity, either with a dominant-negative form of the protein or with targeted small interfering RNA (siRNA), inhibits EMT in many systems, hence, there is delayed wound healing in trauma or inflammation, delayed or no metastasis in cancer, or delayed embryogenesis (Gonzalez & Medici, 2014; Kaufhold & Bonavida, 2014).

In addition to transcription factors, there are other factors involved in the modulation of EMT, such as exosomes, extracellular matrix, hypoxia, and peptides like transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP), Wnt/β-catenin, Notch, Hedgehog, and receptor tyrosine kinases (Gonzalez & Medici, 2014; Feitelson et al., 2015; Chen et al., 2019), some of them being important modulatory mechanisms of cell gene expression impacting on cell proliferation.

- *Autophagy*: Autophagy is a highly conserved cellular degradation and recycling process in all eukaryotic cells (Mizushima et al., 2008; Ravikumar et al., 2010; Feng et al., 2014; Parsych & Klionsky, 2014). Three types of autophagy exist in mammalian cells, namely: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Each type of autophagy is morphologically distinct, yet all three culminate in the delivery of cargo to the lysosome for degradation and recycling (Mizushima et al., 2008; Feng et al., 2014; Parsych & Klionsky, 2014). Microautophagy consists of the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. The function of this process in higher eukaryotes is not known, whereas microautophagy-like processes in fungi are involved in selective organelle degradation (Mizushima et al., 2008; Parsych & Klionsky, 2014). On the other hand, macroautophagy involves the sequestration of cytosolic components within a unique double-membrane cytosolic vesicle, an autophagosome. Sequestration can be either nonspecific, involving the engulfment of bulk cytoplasm, or selective, targeting specific cargoes such as organelles or invasive microbes (Muzushima et al., 2008). Last, chaperone-mediated autophagy involves the direct translocation of unfolded substrate proteins across the lysosome membrane through the action of a cytosolic and lysosomal chaperone hsc70, and the integral membrane receptor LAMP-

2A (lysosome-associated membrane protein type 2A) (Muzushima et al., 2008; Parsych and Klionsky, 2014). Both micro- and macroautophagy have the capacity to engulf large structures through both selective and non-selective mechanisms, whereas CMA degrades only soluble proteins selectively (Mizushima et al., 2008). Of the three types of autophagy, macroautophagy is the most studied, and it occurs under basal conditions. This mechanism plays several roles including maintenance of cellular homeostasis by clearing senescent organelles, misfolded proteins and pathogens, inhibiting the production of reactive oxygen species, eliminating damaged mitochondria and peroxisomes, and reducing DNA damage and chromosomal instability, but at the same time is closely related to cancer development, among other diseases (Levine & Kroemer, 2008; Ravikumar et al., 2010; Sridhar et al., 2012; Parsych & Klionsky, 2014; Feitelson et al., 2015; Sotthibundhu et al., 20162). Following degradation, the breakdown products are released back into the cytosol to recycle the macromolecular constituents and generate energy to maintain cell viability under unfavourable conditions and to protect the cell during various conditions of stress (Feng et al., 2014).

Though autophagy is known to function in several cellular processes, this discussion will highlight its involvement in cancer. The role of autophagy in cancer is complex and highly debated. Therefore, autophagy is regarded as a double-edged sword in cancer cells depending on the cell and tissue types and the stage of tumours. At the early stage of cancer development, it may act as a cancer suppressor by eliminating potentially harmful agents, damaged mitochondria and misfolded proteins, and inhibiting inflammation, hence, preventing the spread of damage (Ravikumar et al., 2010; Feitelson et al., 2015; Chen et al., 2019, Chaves-Dominguez et al., 2020). Conversely, at the advanced stage, it promotes tumour development by producing nutrient substances and releasing adenosine triphosphate (ATP) for maintenance of protein synthesis and other metabolic functions, and the high demand of energy by cancer cells for proliferation, invasion, and metastasis in the stressful microenvironment (Feitelson et al., 2015; Chen et al., 2019, Chaves-Dominguez et al., 2020).

Autophagy is normally inhibited by mTOR, whereas mTOR is inhibited by stress signals such as HIF, dysregulated PI3K/Akt, and elevated p53. Inhibiting mTOR leads to Beclin1/Class III PI3 complex on phagophore in the cytoplasm being activated, thus promoting autophagy (Feitelson et al., 2015; Chen et al., 2019). Besides the aforementioned stresses, autophagy can be induced by

many antitumoral agents, including some polyphenolic compounds (resveratrol, curcumin, rottlerin, genistein, quercetin). Early inhibitors of autophagy include 3-methyladenine, wortmannin and LY294022, which target the class III PI3K. Late-stage inhibitors include the antimalarial drugs bafilomycin A1 (which targets a vacuole adenosine triphosphatase), monensin and chloroquine, both of which prevent the acidification of lysosomes (Feitelson et al., 2015).

Furthermore, both EMT and autophagy share a complex relationship of interdependency in the occurrence and development of cancer. In this sense, while autophagy regulating pathways (such as PI3K/Akt/mTOR, Beclin-1, p53, and JAK/STAT) play a crucial role in EMT, EMT regulatory pathways (including integrin, WNTs, NF-κB, and TGF-β) modulate autophagy to support the viability of potential metastasis of cancer cells (Chen et al., 2019).

- *Hypoxia*: Hypoxia is another essential event in cancer development. This is because hypoxia modulates cell-specific metabolic pathways, thus determining key cell features such as proliferation, migration, and invasive rate (Weider et al., 2017; Paredes et al., 2021). Cancer cells can adapt to a hostile microenvironment (hypoxia, low glucose levels and acidic extracellular pH) that increases genetic instability through the activation of a rapid and intense energy production system (Zhang et al., 2017). Tumoural cells switch their energy metabolism from aerobic, such as mitochondrial oxidative phosphorylation, to anaerobic metabolic pathways even in the presence of a sufficient level of oxygen, being this mechanism known as the Warburg effect (Semanza, 2012; Weider et al., 2017; Paredes et al., 2021). Hypoxia influences a cell's preference for specific metabolic pathways, determining its proliferation, migration, and invasive rate (Weider et al., 2017; Paredes et al., 2021). Hypoxia and pseudohypoxia conditions are linked with the up-regulation of several hypoxia-inducible factors (HIFs) (Carvalho et al., 2016; Zhang et al., 2012).

Three isoforms of HIFs (HIF-1, HIF-2, and HIF-3) have been identified. While HIF-1 and HIF-2 are known transcription activators, HIF-3 has been considered a negative regulator of the hypoxia response pathway (Tolonen et al., 2020). HIF-1 is the main regulator of oxygen by inducing alternative metabolic pathways in cancer cells. HIF-1 has two subunits, namely HIF-1α and HIF-1β (Semanza, 2003; Semanza, 2012; Graham & Presnell, 2017; Tolonen et al., 2020). HIF-1α stimulates the production of growth factors such as transforming growth factor β (TGF-β), insulin-like growth factor 2 (IGF-2), IL-6, IL-8, macrophage migration inhibitory factor (MIF), as well as growth factor receptors (EGFR), resulting in continuous proliferative signalling (Weider et al.,

2017). HIF-1β subunits are non-oxygen-responsive nuclear proteins (Gu et al., 2000). The cell proliferative effect of hypoxia is blocked by HIF inhibitors which enhance the degradation of HIF-1 through the inhibition of heat shock protein 90 (Hsp90), or by inhibiting mTOR (Feitelson et al., 2015). Warburg effect in cancer cells is regulated by mitochondrial nicotinamide adenine dinucleotide (NAD) - dependent deacetylase SIRT3, which destabilizes HIF-1α (Weider et al., 2017). HIF-1α is also known to promote autophagy (Carvalho et al., 2016; Zhang et al., 2017).

3.3.- Tumour immunity phenomenon

Tumour microenvironment (TME) is the environment around a tumour. It consists of tumour cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells), together with innate immune cells including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells and adaptive immune cells (T and B lymphocytes) (Grivennikov et al 2010; Carvalho et al 2016; Baghban et al., 2020). Tumour cells possess antigens that differentiate them from normal cells, and the immune cells mount surveillance against them. They act by either engulfing and presenting tumour antigens, releasing cytokines to activate and recruit other cells, or directly killing them (Ponomarev & Shubina, 2019). However, tumour cells in turn mount surveillance to evade their elimination by immune cells through the tumour immunity phenomenon, which is a complex defence mechanism by tumour cells themselves or through the pro-inflammatory-associated environment to escape immune cells by suppressing their function. This action occurs in the initial phase of neoplastic transformation and may influence tumour progression and the overall prognosis (Huber et al., 2013; Whiteside, 2022). As the tumour progresses, there is a switch in innate immune cells from an anti-tumour role towards a pro-tumorigenic action. In this way, innate immune cells can actively contribute to immune tolerance, thus preventing the rejection of the tumour (Grivennikov et al., 2010). In addition, tumour cells are genetically unstable, they acquire features that render them resistant to pro-inflammatory immune attack to pro-tumour status (Hagerling et al., 2015). Tumour immunity does not only occur at the primary tumour site but also in regional and distant sites, such as lymph nodes and bone marrow (Huber et al., 2013; Whiteside, 2022).

The crosstalk/interaction between immune cells and tumour cells is mediated by increased production of pro-inflammatory mediators (cytokines, chemokines, reactive oxygen species); increased expression of oncogenes, cyclooxygenase 2, 5-lipoxygenase, matrix metalloproteinases, and pro-inflammatory transcription factors such as NF-κB (nuclear factor κB), STAT3 (signal transducer and activator of transcription 3), AP-1 (activator protein 1), and HIF-1α (Carvalho et al., 2014; Pinto et al., 2015; Kawaguchi et al., 2019). These pro-inflammatory mediators potentiate in turn tumour cell proliferation, transformation, metastasis, invasion, angiogenesis, and resistance to chemo and radiotherapy (Carvalho et al., 2014; Pinto et al., 2015; Kawaguchi et al., 2019). Inhibition of anti-tumour response by immune cells can be done in a number of ways as discussed below.

These mediators and factors promote the recruitment and accumulation of immature myeloid cells. Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of immature myeloid cells, immature macrophages, immature granulocytes, and immature dendritic cells. These are potent inhibitors of immune cell function in infection, inflammation, and cancer (Gabrilovich & Nagaraj, 2009; Monu & Frey, 2012). They alter anti-tumour immune responses indirectly by suppressing NK cell-mediated lysis and polarizing tissue macrophage differentiation which enhance tumour progression. It has also been suggested that MDSCs limit the availability of mature and functional dendritic cells, indirectly suppressing T cells activation by inducing T regulatory cells (Tregs) and impair T cell homing to lymph nodes by regulating L-selectin levels (Monu & Frey, 2012).

Tumour-associated macrophages (TAMs) may directly induce tumour cell proliferation by releasing growth factors such as epidermal growth factor receptor (EGFR) (Kumari & Choi, 2022) which remodel the pro-tumour TME through the release of mediators including cytokines and growth factors including vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, and transforming growth factor-β, and NF-κB-mediated factors (Pan et al., 2020; Kumari & Choi, 2022). TAMs act indirectly by affecting different cell types. Macrophages produce extracellular matrix-degrading enzymes (MMPs, cathepsin, and many other types of proteases) to disintegrate extracellular matrix and allow tumour cells to escape. TAMs upregulate the secretion of immunosuppressive cytokines (IL-1Ra) by increasing tumour stemness and promoting metastasis (Kumari & Choi, 2022).

Tumour-associated neutrophils (TAN) in the tumour microenvironment induce angiogenic switch and promote cell growth and invasion by remodelling the extracellular matrix and modulating the biology of the tumour cell in later stages (Hagerling et al., 2015). In advanced tumour mouse models, TANs induce CD8 T-cell apoptosis. TANs and granulocytic myeloid-derived suppressor cells (G-MDSCs) suppress CD8 T-cell proliferation, influence their activation and abolish the anti-tumour effect of CD8 T-cells. TANs inhibit the proliferation of T-cells by releasing arginase 1 (Arg 1) and modulate PD-L1/PD-1 signalling to regulate an immunosuppressive response (Masucci et al., 2019).

Natural killer (NK) cells can either function as anti- or pro-tumour in tumour progression, though they are strictly anti-tumour cells. Tumour cells evade NK-cell immunity by acquiring characteristics which upregulate the function of telomeric repeat-binding factor 2 (TRF 2) to inhibit NK-cell recruitment and NK-cell mediated elimination (Hagerling et al., 2015). Hypoxia reduces the anti-tumour ability of NK cells and attracts T cells suppressive myeloid cells. Elevated cyclooxygenase results in production of prostaglandin E2, which reduces NK-cell infiltration and thus reduces the dendritic cells recruitment factors like chemokine (C motif) ligand 1(XCL1) and chemokine (C-C motif) ligand 5 (CCL5) in the tumour microenvironment (Haas & Obenauf, 2019).

Dendritic cell (DC) functionality in the tumour microenvironment can be affected by the secreted factors from tumour cells. For instance, IL-6 and colony-stimulating factor 1 (CSF-1) promote lineage commitment toward suppressive monocytes, and vascular endothelial growth factor inhibits DC maturation by suppressing NF-κB signalling in haematopoietic progenitors. In addition, TGF-β can inhibit antigen uptake of DCs, hence inhibiting its anti-tumour activity (Haas & Obenauf, 2019).

4.- Mammary cancer biology

Cells react to the stimuli of extracellular signalling molecules that bind to receptors located on the cell membrane or in the cytoplasm. This binding to receptors transfers signals to the nucleus and induces the corresponding gene expression, thus producing biological effects and cellular responses. All these molecular pathways are tightly regulated to avoid aberrant functional features.

However, tumour cells show an overall weakening of the control mechanisms that modulate gene expression. This weakening would lead to tumour-specific features such as excessive cell proliferation, apoptotic resistance, angiogenesis, invasion, and metastasis, consequently leading to the development and progression of cancer (Xia et al., 2018). In this way, the precise comprehension of the most important modulatory mechanisms of cell gene regulation is of the utmost importance to launch effective antitumoural therapies. Some of these mechanisms are succinctly further described next.

- *Wnt/β-catenin signalling pathway*: Also known as the canonical Wnt signalling pathway, it is a developmental modulatory mechanism of the regulation of cell proliferation, differentiation, migration, apoptosis, genetic stability, polarity and stem cell renewal (Pai et al., 2017) (Figure 1). There are also the noncanonical Wnt pathways, which are independent of β-catenin-T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), such as the Wnt/Ca2+ pathway and noncanonical Wnt planar cell polarity, that are involved in cell polarity and migration (Liu et al., 2022). The canonical Wnt pathway (Figure 1) involves the nuclear translocation of β-catenin and the activation of target genes through TCF/LEF transcription factors. These two pathways (canonical and noncanonical) form a network of mutual regulation (Liu et al., 2022).

The Wnt/β-catenin pathway comprises four parts. The extracellular part mediates signals through Wnt proteins (Wnt3a, Wnt1, and Wnt5a). The cell membrane part contains the Wnt receptors frizzled protein and LRP5/6. The cytoplasmic part includes β-catenin, Dvl, glycogen synthase kinase-3β (GSK-3β), AXIN, APC, and casein kinase I (CK1). Finally, the nuclear part includes β-catenin, which translocates to the nucleus, TCF/LEF family members, and β-catenin downstream target genes, such as MMPs and c-Myc (Liu et al., 2022). Once the canonical pathway is activated, it induces the stability of β-catenin and transfers it to the nucleus to facilitate the expression of genes involved in cell proliferation, survival, differentiation, and migration (Liu et al., 2022). The inhibition of the canonical pathway induces the impairment of tissue homeostasis and regeneration (Zhang & Wang, 2020). The dysregulation of Wnt/β-catenin signalling often leads to many serious diseases, including cancer and non-cancer diseases (Liu et al., 2022). The inactivation of negative regulators, or over-expression of the Wnt signalling pathway, results in aberrant Wnt signalling, hence playing crucial roles in tumourigenesis of most tumours, including breast and colorectal cancers (Feitelson et al., 2015; Pai et al., 2017; Zhang & Wang, 2020).

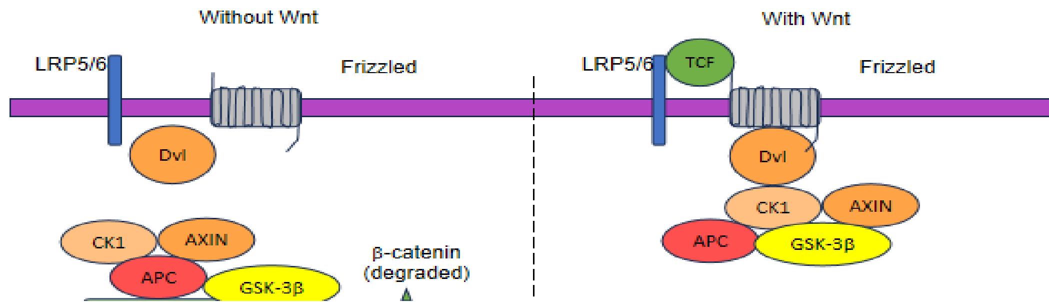

Figure 1: Schematic representation of Wnt/β-catenin pathway. Left: inhibited Wnt pathway by phosphorylation of β-catenin and subsequent degradation; Right: activated Wnt pathway; LRP5/6 (low-density lipoprotein receptor-related protein 5/6), Frizzled receptor, Dvl (dishevelled), β-catenin destruction complex [Axin, APC (adenomatosis polyposis coli), CK1 (casein kinase 1), GSK-3β (glycogen synthase kinase-3β)], TCF (T cell factor), HDAC (histone deacetylase), TLE (transducin-like enhancer). Adapted from Amado et al., 2014.

- *Notch signalling pathway*: The Notch-modulated pathway is detected in breast cancer, gliomas, T-cell leukaemia, salivary, pancreatic, colorectal, lung, cervical and ovarian carcinomas, and embryonic brain tumours (Feitelson et al., 2015). Notch signalling (Figure 2) is activated when a specific ligand binds with the Notch receptor, leading to the subsequent activation of a transcriptional complex involving Hes and Hey genes. Notch signalling regulates the expression of p27cip1/wafl, cyclin D1, c-Myc, p21, Survivin, Snail2, and Nanog and activates the KB pathway (Yuan et al., 2015). During hypoxia, HIFs activate the Notch pathway, being this activation instrumental in launching tumour proliferation, survival and chemoresistance (Feitelson et al., 2015; Yuan et al., 2015). Furthermore, Notch also plays a key role in the modulation of tumour angiogenesis, proliferation, differentiation, and apoptosis (Feitelson et al., 2015; Yuan et al., 2015; Zhou et al., 2022). Notch signalling can both promote and inhibit tumour development in various cancer types. For instance, Notch activation can suppress SIRT1 while activating p53 in Ewing sarcoma, leading to an arrest of the tumour growth (Feitelson et al., 2015; Yuan et al., 2015; Zhou et al., 2022). Notch inhibitors induce tumour arrest. Therefore, combined therapy of

either chemotherapy or radiotherapy with notch inhibitors may improve the response to therapy (Yuan et al., 2015).

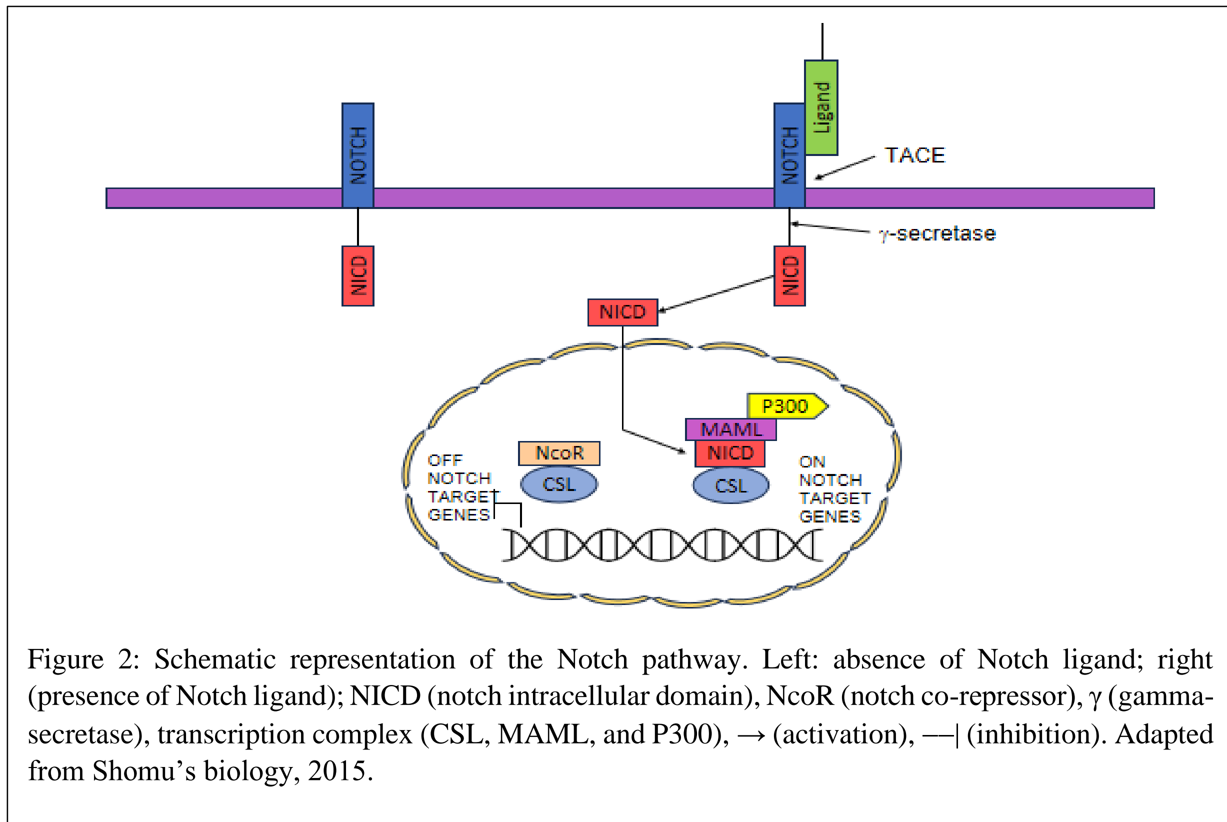

Figure 2: Schematic representation of the Notch pathway. Left: absence of Notch ligand; right (presence of Notch ligand); NICD (notch intracellular domain), NcoR (notch co-repressor), γ (gamma-secretase), transcription complex (CSL, MAML, and P300), → (activation), —| (inhibition). Adapted from Shomu's biology, 2015.

- Hedgehog (Hh) signalling pathway: Hedgehog signalling is an evolutionarily conserved pathway that functions in regulating a variety of developmental and physiological processes in stem cell differentiation, cancer proliferation, and proper segregation in vertebrates and invertebrates (Sari et al., 2018; Jeng et al., 2019). Three types of Hh ligands have been associated with this pathway in humans including Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog. Each Hh ligand exhibits different spatial and temporal expression patterns despite their physiological similarities (Sari et al., 2018; Jeng et al., 2019). Of all the Hh ligands, SHH (Figure 3) is the most studied ligand and acts as a cell-cell signalling factor, modulating cell fates through an autocrine or paracrine mechanism (Sari et al., 2018). The Hh pathway has several main components, namely three Hh homologs, Patched1 (PTCH1 in humans, Ptch1 in mice, and Ptc in *Drosophila melanogaster*), a G-protein-coupled receptor (GPCR)-like receptor Smoothened (SMO in humans

and Smo in mice/*Drosophila melanogaster*), and three transcription factors (GLI1, GLI2, and GLI3) named from the correlation of GLI1 and glioma (Sari et al., 2018).

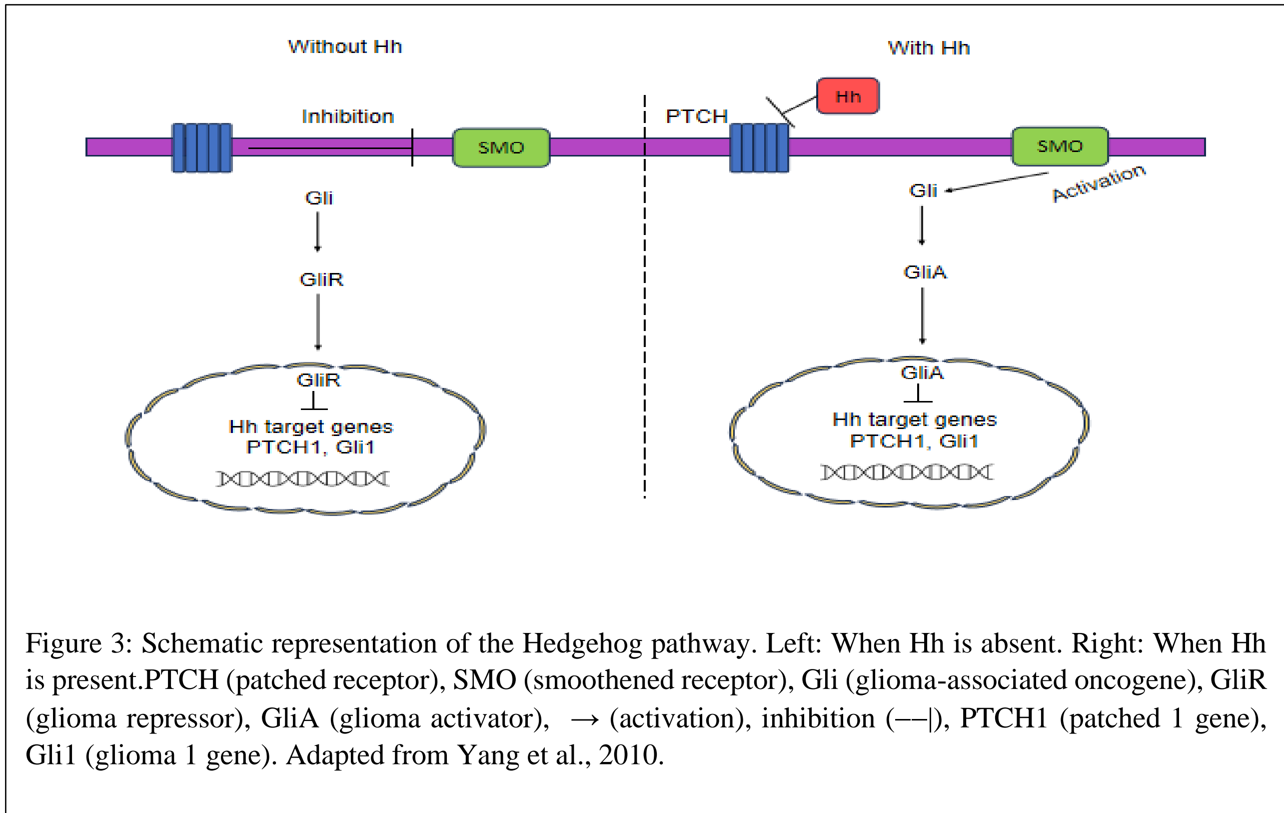

Figure 3: Schematic representation of the Hedgehog pathway. Left: When Hh is absent. Right: When Hh is present.PTCH (patched receptor), SMO (smoothened receptor), Gli (glioma-associated oncogene), GliR (glioma repressor), GliA (glioma activator), → (activation), inhibition (—|), PTCH1 (patched 1 gene), Gli1 (glioma 1 gene). Adapted from Yang et al., 2010.

Hh activation is associated with tissue invasion and increased metastasis in gastric and prostate cancers, just as inhibition of the pathway will reduce tumour proliferation in these cancers. Nevertheless, the aberrant activation of Hh leads to the growth, proliferation, and invasion of tumour cells (Skoda et al., 2018), and the development of congenital defects if activation occurs at the embryonic developmental stage (Feitelson et al., 2015; Sari et al., 2018). It regulates the proliferation of cancer stem cells proliferation in human tumours such as glioblastoma, breast cancer, pancreatic adenocarcinoma, multiple myeloma, and chronic myeloid leukaemia (Feitelson et al., 2015; Sari et al., 2018). Abnormal regulation of the Hh pathway during embryonic development is implicated in various congenital defects (Feitelson et al., 2015; Sari et al., 2018). Moreover, as the Hh pathway plays an essential role in the maintenance of somatic stem cells and pluripotent cells, it is involved in the regenerative proliferation of epithelial stem cells in the lung, tooth, liver, prostate, and bladder. This pathway may be correlated with the maintenance of cancer

stem cells (Sari et al., 2018). It is also involved in the induction of EMT through Wnt, EGF/FGF, Notch and TGF-β1 cascades (Jeng et al., 2019).

- Transforming growth factor-β1 signalling pathway: Transforming growth factor-β1 (TGF-β1) signalling exerts principal functions during embryonic development and homeostasis of adult tissues (Pietrobono et al., 2019) (Figure 4). In epithelial cells, activation of the TGF-β1 signal pathway leads to inhibition of cell proliferation and an increase in extracellular matrix production (Pietrobono et al., 2019).

During carcinogenesis, TGF-β1 can act both as a tumour suppressor or promoter oncogene depending on tumour type and stage (Pietrobono et al., 2019). Several mouse models have also shown that TGF-β1 is also critical for hedgehog-mediated carcinogenesis (Pietrobono et al., 2019).

- NF-κB signalling pathway: NF-κB is responsible for the modulation of the expression of key genes for innate and adaptive immunity, cell proliferation, survival, and lymphoid organ development (Feitelson et al., 2015) (Figure 5). It is activated by pro-inflammatory cytokines, EGF, T- and B-cell mitogens, bacteria, polysaccharides, viruses, viral proteins, double-stranded RNA, and physical and chemical stressors (Feitelson et al., 2015) (Figure 5). Excessive activation of the NF-κB pathway has been documented in various tumour tissues. NF-κB is thus extensively involved in cancer development and progression. It mediates tumour-cell proliferation, survival, and angiogenesis by modulating the expression of target genes, such as TNFα, IL6, BCLXL, BCL2, BCLXS, XIAP, and VEGF (Xia et al., 2018).

In resting cells, complexes formed from NF-KB and IKB generate a shuttle between the cytoplasm and nucleus in a dynamic equilibrium (Xia et al., 2018). When cells are stimulated by extracellular signals such as TNFα, IL1, lipopolysaccharides, viral double-stranded RNA or ionizing radiation, NF-KB is activated and enters the nucleus to bind to target genes (Xia et al., 2018). Furthermore, NF-KB is involved in cellular immunity, inflammation, and stress, as well as the regulation of cell differentiation, proliferation, and apoptosis (Xia et al., 2018). Finally, NF-KB has recently been shown to be activated in cancer stem cells, where it can promote a pro-inflammatory environment, inhibit apoptosis, and stimulate cell proliferation (Hoesel & Schmid, 2013).

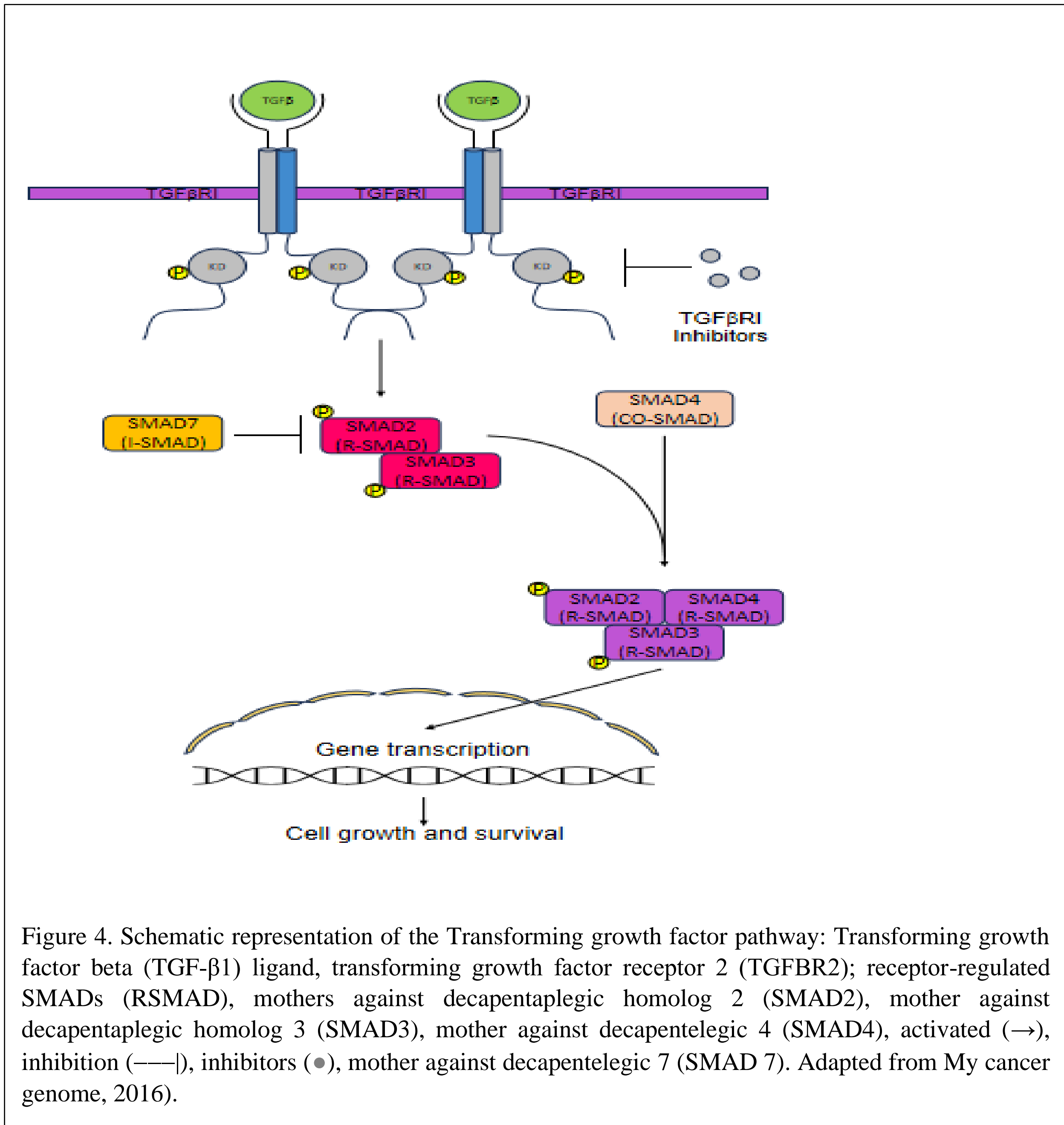

Figure 4. Schematic representation of the Transforming growth factor pathway: Transforming growth factor beta (TGF-β1) ligand, transforming growth factor receptor 2 (TGFBR2); receptor-regulated SMADs (RSMAD), mothers against decapentaplegic homolog 2 (SMAD2), mother against decapentaplegic homolog 3 (SMAD3), mother against decapentelegic 4 (SMAD4), activated (→), inhibition (—–|), inhibitors (●), mother against decapentelegic 7 (SMAD 7). Adapted from My cancer genome, 2016).

- *Insulin-like growth factor (IGF) signalling pathway:* The Insulin-like growth factor (IGF) signalling pathway is a complex and tightly regulated network which is critical for cell proliferation and survival (Iams & Lovly, 2015). This pathway (Figure 6) is composed of three receptor tyrosine kinases (insulin-like growth factor-1 receptor (IGF-1R), insulin-like growth factor-2 receptor (IGF-2R), and insulin receptor (InsR)); three ligands (insulin, IGF-1, and IGF-2); and six serum Insulin-like Growth Factor Binding Proteins (IGFBP's), which serve as

regulators of the pathway. Both IGF-1 and IGF-2 exert their effects through autocrine, paracrine, and endocrine mechanisms, and both can activate IGF-1R signalling (Casa et al., 2008; Iams & Lovly, 2015). IGF-1R is a type-2 tyrosine kinase transmembrane receptor that is normally found as a heterotetramer with two alpha and two beta subunits. IGF-1R binding to IGF-1 or IGF-2 can occur with IGF-1R as a homodimer or as a heterodimer with insulin receptor isoforms A or B (INSR-A, INSR-B). While the heterodimer IGF-1R/INSR can bind insulin, it has been shown to preferentially favour IGF-1-mediated signalling (Casa et al., 2008; Iams & Lovly, 2015).

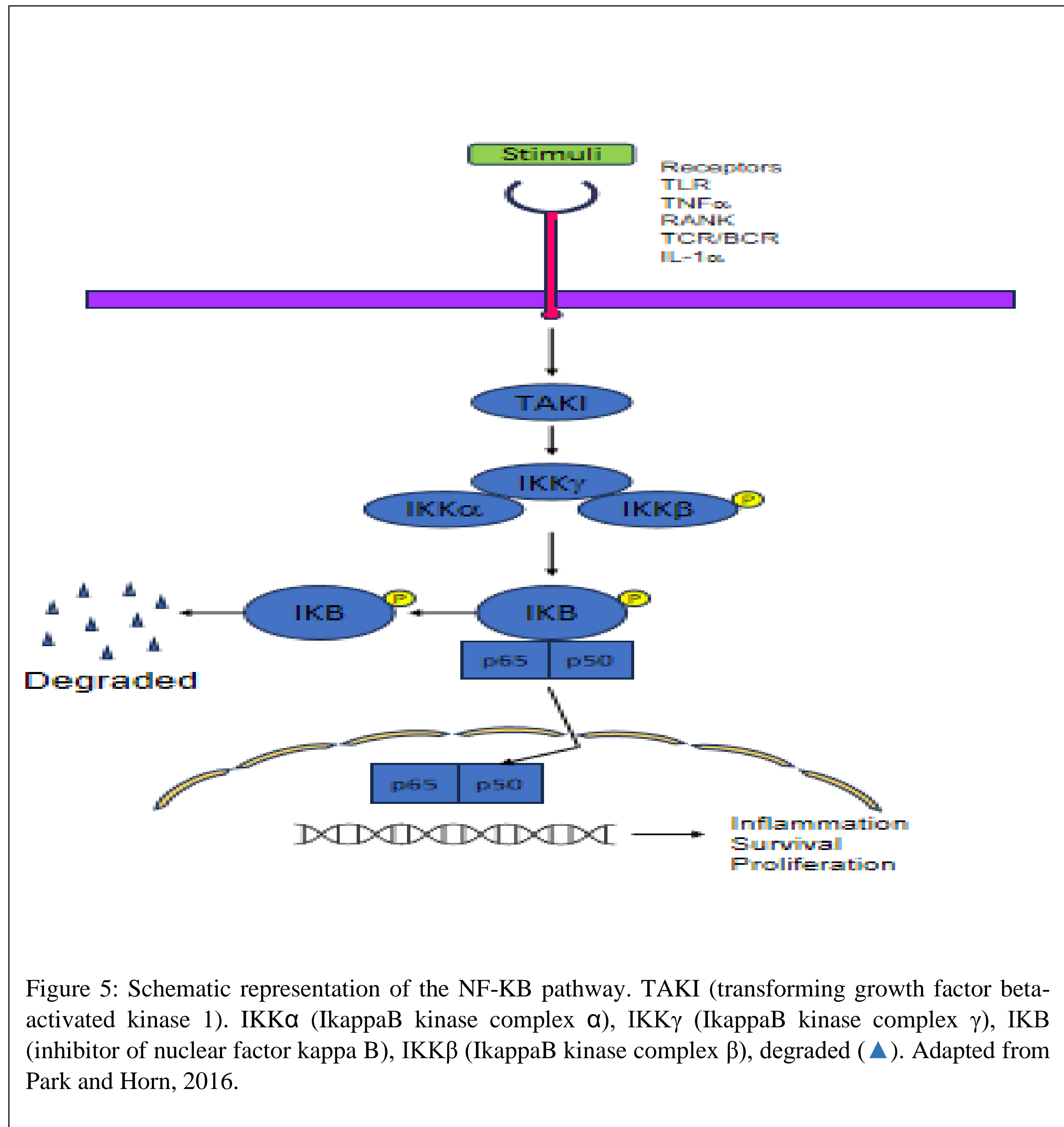

Figure 5: Schematic representation of the NF-KB pathway. TAKI (transforming growth factor beta-activated kinase 1). IKKα (IkappaB kinase complex α), IKKγ (IkappaB kinase complex γ), IKB (inhibitor of nuclear factor kappa B), IKKβ (IkappaB kinase complex β), degraded (▲). Adapted from Park and Horn, 2016.

Insulin-like growth factor (IGF) is produced by hepatocytes, tumour cells, and tumour-associated macrophages (TAM). IGF-1, alongside other growth factors including epidermal growth factor (EGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), stimulates cancer and stromal cell proliferation, migration, survival, and metastasis (Hua et al., 2020). In cancer, once IGF-1 is activated by the ligands (IGF-IR and InsR), it binds to IGF-IR to initiate multiple signalling pathways, which include PI3K/Akt, MAPK, JAK/STAT, Src and focal adhesion kinase (FAK), to regulate cancer cell proliferation, differentiation, survival, EMT, migration, stemness, and promote drug resistance to cancer therapy (Iams & Lovly, 2015; Hua et al., 2020). At the same time, the activation of IGF-IR modulated pathways also inhibits autophagy and anoikis (which is a unique mode of apoptotic cell death that occurs consequentially due to insufficient cell-matrix interactions). Thereby reducing circulating tumour cells in the blood and, subsequently, metastasis (Hua et al., 2020). IGF signalling has significant implications for the treatment and survival of breast cancer patients. This has led many to believe that co-targeting IGF-1R, ER, and HER-2 may circumvent drug resistance (Denduluri et al., 2015). Although it has been noted that the solution may not be so straightforward. This may be because tyrosine-kinases are upregulated in endocrine therapy-resistant breast tumours (Denduluri et al., 2015). Preclinical data in sarcoma tumour models have shown that the IGF-1R pathway is particularly important in tumour growth, metastasis, and angiogenesis in Ewing's sarcoma and rhabdomyosarcoma individuals (Iams & Lovly, 2015).

Aberrant IGF signalling is associated with numerous malignancies, including colon cancer, prostate cancer, pancreatic cancer, melanoma, osteosarcoma, and childhood malignancies, among many others (Denduluri et al., 2015; Hua et al., 2020).

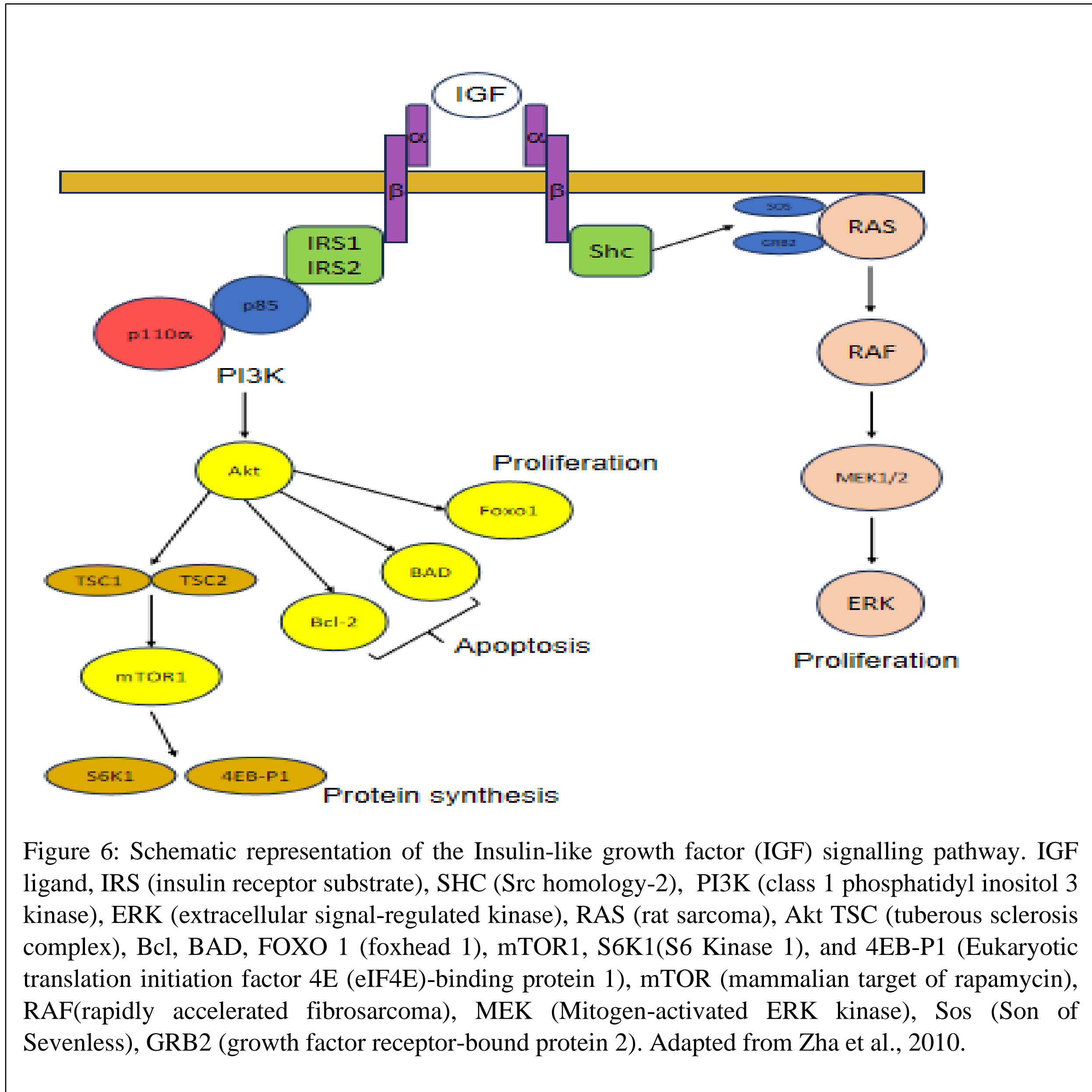

Figure 6: Schematic representation of the Insulin-like growth factor (IGF) signalling pathway. IGF ligand, IRS (insulin receptor substrate), SHC (Src homology-2), PI3K (class 1 phosphatidyl inositol 3 kinase), ERK (extracellular signal-regulated kinase), RAS (rat sarcoma), Akt TSC (tuberous sclerosis complex), Bcl, BAD, FOXO 1 (foxhead 1), mTOR1, S6K1(S6 Kinase 1), and 4EB-P1 (Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1), mTOR (mammalian target of rapamycin), RAF(rapidly accelerated fibrosarcoma), MEK (Mitogen-activated ERK kinase), Sos (Son of Sevenless), GRB2 (growth factor receptor-bound protein 2). Adapted from Zha et al., 2010.

- *PI3K/Akt/mTOR signalling pathway*: The PI3K/Akt/mTOR pathway is a cell-cycle regulating pathway related to nutrient uptake, cell proliferation, and survival in physiological and pathological conditions like cancer (Porta et al., 2014; Pietrobono et al., 2019) (Figure 7).

In breast cancers, aberrations in the PI3K/AKT/mTOR pathway are the most common genomic abnormalities such as PIK3CA gene mutation and the loss-of-function mutations or epigenetic silencing of PTEN. This aberrant activation leads to cancer proliferation, EMT, and resistance to chemotherapy (Feitelson et al., 2015; Pietrobono et al., 2019). Conversely, inhibition of mTOR suppresses EMT and cancer stem cell-like characteristics in colorectal cancer (Feitelson et al., 2015).

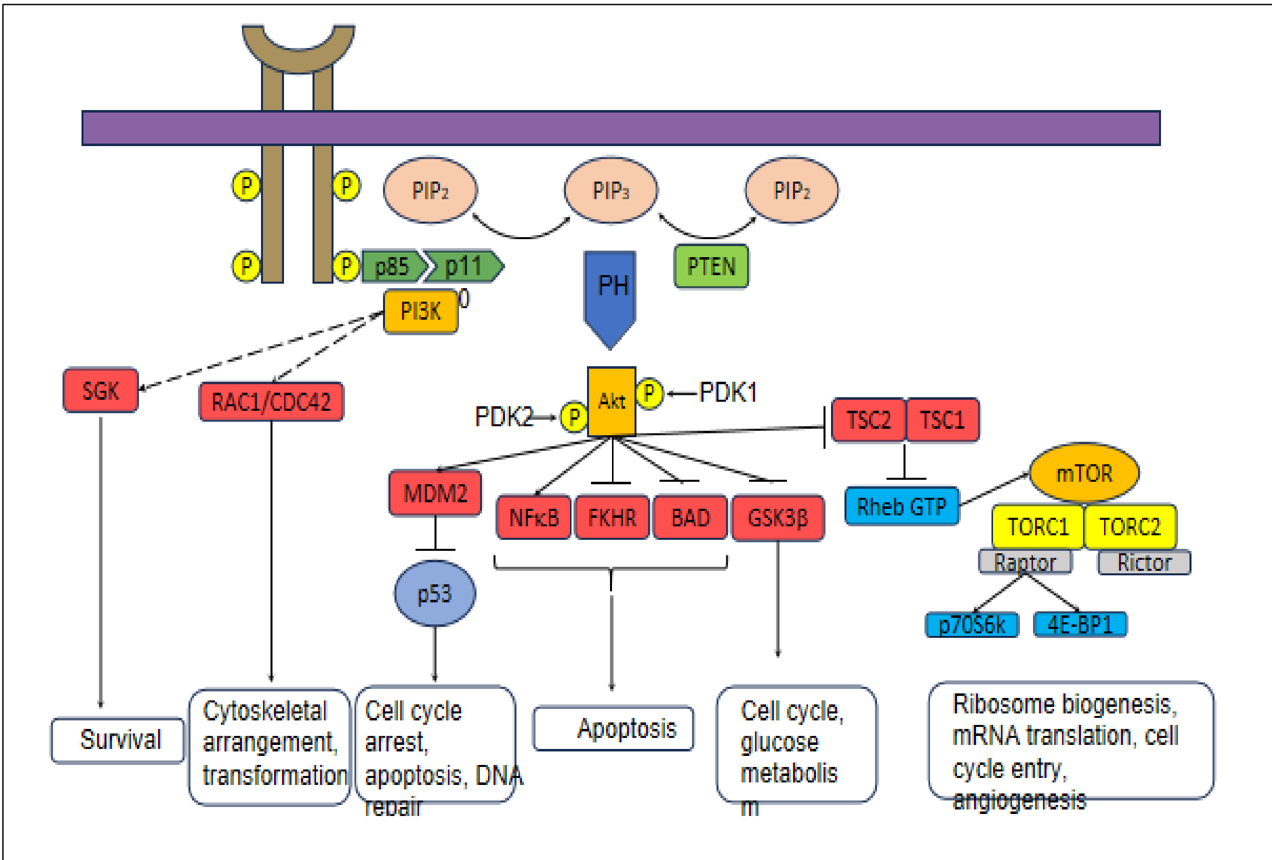

Figure 7: Schematic representation of the PI3K/Akt/mTOR pathway. PI3K (Phosphatidyl-inositol-3-kinase), PIP3(phosphatidylinositol-3,4,5-triphosphate), PIP2(phosphatidylinositol-4,4-isphosphate), PH (plecstrin homology), PDK1 (protein serine/threonine kinase-30 -phosphoinositide-dependent kinase 1), Akt, SGK (serum and glucocorticoid inducible protein kinase), RAC1 (Ras-related C3 botulinum toxin substrate 1), MDMS (murine double minute 2), PTEN(Phosphatase and tensin homolog deleted on chromosome 10) NF-KB, BAD () FKHR (forkhead transcription factor), GSK3β, (Glycogen Synthase Kinase-3), TSC (tuberous sclerosis complex), Rheb GTP, mTOR (mammalian target of rapamycin), 4E-BP1 (Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1). Adapted from Porta et al., 2014.

During cellular stress, the PI3K/Akt pathway occurs by activation of PI3K, which in turn phosphorylates and activates Akt. Akt can have some downstream effects such as activating CREB, inhibiting p27, localizing FOXO in the cytoplasm, activating PtdIns-3ps, and activating mTOR (Porta et al., 2014; Peng et al., 2022). On the other hand, activation of mTOR can influence the transcription of p70 or 4EBP1. The PI3K/AKT pathway can be induced by EGF, sonic hedgehog gene (SHH), IGF-1, insulin, and CaM. Likewise, it is inhibited by various factors including phosphatase and tensin homolog (PTEN), Glycogen synthase kinase-3 beta (GSK3B), and homeobox gene (HB9) (Peng et al., 2022; Porta et al., 2022). In many cancers, this pathway is overactive, thus reducing apoptosis and allowing proliferation (Feitelson et al., 2015; Peng et al., 2019).

OBJECTIVES

Considering the afore-described information, two main objectives were proposed for this PhD thesis dissertation:

1- To evaluate and compare the profile of inflammatory and immune molecules in serum samples from healthy and mammary tumour bitches, followed by the assessment of the putative diagnostic potential of these said molecules as serum biomarkers in canine mammary tumours. To cover this purpose, ninety cluster differentiation (CD) surface markers and 56 cytokines/chemokines were analysed using microarray techniques and further validated by immunoblotting techniques.

2- To determine the gene expression through mRNA quantitation in mammary tissues from healthy and mammary tumour bitches. The target genes chosen for this study included vascular endothelial growth factorA (VEGFA), CD20, progesterone receptor (PGR), hyaluronidase 1 (HYAL1), programmed death ligand 1 (PDL1), epidermal growth factor (EGF), relaxin 2 (RLN2), and matrix metallopeptidase 3 (MMP3).

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Canine Mammary Neoplasia Induces Variations in the Peripheral Blood Levels of CD20, CD45RA, and CD99

Makchit Galadima [1], Iuliia Kotova [2], Ronny Schmidt [2], Josep Pastor [1], Christoph Schröder [2], Joan Enric Rodríguez-Gil [1] and Maria Montserrat Rivera del Alamo [1,*]

1 Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; makchitgaladima@gmail.com (M.G.); josep.pastor@uab.cat (J.P.); juanenrique.rodriguez@uab.cat (J.E.R.-G.)
2 Sciomics GmbH, Karl-Landsteines-Straβe 6, 69151 Neckargemünd, Germany; iuliia90kotova@gmail.com (I.K.); ronny.schmidt@sciomics.de (R.S.); schroeder@sciomics.de (C.S.)
* Correspondence: mariamontserrat.rivera@uab.cat; Tel.: +34-935811045

Abstract: The idea of using tumour biomarkers as diagnostic tools is progressively increasing. Of these, serum biomarkers are of particular interest, as they can provide rapid results. In the present study, serum samples from 26 bitches diagnosed with mammary tumours, plus 4 healthy bitches, were obtained. The samples were analysed using CD antibody microarrays targeting 90 CD surface markers and 56 cytokines/chemokines. A total of five CD proteins, namely CD20, CD45RA, CD53, CD59, and CD99, were selected and further analysed, utilizing immunoblotting techniques to validate the microarray results. CD45RA showed a significantly lower abundance in the serum samples from the bitches carrying mammary neoplasia in comparison to the healthy animals. Regarding CD99, the serum samples from the neoplastic bitches showed it in a significantly higher abundance than those from the healthy patients. Finally, CD20 showed a significantly higher abundance in bitches carrying a malignant mammary tumour in comparison to healthy patients, but no differential expression between malignant and benign tumours was observed. According to these results, both CD99 and CD45RA are indicators of mammary tumour presence, but without distinguishing between malignant and benign.

Keywords: canine mammary tumours; serum biomarkers; CD99; CD45RA; CD20

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1. Introduction

Mammary tumour is a life-threatening condition in both humans and bitches [1–3], although dogs display the highest incidence of mammary tumours among all the mammalian species [1,4,5]. Canine mammary tumour (CMT) is the second most common tumour after skin cancer in dogs [1–3,6], especially in intact bitches [6–10]. About 50% of CMTs are diagnosed as malignant and can metastasize to other organs through the vascular or lymphatics systems [8,11,12]. CMTs are prevalent globally, with a reported incidence rate of 200/100,000 dogs/year, showing lower incidences in countries that practice early spaying [8].

Mammary tumour development, like other neoplasia, is associated with inflammation [13,14], which is a normal quick response to acute tissue damage resulting from physical injury, ischemic injury, toxins, or other types of injury [13,14]. Whatever its origin, inflammation in the tumour microenvironment (TME), mainly chronic, has many cancer-promoting effects on and aids in the proliferation and survival of malignant cells, as well as promotes angiogenesis and metastasis [15]. The TME is composed of innate immune cells (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells), adaptive immune cells (T and B lymphocytes), tumour cells, and their surrounding stroma, which consist of fibroblasts, endothelial cells, pericytes, and mesenchymal cells [13–15]. Immune cells provide diverse molecules to this

TME, such as growth factors, proangiogenic factors, and extracellular-matrix-modifying enzymes, among others [16–18]. Thus, the link between inflammation, mainly chronic, and immune cells in tumorigenesis is evident.

To date, canine mammary diagnoses are usually performed by means of histopathology techniques. However, using tumour biomarkers as diagnostic tools has gained interest [11]. The promising mammary tumour biomarkers in both humans and canines are cancer-associated stroma (CAS) or TMEs, circulating tumour cells and tumour DNA (ctDNA), exosomes and miRNAs, and metabolites [19]. Variations in the expression of these markers may be helpful for achieving an early diagnosis, providing a prognosis, following the course of anti-tumoral therapy, predicting the response or resistance to specific therapies and the surveillant after primary surgery [11,20].

Nevertheless, CMT biomarkers are poorly studied and choosing the most appropriate biomarker remains the biggest challenge in optimizing tumour diagnoses [11,21]. Since early detection is crucial for the evolution of patients with mammary tumours, the determination of these biomarkers is key to evaluating the disease progression and response to treatments [22]. However, tissue biomarkers, although useful, do not provide an improvement in CMT in terms of immediacy, whereas serum biomarkers open up an interesting possibility of improving the celerity of the diagnosis. The literature focused on biomarkers in the peripheral blood is abundant in human research, but it is nearly absent when focusing on CMT.

Against this background, this study aimed at two different goals. The first objective aimed to determine the putative variations in the expressions of both the inflammatory and immune molecules in peripheral blood. On the other hand, it aimed to determine the potential of these molecules as serum biomarkers in canine mammary tumours. For these purposes, serum samples from 30 bitches were analysed through CD antibody microarrays targeting 90 CD surface markers and 56 cytokines/chemokines (Sciomics GmnH, Neckargemünd, Germany). Furthermore, the CD molecules showing differential expressions were validated using immunoblotting techniques.

2. Results

2.1. Animals and Histopathology

Twenty-six patients carrying mammary neoplasia were submitted to the teaching veterinary hospital at the Universitat Autònoma de Barcelona (Bellaterra, Spain) for surgical removal. After performing the surgery, tissue samples were submitted to the laboratory for a histopathology evaluation. The histology analyses yielded a total of 12 bitches with benign mammary tumours, 10 carrying mammary carcinoma, and 4 carrying both mammary carcinoma and adenoma. On the other hand, the 4 healthy bitches were proven, using histopathology, to have normal mammary tissue.

2.2. Microarrays

When comparing the samples from the healthy animals and the samples from the bitches carrying benign mammary tumours, 20 molecules recorded differential protein abundances in terms of either their log FC or p values (Table 1). However, only two proteins showed differential abundances in terms of both the log FC and p-value simultaneously, namely ITAX and CD177.

The proteins with positive log FC values showed a higher abundance in the samples from the bitches with benign mammary tumours, whereas those with negative values showed a higher abundance in the samples from the healthy bitches. $|\log FC| > 1$ and > -1 indicated a differential abundance between the analysed groups. $p < 0.05$ indicated statistically significant differences.

Table 1. Proteins with differential abundance, benign tumours, cancer (n = 12) vs. healthy (n = 4) animal.

Protein	Antibody ID	log FC	AveExp	Adj. *p*-Val	Uniport-Link
CD20	S0288	1.51	13.45	0.270	P11836
ICAM1	S0333	1.25	11.50	0.920	P05362
CD53	S0332	1.23	13.91	0.650	P19397
ICAM1	S0334	1.22	12.28	0.920	P05352
DAF	S0335	1.08	9.49	0.370	P08174
MCP	S0325	1.05	12.60	0.960	P15529
CD20	S0286	−0.96	11.50	0.039	P11836
CD139	S0438	−1.01	14.31	0.950	
PDCD1	S0003	−1.01	12.57	0.960	Q15116
IL1β	S0387	−1.04	14.53	0.950	P01584
IL6	S0530	−1.06	10.01	0.490	P05231
CCL3	S0393	−1.07	14.54	0.920	P10147
IL16	S0529	−1.09	11.88	0.720	Q14005
CD86	S0356	−1.10	10.98	0.960	P42081
TGM2	S0405	−1.14	9.07	0.650	P21980
CD79A	S0354	−1.15	11.66	0.920	P11912
ITAX	S0268	−1.22	11.04	0.036	P20702
KIT	S0001	−1.24	13.51	0.960	P10721
CD24	S0294	−1.33	10.84	0.960	P25063
CD177	S0368	−1.34	10.62	0.039	Q8N6Q3
MPRI	S0369	−1.42	11.61	0.090	P11717
IFNA1	S0402	−2.10	13.94	0.720	P01562

When comparing the samples from the healthy animals and samples from the bitches carrying malignant mammary tumours, 36 molecules recorded differential protein abundances in terms of either their log FC or p values (Table 2). When considering both the log FC and p values, six proteins showed differential abundances in terms of both the log FC and p value simultaneously, namely CD20, CD53, ITAX, FCG3A, CD177, and MPRI.

The proteins with positive log FC values showed a higher abundance in the samples from the bitches with malignant mammary tumours, whereas those with negative values showed a higher abundance in the samples from the healthy bitches. $|\log FC| > 1$ and > -1 indicated differential abundances between the analysed groups. $p < 0.05$ indicated statistically significant differences.

Finally, when comparing the samples from the bitches carrying malignant mammary tumours or benign mammary tumours, 17 molecules recorded differential protein abundances in terms of either their log FC or p values (Table 3). When considering both the log FC and p values, seven proteins showed differential abundances in terms of both the log FC and p value simultaneously, namely PDCD1, IFNA1, CD44, CD20, CD15, IL37, and TNR8.

The proteins with positive log FC values showed a higher abundance in the samples from the bitches with malignant mammary tumours, whereas those with negative values showed a higher abundance in the samples from patients carrying benign mammary tumours. $|\log FC| > 1$ and > -1 indicated differential abundances between the analysed groups. $p < 0.05$ indicated statistically significant differences.

2.3. Immunoblotting

The microarrays analysis yielded an elevated number of differential proteins between the groups of both the CD surface markers and the cytokines/chemokines. Of these, the five most relevant CD molecules were selected for further analyses, utilizing Western blotting for validation, namely CD20, CD45RA, CD53, CD59, and CD99 (Figure 1).

The data obtained from the Western blotting were analysed using two different approaches. On the one hand, the data were analysed by distributing the samples in the healthy and neoplasia groups. On the other hand, the data were distributed in the healthy, benign mammary neoplasia, and malignant mammary neoplasia groups.

Table 2. Proteins with differential abundance, malignant tumours, cancer (n = 14) vs. healthy animal (n = 4).

Protein	Antibody ID	log FC	AveExp	Adj. *p*-Val	Uniport-Link
CD20	S0288	2.57	13.45	0.002	P11836
MCP	S0325	2.05	12.60	0.820	P15529
CD3	S0407	2.05	12.86	0.290	P07766
PD1L1	S002	1.96	12.88	0.820	Q9NZQ7
CD53	S0332	1.92	13.91	0.040	P19397
AMPN	S0378	1.54	13.32	0.710	P15144
ICAM1	S0.334	1.47	12.28	0.480	P05362
CD44	S0424	1.43	14.10	0.190	P16070
CD22	S0292	1.40	11.35	0.420	P20273
CD99	S0360	1.31	11.75	0.430	P14209
CD8A	S0254	1.15	12.13	0.480	P01732
CD44	S0315	1.12	12.40	0.520	P16070
EGLN	S0363	1.02	11.72	0.820	P17813
CD20	S0286	−0.87	11.50	0.049	P11836
CEAM6	S0503	−1.00	12.45	0.630	P40199
IL7	S0388	−1.01	13.42	0.910	P13232
IL16	S0529	−1.02	11.88	0.470	Q14005
CEAM1	S050	−1.02	12.76	0.480	P13688
IL8	S0389	−1.08	11.41	0.190	P10145
LFA3	S0340	−1.10	12.29	0.910	P19256
CEAM5	S0348	−1.12	12.35	0.500	P06731
IL8	S0475	−1.15	10.33	0.420	P10145
ITAX	S0268	−1.16	11.04	0.019	020702
CD24	S0294	−1.16	10.84	0.820	P25063
NTF4	S0397	−1.22	13.88	0490	P34130
CCL7	S0394	−1.22	13.59	0.710	P80098
FCG3A	S0277	−1.23	10.50	0.011	P08637
CD177	S0368	−1.24	10.62	0.044	Q8N6Q3
CD53	S0331	−1.35	12.03	0.250	P19397
TGM2	S0405	−1.36	9.07	0.230	P21980
CCL11	S0382	−1.37	14.23	0.370	P51671
IL15	S0391	−1.39	14.48	0.290	P40933
CD139	S0438	−1.42	14.31	0.480	
IL1b	S0387	−1.43	14.53	0.480	P01584
BDNF	S0381	−1.50	14.15	0.400	P23560
IL18	S0392	−1.52	14.34	0.290	Q14116
MPRI	S069	−1.65	11.61	0.017	P11717
CCL3	S0393	−1.75	14.54	0.230	P10147
CD86	S0356	−2.12	10.98	0.390	P42081

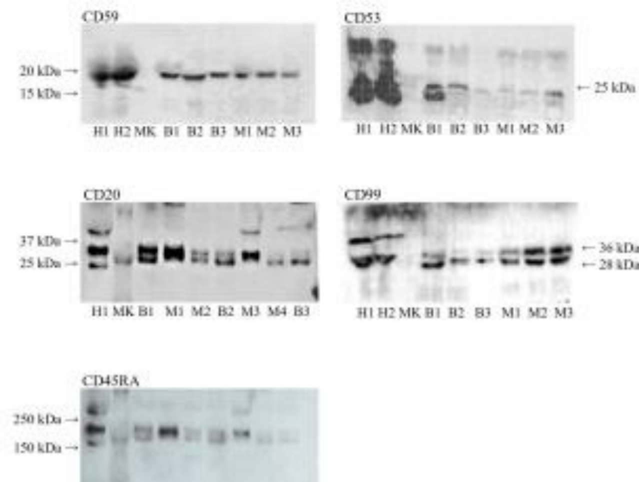

Figure 1. Western blotting images. H: healthy bitches; B: bitches carrying benign mammary tumours; M: bitches carrying malignant mammary tumours; and MK: molecular weight marker.

Table 3. Proteins with differential abundance, malignant (n = 14) vs. benign tumours (n = 12).

Protein	Antibody ID	log FC	AveExp	Adj. *p*-Val	Uniport-Link
PDCD1	S0003	1.82	12.57	0.012	Q15116
IFNA1	S0402	1.72	13.94	0.008	P01562
CD44	S0315	1.31	12.40	0.007	P16070
KIT	S0001	1.24	13.51	0.370	P10721
CD3	S0407	1.11	12.86	0.170	P07766
PD1L1	S0002	1.08	12.88	0.550	Q9NZQ7
CD20	S0288	1.07	13.45	0.011	P11836
MCP	S0325	1.00	12.60	0.640	P15529
IL37	S0473	−0.42	10.27	0.012	Q9NHZ6
PTPRC	S0323	−068	11.29	0.012	P08575
CD38	S0308	−0.78	10.15	0.011	P28907
IL8	S0475	−0.80	10.33	0.021	P10145
NCAM1	S0337	−0.94	11.60	0.013	P13591
CD86	S0356	−1.03	10.98	0.200	P42081
CD15	S0273	−1.04	11.41	0.012	
LFA3	S0340	−1.04	12.29	0.550	P19256
IL37	S0474	−1.11	11.36	0.008	Q9NZH6
TNR8	S0302	−1.13	12.34	0.021	P28908

When comparing the healthy and neoplastic samples, CD99 showed a significant overexpression in the neoplastic samples (Figure 2D), whereas CD45RA showed significantly lower values in the serum from the bitches with mammary neoplasia (Figure 2D).

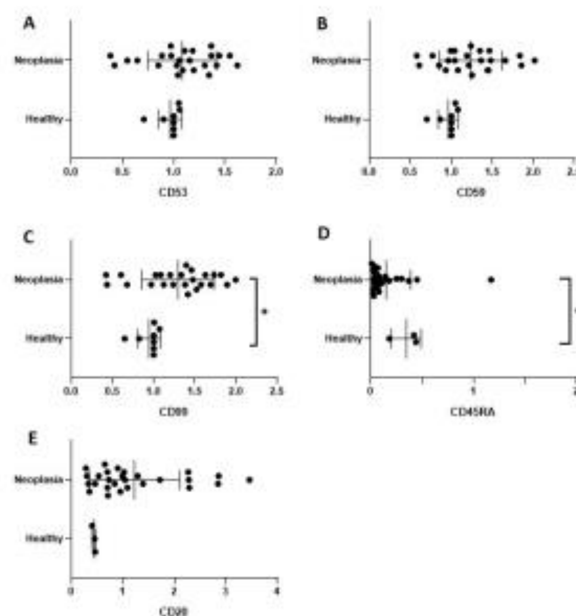

Figure 2. Comparative expression of peripheral blood levels for CD53 (**A**), CD59 (**B**), CD99 (**C**), CD45RA (**D**), and CD20 (**E**) between healthy (n = 4) and mammary neoplasia (n = 26) bitches. Results are expressed in terms of mean ± SD from two immunoblotting replicates. Statistically significant ($p < 0.05$) differences are marked with asterisks.

When comparing the healthy, benign, and malignant samples, CD99 showed a significant overexpression in the neoplastic samples, regardless of if they were benign or malignant, in comparison to the healthy samples (Figure 3D). In contrast, only the carcinoma samples showed a significant overexpression of CD20 (Figure 3E) in comparison to

the healthy bitches. However, no significant difference between the benign and malignant samples for CD20 expression was observed.

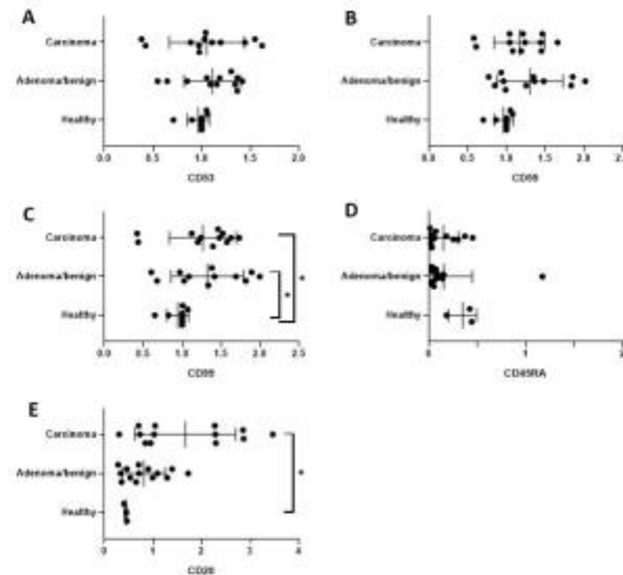

Figure 3. Comparative expression of peripheral blood levels for CD53 (**A**), CD59 (**B**), CD99 (**C**), CD45RA (**D**), and CD20 (**E**) among healthy (n = 4), benign (n = 12), and malignant mammary (n = 14) neoplasia bitches. Results are expressed in terms of mean ± SD from two immunoblotting replicates. Statistically significant ($p < 0.05$) differences are marked with asterisks.

3. Discussion

The present study demonstrated that the presence of mammary neoplasia in bitches induces modifications in the abundance of the cluster of differentiation (CD) 45RA, CD99, and CD20 in the peripheral serum. CD45RA and CD99 were under- and overexpressed in the peripheral serum from the bitches carrying CMTs, respectively, in comparison to the healthy bitches. When comparing benign mammary neoplasia, malignant mammary neoplasia, and healthy animals, CD20 showed a significant increase in the serum from the bitches with malignant CMTs in comparison to the healthy patients, but no differences were observed between malignant and benign CMTs.

CDs are cell surface molecules that provide targets for immunophenotyping [23] and are expressed in leukocytes. CD molecules are involved in diverse biological processes such as cell-to-cell communication and the stimulation of the immune response in front of foreign agents [24]. Focusing on the field of neoplasia, CD molecules have been described to be expressed in neoplasia tissue and have thus been proposed as good diagnosis and prognosis markers in both tissue and peripheral blood samples of neoplasia (see [24] for a review).

Specifically centring on the mammary gland, the presence of CD molecules has been described in both the healthy and neoplastic mammary tissues of women. In this sense, the ductal cellular layer of the mammary gland is composed of a relevant population of immune cells, which include $CD8^+$ and $CD4^+$ T cells [25,26]. Regarding mammary neoplasia tissue, several studies have evaluated the expression of CD molecules in human breast cancer (see [24,27] for reviews), but the literature on CMT is much less abundant [28–30].

In this respect, CD45 is a cell surface glycoprotein family, with CD45RA being one of its numerous isoforms [31–33]. Monocytes and macrophages labelled with this molecule

are known to infiltrate tumour microenvironments [34,35]. Mammary tumours being infiltrated with CD45 have been previously described, although scarcely, in mice, also showing an increased tissue infiltration [36]. Thus, there is a clear correlation between CD45 and tumorigenesis. As stated above, CD45RA was under-expressed in the peripheral blood of the bitches carrying CMTs. A feasible hypothesis for this could be the fact that $CD45^{+}$ cells were displaced to the mammary tumour, maybe in response to the inflammatory reaction occurring in the tumoral area. In fact, the migration of tumorigenic molecules from the peripheral blood to tumorigenic tissue has been already described in the literature on ovarian neoplasia [37]. Therefore, it seems logical to think that $CD45^{+}$ labelled mono-macrophages may migrate from the peripheral blood to tumour tissue. This hypothesis would be reinforced by the fact that $CD45^{+}$ cells have been related to tumoral vasculogenesis [38]. However, further research is needed to confirm this hypothesis, since CD45 expression has not yet been evaluated in CMT.

CD99 is a surface glycoprotein which is expressed in endothelial and haematopoietic lineage cells, among others [39]. In inflammatory processes, this molecule regulates the adhesion and transendothelial migration of haematopoietic cells [40,41]. Its expression has been demonstrated in several types of neoplasia, including mammary tumours in women [42], being suggestive of an increased invasiveness [43], tumour migration [44], and malignancy [45]. Nevertheless, the role of CD99 in tumour development, growth, migration, and metastasis is still under discussion, as it is considered to be both an oncogenic and tumour suppressor, depending on the type of neoplasia (see [39] for a review). In this sense, under-regulation has been associated with tumour progression in osteosarcoma and gastric cancer [46], whereas the over-expression of CD99 has been associated with a higher migration, tumour growth, and metastasis in Ewing's sarcoma [47,48], given that the present results are in agreement with the latter.

Specifically focusing on the peripheral blood, the overexpression of this molecule has been described in neoplasia such as Ewing's sarcoma [49,50]. According to the literature, this is the first time that the peripheral blood levels of CD99 have been analysed for mammary tumours in any species. Remarkably, the overexpression of CD99 in the peripheral blood, together with the low levels of CD45 ($CD99^{+}CD45^{-}$), has been related to a very poor prognosis in children experiencing Ewing's sarcoma [50]. However, this is not applicable in the present study, since our results suggest that $CD99^{+}CD45^{-}$ expression is associated with the presence of CMT, but do not provide a differential diagnosis between malignant and benign CMT. Thus, it cannot be related to tumour progression or metastasis.

CD99 is also related to inflammation (see above). During an inflammatory response, the affected tissue releases pro-inflammatory mediators, which, in turn, induce local changes in the endothelium that will be translated into leukocyte extravasation [51]. This leukocyte extravasation is facilitated by CD99, among other molecules [41]. The association between tumorigenesis and inflammation is well known, and mammary tumours are not an exception. Thus, this increased expression of CD99 in the serum samples from the bitches with CMTs may be related to tumorigenic inflammation.

CD20 is a surface protein expressed in B cells during their development [52]. It is thought to be involved in the control of cell growth by means of regulating calcium influx [53]. In situ, CD20 is overexpressed in ductal carcinoma in women [27] and a correlation between tissue and peripheral expression has also been found in breast cancer [54]. However, CD20 is poorly described in CMT, being described only in canine primary breast lymphoma as an unexpected finding [55], with no literature focusing on the peripheral levels of this molecule. The role of CD20 in breast cancer is controversial. While some studies have affirmed an association between $CD20^{+}$ B-cells and a favourable prognosis in invasive breast cancer [56], others have observed an association with a poor prognosis [57] and reduced disease-free survival [58].

Circulating levels of CD20 have been associated with better prognoses in women with breast cancer [59]. In the present study, no such conclusion can be reached, since the values were higher in the bitches with malignant mammary tumours, but these values were

significantly higher only in comparison to the healthy bitches. Benign mammary tumours yielded a lower mean value in comparison to malignant mammary tumours, but this difference was not significant, probably due to the high dispersion of the data in the latter group. When analysing the results individually, it can be observed that half of the samples yielded low values for the peripheral CD20 and half of the samples yielded considerably high values. We can only hypothesize to figure out the reason for this data dispersion. As stated above, CD20 has yielded controversial results in women's breast cancer, which is probably associated with the different types of mammary neoplasia. It seems logical to think that this could be also a feasible explanation in CMT. No information other than the histology classification was considered. Thus, maybe considering other parameters, such as the presence/absence of metastasis, invasiveness of the tumour, and presence/absence of more than one tumour of the same/different type in the patient, among others, would yield a more accurate result.

Chronic inflammation, in tight cooperation with the immune system, has long been associated with cancer development, including, of course, breast cancer. Chronic inflammation enhances angiogenesis and tissue invasion, which will promote cellular proliferation and cancer progression [15]. On the other hand, it releases carcinogenic molecules [60] that recruit immune and regulatory cells [61]. Immune cells are components of the normal mammary tissue (see above). The appearance of a tumorigenic process provokes modifications in both the qualitative and quantitative compositions of these immune cells in the mammary tissue [62,63]. The present results reinforce the hypothesis that the immune system, in cooperation with inflammation, is involved in CMT.

Although much research has been performed to date, mammary neoplasia is still surrounded by many unanswered questions. Many endogenous factors, some of them associated with chronic inflammation and the immune system, are involved in the development, growth, and migration of these tumours in an intertwining way that is not completely elucidated at all. As more studies are performed, more questions arise, so further research is needed to improve the understanding of mammary tumours, and, in turn, improve the current diagnostic, prognostic, and treatment tools.

One of the objectives of the present study was to evaluate CD surface molecules and cytokines/chemokines as potential CMT biomarkers. Our results allow for the establishment of the presence of new players in this macro-game, but do not allow for the establishment of new diagnostic biomarkers, making further research mandatory.

4. Materials and Methods

4.1. Animals and Sampling

A total of 30 bitches were included in the study. Twenty-six of them were referred to the Fundació Hospital Clinic Veterinari at the Autonomous University of Barcelona (UAB, Bellaterra, Spain) for mammary tumour resections, while 4 bitches referred for elective ovariohysterectomies were used as a control group. The ages of the females with mammary tumours ranged from 7 to 14 years (mean age: 9.72 years), whereas those of the control bitches ranged from 3 to 5 years (mean age: 3.75 years). The samples were always obtained under the signed consent of the owner and followed the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). The specimens were obtained following the guidelines of the Ethical Committee of Animal Care and Research of the UAB (protocol CEEAH number 1127, 20 March 2012).

Before surgery, 3–5 mL of blood was collected through a venipuncture of the jugular vein, deposited into a tube without anticoagulant, and allowed to clot at room temperature. Thereafter, it was centrifuged at $2000\times g$ for 10 min and the serum was aspirated with a Pasteur pipette into Eppendorf tubes. The blood samples were stored at -80° until they were needed for the microarray and immunoblotting techniques.

After surgical resection, the mammary tumours were fixed with 10% paraformaldehyde (Sigma-Aldrich, Barcelona, Spain) and submitted to the laboratory for the histological

diagnosis and typification of the mammary tumours. In the control group, a 1 cm × 1 cm specimen was obtained from the mammary gland of each bitch and further submitted for a histology examination to check for the absence of mammary tumours.

4.2. Microarrays Technique

First, the protein concentrations were determined using a Bicinchoninic Acid (BCA) assay with a BCA commercial kit (Thermo Fisher Scientific, Dreieich, Germany), following the supplier's instructions. Briefly, the serum samples were labelled at an adjusted protein concentration for one hour with scioDye 1 and scioDye 2. Then, the reaction was stopped by adding hydroxylamine. The excess of dye was removed 30 min later and the buffer was exchanged for 1x phosphate-buffered saline (PBS).

Immediately, the samples were analysed in a dual-colour approach using a reference-based design on 30 scioCD antibody microarrays (Sciomics GmbH, Neckargemünd, Germany). These microarrays specifically targeted for 93 different CD surface markers and 25 cytokines/chemokines with 256 monoclonal antibodies (Table A1). Each antibody was represented on the array in four replicates. The arrays were blocked with scioBlock (Sciomics GmbH, Neckargemünd, Germany) on a Hybstation 4800 (Tecan, Austria) and the samples were then incubated competitively using a dual-colour approach. After incubation for three hours, the slides were thoroughly washed with 1x PBSTT, rinsed with 0.1x PBS, as well as with water, and subsequently dried with nitrogen.

Slide scanning was conducted using a Powerscanner (Tecan, Austria) with identical instrument laser power and adjusted PMT settings. Spot segmentation was performed with GenePix Pro 6.0 (Molecular Devices, Union City, CA, USA). Once the microarray technique was applied, the proteins yielding differentiation values among the different types of samples were validated by means of immunoblotting techniques.

4.3. Immunoblotting

First, the protein concentrations of the serum samples were determined through the Bradford method [64], using the commercial kit Bio-Rad Protein Dye Reagent (BioRad, Hercules, CA, USA). Afterwards, the samples were diluted at a final concentration of 1 mg/mL with a homogenization buffer (TRIS HCl 50 mM, EDTA 1 mM, EGTA 10 mM, DTT 25 mM, 1.50% Triton x100, PMSF 1 mM, leupeptin 10 µg/mL, orthovanadate 1 mM, and benzamidine 1 mM). A total of 10 mg of protein from each sample was loaded.

The immunoblotting protocol was performed according to Sirois and Dore [65]. Briefly, the proteins were separated by SDS-PAGE and, after the electrophoresis, transferred using a Trans-Blot Turbo Transfer System (BioRad, El Prat del Llobregat, Spain). The transferred membranes were then probed against the different primary antibodies. Then, the detection was performed by using the corresponding secondary antibody. Afterwards, the membranes were incubated with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, INC, Heidelberg, Germany) for 5 min and further exposed to a radiograph film.

All the sera were assessed using immunoblotting and two replicates for each sample were performed. The densitometric analysis included the duplicated values obtained from each sample.

4.4. Primary Antibodies

Anti-human CD20, anti-human CD45RA, anti-human CD53, anti-human/-mouse CD59, and anti-human CD99 antibodies were used in this experiment as the primary antibodies. They were purchased from ImmunoTools (Friesoythe, Germany). The membranes were incubated overnight at 4 °C, with each corresponding antibody at a 1:1000 (v/v) dilution.

4.5. Secondary Antibodies

After exposure to the primary antibodies, the membranes were incubated with a polyclonal rabbit anti-mouse secondary antibody at a 1:500 (v/v) dilution. The secondary antibody was purchased from Dako (Glostrup, Denmark).

4.6. Image Analysis

The slide scanning of the microarrays was performed utilizing Powerscanner (Tecan, Austria) equipment with identical instrument laser power and adjusted PMT settings. The spot segmentation was performed with Gene Pix Pro 6.0 (Molecular Devices, Union City, CA, USA).

The quantification of the proteins on the X-ray film from the immunoblotting was performed through the ImageJ software (URL: https://imagej.en.softonic.com/?ex=DINS-635.2; accessed on 2 December 2022).

4.7. Statistical Analysis

The acquired raw data from the microarrays technique were analysed using the linear models for the microarray data (LIMMA) package of R-Bioconductor after uploading the median signal intensities. A specialised invariant Lowess method was applied for the normalisation. For the analysis of the samples, a one-factorial linear model was fitted with LIMMA, resulting in a two-sided t-test or F-test based on the moderated statistics.

All the presented p values were adjusted for multiple testing by controlling the false discovery rate according to Benjamini and Hochberg [66]. The proteins were defined as differential for a $|\log FC| > 1$ and a p value < 0.05. The differences in the protein abundances between the different samples or sample groups were presented as log-fold changes (log FC) calculated for the basis 2. In a study comparing samples versus controls, a $\log FC = 1$ means that the sample group has, on average, a $2^1 = 2$-fold higher signal than the control group. $\log FC = -1$ stands for $2^{-1} = 1/2$ of the signal in the sample, as compared to the control group.

The raw data obtained from the immunoblotting technique were analysed by applying a Shapiro–Wilk test, which was used to study the normality distribution. When a parameter did not follow a normal distribution, a non-parametric test was used to assess the differences between the groups. The Kruskal–Wallis test was used when more than two variables were compared with multiple comparison tests and the Mann–Whitney test when two variables were compared. The correlations between the parameters were studied using Pearson or Spearman correlation coefficients, according to their distribution. The statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, CA, USA, (URL: www.graphpad.com).

5. Conclusions

Canine mammary tumours induce variations in the peripheral concentrations of CD20, CD45RA, and CD99. However, these differences are only significant between animals carrying mammary tumours and healthy bitches, thus not allowing for a differentiation between malignant and benign CMT. Further research is warranted to determine the novel serum biomarkers that allow for more accurate and celeritous diagnoses.

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Data Availability Statement: Data are available at the authors' request.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. List of evaluated proteins by means of microarrays.

ANCA	CD45RO	CYTL
BDNF	CD46	Eotaxin-1
CD1a	CD47	GDC
CD2	CD48	GM-CSF
CD3	CD49d	HCD137
CD4	CD50	HLA
CD5	CD52	HLA-ABC
CD6	CD53	HLA-DP
CD7	CD54	HLA-DR
CD8	CD55	HLA-I
CD9	CD56	IFN
CD10	CD57	IFN alpha
CD11a	CD58	IFNg
CD11b	CD59	IFNy
CD11c	CD61	IgE
CD13	CD62L	IL-1
CD14	CD62p	IL-10
CD15	CD63	IL-12B
CD16	CD66e	IL-12p70
CD17	CD69	IL-13
CD18	CD71	IL-15
CD19	CD72	IL-16
CD20	CD79a	IL-18
CD21	CD80	IL-1a
CD22	CD86	IL-1b
CD23	CD95	IL-37
CD24	CD97	IL-4
CD25	CD98	IL-6
CD27	CD99	IL-7
CD28	CD99R	IL-8
CD29	CD105	MICP-2
CD30	CD106	MCP-E
CD31	CD116	MIP-1a
CD33	CD117	MIP-4
CD34	CD123	MPO
CD35	CD139	NT-4
CD36	CD147	NTAL
CD37	CD162	P53
CD38	CD177	P72Syk
CD40	CD222	Pan
CD41	CD235a	PD1
CD41a	CD235ab	PDL1
CD41b	CDw131	RANTES
CD42b	CEACAM1	TNFa
CD43	CEACAM3	TRAIL
CD44	CEACAM5	TSLP
CD45	CEACAM6	TSLPR
CD45RA	CEACAM8	tTG
CD45RB	COX1	CYTL

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Selected gene expression in canine mammary neoplasia

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Article

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Makchit Galadima [1], Mariana Teles [2,*], Josep Pastor [1], Javier Hernández-Losa[3], Joan Enric Rodríguez-Gil [1], and Maria Montserrat Rivera del Alamo [1]

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[1] Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; makchitgaladima@gmail.com (M.G.); josep.pastor@uab.cat (J.P.); juanenrique.rodriguez@uab.cat (J.E.R.G.); mariamontserrat.rivera@uab.cat (M.M.R.A.)
[2] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain; mariana.teles@uab.cat (M.T.)
[3] Department of Pathology, Hospital Universitari Vall d'Hebron, VHIR, 08035 Barcelona; Spain; Javier.hernandez@vallhebron.cat (J.H.L.)

* Correspondence: mariana.teles@uab.cat (M.T.)

Abstract: Canine mammary tumours (CMTs) are the most frequent cancers in intact bitches, being early diagnosis as the main milestone for a good prognosis. Genes expression has been suggested as a putative tool for prognosis and diagnosis. In the present study, 58 paraffined canine mammary tumours from 27 different bitches were included. Thirty-seven tumours were classified as benign, whereas 31 were classified as different types of canine carcinoma. In addition, mammary samples from 3 healthy bitches were also included. The gene expression for vascular endothelial growth factor-α (*VEGFα*), *CD20*, progesterone receptor (*PGR*), hyaluronidase-1 (*HYAL-1*), programmed death-ligand 1 (*PD-L1*), epidermal growth factor (*EGF*), relaxin (*RLN2*) and matrix metalloproteinase-3 (*MMP3*) was assessed through RT-qPCR. All the assessed genes yielded a higher expression in neoplastic mammary tissue compared to healthy tissue. VEGF-α, CD20, PGR and HYAL-1 were over-expressed in CMTs, showing no significant differences between benign and malignant tumours. PD-L1, EGF, relaxin and MMP3 were also overexpressed in both benign and malignant CMTs, however, malignant CMTs showed a statistically significantly higher expression when compared with benign CMTs. The present study concludes that PD-L1, EGF, relaxin and MMP3 may be useful as malignancy biomarkers in CMTs.

Keywords: canine mammary tumour; gene expression; malignancy biomarkers; programmed death-ligand 1; epiderl growth factor; relaxin; matrix metalloprotease-3

1. Introduction

Early diagnosis of tumours, especially those of malignant characteristics, is one of the main milestones in current cancer therapy strategies. Thus, early diagnosis allows the implementation of a precocious treatment and, consequently, the improvement of the prognosis for the patient. Nowadays, the study of tumour-linked gene expression patterns has gained relevance as a useful tool in the diagnosis and prognosis, and especially the treatment approach, of canine mammary tumours (CMTs) [1].

Reverse transcriptase quantitative real-time PCR (RT-qPCR) is the most commonly used method for quantifying mRNA expression since it is sensitive, specific, fast, easy to use, and reproducible. Therefore, RT-qPCR is considered an important tool for transcriptomic analysis in a variety of studies, including those carried out with samples from *Canis lupus familiaris* mammary tumours [2,3]. However, accurate normalization of data is required to reduce errors arising from variations in the quantity and integrity of the RNA template and the efficiency of complementary DNA (cDNA)

synthesis. In addition, it is known that the transcript levels of reference genes can differ with the experimental conditions [4]. Therefore, the choice of the correct reference gene/s to be used in data normalization is a crucial step when carrying out relative gene expression studies. In the present study, the stability of four potential reference genes was evaluated to be used in the normalization of the target genes. These genes included glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), ribosomal protein S19 (*RPS19*), and ribosomal protein L8 (*RPL8*). The choice of the reference genes evaluated in the present study was made taking into account previous studies carried out with samples from mammary tumours from dogs [5,6].

The objective of this study was to assess the gene expression in CMTs of hyaluronidase-1 (*HYAL-1*), *CD20*, matrix metallopeptidase 3 (*MMP3*), vascular endothelial growth factor-α (*VEGFα*), relaxin 2 (*RLN2*), programmed death-ligand 1 (*PD-L1*), epidermal growth factor (*EGF*), and progesterone receptor (*PGR*). These genes were selected because of their importance in the signalling pathways of mammary tumours according to the literature.

2. Results

2.1. Tumours typification

The histology typification of the 58 tumours included in the study yielded a total of 37 benign CMTs and 21 were classified in different categories of malignant tumours (Table 1). Likewise, healthy samples were also confirmed by histology evaluation.

Table 1. Histological classification of the mammary tumours included in the present study.

Malignant (n=21)		**Benign (n=38)**	
Typification	n	Typification	n
Complex adenocarcinoma	2	Adenoma	36
Complex carcinoma	8	Hemangioma	1
Solid carcinoma	1		
Anaplastic carcinoma	2		
Adenosquamous carcinoma	1		
Tubullo-papillary carcinoma	6		
Intraductal-papillary carcinoma	1		

2.2. Estimation of gene efficiency

The efficiency of each gene was estimated by a standard curve generated by using serial dilutions of a pool of cDNA samples. The correlation coefficients were highly linear, and the PCR amplification efficiencies were close to 100 % for all the primers (Table 2). From the four reference genes evaluated to be used in the normalization of data, the *HPRT* gene was

eliminated from the evaluation of best reference genes since, according to CFX Maestro™ Software data, HPRT expression was unstable and therefore it cannot be used as a reference gene. A ranking according to the expression stability of each gene in a given sample set and experimental design is performed with both algorithms. According to NormFinder, stability values for candidate reference genes were: 0.064 for *GADPH*, 0.041 for *RPL8* and 0.028 for *RPS19*. The latter was then considered the most stable reference gene according to the NormFinder algorithm. According to the reference gene selection tool (CFX Maestro™ Software, BioRad), the stability values for candidate reference genes were 1.07 for *GADPH*, 0.57 for *RPL8* and for *RPS19*. Therefore, the combination *RPL8*+*RPS19* was used for RT data normalization.

Table 2. Amplification efficiency of primers in samples from Canis lupus familiaris. *E: Efficiency. The amplification efficiency of each primer was calculated according to the equation E=10(1/-slope).

Gene	Slope	R^2	E*	E (%)
GADPH	-3.25	0.99	2.03	103
HPRT	-3.11	0.99	2.05	105
RPL8	-3.27	0.94	2.02	102
RPS19	-3.25	0.99	2.03	103
HYAL1	-3.16	0.99	2.07	107
MMP3	-3.21	0.99	2.05	105
RLN2	-3.29	0.99	2.01	101
VEGFA	-3.20	0.94	2.05	105
PGR	-3.25	0.99	2.03	103
CD20	-3.29	0.98	2.02	102
PD-L1	-2.87	0.98	2.23	123
EGF	-3.05	0.96	2.13	113

GADPH: Glyceraldehyde-3-phosphate dehydrogenase; HPRT: Hypoxanthine guanine phosphoribosyltransferase; RPL8: Ribosomal protein L8; RPS19: Ribosomal protein S19; HYAL1: Hyaluronidase 1; MMP3: Matrix metallopeptidase 3; RLN2: Relaxin 2; VEGFα: Vascular endothelial growth factor α; PGR: Progesterone receptor; PD-L1: programmed death-ligand 1; EGF: epidermal growth factor.

Concerning the expression levels of the target genes evaluated in the present study, *HYAL1*, *VEGFα*, *CD20* and *PGR* genes presented a similar pattern of response, showing significantly higher levels of expression both in benign and malignant tumours when compared to the control group. However, no statistically significant difference in the expression levels was observed when comparing both types of tumours, benign and malignant. Concerning the mRNA levels of *PDL-1*, *EGF*, *RLN2* and *MMP3*, a similar pattern of response was also depicted. Expression in both benign and malignant tumours was significantly up-regulated when compared to the control group. Moreover, malignant tumours presented significantly higher expression levels when compared to benign tumours (Figure 1).

2.3. Correlation between genes

Several correlations between genes were observed. Tables 3 and 4 show the correlation between genes in benign and malignant CMTs respectively. In this sense, gene expression for *PGR* was positively correlated with *CD20*, *EGF*, *RLN* and *MMP3* gene expression in both benign and malignant CMTs. Gene expression for *EGF* was positively correlated to *RLN2* and *MMP3* in both benign and malignant CMTs. Finally, gene expression for *RLN2* was positively correlated with *CD20* and *MMP3* in both benign and malignant CMTs. Focusing on benign CMTs, gene expression for *VEGF*α was negatively correlated with relaxin and *MMP3*. Gene expression for *EGF* was positively correlated to *CD20* and *HYAL1* expression. Regarding malignant CMTs, gene expression for *CD20* was positively correlated to *MMP3* gene expression. Finally, gene expression for *PD-L1* was negatively correlated with *HYAL1* and *EGF* expression.

Following the evaluation of the linear correlation, a principal components analysis (PCA) was performed. Table 5 shows the relative weighting for the first three components. The first principal component accounted for 80.9% to 88.4% of the total variance. The contributing molecules were *CD20*, *PGR*, *EGF*, *RLN2* and *MMP3*. In the second component, two molecules overweighted, namely *HYAL1* and *PD-L1* with 71.6% and -81.2% respectively. Finally, in the third component, only *VEGF*α was over-represented, being responsible for 86.3% of the total variance. Figure 2 shows the spatial expression of the different components analysed.

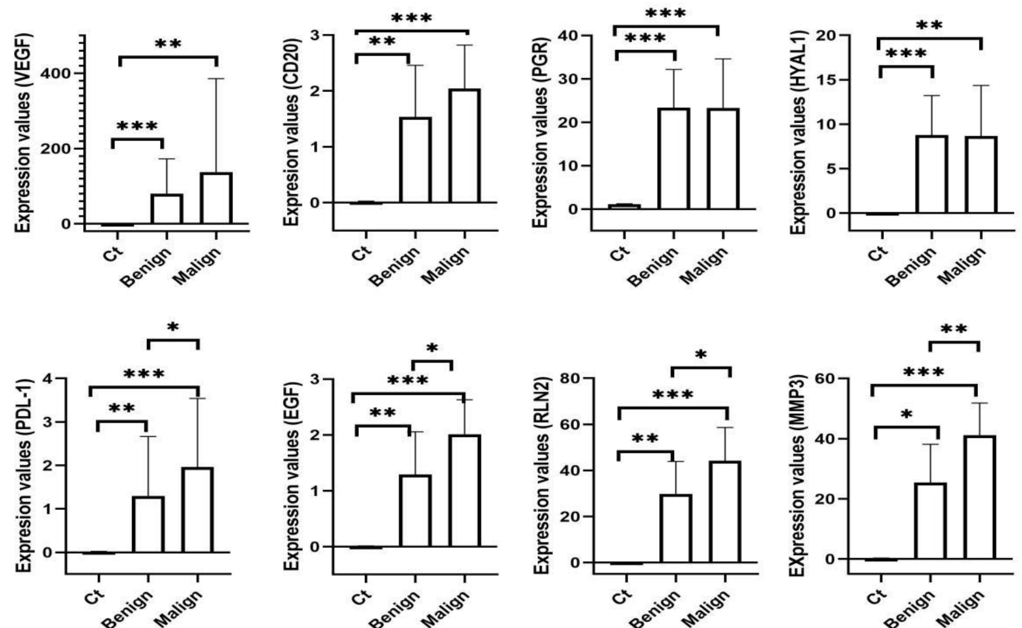

Figure 1. Relative mRNA levels of target genes measured in samples obtained from healthy mammary tissue and mammary tumours. Bars represent the mean value with standard deviation. Statistical significances between groups are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p<0.001$). VEGFα: Vascular endothelial growth factor α; PGR:

Progesterone receptor; HYAL1: Hyaluronidase 1; PD-L1: Programmed death-ligand 1; EGF: Epidermal growth factor; RLN2: Relaxin 2; MMP3: Matrix metallopeptidase 3.

Table 3. Coefficients of linear correlation between gene expressions in benign CMTs.

	VEGF-α	*CD20*	*PGR*	*HYAL1*	*PD-L1*	*EGF*	*RLN2*	*MMP3*
VEGFα	….							
CD20		….						
PGR		0.558**	….					
HYAL1				….				
PD-L1					….			
EGF		0.790***	0.502**	0.418*		….		
RLN2	-0.467**	0.546**	0.435*			0.718***	….	
MMP3	-0.546**		0.464*			0.500**	0.507**	….

Statistically significant correlations between gene expression are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p<0.001$). VEGFα: Vascular endothelial growth factor α; PGR: Progesterone receptor; HYAL1: Hyaluronidase 1; PD-L1: Programmed death-ligand 1; EGF: Epidermal growth factor; RLN2: Relaxin 2; MMP3: Matrix metallopeptidase 3.

Table 4. Coefficients of linear correlation between gene expressions in malignant CMTs.

	VEGF-α	*CD20*	*PGR*	*HYAL1*	*PD-L1*	*EGF*	*RLN2*	*MMP3*
VEGFα	….							
CD20		….						
PGR		0.850***	….					
HYAL1				….				
PD-L1				-0.725**	….			
EGF			0.702***		-0.566*	….		
RLN2		0.725**	0.754***			0.547*	….	
MMP3		0.654**	0.718**			0.662*	0.718**	….

Statistically significant correlations between gene expression are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p<0.001$). VEGFα: Vascular endothelial growth factor α; PGR: Progesterone receptor; HYAL1: Hyaluronidase 1; PD-L1: Programmed death-ligand 1; EGF: Epidermal growth factor; RLN2: Relaxin 2; MMP3: Matrix metallopeptidase 3.

Table 5. Principal components matrix of gene expressions in CMTs.

	Component		
	1	2	3
VEGFα	-0.292	0.295	0.863

CD20	0.838	0.136	-0.132
PGR	0.809	0.047	0.096
HYAL1	0.334	0.716	-0.397
PD-L1	0.296	-0.812	-0.046
EGF	0.825	0.111	0.278
RLN2	0.884	-0.152	0.151
MMP3	-0.292	0.295	0.863

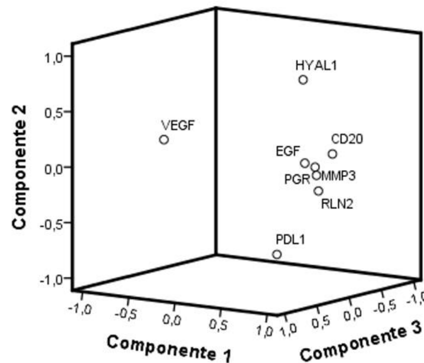

Figure 2. Spatial expression of the different components analysed.

3. Discussion

The present results highlight the differential gene expression of canine mammary neoplasia compared to healthy mammary tissue. The genes evaluated in this study may be classified into two different categories, those related to the presence of mammary neoplasia (namely VEGFα, CD20, PGR and HYAL1) and those indicative of malignancy (namely PDL-1, EGF, RLN2 and MMP3).

Neovascularization is mandatory in tumorigenesis, tumour growth and dissemination [7]. Neovascularization is regulated, among other angiogenic factors, by the vascular endothelial growth factor (VEGF) [see 8 for a review]. Thus, the role of VEGF in tumorigenesis is more than expected. VEGF expression has been widely demonstrated to be directly correlated with tumour growth in breast cancer [9-13]. Focusing on canine mammary tumours, the expression of VEGF has been assessed in both serum and tissue samples. VEGF is over-expressed in bitches with malignant CMTs in comparison with healthy bitches [14-17]. According to the literature, malignant CMTs also over-express VEGFα when compared with benign CMTs [14]. However, the present study shows no statistical difference in the expression of VEGFα between benign and malignant CMTs. A feasible explanation for this difference is the different applied techniques. Thus, in

the present study, VEGFα expression was assessed through RT-qPCR, whereas in the previous study, VEGFα expression was evaluated using immunohistochemistry. The higher expression of VEGFα in both benign and malignant CMTs clearly suggests that this molecule is involved in the neovascularization processes of CMTs regardless of their benign or malignant nature.

CD20 has been scarcely described in CMTs. According to the authors' knowledge, CD20 expression has been described only in one case of canine primary mammary lymphoma [18], with no other research performed in this field. Peripheral levels of CD20 have been previously assessed and compared among healthy bitches, bitches carrying a malignant CMT and bitches carrying a benign CMT by this research group [19]. In that previous study, higher levels of serum CD20 were observed in bitches with CMTs regardless the neoplasia was benign or malignant, with no significant difference among them. These results agree with the present study, where tissue over-expression of CD20 was observed in CMTs in comparison with healthy tissue, with no significant difference between benign and malignant neoplasia. CD20 has been studied in human breast cancer, but its role is not clear. Whereas some studies suggest that CD20 is associated with a good prognosis in human breast cancer [20], others associate it with a bad prognosis [21,22]. In the present study, no such affirmation can be done since most of the patients included did not provide any further feedback after the surgery. Focusing on the present results, it seems clear that CD20 plays a role in canine mammary tumorigenesis, but it is not helpful as a diagnostic biomarker.

The role of ovarian hormones on CMTs, oestrogen and progesterone, is well-known and described in the literature [23], as well as their respective receptors. Regarding progesterone receptor (PGR), the literature describes its presence in mammary tissue as a favourable prognostic indicator [24,25]. The expression of PGR has been assessed in healthy and neoplastic canine mammary tissue through different techniques, always yielding similar results. According to the literature, PGR is expressed by all benign CMTs and approximately two-thirds of malignant MCTs [26-28]. However, the present results disagree with those previously reported. In this study, the overall expression of PGR did not differ between benign and malignant CMTs. A feasible explanation is the fact that, in the present study, malignant CMTs were not classified according to the degree of malignancy due to the low number of samples. According to the literature, simple carcinomas show a higher expression of PGR in comparison with complex carcinomas [28], the lack of differentiation between simple and complex carcinomas in our study would dilute these possible differences.

Hyaluronidases are enzymes which degrade hyaluronic acid, the major constituent of the extracellular matrix (ECM) [29]. The degradation of ECM is a requirement for tumour development, so a putative role of hyaluronidases in tumour development is expected. The gene expression of the hyaluronidase isoform HYAL-1 has been scarcely described in CMTs and the results are controversial. Thus, whereas Varallo et al. [30] have described a higher expression of HYAL-1 in canine mammary carcinoma when compared with healthy tissue, Sakalauskaite et al. [31] observed no difference between carcinoma and healthy adjacent tissue, with the exception of

German Sheperd bitches, which showed a higher expression of HYAL-1 in the adjacent tissue. According to the authors' knowledge, this is the first study that compares the expression of HYAL-1 among healthy mammary tissue, and benign and malignant mammary tumours in bitches. The present results partially agree with those previously obtained by Varallo et al. [30] since malignant CMTs showed a higher expression of HYAL-1 when compared with healthy mammary tissue. However, no significant difference was observed between benign and malignant CMTs. The present results clearly suggest that HYAL-1 may be involved in mammary tumorigenesis, but it is not useful as a malignancy biomarker, being its action probably related to the modification of the ECM. HYAL-1 degrades hyaluronic acid, generating small fragments of hyaluronic acid which are angiogenic [32-34]. *In vitro* studies have also demonstrated the contribution of HYAL-1 in tumour cell proliferation, motility, invasion, tumour growth, metastasis and angiogenesis in breast, bladder, prostate and colon cancer [35-40].

PD-L1 (programmed death-ligand 1) is a transmembrane protein which is expressed on the surface of activated cytotoxic T cells. This molecule inhibits the production of IL-2, and the migration and proliferation of T cells [41]. It also activates the PD-1 protein localized on the surface of T cells, which would lead to immune tolerance [42]. When engaged, the PD-1/PD-L1 complex induces the dysfunction, exhaustion and neutralization of TILs [43]. TILs are known to be recruited to the tumour microenvironment with the aim of killing tumoural cells. Consequently, the overexpression of PD-L1 is considered a protective mechanism developed by neoplasia [44]. The studies assessing its possible role in mammary cancer in women have yielded discordant results. Thus, some studies have observed that this molecule is commonly expressed in triple-negative breast cancers (TNBC) [45] and its higher expression is related to a significantly decreased survival and higher tumour grade [46], whereas a more recent study has shown that only 10% of TNBC are positive to PD-L1 [47].

Focusing on CMTs, the expression of PD-L1 has been scarcely studied. It has been described to be expressed in approximately two-thirds of both benign and malignant CMTs through immunohistochemistry techniques [48]. Likewise, the gene overexpression of PD-L1 has been correlated to metastatic malignant CMTs and shorter survival periods [49]. According to the authors' knowledge, this is the first time that gene expression of PD-L1 is compared between benign and malignant CMTs. In the present study, PDL-1 was significantly over-expressed in CMTs compared to healthy mammary tissue, with malignant CMTs showing the highest tissue expression. These results are suggestive of the putative potential of PD-L1 as a malignancy biomarker of CMTs.

Epidermal growth factor (EGF) is a protein which is involved in the proliferation and differentiation of epithelial cells [50] in addition to angiogenesis, metastasis and apoptosis inhibition [51-52]. The binding of EGF with its receptor (EGFR) induces changes in gene transcription that enhance tumorigenesis [53]. Likewise, EGF overexpression has been associated with tumour invasion and progression [54-56]. Focusing on CMTs, i*n vitro* studies have demonstrated that EGF enhances proliferation, chemotaxis and the production of VEGF, and decreases apoptosis in canine carcinomas [57]. However, *in vivo* studies on EGF have yielded controversial results. Thus,

whereas Klopfleish et al. [58] observed a down-regulation in the gene expression of EGF in metastatic canine mammary carcinomas, Queiroga et al. [59] reported higher concentrations of EGF in neoplastic mammary tissues by enzyme-immunoassay determinations. In the present study, CMTS showed significantly higher expression of EGF than healthy mammary tissue. As stated above, EGF is involved in tumorigenesis. Thus, overexpression was somehow expected in neoplastic tissue. On the other hand, malignant CMTs showed significantly higher expression than benign CMTs, making EGF a potential biomarker for malignancy.

Relaxin is a peptide hormone that, among other functions, promotes both the growth and development of the mammary gland, which are mandatory events to ensure lactation after delivery [60,61]. A not-so-well-known role of this hormone is the one played in mammary neoplasia. In this regard, relaxin enhances the invasiveness of breast cancer *in vitro* [62] and higher serum levels of relaxin have been related to poor prognosis of breast cancer in women [63]. Both serum and tissue expressions have been assessed also in CMTs. However, the results are contradictory. Thus, while some studies [64,65] have observed no correlation between relaxin expression and tumour malignancy, some others have found that relaxin is over-expressed in malignant III-grade CMTs [5]. In the present study, CMTs showed significantly higher expression of RLN2 in comparison with healthy canine mammary tissue. Additionally, malignant CMTs significantly overexpressed RLN2 when compared to benign CMTs. These results demonstrate the potential of RLN2 as a malignancy biomarker in CMTs.

MMPs are proteases that play a crucial role in many biological processes such as the remodelling process of the extracellular matrix, cell proliferation, migration and differentiation, and tissue invasion and vascularization [see 66 for a review]. These biological processes must be accurately balanced. Otherwise, they may cause pathological conditions, including cancer and tumour progression [66]. In fact, MMP members are known to play a relevant role in cancer progression since they facilitate the invasion and metastasis of the original tumour by dissolving the basement membrane and degrading the extracellular matrix [62].

MMPs have been described to be present in the mammary tissue and linked to the extensive remodelling that the mammary gland undergoes from the pre-puberal to the adult stage, and during pregnancy and lactation [see 67 for a review]. MMP3, also known as stromelysin-1, has been described to play a relevant role in the development of the mammary gland, specifically in the lateral branching of ducts during the phases of mid-puberty and early pregnancy [68]. This enzyme has been reported to be involved in angiogenesis, cell growth and cell invasion [69]. These specific features have warranted further research of MMP3's role in tumourigenesis, being this topic beyond the aims of the present manuscript. Regarding breast cancer, MMP3 has been described to be over-expressed in neoplastic mammary tissue [69], to be involved in both mammary cancer invasion and metastasis [70-73] and has also been considered a prognostic factor [74,75].

Focusing on CMT, and according to the consulted literature, over-expression of MMP3 has been described in serum [76] and tissue [65] samples from bitches with mammary carcinoma. The present results confirm that MMP3 plays a role in CMT development since it is over-expressed in both

benign and malignant mammary tumours. On the other hand, the significantly higher expression in malignant CMTs suggests that MMP3 may be involved in further metastatic processes and could be used as a prognostic parameter, agreeing with those findings observed in women. Unfortunately, it was not possible to acquire the follow-up of the patients included in this study, making it impossible to establish a correlation between survival and MMP3 expression. Thus, further research on this molecule is warranted.

Statistical correlations among the different evaluated gene expressions were also assessed. Molecules such as PGR, CD20, EGF, relaxin, and MMP3 showed significant correlations between them in both benign and malignant CMTs. These correlations suggest that they might be involved in the process of mammary tumorigenesis, but not necessarily in the process of malignity. Focusing on correlations in benign CMTs, it is worth mentioning the negative correlation of VEGFα with relaxin and MMP3. As indicated above, neovascularization is needed for tumorigenesis, and tumour growth and dissemination [7]. This is especially relevant to malignant tumours, which greatly depend on the neovascularization to metastasize [77]. Thus, a negative correlation is not surprising in benign CMTs.

Regarding malignant CMTs, a significant negative correlation of PD-L1 with HYAL1 and EGF was also observed. Since PD-L1 has been described to act as a protective mechanism developed by neoplasia [44], this negative correlation is surprising and no feasible explanation can be provided, warranting more research.

Focusing on the PCA, the relevant elements of component 1 are the same molecules which already showed a significant correlation in the previous linear analysis, namely CD20, PGR, EGF, relaxin and MMP3. These results suggest that the combined analysis of these markers, further highlighted by the PCA analysis, may have differential diagnostic potential in CMTs between normal and neoplastic tissue.

The assessment of gene expression in CMTs is also interesting for personalized therapeutic approaches. Some of the evaluated molecules have been suggested or are already being used as therapeutic targets for human cancer. One of these potential targets is PD-L1. Previous studies in human cancer have shown that the blockade of PD-L1 with monoclonal antibodies enhances the destruction of cytotoxic T-lymphocyte-mediated tumour cells and improves the activation of antigen presentation and cytokine release, suggesting a putative potential as a therapeutic target [78,79]. Similar results have been observed in *in vitro* studies in canine B cell lymphoma [2,80] and oral melanoma [81]. Since PD-L1 is significantly over-expressed in malignant CMTs, it seems logical to hypothesize that the blockade of PD-L1 may be a potential therapeutic target for CMTs.

EGF has been a targeted molecule in breast cancer in women since 1998 (HER2), when the first anti-HER2 antibody, trastuzumab, was approved [82]. Several drugs have been further developed since then and are commercially available to treat human mammary cancer [see 83 for a review]. The implementation of anti-HER2 antibodies as targeted therapies has improved the outcome of breast cancer patients (Jacobs et al., 2022). Therefore, the implementation of these drugs in CMTs may also be beneficial.

VEGFα and MMP3 are also promising molecules as therapeutic targets. These molecules are involved in neovascularization, angiogenesis, cell

growth and invasion [8,69]. These biological processes are fundamental for tumour growth and dissemination, thus their blockade may prevent the expansion of the tumour.

Another molecule that warrants further research is PGR. Recent studies have demonstrated that the administration of aglepristone, a progesterone receptor antagonist, inhibits the proliferation index in PGR-positive canine carcinomas [84] and increases the disease-free period [85].

Relaxin has been tested as a co-adjuvant drug in the treatment of cancer, but not as a targeted molecule itself for inhibiting tumour growth. This protein increases the expression and catalytic activity of some MMPs [86-89], which ultimately would facilitate the degradation of the tumour stroma and, consequently, increase the porosity of the tumour to oncolytic drugs [90,91]. In this sense, a previous study [92] has demonstrated that the intratumoral transplantation of tumour cells containing the relaxin gene or haematopoietic cells containing relaxing has an antitumour effect in tumour-bearing mice (Li et al., 2009).

Finally, HYAL-1 has been shown to be involved in several biological processes related to tumorigenesis and metastasis such as cell, growth, migration, invasion and angiogenesis in breast cancer [93,94]. thus, the blockade of this molecule could arrest these processes and, consequently, be used as a therapeutic target CMTs.

4. Materials and Methods

4.1. Animals and sampling

In the present study, a total of 30 bitches referred to the Teaching Veterinary Hospital (Fundació Hospital Clínic Veterinari) at Universitat Autònoma de Barcelona (UAB) and diagnosed with mammary tumours were included. Patients were aged between 6 and 13 years (mean age: 10.3 years) and belonged to different dog breeds. All females were subjected to surgery to remove the mammary tumours. Once removed, the masses were fixed with 10% paraformaldehyde (Sigma-Aldrich, Barcelona, Spain) and submitted to the laboratory for tumour typification. Samples were further embedded in paraffin and four to five 10μm-sections from each paraffined tumour were obtained for RNA extraction.

A total of 58 mammary samples were submitted for histology purposes. In addition, healthy mammary tissue from 3 bitches was also included as a control group. Specimens were obtained according to the guidelines of the Ethical Committee of Animal Care and Research of the UAB (protocol CEEAH number 1127, 20 March 2012).

4.2. Extraction of RNA and synthesis of complementary DNA

Only paraffin-conserved samples with more than 80% of tumour samples confirmed by histology were used. After deparaffinization of 10 μm paraffin-embedded tissue samples, RNA was extracted using the High Pure FFPET RNA Isolation Kit (Roche). Cells were lysed in 100 μL RNA Tissue Lysis Buffer, 16 μL 10% SDS and 40 μL of Proteinase K for 30 min at 85 ºC and shaking at 600 rpm. After this step, 60 μL of Proteinase K was added and

the tissue samples were incubated at 55 ºC and shaken at 600 rpm for 30 min. After cell lysis, the samples were incubated for 15 min at 15 to 25 ºC with the DNAase working solution to enhance the RNA extraction. After this, the samples were centrifuged several times to eliminate debris and, finally, 50 μL of RNA elution buffer was added to obtain the pure total RNA. RNA quantification (ng/μL) was done using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using 1 μg of the total RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The iScript cDNA synthesis kit is a sensitive and easy-to-use first-strand cDNA synthesis kit for gene expression analysis using real-time qPCR.

4.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

The set of genes studied includes indicators of malignancy (HYAL1, MMP3 and RLN2) and indicators of the hormonal response (PGR). Primers' information is given in Table 6. The efficiency of amplification was tested for each primer pair as follows: 5-fold serial dilutions of the cDNA pool were analysed, and E = 10 (− 1/s) was used as the formula for efficiency, where s is the slope generated by the serial dilutions. RT-qPCR was performed in a Bio-Rad CFX384 real-time PCR detection system (Bio-Rad Laboratories). Reactions were done using iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Briefly, 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 60 °C (or 55°C for HYAL1 and VEGFA) for 30 s, and 72 °C for 30 s were run. Expression data, obtained from two independent biological replicates, were used to calculate the threshold cycle (Ct) value.

Table 6. Sequences and efficiencies of primers used for quantitative real-time PCR analysis in samples from *Canis lupus familiaris*.

Gene name	Category	Accession numbers	Forward primer	Reverse primer
GADPH	Housekeeping	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT
HPRT	Housekeeping	AY_283372	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC
RPL8	Housekeeping	XM_532360.2	CCATGAATCCTGTGGAGC	GTAGAGGGTTTGCCGATG
RPS19	Housekeeping	XM_533657.3	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG
HYAL1	Malignity	XM_005632508.4	CACAGGGAAGGGACAGATGT	TGTTCTCCCAGCTTACCCAG
MMP3	Malignity	NM_001002967.1	CCTAGCGCTCTGATGTACCC	GGACTGGATGCCATTCACAT
RLN2	Malignity	NM_001003132.1	TGCTAGGTGTCTGGCTGCTACTAA	CGGACATAATCACGACCACATG
VEGFα	Malignity	NM_001003175.2	GTGCCCACTGAGGAGTTCAAC	CCCTATGTGCTGGCCTTGAT
PGR	Hormonal	NM_001003074.1	CGAGTCATTACCTCAGAAGATTTGTTT	CTTCCATTGCCCTTTTAAAGAAGA

CD20	Malignity	AB210085.1	AACTTTGCAGAACCTCCCCAG	AAGCTGTGAACACTAACGCCT
EGF	Malignity	NM_001003094.3	GTTGAAAGTTCGAGCCCCCT	CAGTTCCAGTTCTCCGGGTC
PD-L1	Malignity	AB898678.1	CCGCCAGCAGGTCACTT	TCCATTGTCACATTGCCACC

GADPH: Glyceraldehyde-3-phosphate dehydrogenase; HPRT: Hypoxanthine guanine phosphoribosyltransferase; RPL8: Ribosomal protein L8; RPS19: Ribosomal protein S19; HYAL1: Hyaluronidase 1; MMP3: Matrix metallopeptidase 3; RLN2: Relaxin 2; VEGFα: Vascular endothelial growth factor α; PGR: Progesterone receptor.

4.4. Standardization strategy

NormFinder and the algorithm that comes with the CFX Maestro™ Software (Reference Gene Selection Tool, Bio-Rad Laboratories) were used to identify the most appropriate reference gene or combination of reference genes among four: GAPDH, HPRT, RPL8, and RPS19. Since the CFX Maestro™ Software was used for the calculations, we used the selection of the best combination references genes defined by the Bio-Rad software. Therefore, the expression levels of the target genes were normalized using the best combination of two reference genes and relative gene expression calculated with the ΔΔCt method using the CFX Maestro™ Software.

4.5. Statistical analysis

Shapiro-Wilk test was used to study the normality distribution between variables because some or all of the variables did not follow a normal distribution ($p<0.005$). A non-parametric Kruskal Wallis test with Dunn's multiple comparison test was used to study if there were statistically significant differences between groups. Spearman coefficient of correlation were studied between genes. Statistically significant differences were when p values were < 0.05. All statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. IBM SPSS statistics version 22 (IBM, USA) was used to study principal component analysis.

5. Conclusions

The gene expression of *VEGFα*, *CD20*, *PGR* and *HYAL1* is overexpressed in both benign and malignant CMTs, thus not allowing to differentiate between them. These molecules are probably related to the tumorigenic process, but not to the malignancy process. Gene expression for *PDL-1*, *EGF*, *RLN2* and *MMP3* was also statistically overexpressed in both benign and malignant CMTs. However, malignant CMTs showed a statistically significant overexpression in comparison to benign tumours, suggesting that these molecules may be useful as malignancy biomarkers in CMTs. Finally, the present results indicate that no combination of parameters provides a 100% specificity to differentiate between malignant and benign CMTs. However, the combination of PGR, CD20, EGF, relaxin, and MMP3 provides a specificity slightly above 80%.

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DISCUSSION

In the present study, serum levels and gene expression of potential biomarkers in the diagnosis and prognosis of CMTs have been assessed. Serum biomarkers are the focus of current research to improve the early detection of different cancers in dogs. Furthermore, gene expression of different cancers can improve the accuracy of the diagnosis, thus enhancing the prognosis by identifying possible therapeutic targets.

The results obtained in this PhD Thesis, presented in two manuscripts, have several implications for the clinical diagnosis and prognosis of canine mammary tumours. The results revealed that the presence of mammary tumours regulates the abundance of CD20, CD45RA, and CD99 in the peripheral blood (Manuscript 1). Similarly, CMTs influence the expression of genes such as *VEGF*α, *CD20*, *PGR*, *HYAL-1*, *PD-L1*, *EGF*, *RLN2* and *MMP3* in the mammary tissue. (Manuscript 2).

In studies 1 and 2, CD20 was assessed in the serum and tissues respectively from healthy and mammary tumour bitches. Both results showed significant over-expression of CD20 in the serum and neoplastic tissues of bitches with CMT in comparison with healthy bitches. Similar expression was evident in women with ductal carcinoma in situ (Goff and Danforth, 2020). In addition, a correlation between tissue and peripheral expression has also been found in breast cancer (Hamed et al., 2022). So far, CD20 is poorly described in CMT, being reported only in canine primary breast lymphoma as an unexpected finding (Rismanchi et al., 2015) with no literature focusing on the peripheral levels of this molecule. However, the role of CD20 in breast cancer is inconclusive. While some studies have affirmed an association between CD20+ B-cells and a favourable prognosis in invasive breast cancer (Schmidt et al., 2008) others have observed an association with a poor prognosis (Olkhanud et al., 2011) and reduced disease-free survival time (Schnellhardt et al., 2020). Nonetheless, blood levels of CD20 have been associated with better prognoses in breast cancer (Jia et al., 2014). Unfortunately, such a conclusion cannot be reached in CMTs. Although yielding a lower mean value in comparison to malignant mammary tumours, no significant differences between benign and malignant CMTs were observed, probably due to the high dispersion of the data in the latter group. When analysing the results individually, it can be observed that half of the samples yielded low values for the peripheral CD20, and half of the

samples yielded considerably high values. We can only hypothesize to figure out the reason for this data dispersion. As stated above, CD20 has yielded controversial results in human breast cancer, which is probably associated with the different types of mammary neoplasia. It seems logical to think that this could be also a feasible explanation in CMT. Regarding neoplastic mammary tissues, no information other than the histology classification was considered. Thus, maybe considering other parameters, such as the presence/absence of metastasis, invasiveness of the tumour, and presence/absence of more than one tumour of the same/different type in the bitch, among others, would yield a more accurate result about CD20. Similarly, in study 2, no such affirmation can be done since most of the patients included did not provide any further feedback after the surgery. Focusing on the present results, it seems clear that CD20 plays a role in canine mammary tumourigenesis, but it is not helpful as a diagnostic biomarker.

CD45, another molecule that was assessed, was significantly under-expressed in the peripheral blood of the bitches with CMTs when compared with healthy bitches. Little information is available on CD45 expression, although it has been demonstrated to be under-expressed in breast cancer patients (Raiter et al., 2021), being the present results in agreement. A feasible hypothesis for this CD45 under-expression could be the fact that CD45+ cells from peripheral blood were displaced to the mammary tumour tissue, maybe in response to the inflammatory reaction occurring in the tumoural area. In fact, this migration of tumourigenic molecules from the peripheral blood to tumourigenic tissue has been already described in the literature on ovarian neoplasia (Negus et al., 1997). Therefore, it seems logical to think that CD45+ labelled mono-macrophages may migrate from the peripheral blood to tumour tissue. This hypothesis would be reinforced by the fact that CD45+ cells have been related to tumoural vasculogenesis (Ma et al., 2016). However, further research is needed to confirm this hypothesis, since CD45 expression has not yet been evaluated in CMT.

Another fascinating component of the results of the studies conducted in PhD thesis was the over-expression of CD99 in bitches with CMT when compared with the healthy bitches. CD99 has been demonstrated in several types of neoplasia, including human breast cancer (Milanezi et al., 2001), being suggestive of increased invasiveness (Kim et al., 2000), tumour migration (Lee et al., 2002), and malignancy (Baccar et al., 2013). Nevertheless, the role of CD99 in tumour development, growth, migration, and metastasis is still inconclusive, since it has been proposed as a tumour

promoter and suppressor depending on the type of neoplasia (Pasello et al., 2018). In this sense, under-regulation has been associated with tumour progression in osteosarcoma and gastric cancer (Manara et al., 2018), whereas the over-expression of CD99 has been associated with a higher migration, tumour growth, and metastasis in Ewing's sarcoma (Kreppel et al., 2006; Rochhi et al., 2010), given that the present results are in agreement with the latter. Specifically focusing on the peripheral blood, CD99 has been over-expressed in Ewing's sarcoma (Benini et al., 2018; Zakzok et al., 2022). According to the literature, this is the first time that the peripheral blood levels of CD99 have been analysed for mammary tumours in any species. Remarkably, the over-expression of CD99, together with the under-expression of CD45 in the peripheral blood, has been related to a very poor prognosis in Ewing's sarcoma (Zakzok et al., 2022). However, this is not applicable in the present study, since our results suggest that CD99+CD45− expression is associated with the presence of CMT, but do not provide a differential diagnosis between malignant and benign CMT. Thus, it cannot be related to tumour progression or metastasis.

Focusing on the study of CMTs gene expression, all the evaluated genes showed significant over-expression in comparison to healthy canine mammary tissue. Thus, VEGFα, CD20, PGR and HYAL-1 showed over-expression in both benign and malignant CMTs with no difference between the type of tumour. On the other hand, PD-L1, EGF, RLN2 and MMP3 showed also over-expression of both types of tumours compared to healthy tissue, but malignant CMTs were over-expressed in comparison with benign ones.

The results showed higher levels of expression of HYAL-1 in both benign and malignant CMTs tissues when compared with the healthy mammary tissues, being the present results in agreement with Varallo et al. (2019) who observed higher expression of HYAL-1 in canine mammary carcinoma when compared to healthy tissue. Likewise, Sakalauskaite et al. (2021) observed no difference between expression in mammary carcinoma and healthy adjacent tissue, with the exception of German Shepherd bitches, which showed a higher expression of HYAL-1 in the adjacent tissue. According to the authors' knowledge, this is the first study that compares the expression of HYAL-1 among healthy mammary tissue, and benign and malignant mammary tumours in bitches. The present results clearly suggest that HYAL-1 may be involved in mammary tumourigenesis, but it is not useful as a malignancy biomarker, being its action probably related to the modification of the extracellular matrix.

MMP3 was also over-expressed in both benign and malignant tissues of CMT when compared to healthy tissues, being the expression also significantly higher in malignant tissues in comparison with benign CMTs. With reference to breast cancer, MMP-3 has been over-expressed in neoplastic mammary tissue (Hadler-Olsen et al., 2013) and has been involved in both cancer invasion and metastasis (Lochter et al., 1997; Sternlicht et al., 1999; Duffy et al., 2000; Slattery et al., 2013). MMP-3 has been considered a prognostic marker (Mehner et al., 2015; AbdRaboh and Bayoumi, 2016). Earlier studies by Lamp et al. (2013) and Pandey et al. (2016) have described MMP-3 in the tissue and serum samples respectively from bitches with mammary carcinoma. This result is similar to that found in breast cancer, thus MMP-3 may be involved in the metastatic process and thus may be considered as a prognostic marker for CMT. Unfortunately, it was not possible to acquire the follow-up of the patients included in this study, making it impossible to establish a correlation between survival and MMP3 expression. Thus, further research on this molecule is warranted.

PD-L1 was also over-expressed in CMTs in comparison with healthy mammary tissue, with malignant CMTs showing significantly higher expression values compared to benign CMTs. Some studies have described the expression of this molecule in triple-negative breast cancers (TNBC) (Beckers et al., 2016), and its higher expression was related to a significantly decreased survival and higher tumour grade (Qin et al., 2015), whereas a more recent study has shown that only 10% of TNBC are positive to PD-L1 (Constantinou et al., 2018). With regards to CMTs, the expression of PD-L1 has been scarcely studied. It has been described to be expressed in approximately two-thirds of both benign and malignant CMTs through immunohistochemistry techniques (Shosu et al., 2016). Similarly, the gene over-expression of PD-L1 has been correlated to metastatic malignant CMTs and shorter survival periods (Ariyarathna et al., 2020). According to the authors' knowledge, this is the first time that gene expression of PD-L1 was compared between benign and malignant CMTs. This result is suggestive of the putative potential of PD-L1 as a malignancy biomarker of CMTs.

Furthermore, PGR was over-expressed in neoplastic mammary tissues when compared to healthy tissues. Results from several studies assessing PGR through different techniques have reported similar expression levels of PGR in healthy and neoplastic canine mammary tissue, being over-expressed in all benign and about two-thirds of malignant CMTs (Geraldes et al., 2000; Martin de

las Mulas et al., 2005; Chang et al., 2009; Guil-Luna et al., 2013). However, the results presented in this study disagree with the aforementioned results. In this study, the overall expression of PGR did not differ between benign and malignant CMTs. A feasible explanation is the fact that, in the present study, malignant CMTs were not classified according to the degree of malignancy due to the low number of samples. According to the literature, simple carcinomas show a higher expression of PGR in comparison with complex carcinomas (Guil-Luna et al., 2014).

This study showed over-expression of VEGFα in CMT tissues, being significantly higher when compared with healthy mammary tissues. Similar studies have assessed VEGFα in both serum and tissues, while this molecule was described to be over-expressed in malignant CMTs when compared with healthy bitches (Qui et al., 2008; Al-Dissi et al., 2010; Moschetta et al., 2015), malignant CMTs also over-express VEGFα when compared with benign CMTs (Qui et al., 2008). However, the present study shows no statistical difference in the expression of VEGFα between benign and malignant CMTs. A feasible explanation for this difference is the different applied techniques. Being that the present study assessed VEGFα expression through RT-qPCR, whereas in the previous study, immunohistochemistry was applied. The higher expression of VEGFα in both benign and malignant CMTs clearly suggests that this molecule is involved in the neovascularization processes of CMTs regardless of their benign or malignant nature. Human breast cancer also expresses VEGF and has been widely demonstrated to correlate directly with tumour growth (Greenberg and Rugo, 2010; Romon et al., 2010; Taneja et al., 2010; Zhang et al., 2010; Koukourakis et al., 2011).

Although *in vitro* studies have described EGF to promote proliferation, chemotaxis and the production of VEGF, and decrease apoptosis in canine carcinomas (Kennedy et al., 2011), results from *in vivo* studies contradict the claim. Thus, whereas Klopfleish et al. (2011) observed a down-regulation in the gene expression of EGF in metastatic canine mammary carcinomas, Queiroga et al. (2009) reported an up-regulation of EGF in neoplastic mammary tissues by enzyme-immunoassay determinations. In the present study, CMTs showed significantly higher expression of EGF than healthy mammary tissue. As stated above, EGF is involved in tumorigenesis. Thus, overexpression was somehow expected in neoplastic tissue. On the other hand, malignant CMTs showed significantly higher expression than benign CMTs, making EGF a potential biomarker for malignancy.

Finally, in the present study, CMTs showed significantly higher expression of RLN2 in comparison with healthy canine mammary tissue, being significantly over-expressed in malignant compared to benign CMTs. Although both serum and tissue expressions have been assessed also in CMTs, the results are contradictory. Hence, while some studies (Lamp et al., 2009; Lamp et al., 2011) have observed no correlation between RLN2 expression and tumour malignancy, some others have demonstrated over-expressed in malignant III grade CMTs (Pawlowski et al., 2013). Although further research is warranted, the present results suggest that RLN2 may be useful as a malignancy biomarker in CMTs.

When evaluating both the linear correlation and the principal components analysis of the studied genes, the results clearly demonstrate the combined action of several molecules and mechanisms behind the appearance of CMTs. Furthermore, these results suggest that the combined analysis of these markers may have differential diagnostic potential in CMTs between normal and neoplastic mammary tissue.

The study of gene expression also offers a better understanding of the molecular pathways behind the appearance and development of CMTs, consequently leading to a better therapeutic approach. In fact, gene therapy has been suggested as a potential therapeutic approach in some neoplasia (Smith and Bird, 2010) and canine mammary neoplasia is not an exception. It has been suggested that targeting genes involved in cellular processes such as proliferation, angiogenesis, apoptosis, DNA damage repair, cycle regulation, signal transduction and survival may improve the therapeutic response to CMTs (see Kabir et al., 2016 for a review).

Some of the genes evaluated in this study are hypothesized to be useful as therapeutic targets in personalized treatments in bitches with CMTs. A promising molecule in the therapeutic field is PD-L1. High levels of this molecule have been associated with metastasis and shorter survival in CMTs (Ariyarathna et al., 2020). Some studies have demonstrated that the blockade of PD-L1 with monoclonal antibodies enhances the destruction of cytotoxic T-lymphocyte-mediated tumour cells and improves the activation of antigen presentation and cytokine release in some human cancers, making this blockade a potential treatment (Callahan et al., 2016; Topalian et al., 2015). In veterinary medicine, similar results have been observed in *in vitro* studies in diverse canine cancers such as B cell lymphoma (Kumar et al., 2017; Aresu et al., 2019), and oral melanoma (Maekawa

et al., 2016). Thus, it seems logical to hypothesize that the blockade of PD-L1 may be also a potential therapy for CMTs.

Two other molecules considered in the literature as potential targets are VEGFα and MMP (see Lee et al., 2018 for a review). VEGFα, among other angiogenic factors, is involved in neovascularization (see Melincovici et al., 2018 for a review), which is essential for tumour growth and dissemination (Galas et al., 2014). Thus, the blockade of VEGFα would avoid neovascularization and, consequently, prevent tumour growth. On the other hand, MMPs are involved in the remodelling process of the extracellular matrix, cell proliferation, migration and differentiation, as well as tissue invasion and vascularization (see Cui et al., 2017 for review). Focusing specifically on MMP3, this molecule plays a role in angiogenesis, cell growth and cell invasion (Hadler-Olsen et al., 2013). Thus, the blockade of MMP3 may prevent the expansion of the tumour.

Another molecule that warrants further research is PGR. Recent studies have demonstrated that the administration of aglepristone, a progesterone receptor antagonist, inhibits the proliferation index in PGR-positive canine carcinomas (Guil-Luna et al., 2011) and increases the disease-free period (Guil-Luna et al., 2017).

HYAL-1 is over-expressed in canine mammary carcinoma (Varallo et al., 2019). The up-regulation of HYAL-1 has been demonstrated to be involved in several processes related to tumorigenesis and metastasis such as cell growth, migration, invasion and angiogenesis in breast cancer (Tan et al., 2011a,b). Thus, its blockade could arrest these processes and, consequently, be used as a therapeutic target in CMTs.

EGF has been a focus of great interest in breast cancer (human epithelial growth factor, HER2). A synergistic interaction with its receptor (EGFR) has been suggested to be involved in oncogenesis (Suo et al., 2002; Witton et al., 2003; DiGiovanna et al., 2005; Wiseman et al., 2005), being this synergy also reported in mouse models for mammary tumorigenesis (Muller et al., 1996; DiGiovanna et al., 1998). HER2 has been a targeted molecule to treat breast cancer since 1998, when the first anti-HER2 antibody, trastuzumab, was approved (Hudziak et al., 1998). Nowadays, several drugs against HER2 are commercially available to treat human mammary cancer (see Jacobs et al., 2022 for a review). The mechanisms of action of these drugs include receptor

downregulation, suppression of proliferation (Cuello et al., 2001; Cho et al., 2003), selective growth arrest and apoptosis in breast cancer cells over-expressing HER2 (Rusnak et al., 2001) among others. The implementation of HER2-targeted therapies has improved the outcome of breast cancer patients (Jacobs et al., 2022). Therefore, the implementation of these drugs in CMTs may also be beneficial.

CONCLUSIONS

1. The presence of a canine mammary tumour induces modifications in the concentration of CD20, CD45RA, and CD99 in the peripheral serum, suggesting that these molecules may be involved in tumourigenesis.

2. CD45RA and CD99 are, respectively, under and over-expressed in peripheral serum from bitches with CMTs when compared with healthy individuals, regardless the tumour was either benign or malignant.

3. CD20 is over-expressed in peripheral serum from bitches with malignant CMTs in comparison to healthy individuals. However, no significant differences were observed between benign and malignant CMTs.

4. CD20, CD45RA and CD99 may be considered tumorigenesis biomarkers, but they cannot differentiate between benign and malignant CMTs.

5. Canine mammary tumours over-express the *HYAL1*, *MMP3*, *PGR*, *RLN2*, *EGF*, *VEGF*α, *PDL1*, *CD20* genes in comparison with normal canine mammary tissue.

6. *VEGF*α, *CD20*, *PGR* and *HYAL1* are indistinctly over-expressed in both benign and malignant CMTs. This precludes them from being potential biomarkers of malignancy in CMTs.

7. *PDL1*, *EGF*, *RLN2* and *MMP3* expression levels are significantly higher in malignant CMTS compared to benign CMTs, suggesting that they can be used as potential malignancy indicators.

8. Gene expressions for *CD20*, *PGR*, *EGF*, *RLN2* and *MMP3* are significantly correlated, suggesting that the combination of these molecules may have potential as malignancy biomarkers in CMTs.

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