UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

IDENTIFICATION OF CIRCULATING MICRORNAS AS BIOMARKERS OF FELINE MAMMARY CARCINOMA

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Para os meus avós

Ilda, Silvina, Agostinho e Francisco,

por serem as estrelas no Céu que me inspiram sempre.

" (…) H*á outra forma de recordar, mais profunda e mais importante: uma forma de memória que nunca ninguém pensou em honrar com um nome. É a memória de um passado que se escreveu a ele próprio em nós, na nossa personalidade, e na vida em que pomos em prática essa personalidade. Normalmente, não nos apercebemos destas memórias; muitas vezes nem são uma coisa de que possamos ter consciência. Mas são essas memórias, mais do que qualquer outra coisa, que fazem de nós aquilo que somos. Essas memórias surgem nas decisões que tomamos, nas ações que executamos e na vida que consequentemente vivemos.*

É na nossa vida, e não nas nossas experiências conscientes, que encontramos as memórias daqueles que já partiram."

Mark Rowlands

em "O Filósofo e o Lobo"

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IDENTIFICATION OF CIRCULATING MICRORNAS AS BIOMARKERS OF FELINE MAMMARY CARCINOMA

Abstract

Feline mammary carcinomas (FMC) are very aggressive and, even after radical mastectomy, are usually fatal due to metastasis. Therefore, diagnostic tools allowing earlier detection and more effective treatment options are urgent. MicroRNAs (miRNAs) regulate gene expression and have been found to be altered in several cancer types, including breast cancer (BC). There are no studies yet evaluating miRNAs in feline cancers, thus this study aimed to elucidate if there are differences in miRNA serum levels between FMC and healthy controls, if they followed the same patterns as in BC and, finally, if they were associated with clinicopathological features.

Serum samples from 45 female cats with FMC and 5 female healthy controls were used for the relative quantification of 5 microRNAs (miR-21, let-7a, miR-10b, miR-200b and miR-200c), which are consistently reported to be dysregulated in BC. Real-time polymerase chain reaction was performed and results were normalized to 2 reference genes (miR-191 and miR-484) by application of the $2^{-\Delta\Delta C_T}$ method.

Circulating miR-200c and let-7a were significantly downregulated (p=0,045 and p=0,04, respectively) in cats with mammary carcinoma, comparatively to healthy cats. Moreover, let-7a levels were significantly associated with overall-survival (p=0,04), histological subtypes (p=0,04) and an inverse correlation was found with serum stromal derived factor-1 (SDF-1) levels (p=0,03; Spearman r=-0,34). Regarding miR-21, high serum levels were significantly associated (p=0,02) with shorter disease-free survival (DFS) and lymph node metastasis (p=0,01), whereas for miR-10b, higher levels were associated to a positive SDF-1 status (p=0,01). Furthermore, higher serum miR-200b levels were significantly associated with shorter DFS (p=0,02), necrosis (p=0,02), histological (p=0,0078) and molecular subtypes (p=0,04), and a positive correlation was found with the tumour size (p=0,04, Spearman r=0,31).

In conclusion, results suggest that miR-200c and let-7a are candidate diagnostic biomarkers for FMC, and let-7a, miR-21 and, particularly miR-200b, appear to have value as prognostic biomarkers. Moreover, miRNA patterns in FMC were similar to what is observed in BC, supporting that the first is a proper model in comparative oncology.

Keywords: Feline mammary carcinoma, microRNAs, biomarkers, serum, comparative oncology.

IDENTIFICAÇÃO DE MICRORNAS CIRCULANTES ENQUANTO BIOMARCADORES DE CARCINOMA MAMÁRIO FELINO

Resumo

Os carcinomas mamários felinos (CMF) são muito agressivos e, mesmo após mastectomia total, são geralmente fatais devido a metastatização. Desta forma, meios de diagnóstico que permitam uma deteção mais precoce e opções terapêuticas mais eficazes são urgentes. Os microRNAs (miRNAs) regulam a expressão génica e encontram-se alterados em vários tipos de cancro, incluindo no cancro da mama humano (CMH). Até à data, não existem estudos sobre miRNAs em cancros felinos, pelo que este projeto teve como objetivos avaliar as diferenças nos níveis séricos de miRNAs entre gatas com carcinoma mamário e saudáveis, se estas seguiam o mesmo padrão que no CMH e se existiam associações com características clínico-patológicas.

Soros de 45 gatas com CMF e de 5 saudáveis foram usados para a quantificação relativa de 5 miRNAs (miR-21, let-7a, miR-10b, miR-200b e miR-200c), os quais são consistentemente encontrados desregulados no CMH. Foi realizado PCR em tempo real e os resultados foram normalizados com 2 genes referência (miR-191 e miR-484), por aplicação do método $2^{-\Delta\Delta C_T}$.

O miR-200c e o let-7a mostraram-se significativamente diminuídos (p=0,045 e p=0,04, respetivamente) nos soros de gatos com CMF, comparativamente aos saudáveis. Adicionalmente, os níveis de let-7a mostraram associações significativas com o tempo de sobrevivência (p=0,04), subtipos histológicos (p=0,04) e uma correlação inversa com os níveis séricos de stromal derived factor-1 (SDF-1) (p=0,03; Spearman r=-0,34). Relativamente ao miR-21, níveis maiores estavam significativamente associados a menor tempo livre de doença (p=0,02) e à presença de metástases nos linfonodos (p=0,01), enquanto para o miR-10b, mostraram associação com positividade para o SDF-1 (p=0,01). Além disso, níveis superiores de miR-200b apresentaram associações significativas com menores períodos de remissão (p=0,02), com a presença de necrose (p=0,02), subtipos histológicos (p=0,0078) e moleculares (p=0,04) e uma correlação positiva com o tamanho do tumor (p=0,04, Spearman r=0,31).

Em conclusão, os resultados sugerem que o miR-200c e o let-7a são candidatos a biomarcadores de diagnóstico de CMF e que os níveis séricos de let-7a, miR-21 e, particularmente de miR-200b, poderão ter valor enquanto biomarcadores de prognóstico. Por fim, o padrão de desregulação dos miRNAs nos CMF foi semelhante ao CMH, apoiando o primeiro como um modelo válido em oncologia comparada.

Palavras-chave: carcinomas mamários felinos, microRNAs, biomarcadores, soro, oncologia comparada.

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List of Abbreviations, acronyms and symbols

AntimiR – Antisense oligonucleotide miRNA construct
Ago – Argonaute protein
AUC – Area under the curve
BC- Breast Cancer
BCL2 – B-cell lymphoma 2
c-onc – Cellular oncogenes
CAF – Cancer-associated fibroblasts
ceRNA – Competing endogenous RNA
CI – Confidence interval
circRNA – Circular RNA
CLL – Chronic lymphocytic leukaemia
COX-2 – Cyclooxygenase 2
CREB1 - Cyclic AMP-responsive element-binding protein 1
CSC – Cancer stem cells
CSF – Cerebrospinal fluid
CT – Computed tomography
CXCR4 – C-X-C motif receptor 4
DFS – Disease-free survival
DNA – Desoxyrribonucleic acid
ECM – Extracellular matrix
ECM-ME – Extracellular matrix-modifying enzymes
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
EMT – Epithelial-mesenchymal transition
ER – Oestrogen receptor
ESE – Embryonic stem cells
ESC – Embryonic stem cells
FCoV – Feline coronavirus
FFPE – Formalin-fixed paraffin-embebed
FMC – Feline mammary carcinoma
FMT – Feline mammary tumour
FNA – Fine needle aspiration
GF – Growth factor
GFR- Growth factor receptor
GLUT-1 – Glucose transporter 1
HER2 – Epidermal growth factor receptor 2
HIF – Hypoxia-inducible factors
HMGA – High motility group A
HOXD10 - Homeobox D10
IL-1 – Interleukin-1
IL-6 – Interleukin-6
IL-10 – Interleukin-10
JAK – Janus protein tyrosine kinase
LA – Luminal A
LB – Luminal B
LN – Lymph node
LNS – Lymph node status
lncRNA – Long non-coding RNA
MDR – Multidrug resistance
MET – Mesenchymal-epithelial transition

miRNA – MicroRNA
miRNA* - MicroRNA star strand
MMP2 – Matrix metalloproteinase 2
MMP9 – Matrix metalloproteinase 9
mRNA – Messenger RNA
MT – Mammary tumour
ncRNA – Non-coding RNA
oncomiR – oncogenic microRNA
oncosuppressor-miR – tumor suppressor microRNA
OS – Overall survival
PCNA – Proliferating cell nuclear antigen
PCR – Polymerase chain reaction
PDCD4 – Programmed cell death 4
piRNA – Piwi-interacting RNA
PR – Progesteron receptor
pre-miRNA – Percursor microRNA
pri-miRNA – Primary microRNA transcript
PT – Primary tumour
RISC – RNA induced silencing complex
RNA – Ribonucleic acid
RNA pol II – RNA polymerase II
ROS – Reactive Oxygen Species
rRNA – Ribosomal RNA
RT-PCR – Real-time polymerase chain reaction
SDF-1 – Stromal-derived factor-1
siRNA – Small interfering RNA
SKI - v-sik sarcoma viral oncogene homolog
snoRNA – Small nucleolar RNA
snRNA – Small nuclear RNA
STAT – Signal transducers and activators of transcription
TGF-α - Transforming growth factor-alfa
TGF-β – Transforming growth factor-beta
TGFβRII - Transforming growth factor-beta receptor II
TGFβI- Transforming growth factor-beta-induced protein
TN – Triple negative
TNBC – Triple negative breast cancer
TopBP1 – Topoisomerase IIbeta binding protein-1
TNF-α – Tumour necrosis factor α
Treg – Regulatory T lymphocytes
tRNA – Transfer RNA
UTR – Untranslated Region
v-onc – Viral oncogene
VEGF – Vascular endothelial growth factor
WHO – World Health Organization
ZEB1 - Zinc Finger E-Box Binding Homeobox 1
ZEB2 - Zinc Finger E-Box Binding Homeobox 2
™ - Trade mark
~ - Aproximately
*- $p<0,05$
**- $p<0,01$

1. Activities Developed at the Internship

The curricular internship was performed at Centro Interdisciplicar de Investigação em Sanidade Animal - CIISA, from Faculty of Veterinary Medicine, University of Lisbon, between September 1st, 2017 and February 1st, 2018. During this period, several methods were performed in order to accomplish the intended objectives, including RNA extraction from 50 serum samples (45 from female cats with mammary carcinoma and 5 from female healthy controls), reverse transcription, pre-amplification and real-time polymerase chain reaction (RT-PCR) by application of the $2^{-\Delta\Delta C_T}$ method (relative quantification). The RT-PCR was performed to quantify serum levels of 5 microRNAs (miR-21, let-7a, miR-10b, miR-200b and miR-200c) and compare their expression levels between diseased and healthy cats, and further evaluate for associations with clinicopathological features. The obtained results were subjected to statistical analyses using the program GraphPad Prism 7.

Besides the above described steps related to the development of the present theses, other procedures were accompanied including cell culture passages of feline and human mammary cancer cell lines and immunohistochemical techniques performed in formalin-fixed paraffin-embebed samples collected from cats with FMC to determine the expression of stromal-derived factor-1 (SDF-)1 and its receptor, C-X-C motif receptor 4 (CXCR4). I also accompanied Professor Jorge Correia in the observation and histological classification of mammary tumour biopsies and their respective lymph nodes, when sent, for diagnosis purposes. Moreover, I participated as co-author in one scientific article and in two abstract submissions to the 2018 Congress of the European Society of Veterinay Oncology, which are displayed at ANNEX I.

2. Introduction

The incidence of cancer in domestic animals is increasing with their extended lifespan. Feline mammary carcinoma (FMC) is the third most common neoplasm in cats, presents highly aggressive behaviour and is usually fatal (Zappuli *et al*., 2015). The diagnosis of mammary tumours in veterinary medicine relies mainly in histopathologic classification, histopathologic grading and clinical staging and, although it was demonstrated that the molecular classification of human breast cancer (BC) can also be applied to cats (Soares, Correia, Peleteiro & Ferreira, 2016), it is still not implemented for routine diagnosis. Nevertheless, the molecular classification based on the expression of progesterone receptor (PR), oestrogen receptor (ER), epidermal growth factor receptor 2 (HER2) and Ki-67 appears to have prognostic significance in cats, similar to what is reported for BC patients (Zappuli *et al*., 2015).

Because most feline mammary tumours are malignant, new tools allowing earlier diagnosis are urgently required (Giménez, Hecht, Craig & Legendre, 2010). Several biomarkers are being investigated, particularly circulating biomarkers that possess the advantage of being obtained through low-invasive procedures, as is the case of microRNAs (miRNAs). miRNAs are small regulatory molecules that are dysregulated in several diseases, including cancer, and are being investigated for their diagnostic potential (Fleischhacker, Bauersachs, Hartman & Weber, 2013). Moreover, specific microRNAs have been associated with better or worse prognosis in BC and, therefore, may be candidate prognostic biomarkers.

Regarding treatment, therapeutic options for FMC are limited and, even if successful, recurrence often occurs (Morris, 2013). Therefore, the search for new molecules is one of the main concerns. MicroRNAs are amongst the potential candidates for cancer therapies and some miRNA-based therapies are already in human clinical trials (Rupaimoole & Slack, 2017).

To our knowledge, this is the first study evaluating microRNAs in feline cancers. Hence, the aim of this project was to provide insight on how microRNAs serum levels are altered in FMC and whether they may be considered diagnostic and/or prognostic biomarkers. Particularly, microRNAs reported to be involved in BC progression and reported to be potential diagnostic and prognostic biomarkers in humans were evaluated.

3. Bibliographic Review

3.1. Oncogenesis

A neoplasm is a set of cells that have undergone heritable genetic changes and are unresponsive to growth control mechanisms, resulting in expansion beyond normal anatomic boundaries. Neoplasms may be benign if the growth is local, expansive and well delimitated; or malign if they are locally invasive and have the capacity to metastize systemically. The last scenario corresponds to cancer (Zachary & McGavin, 2012). However, the term "cancer" is often applied indiscriminately to neoplasms in general, describing therefore a heterogeneous group of diseases characterized by proliferation and uncontrolled growth that result from the accumulation of successive mutations (Withrow, Vail & Page, 2013).

Oncogenesis, also referred as carcinogenesis, describes the process by which normal cells undergo neoplastic transformation and thrive to originate a neoplasm. The events that may contribute to oncogenesis include DNA mutations, chromosomal changes, epigenetic modifications, which may result from chemical, physical and biological agents, disrupted DNA damage responses, environmental conditions or may be heritable. DNA mutations include deletions, insertions, recombinations and amplifications; chromosomal changes include

duplications, deletions, translocations and inversions; and epigenetic events refer to changes in DNA methylation and histone modifications, as well as dysregulations in molecules that influence gene expression, as is the case of microRNAs (Zachary & McGavin, 2012).

3.1.1. Genetic Events

The main trigger factors involved in cancer are genetic alterations, however no single genetic defect can cause cancer alone and, therefore, cancer is a multigenic disease (Vogelstein & Kinzler, 2004). Typically, tumours contain 2 to 8 mutations that drive oncogenesis by conferring selective growth advantages to the cell, the so called "tumour drive mutations". The many other mutations present, which are often the majority, are just "passenger mutations" that do not have effects on the neoplastic process. In solid tumours, there is an average of 33 to 66 mutated genes. Certain tumours, however, present many more or many fewer mutations, for example melanomas and lung tumours usually contain approximately 200 mutations, which probably reflects the involvement of potent mutagens (UV light and nicotine, respectively). Indeed, lung cancers from smokers have 10 times more mutations than those from non-smokers (Vogelstein *et al.*, 2013).

Alterations in two types of genes are the main responsible for oncogenesis: oncogenes and tumour suppressor genes. An oncogene is a gene that, in its native form, is inactive and, when mutated, can lead or contribute to oncogenesis. Most of these genes are involved in cell growth, coding for either growth factors, growth factors receptors, protein kinase receptors or transcription factors, among others (Withrow, Vail & Page, 2013). An activating mutation in just one allele of the oncogene is, in most cases, enough to confer a selective growth advantage. On the contrary, tumour suppressor genes are active in physiologic conditions and mutations that alter their function are involved in oncogenesis. In these genes, mutations in both alleles are typically required (Vogelstein & Kinzler, 2004). Mutations in tumour suppressor genes often lead to failure of cell cycle arrest or apoptosis induction after DNA damage, resulting in proliferation of defected cells and progressive accumulation of DNA alterations (Zachary & McGavin, 2012). Indeed, cancer cells often present aberrant and complex genomes and a great genetic intra-tumour variation. The genomic diversity may range from single nucleotide changes to changes in chromosome copy number, called aneuploidy (Giam & Rancati, 2015).

3.1.2. Epigenetic Events

Epigenetic events are those that alter the phenotype without changing the genotype. The main epigenetic mechanisms regulating gene expression are DNA methylation and histone

acetylation, which can enhance or repress gene expression. Moreover, some regulatory molecules, as microRNAs, can also alter gene expression and contribute to cancer.

DNA methylation is an epigenetic mechanism that involves the addition of a methyl group to CpG dinucleotides by a methyltransferase and, generally, when occurs at promoter regions leads to gene silencing. In cancer cells, a global hypomethylation is commonly observed (Zachary & McGavin, 2012), mainly occurring in DNA repeats, as retrotransposons and endogenous viral elements, which results in their upregulation. Often found in cancer is also the hypermethylation of CpG islands with a consequent transcriptional silencing of genes involved in tumour suppression (Ehrlich, 2002).

Histone modifications consist in post-transcriptional alterations in histones that influence the strength of the bond between histones and the DNA and, hence, disrupts chromatin structure. It can result in a more relaxed chromatin configuration, making DNA more accessible to transcription machinery or can instead lead to the opposite. Histone modifications include methylation, phosphorylation, acetylation, ubiquitination, amongst others and may result in gene activation or repression. For example, acetylation of histone tails is generally associated with gene activation. In several cancer types, specific histone modifications were reported and a global decrease in histone marks has been associated with poor prognosis (Chervona & Costa, 2012).

3.1.3. Heritable Cancer Syndromes

If the alterations described above occur in germline, transmission from one generation to the next occurs and may lead to heritable cancer syndromes. These syndromes are characterized by early onset of the disease, family history of cancer and formation of bilateral tumours in paired organs or multiple tumours in non-paired organs (Zachary & McGavin, 2012).

So far, more than 200 heritable cancer syndromes have been described in humans, accounting for 5% to 10% of all human cancers. In dogs, at least one has been identified, the renal carcinoma and nodular dermatofibrosis of the German Shepherd, characterized by multifocal renal tumours, uterine leiomyomas and skin nodules (Withrow, Vail & Page, 2013).

3.1.4. Carcinogenic Agents

There are several known carcinogenic agents for humans, which are described in the "Report on Carcinogens" (National Toxicology Program, 2016). For example, several pesticides, herbicides and insecticides present carcinogenic potential. In veterinary medicine, a relationship between 2,4-dichlorophenoxyacetic acid and the development of canine lymphoma

was found whereas other insecticides and herbicides have been linked to transitional cell carcinoma in dogs (Withrow, Vail & Page, 2013). Tabaco has long been associated to lung cancer development, but nicotine is not an initiator of oncogenesis but rather is a promoter. However, one of its metabolites, the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is, indeed, a carcinogenic agent (Warren & Singh, 2013). Other examples include cyclophosphamide, a cytotoxic agent, that has been associated to urinary bladder cancer in dogs and humans (Withrow, Vail & Page, 2013) and ingestion of the foetus *Pteridium aquilinum* leads to bovine enzootic hematuria, a disease characterized by urinary bladder neoplasms (Pinto, 2010). The sunlight exposure is also a well known risk factor for the development of squamous cell carcinoma in humans and other animals, particularly in white cats (Withrow, Vail & Page, 2013) and chronic inflammation, with the consequent production of oxygen reactive species, has also been linked to oncogenesis. For instance, there are reports of uveitis and lens rupture with subsequent development of eye tumours in cats. Other example is the vaccine-associated feline sarcoma, a sarcoma which develops secondarily to vaccination at the sites of inoculation (Withrow, Vail & Page, 2013). Moreover, certain hormones are also related to tumour development, as is the case of oestrogen and progesterone in mammary tumours. Indeed, cats sprayed before 6 months of age presented a reduction of 91% in the risk of developing mammary tumours and the use of medroxyprogesterone acetate for preventing oestrus or to treat pseudopregnancy has been associated to increased incidence of mammary tumours in dogs and cats (Zappuli *et al*., 2015).

3.1.5. Oncogenic Infectious Agents

The infectious agents involved in oncogenic processes are mainly viruses, however bacteria and parasites are also reported to be associated with cancer. One example is the close link between *Helicobacter pylori* and gastric neoplasms in humans, although the mechanisms are still not fully elucidated (Wang, Meng, Wang & Qiao, 2014). Relatively to parasites, *Spirocerca lupi* infects dogs and seems to contribute to development of oesophageal sarcomas (van der Merwe *et al*., 2008). Also, *Schistosoma haematobinum* has been associated to urinary bladder cancer in humans and *Cryptosporidium parvum* to gastrointestinal and biliary cancers (Benamrouz *et al.*, 2012).

There are several oncogenic viruses affecting humans and animals, namely retroviruses, hepadnaviruses, herpesviruses, poxviruses and papillomaviruses. Some carry viral oncogenes (v-onc), genes that direct the malignant transformation whereas others, thought the process of integration, activate cellular oncogenes (c-onc) or silence tumour suppressor genes (Withrow, Vail & Page, 2013). The v-onc encode oncoproteins that act as growth factors (GF), growth

factor receptors (GFR), hormone receptors, intracellular signal transducers or transcription factors (Murphy, Gibbs, Horzinek & Studdert, 1999). Papillomaviruses are an example of oncogenic viruses that do not integrate into the cellular genome but encode oncoproteins. For instance, the E6 protein encoded by the human Papillomavirus binds to cellular p53 and functionally inactivates it (Vogelstein & Kinzler, 2004).

3.2. Cancer Biology

3.2.1. Initiation, Promotion and Progression Model

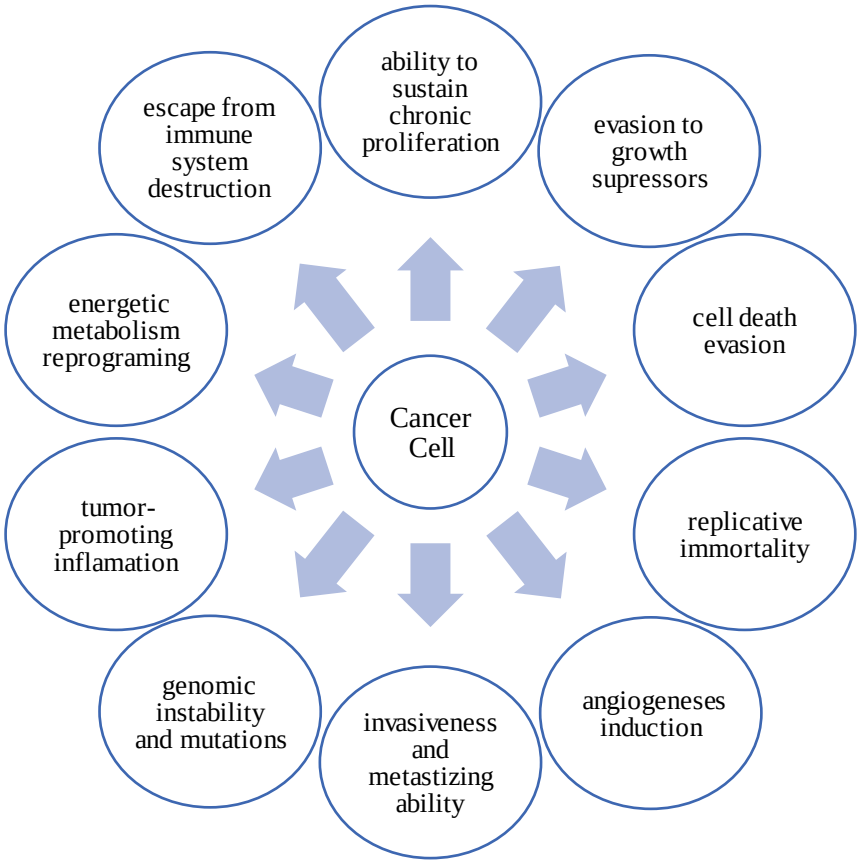
It is believed that most cancers are of clonal origin and that the tumour heterogeneity is due to the progressive accumulation of genetic alterations during tumour growth (Zachary & McGavin, 2012). Therefore, the "initiation, promotion and progression" model proposes a stepwise cancer development that starts with "initiation". This step corresponds to an irreversible genetic alteration, more likely to be a series of mutations rather than a single mutation, which endows a somatic cell with a growth or survival advantages. This alone would not be sufficient to give rise to a tumour, so a second phase, called "promotion" is required, and it is characterized by proliferation of the initiated cells in response to selective stimuli that confer a growth advantage to those cells. Finally, a third phase called "progression" reinforces the cells' malignancy (invasiveness, tissue destruction and metastatic potential), leading to clinical disease (Zachary & McGavin, 2012; Withrow, Vail & Page, 2013).

Metastasis correspond to the formation of new tumours arising from cancer cells that detach from the primary tumour (PT) and colonize distant sites. Metastasis mainly occur through the lymphatic system or the hematogenous route but transcoelomic dissemination can also occur when cancer cells within cavities, such as the abdominal cavity, spread directly to surrounding parietal or visceral surfaces. Certain cancers metastize preferentially to specific sites (Zachary & McGavin, 2012). For metastasis to occur, cancer cells must detach from the primary tumour, which in epithelial cell requires the loss of function of intercellular junction elements as cadherin or catenin and, consequently, loss of adhesion. Then, cancer cells must penetrate the basement membrane and enter into lymphatic or blood circulation (intravasion). Once there is intravasion, cancer cells tend to aggregate forming emboli that, at a certain point, adhere to the basement membrane and extravasion occurs. If the environment is suitable, cancer cells thrive and originate a metastasis. For cancer cells to detach from the PT they must loose adhesion which, in epithelial cells, occurs (Witsch, Sela & Yarden, 2010).

3.2.2. Hallmarks of Cancer

The hallmarks of cancer (Figure 1) are biological abilities acquired by neoplastic cells that allow tumour growth and progression and are common characteristics to different cancer types. Six hallmarks were initially described: ability to sustain chronic proliferation, evasion to growth suppressors, cell death evasion, replicative immortality, angiogenesis induction and invasiveness and metastizing ability (Hanahan & Weinberg, 2000). Later, four new hallmarks were added: genomic instability and mutations, tumour-promoting inflammation, energetic metabolism reprograming and escape from immune system destruction (Hanahan & Weinberg, 2011).

Figure 1: The 10 hallmarks of cancer.



3.2.2.1. Ability to Sustain Chronic Proliferation

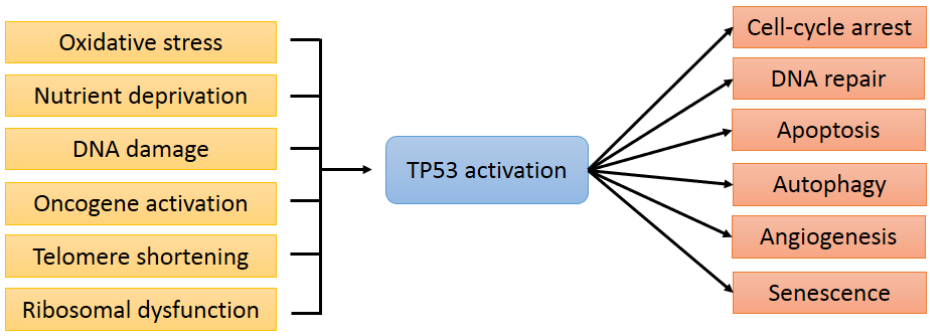
Many, if not all, of the oncogenes are involved in GF and GFR pathways (Goustin, Leof, Shipley & Moses, 1986). The ability of tumours to sustain chronic proliferation is due, mainly, to GF produced by the own cells (autocrine signalling) or by other cells types which are stimulated by cancer cells to do so. GF are essential for clonal expansion, which allows the maintenance of the oncogenic mutations and tumour progression. Alternatively, tumour cells

can become hyper-responsive to GF through overexpression the corresponding receptors (Witsch, Sela & Yarden, 2010).

3.2.2.2. Evasion to Growth Suppression Mechanisms

Evasion to normal mechanisms of growth suppression is essentially based on the loss of function of tumour suppressor genes, as is the case of retinoblastoma 1 (RB1) and TP53. RB1 acts as a transcriptional repressor, regulating the expression of cell-cycle genes and, further, contributes to the maintenance of genomic stability, as is also the case of TP53 (Gonzalo *et al.*, 2005). The tumour suppressor TP53 gene, that encodes the p53 protein, is the most frequently mutated gene in several types of cancer and is involved in various cellular mechanisms including cell cycle regulation, DNA damage repair and cell death (Kamp, Wang & Hwang, 2016). When stimulated by cellular stress inducers, like ionizing radiation, hypoxia, oxidative stress and carcinogens, TP53 is activated leading to cell-cycle arrest and promotion of DNA repair, or induction of apoptosis through several mechanisms (Figure 2). In cancer, TP53 is frequently mutated and, as a result, DNA damage is not repaired nor there is cell cycle arrest or apoptosis induction. Consequently, successive mutations accumulate and are passed to the cell progeny since there are no constrains (Pflaum, Schlosser & Müller, 2014).

Figure 2: TP53 activation and response mechanisms in normal conditions. Adapted from Bieging & Attardi (2012).



3.2.2.3. Cell Death Evasion

Evading cellular death is a mechanism by which neoplastic cells escape apoptosis and necroptosis, two forms of programmed cell death. Apoptosis is a programmed cell death, mediated by caspases, crucial for embryonic development, healing and other normal physiological states, but can also be associated with pathological states such as neurodegenerative diseases, autoimmunity and cancer. It is characterized by membrane

blebbing, cell shrinkage, nuclear condensation called pyknosis, DNA fragmentation, karyorrhexis, which is the fragmentation of the nucleus and, finally, formation of apoptotic bodies and their subsequent phagocytosis (Dasgupta, Nomura, Shuck & Yustein, 2017). Cancer cells undergo constant oncogenic stress, genomic instability and cellular hypoxia, which constitute apoptotic stimuli that should activate the apoptotic intrinsic pathway. However, cancer cells often avoid this process by modulating the apoptotic pathways at several levels: transcriptionally, translationally or post-translationally (Fernald & Kurokawa, 2013). Often, evasion of apoptosis results from loss of proapoptotic tumour suppressor genes such as p53 or by gain of function of antiapoptotic genes, as B-cell lymphoma 2 (BCL2) (Withrow, Vail & Page, 2013).

In addition to the well-studied apoptosis, necroptosis has recently been discovered and described in various cancer types. Necroptosis shares the same morphological features as necrosis (membrane permeabilization and swelling of intracellular organelles), a cell death that occurs due to trauma or injury, however, necroptosis is genetically programmed and triggered by the same stimuli that trigger apoptosis (Dasgupta, Nomura, Shuck & Yustein, 2017). Necrosis and necroptosis, in contrary to apoptosis, promote an inflammatory state, which can be beneficial for tumours, namely by the release of interleucine-1α that directly stimulates cell proliferation (Hanahan & Weinberg, 2011).

3.2.2.4. Limitless Replicative Capacity

There is a limited number of times a cell is able to replicate, the *Hayflick limit*, and when cells reach this limit, replicative senescence is induced. This is related to the shortening of telomeres, specialized DNA structures at the end of chromosomes that convey protection. However, the enzyme that maintains telomeres (telomerase) is repressed in most somatic tissues and, consequently, telomeres are shortened with each progressive cell division. After ~ 50 cell divisions (the *Hayflick limit*), cells enter an irreversible state of cellular senescence with arrest of proliferation (Withrow, Vail & Page, 2013). Cancer cells escape this senescence mainly due to increased telomerase activity in most cancer types. As a result, cancer cells present limitless replicative capacity, also known as immortalization (Kelland, 2007).

3.2.2.5. Angiogenesis Induction

Angiogenesis refers to the formation of new blood vessels from pre-existing ones. It occurs in physiologic condition, as during embryogenesis and wound healing, but it is also observed in several cancer types. Cancer cells exuberant proliferation overcomes the capacity of vascular supply and progressive hypoxia develops. As a result, pro-angiogenic factors are released and

angiogenesis triggered (Tonini, Rossi & Claudio, 2003). The main pro-angiogenic factors include vascular endothelial growth factors (VEGF), platelet-derived growth factor-β (PDGF-β), angiogenin, interleukin-1 (IL-1), EGF, granulocyte colony-stimulating factor, angiopoietins, tumour necrosis factor- α (TNF-α), TGF-α, and TGF-β (Tonini *et al*., 2003; Nishida, Yano, Nishida, Kamura & Kojiro, 2006). VEGF is the most studied pro-angiogenic factor and it is commonly overexpressed in a variety of cancer types. Moreover, besides being critical for angiogenesis, it also promotes lymphangiogenesis (Ferrara, Geber & LeCouter, 2003). Angiogenesis is fundamental for cancer growth and progression since inadequate vascular supply severely limits cancer growth (Nishida *et al.*, 2006).

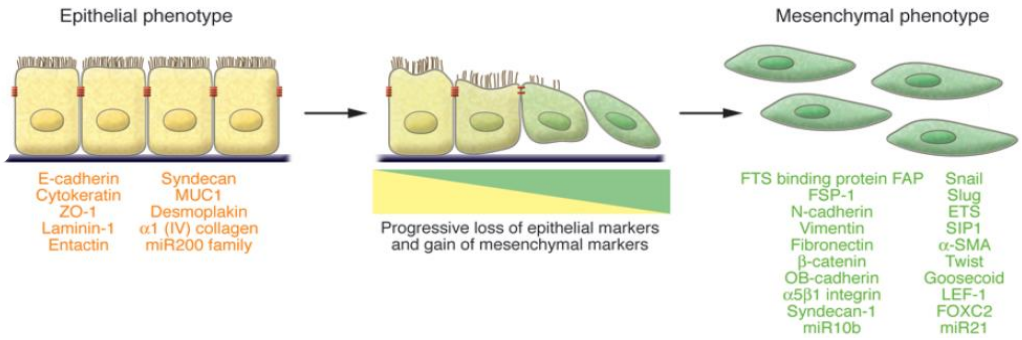
3.2.2.6. Invasiveness and Metastizing Ability

Regarding invasiveness and metastizing ability, hematopoietic tumours are inherently metastatic, whereas in epithelial neoplasms the epithelial-mesenchymal transition (EMT) plays a fundamental role in the metastizing ability. As previously mentioned, the cancer cell must leave the PT, pass through the basement membrane and then through endothelial cells to reach circulation (intravasion). In circulation, cancer cells must resist to anoikis (programmed cell death associated with loss of cellular contact) and evade immune detection so that they can reach and be arrested at distant sites (extravasion). In this new microenvironment, cancer cells need to be able to survive and proliferate in order to evolve into micrometastases and, afterwards, metastasis. The metastatic sites are believed to be previously modulated to receive the cancer cells through effects mediated by the PT, creating a pre-metastatic niche, proper for cancer cells to survive and thrive (Withrow, Vail & Page, 2013).

Tumours may release millions of cells into the circulation everyday but most of them are unable to survive and only a small portion establishes metastasis. Hence, the bigger the PT, the more likely are metastasis to occur because more cells are released into the circulation (Vogelstein et al., 2013) and, thereby, tumour size is of prognostic significance.

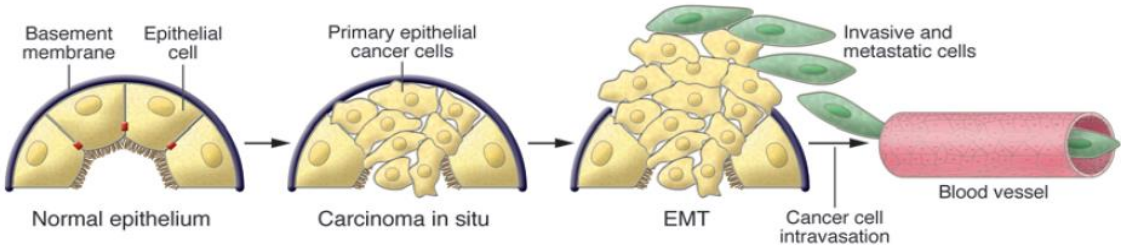
For epithelial cancer cells to gain invasive ability, they must undergo epithelial-mesenchymal transition (EMT). EMT (Figure 3) is the process by which epithelial cells acquire mesenchymal-like characteristics and, as a result, lose intercellular adhesion and display increased motility. Indeed, loss of adhesion molecules as E-cadherin, is a critical feature of EMT (Larue & Bellacosa, 2005). Besides the loss of epithelial intercellular junction elements, EMT implies the loss of apical-basal polarity, cytoskeletal reorganization with changes in cell shape, increased motility, production of extracellular matrix-degrading enzymes that contribute to invasiveness and increased resistance to apoptosis (Lamouille, Xu & Derynck, 2014).

Figure 3: Schematic illustration of epithelial-mesenchymal transition. Adapted from Kalluri & Weinberg (2009).



EMT occurs in 3 distinct biological phenomena: 1) EMT associated with embryo formation and organogenesis; 2) EMT associated with inflammation, wound healing, tissue regeneration and fibrosis; and 3) EMT that occurs in cancer cells and is associated to malignant transformation. In the last scenario, EMT enables cancer cells with increased motility and, thereby, invasive and metastatic potential (Figure 4) (Kalluri & Weinberg, 2009).

Figure 4: EMT in epithelial cancers leads to acquisition of invasiveness and metastizing ability. Adapted from Kalluri & Weinberg (2009).



Processes involved in EMT induction include: activation of transcription factors, expression of certain cell-surface receptors, reorganization and expression of cytoskeletal proteins and altered microRNA expression (Kalluri & Weinberg, 2009).

3.2.2.7. Genomic Instability

Other of the hallmarks, the genomic instability, is a cellular state characterized by an elevated rate of genetic alterations that is commonly observed in cancer. Causative factors include alterations in: DNA replication fidelity, cell cycle checkpoint controls, chromosome segregation in mitosis and mechanisms to repair DNA damage. Genomic instability is a very important feature since it increases the chances of acquiring beneficial mutations and enhances tumour heterogeneity, conveying cancer cells the ability to adapt to harsh environments, progress and acquire features responsible for chemotherapy resistance. Genomic instability can be at the nucleotide or chromosomal level. Indeed, most solid tumours have aneuploidy and

large-scale structural genomic rearrangements that reflect chromosomal instability (Giam & Rancati, 2015). This refers to a high rate of gain/loss of entire chromosomes due to missegregation during mitosis. Several factors may lead to these errors in mitosis including inefficient chromosome congregation, improper chromosome condensation or cohesion, defects in mitotic spindle and defects in mitotic checkpoints. Chromosome instability has been linked with poor prognosis and chemoresistance (Orr & Compton, 2013). Furthermore, genomic instability, besides generating a heterogeneous population of cancer cells and, therefore, a greater adaptational capacity, is also associated with increasing malignancy. For instance, karyotypic and epidemiological analysis in BC show that increasingly aggressiveness is observed through the stepwise accumulation of genetic changes (Lee & Muller, 2010).

3.2.2.8. Evasion from Immune System Destruction

One of the abilities of the immune system is to recognize altered forms of self-proteins that arise during oncogenesis, known as tumour-antigens, in order to eliminate cancer cells. This role is supported by experimental evidence since genetic altered mice with T ou B-cell deficiencies are more prone to spontaneous and chemical carcinogenesis. Moreover, patients with immunosuppressive diseases or post-transplant immunosuppression have increased risk for developing cancer (Cavallo, Giovanni, Nanni, Forni & Lollini, 2011). However, the immune system often fails in this mission because cancer cells develop mechanisms to evade immune system detection or destruction, including active immune suppression by myeloid-derived suppressor cells, induction of regulatory T-cells, impaired dendritic cell activation and production of immunosuppressive cytokines (Withrow, Vail & Page, 2013). One of the main aspects of the immune evasion is based on promotion and maintenance of an immunosuppressive microenvironment, which is mainly mediated by regulatory T lymphocytes (Treg), mesenchymal stem cells and myeloid-derived suppressor cells, under the influence of the tumour. For instance, cancer cells release transforming growth factor-beta (TGF-β), interleukin-10 (IL-10) and indoleamine 2,3-dioxygenase leading to conversion of naive T cells into Treg and, consequently, immunotolerance induction (Cavallo, Giovanni, Nanni, Forni & Lollini, 2011). Moreover, the immune system effectively destroys highly antigenic cancer cells and inadvertently selects weakly antigenic cancer that escape immune detection (Beatly & Gladney, 2015).

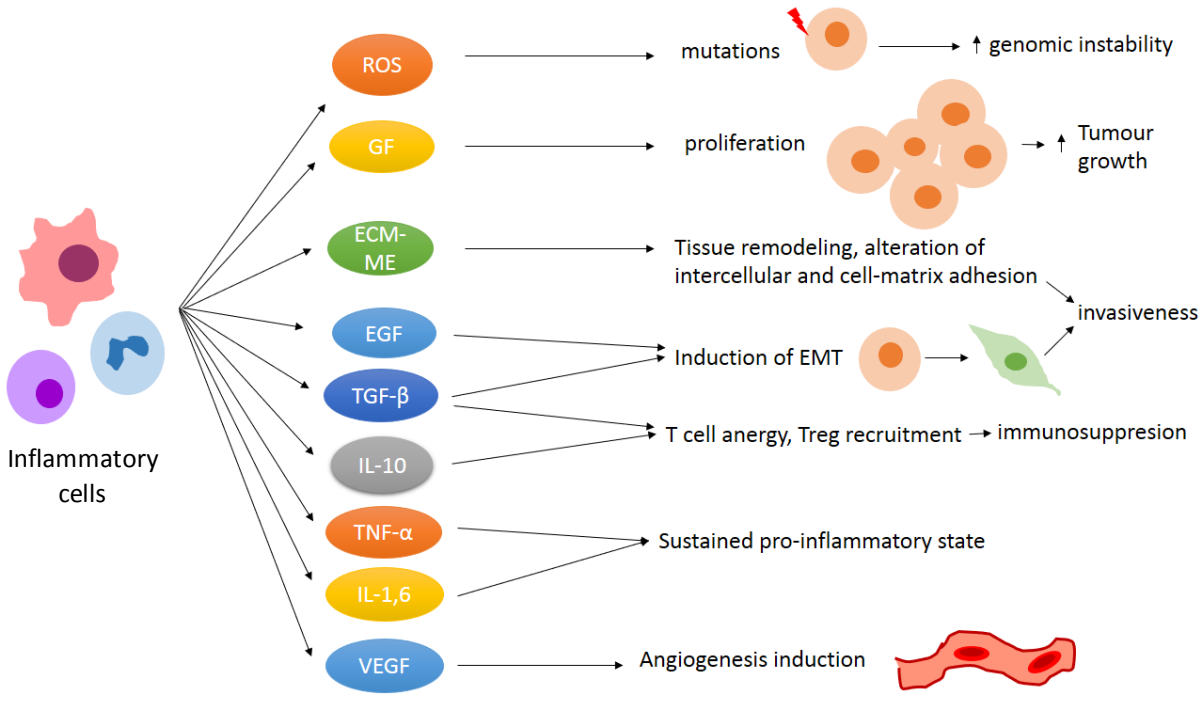
3.2.2.9. Tumour-Promoting Inflammation

Besides evading immune system destruction, cancer cells may even beneficiate from inflammation. Most solid tumours are richly infiltrated with immune cells and, often, this

inflammatory microenvironment promotes tumour growth and progression. Contributing to this perspective is the fact that chronic inflammation is itself a known high-risk factor for cancer development and that administration of anti-inflammatory drugs have improved survival of patients and seemed to aid in the prevention of cancer development (Zhang, Zhu & Li, 2017).

The factors involved in tumour-promoting inflammation (Figure 5) are related to the production of reactive oxygen species (ROS), that cause DNA damage; production of GF; secretion of VEGF and other pro-angiogenic molecules; alteration of intercellular and cell-matrix adhesion molecules due to production of extracellular matrix-modifying enzymes (ECM-ME); induction of EMT, mainly mediated by epidermal growth factor (EGF) and TGF-β; and induction of an immunosuppressive environment to which contribute Interleukin-10 (IL-10) and TGF-β. This pro-inflammatory state is sustained by continuated production of pro-inflammatory molecules, such as interleukin-1 and 6 (IL-1, IL-6) and TNF-α (de Visser, Eichten & Coussens, 2016; Zhang, Zhu & Li, 2017).

Figure 5: Inflammatory-mediated mechanisms that contribute to tumour development and progression.



3.2.2.10. Energetic Metabolism Reprogramming

Despite presenting a markedly increased consumption of glucose and glutamine compared to normal cells, cancer cells often face harsh environments with nutrient scarcity due to their high rates of nutrient consumption associated with inadequate vascular supply. As a result, certain

mutations that enable cancer cells with unconventional modes of nutrient acquisition confer adaptational advantages. For instance, cancer cells might use extracellular soluble proteins from plasma or extracellular fluids as a source of amino acids. They can also recover amino acids through phagocytosis of apoptotic corpses and autophagy of whole organelles, as well as by engulfment of entire cells (Pavlova & Thompson, 2016). Indeed, 3 types of cell-to-cell interaction have been described in cancer cells: cannibalism, entosis and emperipolesis. Cannibalism is the active internalization and destruction of death or living cancer cells by other cancer cells. Entosis is a form of live-cell invasion where the invading cell seems to take the initiative of being internalized, almost like an intracellular parasite. Finally, emperipolesis corresponds to phagocytosis of intact hematopoietic cells. Cases of cell cannibalism have been reported in feline and canine tumours, and were associated to strong EMT phenotype and high malignancy (Ferreira *et al*., 2015). Also, in apparently all tumours, there is an upregulation of glucose transporter-1 (GLUT-1), leading to more efficient glucose uptake from the surrounding environment. This increased glucose uptake by cancer calls compared to the normal ones, can be applied for diagnostic purposes by using positron-emission tomography with a radiolabelled analogue of glucose (Withrow, Vail & Page, 2013). Moreover, cancer cells also have the peculiarity of preferentially utilize glycolysis rather than oxidative phosphorylation to generate energy, even in environments rich in oxygen and with completely functional mitochondria, and this metabolic switch is known as the *Warburg effect* (Liberti & Locasale, 2016).

The high utilization of glucose and glutamine results in accumulation of extracellular lactate and pH decrease, which lead to an immune-permissive environment by attenuating dendritic, macrophages and T-cell activation, induces secretion of pro-angiogenic factors and stimulate hyaluronic acid production by fibroblasts (Pavlova & Thompson, 2016). Hence, the tumour microenvironment is acidic due to lactate accumulation, has low oxygen tension and low glucose levels, all of which increase genetic instability. Moreover, in these conditions, there is an upregulation of hypoxia-inducible factors (HIF) that stimulates the production of GF, GFR, glycotytic enzymes and glucose transporters and, also, promotes autophagy (Feitelson *et al*., 2015).

3.2.3. Genome Theory of Cancer Evolution

Despite the great acceptance of the hallmarks of cancer by the scientific community, many implications based on those concepts failed in the clinical application and some authors started raising issues, such as that the hallmarks of cancer present a reductive vision of cancer, that is far more complex and dynamic than what is displayed as compartmentalized static features. Moreover, properties that are alleged to be unique to cancer cells are, in fact, exhibited by

normal cells during various stages of normal development, as is the case of stem cells concerning the replicative immortality (Horne, Pollick & Heng, 2014). Also, the excessive growth rates of cancer cells do not exceed those of embryos (Bignold, 2007) and, with the genome sequencing, new cancer-associated mutations were unveiled and many cannot be explained by the current hallmarks or, rather, can be apposed to multiple hallmarks (Horne, Pollick & Heng, 2014).

Tumours are in continuous progression and the interactions and traits present at one stage are likely to differ from the next. In fact, at histologic observation, for any cancer type, individual tumours display tremendous graduation of characteristics, which reflects the dynamic progression and evolution of cancer (Bignold, 2007). Furthermore, in the majority of cancer populations where genomic instability prevails, the heterogeneity can be so pronounced that there is no "average profile", which means that even within a single tumour at a single moment in time, there are distinct cancer cell subpopulations (Weinberg, 2014). Therefore, the genome theory of cancer evolution redefines cancer as a constantly evolving process with cycles of genomic aberrations. Cancer evolution can be divided into two phases: the punctuated phase, characterized by extreme heterogeneity and rapid and high-level genome changes which are mainly due to genomic chaos; and the stepwise phase, where genomes that confer better adaptation to a given microenvironment prevail, thrive and remain relatively stable over time (Horne, Pollick & Heng, 2014).

3.2.4. Tumour Microenvironment

As cancer progresses, the surrounding microenvironment co-evolves due to the paracrine stimulation exerted by cancer cells. Therefore, neoplastic cells modulate the tumour stroma to support their own growth and, in return, the tumour microenvironment also influences cancer progression (Figure 6). The tumour microenvironment is the environment surrounding cancer cells and is composed by the extracellular matrix (ECM), blood and lymphatic vessels, signalling molecules and non-malignant cells (Hui & Chen, 2015) such as cancer-associated fibroblasts (CAFs), leukocytes and cancer stem cells (CSC) (Pietras & Östman, 2010).

CAFs directly stimulate tumour proliferation by supplying GF, hormones and cytokines. They also provide many components of the ECM and pro-angiogenic factors and further induce EMT, thereby promoting invasion and metastases (Pietras & Östman, 2010).

Other important elements of the tumour microenvironment are leukocytes that, as previously mentioned, can greatly contribute to tumour progression (see hallmarks of cancer, page 7).

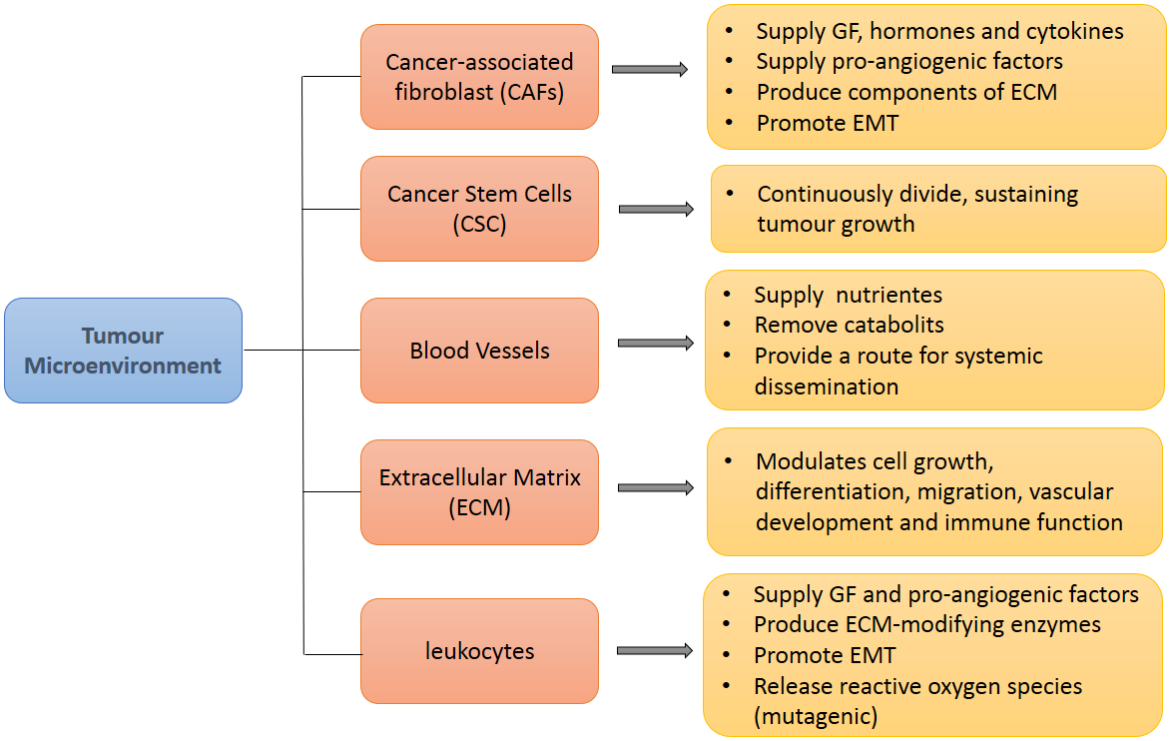
The CSC or tumour-initiating cell hypothesis refers that a subpopulation of cancer cells retains or acquires the ability of self-renewal and are responsible for initiating and maintaining the tumour, with some of the CSC progeny undergoing partial to complete differentiation and losing the ability to support the tumour growth. CSC origin is under debate but it is mainly considered that CSC arise from mutations in stem cells, from de-differentiation of somatic cells that re-acquire stem cell-like properties, or from fusion of neoplastic cells with bone-marrow-derived stem cells (Albini *et al*., 2015).

The ECM is dynamically remodelled and regulates tissue development and homeostasis, conditioning cell growth, migration, differentiation, vascular development and immune function. In cancer, there is a deregulation of the ECM, which contributes to the neoplastic progression. For instance, tumours often display a desmoplastic response, an altered organization of ECM proteins and increased deposition of collagen, that has been associated with poor disease outcome (Pickup, Mouw & Weaver, 2014).

The oxygen tension within a tumour varies from anoxia to hypoxia, in most cases. Hypoxic cells mount adaptive responses to restore tissue perfusion and oxygenation. One of those responses is the release of VEGF, a promoter of angiogenesis. However, the excessive production of angiogenic factors by the tumour in response to the lack of oxygen, leads to abnormal and dysfunctional vasculature, with endothelium lacking tight junctions, not covered by mural cells and with an irregular basement membrane. This results in leakage with increased intra-tumour pressure and poor delivery of oxygen, nutrients and anticancer drugs to cancer cells. Moreover, these more permeable vessels also allow cancer cells to enter in circulation and, eventually, colonize distant sites (Casazza *et al.,* 2013). Moreover, hypoxia and also the acidity that often characterize the tumour microenvironment, can both modulate the metabolism, reconfiguring cancer cells towards glycolysis and inducing EMT (Albini *et al*., 2015).

In this new perspective of a tumour being seen as an ecosystem composed of multiple elements and not just by neoplastic cells, alterations in a single component may cause the reorganization of the whole system, with the tumour microenvironment being able to direct the tumour progression. Consequently, many cancer therapies targeting the tumour microenvironment are arising, mainly in the perspective of serving as a complement to the conventional chemotherapy (Sounni & Noel, 2013).

Figure 6: Elements of the tumour microenvironment and their roles in cancer progression.



3.3. Feline Mammary Carcinomas

3.3.1. Epidemiology

Feline mammary tumours (FMT) are the third most common neoplasm in cats, only preceded by skin and lympho-hemopoietic tumours (Giménez, Hecht, Craig & Legendre, 2010), and account for ~17% of all feline neoplasms. The majority are malignant and hormone-independent carcinomas with aggressive biological behaviour and metastasis are reported in 50 to 90% of the cases, mainly in regional lymph nodes, lungs, liver and pleura (Zappulli et *al.*, 2015). Since only 10% to 20% of FMT are benign, early detection and aggressive therapy have great impact on survival times (Giménez *et al.*, 2010).

Risk factors include breed, gender, hormonal influence and age. Middle-aged to older female cats are the predominantly affected, however 1,5% of the cases occur in males. A breed-associated risk is reported, with higher incidence rates in Siamese and shorthaired cats. Moreover, Siamese cats are significantly younger at the time of diagnosis. Relatively to the hormonal status, despite the risk is not completely eliminated with castration, queens neutered before 1 year of age show a significantly decreased risk of developing FMC (Giménez *et al.*, 2010; Withrow, Vail and Page, 2013) and cats sprayed before 6 months of age had a reduction of 91% on the risk of developing FMT (Zappulli et al., 2015). Moreover, progestogens used for oestrus prevention or treatment of dermatological conditions in cats considerably increased the

risk of mammary tumours development with a dose-related effect, if given regularly (Giménez *et al*., 2010).

3.3.2. Clinical Presentation

FMT are generally presented as discrete and palpable subcutaneous masses or nodules within the mammary gland. Approximately 25% have ulceration and necrosis and the involved nipples are often red and swollen (Giménez *et al*., 2010). Multiple ipsilateral masses along the mammary chain are common and, occasionally, there are masses distributed bilaterally. The mammary glands involved may be enlarged, hot and painful and the drainage lymph nodes (LN) may be visibly or palpably enlarged due to regional metastasis. Regional metastasis occur mainly to the axillary and inguinal superficial LN, however the sternal LN may also be involved. Distant metastasis typically involve the lungs and liver (Morris, 2013).

3.3.3. Diagnosis

The diagnosis of FMT generally includes the physical examination, complete blood count, biochemical analysis, urinalysis, thoracic radiograph, abdominal ultrasound and biopsy or, alternatively, fine needle aspiration (FNA). The complete blood count, biochemical analysis and urinalysis are required mainly in elderly cats to evaluate concurrent diseases; thoracic radiographs (in ventrodorsal, left lateral and right lateral views) and abdominal ultrasound are employed to search for the presence of metastasis and may be replaced by computed tomography, which provides more accurate detection of metastasis. The FNA is performed to collect material for cytology and the biopsy follows for histopathological evaluation, which remains the gold standard method for definitive diagnosis. The surgical extirpation of the mammary gland/chain that serves both the purpose of treatment and diagnosis (Giménez *et al*., 2010). In veterinary medicine, the definitive diagnosis is based on histopathology, or sometimes, cytology. In human BC it is complemented with the molecular classification, which is discussed later.

3.3.3.1. Histopathologic Classification and Grade

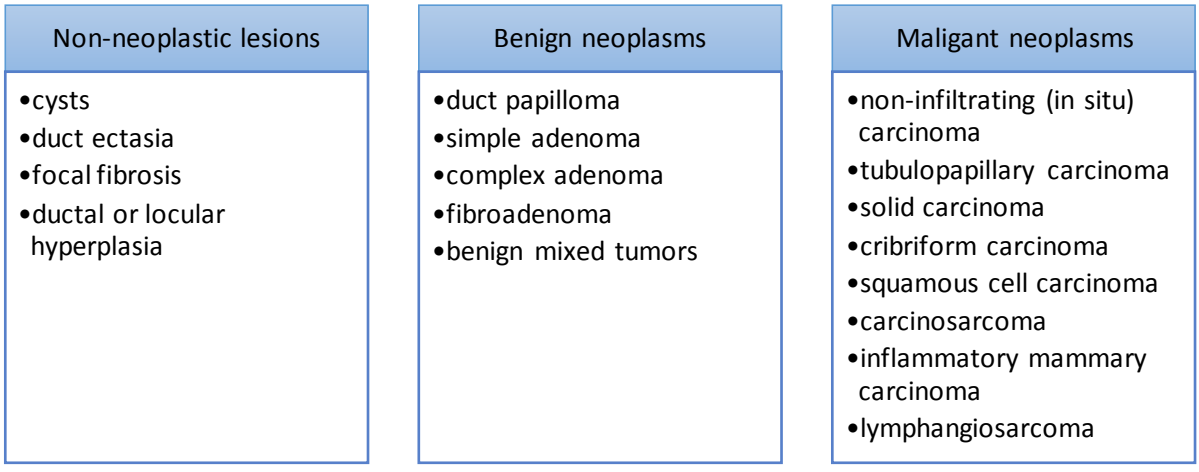
The following histological features are commonly found in tumours: pleomorphism, anaplasia, anisocytosis, anisokaryosis and desmoplastic response. Pleomorphism is the occurrence of multiple forms, shapes and sizes of cells and nucleus; anaplasia consists in a loss of differentiation or atypical differentiation; anisocytosis refer to abnormal cell size while anisokaryosis is abnormal nuclear size. Finally, desmoplastic response corresponds to an abundant fibroblastic proliferation with collagen formation and is observed is some malignant

cancers (Withrow, Vail & Page, 2013). The loss of differentiation observed in cancer cells is often accompanied by loss of function and, even in functional tumours as thyroid adenomas that continue to produce thyroid hormones, these functions are no longer appropriately regulated (Zachary & McGavin, 2012).

Histological differentiation between benign tumours and hyperplasia may be difficult in some cases since both present proliferation of well-differentiated cells. However, benign neoplasms present loss of normal tissue architecture. In contrast to malignant tumours, benign neoplasms usually have expansive growth rather than invasive, and the presence of a fibrous capsule surrounding it is common. In malignant tumours, the loss of tissue architecture is more pronounced, there is anisokaryosis and anisocytosis, increased pleomorphism, higher nuclear:cytoplasmatic ratio, elevated mitotic index, abnormal nuclear chromatin, abnormal mitotic figures, large and/or multiple nuceoli, presence of necrosis, invasiveness of adjacent tissues and distant metastasis (Withrow, Vail & Page, 2013).

The majority of veterinary pathologists follow the World Health Organization (WHO) histological classification of mammary tumours of the cat (Misdorp, Else, Hellmen & Lipscomb, 1999) (Figure 7). This classification can further be supplemented by tumour grading, as discussed later (Hughes & Dobson, 2012).

Figure 7: Histopathologic classification of feline mammary lesions. Adapted from Giménez *et al*. (2010).



One of the main issues of histological classification is that tumours are heterogeneous and the patterns and features of malignancy may vary from one area to another. If heterogeneity is present, generally the most malignant areas are the ones considered for grading purposes (Withrow, Vail & Page, 2013).

Histological classification and grade are fundamental for the diagnosis of FMC, since it is related to the biological behaviour of the tumour, survival times and recurrence rates. The assessment of tumour margins is another point that is essential to determine adequate surgical treatment and to predict the treatment outcome (Withrow, Vail & Page, 2013).

The grading system was initially based on Elston and Ellis recommendations, which scored human breast carcinomas according to the percentage of tubule formation, nuclear pleomorphism and mitotic count. Later, lymphovascular invasion and nuclear form were added (Table 1). Application of this system to FMC revealed significant associations between histopathologic grade and PT size, clinical stage, overall survival (OS) and disease-free survival (DFS), being therefore considered of prognostic significance (Seixas, Palmeira, Pires, Bento & Lopes, 2011). Nevertheless, Mills *et al*. (2015) designed a new grading system specifically for FMC (Table 2) that resulted in superior discrimination of tumours concerning OS.

Table 1: Revised Elston and Ellis grading system. Adapted from Mills *et a*l., 2015.

Histological Feature	**Categories**	**Score**
Tubule Formation	Present in more than 75% of the tumour	1
	Present in 10 to 75% of the tumour	2
	Presence limited or absent (<10%)	3
Nuclear Pleomorphism	Small, regular and uniform nuclei	1
	Moderately increased size and variability	2
	Vesicular chromatin, marked variations in size and shape	3
Mitotic Count (cumulative number in 10 fields, 40X objective)	0-50	1
	51-70	2
	≥71	3
Lymphovascular invasion	Absent	0
	Present	1
Abnormal Nuclear Forms	≤5%	1
	6-25%	2
	≥25%	3
Total Score	**Grade**	
3-5	I – well differentiated	
6-7	II – moderately differentiated	
8-10	III – Poorly differentiated	

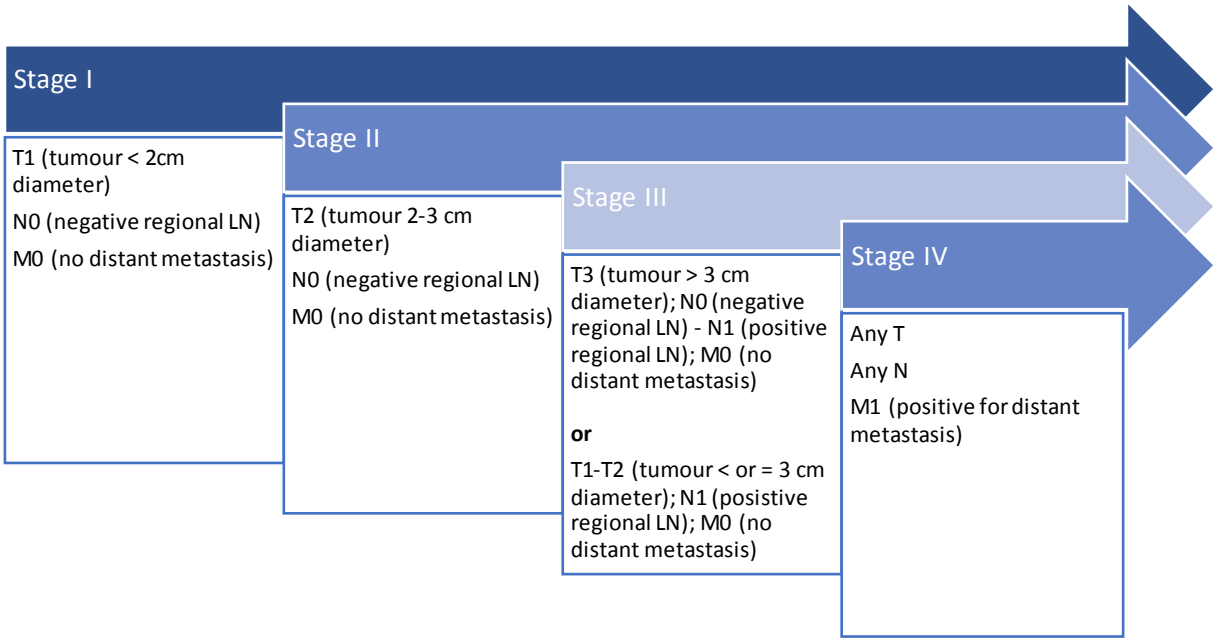
Table 2: New grading system specially conceived for FMC. Adapted from Mills *et al.* (2016).

Histological Feature	Categories	Score
Lymphovascular Invasion	Absent	0
	Present	1
Abnormal Nuclear Form	≤5%	0
	>5%	1
Mitotic Count	≤62	0
	>62	1
Total Score	**Grade**	
0	I – low-grade carcinoma	
1	II – intermediate-grade carcinoma	
2-3	III – high-grade carcinoma	

3.3.3.2. Staging System

The clinical staging of FMC (Figure 8) is based on the WHO's TNM classification of malignant tumours system and, since it is correlated to OS and DFS, it has prognostic significance and influences therapeutic choices (Zappulli *et al.*, 2015). Because so few FMT are benign, complete staging should be performed as a routine (Morris, 2013).

Figure 8: WHO's TNM classification of malignant tumours system. Adapted from Zapulli *et al.* (2015).



3.3.3.3. Molecular Classification

The molecular classification (Table 3) widely used in human medicine categorizes MT according to the expression of specific markers (PR, ER, HER2 and Ki-67), stratifying BC in the following subtypes: Luminal A (LA), Luminal B (LB), HER2-positive and Triple Negative (TN) basal-like (which express cytokeratins) and TN normal-like. This classification correlates with prognosis, aggressiveness and therapy response and, therefore, different molecular subtypes are subjected to different therapeutic strategies (Prat *et al*., 2015; Soares *et al*., 2016a). In humans, the LA subtype is the one with better prognosis whereas HER2-positive and, particularly, TN tumours are associated with poorest prognosis (Hennigs *et al*., 2016), as in cats (Soares *et al*., 2016b).

Table 3: Molecular Classification of BC. Adapted from Dai *et al.* (2015).

Molecular Subtype	IHC status	Grade	Prognosis
Luminal A	ER+, PR+, HER2-, ki67-	I/II	Good
Luminal B	ER+, PR+, HER2-, ki67+	II/III	Intermediate
Luminal B/HER2-positive	ER+, PR+, HER2+, ki67+	II/III	Poor
HER2-positive	ER-, PR-, HER2+	II/III	Poor
TN basal-like	ER-, PR-, HER2-, basal markers +	III	Poor
TN normal-like	ER-, PR-, HER2-, basal markers -	I/II/III	Intermediate

FMC present morphologic and biologic heterogeneity, with distinct prognosis and therapy responses and can also be categorized in the subtypes mentioned above (Silva, 2015). Moreover, the molecular subtypes do correlate with prognosis in cats as it was stated in one study where LA FMC presented the highest OS and DFS while TN basal-like FMC showed the worse survival times (Soares *et al.*, 2016b). According to Silva (2015), the LB subtype was the most prevalent in cats, followed by HER-positive, LA and TN. Even though, FMC are generally highly aggressive and many correspond to TN exhibiting basal-like characteristics and clinical behaviours similar to what is observed in humans, making these neoplasms proper models for TNBC (Wiese, Thaiwong, Yuzbasiyan-Gurkan & Kiupel, 2013)

Although ER and PR are implicated in early stages of mammary tumours, most FMT are ER and PR negative which is consistent to the extremely elevated rate of malignancy and aggressive behaviour observed in MT in cats (Withrow, Vail and Page, 2013).

The feline HER2 homologue is overexpressed in about 30% of FMC and is associated with shorter OS (Soares *et al.*, 2016b). Moreover, Soares *et al.* (2016c) showed that cats with

mammary carcinomas had significantly elevated serum HER2 levels compared to healthy animals and that serum HER2 levels predicted the tumour HER2 status.

The classification of BC according to molecular subtypes is so important in human medicine that it determines what type of non-surgical therapy (Table 4) is to be instituted (Senkus *et al*., 2015). Nonetheless, in veterinary medicine the molecular classification is not yet routinely performed.

Table 4: BC non-surgical treatment recommendations according to the molecular subtype Adapted from Senkus *et al*. (2015).

BC Subtype	**Recommended Treatment**
Luminal A	Endocrine therapy
Luminal B (HER2-)	Endocrine therapy + Chemotherapy
Luminal B (HER2+)	Endocrine therapy + chemotherapy + anti-HER2 therapy
HER2-positive	Chemotherapy + anti-HER2 therapy
TN	Chemotherapy

3.3.3.4. Other Biomarkers

New tools allowing earlier diagnosis and with less invasive procedures are under investigation. Biomarkers are indicators that can be objectively measured and are suggestive of normal or pathologic processes, as well as pharmacologic responses, allowing monitorization of health status or disease processes. Cancer biomarkers should ideally have high specificity and sensitivity, detect early stage cancers and be measurable in non-invasive body fluids as saliva, urine or mammary fluids or, as alternative, in low-invasive samples as blood or serum (Ettinger & Feldman, 2010).

Several biomarkers are being investigated in mammary tumours, such as RON (another tyrosine kinase receptor), signal transducers and activators of transcription (STAT), cyclins, topoisomerase IIβ binding protein 1 (TopBP1), p53, telomerase activity and expression, VEGF, cyclooxygenase-2 (COX-2), CXCR4, SDF-1 and the recently discovered microRNAs, which will be discussed later.

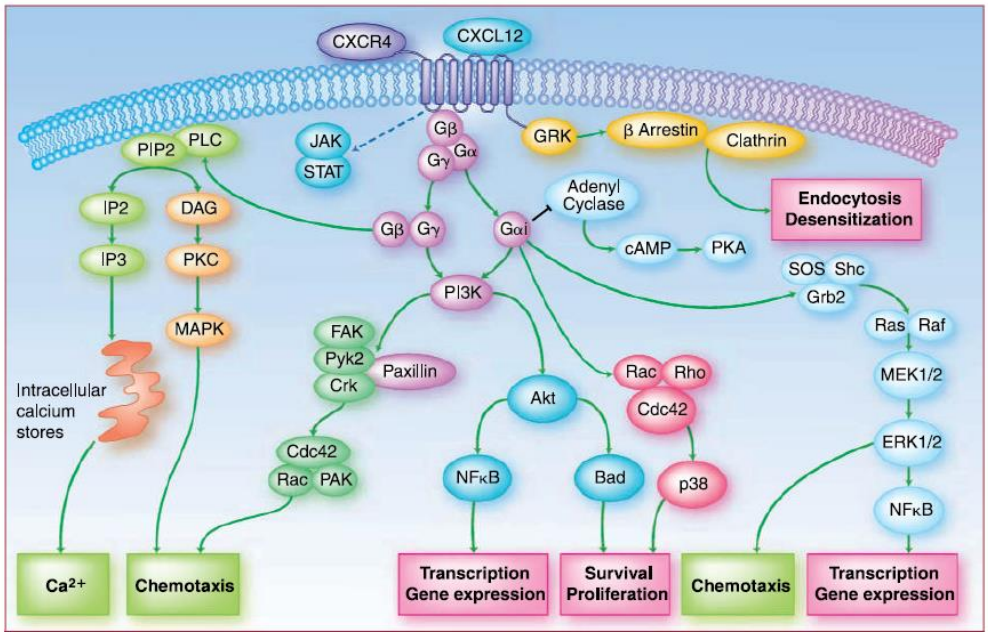
The STAT family encodes for transcription factors involved in control of differentiation, proliferation and apoptosis that are emerging as significant oncogenes; some cyclins are overexpressed in breast carcinomas; and COX-2 expression has been demonstrated in FMC (Hughes & Dobson, 2012). In feline mammary tumours, overexpression of TopBP1 was also

coorelated with histological grade (Morris et al., 2008). Regarding VEGF, it is upregulated in several cancer types, including BC. Alike, in cats with MT, VEGF was found overexpressed and significantly associated with tumour grading and OS (Millanta *et al.*, 2006).

Other biomarkers include proliferation markers as Ki-67, proliferating cell nuclear antigen (PCNA) and AgNORs. PCNA is an auxiliary subunit of DNA polymerase delta, involved in DNA repair and it is significantly increased in FMC compared to benign tumours. AgNORs are nucleolar components that associate with proteins involved in transcription and processing of rRNA and their number and size might correlate with cell proliferation (Hughes & Dobson, 2012). Regarding Ki-67, the ki-67 index of the PT was positively associated with regional and distant metastasis in FMC (Soares *et al.*, 2015).

Recently, the SDF-1/CXCR4 axis has gained attention due to its involvement in cancer. SDF-1 is a chemokine that binds to CXCR4 and this receptor is generally absent in several tissues including in the mammary gland, however it has been found overexpressed in several cancer types, namely BC. Moreover, inducers of CXCR4 expression include VEGF and HIF, both of which are frequently upregulated in cancer (Sun *et al.*, 2010). Interestingly, cells expressing CXCR4 migrate along SDF-1 gradients, with CXCR4-positive cancers metastizing to LN in a SDF-1-dependent manner. Besides being involved in chemotaxis, CXCR4 activation by SDF-1 can trigger other signalling pathways resulting in proliferation and gene transcription, as is illustrated in Figure 9 (Teicher & Fricker, 2010), which can directly contribute to tumour growth (Ferreira, 2017). Results consistently show that CXCR4 expression is common in malignant BC and stronger immunoreactivity for CXCR4 is observed in metastatic samples comparatively to the correspondent PT (Ferrari *et al*., 2012). In cats, the ones with mammary carcinomas had significantly higher serum SDF-1 levels than healthy animals, which was particularly evident for HER2-overxpressing neoplasms, corroborating the involvement of SDF-1 in mammary tumours (Marques, Soares, Santos, Correia & Ferreira, 2017).

Figure 9: Schematic illustration of CXCR4/SDF-1 signal transduction pathways. Adapted from Teicher & Fricker (2010).



However, much research is still needed to clarify if these biomarkers add additional prognostic or diagnostic value to the established molecular classification.

3.3.4. Prognostic Factors

In FMC the following features have been used as prognostic indicators: tumour size, LN status, metastasis (the three of which are used for clinical staging), histological grade and molecular markers (Giménez *et al.*, 2010; Zappulli *et al.*, 2015). Tumour size is the most important prognostic factor in FMC, being correlated with both OS and DFS, with a cut off value of 3 cm diameter. Other relevant features for prognostic include: presence of necrosis, apoptosis index, cutaneous ulceration, infiltrative growth, lymphocytic infiltration, tumour margins and lymphatic invasion, p53 mutation, CXCR4 and VEGF expression (Zappulli *et al.*, 2015). Moreover, microRNAs deregulation has been associated with the prognosis in BC (Yan *et al.*, 2008). However, no data have been reported for cat.

3.3.5. Therapeutic Approach

Treatment options include surgical excision of the tumour, chemotherapy, endocrine therapy and immunotherapy. Surgical treatment is the most widely accepted therapeutic approach to FMC and is the most effective method, nevertheless it is usually not curative since FMC are highly aggressive and tend to recur. Even so, cats undergoing radical mastectomy had longer DFS than those who were subjected to more conservative surgery (Zappulli *et al.*, 2015), so the recommended approach is total unilateral or even bilateral chain mastectomy and also the

removal of the retromammary LN as a routine procedure, whereas the removal of axillary LN remains recommended only when it is enlarged or positive on FNA (Giménez *et al*., 2010; Morris, 2013)

Some authors additionally recommend neutering at the time of tumour removal, however there is yet no consensus. Chemotherapy is sometimes recommended as adjuvant of surgery however response is usually poor once metastasis have occurred and immunomodulators to stimulate host immune response to the tumour have proven unsuccessful so far (Giménez *et al*., 2010; Morris, 2013). Due to the low hormonal receptors expression in FMC, hormonal therapy is also unlikely to be effective (Withrow, Vail and Page, 2013). So, the treatment of FMC relies basically in surgical extirpation of the tumour and, sometimes, adjuvant chemotherapy; however, most cases still recur. Therefore, unravelling new therapeutic targets and conceiving more successful therapies is essential.

3.4. MicroRNAs

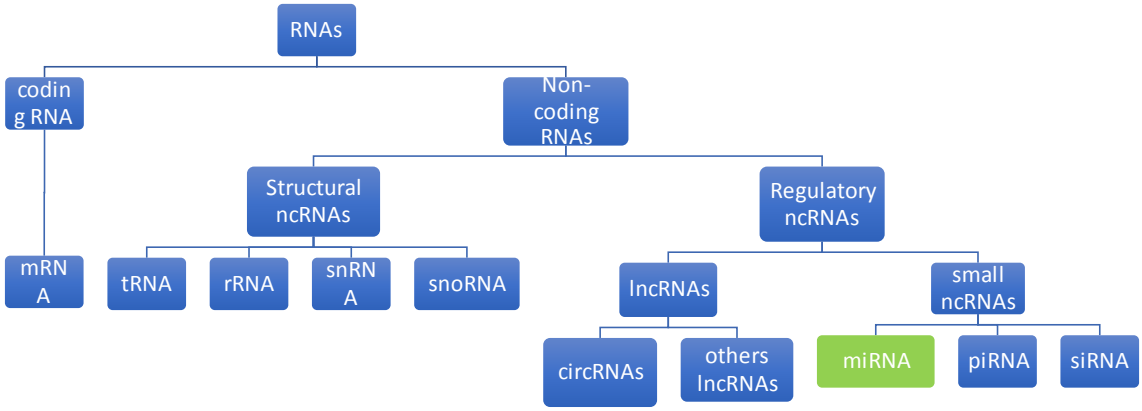
3.4.1. Introduction to Non-coding RNAs

The non-coding sequences of the genome were previously considered to be "junk DNA" (deoxyribonucleic acid) with no functional purpose (Ho *et al*., 2016). With the genome sequencing, it was revealed that, in humans, the exonic sequences correspond only to ~1,2% of the whole genome and that the majority of the genome is composed by non-coding sequences. These non-coding sequences are now known to correspond to introns, untranslated regions (UTR), simple and tandem repeats, transposable elements, pseudogenes, segmental duplications, structural variants, regulatory elements, such as promotors, enhancers, silencers, insulators and locus-control regions; and sequences that originate functional non-coding RNAs (ncRNAs) (Alexander, Fang, Rozowsky, Snyder & Gertein, 2010). It was also verified that the human genome contains approximately 20000 protein-coding genes, which is much lower than what was previously estimated, revealing that complex organisms like humans have similar numbers of protein-coding genes to much simpler organisms, as roundworms. Despite only 1,2% of the genome is transcribed and then translated, the vast majority of the genome, ~ 93%, is in fact transcribed as ncRNAs which implies they must have some roles (Wright & Bruford, 2011; Patrushev & Kovalenko, 2014; Ho *et al*., 2016). Indeed, many ncRNAs have now been acknowledged to play regulatory roles and deregulations in their expression is reported in many diseases.

ncRNAs (Figure 10) are functional molecules and comprise the well-known ribosomal RNA (rRNA) and transfer RNA (tRNA), as well as other structural ncRNAs as small nuclear RNA

(snRNA) and small nucleolar RNA (snoRNA). In addition to these, others were recently described, including microRNAs (miRNA), piwi-interacting RNAs (piRNA), small interfering RNAs (siRNA) and long non-coding RNAs (lncRNA), including circular RNAs (circRNA), which differ in length, functions and biogenesis pathways (Eddy, 2001; Wright & Bruford, 2011; Desvignes *et al*., 2015; Palazzo & Lee, 2015; Herter & Landén, 2017).

Figure 10: Functional classification of RNAs



MicroRNAs were first described in 1993, with the discovery of lin-4 in *C.elegans* (Lee, Feinbaum & Ambros, 1993). They are small segments of ncRNAs, with approximately 18-25 nucleotides length and constitute the dominating class of small RNAs in most somatic tissues. MicroRNAs are evolutionary conserved molecules and many share homologous sequences among fungi, worms, insects and mammals (Wagner, Willenbrock, Nolte & Escobar, 2013). Besides being expressed by all multicellular organisms, even entities as simple as viruses express them (Tycowski *et al*., 2015).

MicroRNAs play an important role in post-transcriptional regulation by causing translational suppression or messenger RNA (mRNA) degradation (Bartell, 2004). Indeed, Guo *et al*. (2011) demonstrated that changes in mRNA closely reflect the expression of miRNA. Moreover, different miRNAs target the same mRNA whereas a single miRNA acts on many targets, thus regulating multiple pathways and affecting the expression of many genes (Hayes, Peruzzi & Lawler, 2014). To further add to this complexity, microRNAs can also interact directly with other miRNAs modulating their function (Wagner *et al*, 2013; Bertoli *et al*., 2015).

So far, evidence suggests that more than 60% of protein-coding genes in the human genome are subject to regulation by miRNAs, making them the most abundant single class of regulatory biomolecules known (Kabir, Delnnoncentes & Bird, 2015). These molecules have specific

expression profiles in different tissues and development stages and play essential roles in diverse biological events, such as cell proliferation and differentiation (Wang & Luo, 2015) and cell death (Su, Yang, Xu, Chen & Yu, 2015). Many human diseases such as Alzheimer's disease, cardiovascular diseases and several cancer types (Wagner *et al*., 2013), such as BC (Frères *et al*., 2015), were associated with dysregulated miRNAs expression. The involvement of miRNAs has also been mentioned in sepsis (Ho *et al*., 2016), inhibition of cutaneous wound healing (Pastar *et al*., 2012), metabolic diseases, obesity and many other disorders (Shi *et al*., 2016).

miRNA deregulation can occur due to 1) genetic alterations, such as chromosomal abnormalities, deletions, insertions, amplifications and translocations in the genome; 2) epigenetic mechanisms, namely DNA methylation and histone modifications; 3) alterations in miRNA processing (altered expression or mutations in genes encoding the mediators of miRNA biogenesis); and 4) altered transcription factor activity which can influence the transcription of miRNA-coding genes (Garzon, Marcucci & Croce, 2010; Ha & Kim, 2014; Iorio & Croce, 2012; Hata & Lieberman, 2016; Rupaimoole & Slack, 2017).

Besides the involvement of miRNA deregulation in cancer, also variations (as single nucleotide polymorphisms) at the miRNA binding sites in the 3'UTR of the target mRNA are a common feature of cancer cells, making the mRNA insensitive to miRNA regulation. As so, various SNPs in miRNA binding sites are associated to cancer risk and may, therefore, be biomarkers of genetic susceptibility (Hayes, Peruzzi & Lawler, 2014).

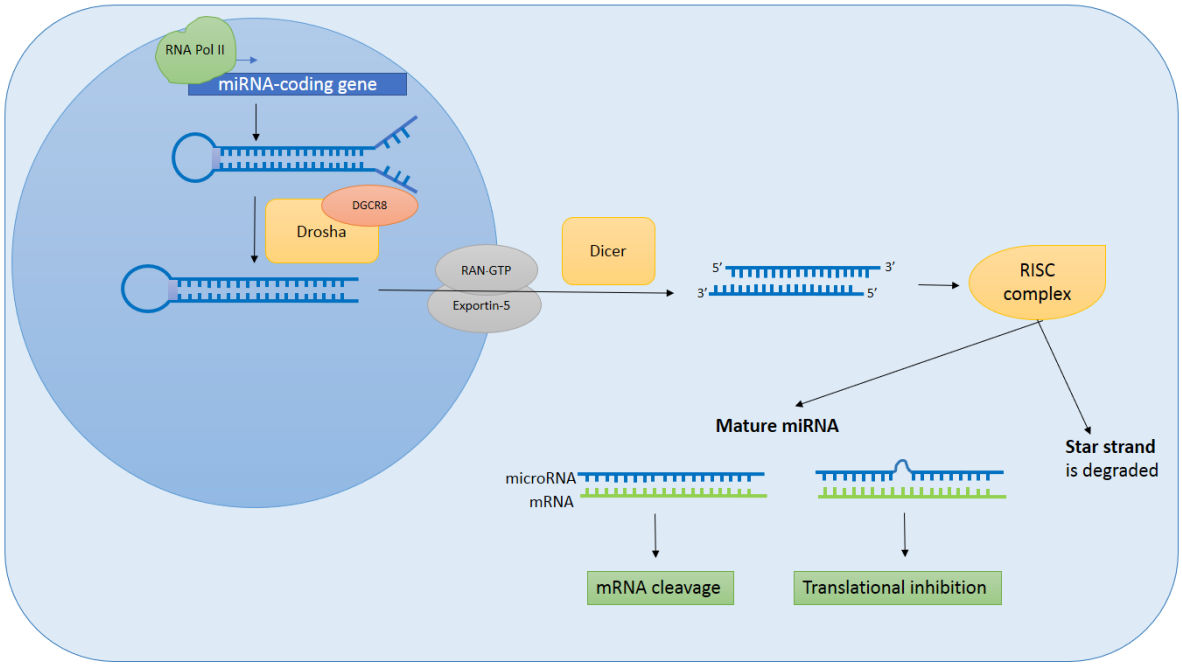
3.4.2. MicroRNAs Biogenesis and Mechanism of Action

miRNAs are encoded in the genome, either in noncoding genes or within introns or UTRs of a protein coding gene (Hammond, 2015). In plants, most of miRNAs-coding loci are independent, non-coding units (approximately 90% are located within exons), whereas in animals the majority (over 70%) is located on introns (Axtell, Westholm & Lai, 2011; Finnegan & Pasquinelli, 2013).

In the canonical pathway (Figure 11), the miRNA-coding gene is transcribed, generally by RNA polymerase II, originating a primary miRNA transcript (pri-miRNA). The hairpin is excised by the Microprocessor (which includes Drosha, a RNase III enzyme, and its cofactor, DGCR8) and this cleavage results in a precursor miRNA (pre-miRNA) (Finnegan & Pasquinelli, 2013; Hammond, 2015). The previously described steps occur in the nucleus and, afterwards, the pre-miRNA is exported to the cytoplasm via Exportin-5 that forms a complex with GTP-binding nuclear protein RAN·GTP and the pre-miRNA. After transportation through the nuclear pore

complex, GTP is hydrolyzed resulting in the release of the pre-miRNA into the cytosol (Ha & Kim, 2014). It is then cleaved by another RNase III enzyme, called Dicer, originating a duplex RNA (miRNA/miRNA* duplex). The RNA duplex is loaded onto Argonaute (Ago), that together with other protein factors forms the RISC (RNA induced silencing complex). There is unwinding of the duplex and one strand corresponds to the mature miRNA whereas the other strand, designated star strand or passenger strand (miRNA*), is typically degraded (Garzon *et al*. 2010; Ha & Kim, 2014; Hata & Lieberman, 2015;). The incorporation of the mature miRNA into RISC directs the complex to the 3'-UTR of the target mRNAs leading to 1) translational repression, if there is a low degree of complementarity between the miRNA and its target; or 2) mRNA degradation, if they exhibit high complementarity. The first mechanism is the predominant in animals and the second is the prevalent regulation mechanism in plants (Humphries & Yang, 2015; Bhat, Jarmolowski & Szweykowska-kulinska, 2016). Besides their established function in repressing translation, it has recently been described the involvement of nuclear miRNAs in positive or negative regulation of transcription by interacting directly with promoters (Patrushev & Kovalenko, 2014).

Figure 11: Schematic illustration of microRNA biogenesis.



The dysregulation of the enzymes required for miRNA biogenesis is thought to be a common feature in tumors (Yu & Li, 2015; Zamani-Ahmadmahmudi, 2016) and reduced Dicer or Drosha mRNA correlates with worse outcome in breast, lung, skin, endometrial and ovarian cancers (Hata & Lieberman, 2016).

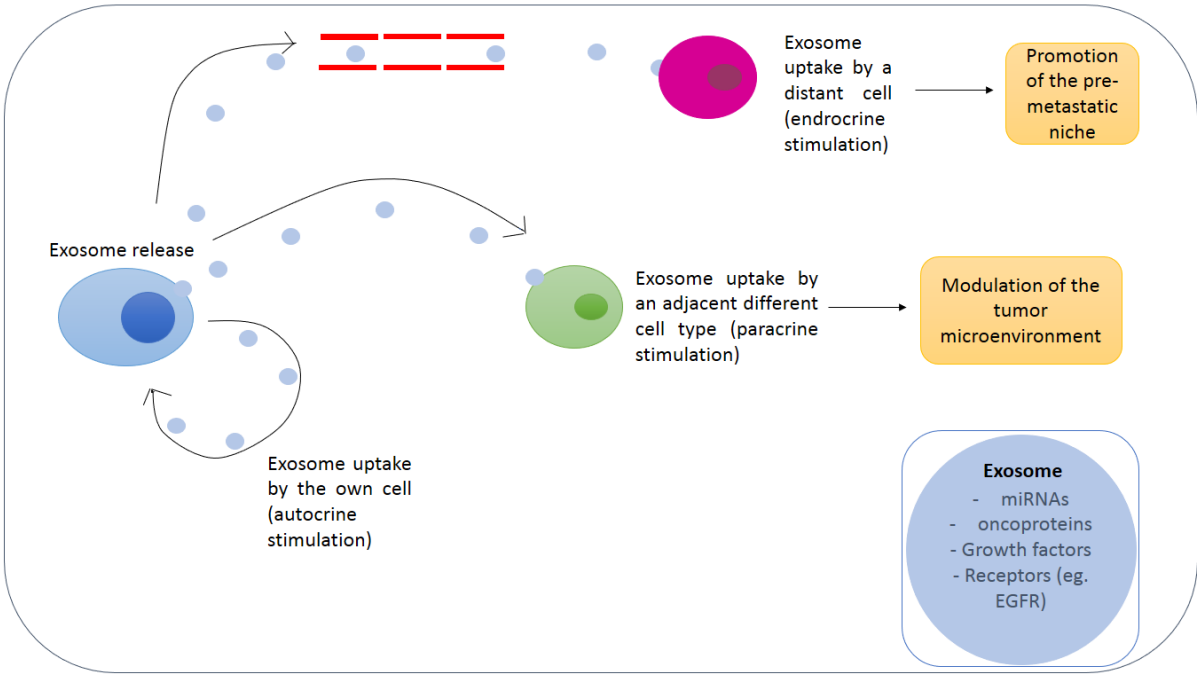
Other alternative (non-canonical) biogenesis pathways exist, as is the case of the miRtron pathway, which is Drosha-independent because the miRtrons are directly processed by the splicing machinery, bypassing the necessity of Drosha cleavage (Westholm & Lai, 2011; Iorio & Croce, 2012; Wen, Ladewig, Mohammed & Lai, 2015). Other non-canonical pathways are still being revealed and there is one that is Dicer-independent and some miRNAs are even produced by the cleavage of snoRNAs and tRNAs (Ha & Kim, 2014).

Derivations in the processing of pre-miRNAs lead to the formation of many isoforms, called isomiRs, differing in size, primary structure and functional activity (Patrushev & Kovalenko, 2014).

miRNAs are found in all tissues and body fluids, including blood, serum, plasma, urine, saliva, semen, pleural and ascitic effusion (Graveel, Calderone, Westerhuis, Winn & Sempere, 2015; Tiberio, Callari, Angeloni, Daidone & Appierto, 2015) and, for diagnosis purposes, they present the advantages of being very stable. Indeed, their detection in body fluids appears to have superior accuracy than mRNA profiling due to their high tissue specificity, stability and aberrant expression in different tumor types (Amorim, Salta, Henrique & Jerónimo, 2016). miRNAs can be secreted from cells and found in body fluids within exosomes or carried by proteins or lipoproteins. Additionally, passive leakage of miRNAs from cells due to injury, apoptosis and necrosis can also occur (Ling, Fabbri & Calin, 2013).

Most of miRNAs from serum and saliva are within exosomes which are bi-layered nanovesicles that are formed via inward budding of endosomal membranes. Most cells, either normal or diseased, release exosomes into the extracellular space and body fluids. Those exosomes may then suffer uptake by another cell thereby transferring miRNAs (and other molecules) from one cell to another (Figure 12). Since tumor exosomes can enter circulation and therefore be transported systemically, microRNAs produced by a cancer cell can influence the environment and cellular events at very distant sites. The tumor microenvironment is particularly exosome-enriched and cancer cells secrete at least 10-fold more exosomes than normal cells. For that reason, tumor-derived exosomes have been extensively studied for their roles in cancer development and treatment failure (Yu, Cao, Shen & Feng, 2015; Shao *et al.*, 2016). Exosomes are released by neoplastic cells, but stromal cells may themselves release exosomes that contribute to tumour progression (Meehan & Vella, 2015).

Figure 12: Functional roles of exosomes in cancer progression.



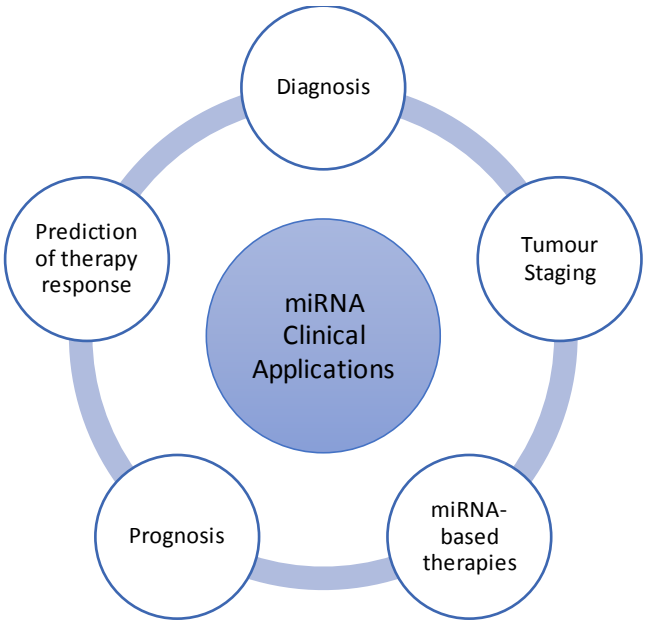
3.4.3. MicroRNAs Clinical Applications

The presence of miRNA in circulation and their dysregulated expression in diseases makes them potential sensitive and non-invasive biomarkers for diagnosis, prediction of therapy response and prognosis in many pathologies (Figure 13), particularly in malignancies (Fleischhacker, Bauersachs, Wehner, Hartman & Weber, 2013). For instance, patients with BC showed significant higher serum miR-155, miR-10b, and miR-195 levels, whereas miR-34b was downregulated, in comparison to controls. Moreover, higher levels of miR-10b were correlated with the existence of distant metastasis and, therefore, poor prognosis (Hagrass *et al.*, 2015). miRNA signatures were also proposed as prognostic biomarkers in lung squamous cell carcinoma (Gao, Wu, Yu & Li, 2016), hepatocellular carcinoma (Ji et *al.*, 2016), colorectal cancer (Yang *et al.*, 2016) and many other cancer types. Some microRNAs have also be suggested as biomarkers for prediction of chemoresistance (Komatsu *et al.*, 2016).

MicroRNAs are also potential targets for treatment, however they have not been implemented in the clinical practice yet due to the lack of concordance across studies; and this can be a result of methodological heterogeneity affecting several steps such as sample preparation, profiling, validation and normalization. To overcome this limitation, optimization of those processes is required (Vigneron *et al.*, 2016). Accordingly, Tiberio *et al.* (2015) reported various preanalytical and analytical factors which were responsible for affecting the detection of miRNAs, including the starting material, hemolysis interference, the extraction methods chosen, detection platforms used for miRNA measurement, normalization and data analysis as

well as individual factors (race, gender, level of physical activity and others). Nevertheless, there are already miRNA-based therapies in human clinical trials (Rupaimoole & Slack, 2017).

Figure 13: miRNA potential clinical applications.



3.4.4. MicroRNAs in Veterinary Medicine

Comparatively to human medicine, there is very few information on miRNA in veterinary medicine. Mature miRNAs for humans, mice, dogs, pigs, cows and horses are listed in the miRbase (http:mirbase.org/), however no data is available for cats. Nevertheless, one study identified putative miRNAs from cats (Sathyamurthy & Swamy, 2010), and other profiled miRNA expression in feline and canine kidneys and further compared the differences found between renal cortex and medulla in both species (Ichii *et al*., 2014). More recently, the feline miRNAome, in normal tissues, was characterized by high-throughput sequencing (Laganà *et al*., 2017).

Some studies portrait miRNAs roles in viral infections, as they can enhance viral transcription and replication and regulate host-pathogen interactions. Specifically, miRNAs are known to play an important role in infections caused by Influenza A virus, Venezuelan equine encephalitis virus, Marek's disease virus, Aujeszjy's disease, rabies and also in prion diseases such as scapie and bovine spongiform encephalopathy (Liu *et al*., 2016; Samir, Vaas & Pessler, 2016). Also, evaluation of miRNAs expression in lungs and trachea of dogs infected with canine influenza virus showed a differential miRNA expression between the infected and non-infected dogs, with five miRNAs (miR-376b, miR-376c, miR-433, miR-487a and miR-490) being

exclusively expressed in the infected samples (Zhao *et al*., 2014). Viral infections clearly alter the miRNA expression profiles of the host, but some viruses also possess their own microRNAs, with Herpesviruses encoding the largest number of them (Tycowski *et al*., 2015).

MiRNAs have also been implicated in parasitic infections, as it was stated by Tritten *et al*. (2014), in which study, miRNAs derived from *Dirofilaria immitis* parasites were detected in peripherical blood of infected dogs and thus provide a diagnosis of the infection. More recently, *Dirofilaria immitis* was found to exhibit sex and stage-specific miRNA profiles (Tritten, Clarke, Timmins, McTier & Geary, 2016). Similarly, *Toxocara canis*, a roundworm causative of toxocariasis in humans, dogs and other animals, exhibits miRNAs that are exclusively transcribed in male or female parasites (Ma *et al*., 2016).

Regarding hepatobiliary diseases, Dirksen *et al*. (2016) suggested a panel of five miRNAs (miR-21, miR-122, miR-126, miR-200c, and miR-222) that allows distinction between parenchymal, biliary and neoplastic hepatobiliary diseases in dogs. These findings are of most value since the current biochemical analysis do not enable this discrimination.

Many other diseases in dogs and cats were associated to dysregulated microRNAs, such as acute pancreatitis (Rouse *et al*., 2017), atrial fibrillation (Zhang *et al*., 2015), myxomatous mitral valve disease (Li, Freeman, Rush & Laflamme, 2015), Golden Retriever muscular dystrophy (Jeanson-Leh *et al*., 2014), diabetes (Fleischhacker et al., 2013), as displayed in Table 5.

Table 5: miRNAs up and downregulated in non-oncologic diseases of dogs and cats

Species	Disease	Samples analyzed	miRNAs ↑	miRNAs ↓	Ref
Dog	Kidney disease	Urine from 47 dogs with kidney disease and 37 healthy controls		miR-26a miR-10a/b	Ichii *et al*., 2017
Dog	Pancreatic injury	Serum collected from 4 dogs before and after drug-induced pancreatic injury	miR-216a miR-206b miR-217 miR-375 miR-148[a]		Rouse *et al*., 2017
Dog	Meningoence-phalomyelitis of unknown origin	Cerebrospinal fluid from 10 dogs with meningoenchephalomyelitis of unknown origin and 8 dogs with non-inflamatory neurological diseases	miR-21 miR-181		Gaitero *et al*., 2016
Dog	Atrial Fibrillation	Tissue from 6 dogs with induced atrial tachypacing and 6 controls	miR-208b miR-206 miR-21 miR-224 miR-451 miR-450b	miR-129 miR-138a miR-340 miR-7 miR-449 miR-203 miR-205 miR-137 miR-124 miR-202	Zhang *et al*., 2015

Table 5: miRNAs up and downregulated in non-oncologic diseases of dogs and cats

(Continuation)

Species	Disease	Samples analyzed	miRNAs ↑	miRNAs ↓	Ref
Dog	Chronic Degenerative valvular disease	Plasma collected from 15 dogs with chronica degenerative valvular disease and 8 controls		miR-30b (in stage B) miR-133b (in stage C)	Hulanicka *et al.*, 2014
Dog	Muscular Dystrophy	Serum collected from 3 dogs with the disease, 3 carriers of the mutation and 3 controls.	miR-1 miR-133a miR-206		Mizuno *et al.*, 2011
Dog	Hepatobiliary diseases	Serum collected from 6 dogs with acute hepatitis, 6 with chronic hepatitis, 5 with mucoceles, 6 with billiary diseases, 5 with congenital postosystemic shunts, 6 with hepatocelular adenoma, 6 with hepatocelular carcinoma, 6 with hepatic lymphoma and 11 controls	miR-122 (in parenchimal, biliary and neoplastic diseases) mir-21 (in mucoceles, chronic hepatitis, hepatocelular carcinoma and lymphoma) miR-222 (in mucoceles and hepatocelular carcinoma)		Dirksen *et al.*, 2016
Dog	Liver damage with hepatotoxicants	Serum from 2 dogs with induced liver necrosis and controls	miR-122 miR-885		Koenig *et al.*, 2016
Cat	Toxoplasmosis	Liver samples from 6 infected cats and 6 controls	miR-21 miR-17 miR-223 miR-27 miR-126 miR-486		Cong *et al.*, 2017
Cat	hypertrophic cardiomyopathy	Serum from 11 diseased cats and 12 controls	miR-381-3p miR-486-3p miR-4751 miR-476c-3p miR-5700 miR-513a-3p miR-320 miR-1246		Weber *et al.*, 2015
Cat	type 2 diabetes mellitus	Serum collected from 8 diabetic, 4 diabetic in remission and 8 healthy cats	miR-122 miR-193b miR-483		Fleischhacker *et al.*, 2013
Cat	Toxoplasmosis	Liver tissue from 6 cats experimentally infected with *Toxoplasma gondi* and 6 controls	miR-21a-5p miR-20a-5p miR-17-5p miR-223-3p miR-27a-5p miR-126 miR-486 miR-30e-3p miR-142a-3p miR-106b-3p		Cong *et al.*, 2017

Besides being implicated in multiple diseases, miRNAs also present differential expressions during normal development stages of certain tissues as it was referred by Genini *et al. (*2014). They showed that fifty miRNAs were differentially expressed during normal retinal development in dogs (between 3 and 7 weeks). Likewise, another study reported that mature and prepubertal dog testes exhibited significantly different miRNAs patterns (Kasimanickam & Kasimanickam, 2015). There are also miRNAs alterations attributed to certain external stimulus. Indeed, chronic stress exposure was found to alter miRNAs expression in dogs (Luo *et al*., 2016). In fact, miRNAs are involved in nearly all biological processes and even a diet alteration can modify its expression as it was verified in a study enrolling overweight pet dogs in a weight loss program based on calorie restriction and physical training or calorie restriction alone. In the last scenario, various miRNAs showed significant alterations before and after the program implementation (Uribe *et al*., 2016).

3.4.5. MicroRNAs in Oncology

miRNAs are classified as oncogenic miRNAs (oncomiRs) if they inhibit the expression of tumor suppressor genes; or as tumor suppressor miRNAs (oncosupressor-miRs) if they target oncogenes. In cancer, oncomiRs are usually upregulated while oncosupressor-miRs are downregulated (Bertoli *et al*., 2015). However, miRNAs may present a dual function, based on the tumor type and cellular context, and some exhibit irregular patterns of expression (Amorim *et al*., 2016). For instance, miR-520c and miR-373 are characterized as oncomiRs in certain types of cancer and as tumor supressors in others. One study evaluated this difference and found they could dual-regulate cellular functions, by affecting the matrix metallopeptidase 2 (MMP2) or 9 (MMP9) genes in different cell types. Therefore, they seem to have different regulation pathways according to the cell type (Lu *et al*., 2015).

Currently, miRNAs are being studied for their multiple roles in cancer including in tumorigenesis, tumor growth, angiogenesis and therapy-resistance (Graveel *et al*., 2015; Liu *et al*., 2015; Zhang *et al*., 2015; Amorim *et al*., 2016). Additionally, miRNAs are involved in regulation of the self-renewal and differentiation properties of cancer stem cells, regulation of EMT (Bertili *et al*., 2015), destruction of vascular endothelial barrier (Signh, Pochampally, Watabe, Lu & Mo, 2014; Zhou *et al*., 2015), enhancement of the pre-metastatic niche (Fong *et al*., 2015), modulation of the tumor immune response (Paladini *et al*., 2016) and, also, modulation of the tumor microenvironment (Chan, Manley, Lee & Singh, 2014; Suzuki, Katsura, Matsuyama & Miyazono, 2015, Wang, Chen, Liu & Tian, 2016). Furthermore, altered metabolism is a well-known common feature in tumors and many miRNAs were reported to

regulate the expression of glucose transporters and enzymes involved in glycolysis, as well as the metabolism of lipids and amino acids (Chan *et al*., 2014). Additionally, miRNA-coding genes are frequently located at fragile sites and in cancer-associated genomic regions (Rossi, Sevignani, Nnadi, Siracusa & Calin, 2008) and there are miRNAs targeting components of the epigenetic machinery, the so-called epi-miRNAs, as is the case of miR-29 family (Amodio *et al*., 2015). All these data reinforce the crucial involvement of miRNAs in oncogenesis.

Due to the altered miRNAs expression observed in cancer cells and due to the specific expression signature exhibited in distinct tumor types, miRNAs show great potential as cancer biomarkers for diagnostic purposes (Sun *et al*., 2015; Xiaoli, Yawei, Lianna, Haifeng & Hui, 2015; Thakur, Grover, Gupta, Yadav & Das, 2016). Additionally, miRNAs can be useful prognostic biomarkers and predictive indicators in several types of cancer, since they are correlated with the clinical outcome, overall survival and disease-free survival (Guo *et al*., 2015; Xiaoli *et al*., 2015; Liao, Wang, Li & Jiang, 2017).

microRNAs are also promising targets for cancer therapy. As they can act as tumor suppressors or oncomiRs, two therapeutic potentials exist: miRNA replacement therapy or down-regulation (silencing) of miRNAs (Gambari, Brognara, Spandidos & Fabbri, 2016). Liu *et al*. (2016) conducted a study in which they showed that artificial miRNA can be used to effectively suppress growth and invasion of BC both *in vitro* and *in vivo*. Another study, used anti-miRNA nanoparticles, resulting in knockdown of miR-21 and consequently tumor growth inhibition. Moreover, no nanoparticle accumulation in healthy organs and tissues was detected and, as so, there were no detectable side effects (Shu *et al*., 2015). More interesting results may be obtained by the application of combined treatments (targeting multiple miRNAs) and the combined administration of conventional antitumoral drugs with miRNA therapeutic agents shows particularly promising results (Gambari *et al*., 2016).

These small molecules may even be useful as biomarkers of chemotherapy response since higher circulating levels of some miRNA, were associated with relapsing, others with good clinical outcomes and other with resistance to multiple forms of cancer treatment, including chemotherapy, anti-endocrine therapy and radiotherapy (Casey, Sweeney, Brown, & Kerin, 2016). It was also revealed that plasma miRNAs profiles before chemotherapy were correlated with the posterior response to treatment in lung cancer patients (Kjersem *et al*., 2013). There is evidence of the importance of miRNAs in multidrug resistance (MDR), since miRNAs can modulate various drug resistance mechanisms, such as overexpression of MDR transporters, defects in cell-cycle, apoptosis and autophagy, alteration of drug metabolism or drug targets and interference with DNA repair (An, Sarmiento, Tan & Zhu, 2017).

Regarding canine and feline cancers, few articles address miRNAs. Nevertheless, in dogs, the results of a comprehensive bioinformatics study revealed that there were significantly more miRNA genes in cancer-associated genomic regions than in all the other regions, similarly to what is observed in the human genome (Zamani-Ahmadmahmudi, 2016), emphasizing the importance and the requirement for more research in this field.

Deregulation of miRNA expression was reported in several canine cancers (Table 6) but, to our knowledge, no studies were yet performed for feline cancer.

Regarding mammary tumours, canine mammary cancer cell lines showed an upregulation of miR-143, miR-145, miR-199, miR-214 and miR-947. In addition, a downregulation of miR-138a, miR-221, miR-222, miR-301a and miR-18b was also identified (Osaki *et al.*, 2016). Others reported miR-21 and miR-29b to be significantly upregulated in canine mammary tumors comparatively to normal mammary tissue (Boogs *et al*., 2008). From a different perspective, analysis of the miRNA expression in canine mammary cancer stem-like cells in comparison to differentiated tumor cells revealed dissimilar expression patterns, with 24 miRNAs being downregulated and 9 upregulated (Rybicka *et al*., 2015). A distinct expression profile in samples from metastatic and primary mammary tumors in dogs was also reported (von Deetzen *et al*., 2014).

Deregulation of microRNAs has also been reported in the following canine cancers: prostate cancer (Kobayashi *et al.*, 2017), B and T-cell lymphoma (Mortarino *et al.*, 2010; Albonico *et al*., 2013; Fukiwara-Igarashi *et al.*, 2015), chronic lymphocytic leukemia (Gioia *et al*., 2011), disseminated histiocytic sarcoma (Borresen *et al*., 2016), hemansiosarcoma (Heishima *et al*., 2015; Grimes *et al*., 2016), osteosarcoma (Fenger *et al.*, 2016), uveal melanoma (Starkey *et al.*, 2017), bladder transitional cell carcinoma (Vinall, Kent & deVere White, 2012) and hepatocellular carcinoma (Dirksen *et al*., 2016).

Table 6: miRNAs up and downregulated in different canine cancers

Species	Cancer type	Samples analyzed	miRNAs ↑	miRNAs ↓	Ref
Dog	Mammary cancer	Tissue from 6 canine mammary tumours and 3 normal mammary glands from healthy dogs	miR-21 miR-29b in all tumor types, miR-181b let-7f in tubular papillary carcinomas	miR-15a and miR-16 (ductal carcinoma)	Boggs *et al.*, 2008
Dog	Mammary cancer	mammary tissue from 30 dogs with benign and malign mammary tumours versus 10 normal mammary gland tissues	miR-210 miR-21 miR-143 miR-194 miR-203	miR-125a	von Deetzen *et al.*, 2014

Table 6: miRNAs up and downregulated in different canine cancers (Continuation)

Species	Cancer type	Samples analyzed	miRNAs ↑	miRNAs ↓	Ref
Dog	Mammary cancer	1 canine mammary tumour cell line	miR-143 miR-145 miR-199 miR-214 miR-947	miR-138a miR-221 miR-222 miR-301a miR-18b	Osaki *et al.*, 2016
Dog	Lymphoma	Sera of 61 dogs with lymphoma and 40 control dogs	miR-423a	let-7b miR-223 miR-25 miR-92a	Fujiwara-Igarashi *et al.*, 2015
Dog	B-cell lymphoma	15 Fresh frozen lymphoma samples and 3 lymph node samples from dogs without hematopoietic diseases; 7 formalin-fixed paraffin embebed (FFPE) lymphoma samples and 5 FFPE non-neoplastic lymph node samples.	miR-17-5p		Mortarino *et al.*, 2010
	T-cell lymphoma		miR-181	miR-29b	
Dog	Hemangiosarcoma	7 splenic hemangiosarcoma tissues and 7 samples of normal splenic tissue; 3 hemangiosarcoma cell lines and 1 normal canine endothelial cell line		miR-214	Heishima *et al.*, 2015
Dog	Disseminated histiocytic sarcoma and carcinoma	Whole blood from dogs with Disseminated histiocytic sarcoma or carcinoma and 7 healthy dogs		let-7g	Borresen *et al.*, 2016
Dog	Osteosarcoma	72 canine fresh osteosarcoma samples; 2 canine osteosarcoma cell lines and one normal canine osteoblasts culture	miR-9		Fenger *et al.*, 2016
Dog	Hepatocellular carcinoma	Tissues from 6 dogs with hepatocellular carcinoma, 40 with other hepatobiliary diseases and 10 from normal canine liver	miR-200c miR-122 miR-21 miR-222		Dirksen *et al.*, 2016

Table 6: miRNAs up and downregulated in different canine cancers (Continuation)

Species	Cancer type	Samples analyzed	miRNAs ↑	miRNAs ↓	Ref
Dog	Transitional cell carcinoma (urinary bladder)	4 normal, 13 bladders with non-neoplasic inflammatory disease and 18 transitional cell carcinoma FFPE samples.	miR-34a miR-106b miR-16 miR-103b		Vinall *et al.*, 2012
Dog	Oral Malignant melanoma	23 canine oral malignant melanoma tissue samples and 4 canine malignant melanoma cell lines in comparison to 11 normal oral mucosa tissues		miR-145	Noguchi *et al.*, 2012
Dog	Oral malignant melanoma	26 oral canine malignant melanoma tissue samples, 4 canine malignant melanoma cell lines and 11 normal oral mucosa tissues	miR-520c-3p	miR-205 miR-203 miR-126 miR-200a	Noguchi *et al.*, 2013
Dog	Uveal melanoma (metastasizing melanoma versus non-metastasizing)	8 metastasizing and 12 non-metastasizing uveal melanoma FFPE tissue samples	miR-124 miR-130b miR-155 miR-182 miR-362 miR-4430 miR-4454 miR-4742-5p miR-500a miR-548c	miR-2287 miR-2381 miR-2398 miR-2411	Starkey *et al.*, 2017
Dog	Thyroid and mammary carcinoma, osteosarcoma, histiocytic sarcoma, chondrosarcoma, hemangiosarcoma	Plasma samples from 169 dogs with oncogenic diseases and 22 controls	miR-214		Heishima *et al.*, 2017
	Mammary, hepatocellular, squamous cell, thyroid, transitional cell carcinomas and adenocarcinomas, osteosarcoma, mast cell tumor, melanoma, hemangiosarcoma		miR-126		

3.4.6. MicroRNA-based Therapies

Because cancer is a multigenic disease, miRNA-based therapies offer the advantage of one miRNA being able to target multiple genes and resistance to miRNA-based therapies would require multiple mutations in multiple genes, which is unlikely (Ling, Fabbri & Calin, 2013).

Besides, miRNA also regulate genes encoding for epigenetic machinery and thus are capable of regulating DNA methylation, for example, that is altered in cancer. However, as previously mentioned, miRNAs can exert different effects in different cell types and miRNA-based therapies can have potential deleterious effects in certain tissues. As so, means to deliver miRNAs only to targeted cells are required.

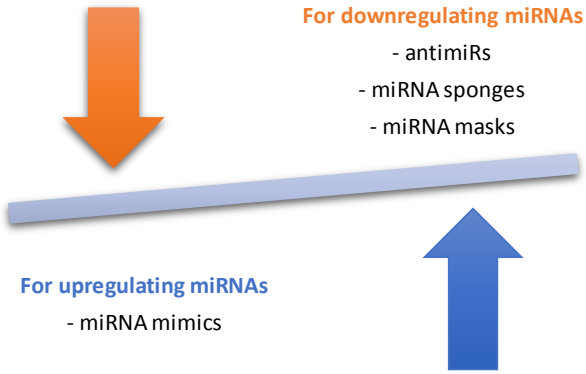
Although a deeper understanding is needed, miRNA research opens new optimistic perspectives for cancer therapy, as well for many other diseases. Indeed, some miRNA-based therapies developed for human diseases are already in clinical trials, including an antimiR-122 for hepatitis C, antimiR-103/107 for type 2 diabetes, antimiR-155 for cutaneous T-cell lymphomas and mycosis fungoides, miR-29 mimic for scleroderma, miR-16 mimic for mesothelioma and non-small cell lung cancer and, finally, miR-34 mimic for various solid tumours (Rupaimoole & Slack, 2017).

miRNA-based therapeutics (Figure 14) present two possible approaches: miRNA replacement therapy, to replace depleted miRNAs; or miRNA inhibition therapy when the therapeutic target is an overexpressed microRNA (Broderick & Zamore, 2011). miRNA replacement therapy relies on miRNA mimics which are synthetic double-stranded oligonucleotides that possess the miRNA sequence of interest and exert the corresponding effect, in other words, they behave like endogenous miRNAs. These molecules suffer endogenous processing into the final therapeutic product by RISC, resulting in single strand oligonucleotides, mimicking the miRNA mature strand (Bader, Brown, Stoudemire & Lammers 2011). Initially, naked RNAs were used however they have a very short half-time due to the abundance of ribonucleases in the blood stream. Therefore, for improving stability, as well as delivery and efficiency, chemical modifications and new approaches were developed (Rupaimoole & Slack, 2017).

On the other hand, miRNA inhibition therapy relies on antimiRs (antisense oligonucleotides), miRNA sponges or miRNA masks. AntimiRs are single stranded antisense oligonucleotides targeting oncomiRs, that bind to the matching miRNA, blocking its function. miRNA sponges are long nucleic acids (DNA constructs) with strong promoters and multiple miRNA binding motives, thereby sequestrating the targeted miRNAs and consequently upregulating the expression of the respective mRNA targets. The miRNA sponges present the advantage of being able to inhibit various miRNAs simultaneously (Hayes, Peruzzi & Lawler, 2014). Moreover, inserting different miRNA binding sites generates a sponge that can inhibit multiple miRNAs with different seed sequences (Jung *et al*., 2015). The miRNA sponge technology may rely on viral or non-viral vectors to deliver the miRNA sponge cassette to the cells (Tay, Lim, Zhu, Hin & Wang, 2014). Interestingly, miRNA sponges were developed before the discovery of naturally occurring RNA products that act like miRNA sponge, the competing endogenous

RNAs (ceRNAs). Various types of RNAs were reported to act as ceRNAs: circular RNAs, pseudogene-derived lncRNAs, others lncRNAs and viral ncRNAs (Thomson & Dinger, 2016). Finally, miRNA masks are modified single stranded RNAs complementary to a target site in a given mRNA, therefore masking that binding site and preventing the action of miRNAs (Murakami & Miyagisgi, 2014).

Figure 14: Potential miRNA-based therapies



Furthermore, certain compounds can interfere with miRNA's biogenesis machinery. For instance, enoxacin, a fluoroquinolone antibiotic, interacts with a component of RISC, thereby altering the miRNA/mRNA regulation however it does not target one specific miRNA (Baumann & Winkler, 2014).

Regarding veterinary medicine, Anis *et al*. (2016) evaluated the use of miRNAs against feline infectious peritonitis, a disease caused by the feline coronavirus (FCoV). They constructed three types of lentivirus, each expressing a different anti-FCoV miRNA (miR-L2, miR-L1 and miR-N) and verified that they successfully integrate the miRNA-coding DNA into the genome of feline cells, in vitro. Furthermore, all the lentivirus caused a reduction in FCoV production compared with negative controls and the inhibition of viral replication was most significant in feline cells expressing miR-L2, with a reduction of 92% in virus production. In another study, the production of an attenuated vaccine, using an influenza virus as a model which was engineered to express an artificial miR-93, resulted in the production of mature miRNAs in mammalian cells. Intranasal immunization of mice with that virus conferred cross-protective immunity against heterologous influenza virus stains (Li *et al*., 2015).

miRNAs have also been tested as therapeutic tolls in bone defects. Bone marrow stem cells were transfected with lentiviral vectors encoding miR-31, anti-mir-31 and a negative control. The cells were then implanted into bone defects in dogs. Over time, computed tomography (CT) scans were performed and the results were confirmed after 16 weeks by histological analysis. Results revealed a much better recovery for the anti-miR-31 group, showing that suppression

of miR-31 expression can be used to efficiently repair bone defects in dogs (Deng, Zhou, Gu & Fan, 2014).

3.4.7. Importance of MicroRNAs in Comparative Oncology

The progresses made in veterinary medicine may be translated to human medicine with cats and dogs serving as models of spontaneous occurring tumours, opposing to the traditional murine models. In this perspective, miRNAs provide a novel opportunity for comparative oncology studies, because they are encoded by highly conserved genes across mammalian species. For instance, conserved miRNA signatures in specific heart structures across rat, Beagle dog and Cynomolgus monkey were identified (Vacchi-Suzzi *et al*., 2013). Also, the characterization of the feline miRNAome in normal tissues revealed that from the 31 miRNA clusters found in cats, 28 were partially or totally conserved comparatively to humans (Laganà *et al.*, 2017). On the other hand, once some miRNAs were identified as being specific to a particular group of organisms or even to a single species, it is possible that those miRNAs may be related with phenotypic diversity (Glazov, McWilliam, Barris & Dalrymple, 2008). In fact, there is substantial evidence that miRNAs play an important role in evolution and the number of miRNAs in the genome seems to correlate with morphological complexity of the organisms (Dweep *et al.*, 2013). Nevertheless, most of the currently known miRNAs are conserved among species. For instance, it was reported that 50% of the mature feline miRNAs evaluated, displayed 100% homology to human sequences (Weber *et al.*, 2015). Also, an evolutionary analysis of miR-675, showed that both pre-miRNA and mature miRNAs were well-conserved phylogenetically between human, canine and equine species (Guo *et al*., 2014). Moreover, due to the high homology between miRNAs in dogs and humans, many of human miRNA assays can be applied for analysis of canine miRNAs expression (Uhl, Krimer, Schliekelman, Tompkins & Suter, 2011; Wagner *et al.*, 2013). Humans and dogs also share similar miRNA profiles in some types of cancer, including mammary tumors, melanomas, osteosarcomas and lymphomas (Wagner *et al.*, 2013). Because most miRNAs are evolutionary conserved with cats and dogs sharing many physiological mechanisms, pathological conditions and similar environmental risks with humans, it is reasonable to assume them as proper models of spontaneous occurring tumors for microRNAs studies. Indeed, different research groups found similar miRNAs signatures between human and canine tumors.

Kabir *et al*. (2015) identified a group of miRNAs (miR-21, miR-155, miR-9, miR-34a, miR143/145 and miR-31) that was altered in both canine mammary tumors and human breast cancer. Regarding malignant melanoma, miR-145 was reported to be significantly downregulated in canine malignant melanoma tissues and cell lines as well as in human melanoma cells. Also, the ectopic expression of miR-145 led to growth inhibition in both canine

and human melanoma cells (Noguchi *et al*., 2012). Downregulation of miR-205 and miR-203 was also reported in human and canine malignant melanoma cells (Noguchi *et al*., 2013) and as miR-203 directly targeted cyclic AMP-responsive element-binding protein 1 (CREB1), effects of transfection with miR-203 and knockdown with CREB1 were investigated. Both displayed the same outcome: suppression of canine and human melanoma cells, therefore showing that the expression pattern and function of miR-203 was homologous between dogs and humans (Noguchi *et al.*, 2014). More recently, Noguchi *et al*. (2015) showed that there was DNA methylation of the CpG islands upstream of the miR-203 coding region in human, canine melanoma cells and in canine melanoma specimens, opposing to human normal melanocytes.

In addition, the downregulation of miRNAs at the 14q32 locus significantly contributes to osteosarcoma pathology. Thayanithy *et al*. (2012) examined the role of epigenetic events controlling the transcription of that locus by using a histone deacetylase inhibitor and a DNA methylation inhibitor in both human and canine osteosarcoma cell lines and obtained comparable effects of cytotoxicity. Moreover, cell lines presenting more aggressive gene expression profiles, were more sensitive to the compounds.

Finally, microRNA homology was also found between human, mouse and dog stem cells. Hayes *et al*. (2008) characterized canine embryonic stem cells (ESC) and showed that they expressed a cluster of miRNAs associated with pluripotency (miR-302b, miR-302c and miR-367) typical of human and mouse ESC.

4. Objectives and Hypothesis

In dogs there are some articles evaluating miRNAs in cancer but, to our knowledge, this is the first study addressing microRNAs in feline cancers. Therefore, the objectives of the present research were:

1. To evaluate the usefulness of microRNAs as diagnostic biomarkers for FMC;
2. To investigate the value of microRNAs as prognostic biomarkers for FMC;
3. To search for significant associations between serum microRNAs levels and clinicopathological characteristics (presence or absence of metastasis, necrosis, lymphatic invasion, lymphocytic infiltration and multiple mammary tumours; clinical stage, histological classification, histological grade, PT size, LNS, serum SDF-1 levels, CXCR4 status of the PT and metastasis, OS and DFS);
4. To compare our results to what is reported in humans, in order to give insight in whether cats are a suitable model for comparative oncology, in what concerns microRNAs.

We hypothesized that quantification of the chosen microRNAs (miR-21, let-7a, miR-200b, miR-200c, miR-10b and miR-121) in serum would allow discrimination of healthy cats from

cats with FMC and/or could be associated with clinicopathological features and prognosis. We further speculate that dysregulation of microRNA serum levels could be similar to what is observed in humans, due to the high homology between FMC and BC in several features.

5. Materials and Methods

5.1. Sample Characterization

In the present study, 50 serum samples were used, 45 from female cats with mammary carcinomas and 5 from healthy controls. These samples were collected, together with the PT and drainage LN, at Hospital Escolar Veterinário – Faculdade de Medicina Veterinária, Universidade de Lisboa, with the written consent of the owners, as described by Soares *et al.* (2016a). The exclusion criteria were the presence of concomitant diseases. For previous studies, tumours were characterized regarding the presence of necrosis, ulceration, lymphatic invasion, lymphocytic infiltration and metastasis, LNS, PT size, histological classification (Table 7), molecular classification, Ki-67, HER2, PR, ER and OS and DFS were also recorded. Later, CXCR4 and SDF-1 status of the PT and metastasis were also evaluated, as well as serum SDF-1 levels which were classified as negative or positive according with a cut off value of 2 ng/ml (Marques, Soares, Santos, Correia & Ferreira, 2017). After collection and preparation, serum samples were stored at -80ºC until use.

Table 7: Histopathological classification of the mammary tumours collected.

Histopatological classification	Tubulopapillary carcinoma	Solid carcinoma	Tubular carcinoma	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma
Number of cases	15	9	9	5	5	2

5.2. Experimental Design

Serum samples collected from cats with MT were stored at the Pathology department of Faculdade de Medicina Veterinária – Universidade de Lisboa, and used to quantify miRNA levels by the sequential procedures illustrated in Figure 15.

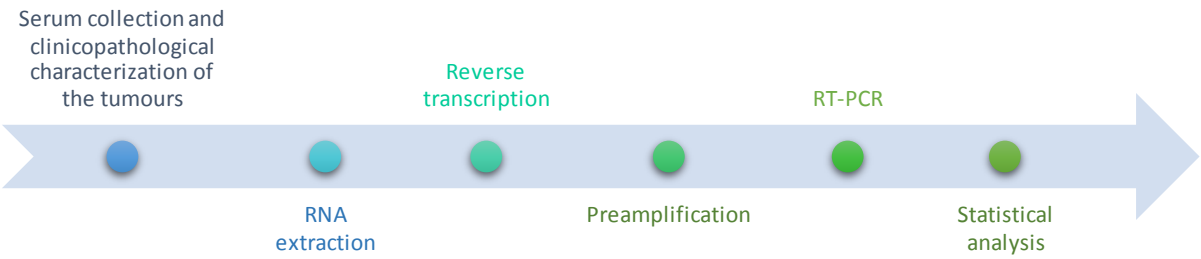
The miRNAs chosen to be evaluated (Table 8) were selected based on data reported in humans (number of published articles, consistency of results, considered as potential biomarkers for BC) and on the homology of sequences between the feline and the equivalent human miRNA, evaluated by Weber *et al.* (2015). Moreover, the selected miRNAs present 100% of sequence homology for the corresponding mature sequences and, thereby, the primers used were commercial primers designed for human microRNAs, the miScript Primer Assays (Qiagen ™, Germany).

Table 8: Selected microRNAs and their potential value as biomarkers in BC

microRNA	Dysregulation in BC	Involvement in the hallmarks of cancer	Reported clinical associations	Potential biomarker	References
let-7a	Downregulated	Insensitivity to antigrowth signals, replicative immortality (stemness), apoptosis evasion	Associated with metastasis, poor OS and DFS, and larger tumour sizes	Diagnostic and prognostic biomarker	Quesne & Caldas, 2010; Feng *et al.*, 2012 Serguienko *et al.*, 2014; Elghoroury *et al.*, 2017;
miR-21	Upregulated	Cell proliferation, apoptosis evasion, invasiveness and metastizing ability	Associated with higher clinical stages and histological grades, LN metastasis and short OS and DFS	Diagnostic and prognostic biomarker	Yan *et al.*, 2008; Asaga et al., 2011; Wang *et al.*, 2015
miR-200b	Downregulated	Invasiveness and metastizing ability (regulates EMT), sustained cell growth, evasion to apoptosis	Associated with poor OS and DFS, with invasiveness and metastasis	Prognostic biomarker	Ye *et al.*, 2014; Quesne & Caldas, 2010
miR-200c	Downregulated	Invasiveness and metastizing ability (regulates EMT), evasion to apoptosis	Associated with LN metastasis and poor OS	Prognostic biomarker	Quesne & Caldas, 2010; Berber *et al.*, 2014; Ren *et al.*, 2014; Kawaguchi *et al.*, 2017
miR-10b	Upregulated in metastatic BC	Invasiveness and metastizing ability (involved in cell migration and ECM remodelling)	Strongly associated with metastatic behaviour	Prognostic biomarker	Ma, 2010; Ahmand et al., 2014; Singh et al., 2014; Kim *et al.*, 2016

There is not accordance across studies concerning to what endogenous controls should be used for normalization of miRNA expression. Nonetheless, the miRNAs used as reference genes (miR-191 and miR484) were selected because they were reported as reliable serum reference genes for BC by Hu *et al.* (2012).

Figure 15: Procedures performed to quantify miRNA levels in serum of cats with mammary carcinoma and healthy animals.



5.3. RNA Extraction

Extraction of total RNA from serum samples was performed using the miRNeasy Serum/Plasma kit (Qiagen®, Germany) and following the manufacturer's recommendations. A volume of 10 µl from each serum sample was diluted in 40 µl of PBS and 250 µl of Qiazol Lysis Reagent was added. After incubation at room temperature for 5 minutes, 50 µl of chloroform was added, then shacked vigorously and left at room temperature for 3 minutes. Afterwards, it was centrifuged at 12000g, for 15 minutes, at 4ºC and subsequently the aqueous superior phase was transferred to a new 2ml tube. Followed an addition of 225 µl of 100% ethanol and all the solution was transferred to a RNEasy MinEtute spin column and centrifuged at 8000g for 15 seconds, discarding the flow-throw. 700 µl of Buffer RWT was pipetted to the columns, centrifuged at 8000g during 15 seconds at room temperature and the flow-throw was discarded. A volume of 500 µl of Buffer RPE was pipetted to the column, centrifuged at 8000g, for 15 seconds and the flow-throw was discarded. Then, 500 µl of 80% ethanol was added to the column and centrifuged at 8000g, for 2 minutes, discarding the tube with the flow-throw. The column was placed in a new 2 ml tube and it was centrifuged, with the lids open, at 17000g for 5 minutes and at the end the tube was discarded together with the flow-throw. The column was placed in a new 1,5 ml tube and 14 µl of RNase-free water was added directly to the centre of the column membrane. It was centrifuged at 17000g for 1 minute and the column was discarded. For measuring the total RNA concentration, 1 µl of the final solution was used (NanoDrop 2000c Thermo Scientific, United States of America).

5.4. Reverse Transcription Reaction

The reverse transcription of the total RNA obtained from the previous procedure was performed with the miScript II RT Kit (Qiagen®, Germany). A master mix was prepared on ice, by adding 2 µl of Hispec Buffer, 1 µl of Nucleics Mix, 4,5 µl RNase-free water and 1 µl of miScript Reverse Transcriptase Mix. A volume of 8,5 µl from the master mix was pipetted to each tube and the respective template RNA was added, mixing. Afterwards, the samples were placed in the thermocycler Doppio (VWR™, USA) to be submitted to the following program: 37ºC during 60 min followed by 95ºC for 5 minutes. After the reverse transcription being concluded, 1 µl of each sample was used to read the absorbance on NanoDrop 2000c; and a dilution was performed in 36 µl de of RNase-free water.

5.5. Preamplification Reaction

Due to the small volume of sample initially (10 µl) used and in order to optimize the subsequent steps, a preamplification reaction was performed prior to the real-time PCR (RT-PCR), using

the miScript PreAmp PCR Kit (Qiagen®, Germany). A master mix was prepared at room temperature, by adding 5 µl of miScript PreAmp Buffer, 2 µl of HotStaTaq DNA Polymerase, 5 µl of miScript PreAmp Primer Mix, 7 µl of RNase-free water and 1 µl of miScript Universal Primer. From the mater mix 20 µl was placed in each tube followed by the addition of 5 µl of the respective template complementary DNA (cDNA) obtained from the reverse transcription reaction. The samples were placed in the thermocycler Doppio (VWR™, USA) under the following program: initial step of 15 minutes at 95ºC and 12 cycles of 30 seconds at 94ºC and 3 minutes at 60ºC. The preamplified cDNA was diluted in 100 µl of RNase-free water and stored at freezing temperature (-20ºC) until the RT-PCR was performed.

5.6. RT-PCR

RT-PCR plates of 96 wells were used. In order to obtain valid and comparable results between plates and within the same plate, samples of healthy cats and cats with FMC were displayed in each plate, with duplicates, and 3 miRNAs (one miRNA of interest and 2 endogenous controls) were measured in each plate. Negative controls were always performed for each miRNA in duplicates.

We used miScript SYBR® Green PCR Kit (Qiagen®, Germany) and 7 miScript Primer Assays (Qiagen®, Germany), each specific for every miRNA chosen. The reaction mix was prepared by adding 12,5 µl pf QuantiTect SYBR Green PCR Master Mix, 2,5 µl of miScript Universal Primer and 2,5 µl of the respective miScript Primer Assay. The 6,5 µl of RNase-free water was added to 1,5 µl of cDNA. A volume of 17,5 µl of the reaction mix was deposited in each well, followed by 7,5 µl of the cDNA with the water. The plates were sealed, centrifuged at 1000g for 1 minute, at room temperature. The RT-PCR instrument (StepOnePlus™ Real-Time PCR system) was programmed according to the manufacturer's recommendations: 95ºC during 15 minutes followed by 40 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds and 70ºC for 30 seconds. A melting curve analysis was also performed.

5.7. Normalization and Analysis of RT-PCR Data

A relative quantification of the microRNAs was intended, therefore two reference miRNAs (miR-191 and miR-484) were selected and their amounts measured. To quantify the relative changes of gene expression in the samples from cats with FMC comparatively to the healthy controls, we applied the $2^{-\Delta\Delta C_T}$ method which includes a normalization to an endogenous reference gene, or in this case two, allowing correction of results for differing amounts of RNA input (Kenneth & Schmittgen, 2001). The calculations were performed as referred by

Schmittgen & Livak (2008). Briefly, the fold change in expression between diseased and healthy animals is given by the $2^{-\Delta\Delta C_T}$ that is obtained by the following equation:

$$2^{-\Delta\Delta C_T} = \Delta C_{T\,(\text{sample A})} - \text{mean}\ \Delta C_{T\,(\text{samples from healthy controls})}$$

The ΔC_T was calculated as follows:

$$\Delta C_{T\,(\text{sample A})} = C_{T\,(\text{gene of interest})} - \text{Mean}\ C_{T\,(\text{reference genes})}$$

$2^{-\Delta\Delta C_T}$ values <1 imply there is a reduction in the expression rather than an upregulation, so the inverse of $2^{-\Delta\Delta C_T}$ needs to be calculated for providing the fold change reduction (Schmittgen & Livak, 2008). To represent individual samples, $2^{-\Delta C_T}$ values were used, as recommended by Schmittgen & Livak (2008).

5.8. Statistical Analyses

For statistical analysis, the program GraphPad Prism 7 (USA) was used and a *p* value of 0,05 was considered significant. To identify outliers, the ROUT analysis was performed, with outliers being excluded in each analysis.

Normality was tested with the Kolmogorov-Smirnov test, since the application of parametric or non-parametric tests relies upon the presence of a normal distribution or not. Although some authors refer that, for large samples (over 30 or 40 observations), one can assume that the distribution approaches normality, others reinforce the importance of evaluating the distribution independently of the sample size. When the sample does not follow a normal distribution, non-parametric tests are indicated since they are more conservative and are associated to less possibility of obtaining incorrect conclusions (Ghasemi & Zahediasl, 2012; Nahm, 2016).

The non-parametric Mann-Whitney test was used for comparing the miRNA expression in diseased and healthy animals and ROC analysis was applied to determine the sensitivity and specificity of the microRNAs in discriminating the FMC group from the control group.

Mann-Whitney test was also used to search for associations between miRNA expression and the CXCR4 and SDF-1 status, the size class, the presence or absence of multiple mammary tumours, necrosis, lymphatic invasion, lymphocytic infiltration, and LNS. To search for associations between miRNA expression and molecular subtypes, clinical stages and histological subtypes of FMC, the non-parametric one-way ANOVA Kruskal-Wallis test was used. For correlating the PT size and SDF-1 serum levels with miRNA expression, the non-parametric Spearmen test was performed. Finally, for comparison of survival curves (OS and DFS), the long-rank(Mantel-Cox) test was used.

6. Results

Although for the majority of miRNAs, two reference genes (miR-191 and miR-484) were used for relative quantification, for miR-200c only miR-191 was employed as reference gene due to contaminations of miR-484 negative controls.

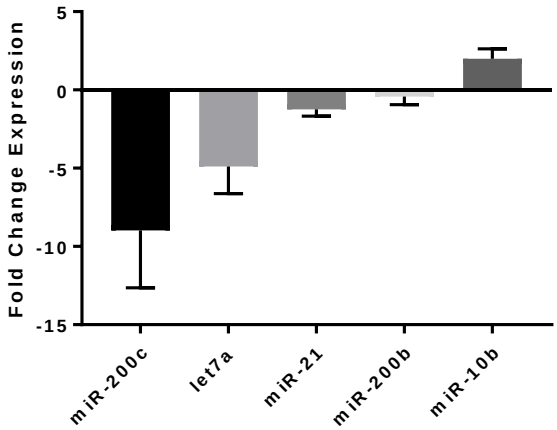
The normality was tested for all miRNAs, using the Kolmogorov-Smirnov test, and none followed a normal distribution (p<0,0001). Therefore, non-parametric tests were applied and results were considered statistically significant when p<0,05. In graphical representations, * indicates p<0,05 while ** indicates p<0,01.

In the OS and DFS curves, cats with MT were stratified in two groups: cats with the serum miRNA upregulated and cats with the serum miRNA downregulated, comparing to healthy cats.

All the descriptive statistics, detailed results of statistical tests and graphical representations of not-significant variables are displayed in ANNEX II.

The fold expression of each miRNA in FMC comparatively to controls is illustrated at Figure 16. The miRNA-200c was downregulated 8,98-fold, the let-7a had a downregulation of 4,89-fold, the miR-21 of 1,24 and the miR-200b of 0,42-fold. Only the miR-10b was upregulated 1,98-fold.

Figure 16: Fold change expression of each miRNA in cats with MT. This figure illustrates the number of times each miRNA is increased or diminished in cats with MT comparing to controls



6.1. Results obtained for let-7a

Statistical significant differences (p=0,04) were found between serum let-7a levels in cats with MT (mean $\pm$ standard deviation: 0,03 $\pm$ 0,04; n=37) and healthy controls (0,10 $\pm$ 0,09;

n=5), with the mean fold expression in cats with mammary carcinomas, given by $2^{-\Delta\Delta C_T}$, being 4,89-folds lower than in the control group (Figure 17). In addition, the ability of serum let-7a levels to discriminate cats with MT from healthy controls presented a specificity of 80% and sensitivity of 64.86% (AUC=0,78; *p*=0,04; 95% CI=47,46% to 79,79%) (Figure 18).

Figure 17: Serum let-7a levels in cats with MT and healthy controls

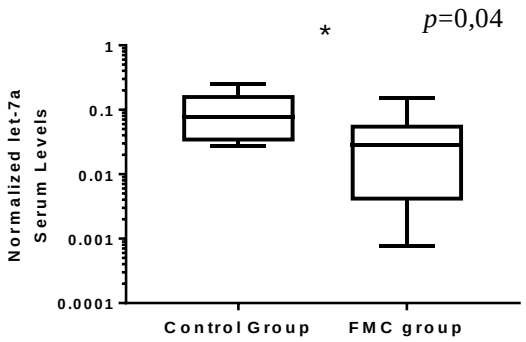
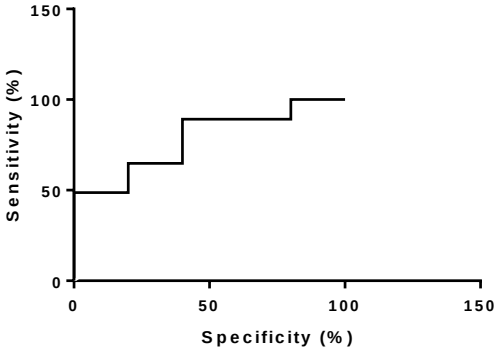
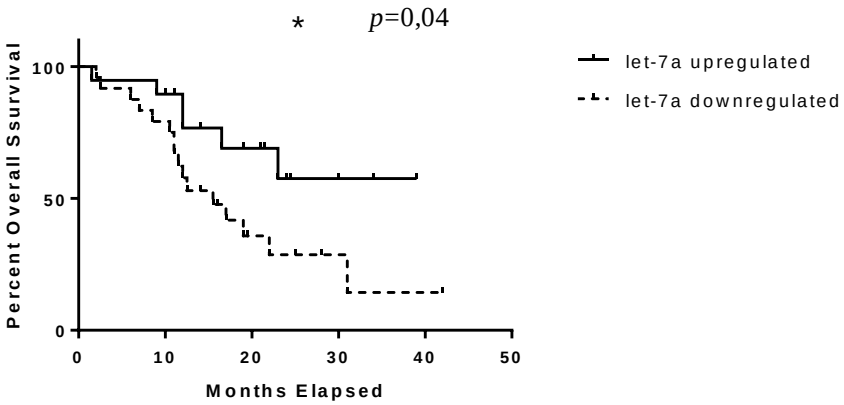


Figure 18: ROC curve for serum let-7a levels



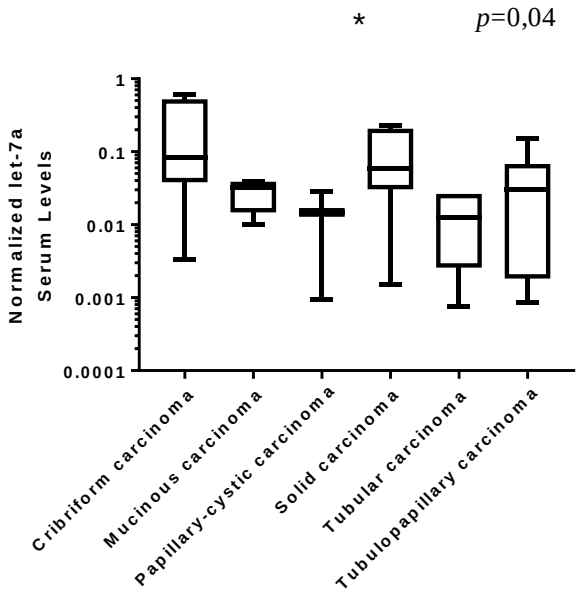
Regarding OS, survival curve analysis showed that serum let-7a levels are significantly associated with survival times of cats with mammary carcinomas (*p*=0,04). Indeed, cats with higher serum let-7a lived longer (mean survival=18,05 months; *n*=19) comparing to those showing lower levels of let-7a (mean survival=15,27 months; *n*=22) (Figure 19).

Figure 19: OS of cats with serum let-7a up and downregulated



Concerning the histological classification (*p*=0,04), serum let-7a levels were higher in cats with cribriform carcinomas (0,240 ± 0,26; *n*=5) and in solid carcinomas (0,10 ± 0,09; *n*=8), than in the other subtypes: mucinous carcinoma (0,03 ± 0,01; *n*=4), papillary-cystic carcinoma (0,01 ± 0,01; *n*=2), tubular carcinoma (0,01 ± 0,01; *n*=7) and tubulopapillary carcinoma (0,04 ± 0,04; *n*=15) (Figure 20).

Figure 20: Serum let-7a levels in cats with different histological subtypes of FMC



Additionally, an inverse correlation was found between serum SDF-1 levels and let-7a (p=0,03; Spearman r = -0,34) (Figure 21). No other associations reached significance for this miRNA. Nevertheless, although not statistically significant, an inverse association between let-7a levels and PT SDF-1 status (p=0,10) was found, with negative SDF-1 status being associated with higher serum let-7a levels (0,07 $\pm$ 0,07; n=15) and vice-versa (0,03$\pm$ 0,03; n=24) (Figure 22).

Figure 21: Correlation of serum SDF-1 concentration with serum let-7a levels

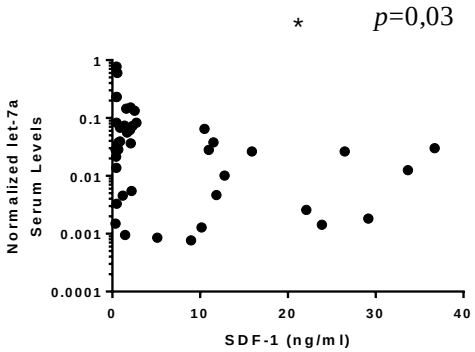
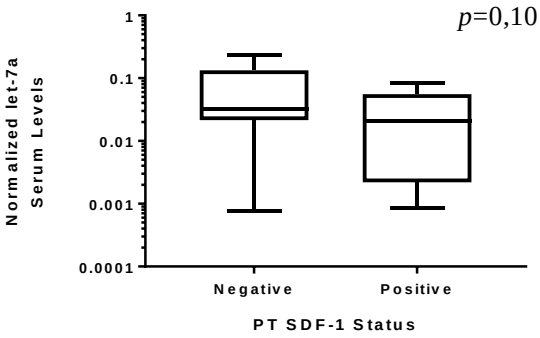
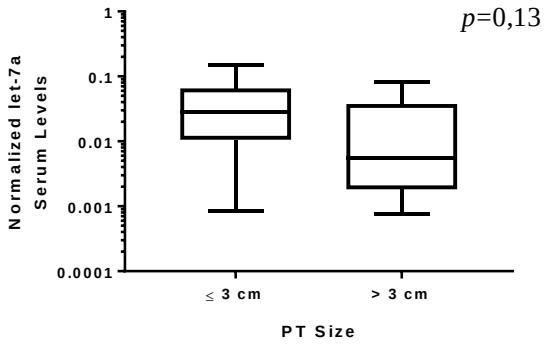


Figure 22: Serum let-7a levels according with the SDF-1 status of the PT



Moreover, also approaching significance was the PT size (p=0,13). PT sizes $\leq$ 3 cm were associated with higher levels of let-7a (0,04 $\pm$ 0,03; n=26), while PT > 3cm were associated with lower circulating levels of let-7a (0,02$\pm$ 0,03; n=11) (Figure 23).

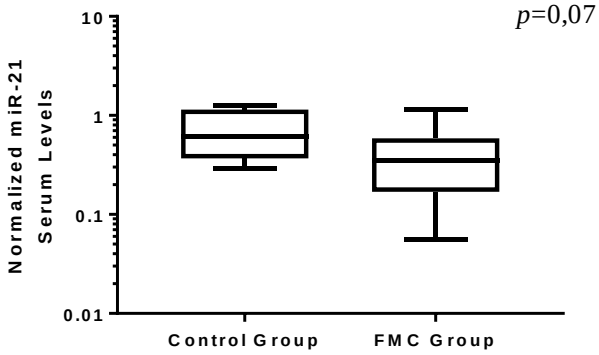
Figure 23: Serum let-7a levels according with the PT size stratified into two classes



6.2. Results obtained for miR-21

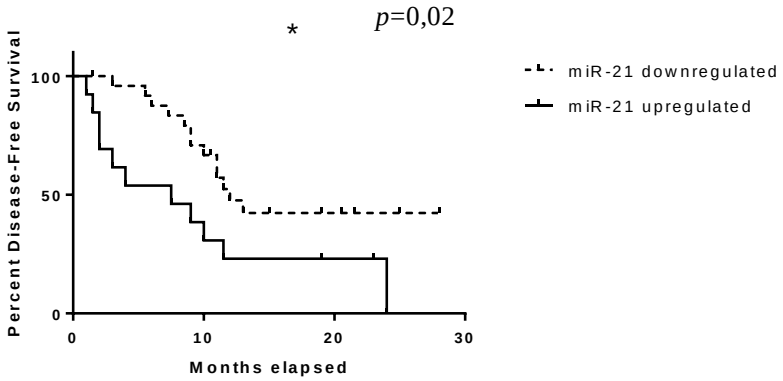
Serum miR-21 levels were found to be lower in cats with MT (0,42 ± 0,29; *n*=37), comparing to the control group (0,73 ± 0,42; *n*=5) (*p*=0,07), with a mean fold expression of -1,24 (Figure 24).

Figure 24: Serum miR-21 levels in cats with MT and healthy controls



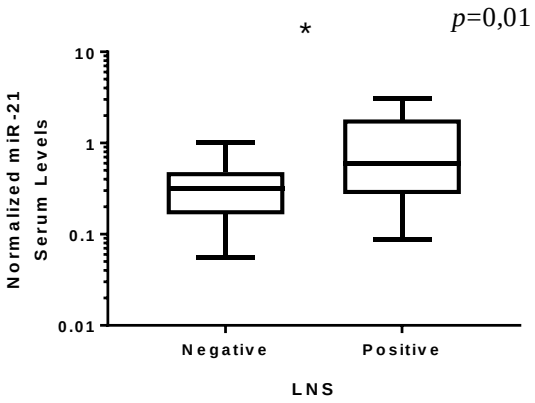
Survival curves analysis showed that miR-21 upregulation was significantly associated (*p*=0,02) with poor DFS, with cats showing higher serum miR-21 levels presenting a mean DFS of 8,39 months (*n*=15), whereas those with a downregulation of miR-21 had longer DFS (mean DFS: 12,47 months, *n*=27) (Figure 25).

Figure 25: DFS of cats with serum miR-21 up and downregulated



Association of miR-21 and the LNS also reached statistical significance (p=0,01). Positivity to LNS was associated with higher circulating miR-21 levels (1,00 $\pm$ 0,92; n=16), contrasting with the negative LNS (0,36 $\pm$ 0,27; n=22) (Figure 26).

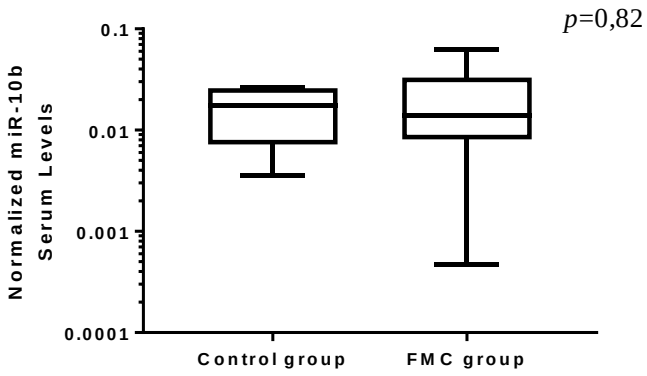
Figure 26: Association of LNS with serum miR-21 levels



6.3. Results obtained for miR-10b

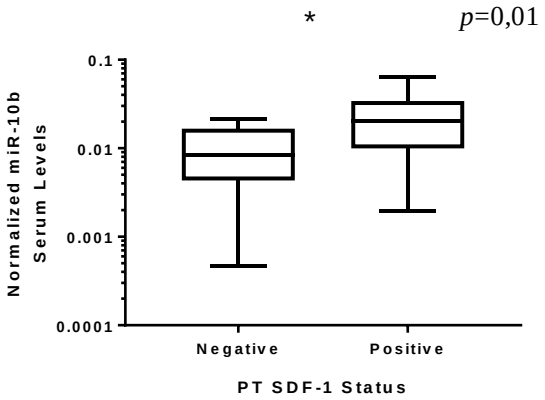
No significant differences (p=0,82) were found for serum miR-10b levels between the FMC group (0,02 $\pm$ 0,02; n=40) and healthy controls (0,02 $\pm$ 0,01; n=5) (Figure 27). However, there as a slightly tendency for being upregulated in diseased cats (1,98 fold higher).

Figure 27: Serum miR-10b levels in FMC and healthy groups



Regarding the SDF-1 status in the PT, positivity was significantly associated (p=0,01) with higher serum miR-10b levels (0,02 ± 0,02; n= 25), whereas cats with negative SDF-1 status presented lower miR-10b levels (0,01 ± 0,01; n=12) (Figure 28).

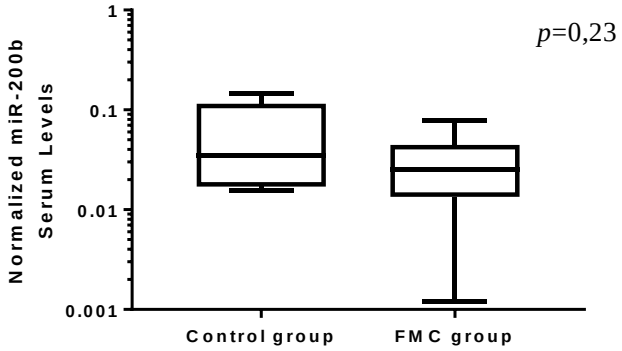
Figure 28: Serum miR-10b levels according with the SDF-1 status of the PT



6.4. Results obtained for miR-200b

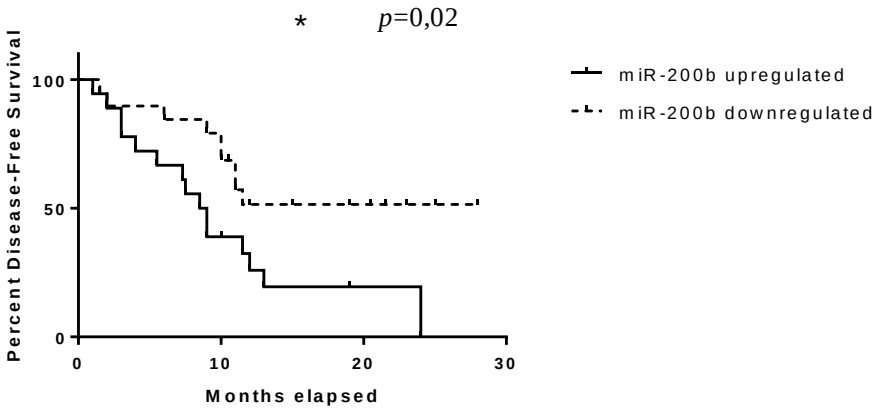
Despite no statistically significant differences (p=0,23) were found between the FMC (0,03 ± 0,02; n=40) and the control group (0,06 ± 0,06; n=5), a mean downregulation of 0,42-fold for miR-200b was found in cats with MT (Figure 29).

Figure 29: Serum miR-200b levels in FMC and healthy groups



On the other hand, miR-200b serum levels were significantly predictive of DFS (p=0,02). Cats with higher miR-200b levels had worse DFS (9,02 months, n=18) than cats with miR-200b downregulation (12,71 months, n=21) (Figure 30).

Figure 30: DFS of cats with serum miR-200b up and downregulated



A positive correlation was found between miR-200b serum levels and the PT size (p=0,04, Spearman r=0,31; n=45) (Figure 31). Also, when stratifying by size, using a cut off value of 3 cm, miR-200b serum levels are increased in PT > 3 cm (0,05 $\pm$ 0,04; n=12), comparing to tumours $\leq$ 3cm (0,03 $\pm$ 0,02; n=30) with a p value of 0,05 (Figure 32).

Figure 31: Correlation of serum miR-200b levels with the PT size

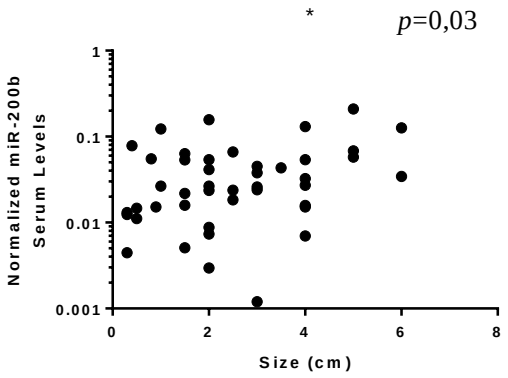
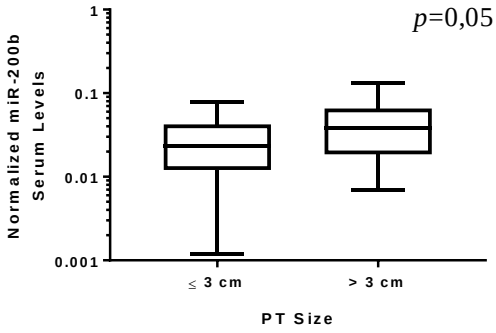
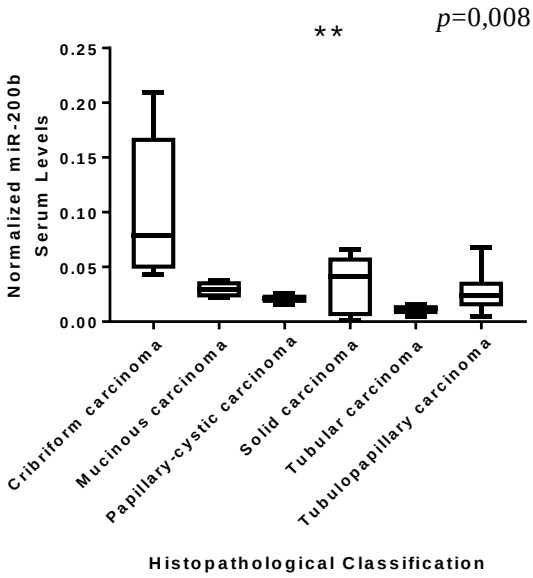


Figure 32: Serum miR-200b levels according with the PT size stratified into two classes



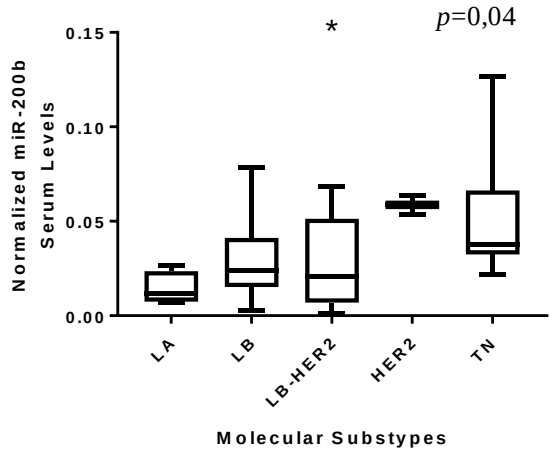
Amongst the different histological subtypes, significant differences in serum miR-200b levels were detected (p=0,008). Cats with cribriform carcinoma (0,10 ± 0,07; n=5) showed the highest serum miR-200 levels, followed by cats with solid carcinoma (0,03 ± 0,03; n=9), mucinous carcinoma (0,03 ± 0,01; n=4), tubulopapillary carcinoma (0,03 ± 0,02; n=13), papillary-cystic carcinoma (0,02 ± 0,01; n=2) and, finally, tubular carcinoma (0,01 ± 0,004; n=7) (Figure 33).

Figure 33: Serum miR-200b levels in cats with different histological subtypes of FMC



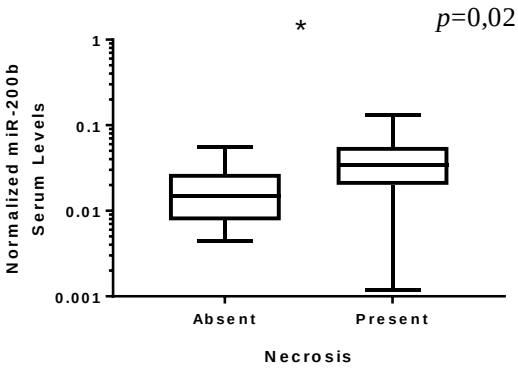
Differences in miR-200b serum levels were also found amongst the different molecular subtypes, with a p value of 0,04. Cats with HER2-positive (0,06 ± 0,01; n=2) and TN tumours (0,05 ± 0,04; n=7) showed higher miR-200b levels than the other molecular subtypes: LA (0,01 ± 0,01; n=4), LB (0,03 ± 0,02; n=16) and LB-HER2 (0,03 ± 0,02; n=12) (Figure 34).

Figure 34: Serum miR-200b levels in cats with MT stratified by molecular subtypes



Moreover, higher circulating levels of miR-200b were significantly associated (p=0,02) with the tumour necrosis (0,04 ± 0,04; n=25), whereas cats with no necrosis on the PT presented lower levels (0,02 ± 0,02; n=16) (Figure 35).

Figure 35: Serum miR-200b levels is cats according with the presence or absence of necrosis in the PT



6.5. Results obtained for miR-200c

A significant downregulation (8,98-fold) of miR-200c was found in cats with MT (0,05 ± 0,06; n=37), comparatively to controls (0,11 ± 0,07; n=5), with a p value of 0,045 (Figure 36). The ROC analysis showed an AUC of 0,78, with a specificity of 80% and sensitivity of 62,16% (95% CI=0,5787 to 0,978) (Figure 37).

Figure 36: Serum miR-200c levels in cats with MT and healthy controls

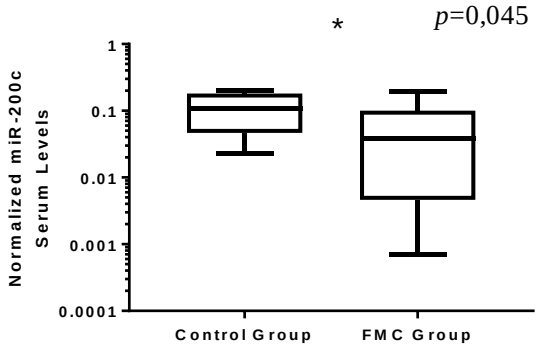
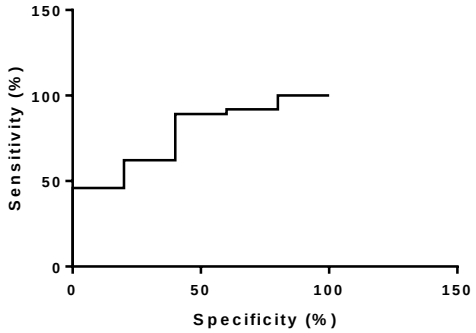
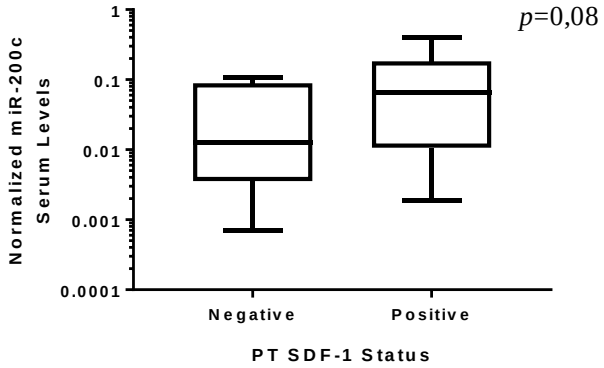


Figure 37: ROC curve for serum miR-200c levels



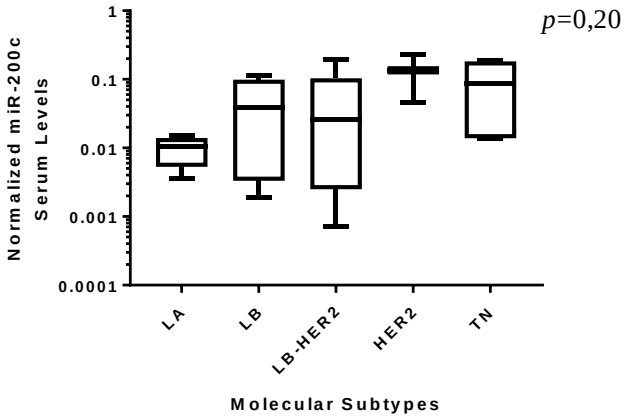
Although not statistically significant, a clear tendency (p=0,08) was found between cats with higher serum miR-200c levels and positive PT SDF-1 status (0,10 ± 0,11; n=26), whereas those with negative SDF-1 status presented lower circulating miR-200c levels (0,04 ± 0,04; n=14) (Figure 38).

Figure 38: Serum miR-200c levels according with the SDF-1 status of the PT



Despite not being statistically significant (p=0,20), cats with HER2-positive (0,14 ± 0,13; n=2) and TN tumours (0,09 ± 0,07; n=7) had higher serum miR-200c levels than cats with MT from the remaining subtypes: LA (0,01 ± 0,005; n=4), LB (0,05 ± 0,05; n=15) and LB-HER2 (0,06± 0,07; n=10) (Figure 39).

Figure 39: Serum miR-200c levels of cats with MT stratified by molecular subtypes



7. Discussion

In accordance to what is consistently reported for many cancer types in humans (Thammaiah & Jayaram, 2016), let-7a was found to be significantly downregulated in FMC (4,89-fold lower than in control cats). Let-7a is a known tumour suppressor miRNA that directly represses the following oncogenes: STAT3, RAS family, c-MYC, high-mobility group A (HMGA), Janus Protein Tyrosine Kinase (JAK), BLIMP1 and others involved in cell cycle regulation, apoptosis and cell adhesion (Wang et al., 2012; Blazeau, Menezes, Cao & Hagan, 2017). Indeed, Kim *et al*. (2012) reported that the use of let-7a mimics led to decreased proliferation, migration and invasion of BC cells, suggesting that the commonly found downregulation of let-7a contributes to tumour growth and progression. In accordance, other studies showed that BC cells transfected with let-7a presented repression of cell proliferation, migration and invasion, as well as increased sensitivity to doxorubicin (Serguienko *et al.*, 2014; Liu *et al.*, 2015).

Furthermore, the high expression of two RNA binding proteins (LIN28A and LIN28B) in undifferentiated tissues, inhibits the biogenesis of the let-7 family. As differentiation occurs, the expression of these proteins is lost and a subsequent increase of let-7a levels occurs. However, in cancer cells, there is a high expression of LIN28A and LIN28B that leads to pluripotency and de-differentiation and diminishes let-7 levels (ANNEX III) which, in turn, results in activation of several oncogenes (Blazeau, Menezes, Cao & Hagan, 2017). Our results show, for the first time, that let-7a (a member of let-7 family) is also significantly downregulated in serum from cats with MT and, as a result, might be useful for diagnosis purposes and, eventually, as a therapeutic target.

In the present study, a significant association between serum let-7a levels and OS in FMC was also uncovered, with lower serum let-7a levels being associated to shorter OS. Corroborating our results, Liu *et al*. (2016) also reported lower serum let-7a levels to reflect shorter OS and

DFS in colorectal cancer patients and Feng *et al.* (2012) reported lower let-7a tumour expression to be associated with poor OS in BC patients.

We further found let-7a and both serum and tissue SDF-1 status to be inversely associated. In accordance, Xiao *et al.* (2017) treated pancreatic cancer cells with SDF-1 and observed a 50% reduction of let-7a levels. Also, Chen *et al.* (2013), reported that let-7a was downregulated by SDF-1-mediated CXCR4 activation and identified the transcription factor Yin Yang 1 as a link between let-7a and the SDF-1/CXCR4 axis. Therefore, it appears that SDF-1 causes a reduction of let-7a levels, although the exact mechanism is not fully elucidated.

Furthermore, we found that cats with PT >3 cm had lower serum let-7a levels. Accordingly, high Lin28 tissue expression and, consequently, low let-7a were associated with larger tumour size in BC patients (Feng *et al.*, 2012) and in other types of cancer as well, as is the case of colorectal cancer, where lower let-7a expression, both in tissue and serum, was reported to correspond to larger tumours (Liu et al., 2016).

Concerning miR-21, this molecule is considered an oncomiR since it targets programmed cell death 4 (PDCD4), BCL2 and PTEN, which are tumour suppressor genes involved in apoptosis induction and cell cycle arrest (Buscaglia & Li, 2011). Indeed, overexpression of miR-21 is commonly reported in many cancer types, including in tissue and serum samples from BC patients (Yan *et al*., 2011; Li *et al*., 2013; Medimegh *et al*., 2014; Shi, 2016; Han *et al*., 2017). Against our expectations, we found the opposite in cats with MT. However, and in accordance with our results, Shin *et al.* (2015), that also used miR-484 as a reference gene for normalization, reported a downregulation of miR-21 in BC patients, both in serum and tumour samples. Moreover, bioinformatic analysis of miR-21 targets revealed pairing sequences with STAT3, a transcription factor, that is frequently activated in tumours (Zhang *et al*., 2016). Using cells transfected with miR-21 mimics and inhibitors it was observed that miR-21 overexpression led to a decreased STAT3 expression, thereby reducing cell proliferation. These findings suggest that even though miR-21 is considered an oncomiR due to its roles in inhibiting tumour suppressor genes, it may also block the expression of the STAT3 oncogene and, thus, possesses a dual function (Zhang *et al*., 2016). Moreover, a previous study (Yan *et al.*, 2008), also predicted the following molecules as miR-21 targets: RAB6A and RAB6B oncogenes, transforming growth factor-beta-induced protein (TGFβI), transforming growth factor beta receptor II (TGFβRII) and v-sik sarcoma viral oncogene homolog (SKI). Since microRNAs inhibit the translation or induce mRNA cleavage of their targets, miR-21 may repress the expression of the mentioned oncogenes. As so, a downregulation of miR-21 would result in the expression of the above oncogenes and, as a result, contribute to oncogenesis and tumour

progression. Nevertheless, more data is required to fully elucidate the roles of miR-21. Also, it can not be excluded that the divergence of our results from the majority of studies in humans can also be due to the lack of accordance in which miRNA to be used for normalization, or due to degradation of RNA during collection, preparation and handling of sera. Indeed, although miRNAs are much more stable than mRNA, one study evaluated miRNA levels in canine serum and plasma stored at room temperature for 1 hour comparatively to storage for 24 hours and found the levels to be significantly different, which indicates that miRNA degradation occurs over time and that minor differences in sample collection and processing can lead to different results (Enelund, Nielson & Cirera, 2017). In addition, each miRNA precursor gives rise to various mature miRNAs, the previously mentioned isomiRs. Although quantitative PCR methods applied to miRNAs possess high sensitivity, they can also pick up signals from related isomiRs, misleading the quantification of the targeted miRNA. This can be an important issue, since these isoforms of the same seed sequence may have different functions and targets and can even allow distinction between tissue type and disease subtypes. RNA sequencing is the only technique that avoids this issue (Magee, Telonis, Cherlin, Rigoutsos & Londin, 2017). Finally, the serum miR-21 downregulation in FMC may be a specificity of cats, since, so far, there are no studies evaluating miR-21 functions in felines.

Nevertheless, regarding the LNS, there was a significant association with serum miR-21 levels, implying that an upregulation of miR-21 is associated with lymph node metastasis and, therefore, a worse prognosis. This finding is as would be expected, since most studies in human BC refer so (Yan *et al.*, 2008; Asaga *et al.*, 2011). Furthermore, statistically significant results were also obtained for the association of miR-21 and DFS, with higher serum levels predicting shorter DFS in cats with MT. Accordingly, in BC patients, several studies reported that miR-21 upregulation was significantly predictive of poor OS (Jinling, Sijing, Jie & Guinian, 2016) and shorter DFS (Mackenzie *et al*., 2014; Wang, Zhang, Pan, Ma & Zhang, 2015), corroborating our results in cats. So, despite being downregulated in cats with MT comparing to healthy controls, higher serum miR-21 levels within the diseased group seem to reflect poor prognosis, as is reported in BC patients.

Regarding miR-10b, although not statistically significant, an upregulation of 1,98-fold was detected serum of cats with MT. In BC patients, miR-10b upregulation seems to closely reflect metastatic behaviour and metastatic BC cell lines present much higher expression levels of miR-10b than non-metastatic BC cell lines and normal epithelial mammary cells (Ma, 2010). Acordingly, miR-10b knockout mice showed delayed onset of metastasis and fewer cancer circulating cells, supporting the role of miR-10b in metastasis promotion (Kim *et al*., 2016).

Also, overexpression of miR10b in non-metastatic BC cells led to invasion and metastasis in a mice model (Ma, Teruya-Feldstein & Weinberg, 2007). One of the reasons for the importance of miR-10b in metastasis is that miR-10b can directly suppress Homeobox 10 (HOXD10) translation (Singh, Pochampally, Watabe, Lu & Mo, 2014), which is responsible for inhibiting the transcription of genes involved in cell migration and ECM remodelling (Ma, 2010). Moreover, M'hamed *et al*. (2015) showed that miR-10b is involved in downregulating the expression of BRCA1, a tumour suppressor gene that is often inactivated in TNBC. However, we did not find significant associations of miR-10b with metastasis in FMC. Contradicting the majority of studies, Moriarty *et al*. (2010) inhibited miR-10b in BC cells and observed increased migration, and they pointed out this might be explained by the hypothesis that miRNAs functions depend on the environmental context. Indeed, Erhard *et al*. (2014), showed that, depending on the cellular context, miRNA/target interactions may or may not occur, even if the miRNA and its target are both being expressed. What seems to be the major contributors to the "cellular context" are the quantities of the miRNA and its target mRNAs in each cell. Additionally, since each miRNA possesses several targets, the availability of each target mRNA determines the final effect. The complexity of these interactions can further be enhanced by the involvement of RNA-binding proteins, which may prevent or induce miRNA binding to the corresponding mRNAs (Erhard *et al*., 2014). Therefore, results obtained across studies appear, sometimes, contradicting and this may explain why we did not find significant associations with the presence of metastasis. Nevertheless, further studies are required to fully elucidate the value of miR-10b value in feline metastatic disease.

A significant association was found between higher serum miR-10b levels and positivity for SDF-1 in the PT. Although there are no studies reporting or explaining this association, CXCR4 activation by SDF-1 is linked to EMT in cancer cells and elicits the dissemination of cancer cells through the lymphatic system, mediated by a SDF-1 gradient (Karlsson, Gonzalez, Welin & Fuxe, 2017). Moreover, inflammatory cytokines, as TNF- and IL-1, produced by tumour-infiltrating leukocytes, activate SDF-1/CXCR4 axis, which upregulates VEGF and, consequently, contributes to angiogenesis (Zhang, Zhu & Li, 2017). So, SDF-1 is positively related to metastatic dissemination and angiogenesis. miR-10b is considered a pro-metastatic microRNA and, interestingly, it was also reported to be positively correlated with microvessel density in mammary tumours and, hence, mir-10b appears to be related to angiogenesis (Liu, Guan, Wang & Niu, 2017). Therefore, miR-10b and SDF-1 may, somehow, be related since miR-10b is considered a pro-metastatic and, possibly, pro-angiogenic miRNA and SDF-1 is

also involved in migration of cancer cells and angiogenesis promotion, partially corroborating our data.

Our results showed a downregulation of miR-200b (of 0,42-fold) and a significant downregulation of miR-200c (of 9,98-fold) in serum of cats with MT, as is typically reported in BC patients. It is as would be expected since the miR-200 family has tumour suppressive roles and is responsible for the maintenance of an epithelial phenotype, preventing EMT. Indeed, induced overexpression of miR-200b or knockdown of its target oncogenes RAB21, RAB23, RAB18 and RAB3B resulted in repression of proliferation and invasion in BC cell lines (Ye *et al.*, 2014). Ren *et al*. (2014), also showed that miR-200 overexpression resulted in increased apoptosis *in vivo*. Additionally, miR-200 target the mRNA of E-cadherin transcriptional repressors, the Zinc Finger E-Box Binding Homeobox 1 (ZEB1) and 2 (ZEB2), which means high levels of miR-200 promote E-cadherin overexpression, essential for maintaining an epithelial phenotype (Park, Gaur, Lengyel & Peter, 2008). Therefore, miR-200 upregulation, which maintains an epithelial phenotype, has been considered as a good prognostic factor. Indeed, in BC, miR-200 expression is typically lost in more aggressive TN carcinomas (Howe, Cocjrane, Cittelly & Richer, 2012; Berber *et al*., 2014). Moreover, lower miR-200b levels were also associated with poor OS, shorter DFS (Ye *et al*., 2014) and with LN metastasis (Berber *et al*., 2014). However, we found the opposite: that higher levels, within the diseased group, were significantly associated with poor prognosis (shorter DFS, larger PT size, presence of necrosis). Moreover, higher serum miR-200b levels were found in cats with TN or HER2-overexpressing subtypes, which are considered of poor prognosis. A significant association was also found with histopathologic subtypes, with elevated serum levels in cribriform and solid carcinomas, which are generaly considered the more aggressive. Therefore, all our findings associate higher serum miR-200b levels with aggressive features and poor prognosis in FMC. Accordingly, Antolín *et al*. (2015) also found miR-200c to be downregulated in blood samples of BC patients and, within the diseased group higher miR-200c levels were associated with higher stage, poor OS and shorter DFS, which is in contrast with the previously referred studies but corroborates our data. Also, Le *et al*. (2014) showed that miRNAs from the miR-200 family are secreted by mouse metastatic MT cells and, when they injected those cells overexpressing miR-200c in mice with mammary tumours, they observed an increased number of macometastasis in lungs. Moreover, antagonizing miR-200c led to inhibition of this prometastatic effect. What can explain these data is the fact that, although initially PT cells require the acquisition of mesenchymal traits for invasiveness and motility and thus escape from the PT, once they reach distant sites, the reserve process (mesenchymal-epithelial

transition – MET), that is promoted by miR-200, is what seems to facilitate colonization (Le *et al*., 2014). In fact, metastatic lesions of carcinomas often share the same epithelial nature of their PT counterpart and, sometimes, the PT is more undifferentiated than metastasis, themselves. Moreover, several studies have reported that distant metastasis of BC express E-cadherin and that there is E-cadherin re-expression at metastatic lesion arising from low or negative E-cadherin PT (Yao, Dai & Peng, 2011; Gunasinghe, Wells, Thompson & Hugo, 2012). Therefore, some authors hypothesize that EMT is a critical step for the initial transformation of benign tumours into invasive carcinomas, but MET is what conveys the ability to colonize the metastatic niches (Yao, Dai & Peng, 2011) (ANNEX IV). So, higher serum miR-200b levels may reflect the phase when MET must occur for metastasis to prevail and, as a result, are associated with poor prognosis.

Concerning miR-200c, significant associations with clinicopathological features were not found. It may be due to the fact that, for miR-200c, only one reference gene (miR-191) was used for normalization, due to contaminations in the negative controls of miR-484. Therefore, miR-191 alone may not be as reliable for normalization as the combination of miR-191 with miR-484.

Nevertheless, higher serum miR-200c levels were associated, almost significantly, with a positive SDF-1 status in the PT; and cats with TN e HER2-ovexerpressing tumours presented higher serum miR-200c levels. The association between the molecular classification and serum miR-200c levels follows the same pattern as for miR-200b, reinforcing that the upregulation of the miR-200 family is associated with poor prognosis. Regarding the SDF-1, the literature reports the opposite of our results. Beji et al. (2017) showed that CXCR4 upregulation was mediated by the miR-200c/ZEB1 pathway and SDF-1 treatment in mice led to reduction of miR-200c levels. Our results may, therefore, reflect a state were SDF-1 is being produced at the PT as a negative feedback response to higher serum miR-200c levels, but further research is required to fully explain the role of miR-200c in cat.

To better show the similarities of the microRNAs evaluated between women with BC and the female cats enrolled in this study, the results are systematized in the ANNEX V.

8. Conclusion

This study aimed to reduce a gap in scientific literature, since there are no published papers evaluating miRNAs in feline cancers. A dysregulation of serum microRNAs in FMC comparing to healthy controls was found, as expected. Particularly, miR-200c and let-7a were significantly downregulated in FMC and allowed discrimination of healthy and diseased cats, making them

putative diagnostic biomarkers of FMC. Moreover, lower serum let-7a levels were significantly associated with poor OS, reflecting the potential of let-7a to be used as a prognostic biomarker. Regarding miR-21, high serum levels were significantly associated with poor DFS and lymph node metastasis, which suggests miR-21 is correlated with metastization and is a poor prognostic factor in FMC. Furthermore, miR-200b was significantly predictive of DFS, associated with the presence of necrosis and positively correlated with the tumour size. Also, increased miR-200b levels were found in the more aggressive molecular and histological subtypes. As a result, miR-200b appears to be a good prognostic biomarker in FMC.

Besides evaluating the miRNA serum levels in FMC and associate them with clinicopathological features, this study also aimed to compare the results obtained to what is reported in humans. Most of our finding were, in fact, in accordance with what is reported for BC patients, however miR-10b in FMC did not appear to reflect metastatic behaviour.

In conlcusion, this study verified there is a dysregulation of microRNAs in FMC, identified potential diagnostic (let-7a and miR-200c) and prognostic (let-7a, miR-21 and miR-200b) biomarkers and reinforced FMC as a suitable model for BC in comparative oncology studies.

Nevertheless, further studies are required in FMC, since this was the first study addressing miRNAs in any feline cancer. Namely, it would be interesting to evaluate other miRNAs considered as biomarkers in BC, investigate if there is indeed a direct correlation between tissue and serum microRNAs levels and, most important, consider the application of miRNA mimics or antagonists in cell cultures, followed by in vivo studies accordingly with the results, in order to develop miRNA-based therapies.

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ANNEX I – Participations as Co-author in Scientific Articles and Absctracts for Congresses

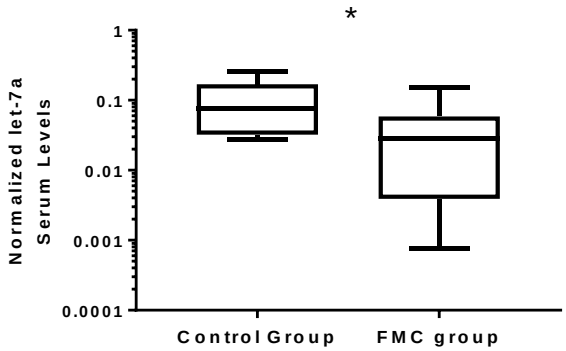
Marques, C., Soares, M., Santos, A., Correia, J., Ferreira, F. 2017. Serum SDF-1 levels are a reliable diagnostic marker of feline mammary carcinoma, discriminating HER2-overexpressing tumors from other subtypes. *Oncotarget*, 8 (62): 105775-89. doi:10.18632/oncotarget.22398; Impact factor: 5.168

Development of a predictive miRNA signature for feline mammary carcinoma. ESVONC European Society of Veterinary Oncology Congress 2018 - Gran Canaria.

The chemokine CXCL12 and its receptor CXCR4 exhibit distinct expression profiles in primary tumors and metastases from cats with mammary carcinoma. ESVONC European Society of Veterinary Oncology Congress 2018 - Gran Canaria.

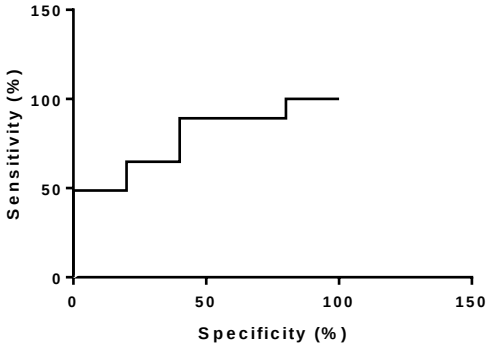
ANNEX II – All Results of the Statistical Analysis

Results for let-7a



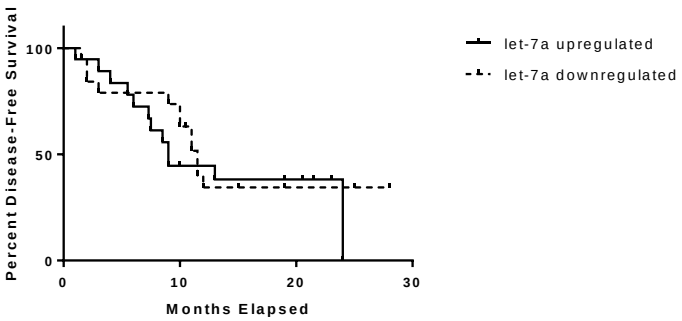
Mann Whitney test	
P value	0,0407
One- or two-tailed P value?	Two-tailed
Median of column A	0,07667, n=5
Median of column B	0,02801, n=37

	Control Group	FMC group
Number of values	5	37
Minimum	0,02741	0,0007667
25% Percentile	0,03214	0,003919
Median	0,07667	0,02801
75% Percentile	0,1686	0,05882
Maximum	0,2545	0,1516
Mean	0,09564	0,03475
Std. Deviation	0,09201	0,03682
Std. Error of Mean	0,04115	0,006053
Lower 95% CI	-0,01861	0,02248
Upper 95% CI	0,2099	0,04703
Mean ranks	32	20,08

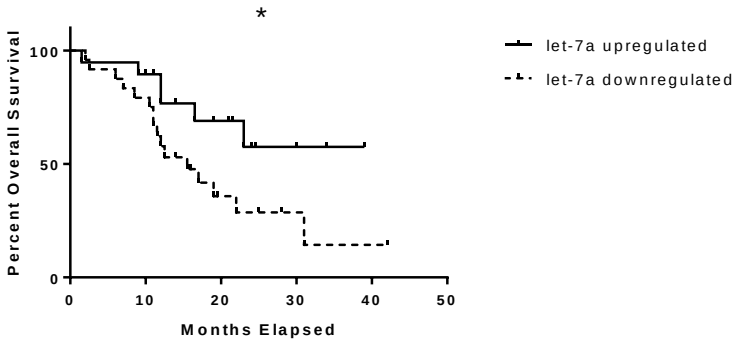


Area under the ROC curve	
Area	0,7838
Std. Error	0,09624
95% confidence interval	0,5952 to 0,9724
P value	0,0414
Controls (Control Group)	5
Patients (FMC group)	37

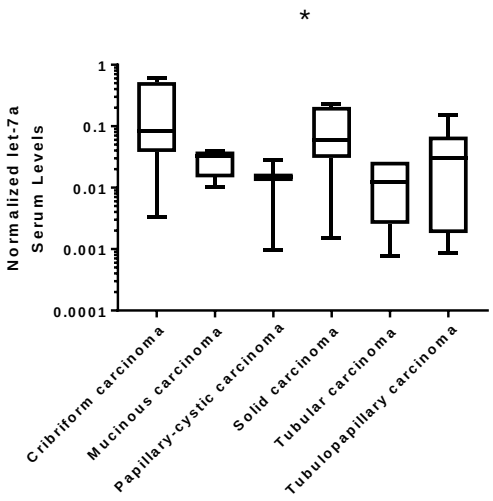
Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.0008103	2,703	0,0684% to 14,16%	100	47,82% to 100%	
< 0.0009033	5,405	0,6615% to 18,19%	100	47,82% to 100%	
< 0.00112	8,108	1,704% to 21,91%	100	47,82% to 100%	
< 0.001358	10,81	3,025% to 25,42%	100	47,82% to 100%	
< 0.001465	13,51	4,537% to 28,77%	100	47,82% to 100%	
< 0.001663	16,22	6,193% to 32,01%	100	47,82% to 100%	
< 0.002202	18,92	7,962% to 35,16%	100	47,82% to 100%	
< 0.002939	21,62	9,827% to 38,21%	100	47,82% to 100%	
< 0.003919	24,32	11,77% to 41,2%	100	47,82% to 100%	
< 0.0046	27,03	13,79% to 44,12%	100	47,82% to 100%	
< 0.005067	29,73	15,87% to 46,98%	100	47,82% to 100%	
< 0.007815	32,43	18,01% to 49,79%	100	47,82% to 100%	
< 0.01136	35,14	20,21% to 52,54%	100	47,82% to 100%	
< 0.01317	37,84	22,46% to 55,24%	100	47,82% to 100%	
< 0.01763	40,54	24,75% to 57,9%	100	47,82% to 100%	
< 0.02392	43,24	27,1% to 60,51%	100	47,82% to 100%	
< 0.02641	45,95	29,49% to 63,08%	100	47,82% to 100%	
< 0.02694	48,65	31,92% to 65,6%	100	47,82% to 100%	
< 0.02771	48,65	31,92% to 65,6%	80	28,36% to 99,49%	2,432
< 0.02822	51,35	34,4% to 68,08%	80	28,36% to 99,49%	2,568
< 0.02843	54,05	36,92% to 70,51%	80	28,36% to 99,49%	2,703
< 0.02921	56,76	39,49% to 72,9%	80	28,36% to 99,49%	2,838
< 0.03092	59,46	42,1% to 75,25%	80	28,36% to 99,49%	2,973
< 0.03421	62,16	44,76% to 77,54%	80	28,36% to 99,49%	3,108
< 0.03672	64,86	47,46% to 79,79%	80	28,36% to 99,49%	3,243
< 0.03721	64,86	47,46% to 79,79%	60	14,66% to 94,73%	1,622
< 0.0377	67,57	50,21% to 81,99%	60	14,66% to 94,73%	1,689
< 0.03863	70,27	53,02% to 84,13%	60	14,66% to 94,73%	1,757
< 0.04769	72,97	55,88% to 86,21%	60	14,66% to 94,73%	1,824
< 0.05882	75,68	58,8% to 88,23%	60	14,66% to 94,73%	1,892
< 0.06316	78,38	61,79% to 90,17%	60	14,66% to 94,73%	1,959
< 0.06628	81,08	64,84% to 92,04%	60	14,66% to 94,73%	2,027
< 0.07036	83,78	67,99% to 93,81%	60	14,66% to 94,73%	2,095
< 0.07333	86,49	71,23% to 95,46%	60	14,66% to 94,73%	2,162
< 0.07525	89,19	74,58% to 96,97%	60	14,66% to 94,73%	2,23
< 0.07972	89,19	74,58% to 96,97%	40	5,274% to 85,34%	1,486
< 0.08288	89,19	74,58% to 96,97%	20	0,5051% to 71,64%	1,115
< 0.08304	91,89	78,09% to 98,3%	20	0,5051% to 71,64%	1,149
< 0.1082	94,59	81,81% to 99,34%	20	0,5051% to 71,64%	1,182
< 0.1425	97,3	85,84% to 99,93%	20	0,5051% to 71,64%	1,216
< 0.203	100	90,51% to 100%	20	0,5051% to 71,64%	1,25



Comparison of Survival Curves	
Log-rank (Mantel-Cox) test	
Chi square	0,2469
P value	0,6192

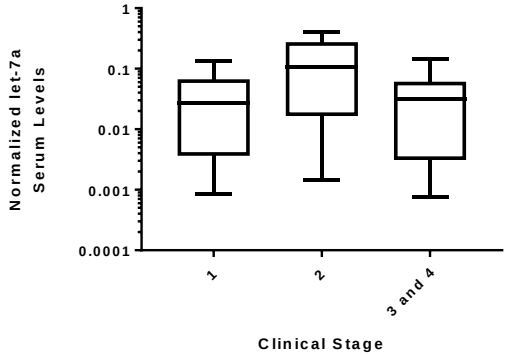


Log-rank (Mantel-Cox) test	
Chi square	4,254
P value	0,0392



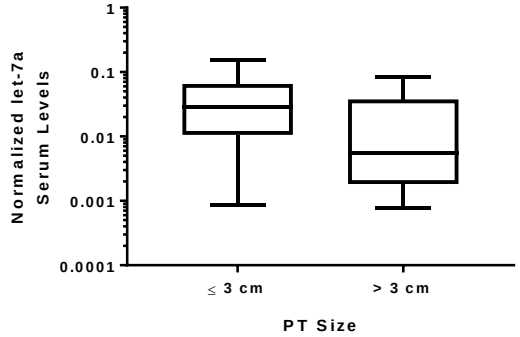
Kruskal-Wallis test	
P value	0,0408
Number of treatments (columns)	6
Number of values (total)	41

	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma	Solid carcinoma	Tubular carcinoma	Tubulopapillary carcinoma
Number of values	5	4	2	8	7	15
Minimum	0,003298	0,01015	0,0009528	0,001501	0,0007667	0,0008539
25% Percentile	0,03806	0,01472	0,0009528	0,03047	0,00258	0,001824
Median	0,08309	0,0325	0,01469	0,05882	0,01257	0,02999
75% Percentile	0,5219	0,03871	0,02842	0,2059	0,02635	0,0679
Maximum	0,5983	0,03942	0,02842	0,2311	0,02647	0,1516
Mean	0,2406	0,02864	0,01469	0,09745	0,01257	0,03843
Std. Deviation	0,2642	0,01318	0,01943	0,09051	0,0106	0,04292
Std. Error of Mean	0,1182	0,006589	0,01374	0,032	0,004006	0,01108
Lower 95% CI	-0,08743	0,007677	-0,1598	0,02178	0,002766	0,01466
Upper 95% CI	0,5687	0,04961	0,1892	0,1731	0,02237	0,06219
Mean ranks	31,4	21,25	11,5	27,63	12,14	19,33



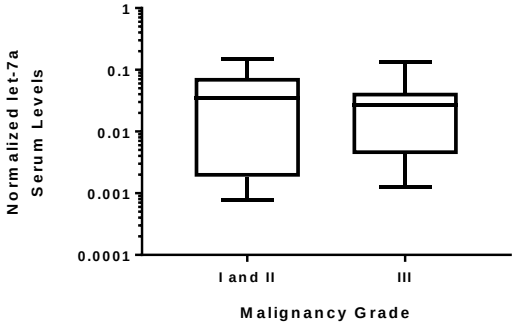
Kruskal-Wallis test	
P value	0,2567
Number of groups	3

	1	2	3 and 4
Number of values	10	6	20
Minimum	0,000854	0,001429	0,000767
25% Percentile	0,003643	0,01648	0,0031
Median	0,02718	0,1066	0,03092
75% Percentile	0,06695	0,2738	0,06078
Maximum	0,1334	0,405	0,1449
Mean	0,03748	0,1452	0,03644
Std. Deviation	0,042	0,1534	0,03764
Std. Error of Mean	0,01328	0,06263	0,008416
Lower 95% CI	0,007435	-0,0158	0,01882
Upper 95% CI	0,06752	0,3062	0,05405
Mean ranks	16,3	24,83	17,7



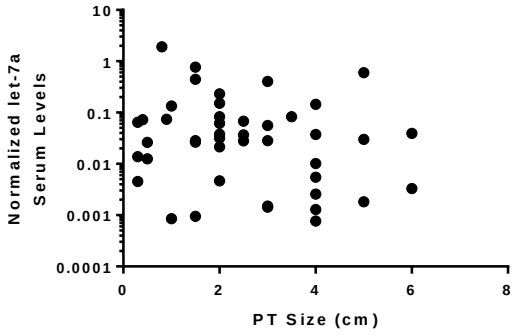
	≤ 3 cm	> 3 cm
Number of values	26	11
Minimum	0,0008539	0,0007667
25% Percentile	0,01059	0,001824
Median	0,02843	0,005476
75% Percentile	0,06547	0,03755
Maximum	0,1516	0,08309
Mean	0,04117	0,01959
Std. Deviation	0,03924	0,02589
Std. Error of Mean	0,007695	0,007807
Lower 95% CI	0,02532	0,00219
Upper 95% CI	0,05702	0,03698
Mean ranks	20,77	14,82

Mann Whitney test	
P value	0,1321
One- or two-tailed P value?	Two-tailed
Median of column A	0,02843, n=26
Median of column B	0,005476, n=11

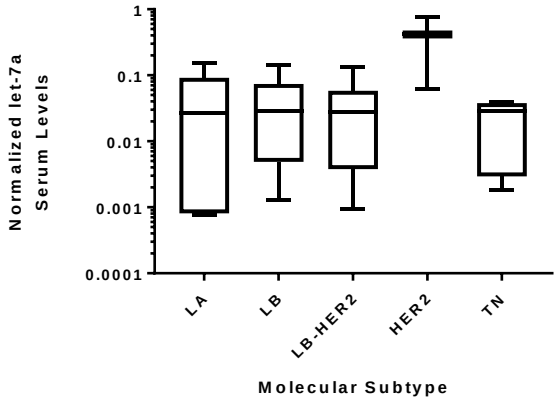


Mann Whitney test	
P value	0,6311
One- or two-tailed P value?	Two-tailed
Median of column A	0,0347, n=12
Median of column B	0,02724, n=26

	I and II	III
Number of values	12	26
Minimum	0,000767	0,001287
25% Percentile	0,00185	0,004319
Median	0,0347	0,02724
75% Percentile	0,07358	0,04238
Maximum	0,1516	0,1334
Mean	0,04984	0,03203
Std. Deviation	0,05374	0,0324
Std. Error of Mean	0,01551	0,006354
Lower 95% CI	0,0157	0,01894
Upper 95% CI	0,08399	0,04511
Mean ranks	20,83	18,88

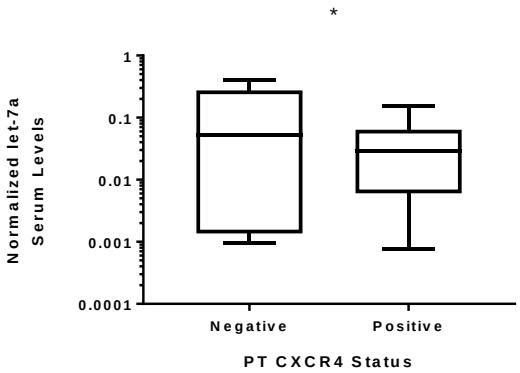


Spearman Correlation	
r	-0,1683
95% confidence interval	-0,4473 to 0,1405
P value	
P (two-tailed)	0,2692
Number of XY Pairs	45



	LA	LB	LB-HER2	HER2	TN
Number of values	5	16	10	2	6
Minimum	0,000767	0,001287	0,000953	0,06166	0,001824
25% Percentile	0,0008105	0,004863	0,00378	0,06166	0,00293
Median	0,02635	0,02903	0,02745	0,416	0,02822
75% Percentile	0,09171	0,07358	0,05814	0,7704	0,03801
Maximum	0,1516	0,1449	0,1334	0,7704	0,03942
Mean	0,04228	0,04123	0,03596	0,416	0,02309
Std. Deviation	0,06274	0,04205	0,04053	0,5011	0,01657
Std. Error of Mean	0,02806	0,01051	0,01282	0,3544	0,006764
Lower 95% CI	-0,03562	0,01882	0,006968	-4,086	0,005702
Upper 95% CI	0,1202	0,06364	0,06496	4,918	0,04047

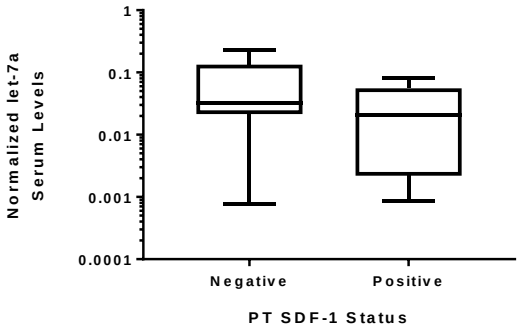
Kruskal-Wallis test	
P value	0,4095
Number of groups	5
Kruskal-Wallis statistic	3,974
Number of treatments (columns)	5
Number of values (total)	39



Mann Whitney test	
P value	0,4933
One- or two-tailed P value?	Two-tailed
Median of column A	0,05191, n=6
Median of column B	0,02843, n=32

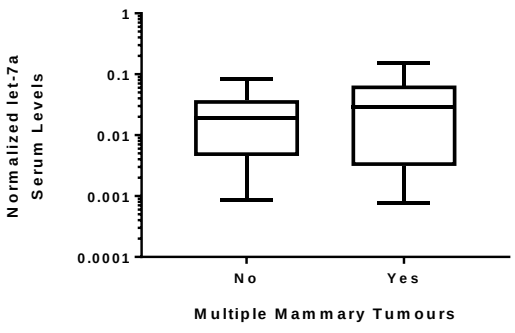
	Negative	Positive

Number of values	6	32
Minimum	0,000953	0,000767
25% Percentile	0,001364	0,006033
Median	0,05191	0,02843
75% Percentile	0,2746	0,06391
Maximum	0,405	0,1516
Mean	0,1237	0,04112
Std. Deviation	0,1624	0,04174
Std. Error of Mean	0,06631	0,007378
Lower 95% CI	-0,04673	0,02607
Upper 95% CI	0,2942	0,05617
Mean ranks	22,5	18,94



Mann Whitney test	
P value	0,1034
One- or two-tailed P value?	Two-tailed
Median of column A	0,03185, n=15
Median of column B	0,02089, n=24

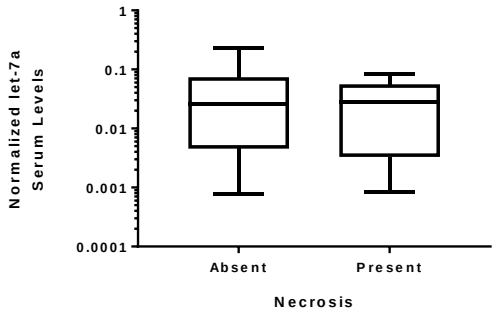
	Negative	Positive
Number of values	15	24
Minimum	0,000767	0,000854
25% Percentile	0,02149	0,002193
Median	0,03185	0,02089
75% Percentile	0,1334	0,05571
Maximum	0,2301	0,08298
Mean	0,06556	0,02823
Std. Deviation	0,06847	0,02813
Std. Error of Mean	0,01768	0,005742
Lower 95% CI	0,02764	0,01635
Upper 95% CI	0,1035	0,04011
Mean ranks	23,8	17,63



Mann Whitney test	

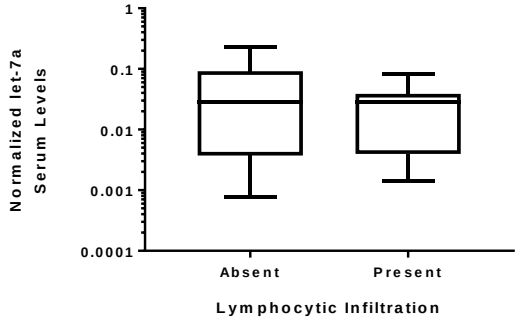
P value	0,4286
One- or two-tailed P value?	Two-tailed
Median of column A	0,01946, n=12
Median of column B	0,02921, n=26

	No	Yes
Number of values	12	26
Minimum	0,000854	0,000767
25% Percentile	0,00457	0,003119
Median	0,01946	0,02921
75% Percentile	0,03753	0,06547
Maximum	0,08309	0,1516
Mean	0,02664	0,04274
Std. Deviation	0,02934	0,04428
Std. Error of Mean	0,00847	0,008684
Lower 95% CI	0,007999	0,02485
Upper 95% CI	0,04528	0,06062
Mean ranks	17,33	20,5



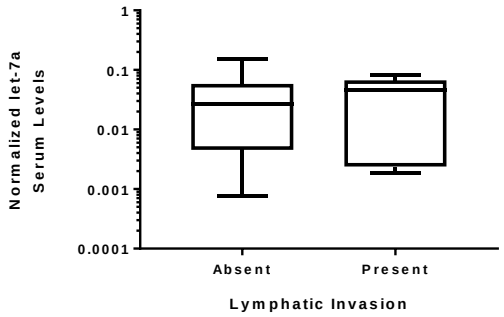
Mann Whitney test	
P value	0,8828
One- or two-tailed P value?	Two-tailed
Median of column A	0,02647, n=15
Median of column B	0,02842, n=23

	No	Yes
Number of values	15	23
Minimum	0,000767	0,000854
25% Percentile	0,00454	0,003298
Median	0,02647	0,02842
75% Percentile	0,07384	0,05597
Maximum	0,2311	0,08309
Mean	0,05257	0,03139
Std. Deviation	0,06758	0,02771
Std. Error of Mean	0,01745	0,005779
Lower 95% CI	0,01515	0,0194
Upper 95% CI	0,09	0,04337
Mean ranks	19,87	19,26



Mann Whitney test	
P value	0,6863
One- or two-tailed P value?	Two-tailed
Median of column A	0,02822, n=18
Median of column B	0,02842, n=21

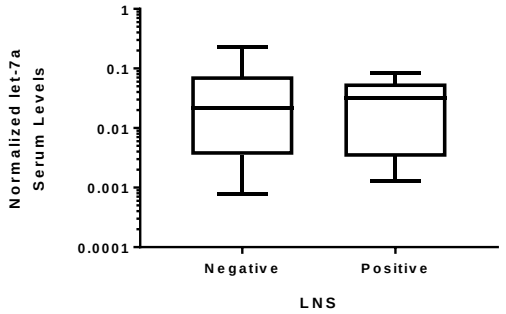
	Absent	Present
Number of values	18	21
Minimum	0,000767	0,001429
25% Percentile	0,003727	0,003979
Median	0,02822	0,02842
75% Percentile	0,09161	0,03863
Maximum	0,2311	0,08309
Mean	0,06263	0,03006
Std. Deviation	0,07688	0,02551
Std. Error of Mean	0,01812	0,005566
Lower 95% CI	0,0244	0,01845
Upper 95% CI	0,1009	0,04168
Mean ranks	20,83	19,29



Mann Whitney test	
P value	0,5978
One- or two-tailed P value?	Two-tailed
Median of column A	0,02724, n=32
Median of column B	0,04676, n=6

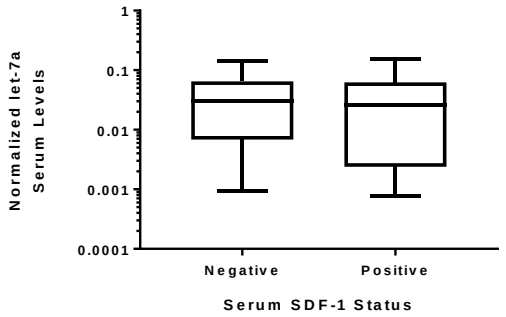
	Absent	Present

Number of values	32	6
Minimum	0,000767	0,001824
25% Percentile	0,00457	0,002391
Median	0,02724	0,04676
75% Percentile	0,05835	0,06699
Maximum	0,1516	0,08298
Mean	0,03713	0,04043
Std. Deviation	0,04217	0,03297
Std. Error of Mean	0,007455	0,01346
Lower 95% CI	0,02193	0,00583
Upper 95% CI	0,05234	0,07502
Mean ranks	19,06	21,83



Mann Whitney test	
P value	0,8000
One- or two-tailed P value?	Two-tailed
Median of column A	0,02149, n=21
Median of column B	0,03185, n=15

	Negative	Positive
Number of values	21	15
Minimum	0,000767	0,001287
25% Percentile	0,00356	0,003298
Median	0,02149	0,03185
75% Percentile	0,07388	0,05597
Maximum	0,2301	0,08298
Mean	0,04784	0,03273
Std. Deviation	0,06425	0,02752
Std. Error of Mean	0,01402	0,007107
Lower 95% CI	0,01859	0,01749
Upper 95% CI	0,07708	0,04797
Mean ranks	18,1	19,07



Mann Whitney test	
P value	0,4379
One- or two-tailed P value?	Two-tailed
Median of column A	0,03014, n=16
Median of column B	0,02641, n=22

	Negative	Positive
Number of values	16	22
Minimum	0,000953	0,000767
25% Percentile	0,006847	0,002391
Median	0,03014	0,02641
75% Percentile	0,06491	0,06241
Maximum	0,1449	0,1516
Mean	0,03981	0,03609
Std. Deviation	0,03832	0,04279
Std. Error of Mean	0,009581	0,009124
Lower 95% CI	0,01939	0,01711
Upper 95% CI	0,06023	0,05506
Mean ranks	21,19	18,27

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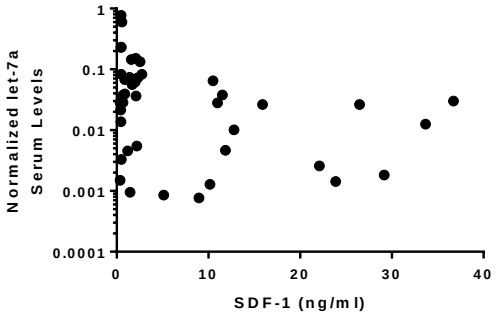
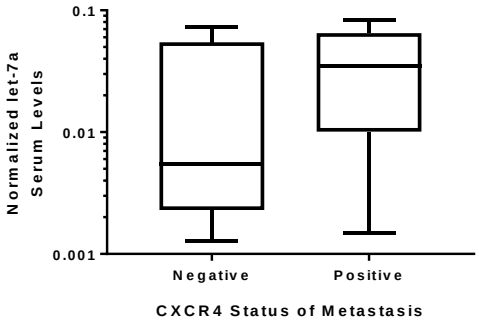


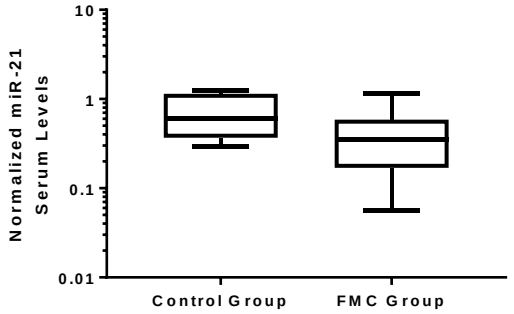
Spearman r	
r	-0,3362
95% confidence interval	-0,5869 to -0,02668
P (two-tailed)	0,0295
Number of XY Pairs	42



Mann Whitney test	
P value	0,3827
One- or two-tailed P value?	Two-tailed
Median of column A	0,005476, n=5
Median of column B	0,0347, n=12

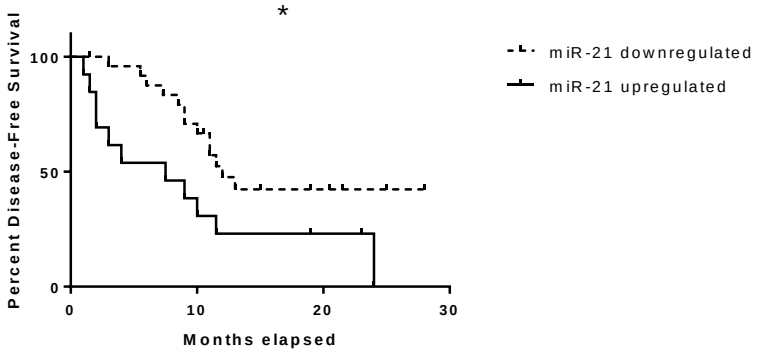
	Negative	Positive
Number of values	5	12
Minimum	0,001287	0,001501
25% Percentile	0,002293	0,01011
Median	0,005476	0,0347
75% Percentile	0,0547	0,06491
Maximum	0,07283	0,08309
Mean	0,02389	0,03834
Std. Deviation	0,03094	0,02906
Std. Error of Mean	0,01384	0,008388
Lower 95% CI	-0,01453	0,01988
Upper 95% CI	0,06231	0,0568
Mean ranks	7,2	9,75

Results for miR-21

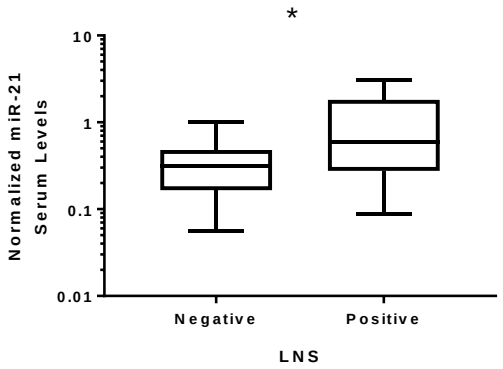


Mann Whitney test	
P value	0,0727
One- or two-tailed P value?	Two-tailed
Median of column A	0,6117, n=5
Median of column B	0,3527, n=37

	Control Group	FMC Group
Number of values	5	37
Minimum	0,294	0,05603
25% Percentile	0,3679	0,169
Median	0,6117	0,3527
75% Percentile	1,144	0,5884
Maximum	1,255	1,151
Mean	0,7273	0,4176
Std. Deviation	0,4047	0,2869
Std. Error of Mean	0,181	0,04717
Lower 95% CI	0,2248	0,322
Upper 95% CI	1,23	0,5133
Mean ranks	30,8	20,24

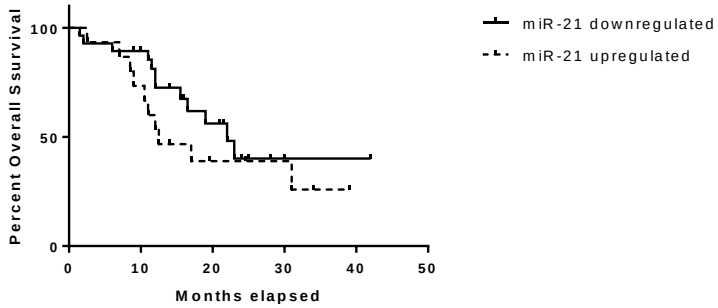


Log-rank (Mantel-Cox) test	
Chi square	5,117
df	1
P value	0,0237

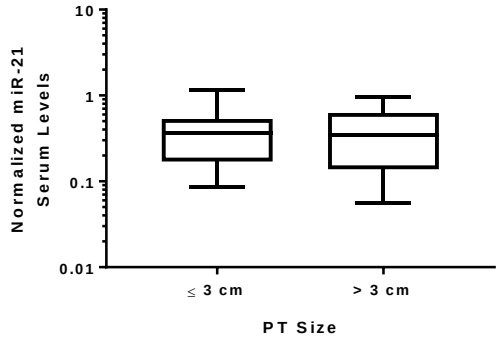


Mann Whitney test	
P value	0,0101
One- or two-tailed P value?	Two-tailed
Median of column A	0,3165, n=22
Median of column B	0,5918, n=16

	Negative	Positive
Number of values	22	16
Minimum	0,05603	0,08697
25% Percentile	0,1654	0,2764
Median	0,3165	0,5918
75% Percentile	0,4797	1,812
Maximum	1,006	3,044
Mean	0,36	1,002
Std. Deviation	0,2659	0,9183
Std. Error of Mean	0,05669	0,2296
Lower 95% CI	0,2421	0,5124
Upper 95% CI	0,4779	1,491
Mean ranks	15,59	24,88

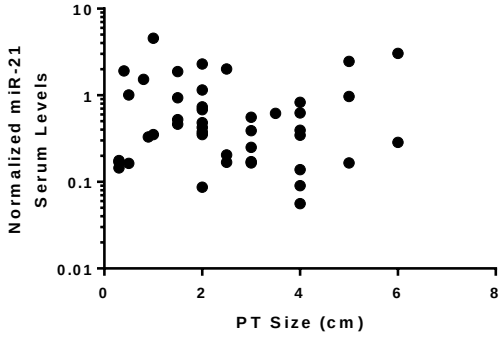


Comparison of Survival Curves	
Log-rank (Mantel-Cox) test	
Chi square	0,9977
df	1
P value	0,3179

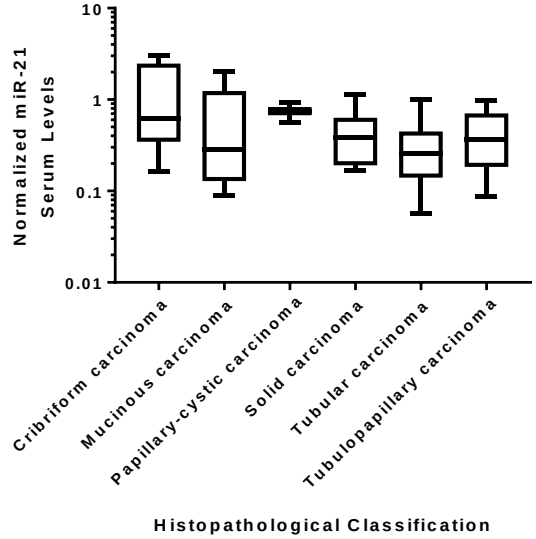


Mann Whitney test	
P value	0,6877
One- or two-tailed P value?	Two-tailed
Median of column A	0,3647, n=26
Median of column B	0,3465, n=11

	≤ 3 cm	> 3 cm
Number of values	26	11
Minimum	0,08697	0,05603
25% Percentile	0,1707	0,1382
Median	0,3647	0,3465
75% Percentile	0,5328	0,6257
Maximum	1,151	0,9702
Mean	0,4205	0,411
Std. Deviation	0,2832	0,3096
Std. Error of Mean	0,05554	0,09335
Lower 95% CI	0,3061	0,203
Upper 95% CI	0,5349	0,619
Mean ranks	19,48	17,86

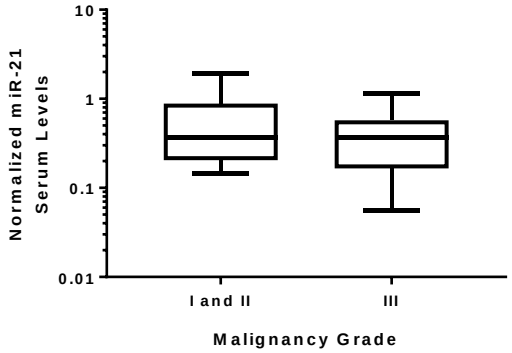


Spearman r	
r	-0,06161
95% confidence interval	-0,3567 to 0,2446
P (two-tailed)	0,6877
Number of XY Pairs	45



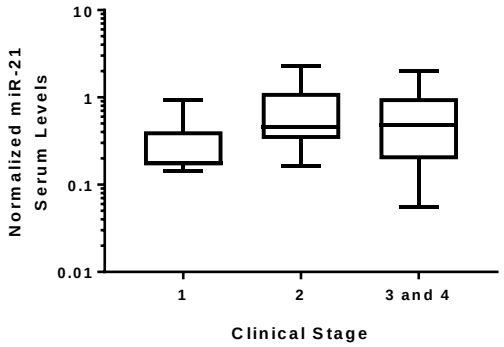
Kruskal-Wallis test	
P value	0,2815
Number of groups	6
Kruskal-Wallis statistic	6,262

	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma	Solid carcinoma	Tubular carcinoma	Tubulopapillary carcinoma
Number of values	5	5	2	8	8	12
Minimum	0,1659	0,09029	0,558	0,1683	0,05603	0,08697
25% Percentile	0,3452	0,1281	0,558	0,1911	0,1399	0,1837
Median	0,6187	0,2865	0,7462	0,3885	0,2552	0,3647
75% Percentile	2,475	1,238	0,9345	0,6311	0,4475	0,7071
Maximum	3,044	2,011	0,9345	1,151	1,006	0,9702
Mean	1,252	0,6037	0,7462	0,4603	0,3395	0,4372
Std. Deviation	1,199	0,7992	0,2662	0,3276	0,3053	0,2861
Std. Error of Mean	0,5362	0,3574	0,1882	0,1158	0,1079	0,0826
Lower 95% CI	-0,2368	-0,3887	-1,646	0,1864	0,08425	0,2553
Upper 95% CI	2,741	1,596	3,138	0,7342	0,5947	0,619
Mean ranks	28,3	17,7	31	21,13	14,5	20,25



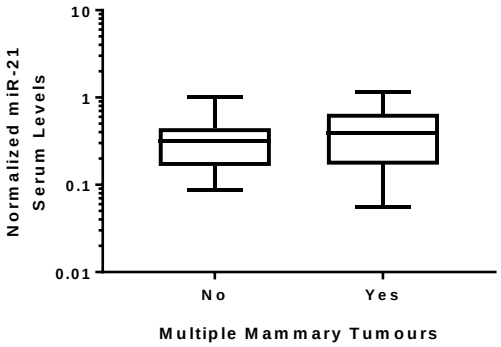
Mann Whitney test	
P value	0,3722
One- or two-tailed P value?	Two-tailed
Median of column A	0,3708, n=12
Median of column B	0,3647, n=26

	I and II	III
Number of values	12	26
Minimum	0,1452	0,05603
25% Percentile	0,2042	0,1659
Median	0,3708	0,3647
75% Percentile	0,8844	0,5732
Maximum	1,906	1,151
Mean	0,5766	0,4015
Std. Deviation	0,5063	0,2863
Std. Error of Mean	0,1462	0,05615
Lower 95% CI	0,2549	0,2859
Upper 95% CI	0,8983	0,5172
Mean ranks	21,92	18,38



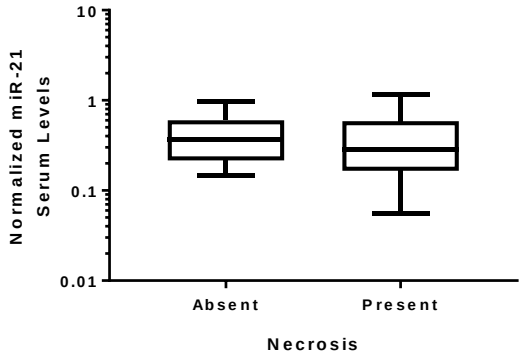
Kruskal-Wallis test	
P value	0,1697
Kruskal-Wallis statistic	3,547

	1	2	3 and 4
Number of values	9	6	22
Minimum	0,1452	0,1659	0,05603
25% Percentile	0,1661	0,3349	0,1961
Median	0,1767	0,4543	0,4766
75% Percentile	0,4085	1,125	0,9791
Maximum	0,9345	2,297	2,011
Mean	0,3228	0,7496	0,6553
Std. Deviation	0,2551	0,7798	0,57
Std. Error of Mean	0,08504	0,3184	0,1215
Lower 95% CI	0,1267	-0,06885	0,4025
Upper 95% CI	0,5189	1,568	0,908
Mean ranks	13,22	22,42	20,43



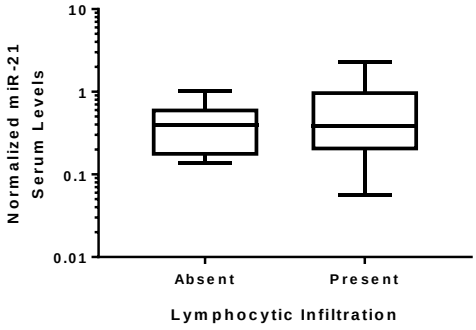
Mann Whitney test	
P value	0,3150
One- or two-tailed P value?	Two-tailed
Median of column A	0,3196, n=12
Median of column B	0,3952, n=25

	No	Yes
Number of values	12	25
Minimum	0,08697	0,05603
25% Percentile	0,1644	0,1704
Median	0,3196	0,3952
75% Percentile	0,4465	0,653
Maximum	1,006	1,151
Mean	0,3524	0,449
Std. Deviation	0,2584	0,2996
Std. Error of Mean	0,07459	0,05992
Lower 95% CI	0,1882	0,3253
Upper 95% CI	0,5165	0,5727
Mean ranks	16,38	20,26



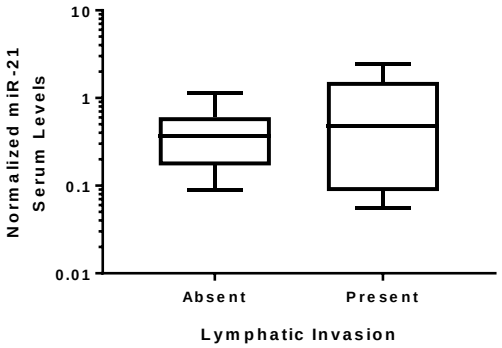
Mann Whitney test	
P value	0,4900
One- or two-tailed P value?	Two-tailed
Median of column A	0,3647, n=16
Median of column B	0,2865, n=21

	No	Yes
Number of values	16	21
Minimum	0,1452	0,05603
25% Percentile	0,2151	0,1659
Median	0,3647	0,2865
75% Percentile	0,6004	0,5884
Maximum	0,9702	1,151
Mean	0,4381	0,4021
Std. Deviation	0,2547	0,3146
Std. Error of Mean	0,06368	0,06865
Lower 95% CI	0,3023	0,2589
Upper 95% CI	0,5738	0,5453
Mean ranks	20,44	17,9



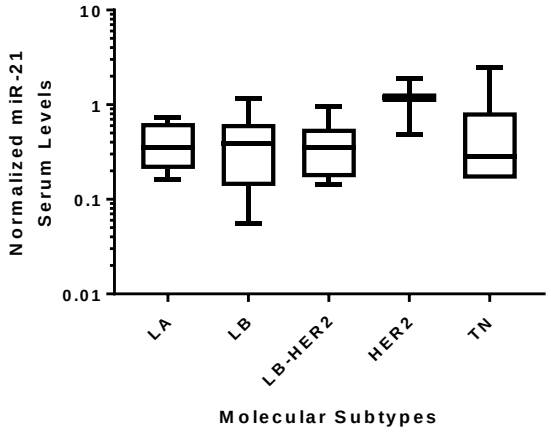
Mann Whitney test	
P value	0,5047
One- or two-tailed P value?	Two-tailed
Median of column A	0,3952, n=19
Median of column B	0,384, n=22

	No	Yes
Number of values	19	22
Minimum	0,1382	0,05603
25% Percentile	0,1683	0,1961
Median	0,3952	0,384
75% Percentile	0,6257	1,015
Maximum	1,006	2,297
Mean	0,4253	0,6861
Std. Deviation	0,2704	0,6732
Std. Error of Mean	0,06204	0,1435
Lower 95% CI	0,2949	0,3876
Upper 95% CI	0,5556	0,9845
Mean ranks	19,63	22,18



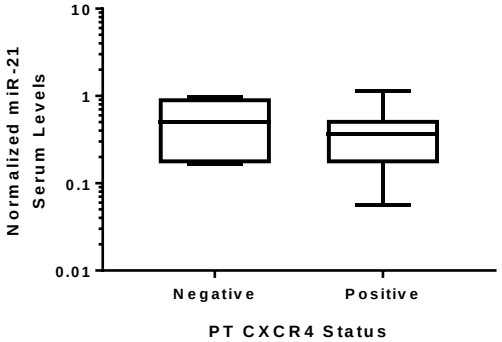
Mann Whitney test	
P value	0,6503
One- or two-tailed P value?	Two-tailed
Median of column A	0,3647, n=32
Median of column B	0,4839, n=7

	Absent	Present
Number of values	32	7
Minimum	0,09029	0,05603
25% Percentile	0,1701	0,08697
Median	0,3647	0,4839
75% Percentile	0,6035	1,531
Maximum	1,151	2,466
Mean	0,4296	0,8146
Std. Deviation	0,2851	0,8919
Std. Error of Mean	0,0504	0,3371
Lower 95% CI	0,3268	-0,01028
Upper 95% CI	0,5324	1,639
Mean ranks	19,59	21,86



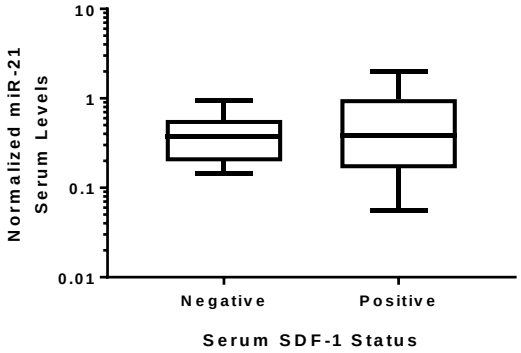
Kruskal-Wallis test	
P value	0,6650
Kruskal-Wallis statistic	2,387
Number of values (total)	39

	LA	LB	LB-HER2	HER2	TN
Number of values	4	15	11	2	7
Minimum	0,1639	0,05603	0,1452	0,4839	0,1659
25% Percentile	0,2096	0,1382	0,1711	0,4839	0,1659
Median	0,3496	0,3912	0,3522	1,183	0,2865
75% Percentile	0,6388	0,6257	0,558	1,882	0,8273
Maximum	0,7342	1,151	0,9702	1,882	2,466
Mean	0,3993	0,4383	0,4289	1,183	0,6493
Std. Deviation	0,2398	0,3285	0,2985	0,9887	0,8364
Std. Error of Mean	0,1199	0,08483	0,09	0,6991	0,3161
Lower 95% CI	0,01775	0,2564	0,2284	-7,7	-0,1242
Upper 95% CI	0,7809	0,6203	0,6294	10,07	1,423
Mean ranks	18,5	19,2	19,91	32	19,29



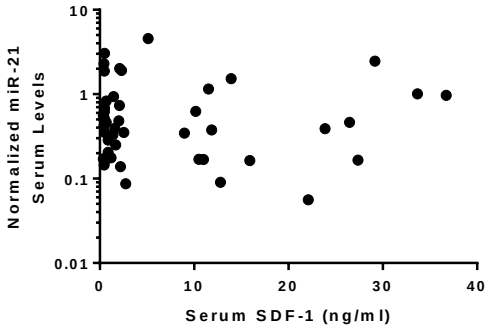
Mann Whitney test	
P value	0,4712
One- or two-tailed P value?	Two-tailed
Median of column A	0,5052, n=6
Median of column B	0,3647, n=30

	Negative	Positive
Number of values	6	30
Minimum	0,1659	0,05603
25% Percentile	0,1698	0,1694
Median	0,5052	0,3647
75% Percentile	0,9434	0,5328
Maximum	0,9702	1,151
Mean	0,542	0,4021
Std. Deviation	0,3689	0,2695
Std. Error of Mean	0,1506	0,0492
Lower 95% CI	0,1549	0,3015
Upper 95% CI	0,9292	0,5027
Mean ranks	21,42	17,92

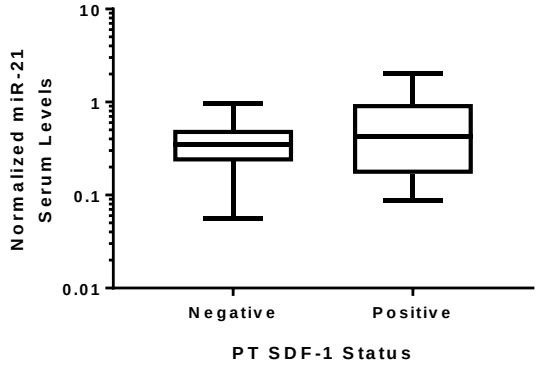


Mann Whitney test	
P value	0,8880
One- or two-tailed P value?	Two-tailed
Median of column A	0,3739, n=18
Median of column B	0,384, n=22

	Negative	Positive
Number of values	18	22
Minimum	0,1452	0,05603
25% Percentile	0,1975	0,1654
Median	0,3739	0,384
75% Percentile	0,5732	0,9791
Maximum	0,9345	2,011
Mean	0,4173	0,6086
Std. Deviation	0,2348	0,5862
Std. Error of Mean	0,05533	0,125
Lower 95% CI	0,3006	0,3487
Upper 95% CI	0,5341	0,8685
Mean ranks	20,19	20,75

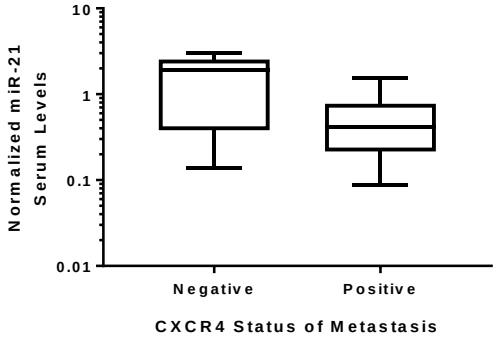


Spearman r	
r	-0,06661
95% confidence interval	-0,361 to 0,2399
P value	
P (two-tailed)	0,6638
Number of XY Pairs	45



Mann Whitney test	
P value	0,4038
One- or two-tailed P value?	Two-tailed
Median of column A	0,3524, n=14
Median of column B	0,428, n=26

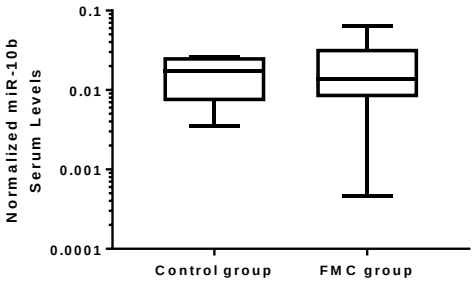
	Negative	Positive
Number of values	14	26
Minimum	0,05603	0,08697
25% Percentile	0,2292	0,1694
Median	0,3524	0,428
75% Percentile	0,5034	0,9523
Maximum	0,9702	2,011
Mean	0,3968	0,6424
Std. Deviation	0,2441	0,5998
Std. Error of Mean	0,06523	0,1176
Lower 95% CI	0,2559	0,4001
Upper 95% CI	0,5377	0,8847
Mean ranks	18,36	21,65



Mann Whitney test	
P value	0,1037
One- or two-tailed P value?	Two-tailed
Median of column A	1,906, n=5
Median of column B	0,4208, n=12

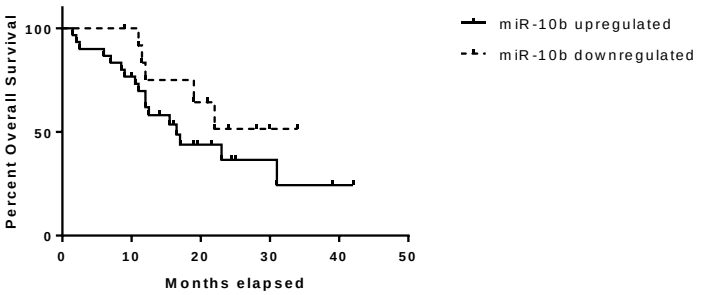
	Negative	Positive
Number of values	5	12
Minimum	0,1382	0,08697
25% Percentile	0,3819	0,216
Median	1,906	0,4208
75% Percentile	2,528	0,7751
Maximum	3,044	1,531
Mean	1,545	0,5494
Std. Deviation	1,164	0,4314
Std. Error of Mean	0,5206	0,1245
Lower 95% CI	0,09969	0,2753
Upper 95% CI	2,99	0,8235
Mean ranks	12,2	7,667

Results for miR-10b

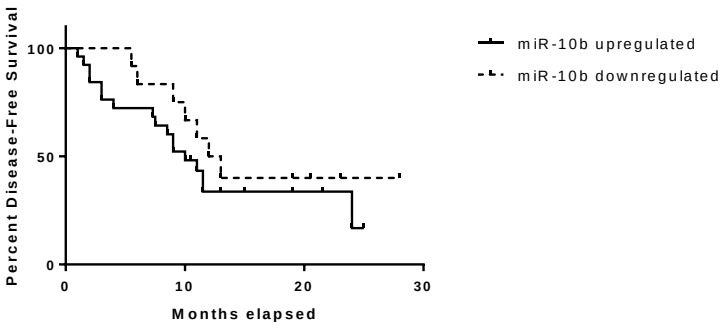


Mann Whitney test	
P value	0,8200
One- or two-tailed P value?	Two-tailed
Median of column A	0,01745, n=5
Median of column B	0,01386, n=40

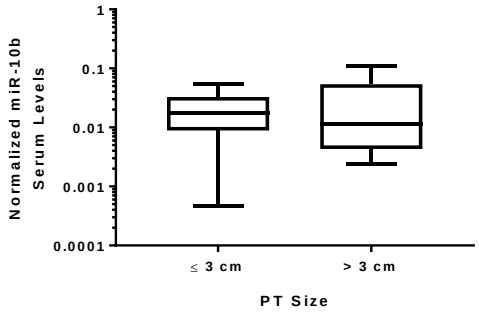
	Control group	FMC group
Number of values	5	40
Minimum	0,003542	0,0004674
25% Percentile	0,007232	0,008123
Median	0,01745	0,01386
75% Percentile	0,02594	0,03304
Maximum	0,02605	0,0633
Mean	0,01676	0,02116
Std. Deviation	0,009719	0,01708
Std. Error of Mean	0,004346	0,0027
Lower 95% CI	0,004691	0,0157
Upper 95% CI	0,02883	0,02662
Mean ranks	21,6	23,18



Comparison of Survival Curves	
Log-rank (Mantel-Cox) test	
Chi square	1,601
df	1
P value	0,2057

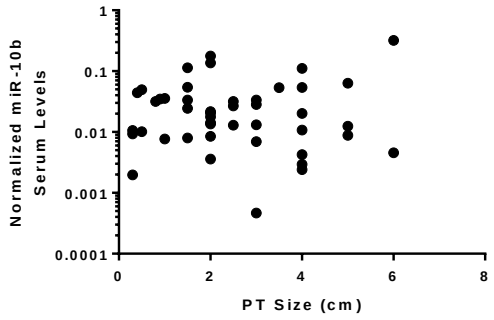


Comparison of Survival Curves	
Log-rank (Mantel-Cox) test	
Chi square	1,017
df	1
P value	0,3132

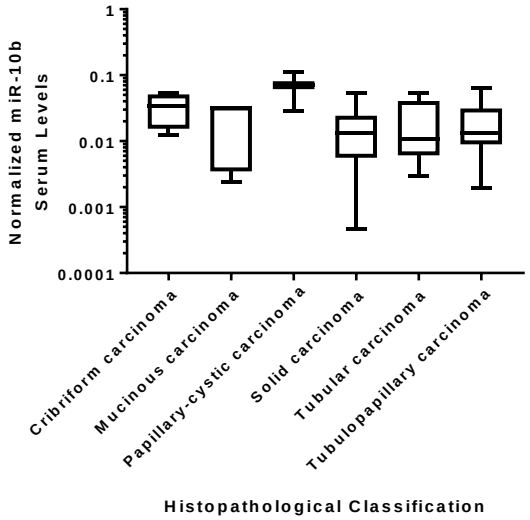


Mann Whitney test	
P value	0,8322
One- or two-tailed P value?	Two-tailed
Median of column A	0,01767, n=29
Median of column B	0,01162, n=12

	≤ 3 cm	> 3 cm
Number of values	29	12
Minimum	0,000467	0,002401
25% Percentile	0,008909	0,004318
Median	0,01767	0,01162
75% Percentile	0,03263	0,05381
Maximum	0,05439	0,1108
Mean	0,02101	0,029
Std. Deviation	0,01451	0,03409
Std. Error of Mean	0,002694	0,009841
Lower 95% CI	0,01549	0,007344
Upper 95% CI	0,02652	0,05066
Mean ranks	21,28	20,33

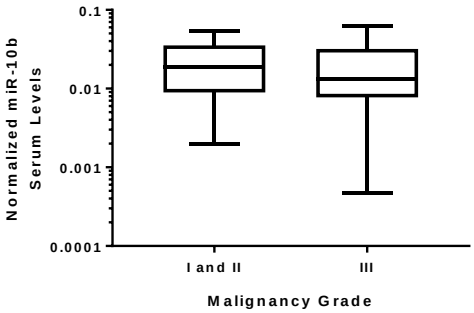


Spearman r	
r	-0,05264
95% confidence interval	-0,3488 to 0,2531
P value	
P (two-tailed)	0,7313
Number of XY Pairs	45



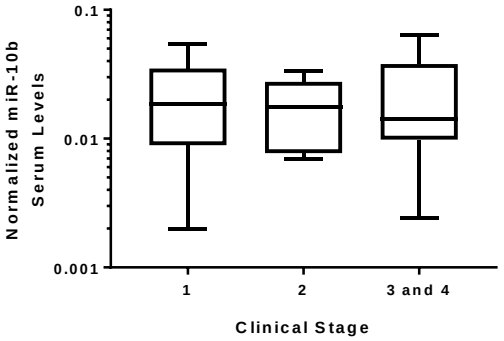
Kruskal-Wallis test	
P value	0,3910
Number of groups	6
Kruskal-Wallis statistic	5,209
Number of values (total)	41

	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma	Solid carcinoma	Tubular carcinoma	Tubulopapillary carcinoma
Number of values	4	5	2	9	9	12
Minimum	0,01245	0,002401	0,02828	0,0004674	0,002937	0,001978
25% Percentile	0,01547	0,003481	0,02828	0,005614	0,006117	0,008955
Median	0,0343	0,03179	0,07068	0,01311	0,01058	0,01327
75% Percentile	0,05125	0,03345	0,1131	0,02428	0,04059	0,03122
Maximum	0,05364	0,03346	0,1131	0,05439	0,05387	0,0633
Mean	0,03367	0,02113	0,07068	0,01671	0,02123	0,01969
Std. Deviation	0,01863	0,01614	0,05996	0,0164	0,01938	0,0172
Std. Error of Mean	0,009315	0,007219	0,0424	0,005468	0,006461	0,004964
Lower 95% CI	0,004027	0,001085	-0,468	0,0041	0,006332	0,008767
Upper 95% CI	0,06331	0,04117	0,6094	0,02932	0,03613	0,03062
Mean ranks	28,75	20,4	34,5	17,44	19,56	20,17



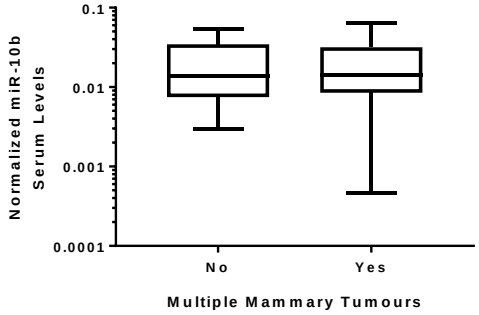
Mann Whitney test	
P value	0,6943
One- or two-tailed P value?	Two-tailed
Mann-Whitney U	154
Median of column A	0,01894, n=12
Median of column B	0,01335, n=28

	I and II	III
Number of values	12	28
Minimum	0,001978	0,000467
25% Percentile	0,008955	0,007738
Median	0,01894	0,01335
75% Percentile	0,03543	0,03181
Maximum	0,05387	0,0633
Mean	0,02217	0,02073
Std. Deviation	0,01665	0,01754
Std. Error of Mean	0,004807	0,003315
Lower 95% CI	0,01159	0,01392
Upper 95% CI	0,03275	0,02753
Mean ranks	21,67	20



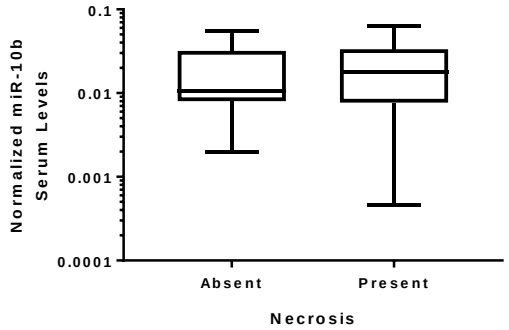
Kruskal-Wallis test	
P value	0,8877
Number of groups	3
Number of values (total)	36

	1	2	3 and 4
Number of values	10	5	21
Minimum	0,001978	0,00693	0,002401
25% Percentile	0,008902	0,007715	0,009815
Median	0,01872	0,01767	0,01413
75% Percentile	0,03501	0,02756	0,03795
Maximum	0,05439	0,03344	0,0633
Mean	0,02248	0,01765	0,02369
Std. Deviation	0,01697	0,01077	0,01892
Std. Error of Mean	0,005366	0,004818	0,004129
Lower 95% CI	0,01034	0,004268	0,01508
Upper 95% CI	0,03461	0,03102	0,0323
Mean ranks	18,6	16,4	18,95



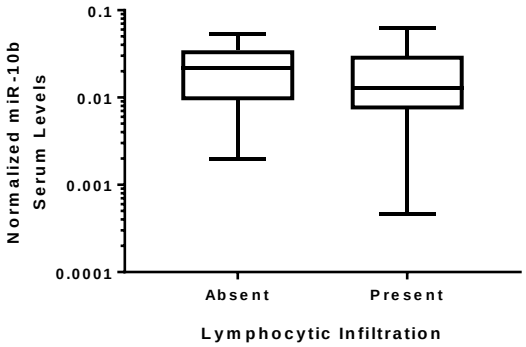
Mann Whitney test	
P value	0,9773
One- or two-tailed P value?	Two-tailed
Median of column A	0,01359, n=13
Median of column B	0,01413, n=27

	No	Yes
Number of values	13	27
Minimum	0,002937	0,000467
25% Percentile	0,007464	0,008499
Median	0,01359	0,01413
75% Percentile	0,03454	0,03179
Maximum	0,05364	0,0633
Mean	0,02153	0,02098
Std. Deviation	0,01738	0,01726
Std. Error of Mean	0,004821	0,003322
Lower 95% CI	0,01102	0,01416
Upper 95% CI	0,03203	0,02781
Mean ranks	20,62	20,44



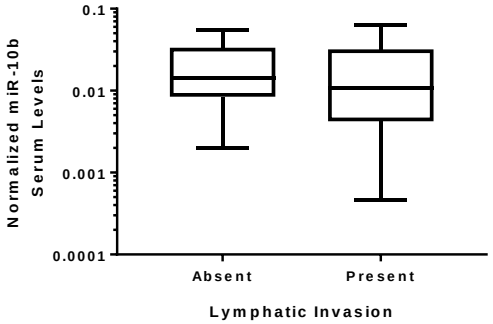
Mann Whitney test	
P value	0,6191
One- or two-tailed P value?	Two-tailed
Median of column A	0,01058, n=15
Median of column B	0,01767, n=25

	Absent	Present
Number of values	15	25
Minimum	0,001978	0,000467
25% Percentile	0,007997	0,007715
Median	0,01058	0,01767
75% Percentile	0,03182	0,03345
Maximum	0,05439	0,0633
Mean	0,01955	0,02213
Std. Deviation	0,01704	0,01738
Std. Error of Mean	0,004399	0,003476
Lower 95% CI	0,01011	0,01495
Upper 95% CI	0,02898	0,0293
Mean ranks	19,27	21,24



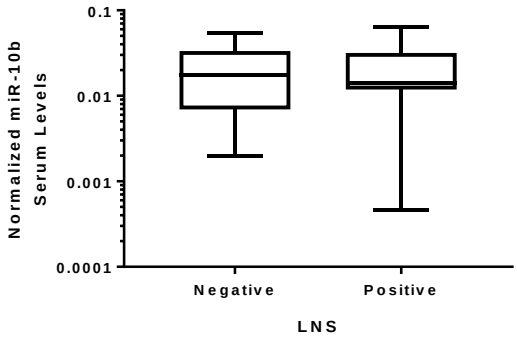
Mann Whitney test	
P value	0,2944
One- or two-tailed P value?	Two-tailed
Median of column A	0,02169, n=19
Median of column B	0,01295, n=21

	Absent	Present
Number of values	19	21
Minimum	0,001978	0,000467
25% Percentile	0,009319	0,007291
Median	0,02169	0,01295
75% Percentile	0,03479	0,03003
Maximum	0,05387	0,0633
Mean	0,02342	0,01912
Std. Deviation	0,01589	0,01823
Std. Error of Mean	0,003646	0,003978
Lower 95% CI	0,01576	0,01082
Upper 95% CI	0,03108	0,02742
Mean ranks	22,58	18,62



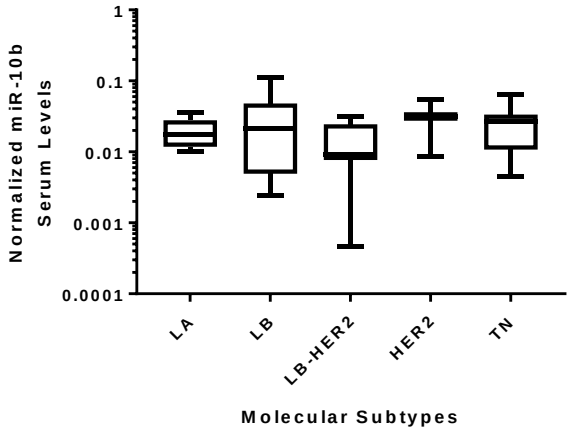
Mann Whitney test	
P value	0,6010
One- or two-tailed P value?	Two-tailed
Median of column A	0,01413, n=33
Median of column B	0,0108, n=7

	Absent	Present
Number of values	33	7
Minimum	0,001978	0,000467
25% Percentile	0,008415	0,004237
Median	0,01413	0,0108
75% Percentile	0,03345	0,03182
Maximum	0,05439	0,0633
Mean	0,02142	0,01995
Std. Deviation	0,0163	0,02184
Std. Error of Mean	0,002837	0,008254
Lower 95% CI	0,01564	-0,0002497
Upper 95% CI	0,0272	0,04015
Mean ranks	20,97	18,29



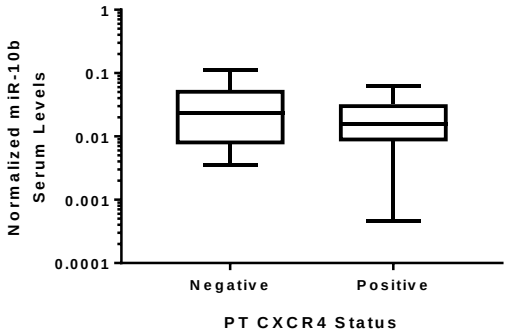
Mann Whitney test	
P value	0,6491
One- or two-tailed P value?	Two-tailed
Median of column A	0,01767, n=23
Median of column B	0,01413, n=13

	Negative	Positive
Number of values	23	13
Minimum	0,001978	0,000467
25% Percentile	0,00693	0,01187
Median	0,01767	0,01413
75% Percentile	0,03346	0,0318
Maximum	0,05439	0,0633
Mean	0,02165	0,02253
Std. Deviation	0,01788	0,01708
Std. Error of Mean	0,003729	0,004738
Lower 95% CI	0,01391	0,0122
Upper 95% CI	0,02938	0,03285
Mean ranks	17,87	19,62



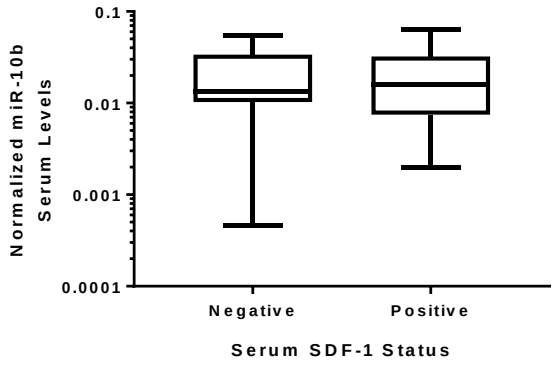
Kruskal-Wallis test	
P value	0,4175
Number of treatments (columns)	5
Number of values (total)	41

	LA	LB	LB-HER2	HER2	TN
Number of values	5	16	11	2	7
Minimum	0,01009	0,002401	0,000467	0,008499	0,004561
25% Percentile	0,01184	0,00491	0,007651	0,008499	0,0108
Median	0,01767	0,0211	0,009319	0,03144	0,02687
75% Percentile	0,02792	0,04804	0,02452	0,05439	0,03346
Maximum	0,03564	0,1108	0,03182	0,05439	0,0633
Mean	0,01944	0,02923	0,01314	0,03144	0,02641
Std. Deviation	0,009849	0,02868	0,01044	0,03245	0,0199
Std. Error of Mean	0,004405	0,00717	0,003147	0,02294	0,007521
Lower 95% CI	0,007209	0,01395	0,00613	-0,2601	0,008005
Upper 95% CI	0,03167	0,04452	0,02015	0,323	0,04481
Mean ranks	22,6	22,69	15	25	24,29



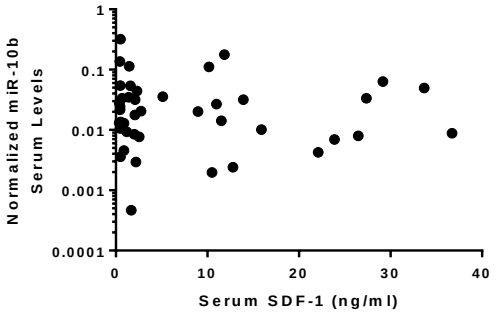
Mann Whitney test	
P value	0,6709
One- or two-tailed P value?	Two-tailed
Median of column A	0,02328, n=6
Median of column B	0,0159, n=34

	Negative	Positive
Number of values	6	34
Minimum	0,003578	0,000467
25% Percentile	0,007519	0,008374
Median	0,02328	0,0159
75% Percentile	0,05436	0,03223
Maximum	0,1131	0,0633
Mean	0,03447	0,02205
Std. Deviation	0,04061	0,01754
Std. Error of Mean	0,01658	0,003008
Lower 95% CI	-0,008149	0,01593
Upper 95% CI	0,07709	0,02817
Mean ranks	22,5	20,15

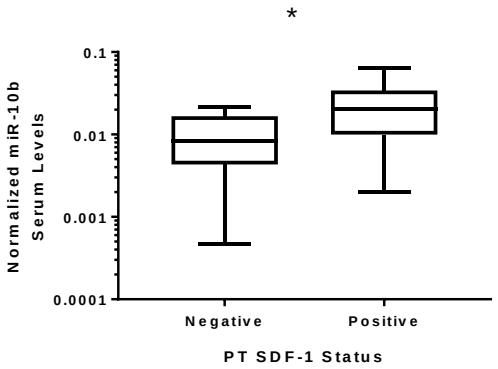


Mann Whitney test	
P value	0,5999
One- or two-tailed P value?	Two-tailed
Median of column A	0,01335, n=18
Median of column B	0,0159, n=22

	Negative	Positive
Number of values	18	22
Minimum	0,000467	0,001978
25% Percentile	0,01026	0,007471
Median	0,01335	0,0159
75% Percentile	0,03379	0,03223
Maximum	0,05439	0,0633
Mean	0,022	0,02047
Std. Deviation	0,01758	0,01704
Std. Error of Mean	0,004143	0,003634
Lower 95% CI	0,01326	0,01291
Upper 95% CI	0,03074	0,02803
Mean ranks	21,61	19,59

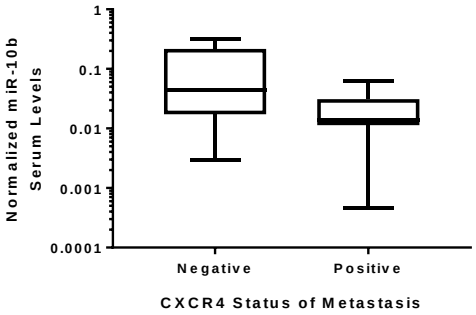


Spearman r	
r	-0,135
95% confidence interval	-0,4196 to 0,1738
P value (two-tailed)	0,3767
Number of XY Pairs	45



Mann Whitney test	
P value	0,0141
One- or two-tailed P value?	Two-tailed
Median of column A	0,008415, n=12
Median of column B	0,02052, n=25

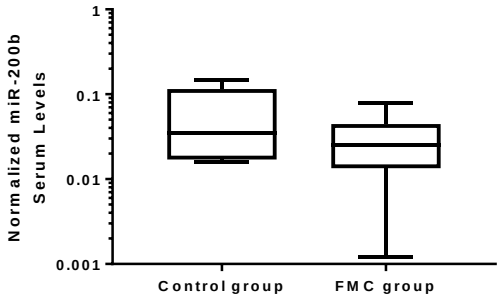
	Negative	Positive
Number of values	12	25
Minimum	0,000467	0,001978
25% Percentile	0,004318	0,009947
Median	0,008415	0,02052
75% Percentile	0,01665	0,03412
Maximum	0,02169	0,0633
Mean	0,009993	0,02378
Std. Deviation	0,006921	0,01713
Std. Error of Mean	0,001998	0,003427
Lower 95% CI	0,005596	0,01671
Upper 95% CI	0,01439	0,03085
Mean ranks	12,75	22



Mann Whitney test	
P value	0,1600
One- or two-tailed P value?	Two-tailed
Median of column A	0,04408, n=5
Median of column B	0,01386, n=12

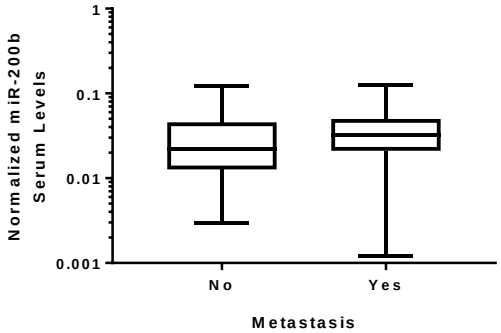
	Negative	Positive
Number of values	5	12
Minimum	0,002937	0,000467
25% Percentile	0,01736	0,01134
Median	0,04408	0,01386
75% Percentile	0,2157	0,03094
Maximum	0,3205	0,0633
Mean	0,102	0,02255
Std. Deviation	0,1284	0,01888
Std. Error of Mean	0,05742	0,005449
Lower 95% CI	-0,05738	0,01056
Upper 95% CI	0,2615	0,03454
Mean ranks	11,8	7,833

Results for miR-200b



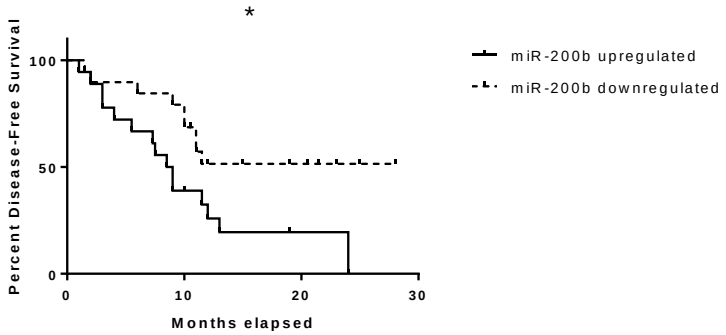
Mann Whitney test	
P value	0,2343
One- or two-tailed P value?	Two-tailed
Median of column A	0,03448, n=5
Median of column B	0,02504, n=40

	Control group	FMC group
Number of values	5	40
Minimum	0,01569	0,001198
25% Percentile	0,017	0,01344
Median	0,03448	0,02504
75% Percentile	0,115	0,04461
Maximum	0,1458	0,07836
Mean	0,05971	0,02984
Std. Deviation	0,05545	0,02075
Std. Error of Mean	0,0248	0,00328
Lower 95% CI	-0,009136	0,0232
Upper 95% CI	0,1286	0,03647
Mean ranks	29,8	22,15

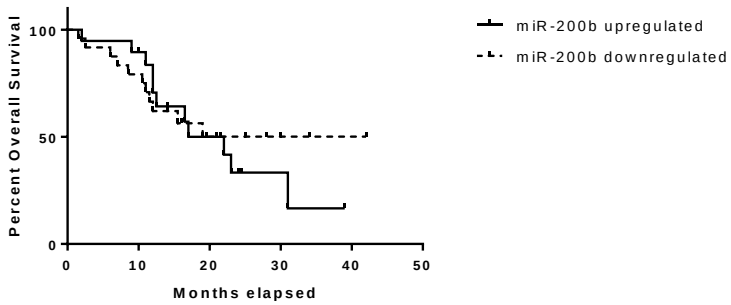


Mann Whitney test	
P value	0,2556
One- or two-tailed P value?	Two-tailed
Median of column A	0,02179, n=21
Median of column B	0,03244, n=17

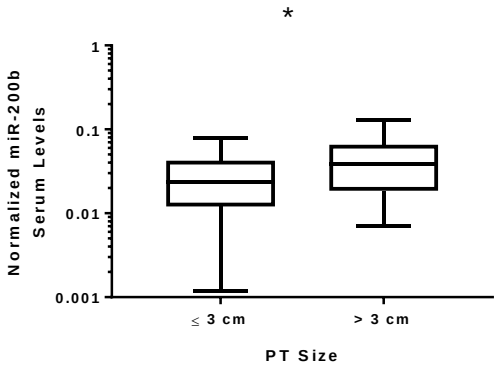
	No	Yes
Number of values	21	17
Minimum	0,002965	0,001198
25% Percentile	0,01271	0,02102
Median	0,02179	0,03244
75% Percentile	0,04564	0,05006
Maximum	0,1228	0,1265
Mean	0,03064	0,03851
Std. Deviation	0,02836	0,03022
Std. Error of Mean	0,006188	0,00733
Lower 95% CI	0,01774	0,02297
Upper 95% CI	0,04355	0,05405
Mean ranks	17,62	21,82



Log-rank (Mantel-Cox) test	
Chi square	5,398
df	1
P value	0,0202

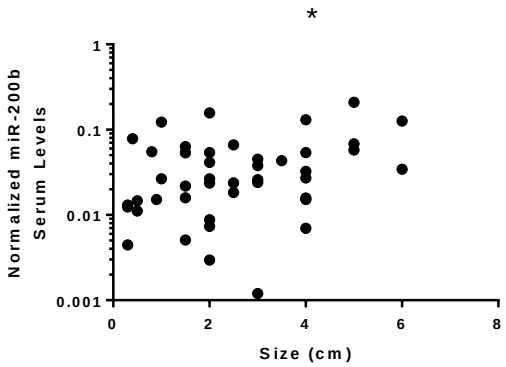


Log-rank (Mantel-Cox) test	
Chi square	0,09665
df	1
P value	0,7559

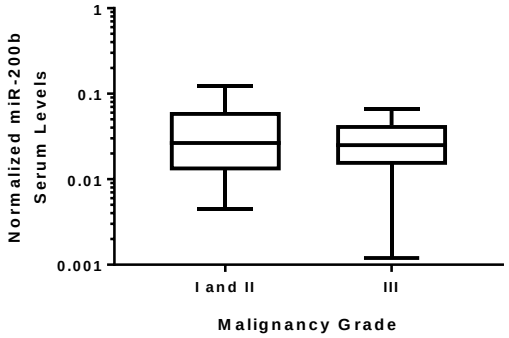


Mann Whitney test	
P value	0,0521
One- or two-tailed P value?	Two-tailed
Median of column A	0,02371, n=30
Median of column B	0,03888, n=12

	≤ 3 cm	> 3 cm
Number of values	30	12
Minimum	0,001198	0,006979
25% Percentile	0,01207	0,01862
Median	0,02371	0,03888
75% Percentile	0,04233	0,06567
Maximum	0,07836	0,1305
Mean	0,02794	0,05102
Std. Deviation	0,02091	0,04055
Std. Error of Mean	0,003818	0,01171
Lower 95% CI	0,02014	0,02525
Upper 95% CI	0,03575	0,07678
Mean ranks	19,17	27,33

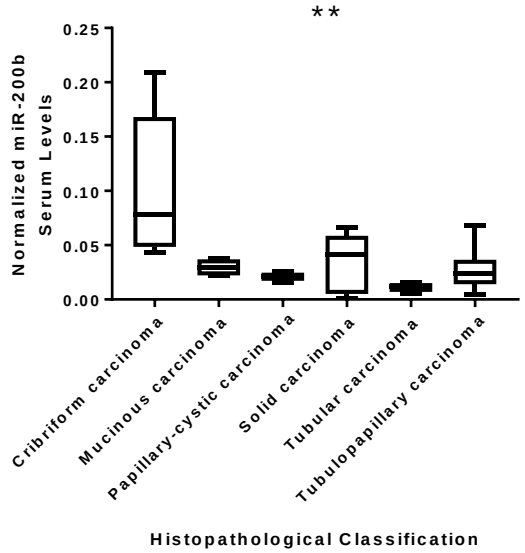


Spearman r	
r	0,3145
95% confidence interval	0,01411 to 0,5628
P value	
P (two-tailed)	0,0354
Number of XY Pairs	45



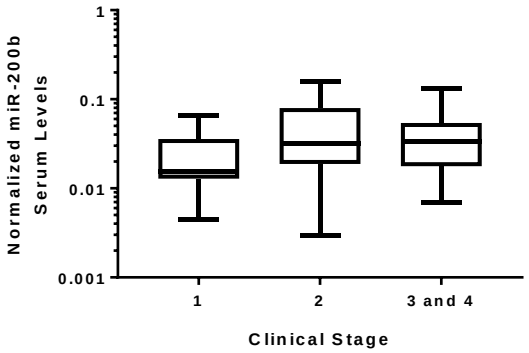
	I and II	III
Number of values	13	28
Minimum	0,004446	0,001198
25% Percentile	0,01271	0,01478
Median	0,02652	0,02504
75% Percentile	0,06115	0,04291
Maximum	0,1228	0,06626
Mean	0,0389	0,02895
Std. Deviation	0,03504	0,01885
Std. Error of Mean	0,009718	0,003562
Lower 95% CI	0,01772	0,02164
Upper 95% CI	0,06007	0,03626
Mean ranks	22,23	20,43

Mann Whitney test	
P value	0,6681
One- or two-tailed P value?	Two-tailed
Median of column A	0,02652, n=13
Median of column B	0,02504, n=28



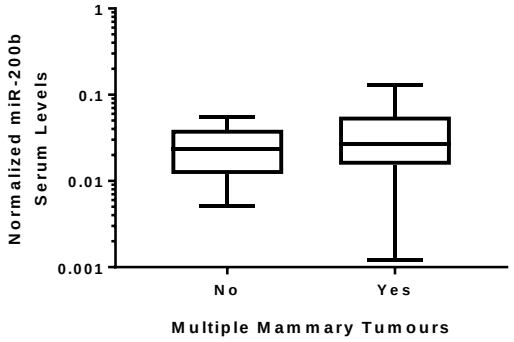
Kruskal-Wallis test	
P value	0,0078
Number of values (total)	40

	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma	Solid carcinoma	Tubular carcinoma	Tubulopapillary carcinoma
Number of values	5	4	2	9	7	13
Minimum	0,0434	0,02179	0,01594	0,001198	0,005082	0,004446
25% Percentile	0,04842	0,02229	0,01594	0,005163	0,006979	0,0141
Median	0,07836	0,02908	0,02096	0,04143	0,01239	0,02409
75% Percentile	0,168	0,03697	0,02599	0,05863	0,01514	0,03656
Maximum	0,2095	0,03785	0,02599	0,06626	0,01579	0,06832
Mean	0,1022	0,02945	0,02096	0,03423	0,01159	0,02772
Std. Deviation	0,06804	0,007859	0,007106	0,02569	0,004167	0,01855
Std. Error of Mean	0,03043	0,00393	0,005025	0,008563	0,001575	0,005145
Lower 95% CI	0,01777	0,01694	-0,04288	0,01448	0,00774	0,01651
Upper 95% CI	0,1867	0,04195	0,08481	0,05398	0,01545	0,03893
Mean ranks	35,8	22,25	18	21,78	9	19,77



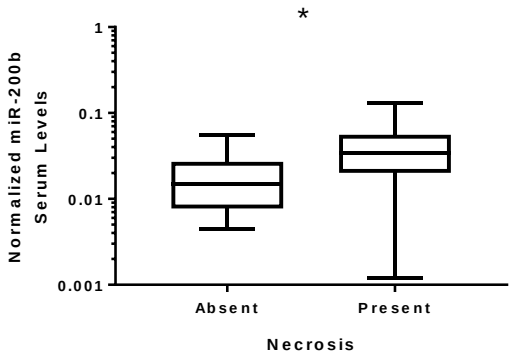
Kruskal-Wallis test	
P value	0,3123
Number of values (total)	38

	1	2	3 and 4
Number of values	10	6	22
Minimum	0,004446	0,002965	0,006979
25% Percentile	0,01287	0,01881	0,01775
Median	0,01556	0,03218	0,0334
75% Percentile	0,03581	0,07964	0,05427
Maximum	0,06626	0,1571	0,1305
Mean	0,02537	0,05039	0,03865
Std. Deviation	0,02161	0,0549	0,02862
Std. Error of Mean	0,006832	0,02241	0,006101
Lower 95% CI	0,009917	-0,007222	0,02596
Upper 95% CI	0,04083	0,108	0,05134
Mean ranks	14,9	21,33	21,09



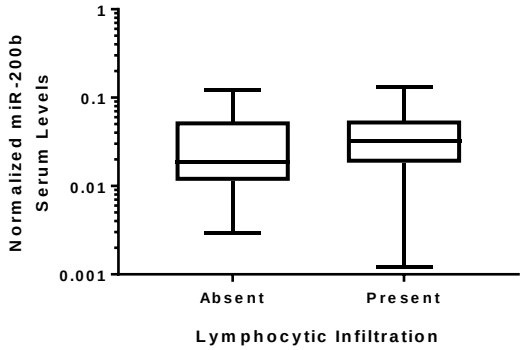
Mann Whitney test	
P value	0,2455
One- or two-tailed P value?	Two-tailed
Median of column A	0,02363, n=13
Median of column B	0,0266, n=29

	No	Yes
Number of values	13	29
Minimum	0,005082	0,001198
25% Percentile	0,01207	0,01548
Median	0,02363	0,0266
75% Percentile	0,03927	0,05585
Maximum	0,05511	0,1305
Mean	0,02515	0,03875
Std. Deviation	0,01567	0,03313
Std. Error of Mean	0,004345	0,006152
Lower 95% CI	0,01568	0,02614
Upper 95% CI	0,03462	0,05135
Mean ranks	18,15	23



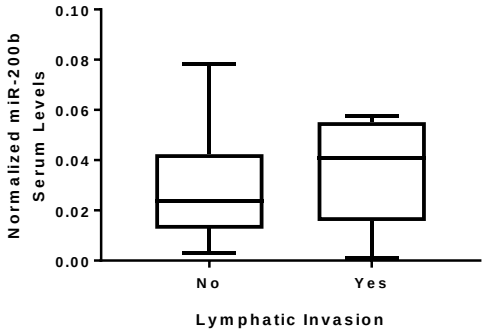
Mann Whitney test	
P value	0,0195
One- or two-tailed P value?	Two-tailed
Median of column A	0,01491, n=16
Median of column B	0,03436, n=25

	No	Yes
Number of values	16	25
Minimum	0,004446	0,001198
25% Percentile	0,007722	0,0201
Median	0,01491	0,03436
75% Percentile	0,02696	0,05576
Maximum	0,05511	0,1305
Mean	0,02148	0,04391
Std. Deviation	0,01765	0,03625
Std. Error of Mean	0,004412	0,007251
Lower 95% CI	0,01208	0,02895
Upper 95% CI	0,03088	0,05888
Mean ranks	15,56	24,48



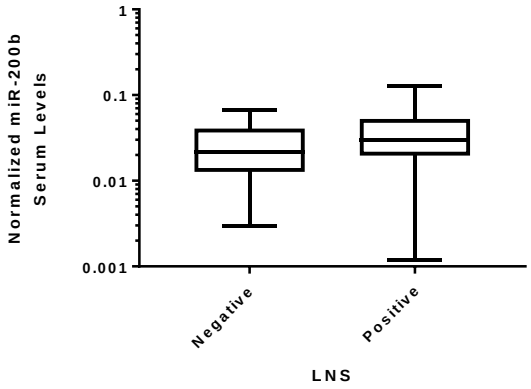
Mann Whitney test	
P value	0,2404
One- or two-tailed P value?	Two-tailed
Median of column A	0,01887, n=20
Median of column B	0,03244, n=23

	No	Yes
Number of values	20	23
Minimum	0,002965	0,001198
25% Percentile	0,01143	0,0184
Median	0,01887	0,03244
75% Percentile	0,05372	0,05511
Maximum	0,1228	0,1305
Mean	0,03232	0,0403
Std. Deviation	0,03079	0,0332
Std. Error of Mean	0,006884	0,006922
Lower 95% CI	0,01791	0,02595
Upper 95% CI	0,04673	0,05466
Mean ranks	19,55	24,13



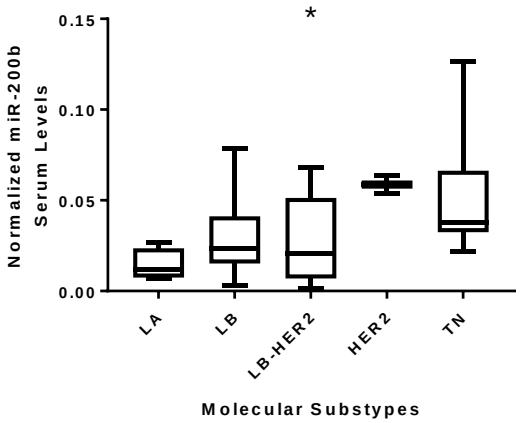
Mann Whitney test	
P value	0,3265
One- or two-tailed P value?	Two-tailed
Median of column A	0,02379, n=33
Median of column B	0,04069, n=7

	No	Yes
Number of values	33	7
Minimum	0,002965	0,001198
25% Percentile	0,01271	0,01579
Median	0,02379	0,04069
75% Percentile	0,04242	0,05511
Maximum	0,07836	0,05771
Mean	0,02839	0,03668
Std. Deviation	0,0206	0,02162
Std. Error of Mean	0,003587	0,008172
Lower 95% CI	0,02108	0,01668
Upper 95% CI	0,03569	0,05667
Mean ranks	19,64	24,57



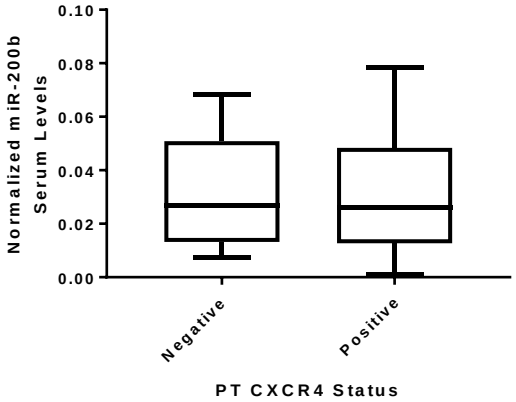
Mann Whitney test	
P value	0,2537
Median of column A	0,02179, n=21
Median of column B	0,02976, n=16

	Negative	Positive
Number of values	21	16
Minimum	0,002965	0,001198
25% Percentile	0,01271	0,01971
Median	0,02179	0,02976
75% Percentile	0,04062	0,05258
Maximum	0,06626	0,1265
Mean	0,02687	0,0382
Std. Deviation	0,01931	0,03119
Std. Error of Mean	0,004215	0,007797
Lower 95% CI	0,01807	0,02158
Upper 95% CI	0,03566	0,05482
Mean ranks	17,19	21,38



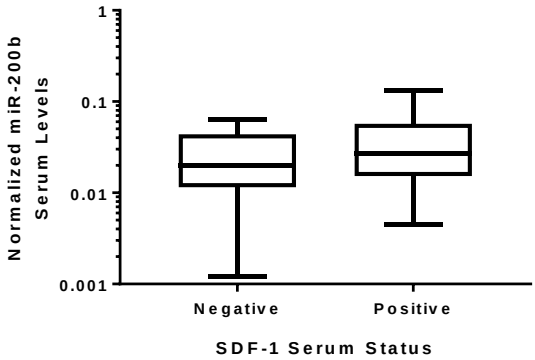
Kruskal-Wallis test	
P value	0,0396
Number of values (total)	41

	LA	LB	LB-HER2	HER2	TN
Number of values	4	16	12	2	7
Minimum	0,006979	0,002965	0,001198	0,05382	0,02179
25% Percentile	0,007436	0,01515	0,006909	0,05382	0,03244
Median	0,01173	0,02371	0,02096	0,05863	0,03785
75% Percentile	0,02355	0,04125	0,05133	0,06343	0,06626
Maximum	0,02652	0,07836	0,06832	0,06343	0,1265
Mean	0,01424	0,02765	0,02721	0,05863	0,05385
Std. Deviation	0,008815	0,01958	0,02278	0,006797	0,03552
Std. Error of Mean	0,004408	0,004895	0,006575	0,004807	0,01343
Lower 95% CI	0,0002127	0,01722	0,01274	-0,002447	0,02099
Upper 95% CI	0,02827	0,03808	0,04168	0,1197	0,0867
Mean ranks	11,25	19,56	18,58	35	30



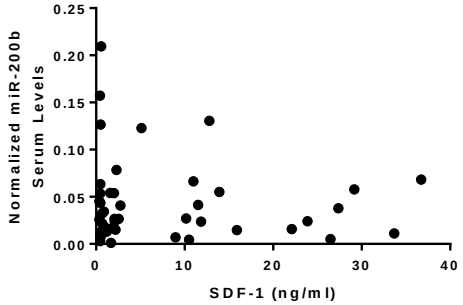
Mann Whitney test	
P value	0,8347
One- or two-tailed P value?	Two-tailed
Median of column A	0,02689, n=6
Median of column B	0,02599, n=33

	Negative	Positive
Number of values	6	33
Minimum	0,00736	0,001198
25% Percentile	0,01322	0,01271
Median	0,02689	0,02599
75% Percentile	0,05084	0,04842
Maximum	0,06832	0,07836
Mean	0,03161	0,02996
Std. Deviation	0,02311	0,02083
Std. Error of Mean	0,009433	0,003625
Lower 95% CI	0,007357	0,02258
Upper 95% CI	0,05586	0,03735
Mean ranks	21	19,82

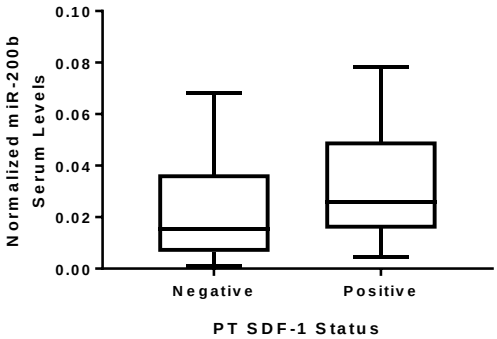


Mann Whitney test	
P value	0,1677
One- or two-tailed P value?	Two-tailed
Median of column A	0,0201, n=18
Median of column B	0,02684, n=24

	Negative	Positive
Number of values	18	24
Minimum	0,001198	0,004446
25% Percentile	0,01149	0,0153
Median	0,0201	0,02684
75% Percentile	0,0438	0,05706
Maximum	0,06343	0,1305
Mean	0,02606	0,04074
Std. Deviation	0,01902	0,03381
Std. Error of Mean	0,004483	0,006902
Lower 95% CI	0,0166	0,02646
Upper 95% CI	0,03552	0,05501
Mean ranks	18,44	23,79

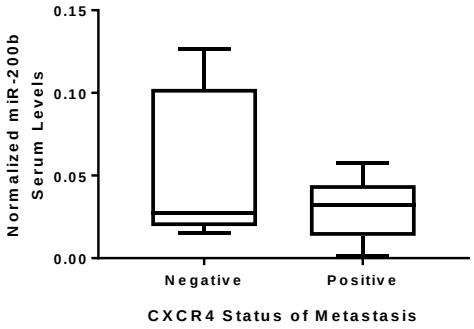


Spearman r	
r	-0,02543
95% confidence interval	-0,3246 to 0,2784
P value	
P (two-tailed)	0,8683
Number of XY Pairs	45



Mann Whitney test	
P value	0,1329
One- or two-tailed P value?	Two-tailed
Median of column A	0,01547, n=14
Median of column B	0,02599, n=25

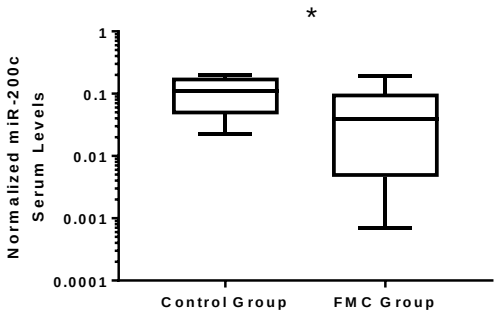
	Negative	Positive
Number of values	14	25
Minimum	0,001198	0,004446
25% Percentile	0,006505	0,01556
Median	0,01547	0,02599
75% Percentile	0,03662	0,04941
Maximum	0,06832	0,07836
Mean	0,02313	0,03265
Std. Deviation	0,02043	0,02039
Std. Error of Mean	0,005459	0,004078
Lower 95% CI	0,01134	0,02424
Upper 95% CI	0,03492	0,04107
Mean ranks	16,29	22,08



Mann Whitney test	
P value	0,5028
One- or two-tailed P value?	Two-tailed
Median of column A	0,02708, n=5
Median of column B	0,03244, n=13

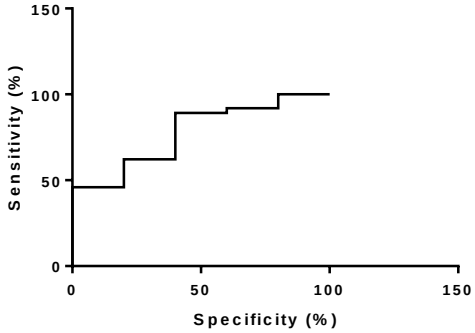
	Negative	Positive
Number of values	5	13
Minimum	0,01514	0,001198
25% Percentile	0,01946	0,0136
Median	0,02708	0,03244
75% Percentile	0,1024	0,04421
Maximum	0,1265	0,05771
Mean	0,05418	0,03068
Std. Deviation	0,04743	0,01857
Std. Error of Mean	0,02121	0,005151
Lower 95% CI	-0,004719	0,01946
Upper 95% CI	0,1131	0,04191
Mean ranks	11	8,923

Results for miR-200c



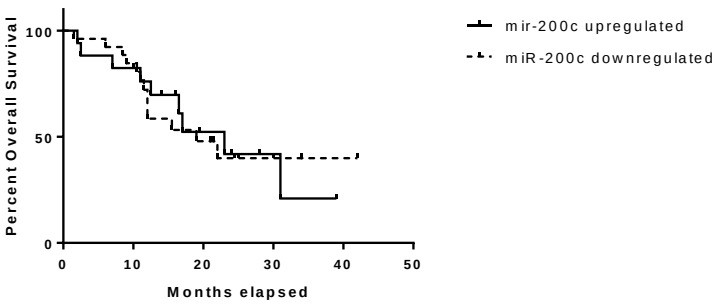
Mann Whitney test	
P value	0,0452
One- or two-tailed P value?	Two-tailed
Median of column A	0,1091, n=5
Median of column B	0,03879, n=37

	Control Group	FMC Group
Number of values	5	37
Minimum	0,02282	0,000705
25% Percentile	0,04669	0,004625
Median	0,1091	0,03879
75% Percentile	0,1809	0,1006
Maximum	0,2021	0,1959
Mean	0,1129	0,05446
Std. Deviation	0,07084	0,05697
Std. Error of Mean	0,03168	0,009365
Lower 95% CI	0,0249	0,03547
Upper 95% CI	0,2008	0,07345
Mean ranks	31,8	20,11

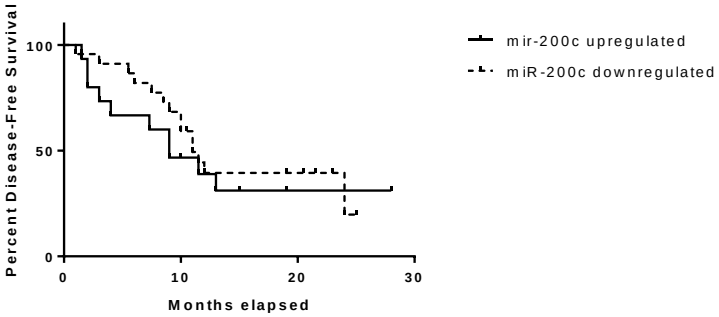


Area under the ROC curve	
Area	0,7784
Std. Error	0,1019
95% confidence interval	0,5787 to 0,978
P value	0,0455
Controls (Control Group)	5
Patients (FMC Group)	37

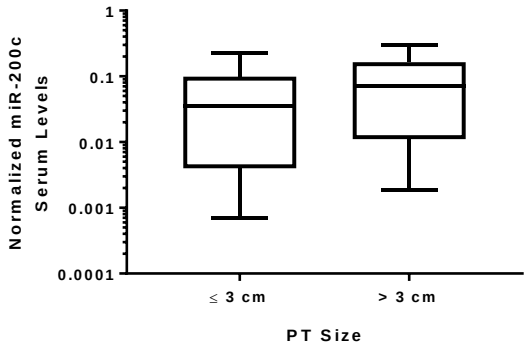
Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.000923	2,703	0,0684% to 14,16%	100	47,82% to 100%	
< 0.001504	5,405	0,6615% to 18,19%	100	47,82% to 100%	
< 0.002144	8,108	1,704% to 21,91%	100	47,82% to 100%	
< 0.002627	10,81	3,025% to 25,42%	100	47,82% to 100%	
< 0.002898	13,51	4,537% to 28,77%	100	47,82% to 100%	
< 0.003145	16,22	6,193% to 32,01%	100	47,82% to 100%	
< 0.00349	18,92	7,962% to 35,16%	100	47,82% to 100%	
< 0.00383	21,62	9,827% to 38,21%	100	47,82% to 100%	
< 0.004625	24,32	11,77% to 41,2%	100	47,82% to 100%	
< 0.006641	27,03	13,79% to 44,12%	100	47,82% to 100%	
< 0.009126	29,73	15,87% to 46,98%	100	47,82% to 100%	
< 0.01042	32,43	18,01% to 49,79%	100	47,82% to 100%	
< 0.01106	35,14	20,21% to 52,54%	100	47,82% to 100%	
< 0.01258	37,84	22,46% to 55,24%	100	47,82% to 100%	
< 0.01377	40,54	24,75% to 57,9%	100	47,82% to 100%	
< 0.01451	43,24	27,1% to 60,51%	100	47,82% to 100%	
< 0.01897	45,95	29,49% to 63,08%	100	47,82% to 100%	
< 0.02907	45,95	29,49% to 63,08%	80	28,36% to 99,49%	2,297
< 0.03706	48,65	31,92% to 65,6%	80	28,36% to 99,49%	2,432
< 0.03885	51,35	34,4% to 68,08%	80	28,36% to 99,49%	2,568
< 0.04097	54,05	36,92% to 70,51%	80	28,36% to 99,49%	2,703
< 0.04459	56,76	39,49% to 72,9%	80	28,36% to 99,49%	2,838
< 0.04932	59,46	42,1% to 75,25%	80	28,36% to 99,49%	2,973
< 0.06153	62,16	44,76% to 77,54%	80	28,36% to 99,49%	3,108
< 0.07452	62,16	44,76% to 77,54%	60	14,66% to 94,73%	1,554
< 0.08175	64,86	47,46% to 79,79%	60	14,66% to 94,73%	1,622
< 0.08648	67,57	50,21% to 81,99%	60	14,66% to 94,73%	1,689
< 0.09329	70,27	53,02% to 84,13%	60	14,66% to 94,73%	1,757
< 0.09891	72,97	55,88% to 86,21%	60	14,66% to 94,73%	1,824
< 0.1006	75,68	58,8% to 88,23%	60	14,66% to 94,73%	1,892
< 0.1023	78,38	61,79% to 90,17%	60	14,66% to 94,73%	1,959
< 0.1032	81,08	64,84% to 92,04%	60	14,66% to 94,73%	2,027
< 0.1047	83,78	67,99% to 93,81%	60	14,66% to 94,73%	2,095
< 0.1071	86,49	71,23% to 95,46%	60	14,66% to 94,73%	2,162
< 0.1088	89,19	74,58% to 96,97%	60	14,66% to 94,73%	2,23
< 0.1107	89,19	74,58% to 96,97%	40	5,274% to 85,34%	1,486
< 0.1361	91,89	78,09% to 98,3%	40	5,274% to 85,34%	1,532
< 0.1709	91,89	78,09% to 98,3%	20	0,5051% to 71,64%	1,149
< 0.1845	94,59	81,81% to 99,34%	20	0,5051% to 71,64%	1,182
< 0.1915	97,3	85,84% to 99,93%	20	0,5051% to 71,64%	1,216
< 0.199	100	90,51% to 100%	20	0,5051% to 71,64%	1,25



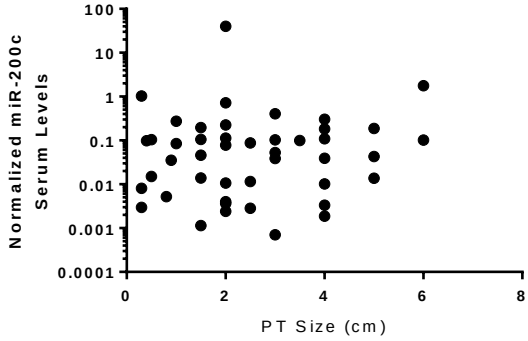
Log-rank (Mantel-Cox) test	
Chi square	0,009563
df	1
P value	0,9221
P value summary	ns



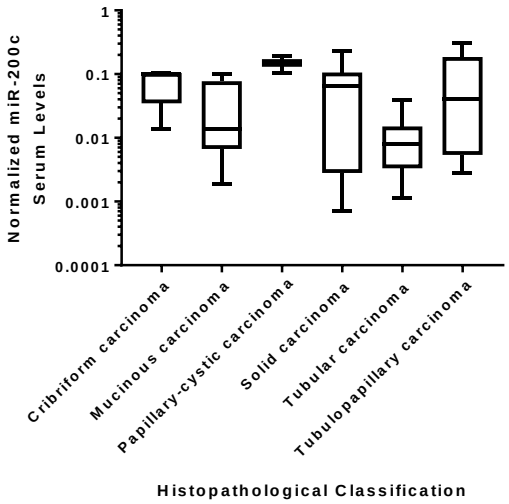
Comparison of Survival Curves	
Log-rank (Mantel-Cox) test	
Chi square	0,3473
df	1
P value	0,5556
P value summary	ns



Mann Whitney test	
P value	0,2578
One- or two-tailed P value?	Two-tailed
Median of column A	0,03532, n=27
Median of column B	0,0711, n=12

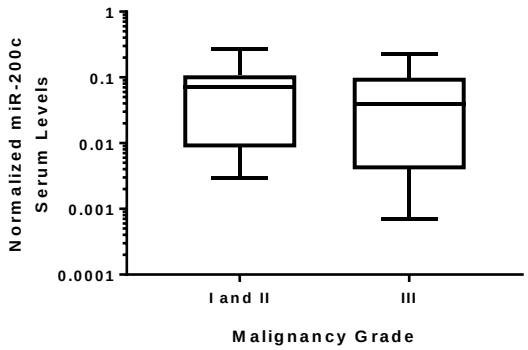


Spearman r	
r	0,07999
95% confidence interval	-0,2272 to 0,3727
P value	
P (two-tailed)	0,6014
Number of XY Pairs	45



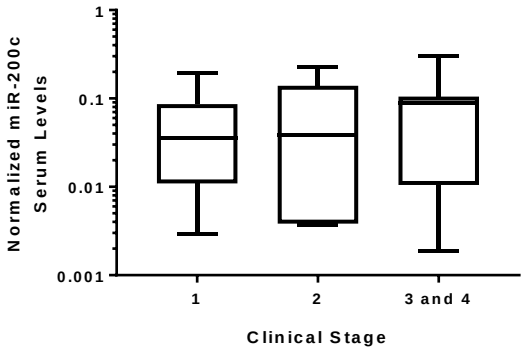
Kruskal-Wallis test	
P value	0,1449
Number of groups	6
Number of values (total)	38

	Cribriform carcinoma	Mucinous carcinoma	Papillary-cystic carcinoma	Solid carcinoma	Tubular carcinoma	Tubulopapillary carcinoma
Number of values	4	5	2	8	7	12
Minimum	0,01365	0,001867	0,1026	0,0007049	0,001141	0,002832
25% Percentile	0,0349	0,006684	0,1026	0,002818	0,003326	0,005396
Median	0,09891	0,01389	0,1493	0,06558	0,008039	0,04091
75% Percentile	0,104	0,07726	0,1959	0,1063	0,01512	0,1857
Maximum	0,1056	0,102	0,1959	0,2256	0,03891	0,3034
Mean	0,07927	0,03635	0,1493	0,07053	0,01171	0,09692
Std. Deviation	0,04386	0,04149	0,06599	0,0764	0,01285	0,1103
Std. Error of Mean	0,02193	0,01855	0,04666	0,02701	0,004857	0,03185
Lower 95% CI	0,009481	-0,01516	-0,4436	0,006661	-0,0001718	0,02682
Upper 95% CI	0,1491	0,08787	0,7422	0,1344	0,0236	0,167
Mean ranks	25,25	17	32,5	19,38	11,29	21,33



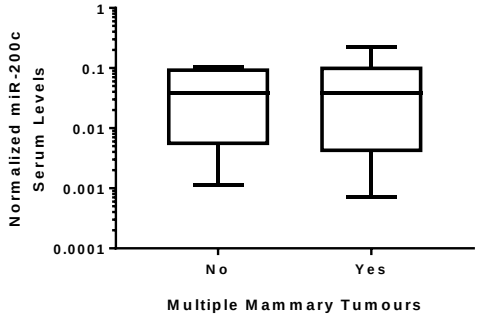
Mann Whitney test	
P value	0,3275
One- or two-tailed P value?	Two-tailed
Median of column A	0,07084, n=12
Median of column B	0,03879, n=27

	I and II	III
Number of values	12	27
Minimum	0,002963	0,000705
25% Percentile	0,008583	0,004007
Median	0,07084	0,03879
75% Percentile	0,1078	0,09917
Maximum	0,2749	0,2256
Mean	0,08241	0,05654
Std. Deviation	0,08465	0,06397
Std. Error of Mean	0,02444	0,01231
Lower 95% CI	0,02862	0,03124
Upper 95% CI	0,1362	0,08185
Mean ranks	22,75	18,78



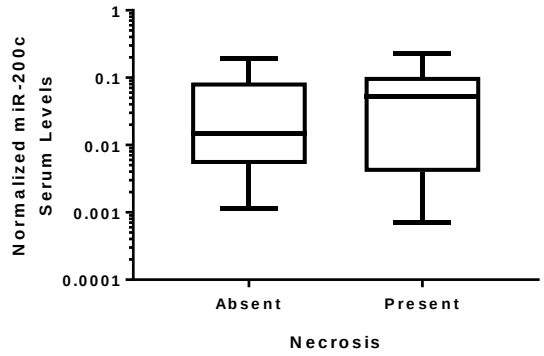
Kruskal-Wallis test	
P value	0,7171
Exact or approximate P value?	Approximate
Number of values (total)	36

	1	2	3 and 4
Number of values	9	5	22
Minimum	0,002963	0,003653	0,001867
25% Percentile	0,01096	0,00383	0,01052
Median	0,03532	0,03879	0,08856
75% Percentile	0,08648	0,1391	0,1049
Maximum	0,1959	0,2256	0,3034
Mean	0,05449	0,06492	0,07828
Std. Deviation	0,06175	0,09237	0,07619
Std. Error of Mean	0,02058	0,04131	0,01624
Lower 95% CI	0,007018	-0,04978	0,04449
Upper 95% CI	0,102	0,1796	0,1121
Mean ranks	16,89	16,4	19,64



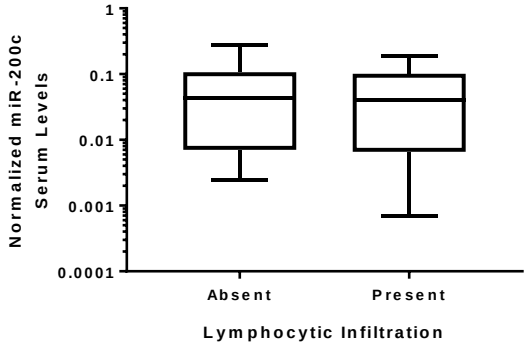
Mann Whitney test	
P value (two-tailed)	0,7272
Median of column A	0,03879, n=11
Median of column B	0,03891, n=27

	No	Yes
Number of values	11	27
Minimum	0,001141	0,000705
25% Percentile	0,005243	0,004007
Median	0,03879	0,03891
75% Percentile	0,09917	0,1056
Maximum	0,1037	0,2256
Mean	0,04638	0,06409
Std. Deviation	0,04259	0,06927
Lower 95% CI	0,01776	0,03669
Upper 95% CI	0,07499	0,0915
Mean ranks	18,45	19,93



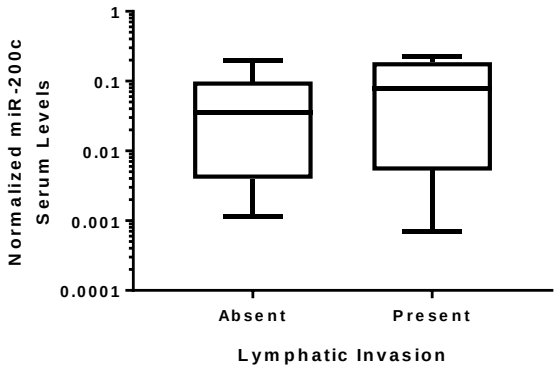
Mann Whitney test	
P value	0,3911
One- or two-tailed P value?	Two-tailed
Median of column A	0,01512, n=15
Median of column B	0,05249, n=23

	Absent	Present
Number of values	15	23
Minimum	0,001141	0,000705
25% Percentile	0,005243	0,004007
Median	0,01512	0,05249
75% Percentile	0,08502	0,1026
Maximum	0,1959	0,2256
Mean	0,04503	0,06805
Std. Deviation	0,05602	0,06627
Std. Error of Mean	0,01446	0,01382
Lower 95% CI	0,014	0,0394
Upper 95% CI	0,07605	0,09671
Mean ranks	17,53	20,78



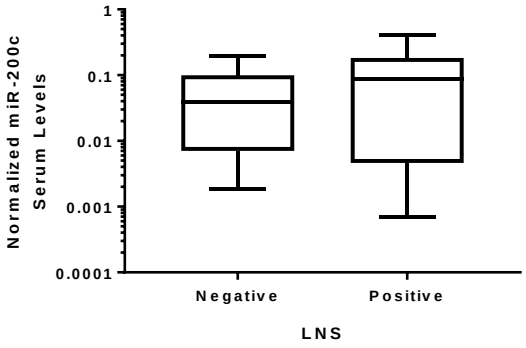
Mann Whitney test	
P value	0,6129
One- or two-tailed P value?	Two-tailed
Median of column A	0,04391, n=18
Median of column B	0,04097, n=20

	Absent	Present
Number of values	18	20
Minimum	0,002421	0,000705
25% Percentile	0,007031	0,006588
Median	0,04391	0,04097
75% Percentile	0,1063	0,1013
Maximum	0,2749	0,187
Mean	0,07486	0,05823
Std. Deviation	0,08392	0,05831
Std. Error of Mean	0,01978	0,01304
Lower 95% CI	0,03313	0,03094
Upper 95% CI	0,1166	0,08552
Mean ranks	20,5	18,6



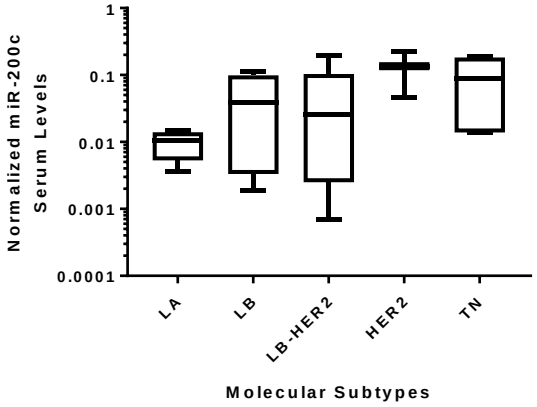
Mann Whitney test	
P value	0,3167
One- or two-tailed P value?	Two-tailed
Median of column A	0,03532, n=31
Median of column B	0,07848, n=7

	Absent	Present
Number of values	31	7
Minimum	0,001141	0,000705
25% Percentile	0,004007	0,005243
Median	0,03532	0,07848
75% Percentile	0,09917	0,187
Maximum	0,1959	0,2256
Mean	0,04912	0,1026
Std. Deviation	0,05032	0,09405
Std. Error of Mean	0,009038	0,03555
Lower 95% CI	0,03066	0,01559
Upper 95% CI	0,06758	0,1896
Mean ranks	18,61	23,43



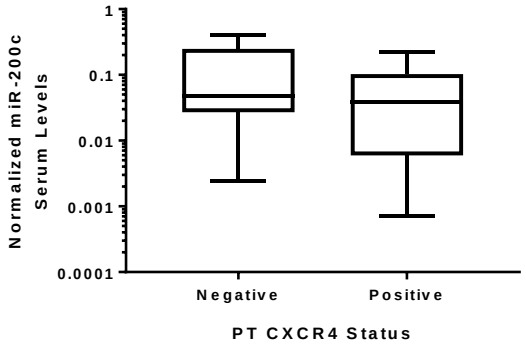
Mann Whitney test	
P value	0,5109
One- or two-tailed P value?	Two-tailed
Median of column A	0,03885, n=22
Median of column B	0,08856, n=14

	Negative	Positive
Number of values	22	14
Minimum	0,001867	0,000705
25% Percentile	0,007031	0,00464
Median	0,03885	0,08856
75% Percentile	0,09989	0,1833
Maximum	0,1959	0,4041
Mean	0,05186	0,1072
Std. Deviation	0,05203	0,1246
Std. Error of Mean	0,01109	0,03331
Lower 95% CI	0,0288	0,03524
Upper 95% CI	0,07493	0,1792
Mean ranks	17,55	20



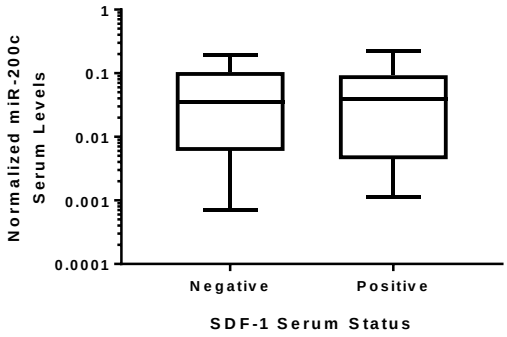
Kruskal-Wallis test	
P value	0,1968
Number of groups	5
Number of values (total)	38

	LA	LB	LB-HER2	HER2	TN
Number of values	4	15	10	2	7
Minimum	0,003653	0,001867	0,000705	0,04614	0,01365
25% Percentile	0,005293	0,003326	0,002508	0,04614	0,01389
Median	0,01042	0,03879	0,02554	0,1359	0,08793
75% Percentile	0,014	0,09917	0,1034	0,2256	0,1821
Maximum	0,01512	0,1124	0,1959	0,2256	0,187
Mean	0,009903	0,04933	0,05503	0,1359	0,09129
Std. Deviation	0,004723	0,04539	0,06581	0,1269	0,07193
Std. Error of Mean	0,002362	0,01172	0,02081	0,08975	0,02719
Lower 95% CI	0,002388	0,02419	0,007956	-1,004	0,02476
Upper 95% CI	0,01742	0,07446	0,1021	1,276	0,1578
Mean ranks	12,5	18,4	17,5	30	25,71



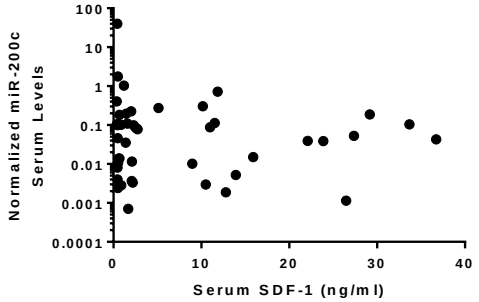
Mann Whitney test	
P value	0,4448
One- or two-tailed P value?	Two-tailed
Median of column A	0,04776, n=6
Median of column B	0,03885, n=32

	Negative	Positive
Number of values	6	32
Minimum	0,002421	0,000705
25% Percentile	0,0271	0,005942
Median	0,04776	0,03885
75% Percentile	0,248	0,1025
Maximum	0,4041	0,2256
Mean	0,1222	0,05963
Std. Deviation	0,1536	0,06207
Std. Error of Mean	0,0627	0,01097
Lower 95% CI	-0,03895	0,03725
Upper 95% CI	0,2834	0,08201
Mean ranks	22,83	18,88

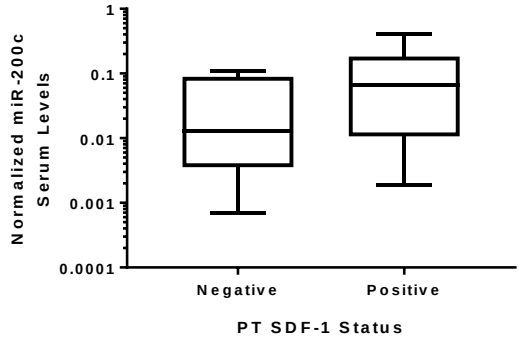


Mann Whitney test	
P value	0,8618
One- or two-tailed P value?	Two-tailed

	Negative	Positive
Number of values	17	21
Minimum	0,000705	0,001141
25% Percentile	0,006023	0,004448
Median	0,03532	0,03891
75% Percentile	0,1041	0,09329
Maximum	0,1959	0,2256
Mean	0,0608	0,05748
Std. Deviation	0,06441	0,0628
Std. Error of Mean	0,01562	0,0137
Lower 95% CI	0,02769	0,02889
Upper 95% CI	0,09392	0,08607
Mean ranks	19,88	19,19

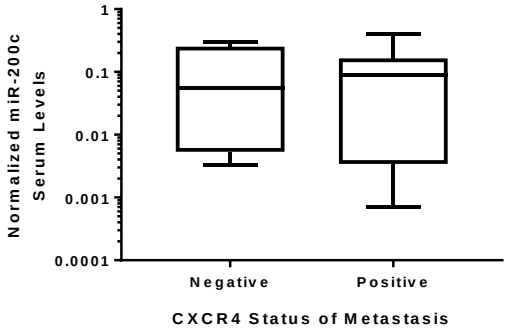


Spearman r	
r	-0,1298
95% confidence interval	-0,4153 to 0,1788
P value	
P (two-tailed)	0,3953
Number of XY Pairs	45



Mann Whitney test	
P value	0,0762
One- or two-tailed P value?	Two-tailed
Median of column A	0,01287, n=14
Median of column B	0,06549, n=26

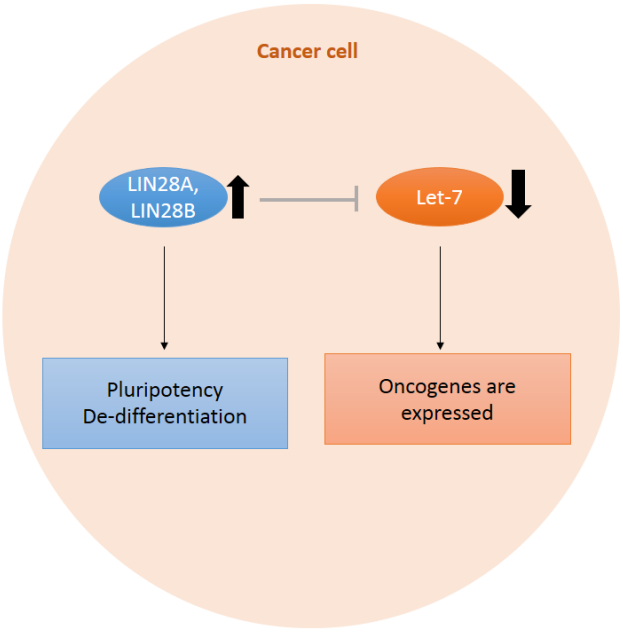
	Negative	Positive
Number of values	14	26
Minimum	0,000705	0,001867
25% Percentile	0,003571	0,01064
Median	0,01287	0,06549
75% Percentile	0,08856	0,1833
Maximum	0,1086	0,4041
Mean	0,03754	0,09969
Std. Deviation	0,04241	0,1088
Std. Error of Mean	0,01134	0,02133
Lower 95% CI	0,01305	0,05576
Upper 95% CI	0,06202	0,1436
Mean ranks	16	22,92



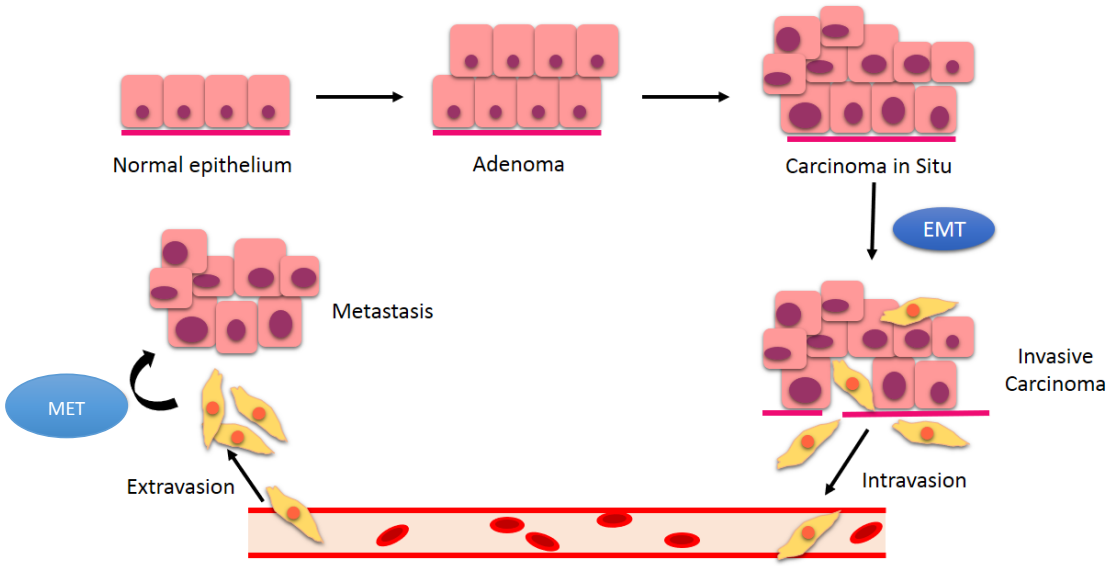
Mann Whitney test	
P value	0,9527
One- or two-tailed P value?	Two-tailed
Median of column A	0,05508, n=4
Median of column B	0,08883, n=12

	Negative	Positive
Number of values	4	12
Minimum	0,003326	0,000705
25% Percentile	0,00537	0,003435
Median	0,05508	0,08883
75% Percentile	0,2522	0,1646
Maximum	0,3034	0,4041
Mean	0,1042	0,09886
Std. Deviation	0,1396	0,1181
Std. Error of Mean	0,06982	0,03409
Lower 95% CI	-0,118	0,02382
Upper 95% CI	0,3264	0,1739
Mean ranks	8,75	8,417

ANNEX III – Schematic illustration of let-7a involvement in oncogenesis



ANNEX IV: Role of EMT and MET in metastasis formation



ANNEX V – MicroRNAs in Human Breast Cancer and Feline Mammary Carcinoma

microRNA	In Human Breast Cancer	Clinical Associations	Biomarker of	In Feline Mammary Carcinoma	Clinical Associations	Biomarker of
let-7a	Downregulated	• Metastasis • Overall survival • Tumour size	Diagnosis and prognosis	Downregulated	• Overall survival • Histopathological subtypes	Diagnosis and prognosis
miR-21	Upregulated	• Stage and grade • Lymph node metastasis • Disease-free survival • Overall survival	Diagnosis and prognosis	Downregulated (not significant)	• Lymph node metastasis • Disease-free survival	Prognosis
miR-200b	Downregulated	• Metastasis • Overall survival • Disease-free survival	Prognosis	Downregulated (not significant)	• Disease-free survival • Presence of necrosis • Histopathologic and molecular subtypes • Tumour size	Prognosis
miR-200c	Downregulated	• Lymph node metastasis • Overall survival	Diagnosis and prognosis	Downregulated	-	Diagnosis
miR-10b	Upregulated	• Metastasis	Prognosis	Upregulated (not significant)	-	-