Serum levels and tumour expression of leptin and leptin receptor as promising clinical biomarkers of specific feline mammary carcinoma subtypes

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Abstract: Obesity is a risk factor for human breast cancer, being associated with increased serum levels of leptin. In cat, although obesity is a common nutritional disorder, the role of leptin and its receptor in mammary carcinoma is unknown. In this study, serum levels of leptin and leptin receptor (ObR) were evaluated in 58 cats with mammary carcinoma and compared with healthy controls by ELISA, as tumour expression by immunohistochemistry. Results showed that the free leptin index is decreased in cats with mammary carcinoma (p=0.0006), particularly for those with luminal B and HER2-positive disease that showed significantly lower serum leptin levels (p<0.0001 and p<0.005, respectively). Serum leptin levels above 4.17 pg/mL were associated with ulcerating tumours (p=0.0005) and shorter DFS (p=0.0217). Elevated serum ObR levels were found in all cats with mammary carcinoma (p<0.0001), with levels above 16.89 ng/mL being associated with smaller tumours (p=0.0118), ER-negative status in HER2-positive tumours (p=0.0291) and increased serum levels of CTLA-4 (p=0.0056), TNF-α (p=0.0025), PD-1 (p=0.0023) and PD-L1 (p=0.0002). In tumour samples, leptin was overexpressed in luminal B and triple-negative carcinomas (p=0.0046), whereas ObR was found overexpressed in luminal B samples (p=0.0425). Altogether, our results reinforce the importance of feline mammary carcinoma in comparative oncology.

Keywords: feline mammary carcinoma, free leptin index, leptin, leptin receptor, immunosuppression, biomarkers

1. Introduction

The feline mammary carcinoma (FMC) is a high prevalence disease (12 to 40% of all tumours in cat) that shows similar clinicopathological features to human breast cancer [1], supporting its use in comparative oncology studies [2] [3]. Likewise, obesity is a common nutritional disorder in cat, with higher prevalence in indoor and sterilized animals above three years of age [4]. In humans, obesity induces a chronic inflammatory status, being a risk factor for breast cancer [5] [6] [7].

Leptin is a 16 kDa adipocytokine, encoded by the *obese* gene and involved in the central regulation of food intake, energy homeostasis, modulation of reproductive function and peripheral metabolic processes, such as breast/mammary gland development, cellular proliferation and angiogenesis. In tissues and serum, leptin expression is modulated by fat mass, with healthy cats showing lower serum leptin levels than obese animals [4], as reported in humans [5] [6]. Interestingly, although this protein is mainly secreted by adipocytes, it can be also expressed by pathologically altered cells, such as cancer cells [8] [9]. Thus, malignant cells can regulate their metabolic activities [10], promoting uncontrolled cell growth, migration, invasion and angiogenesis [11] [7], and downregulating apoptosis through a Bcl-2-dependent mechanism [10] [12]. Accordingly, leptin overexpression is detected in breast cancer cells and neighbouring adipocytes, contrasting with normal breast glandular epithelial cells [13] [7], suggesting an oncogenic role for this adipocytokine [6]. Furthermore, studies in human breast cancer patients showed that leptin overexpression has paracrine effects, not always reflected in serum levels, although associated with more aggressive

tumours and therapy resistance [13]. Additionally, in overweight human patients a positive correlation was found between leptin overexpression in the tumour microenvironment and oestrogen receptor (ER) positive breast cancer, and with a human epidermal growth factor receptor 2 (HER 2)-positive status frequently related to a more invasive tumour phenotype [14].

In parallel, the leptin receptor (ObR, 150-190 kDa) was found to be involved in innate and adaptive immunity [15], being expressed in several organs, including breast and peripheral tissues, as well as in adipocytes [16] [17] and immune cells. ObR is constituted by an extracellular N-terminus domain, a transmembrane domain, and a cytoplasmic C-terminus domain. Upon leptin ligation, ObR homodimerizes and the associated JAK monomer is auto phosphorylated to activate the downstream signalling pathways [8]. The soluble ObR form is a 146 kDa protein [18] that could be generated by cellular apoptosis or by the proteolytic cleavage of the extracellular anchored protein domain, with this shedding being more frequent in shorter intracellular isoforms. In serum, the ObR modulates the leptin bioavailability, being decreased in obese humans [8]. In breast cancer patients, ObR is overexpressed independently of the ER status [6], being correlated with low overall survival (OS) [9]. Furthermore, the ratio between leptin/ObR serum levels (free leptin index – FLI) is considered an useful predictor of leptin activity, reflecting the individual metabolic status [19] and when increased, is an important risk factor for breast cancer development [20]. In parallel, studies in breast cancer patients found an association between leptin and ObR overexpression with a chronic inflammatory status, conditioning T-cell immune responses (increase Th1- and decrease Th2-responses) [21] and the activation of immune checkpoint inhibitors [16]. Indeed, some studies in humans have shown a positive correlation between overexpression of leptin and ObR with several immunomodulatory molecules (e.g. Cytotoxic T-Lymphocyte Associated Protein 4 - CTLA-4; Tumor Necrosis Factor α - TNF-α; Programmed Cell Death-1 - PD-1 and Programmed Cell Death-ligand 1 - PD-L1) [22] [23]. While the CTLA-4 is a protein related to the inflammatory response that is increased in breast cancer patients, contributing for the immune response downregulation [24], the TNF-α is a pro-inflammatory cytokine that induces apoptosis promoted by the absence of leptin [25]. Moreover, the overexpression of PD-1 in T-cells is associated with ObR overexpression in humans with distinct tumour types [26], induced through the AKT pathway activation by oestrogens [27] and being responsible for the PD-1 mediated T-cell dysfunction [28].

As mentioned above, obesity is associated with increased leptin levels, which induces resistance to chemotherapy [29] [30]. Therefore, the leptin/ObR axis has been widely studied [31] as a target for a adjuvant therapy, not only in ER-positive tumour status [29], but also in triple-negative tumours [32], in which the lack of hormonal receptors reduces the therapeutic options. The use of leptin antagonists, allows the blocking of the leptin receptor, leading to a downregulation of the leptin downstream pathways, some of them known as oncogenic (e.g. Wnt and STAT3) [29] [32] [31].

To the best of our knowledge, this study is the first to evaluate the leptin and ObR serum levels and tissue expression in cats with mammary carcinoma. The main goals of this study were as follows: 1) to compare the serum leptin and ObR levels between cats with mammary carcinoma stratified by molecular subtype and healthy controls, using ELISA; 2) to investigate the leptin and ObR expression in mammary carcinoma samples and compare with normal mammary tissues, using immunohistochemistry (IHC); 3) to search for associations between serum leptin/ObR levels and leptin/ObR IHC scores in tumour mammary tissues; 4) to test for associations between serum leptin/ObR levels and several clinicopathological features, in order to evaluate the utility of leptin and ObR as diagnostic and/or prognosis biomarkers or as promising drug targets for feline mammary carcinoma.

2. Materials and Methods

2.1. Animal population

Tumour tissue and serum samples were collected from 58 female cats with fully documented history of FMC that underwent mastectomy and 24 serum samples from healthy cats presented for elective ovariohysterectomy, at the Teaching Hospital of the Faculty of Veterinary Medicine,

96 University of Lisbon. The animals were anesthetized before surgical procedures and blood samples
97 were collected with no interference in animal well-being, with the procedures involving
98 manipulation of animals being consented by the owners. Briefly, all tissue samples were embedded
99 in paraffin after fixation in 10% buffered neutralized formalin (pH 7.2), during 24-48 hours, while
100 serum samples were separated from clotted blood by centrifugation (1500g, 10min, 4ºC) and stored
101 at -80ºC until further use. All samples that showed haemolysis were discarded, as recommended [1]
102 [33].
103   For each animal enrolled in the study, the clinicopathological data were recorded, including age,
104 breed, body weight, reproductive status and contraceptive administration, treatment (none,
105 mastectomy or mastectomy plus chemotherapy), number, location and size of tumoral lesions,
106 histopathological classification (ER status, PR status, HER2 status and Ki-67 index), malignancy
107 grade, presence of tumour necrosis, lymphatic invasion, lymphocytic infiltration, cutaneous
108 ulceration, regional lymph node involvement and clinical stage (TNM system) (Table 1). Regarding
109 the molecular subtyping of feline mammary carcinomas [34], animals were stratified in luminal A
110 (n=10), luminal B (n=17), HER2-positive (n=15) and triple-negative (n=16) groups.

111   **Table 1.** Clinicopathological features of female cats with mammary carcinomas enrolled in this
112   study (n=58).

Clinicopathological feature	Number of animals (%)	Clinicopathological feature	Number of animals (%)
Breed		**Size**	
Undifferentiated	44 (75.9%)	<2cm	22 (37.9%)
Siamese	7 (12.1%)	≥2cm	36 (62.1%)
Persian	5 (8.6%)	**Animal Weight**	(23 unknown)
Norwegian Forest	2 (3.4%)	<3kg	6 (10.3%)
Age		3 – 5kg	24 (41.4%)
<8years old	4 (6.9%)	>5kg	5 (8.6%)
≥8 years old	54 (93.1%)	**Treatment**	(3 unknown)
Reproductive status	(1 unknown)	Mastectomy	49 (84.5%)
Spayed	20 (34.5%)	Mastectomy + Chemo	4 (6.9%)
Pill	21 (36.2%)	None	2 (3.4%)
Both	9 (15.5%)	**Multiple tumours**	
Any	7 (12.1%)	Yes	35 (60.3%)
Lymph node status	(4 unknown)	No	23 (39.7%)
Positive	19 (32.8%)	**Malignancy grade**	(1 unknown)
Negative	35 (60.3%)	I	3 (5.2%)
Stage (TNM)		II	8 (13.8%)
I	15 (25.9%)	III	46 (79.3%)
II	6 (10.3%)	**Necrosis**	
III	31 (53.4%)	Yes	42 (72.4%)
IV	6 (10.3%)	No	16 (27.6%)
Lymphatic invasion		**Lymphocytic infiltration**	(2 unknown)
Yes	7 (12.1%)	Yes	37 (63.8%)
No	51 (87.9%)	No	19 (32.8%)
HER2 status		**Tumour ulceration**	
Positive	14 (24.1%);	Yes	8 (13.8%)
Negative	44 (75.9%)	No	50 (86.2%)
ER status		**Ki67 index**	(1 unknown)
Positive	31 (53.4%)	Low (<14%)	18 (31%)
Negative	27 (46.6%)	High (≥14%)	39 (67.2%)
PR status			

Positive	36 (62.1%)
Negative	22 (37.9%)

113 TNM – Tumor, Node, Metastasis; ER – Estrogen Receptor; PR – Progesterone Receptor.

114 *2.2. Measurement of serum leptin, ObR, CTLA-4, TNF-α, PD-1 and PD-L1 levels*

115   The serum levels of leptin, ObR, CTLA-4, TNF-α, PD-1 and PD-L1 were measured by using
116 commercial ELISA-based kits from R&D Systems (Minneapolis, USA; DY389, DY398-05, DY476,
117 DY2586, DY1086, DY156, respectively) and following the manufacture's recommendations. After
118 collection, the serum samples were kept stored at -80ºC and thawed shortly before use. For each
119 assay, a standard curve was generated by using serial dilutions of the recombinant proteins from kits.
120 For leptin, ObR, PD-1 and PD-L1 the r^2 values were calculated using a quadratic regression ($r^2= 0.9976$,
121 for leptin, $r^2= 0.9632$, for ObR, $r^2=0.99$ for PD-1 and $r^2=0.96$ for PD-L1), whereas serum CTLA-4 and
122 TNF-α concentrations were determined by using a curve-fitting equation ($r^2>0.99$), as previously
123 reported [35].
124   Briefly, a 96-well plate was prepared by adding the capture antibody to each well and incubate
125 overnight. Plates were then treated with 1% bovine serum albumin (BSA) in phosphate buffered
126 saline (PBS), for 1 hour, to prevent nonspecific binding. Standards and diluted serum samples were
127 added to sample wells and incubated for 2 hours at room temperature (RT), before the incubation of
128 the detection antibody for 2 hours at RT. Afterwards, the streptavidin-conjugated to horseradish
129 peroxidase (HRP) was added to each well and incubated at RT, for 20 minutes, before the addition of
130 the substrate solution in 1:1 H_2O_2 and tetramethyl-benzidine to each well (20 minutes, at RT, in the
131 dark). The reaction was interrupted by adding a stop solution ($2NH_2SO_4$) and the absorbance was
132 measured by a spectrophotometer (FLUOStar OPTIMA, Microplate Reader, BMG, Ortenberg,
133 Germany), using 450 nm as the primary wavelength and 570 nm as a reference wavelength.

134 *2.3. Assessment of the leptin and ObR status by immunohistochemistry (IHC)*

135   Initially, the feline mammary carcinoma formalin fixed paraffin-embedded (FFPE) samples were
136 stained with haematoxylin-eosin to select a representative tumour area and a normal tissue area to
137 be used as control (n=20). FFPE samples were sectioned in slices with 3 µm thickness (Microtome
138 Leica RM135, Newcastle, UK) and mounted on a glass slide (SuperFrost Plus, Thermo Fisher
139 Scientific, Massachusetts, USA). On PT-Link module (DAKO, Agilent, Santa Clara, USA), samples
140 were deparaffinized, hydrated and antigen retrieval was performed, for 20 minutes, at 96ºC, using
141 Tris-EDTA buffer, pH 9.0 (EnVision™ Flex Target Retrieval Solution High pH, Dako). Then, slides
142 were cooled for 30 minutes at RT and immersed twice, for 5 minutes in distilled water. IHC technique
143 was performed with commercial solutions from the Novolink™ Max Polymer Detection System Kit
144 (Leica Biosystems, Newcastle UK). Before antibody incubation, tissue samples were treated to block
145 the endogenous peroxidase activity for 15 minutes, and the unspecific antigenic recognition was
146 inhibited for 10 minutes. Finally, tissue samples were incubated at RT for 1 hour, in a humidified
147 chamber, with the following primary antibodies: anti-leptin antibody (ab3583, AbCAM, Cambridge,
148 UK) and anti-ObR antibody (ab104403, AbCAM), both diluted at 1:200. The slides were washed twice,
149 for 5 minutes, between all the incubation steps, using a PBS solution at pH 7.4. Then, the detection
150 polymer was incubated for 30 minutes, at RT, and detection was performed using diaminobenzidine
151 (DAB substrate buffer and DAB Chromogen, Leica Biosystems) for 5 minutes. Later, samples were
152 counterstained with Gills haematoxylin (Merck, New Jersey, USA) for 5 minutes, dehydrated in an
153 ethanol gradient and xylene, and mounted using Entellan mounting medium (Merck).
154   To assess leptin and ObR immunoreactivity were used a scoring system previously reported [6]
155 [13] [36] and the H-Score published by the American Society of Clinical Oncology (ASCO). The final
156 IHC score was obtained by multiplying the positive cells (0=absence of staining; 1=all cells stained),
157 by the highest staining intensity (Table 2), varying from 0 to 3, with tissue samples scored as 0
158 considered negative, while samples scored as 3 designated highly reactive. All slides were subjected
159 to blind scoring, by two independent and experienced pathologists.

160
161   **Table 2.** Scoring criteria of immunostaining assay for leptin and ObR. Three microscopic fields were analyzed at 400x magnification.

	Staining intensity	
	Score	Interpretation
Stained tumour cells	0	No staining
(0-1)	1	Weak
	2	Moderate
	3	Strong*
Final IHC score = stained tumour cells x staining intensity score (0 – 3)		

162   * High protein reactivity

163   *2.4. Statistical analysis*

164   Statistical analysis was carried out using the GraphPad Prism software, version 5.04 (California,
165   USA), with two-tailed p-values less than 0.05 considered statistically significant for a 95% confidence
166   level (*p<0.05, **p<0.01 and ***p<0.001).
167   The non-parametric Kruskal-Wallis test was performed to compare leptin and ObR results
168   between healthy cats and cats with mammary carcinoma stratified by tumour subtype. Receiver-
169   operating characteristic (ROC) curves were performed to choose the optimal cut-off value for serum
170   leptin and ObR levels, and to determine the specificity and sensitivity of the technique to diagnose
171   the disease. The non-parametric Mann-Whitney test was used to compare the serum levels of both
172   proteins with several clinicopathological features. Survival analysis was performed using the Kaplan-
173   Meier test to evaluate the disease-free survival (DFS) in cats with mammary carcinomas. The
174   correlations between serum ObR levels and serum concentrations of the inflammatory proteins
175   CTLA-4, TNF-α, PD-1 and PD-L1 were investigated using the Spearman's rank correlation
176   coefficient.

177   **3. Results**

178   *3.1. Cats with mammary carcinoma showed a reduced Free Leptin Index*

179   The Free Leptin Index (FLI) was determined in the serum samples of cats with mammary
180   carcinomas and compared with healthy animals. Results obtained showed that cats with disease had
181   a significantly lower FLI than control group (0.44 vs. 0.86, p=0.0006, Figure 1).
182   In addition, results revealed that body weight did not influence serum leptin and ObR levels,
183   both in the controls (p=0.0760 and p=0.8432, respectively, data not shown) and in the cancer group
184   (p=0.3294 and p=0.9722, respectively, data not shown).

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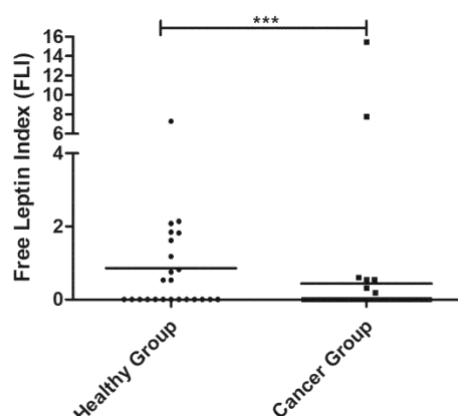
186 **Figure 1. -** Dot plot diagram showing that the free leptin index (FLI) was significantly elevated in
187 healthy animals that in cats with mammary carcinoma (p=0.0006).

188 *3.2. Cats with Luminal B or HER2-positive mammary carcinomas showed decreased serum leptin levels*

189 Regarding serum leptin levels, results obtained showed that cats with luminal B or HER2-
190 positive mammary carcinomas had lower serum leptin levels than healthy animals (0.00 pg/mL vs.
191 13.89 pg/ml, p<0.001; 0.83 pg/mL vs. 13.89 pg/mL, p<0.05, respectively, Figure 2A). The optimal cut-
192 off value to predict mammary carcinoma was 4.17 pg/ml with an area under the ROC curve (AUC)
193 of 0.7045±0.0757 (95% CI: 0.5561-0.8528, p=0.0103; sensitivity=96.9%; specificity=43.5%; Figure 2B).
194 Further statistical analysis showed that elevated serum leptin levels were associated with tumour
195 ulceration (p=0.0005, Figure 2C) and shorter DFS (117 vs. 314 days, p=0.0217, Figure 2D).

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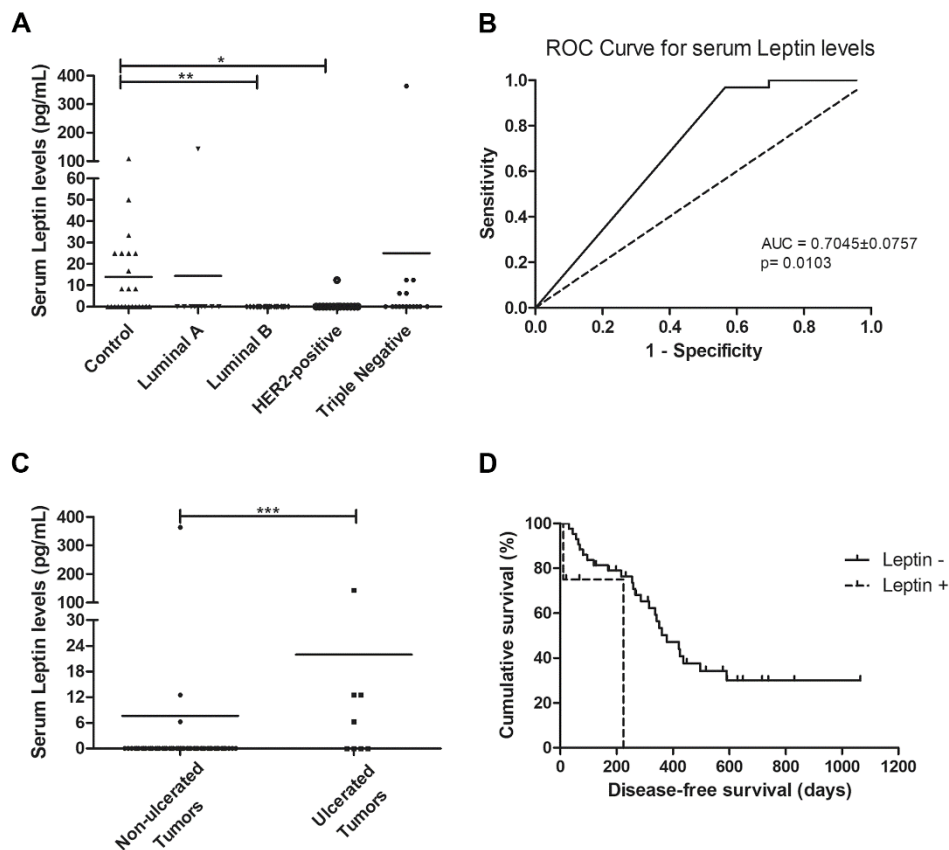
197 **Figure 2.** – Cats with luminal B and HER2-positive mammary carcinomas showed decreased serum leptin
198 levels, as cats had ulcerated tumours, with serum leptin levels above the cut-off level of 4.17 pg/mL being
199 associated with shorter disease-free survival. **A)** Dot plot diagram showing the distribution of serum leptin levels
200 (pg/mL) among healthy animals (control) and cats stratified by mammary carcinoma subtype. Significant
201 decreased serum levels of leptin were found in cats presenting luminal B or HER2-positive subtypes in
202 comparison to healthy animals (p=0.0025). **B)** The optimal cut-off of serum leptin levels to predict mammary
203 carcinoma was determined to maximize the sum of the sensitivity and specificity (4.17 pg/mL;
204 AUC=0.7045±0.0757, 95% CI: 0.5561-0.8528, p=0.0103; sensitivity=96.9%; specificity=43.5%). **C)** Dot plot diagram
205 showing that serum leptin levels were significantly higher in cats with ulcerated tumours (p=0.0005). **D)** Cats
206 with mammary carcinoma and serum leptin levels higher than 4.17 pg/mL had a lower DFS (p=0.0217). *p<0.05;
207 **p<0.001; ***p<0.0001.

208 *3.3. Cats with mammary carcinoma showed elevated serum levels of ObR and of mediators of inflammation*

209   Considering the above results, the serum levels of the ObR were also evaluated. When the
210 animals were grouped according to the tumour subtype, a significant difference was found between
211 the mean ranks of at least one pair of groups ($p < 0.0001$). Results revealed that serum ObR levels were
212 significantly higher in animals with mammary carcinoma than in controls, independently of
213 molecular subtype (control group 15.67 ng/ml; luminal A 23.04 ng/ml, $p < 0.0001$; luminal B 20.18
214 ng/ml, $p < 0.001$; HER2-positive 28.99 ng/ml, $p < 0.0001$; triple-negative 21.70 ng/ml, $p < 0.0001$; Figure
215 3A). Furthermore, the optimal cut-off value calculated for cats with mammary carcinoma was 16.89
216 ng/ml, with an AUC of 0.9408 ± 0.0288 (95% CI: 0.8842-0.9973, $p < 0.0001$; sensitivity=94.8%;
217 specificity=87.0%; Figure 3B).
218   In addition, elevated serum ObR levels were associated with smaller tumours (p=0.0118, Figure
219 3C) and with cats had HER2-positive mammary tumours showing an ER-negative status (p=0.0291,
220 Figure 3D).
221
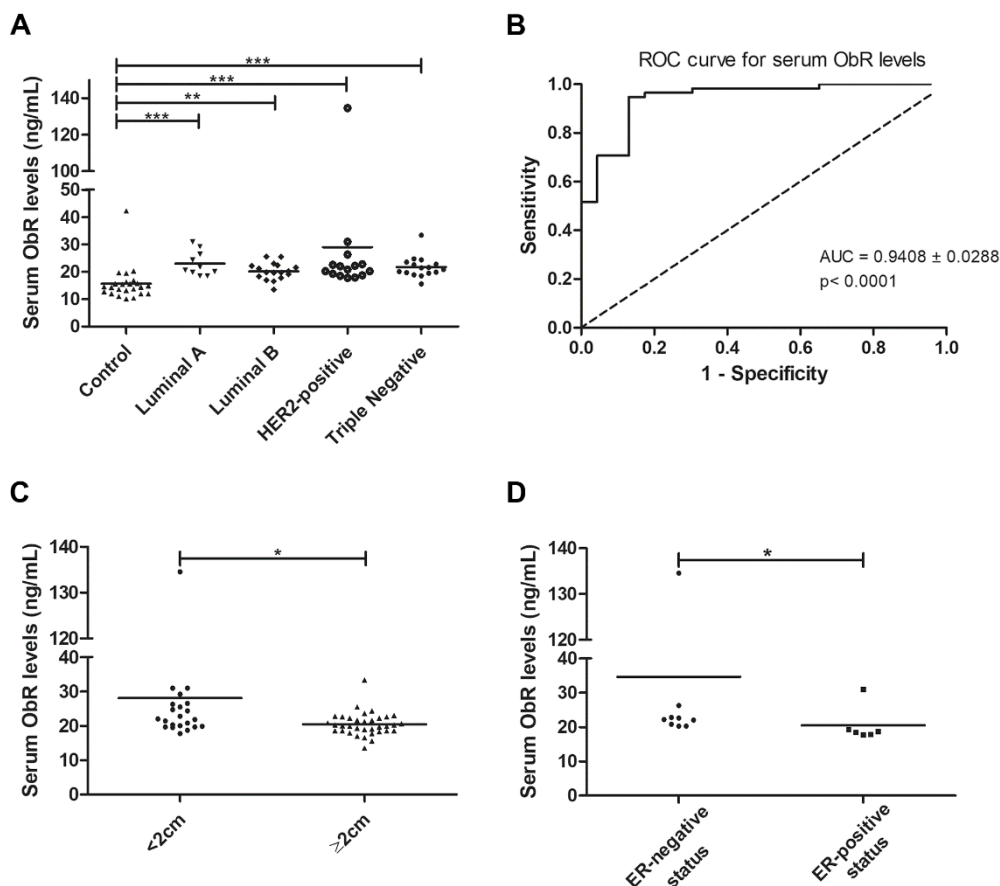
223 **Figure 3.** – Cats with mammary carcinoma showed elevated serum ObR levels, with serum
224 concentrations above 16.89 ng/mL being associated with smaller tumours and with HER-2 positive
225 mammary carcinomas showing an ER-negative status. **A)** Dot plot diagram showing the distribution
226 of serum ObR levels (ng/mL) in heathy animals (control) and in cats with mammary carcinoma
227 stratified by molecular subtype. Significant higher serum levels of ObR were found in all tumour
228 subtypes in comparison to healthy animals ($p < 0.0001$). **B)** The optimal cut-off value of serum ObR
229 levels to predict cats with mammary carcinoma was 16.89 ng/mL with an AUC of 0.9408 ± 0.0288 (95%
230 CI: 0.8842-0.9973, $p < 0.0001$; sensitivity=94.8%; specificity=87.0%). **C)** Dot plot diagram showing that
231 serum ObR concentrations were significantly low in tumours larger than 2 cm (p= 0.0118). **D)** Dot plot

232   diagram for animals with HER2-positive mammary carcinoma displaying a positive association
233   between higher serum ObR levels and ER-negative status (p=0.0291). *p<0.05; **p<0.001; ***p<0.0001.

234   Moreover, the data obtained also showed a positive correlation between serum ObR
235   levels and serum CTLA-4 (r=0.38, p=0.0056, Figure 4A), TNF-α (r=0.40, p=0.0025, Figure 4B),
236   PD-1 (r=0.42, p=0.0023, Figure 4C) and PD-L1 (r=0.50, p=0.0002, Figure 4D) levels.
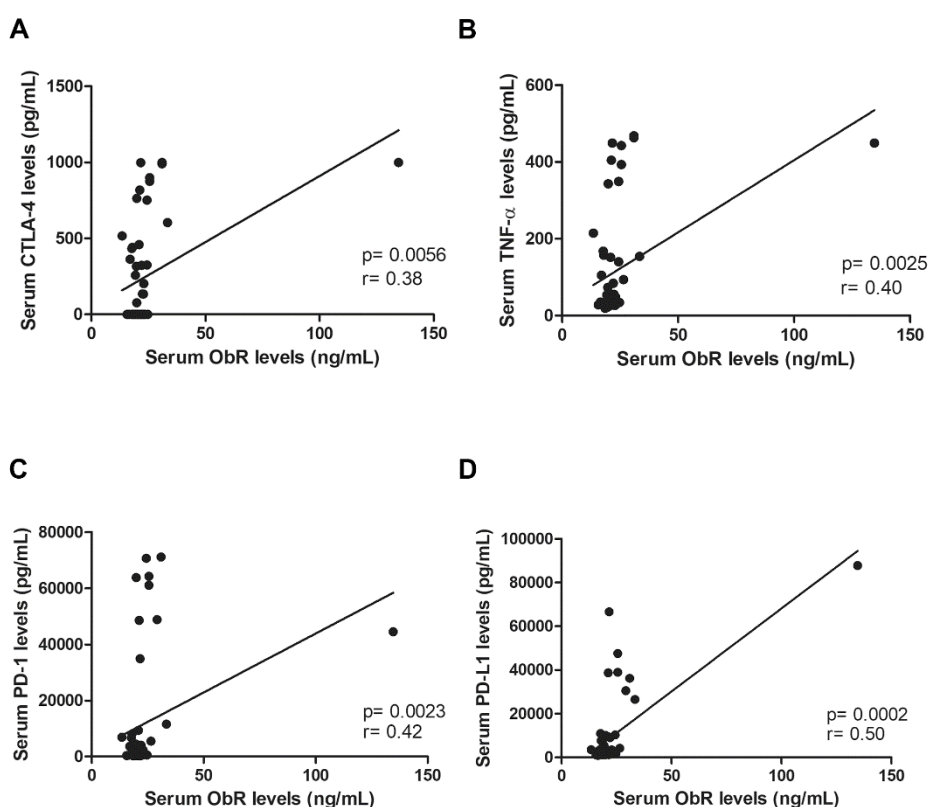
237

238   **Figure 4.** – Serum ObR levels showed a positive correlation between serum ObR levels and **A)** serum
239   CTLA-4 levels (p=0.0056), **B)** serum TNF-α levels (p=0.0025), **C)** serum PD-1 levels (p=0.0023) and **D)**
240   serum PD-L1 levels (p=0.0002).

241   *3.4. Leptin and ObR are overexpressed in Luminal B and triple-negative mammary carcinomas*

242   The obtained results revealed that cats with luminal B or triple-negative mammary carcinoma
243   showed a higher leptin IHC score than controls (1.93 vs. 1.34, p<0.05; 2.00 vs. 1.34, p<0.05,
244   respectively; Figures 5A, 6A and 6B). Regarding the leptin receptor, the IHC score was also
245   significantly higher in animals with a luminal B tumour subtype than healthy animals (2.50 vs. 1.75;
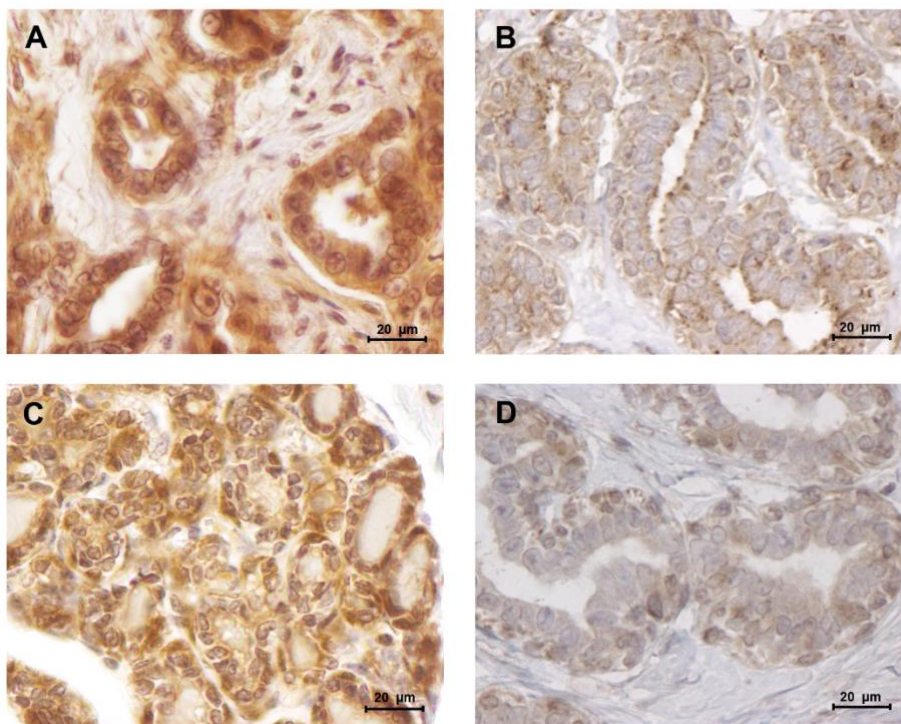246   p=0.0425; Figures 5B, 6C and 6D).

247
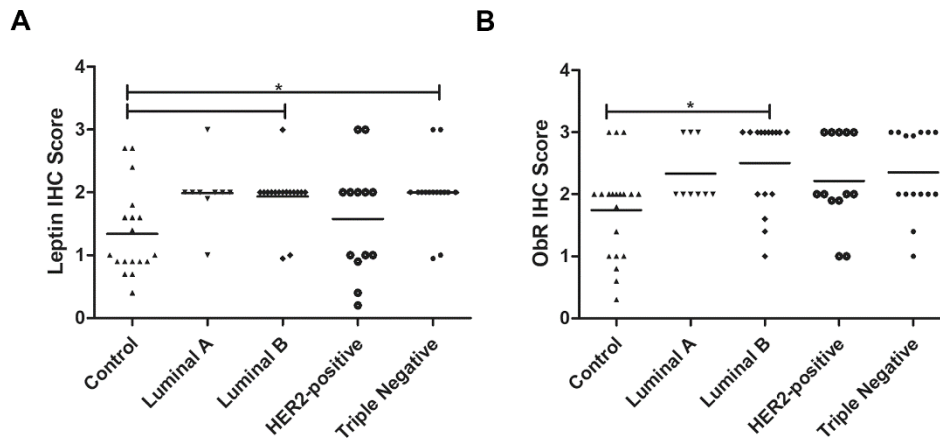
248 **Figure 5.** – Final IHC scores for leptin **(A)** and ObR **(B)** in cats with mammary carcinoma stratified by
249 tumour subtype and controls. **A)** Leptin expression was significantly higher in luminal B and triple-
250 negative subtypes (p=0.0046). **B)** Expression of ObR was statistically higher in luminal B tumour
251 subtype (p=0.0425).

252

253 **Figure 6.** – Leptin and ObR are overexpressed in luminal B mammary carcinomas. **A)** Leptin
254 overexpression in a luminal B mammary carcinoma (IHC score of 1.93) contrasting with **B)** a low
255 staining intensity detected in normal mammary tissues (IHC score of 1.34). **C)** Luminal B mammary
256 tumours showed a higher staining intensity for ObR (IHC score of 2.50), **D)** than normal mammary
257 tissues (IHC score of 1.75). (400x magnification)

258   In addition, our findings revealed that serum ObR levels are negatively correlated with
259 the ObR IHC score, with cats presenting higher serum ObR levels showing mammary
260 tumours with lower ObR IHC scores (p=0.0103, Figure 7).
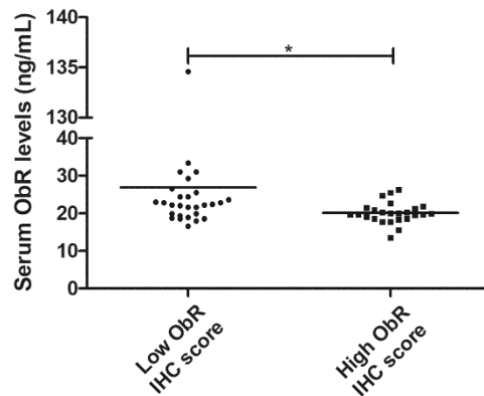
Figure 7. – Dot plot diagram showing a negative correlation between serum ObR levels and tumour ObR IHC score (p= 0.0103).

4. Discussion

Although spontaneous feline mammary carcinoma has been proposed as a suitable model for the study of human breast cancer, the role of the leptin/ObR axis has never been exploited in cats with mammary carcinoma. In humans, previous studies showed that leptin and ObR overexpression are associated with pro-inflammatory and pro-tumorigenic effects, particularly in overweight women [7] [5].

The results obtained in this study showed that cats with mammary carcinoma have a reduced Free Leptin Index (FLI) in comparison to healthy animals (p=0.0006), suggesting that diseased animals may have decreased soluble leptin levels, as reported in pre-menopausal women with breast cancer [37], and colon cancer patients [38], indicating that serum leptin may be recruited by mammary cancer cells to promote tumour growth and cell migration [11]. Indeed, cats with luminal B or HER2-positive mammary carcinoma showed significant lower serum leptin levels when compared with controls (p<0.001 and p<0.05), revealing that serum leptin levels are downregulated in tumours with PR-positive status [6] and/or HER2-positive status [14]. In contrast, cats with luminal A showed elevated serum leptin levels, suggesting that ER overexpression in the tumour may promotes leptin expression [6]. Regarding the elevated serum leptin levels found in cats with triple-negative mammary carcinomas, studies demonstrated that leptin induces cell proliferative capacity (e.g. via Wnt/β-catenin pathway) [30] and promotes cell survival by interacting with Bcl-2 proteins, being associated with more aggressive tumours [39]. Indeed, elevated serum leptin levels were significantly associated with tumour ulceration (p=0.0005) and shorter DFS (p=0.0217), as reported for breast cancer patients [6] [7].

In parallel, serum ObR levels can also affect the FLI [40], and as reported in breast cancer patients [6] [41], all cats with mammary carcinoma showed higher serum ObR levels than healthy controls (p<0.0001), being correlated with smaller tumour size (p=0.0118) and suggesting that ObR shedding occurs in small tumours, modulating the serum levels of free leptin [15]. Indeed, our results further support the hypothesis that malignant cells in larger tumours maintain the ObR expression on its surface to increase their survival and growth [8]. Interestingly, the higher serum ObR levels were found in cats with mammary carcinomas showing both HER2-positive and ER-negative status

293 (p=0.0291), as reported for human breast cancer patients [6], confirming the crosstalk between the
294 leptin/ObR axis and the EGFR downstream signalling pathway [42].

295   In addition, this study discloses the utility of leptin and ObR as promising diagnostic biomarkers
296 for feline mammary carcinoma, with a cut-off value of 4.17 pg/mL determined for serum leptin levels
297 to predict feline mammary carcinoma (AUC=0.7045±0.0757, sensitivity=96.9%, specificity=43.5%),
298 whereas a cut-off value of 16.89 ng/mL was calculated for serum ObR levels (AUC=0.9408±0.0288,
299 sensitivity=94.8%, specificity=87.0%) to differentiate animals with FMC from healthy cats.

300   Interestingly, we also found that serum ObR levels were positively correlated with serum CTLA-
301 4 (p=0.0056), TNF-α (p=0.0025), PD-1 (p=0.0023) levels as reported in breast cancer [26], and with
302 serum PD-L1 levels (p=0.0002). Indeed, previous studies showed that activation of the leptin/ObR
303 axis can result in a chronic inflammatory status [22] [43], a well-known risk factor for breast cancer,
304 with leptin being involved in CD4+ T-regulatory cells differentiation due to ObR overexpression on
305 lymphocyte plasm membrane [44]. These activated CD4+ T-regulatory cells express CTLA-4 [22] and
306 PD-1, two immune-inhibitory checkpoint molecules that downregulate T-cell immune responses [24],
307 leading to tumoral development [45] and contributing to cell growth [46]. On the other hand, in an
308 attempt to control the tumorigenesis process, CD4+ T-regulatory cells secrete TNF-α [25], a molecule
309 that shows a dual role in immunomodulation, being also expressed by cancer cells [47] acting as an
310 autocrine growth factor [48]. Altogether, these findings provide support to the crosstalk between the
311 leptin/ObR axis and tumour immunoediting mechanisms, contributing to an immunosuppressive
312 status in cats with mammary carcinoma [35].

313   The immunostaining analysis of the tumour and normal tissue samples revealed that luminal B
314 and triple-negative mammary carcinoma subtypes (p<0.05) showed leptin overexpression, whereas
315 a strong ObR expression was detected in luminal B mammary carcinomas (p=0.0425), as described in
316 human breast cancer [13], with several studies suggesting that leptin and ObR are overexpressed in
317 tumoral tissues, due to hypoxia and/or as a response to insulin, IgF-1 and/or to oestradiol [41]. In
318 addition, the higher IHC scores for leptin found in luminal B carcinomas also support the previously
319 reported association between the expression of this adipocytokine and aromatase expression, an
320 enzyme that catalyses the conversion of androgen into oestrogen to promote tumour development
321 via an ER-dependent mechanism [6]. The overexpression of leptin detected in triple-negative
322 mammary carcinomas is also in concordance with previous results in triple-negative breast cancer,
323 where leptin signalling is crucial for tumour growth [16] [39], being associated with ERK and Akt
324 pathways, both involved in breast cancer cells proliferation [11]. Finally, our results demonstrated
325 that cats with low ObR-expressing mammary tumours had higher serum ObR levels, indicating a
326 negative feedback between tumour microenvironment and serum, probably due to a shedding
327 mechanism that leads to a reduction of serum leptin levels [11] [40]. Furthermore, the data obtained
328 emphasizes the possibility to block the leptin/leptin receptor axis, as an adjuvant therapy in cats with
329 luminal B and triple-negative tumour mammary carcinoma subtypes, as reported for breast cancer
330 patients [29] [32] [31].

331

5. Conclusion

333   This study evaluated the serum leptin/ObR levels in cats with mammary carcinomas providing
334 rationale for their use as diagnostic biomarkers and, additionally, the utility of serum leptin levels as
335 prognostic indicator for the disease-free survival. Indeed, cats with mammary carcinoma showed a

336 significant reduction in the FLI, coupled with decreased serum leptin levels in cats that had luminal
337 B or triple-negative mammary carcinoma and significant increased serum ObR levels, independently
338 of the tumour subtype. As discussed, serum leptin levels above the cut-off value of 4.17 pg/mL were
339 associated to a shorter DFS, whereas serum ObR levels above 16.89 ng/mL were associated to an
340 immunosuppressive status. In tumour tissue samples, leptin is highly expressed in luminal B and
341 triple-negative mammary carcinomas, with ObR being overexpressed in luminal B subtype.
342 Altogether, the data presented extend the knowledge about the similarities between FMC and human
343 breast cancer, further supporting the utility of spontaneous feline mammary carcinoma as a model
344 for comparative oncology studies.

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357

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