Journal of Steroid Biochemistry & Molecular Biology 115 (2009) 9–13

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Positive correlation of steroid hormones and EGF in canine mammary cancer

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ARTICLE INFO

Article history:
Received 6 November 2008
Received in revised form 24 January 2009
Accepted 28 January 2009

Keywords:
Canine mammary tumours
Inflammatory mammary carcinoma
Epidermal growth factor
Progesterone
Estradiol
Androstenedione
Dehydroepiandrosterone

ABSTRACT

There are no published studies focused on the potential crosstalk between steroid hormones and EGF in canine mammary tumourigenesis. The objective was to investigate the role of EGF in canine mammary tumours (CMT) and the relationship with steroid hormones. Sixty-three CMT (39 malignant including 10 inflammatory mammary carcinomas (IMC); 19 benign and 5 dysplasias), and 13 normal mammary glands from dogs without history of neoplastic disease were analysed. Levels of EGF and steroid hormones [progesterone (P4); 17β-estradiol (E2); androstenedione (A4) and dehydroepiandrosterone (DHEA)], were analysed by EIA in CMT homogenates. Levels of EGF were significantly higher in malignant compared with benign tumours, dysplasias and normal mammary glands ($p < 0.001$). IMC presented the highest EGF levels, with statistical significant difference between IMC and non-IMC cases ($p < 0.05$). Steroid hormone levels were also significantly higher in malignant tumours compared with benign tumours, dysplasias and normal mammary glands ($p < 0.001$). In malignant tumours (non-IMC and IMC), a strong correlation was observed between EGF and: P4 ($r = 0.452$; $p = 0.003$); E2 ($r = 0.624$; $p = 0.023$); A4 ($r = 0.496$; $p = 0.038$); DHEA ($r = 0.431$; $p = 0.005$). These results suggest that EGF is implicated in canine mammary tumourigenesis. The positive correlation observed, opens an interesting perspective of interaction that should be further investigated.

1. Introduction

Mammary tumours constitute the most frequent neoplasm that occurs in the adult female dog. Among mammary tumours, approximately 50% are malignant neoplasms [1], that can metastasise to different organs [2,3].

Inflammatory mammary carcinoma (IMC) is the most aggressive type of malignant mammary tumour that affects female dogs. Inflammatory mammary carcinoma has been proposed by our group, as a model to study human inflammatory breast carcinoma (IBC) [4–6]. Both IMC and IBC neoplastic cells are highly angiogenic, invasive and metastatic. These characteristics have been identified as the responsible for their extremely aggressive and lethal clinical course. Inflammatory mammary carcinoma and IBC are diagnosed on the basis of a rapid progression of signs, such as localized or generalized induration, redness, edema and pain of the mammary gland [4]. Clinical diagnosis might be confirmed histopathologically and the unique histopathologic hallmark is the presence of dermal lymphatic invasion by neoplastic emboli, which compromise the lymphatic drainage and cause edema [5,7].

The role of different hormones in canine IMC has been subject of research for the last 5 years [8,9]. However, little is known about the implication of non-hormonal growth factors in canine IMC.

It has been demonstrated that several peptide growth factors are involved in tumour mammary proliferation both in humans and dogs [10,11]. These factors are probably locally synthesized and they might regulate tumour growth by an autocrine/paracrine mechanism [11–13].

Epidermal growth factor (EGF) is a 6-kDa heat and acid stable polypeptide which acts as a potent mitogen for many normal and malignant cells, including breast tumour cells. It has been detected specific mRNA for EGF in breast tumours and EGF has been demonstrated to be a mitogen in human breast cancer cells in culture [14]. In human breast cancer cells, the production of EGF was reported to be regulated by hormones and growth factors, and an interaction with several steroid hormones at different molecular levels and through distinct pathways has been described [15–17].

Despite all the evidences of EGF implication in human breast cancer, its role in canine mammary tumours (CMT) is unknown. Steroid hormones play an important role in dog mammary tumourigenesis [18,19] however, there are no studies investigating the

Abbreviations: EGF, epidermal growth factor; P4, progesterone; E2, 17β-estradiol; A4, androstenedione; DHEA, dehydroepiandrosterone.

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doi:10.1016/j.jsbmb.2009.01.018

crosstalk between steroid hormones and EGF tissue levels in canine mammary tumours.

The objective of the present study was to investigate the role of EGF in CMT and its relationship with the steroid hormones analysed: [progesterone (P4); 17β-estradiol (E2); androstenedione (A4) and dehydroepiandrosterone (DHEA)].

2. Materials and methods

2.1. Animals and tumours

Thirty-six female dogs received at the Veterinary Teaching Hospital of Madrid with spontaneous canine mammary tumours were prospectively included. The animals ranged in age from 5 to 13 years and were of different breeds. Only bitches in anoestrus phase of cycle were included, which was confirmed by cytology of vaginal smear. Thirteen normal mammary glands (incisional biopsies obtained by tru-cut needle) from 10 beagle female dogs, aged 6–11 years and without history of mammary or endocrine disorder were used as controls.

Clinical staging was performed in animals with malignant tumours, using a modified TNM system [20], which classify the animals in four clinical stages: local, local advanced (inflammatory mammary carcinomas), regional and distant metastasis.

On each mammary tumour, the following clinical characteristics were evaluated: rate of growth (slow, medium, fast), size (T1 < 3 cm; T2 ≥ 3 and <5 cm; T3 ≥5 cm), adherence to skin and/or underlying tissues and skin ulceration.

Surgical excision of the tumours was done in animals in local and regional stages. In dogs diagnosed with IMC, tumour samples were collected by tru-cut biopsy or after animal death (natural or euthanasia).

2.2. Sampling procedures

2.2.1. Tissue samples

A total of 63 mammary samples were obtained. The samples were separated in two adjacent fragments for histopathology and enzyme-immunoassay (EIA) determinations.

2.2.2. Histopathology and EIA procedures

Samples for histopathology were fixed in 10% buffered formalin; paraffin embedded and cut in 4 μm sections, following routinary methods. Histopathological diagnosis of the tumours was made on H&E sections using the World Health Organization nomenclature for canine mammary tumours and dysplasias [21].

For EIA samples, skin and fat were removed and 0.5 g of mammary tissue were homogenized in 4 ml phosphate-buffered saline (PBS, pH 7.4), centrifuged at 3500 rpm, 4 °C for 25 min, and the supernatant was decanted and aliquoted individually (−30 °C) until hormone determinations.

Epidermal growth factor concentrations in tissue homogenates were determined using a commercially available EIA kit (CytElisa, Human EGF kit, Sciences Inc®), following manufacturer's instructions.

Concentrations of P4, E2, A4 and DHEA in mammary tissue homogenates were assayed by competitive enzyme-immunoassay as reported previously [19]. EGF, P4, A4 and DHEA concentrations were expressed in ng/g. The concentrations of E2 were expressed in pg/g.

2.3. Statistical analysis

The statistical software SPSS (SPSS Inc., Chicago, USA) version 12.0 was used for statistical analysis. ANOVA tests and Student's *t*-tests were used for continuous variables. Analyses of variance (*F*-test, pooled *t*-test, if variances are assumed to be equal, or Welch test or separate *t*-test if variances are not equal) were used to study the differences in means of continuous variables. All values are expressed as means ± S.E.M. In all statistical comparisons, $p < 0.05$ was accepted as denoting significant differences.

3. Results

3.1. Histopathological diagnosis

Seventy-six mammary samples (13 normal mammary gland, 5 dysplasias, 19 benign tumours and 39 malignant neoplasias) were histopathologically diagnosed. Among the 39 malignant neoplasias, 10 were from dogs diagnosed with IMC. The group of dysplasias consisted in four lobular and one cystic hyperplasia. Benign tumours included simple ($n = 3$) and complex adenomas ($n = 7$), and mixed benign tumours ($n = 9$). Malignant tumours non-IMC included simple ($n = 7$) and complex carcinomas ($n = 10$), solid carcinomas ($n = 7$) and carcinossarcomas ($n = 5$). Samples of normal mammary gland obtained from the control beagles ($n = 13$) were absent of any histological alteration.

3.2. Hormonal determinations

3.2.1. Epidermal growth factor and steroid hormone levels in mammary tissue homogenates

EGF levels in tissue homogenates were positively and significantly associated with malignancy of the tumours ($p < 0.001$) as is shown in Table 1. The tumours that displayed the highest EGF tissue levels were inflammatory mammary carcinomas. The mean EGF levels in IMC was significantly higher the mean EGF levels in malignant tumours non-IMC. The mean EGF levels in malignant tumours (non-IMC and IMC) were significantly higher than the mean values in benign tumours, in dysplasias and in normal mammary glands (Table 1). Nevertheless, the mean values among benign tumours, dysplasias and normal mammary glands were not significantly different ($p = 0.323$).

Progesterone, E2, A4 and DHEA tissue levels were positively related to malignancy as described in Table 1. All of these hormones were significantly higher in malignant tumours compared with benign tumours and normal mammary gland ($p < 0.001$). Similarly to the described for EGF, differences in the mean values of these hormones among benign tumours, dysplasias and normal mammary glands did not reach the statistical significance (Table 1). Inflammatory mammary tumours displayed the highest tissue levels for all the steroid hormones analysed.

3.2.2. Correlation between EGF, P4, E2, A4 and DHEA tissue levels in malignant tissue homogenates (non-IMC and IMC)

In malignant tumours (non-IMC and IMC), a positive and statistically significant correlation was found between EGF and: P4 ($n = 39$; $r = 0.452$; $p = 0.003$); E2 ($n = 38$; $r = 0.624$; $p = 0.023$); A4 ($n = 39$; $r = 0.496$; $p = 0.038$; and DHEA ($n = 39$; $r = 0.431$; $p = 0.005$) in tissue homogenates. In benign tumours and dysplasias, the positive correlation between EGF and the steroid hormones analysed was not statistically significant.

3.2.3. Clinical and histopathological characteristics and EGF tissue levels in malignant tumours non-IMC

The associations between clinico-pathological features and EGF tissue levels in malignant tumours non-IMC are shown in Table 2. Tumour size and rate of growth were positively and significantly related with EGF levels in malignant tissue homogenates. The EGF tissue levels were considerably higher in malignant tumours with adherence to underlying tissues and skin ulceration, compared to

Table 1
Epidermal growth factor, P4, E2, A4 and DHEA levels in tissue homogenates and neoplastic mammary tissues.

	Normal mammary tissues	Dysplasias	Benign tumours	Malignant tumours		p^*
				Non-IMC	IMC	
EGF (ng/g)	317.5 ± 53.97^a (n = 13)	577.49 ± 259.6^a (n = 5)	765.1 ± 102.9^a (n = 19)	1803.43 ± 188.1^b (n = 29)	2809.05 ± 614.7^c (n = 10)	<0.001
P4 (ng/g)	1.69 ± 0.18^a (n = 13)	3.82 ± 1.63^a (n = 5)	1.81 ± 0.27^a (n = 19)	8.50 ± 1.42^b (n = 29)	16.12 ± 1.22^c (n = 10)	<0.001
E2 (pg/g)	67,8 ± 14.13^a (n = 13)	127,91 ± 9.93^a (n = 5)	107.17 ± 9.22^a (n = 19)	276.4 ± 32.52^b (n = 28)	593.5 ± 51.84^c (n = 10)	<0.001
A4 (ng/g)	22.17 ± 6.22^a (n = 13)	47.44 ± 14.29^a (n = 5)	28.2 ± 4.64^a (n = 19)	116.3 ± 10.55^b (n = 29)	538.7 ± 44.08^c (n = 10)	<0.001
DHEA (ng/g)	35.73 ± 4.21^a (n = 13)	72.37 ± 9.46^a (n = 5)	62.73 ± 6.21^a (n = 19)	230.73 ± 14.85^b (n = 29)	791.19 ± 27.71^c (n = 10)	<0.001

Values of EGF, P4, E2, A4 and DHEA with distinct superscript letters indicate statistical significant differences between groups [(normal mammary gland, dysplasias, benign tumours, malignant tumours (non-IMC and IMC)]. Duncan test (p < 0.05).

* ANOVA test (p-value < 0.05).

those tumours not adhered to underlying tissues or without skin ulceration. Solid carcinoma and carcinossarcoma were the histological types with the highest EGF tissue levels. Simple carcinomas presented the lowest values.

3.2.4. Clinical characteristics and P4, E2, A4 and DHEA tissue levels in malignant tumours non-IMC

The relationship between tissue levels of P4, E2, A4 and DHEA and the clinical characteristics: rate of growth, tumour size; adherence to skin and to underlying tissues and skin ulceration was investigated. The results were similar to previously reported for these hormones [19], showing a positive statistical relationship (p < 0.05) between the steroid hormones analysed and the clinical characteristic mentioned above (data not shown).

The histological type that displayed the highest values for the P4, E2, and DHEA was the solid carcinoma, whereas carcinossarcoma presented the highest A4 tissue content. Figs. 1–4 show the P4, E2, A4 and DHEA concentrations in the malignant histological types analysed.

Table 2
Association of tissular EGF concentrations with clinico-pathologic parameters in the malignant non-IMC mammary tumors examined.

Clinico-pathologic parameters	Number of samples	EGF (ng/g)	p^*
Rate of growth			
Slow	8	884.63 ± 188.98^a	<0.001
Medium	7	1130.82 ± 151.3^a	
Fast	14	2626.42 ± 206.73^b	
Tumor size (cm)			
T1 < 3 cm	10	827.72 ± 211.42^a	0.001
T2 ≥ 3 cm and <5 cm	9	1850.05 ± 333.29^b	
T3 ≥ 5 cm	10	2618.70 ± 270.90^b	
Adherence to skin			
No	12	1480.96 ± 247.53	0.217
Yes	17	1954.91 ± 257.49	
Adherence to underlying tissues			
No	16	1427.85 ± 198.99	0.050
Yes	13	2151.01 ± 301.86	
Skin ulceration			
No	21	1321.89 ± 156.32	<0.001
Yes	8	2984.77 ± 210.51	
Histological diagnosis			
Complex carcinoma	10	1411.67 ± 188.15^a	<0.001
Simple carcinoma	7	942.28 ± 132.81^a	
Solid carcinoma	7	2396.73 ± 321.76^b	
Carcinossarcoma	5	3081.65 ± 359.56^b	

In the parameters rate of growth, tumour size and histological diagnosis, presence of distinct superscript letters in EGF values denote statistical significant differences among the respective subclasses. Duncan test (p < 0.05).

* ANOVA test (p-value < 0.05).

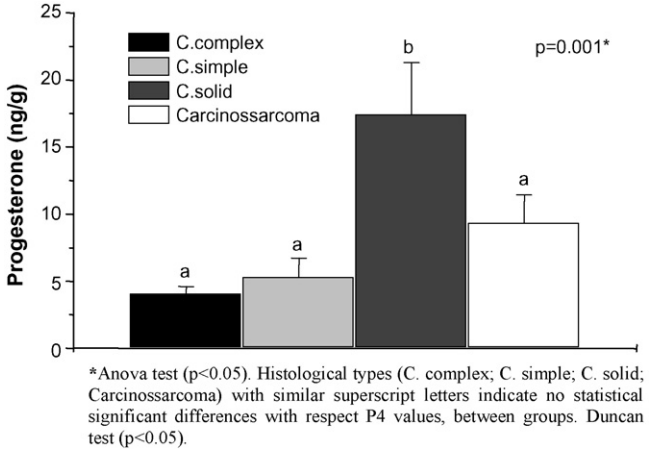

Fig. 1. Progesterone tissue levels in the histological types of malignant tumours non-IMC. *ANOVA test (p < 0.05). Histological types (C. complex; C. simple; C. solid; carcinossarcoma) with similar superscript letters indicate no statistical significant differences with respect to P4 values, between groups. Duncan test (p < 0.05).

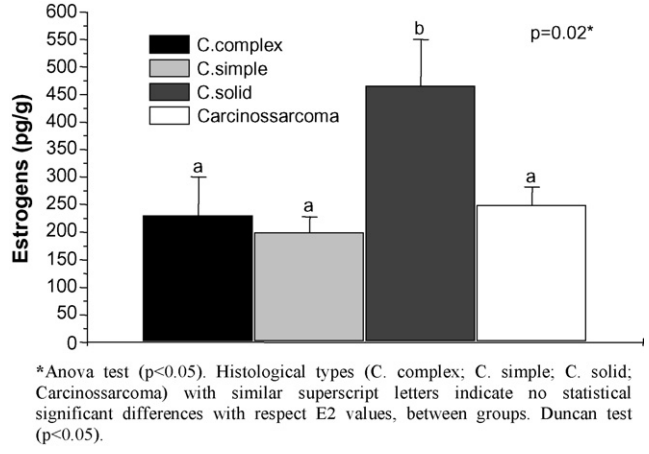

Fig. 2. Estrogen tissue levels in the histological types of malignant tumours non-IMC. *ANOVA test (p < 0.05). Histological types (C. complex; C. simple; C. solid; carcinossarcoma) with similar superscript letters indicate no statistical significant differences with respect to E2 values, between groups. Duncan test (p < 0.05).

4. Discussion

The association between breast cancer incidence and endogenous ovarian hormones has been known since several decades both in woman [22,23] and in the female dog [24].

Inflammatory mammary carcinoma (dog) and inflammatory breast cancer (women) are the most aggressive type of tumours among all the malignant mammary neoplasms. The two entities

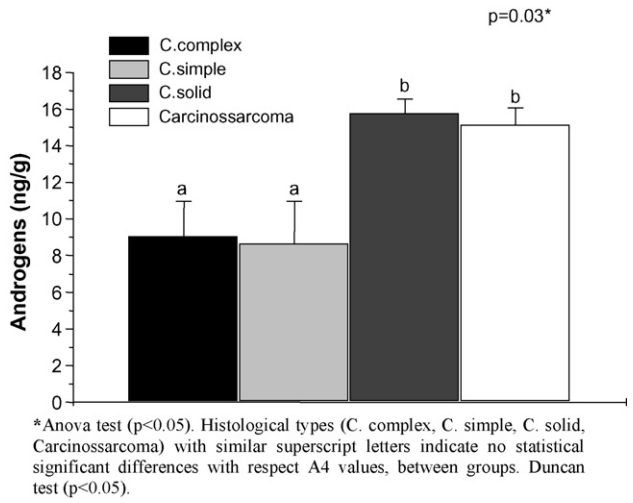

Fig. 3. Androgens tissue levels in the histological types of malignant tumours non-IMC. *ANOVA test ($p < 0.05$). Histological types (C. complex; C. simple; C. solid; carcinossarcoma) with similar superscript letters indicate no statistical significant differences with respect to A4 values, between groups. Duncan test ($p < 0.05$).

are very similar with respect to the clinical and histopathological hallmarks [4,6]. IMC and IBC are highly metastatic and angioinvasive [5,25], which certainly contributes for the great tumour aggressiveness and invasiveness. Our findings reveal that canine IMC presented the highest EGF tissular levels with a statistical significant difference compared with malignant tumours non-IMC. Interestingly, among malignant tumours non-IMC, the highest EGF tumoural content was found in the most clinical aggressive histological type of tumour (carcinossarcoma), which indicates a remarkable link between EGF tissular levels and malignant aggressiveness.

Recently, it has been demonstrated a role for EGF as a determinant contributor for the metastatic phenotype in human breast cancer. Mosadegh et al. [26] observed that CXCL12-induced chemotaxis of MDA-MB-231 metastatic breast cancer cells is dependent of EGF. This finding may provide some insights into the mechanism by which metastatic cells can escape the tumour and metastasise to distant sites [26].

In our opinion, and similarly to the observed in human breast cancer [26,27], in CMT of elevated aggressiveness, the presence of elevated concentrations of EGF might act in the metastatic cells in a paracrine or autocrine manner, inducing motility and powering the metastatic process.

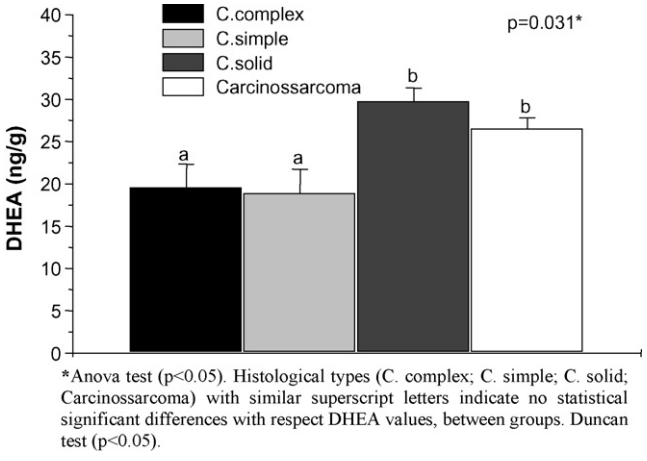

Fig. 4. Tissue levels of DHEA in the histological types of malignant tumours non-IMC. *ANOVA test ($p < 0.05$). Histological types (C. complex; C. simple; C. solid; carcinossarcoma) with similar superscript letters indicate no statistical significant differences with respect to DHEA values, between groups. Duncan test ($p < 0.05$).

The hormonal dependency of CMT is known since several years. The first evidence observed of this dependency was the effect of overiectomy in the prevention of mammary tumours occurrence [2,28]. Furthermore, several reports have been described the presence of estrogen and progesterone receptors in the normal and neoplastic canine mammary gland [29,30]. However, the majority of canine malignant mammary tumours, including IMC, did not express estrogen receptor alfa (ERα) [9,31]. In fact, studies focused on the CMT steroid receptor profile [9,30–32] indicate that poorly differentiated malignant tumours expressed lower concentrations of steroid receptors than did benign or well-differentiated malignant tumours. Regarding the estrogen receptor (ER), some authors have been described the inexistence of this receptor in malignant tumours [29], while others indicate the presence of estrogen receptor beta (ERβ) as the most probably pathway through which estrogens exert their effects in the canine neoplastic mammary gland [9].

In humans, estrogens and EGF are important mitogens for breast cancer cells. The effect of over some mammary cells and tissues is similar to that observed for estrogens, suggesting a crosstalk between their signalling pathways [33]. It is noteworthy to note that this crosstalk exist even in ER negative cells, as recently demonstrated [33,34]. Our results show that EGF tissue levels in malignant mammary tumours (non-IMC and IMC) are highly elevated as well as the estrogen tissue concentrations in the same cases. Likewise, a positive and significant correlation between EGF and estrogens in malignant tumours (non-IMC and IMC) has been observed in the present study. In humans, it has been proposed that breast cancer cells might overcome the loss of ERα-dependent signalling cascades by switching to an EGF-dependent signalling pathway [35]. Our findings allow us to suggest that a similar mechanism could also operate in CMT.

Progesterone is an important steroid hormone that controls cell proliferation and differentiation of the mammary gland in mammalians [36]. It is also known that P4 positively acts in the crosstalk with the EGF signalling pathway in human breast cancer cells at multiple levels [37,38]. One of the mechanisms involved is the STAT5, a latent transcription factor and known target of P4 in mammary cells that is used by the EGF–EGFR complex in cell signalling [15].

Canine neoplastic mammary gland expresses progesterone receptors [29,30] and it has been described a high P4 tissue levels in canine malignant IMC [8,9] and non-IMC tumours [19].

One intriguing finding is the occurrence of canine IMC predominantly in the luteal phase (where P4 levels are elevated) [4,5,9]. In breast cancer cells, progesterone pre-treatment potentiates the EGF signalling pathway in the cell line ZR-75 [39]. Also other studies demonstrated that in human breast cancer cells, progesterone sensitizes the cells to the EGF-induced proliferative effects [40,41].

In the present study, malignant tumours (IMC and non-IMC) presented the highest P4 and EGF tissue concentrations. We also observed a positive and significant correlation between P4 and EGF tissue levels in these tumours. These findings might suggest that a mechanism involving the P4 sensitization of neoplastic cells to the EGF proliferative and angiogenic effects could be present in CMT. This mechanism proposed might contribute for the acquisition of a metastatic phenotype in dog malignant neoplasias.

It has been recently documented the crosstalk of EGF and androgens [17,42] in human breast cancer. In dog mammary tumours, to our knowledge, this crosstalk has never been described.

The present study indicates that malignant tumours present elevated A4 and DHEA tissue levels in comparison with benign tumours or normal mammary gland. Our findings are similar to previously reported by our group [8,9,19]. However this is the first

time, to our knowledge, that a study investigates the simultaneous presence of A4, DHEA and EGF in dog mammary neoplasias.

Based on our results, we can conclude that EGF is implicated in canine mammary tumourigenesis and that an interesting crosstalk might exists between EGF and P4, E2, A4 and DHEA. However, the complexity of the described crosstalk between EGF and steroid hormones needs to be further investigated. As previously mentioned by others, individual signalling events do not simply create linear pathways, rather they are part of a complex and interdependent cellular network [43]. Further elucidation of this molecular and cellular communication system and its physiological as well as pathophysiological significance will be crucial on the next future for the molecular understanding of the mammary cancer in the dog.

Acknowledgement

We thank Dr. Pedro Cuesta, the processing Data Center of the Complutense University, Madrid, Spain, for his assistance with the statistical work.

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