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Contribution to the Study of Feline Endometrial Adenocarcinomas

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ABSTRACT

Endometrial adenocarcinomas are considered rare tumours in domestic animals, though, in the cat, these are often described as the uterine tumours most frequently diagnosed. In fact, in recent years we have been witnessing to an increase in the number of publications in the topic, raising the question of the increasing prevalence of these tumours. Nevertheless, knowledge of these neoplasms is still scarce. The present study foresaw to contribute to the understanding of feline endometrial adenocarcinomas (FEA).

The study of neoplastic disease of the genital tract in companion animal is of great interest, either from the prospect of fertility or from the clinicopathological point of view. Thus, we believe that this work will contribute to the recognition of FEA as an entity with clinical relevance that should be considered for the differential diagnosis within the causes for uterine disease.

In the present study, the morphological and histopathological features of FEA are described and the immunohistochemical profile of the papillary serous type determined, regarding a broad panel of antibodies. Normal uterine samples in follicular and luteal stages of the feline estrous cycle are also used as controls, to establish the normal pattern of expression, because seldom existed available descriptions for most of the antibodies/markers used herein. We believe that the findings report in this study would greatly facilitate the routine histopathological diagnosis. Dedifferentiation markers are suggested with the immunophenotyping of the tumours under study. Also, putative molecular mechanisms underlying carcinogenesis and proliferation of the cat endometrium are unveiled.

Regarding the immunophenotype of the papillary serous FEA, data gathered herein suggests that proliferation in those tumours accompanies estrogen receptor α (ER-α) down-regulation, possibly following activation of pathways mediated by local growth factors. Moreover, FEA retained combined expression of cytokeratin (CK) 7 and CK20, as evidenced in normal endometrial epithelia, although the CK7 expression was decreased. Cyclooxygenase – 2 (COX-2) scores were decreased in FEA; however, evidence for the loss of compartmentalization in COX-2 expression by the neoplastic epithelium most likely reflects the involvement of this enzyme in feline endometrial carcinogenesis. The majority of the cases under study were positive to c-erbB-2 immunolabeling; however, a decrease in the expression

of this protein was observed compared to the follicular stage of normal endometrium. Comparing the results of c-erbB-2 scoring with those of the proliferation marker Ki-67, it was suggested that the former might not be involved in FEA proliferation events. Data also suggest that the expression of c-erbB-2 in the endometrium is influenced by functional changes occurring during the estrous cycle and feline endometrial carcinogenesis.

RESUMO

Os adenocarcinomas do endométrio são considerados tumores raros nos animais domésticos, embora na gata sejam muitas vezes descritos como os tumores uterinos mais frequentemente diagnosticados. De fato, nos últimos anos temos vindo a assistir a um aumento no número de publicações sobre o tema, levantando a questão do aumento da prevalência destes tumores. No entanto, o conhecimento dessas neoplasias ainda é escasso. O presente estudo pretende contribuir para a compreensão dos adenocarcinomas do endométrio da gata (FEA).

O estudo das doenças neoplásicas do trato genital dos animais de companhia é de grande interesse, quer na perspetiva da fertilidade, quer do ponto de vista clínico-patológico. Assim, acreditamos que este trabalho contribuirá para o reconhecimento dos FEA como uma entidade com relevância clínica que deve ser considerada no diagnóstico diferencial da doença uterina.

No presente estudo descrevemos as características morfológicas e histopatológicas dos FEA, assim como o perfil imunohistoquímico do tipo papilar seroso, em relação a um amplo painel de anticorpos. Amostras de úteros normais nas fases folicular e lútea do ciclo éstrico da gata são também utilizadas como controlos, para estabelecer o padrão normal de expressão, pois raramente existem descrições disponíveis para a maior parte dos anticorpos / marcadores aqui utilizados. Acreditamos que as conclusões do nosso trabalho facilitarão o diagnóstico histopatológico de rotina. Marcadores de indiferenciação são sugeridos com a imunofenotipagem dos tumores em estudo. Além disso, sugerimos mecanismos moleculares considerados como subjacentes à carcinogénese e proliferação do endométrio da gata.

Em relação ao imunofenótipo dos FEA papilares serosos, os dados aqui recolhidos sugerem que a proliferação desses tumores acompanha a subregulação do recetor de estrogénios α (ER-α), possivelmente após a ativação de vias mediadas por fatores de crescimento locais. Adicionalmente, os FEA mantiveram a expressão combinada das citoqueratinas (CK) 7 e CK20, como evidenciado no epitélio do endométrio normal, embora a expressão da CK7 estivesse diminuída. Os scores da ciclooxigenase - 2 (COX-2) encontravam-se diminuídos nos FEA; no entanto, a perda de compartimentalização da COX-2 no epitélio neoplásico reflete provavelmente o envolvimento desta enzima na carcinogénese do endométrio da

gata. A maioria dos casos em estudo foram positivos para a imunomarcação com o anticorpo anti-c-erbB-2; no entanto, foi observada uma diminuição na expressão desta proteína em comparação com a fase folicular do endométrio normal. Comparando os resultados do score de c-erbB-2 com aqueles do marcador de proliferação Ki-67, foi sugerido que o primeiro não deverá estar envolvido em eventos de proliferação FEA. Os dados também sugerem que a expressão de c-erbB-2 no endométrio é influenciada por alterações funcionais que ocorreram durante o ciclo éstrico e a carcinogénese do endométrio da gata.

Symbols, Abbreviations and Acronyms

CEH – Cystic endometrial hyperplasia

CEH/P – Cystic endometrial hyperplasia / pyometra complex

c-erbB-2 (HER2) – Human epidermal growth factor receptor type 2

CI – Confidence interval

CK – Cytokeratin

cm – Centimeter

COX – Cyclooxygenase

DAB – 3,3'- diaminobenzidine

DGE – Deep glandular epithelium

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

ER – Estrogen receptor

FEA – Feline endometrial adenocarcinoma (s)

FS – Folicular stage

FSH – Follicle stimulating hormone

GnRH – Gonadotropin releasing hormone

HPF – High power field

IGF-1 – Insulin-like growth factor 1

kDa – KiloDalton

L – Litre

LH – Luteinizing hormone

LS – Luteal stage

ml - Milliliters

mm – Millimeters

µm - Micrometers

mRNA – Messenger ribonucleic acid

n – Number

NE – Normal epithelium

OVH – Ovariohysterectomy

PBS – Phosphate buffered saline

PCNA – Proliferating cell nuclear antigen

PDP – Progesterone-dependent secretory protein

pg – Picogram

PG – Prostaglandin (s)

PLA_2 – Phospholipase A_2

PR – Progesterone receptor

SE – Surface epithelium

SGE – Superficial glandular epithelium

TGF-α – Transforming growth factor alpha

TNF- α – Tumour necrosis factor alpha

VEGF – Vascular endothelial growth factor

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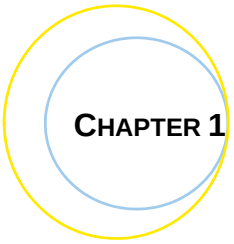

CHAPTER 1

GENERAL INTRODUCTION

AIMS

General Introduction

The survival of a species depends on homeostasis of all organic systems, the reproductive tract being one of the most important. In the past few decades, diseases of the reproductive tract have not changed much but the understanding of the molecular events underlying those diseases, the new advanced methods of treatment and the predictive value of morphological and molecular markers in the outcome of reproductive system pathology largely improved. A great emphasis has been given to production animals since its reproduction is essential to continued supply of food. However, with the increasing social (and economic) importance of companion animals, a lot of effort was taken to understand and treat diseases of the reproductive tract of dogs and cats (Foster, 2007).

The ultimate role of the female reproductive tract is to provide location for the conception, to guarantee the embryo survival, and to succeed on the development and release of viable offspring (Foster, 2007; Ponnampalam et al., 2004). Diseases of the reproductive tract disrupt all these biological functions.

In order to understand the pathology of an organic system or a particular organ, it is of utmost importance to recognize its normal physiology and morphology. Therefore in this chapter, we will focus in the morphological and physiological aspects of the normal reproductive tract of queens.

Anatomy of the Genital Tract

The reproductive tract of queens, as in other mammal females, consists of paired ovaries for the oogenesis – the primary reproductive organs -, and a tubular duct system – the secondary reproductive organs - to facilitate fertilization of ova and to support pregnancy (Rastogi, 2007; Williamson, 1988).

During ovarian development - a gene regulated mechanism – the germ cells undergo meiosis and the supporting cells surround the oocytes to become the cells of the follicles. The development of the paramesonephric (Müllerian) ducts determines the differentiation of the tubular genitalia according to a female phenotype, originating the uterine tube, uterus and upper part of the vagina. The genital tubercle develops into the distal vagina and vulva (Inomata et al., 2009).

OVARIES

The normal ovaries are located caudal and laterally to the caudal poles of the kidneys; their macroscopic appearance varies with the stage of the estrous cycle (Davidson and Baker, 2009).

The ovary has two main functions namely the development and release the ova and to produce hormones, such as estrogen and progesterone. Ovarian hormones influence animal behaviour and affect all organic systems. The hypothalamus and pituitary gland control the ovarian function through the release of gonatropin releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Foster, 2007).

TUBULAR GENITALIA

Four regions compose the uterine tube: infundibulum (which surrounds the ovary), ampulla, isthmus and the uteral-tubal junction. Fertilization occurs in the uterine tube; afterwards, the conceptus moves into the uterus (Foster, 2007). In carnivores, for a short period after the entrance in the uterus, the young embryo free floats within the uterine cavity, allowing the implantation of multiple embryos at equidistant distances from each other (Lamm and Makloski, 2012).

The uterus develops from the paramesonephric ducts of the embryo (König and Liebich, 2007). As age and physiological activity influence uterine anatomy (König and Liebich, 2007), general descriptions of uterine anatomy usually refer to mature and non-pregnant uterus (König and Liebich, 2007).

Cats have a bicornuate uterus, Y-shaped, with two horns longer than the uterine body (Foster, 2007). The uterus is an abdominopelvic organ, located from the pelvis to the kidneys, dorsal to the intestines (König and Liebich, 2007). It receives the uterine tubes anteriorly and opens to the vagina posteriorly (Sisson, 1910). Two major double folds of peritoneum attach the uterus (as well as the ovaries and the uterine tubes) to the lumbar wall of the abdomen and the lateral walls of the pelvic cavity – the broad ligaments of the uterus (König and Liebich, 2007; Sisson, 1910). The uterine horns are entirely located in the abdomen, pressed up against the sublumbar muscles by the intestine (Sisson, 1910). The dorsal border is attached to the broad ligament, while the ventral border remains free (Sisson, 1910). The dorsal surface of the uterine body is related to the colon and rectum, whereas its ventral surface is related to the bladder (Davidson and Baker, 2009;

Sisson, 1910). The cervix of the uterus is the most posterior segment in contact to the vagina (Sisson, 1910). It is located slightly cranial to the bladder trigone, with which is ventrally related along with the urethra, and is best seen when under hormonal influence, either by estrogens or progesterone (Davidson and Baker, 2009). The cervix of the queen cannot be directly observed by gynaecologic examination due to a thin and undistensible cranial vagina (Zambelli et al., 2015).

The broad ligaments extends from the sublumbar region and the lateral pelvic walls to the dorsal border of the uterine horns and the lateral margins of the uterine body. The outer layer of each forms a fold – the round ligament of the uterus – which blends to the parietal peritoneum; the anterior extremity is located above the extremity of the uterine horns and is free. The vessels and nerves that irrigate the uterus travel through the broad ligament. The principal arteries that supply the uterus are the uterine and the uterine branch of the ovarian artery. Additionally, there is a branch of the internal pubic artery. The homologous veins accompanies the arteries. The lymphatics drain to the internal iliac and lumbar lymph nodes. The nerves are derived from the sympathetic through the uterine and the pelvic plexuses (Sisson, 1910).

The cervix is oriented obliquely between the uterine body and vagina, forming an obtuse angle with the vagina. It is located 40–45 mm anterior to the vulva (Zambelli and Cunto, 2005). The major functions of the cervix include: providing a functional barrier, protecting the uterus from the external environment; holding the products of conception during pregnancy; and transport the sperm during breeding (Chatdarong et al., 2006; Foster, 2007; Zambelli and Cunto, 2005). The feline cervix cannot be visualized unless with endoscopy (Zambelli et al., 2015). In cats, the cervix tends to open dorsally and has a relatively smooth mucosa (Foster, 2007). The mucosa of the uterine cervix is elevated into longitudinal folds that may become subdivided into secondary and tertiary folds (Bacha and Bacha, 2012).

The vestibule has 1-2 cm in length and is large in comparison to the vagina; the latter has a narrow lumen, which is further constricted by a prominent dorsal median fold. The vaginal fornix is located ventrolateral to the cervix (Zambelli and Cunto, 2005). Vaginal and cervical modifications during the stages of the estrous cycle are described regarding anatomical variations (Zambelli et al., 2004).

External Genitalia

The vulva is the terminal part of the genital tract and includes the vestibule, labia, and clitoris (Sisson, 1910). Amongst with the vagina, the vulva protects the uterus and cervix, reducing the contamination. These stuctures also provide a passageway to foetus at parturition (Foster, 2007).

Histology of the Genital Tract

Ovaries

The ovary is a compact organ, consisting of two portions – cortex and medulla - surrounded by an outer layer of epithelium. The cortex is composed by follicles in different stages of development, stromal connective tissue, interstitial glands and blood vessels, whereas the medulla presents many large blood vessels, lymphatics, nerves and loosely connective tissue. In this region, remnants of the mesonephric tubules – the rete ovarii – are observed (Bacha and Bacha, 2012).

Within the ovary, follicular units composed of specialized cells surround each ova (or oocyte). The follicle plays a major function in the normal development of the oocyte. The process by which follicles mature is referred to as folliculogenesis, which is under the control of the hypothalamic-pituitary-ovary axis. The ovary itself produces several growth factors also responsible for follicular development. During folliculogenesis, follicular morphology changes according to oocyte growth and differentiation of surrounding cells (Bristol-Gould and Woodruff, 2006).

Follicles are classified as primordial, primary, secondary and antral follicles, according to their timeline evolution (Reynaud et al., 2009; Uchikura et al., 2010). Throughout the follicular progression, there is an increase of the follicle diameter, oocyte diameter and zona pellucida thickness (Reynaud et al., 2009). Primordial / primary follicles are characterized by oocytes surrounded by a single layer of granulosa cells. Secondary follicles contain two or more layers of granulosa cells and a theca cell is deposited on the side opposite to the basement membrane from granulosa cells (Bristol-Gould and Woodruff, 2006; Uchikura et al., 2010). The theca differentiates into a cellular, vascular inner layer, the theca interna**,** and an outer layer of connective tissue, the theca externa (Bacha and Backa, 2012). Oocyte in antral follicles are surrounded by multi-layered granulosa cells; the main characteristic of these follicle is the appearance of a fluid-filled antrum. Multi-oocyte

follicles are characterized by two or more oocytes. Throughout the estrous cycle, follicles can be generally classifies as quiescent, growing or atretic (Bristol-Gould and Woodruff, 2006; Uchikura et al., 2010). Although many primordial follicles begin the process of growth and differentiation, few become mature. The majority undergo a degenerative regression, becoming atretic (Bacha and Bacha, 2012).

The oocyte release at ovulation following the rupture of the follicular walls – ovulation – leads to the formation of a blood clot within the follicular space, which will be posteriorly infiltrated by mural granulosa cells – corpus hemorrhagicum – that will transform, together the thecal cells, into luteal cells – corpus luteum. The regression of the corpus luteum leaves a scar called corpus albicans (Bacha and Bacha, 2012).

TUBULAR GENITALIA

The structure of the uterine wall includes three layers: the endometrium, the myometrium and the perimetrium (Bacha and Bacha, 2012). The endometrium is lined by a simple cuboidal or columnar layer of epithelial cells, supported by a stromal component, the lamina propria, where variable numbers of straight, simple, branched glands are also present and open into the lumen (Aughey and Frye, 2001; Bacha and Bacha, 2012; Foster, 2007). The glandular epithelium is similar to the surface epithelium (Junqueira and Carneiro, 2004). Connective tissue from the lamina propria, mainly composed by fibres of collagen type III, is rich in fibroblasts and extracellular matrix is abundant (Junqueira and Carneiro, 2004). The inflammatory and immune cells residing in the endometrium (Foster, 2007) varies according to the stage of estrous cycle. The work of Tavares Pereira (2012) showed that the pattern distribution of resident B and T lymphocytes and macrophages are different according to the layer of endometrium and also that their numbers change during the oestrus cycle, suggesting that immune cells distribution may be related with the functions of the organ (Tavares Pereira, 2012).

The endometrium can be divided in three anatomical layers: 1) a superficial area, encompassing the area of the surface epithelium and the opening of glandular crypts (equivalent to the superficial component of the named *stratum functionalis* in humans); 2) a basal area, surrounding the deep endometrial glands, closer to the myometrium (equivalent to the named *stratum basalis* in humans); and 3) an intermediate, between the former two layers (equivalent to the remainder of the

stratum functionalis), with a variable number of glandular elements depending on the stage of the estrous cycle (Payan-Carreira et al., 2014).

The myometrium is composed of a deep inner layer and an outer layer of circular and longitudinal smooth muscle, respectively. These two layers are separated by the *stratum vasculare*, a layer of connective tissue with large blood vessels (Aughey and Frye, 2001; Bacha and Bacha, 2012).

The perimetrium or serosa is the outer loose connective tissue layer, which is continuous with the broad ligament (Aughey and Frye, 2001).

The cervix is lined by simple columnar epithelium with goblet cells. Glandular tissue fades in the cervix, extending to the cervical outer orifice. The muscularis is composed by an inner circular and an outer longitudinal layer of smooth muscle (Bacha and Bacha, 2012).

The mucosa of the vagina is lined by a stratified squamous epithelium. The inner layer of the muscularis is thick and consists of circularly arranged smooth muscle, while the outer layer is thin and consists of longitudinally organized smooth muscle. In the queen, a thin layer of longitudinal muscle occurs internal to the circular layer. An adventitia or serosa is present (Bacha and Bacha, 2012).). The cervical and vaginal epithelia undergo histologic variations during the stages of the estrous cycle (Mills et al., 1979).

External Genitalia

The mucosal epithelium of the vulva is stratified squamous. The major vestibular glands are bilateral, mucus-secreting, tubuloacinar glands in the submucosa. Minor vestibular glands are small, branched, tubular, mucous glands distributed through the vestibular mucosa. The integument of the labia (lips of the vulva) has a structure like that of the external skin. It is well endowed with both sebaceous and tubular apocrine glands. The clitoris consists of erectile tissue (corpus cavernosum clitoridis), a glans**,** and a prepuce (Bacha and Bacha, 2012).

Feline Estrous Cycle

The estrous cycle refers to the physiological cyclic changes, both organic and behavioral, induced by different hormonal profiles. Regular estrous cycles start after sexual maturity, which in domestic cats is achieved when the female attains

approximately 80% of the adult body weight, normally between 5 and 12 months of age, if the photoperiod is adequate (Feldman and Nelson, 2004; Schmidt, 1986). The age at the onset of estrous cycles also depends on the breed and on the as the date of birth (Schmidt, 1986). Cats are seasonally polyestrous (Feldman and Nelson, 2004; Kutzler, 2007; Schmidt, 1986), the seasonality being determined by day length (Gimenez et al., 2009; Kutzler, 2007; Leyva et al., 1989). Cats are long day breeders. Therefore, in the North Hemisphere, a prolonged anestrus occurs from September to January, when the length of the day is shorter (Kutzler, 2007). However, anestrus may be minimal or non-existent in household animals, maintained with fourteen hours of light and moderate environmental temperatures (Tsutsui and Stabenfeldt, 1993). Additionally, audiovisual and olfactory signs presented by the females in estrus affect queens' behavior (da Silva et al., 2006).

Cats show a distinctive peculiarity on their estrous cycle: in felids, ovulation is not spontaneous but triggered by mating (Kutzler, 2007; Schmidt, 1986; Wildt et al., 1981). However, in some cases (that may reach up to 25%) spontaneous ovulations can occur, mainly in very young or older queens living in larger groups, possible triggered by visual and pheromone stimuli (Goericke-Pesch, 2010; Gudermuth et al., 1997).

Consequently, cats may present two types of estrous cycle: the anovulatory cycles, when ovulation is not achieved, and the ovulatory cycles, often associated with mating (FIGURE 1). The anovulatory cycle is characterized by successive follicular stages (representing the proestrus and estrus), that lasts for 1 to 7 days (although it may last up to 16-20 days, according to Fontbonne and Garnier, 1998), separated by a short interestrous interval (ranging from 9 to 19 days), equivalent to the period of atresia of one follicular wave and the emergence of the subsequent one; in these cycles, no luteal stage exists (Tsutsui and Stabenfeldt, 1993).

The ovulatory cycle occurs when mating or spontaneous ovulation take place (Borges et al., 2015). This cycle is characterized by periods of ovarian follicular development coincident with sexual activity – follicular stage -, where estrogens dominate, alternating with periods of dominance of progesterone, produced byactive *corpora lutea* is active – the luteal stage (or diestrus) (Tsutsui and Stabenfeldt, 1993). In cats, the non-pregnant diestrus last for 35-40 days, and in that case the interestrous interval is about 45 to 50 days. But if the female conceives, progesterone dominance is maintained for an average of 65 days, until parturition.

During the reproductive season, the female may resume cyclic ovarian activity in about 3 weeks (Fontbonne and Garnier, 1998; Verhage et al., 1976).

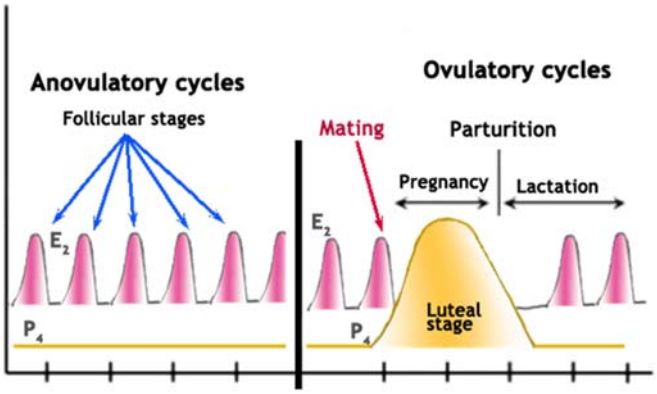

FIGURE 1: Draft of the anovulatory and ovulatory cycles in cats. In pink are depicted the follicular stages and in yellow the luteal stage.

MORPHOLOGICAL CHANGES CHARACTERIZING THE FELINE ESTROUS CYCLE STAGES

The phases of the estrous cycle are characterized based on clinical and behavioral signs and hormonal changes (Feldman and Nelson, 2004; Malandain et al., 2011; Mills et al., 1979), which can be surveyed through changes in vaginal cytology features and ultrasonography in vivo, or by direct inspection of the ovaries at laparoscopy or after the surgical excision of the genital tract. These are reliable tools for staging of the estrous cycle (Davidson and Baker, 2009; Malandain et al., 2011; Schmidt et al., 1983; Wildt et al., 1981).

ENDOCRINOLOGY OF FELINE ESTROUS CYCLE

In the beginning of the breeding season, follicular growth is stimulated at the ovaries, in response the positive stimulus of hypophyseal FSH. In consequence of the follicular development during proestrus and estrus, blood estrogen concentrations increase, and in the follicular stage they may exceed 20 pg/ml (Wildt et al., 1981). LH coordinates the later stages of follicle maturation and ovulation. In cats, pre-ovulatory surge of LH is triggered by the coitus, and is dependent on multiple, recurrent stimulation at the level of the vagina. A sustained elevation in estradiol concentration is a prerequisite for ovulatory LH release (Banks and Stabenfeldt, 1982). A reduced number of copulations might fail to elicit an LH adequate response, impairing ovulation or only allowing partial ovulation (Concannon et al., 1980). Ovulatory LH response, in amount and duration, varies

within animals and intra-individually (Banks and Stabenfeldt, 1982; Glover et al., 1985).

In anovulatory cycles, successive waves of follicles arise at the ovaries, inducing the occurrence of sequential follicular stages, separated by short periods devoid of the stimulation of sex steroids.

In the ovulatory cycles, after ovulation estradiol values decline significantly (Banks and Stabenfeldt, 1982; Shille et al., 1983), closely followed by the increased in the progesterone secretion. In cats, the progesterone levels peak around day 21; thereafter, in non-pregnant cycles, the progesterone drops and reach the basal levels between days 35 and 40 (Fontbonne and Garnier, 1998).

Morphological Changes in the Ovaries and Uterus During the Estrous Cycle

Grossly, two stages may be found in the non-pregnant estrous cycle of the cat: (1) the follicular stage, also named as proliferative or estrogenic - the beginning of the proliferative stage occurs when the follicular teca interna starts to secrete estrogens, which plasma concentrations gradually rise (Shille et al., 1979; Chatdarong et al., 2005); (2) the luteal stage, also named as secretory or progestagenic.

These two stages are characterized by diverse features (whether at a macro or microscopic level) in different segments of the genital tract of the queen that may be used to grossly stage the cycle; besides the use of vaginal cytology, which gives the measure of the estrogen impregnation in the tissues, the most often used are the inspection and the histology of the ovaries and uterus.

The follicular growth may be perceived at the ovarian surface. In the ovaries, follicles growth from less than 1 mm in early proestrus to 1.5 mm at the start of the estrus (Feldman and Nelson, 2004) (Figure 2 A). They appear as multiple cavitary structures, which size varies with the development stage (Chatdarong et al., 2005; Gatel et al., 2015; Karja et al., 2002; Liman et al., 2013). At ovulation, they became less tense and when the follicular wall is ruptured, and their content evades, the follicular walls collapse slightly. Afterwards, proliferating lutein/luteinic cells occupy the follicular cavity and forms the corpora lutea. These are seen as compact round structures at the ovarian surface, in dark red color, giving the ovary the appearance of a berry (Figure 2 B).

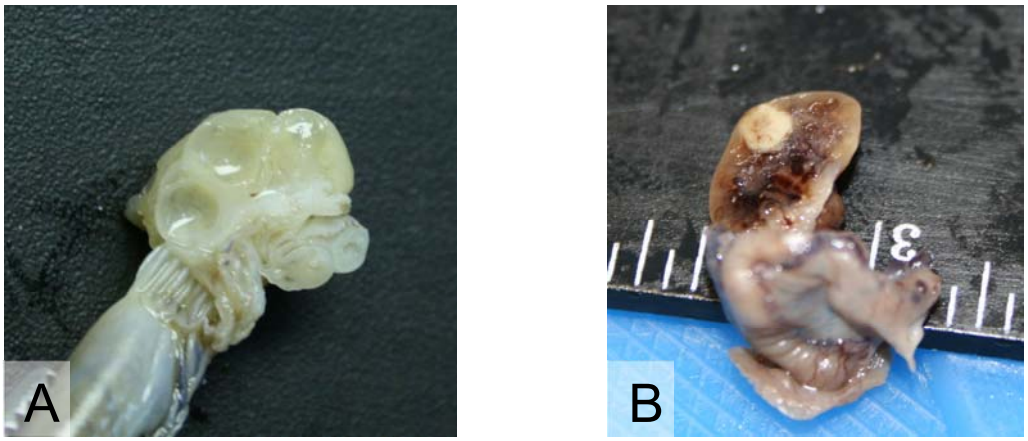

FIGURE 2: Macroscopic features of the feline ovary. In the follicular stage (A), multiple follicles are visible at cut surface. (B) In the luteal stage, a corpus luteum is evident.

The histological evaluation of the ovary sustains and details the gross changes described before (FIGURE 3 A-B). The follicles are lined by single to multiple layers of granulosa cells. In the ovarian cortex, a large number of pre-antral follicles are frequently found. Starting in proestrus, antral follicles increase in size as the follicular cavity enlarges. Around ovulation time, a small number of flatted granulosa cells are disposed around the antrum. Large, well-developed corpora lutea, containing prismatic lutein cells characterize the active corpora lutea, bulging at the ovarian surface; these are the main structures observed during the luteal stage. In the early luteal stage, luteal cells are closely packed and have amphophilic to eosinophilic cytoplasm; in the late luteal stage, luteal cells become vacuolated. Regressing corpora lutea also lost prominence at the surface of the ovary. Small, atretic corpora lutea from previous cycles may also be present in the ovary (Liman et al., 2013).

The uterine gross morphology does not vary much between the follicular and the luteal stage (Chatdarong et al., 2005; Gatel et al., 2015) (FIGURE 4 A-B). In general, the uterus shows a slightly curved shape and a wavy luminal cavity (Chatdarong et al., 2005) with scant fluid present (Davidson and Baker, 2009). During the normal estrous cycle, the macroscopic width of the uterine horns is maximum in the luteal stage (Davidson and Baker, 2009; Chatdarong et al., 2005). In this stage, the uterine horns became coiled or markedly curved, as the cycle progress from early to mid-luteal stage, accompanying the changes in progesterone concentration (Chatdarong et al., 2005).

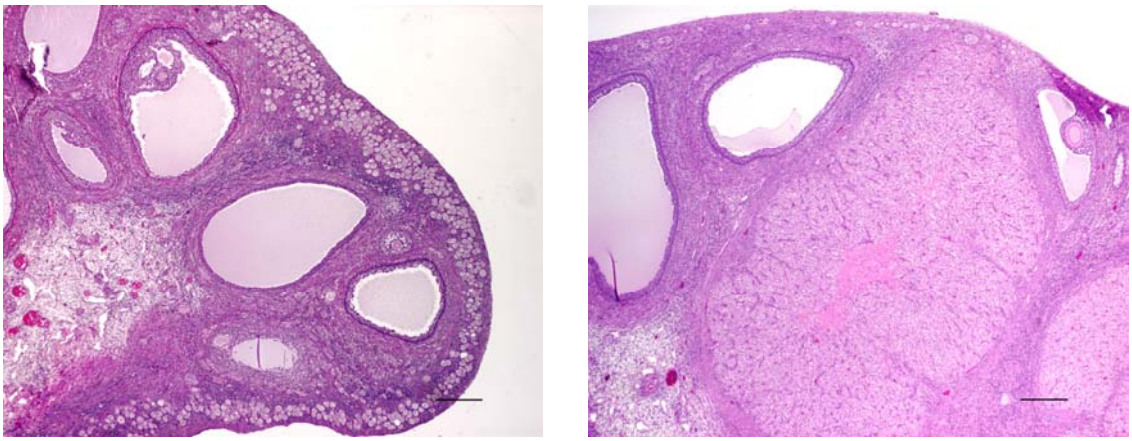

FIGURE 3: Microscopic features of the feline ovaries during the follicular stage (A) and the luteal stage (B). Bar = 300 µm. Multiple follicles and a complete corpus luteum (and half of one in the right bottom) are observed, respectively.

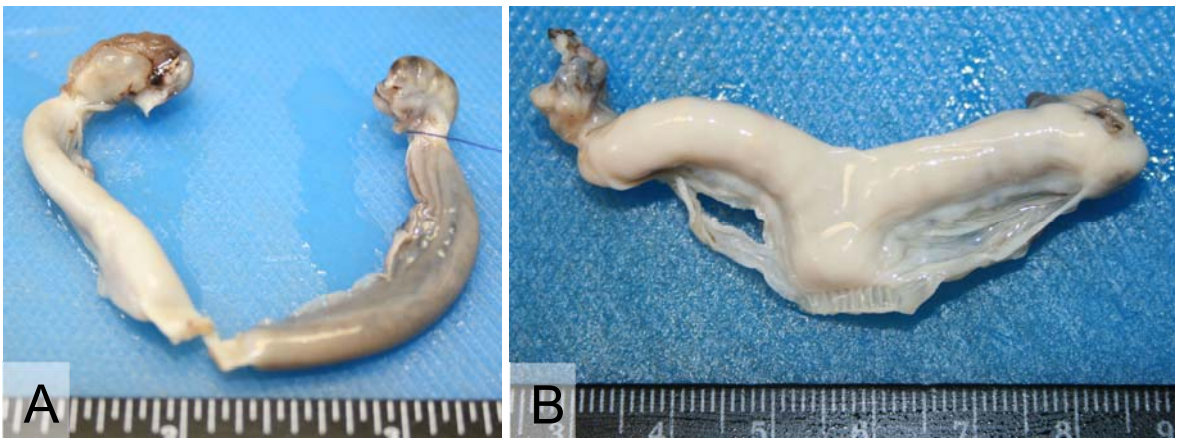

FIGURE 4: Macroscopic features of the feline uterus during the follicular stage (A) and the luteal stage (B). The uterine horns are curved and their width is maximum in the luteal stage.

The major differences between the stages on respect to the uterus are observed microscopically (FIGURE 5 A-B). During the follicular stage, as it happens in other mammalian species, the endometrium is thin, lined a single layer of cuboidal or prismatic cells. In the early follicular stage, the surface epithelial cells are columnar and eosinophilic, contrasting to the vacuolated appearance in the late stage (Liman et al., 2013). Uterine glands are simple and straight (Chatdarong et al., 2005; Rastogi, 2007), the epithelium composed by prismatic cells (Liman et al., 2013). The beginning of the follicular stage is characterized by endometrial growth, which was previously lost (Bacha and Bacha, 2012; Junqueira and Carneiro, 2004).

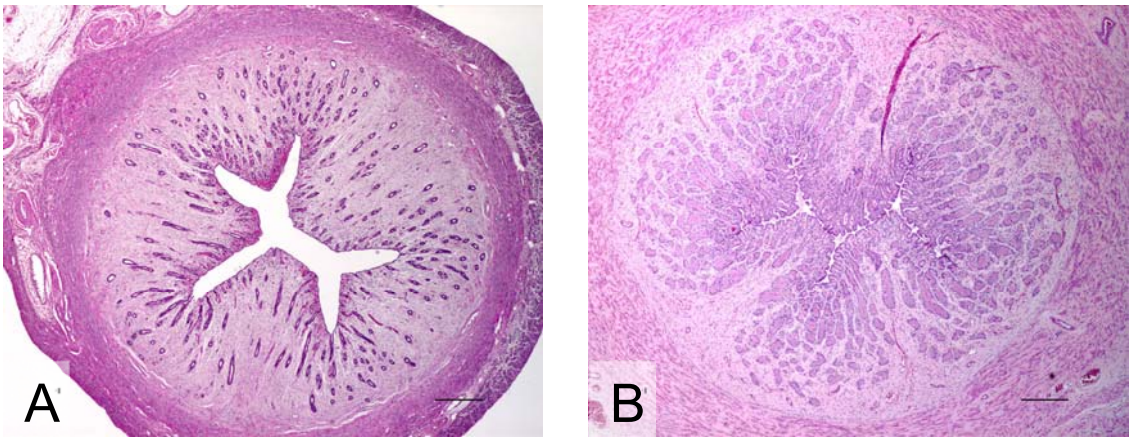

FIGURE 5: Microscopic features of the feline uterus during the follicular stage (A) and the luteal stage (B). Bar = 300 µm. The endometrium reach is maximum thickness in the luteal stage; the endometrial glands show a secretory acidophilic activity.

In the luteal stage, the surface epithelium is composed by high columnar cells, with pseudostratification or single cells (Chatdarong et al., 2005; Liman et al., 2013). The glands grow in diameter and are abundantly coiled, following progesterone stimulus (Chatdarong et al., 2005; Liman et al., 2013; Rastogi, 2007). The epithelial cells of deep glandules are taller than those of superficial glandules and surface epithelium (Liman et al., 2013). During this period, secretory acidophilic activity of the endometrial glands reach the maximum, coinciding with the peak in blood progesterone luteum (Bacha and Bacha, 2012; Liman et al., 2013). In this stage, the endometrium reach is maximum thickness, as the result of the growth of the mucosa and secretion accumulation (Junqueira and Carneiro, 2004). Additionally, the myometrium, which also respond to hormonal production, is thick and composed by hypertrophied smooth muscle cells (Liman et al., 2013).

Despite this division in phases, cyclic structural changes are progressive and continuous; the division of the estrous cycle in stages depends on the differential secretion of ovarian hormones, which is reflected in different functional and clinical conditions (Junqueira and Carneiro, 2004). In TABLE 1, we present a resume of the follicular and luteal stages of the feline estrous cycle (adapted from Chatdarong et al., 2005; Junqueira and Carneiro, 2004).

TABLE 1: Resume of the Principal Features of the Follicular and Luteal Stages of the Estrous Cycle (adapted from Chatdarong et al., 2005; Junqueira and Carneiro, 2004).

	FOLLICULAR STAGE	LUTEAL STAGE
PITUITARY HORMONES	FSH	LH
OVARY DESCRIPTION	Follicular growth; dominant follicle reach preovulatory stage	Ovulation and development of corpus luteum
OVARY HORMONES	Estrogens	Progesterone
UTERINE FEATURES	Growth of the endometrium	Additional growth of the endometrium; the glands become maximum coiled
UTERINE GROSS MEASUREMENTS	Length: 4.46 ± 0.77 cm Width: 0.56 ± 0.11 cm	Length: 4.74 ± 0.87 cm Width: 0.70 ± 0.16 cm
UTERINE MICROSCOPIC MEASUREMENTS (THICKNESS)	Endometrium: 461.63 ± 206.00 µm Myometrium: 629.74 ± 288.02 µm	Endometrium: 398.70 ± 160.39 µm Myometrium: 619.33 ± 191.40 µm

CYCLIC CHANGES OF LOCAL FACTORS IN THE ENDOMETRIUM: THE ENDOMETRIAL CYCLE

The endometrium undergoes a series of precisely determined morphological changes in response to the stimulation of the ovarian sex steroids, coordinated by the effects of estrogens or progesterone over their own receptors at the endometrium which triggers a dynamic interplay of local factors that ultimately determines the endometrial pattern of proliferation, differentiation, apoptosis, regeneration or resident immune cells infiltration, among other (Strowitzki et al., 2006). These are commonly named, in humans, as the endometrial cycle. This phenomena also exist in other mammal species, including the cat.

The feline endometrium undergoes cyclic changes throughout the estrous cycle responding to the ovarian steroid hormones. The proliferative stage is under the influence of estrogen whereas after ovulation, progesterone controls the secretory stage. This shift in hormonal balance promotes the transition between the proliferative pattern of the endometrium to a secretory lining, with the corresponding morphological changes. If conception is achieved, the endometrium remains as decidua, which becomes part of the placenta. On the other hand, if the endometrium doesn't receive the conceptus undergoes reabsorption.

The orchestration of the endometrial cycle is determined by the availability and activation of estrogen and progesterone receptors (ER and PR, respectively) in the organ, which in their turn influence a myriad of smaller molecules, such as interleukins and growth factors. The information regarding the molecules playing a

role in the endometrial cycle in cats is still limited, whether concerning the normal, physiological cycle or the diseased endometrium.

There are two forms of estrogen receptors ER-α and ER-β, without which reproductive functions are severely impaired. The overall proliferative response to estrogen is the result of a balance between ER-α and ER-β signaling. The ligand-dependent pathway of activating ER is not exclusive, however, since growth factor signaling leads to activation of kinases that may phosphorylate and thereby activate ER or associated co-regulators in the absence of ligand (Heldring et al., 2007). The amount of nuclear ER in feline uterus increases during proestrus and estrus and decreases after coitus. This suggest that progesterone decreases ER in feline uterus (Li et al., 1992; West et al., 1976; West et al., 1977).

The uterus respond to progesterone, increasing the growth and secretion of endometrial glands (West et al., 1976; West et al., 1977). Progesterone has both an antiestrogenic and progestagenic effect within the uterine epithelial cells. Progesterone receptors can be cytoplasmic or nuclear and progesterone is able to influence subcellular compartmentalization of PR (Li et al., 1992).

Estrogen receptors also have important interrelationships with the progesterone receptor system in modulation of responses (Katzenellenbogen, 1996). The studies of Kraus and collaborators (1995) showed that PR occupied by its ligand can suppress estradiol-stimulated ER activity, through interference with the ability of ER to interact with the transcription complex below in the molecular cascade.

Progesterone diminishes PR, however progesterone doesn't deplete totally the levels of ER and PR (Li et al, 1992). During pregnancy, the fall in the amount of nuclear ER in the uterus is probably related to the rise in progesterone more than the fall in estrogen. The increasing serum levels of progesterone observed after coitus diminishes cytoplasmic PR and raises nuclear PR; the continued presence of high levels of progesterone results in downregulation of PR system (Verhage et al., 1984).

It has been proposed that estradiol effects were mediated by ER localized in the uterine epithelial cells of the cat endometrium, whereas the existing PR would mediate the progesterone effects in the stromal compartment. However the putative crosstalk between epithelial and stromal compartment was proposed as being

involved in estrogen and progesterone effects in cycling feline endometrium (Li et al., 1992).

Prostaglandin (PG) synthesis is under the regulation of ovarian sex steroids in feline endometrium, as it also occurs in other species. Epithelial cells are the main source of $PGF_{2\alpha}$, whereas stromal cells mainly secrete PGE_2. However, ovarian steroids amplification of PG synthesis is limited to epithelial cells. Estrogens significantly raise $PGF_{2\alpha}$, enhanced by progesterone, which means that it is involved in a local regulation at the diestrus in cats. It is proposed that PGE_2 secretion increased by progesterone promotes a luteotrophic effect on feline *corpora lutea* (Siemieniuch et al., 2010). In another study, using only spontaneously ovulated queens, a higher expression levels of the genes involved in PG synthesis were related to the highest progesterone level at the mid diestrus (Siemieniuch et al., 2013), reinforcing the idea that estrogens and progesterone affect PG synthesis in the feline endometrium. However, ovarian sex steroids did not altered $PGF_{2\alpha}$ output neither its synthase at the gene expression level. Technical issues have been pointed as the main reason for the discrepancies between both studies. On the contrary, the combination of exogenous estrogen and progesterone (added to endometrial explants, imitating normal period shortly after ovulation) augmented PGE_2 secretion at estrus and mid dioestrus, whereas progesterone alone (mimetizing the luteal stage) augmented PGE_2 secretion at estrus. This supports the idea that PGE_2 is a luteotrophic factor released by the endometrium at the time of progesterone dominance (Siemieniuch et al., 2013).

The mechanisms underlying the PG synthesis dependency on steroid hormone activation are still poorly understood; however, it is proposed that the rate-limiting step in PG production may be the availability of arachidonic acid and that different molecular mechanisms may apply for steroids affecting synthesis and secretion of PG by cat endometrium (Siemieniuch et al., 2013).

Oxytocin is secreted by early/developing corpus luteum in cultured luteal cells and these cells express its receptors. The feline uterus also presents oxytocin receptors, suggesting that it responds to the ovarian produced oxytocin. These receptors are abundantly expressed during estrus and early/developing luteal stage, especially in stromal cells, being an important driver in the pathway of PGE_2 synthesis. The importance of PGE_2 in differentiation of stromal cells into decidua and implantation suggests that oxytocin has a major role in fostering implantation.

Furthermore, PGE_2 is essential for changes in uterine vasculature during implantation and can be important for prevent luteolysis. The epithelial cells also secret $PGF_{2\alpha}$ through oxytocin receptors (Siemieniuch et al., 2011).

Tumour necrosis factor alpha (TNF-α) enhances PG secretion in feline epithelial cells but not stromal cells, under the influence of phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2). In the luteal stage, TNF-α immunolabelling is lower and restricted mostly to the stromal cells and into a lesser extend to surface and glandular epithelium, whereas in estrus the immunoreaction is moderate to strong and develops both in surface and in epithelial cells (Fontinha et al., 2015; Jursza et al., 2014). In feline endometrium, TNF-α receptors were only localized in epithelial cells (Jursza et al., 2014).

Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) seem to be involved in an autocrine/paracrine way. These growth factors were found in normal feline endometrium, during treatment with estradiol and progesterone, as well as in pregnant uterus (Boomsma et al., 1997).

Chatdarong and collaborators (2005) tried to establish a correlation between the PCNA (proliferating cell nuclear antigen) index and the stage of the estrous cycle in cats, as it is proposed in other species. However, PCNA expression in feline uterus seems to be unrelated to the stages of the estrous cycle (Chatdarong et al., 2005).

Interestingly, several molecules were identified in the feline endometrium specifically in each steroid-associated stage. A progesterone-dependent secretory protein (PDP), an uterine specific protein, was synthetized in feline endometrium and released into the uterine lumen during early stages of pregnancy, which has be hypothesize to play a role during attachment and implantation (Boomsma and Verhage, 1987). Progesterone-dependent uterine secretory protein is diffusely localized in the cytoplasm of the epithelial cells of the deep uterine glands, where it is synthesized (Verhage et al., 1989). It was also identified a high molecular weight protein dependent on estrogens (CUPED) in the cat endometrium, present in secretory granules of epithelial cells of uterine glands and in the lumen of deep uterine glands, during periods of estrogen dominance (estrus). This protein is also maintained high during the period extending from peri-ovulation and the first few days of pregnancy, being suggested that, although it may act at the time of sperm transport through the uterus and by the time of the unplanted blastocyst, its main

role would relate to the nutrition of developing blastocysts, to coating the uterine epithelium in preparation for implantation and in sperm capacitation or in maintaining sperm viability (Murray et al., 1986; Murray et al., 1985; Murray and Verhage, 1985).

Survivin, Bcl-2 and Bax are apoptosis-related proteins; apoptosis is an important mechanism in endometrial homeostasis. These proteins are expressed in feline endometrium with cyclic variations, playing an important role in endometrial growth and regression. The maximum intensity of Bcl-2 immunolabeling is observed in late proliferative stage, decreasing in the early luteal stage. This is in accordance with the shift in Survivin and Bax expression, which arise during the secretory stage. Survivin is expressed in the cytoplasm and nucleus of superficial and deep uterine glands in the luteal stage, whilst it is only located in the cytoplasm during follicular stage and anestrus. These findings suggest that Survivin may be involved in controlling uterine cell survival and proliferation during feline estrous cycle. Survivin is also expressed in particular stromal cells, endothelial and smooth muscle cells of blood vessels, suggesting that this protein plays a significant role in angiogenesis. Bcl-2 was only found in the cytoplasm of either epithelial or stromal cells of feline endometrium, evidencing increased expression during the follicular stage, suggestive of an anti-apoptotic role for Bcl-2 in feline uterus. Bax immunoreaction was demonstrated predominantly in the cytoplasm of the glandular epithelial cells in the luteal stage; the protein has a diffuse pattern of expression. These findings are consistent with the role of this protein in the increasing of apoptotic susceptibility. Stromal cells of feline endometrium with a Bcl-2/Bax positive phenotype represent macrophages (Liman et al., 2013). Studies on apoptosis performed by our group demonstrated a higher expression of activated Caspase 3 in the proliferative stage of feline normal endometrium (Fontinha, 2014).

Uterine Pathology in the Queen

Impairment of the reproductive function and the clinical syndromes related with uterine inflammatory conditions are the more representative causes for uterine disease in feline practice. Abnormalities of reproductive function generally follow disturbances of the normal hormonal and physiologic patterns. Such disturbances can arise from a number of uterine diseases. The most common lesions of uterine pathology can be grossly divided in congenital abnormalities, displacement,

hypertrophy, hyperplasia and metaplasia, inflammation and neoplasia (Van Dijk et al., 2007).

Developmental anomalies are rare in cats. In one study of 53,258 female cats around United States and Canada, 0.09% of the queens had a gross uterine, namely unicornuate uterus, segmental uterine horn agenesis and uterine horn hypoplasia (McIntyre et al., 2010). These kind of abnormalities can be found as an extraordinary finding during hysterectomy or laparotomy, but they also can be at the origin of ambiguous abdominal pain and distension, as a consequence of fluid accumulation upstream the obstruction point (Colaço et al., 2012).

Cystic endometrial hyperplasia is a frequent condition in cats (Kempisty et al., 2013). This lesion generally arises from disturbances at the functioning of the endometrial glands and frequently progress to pyometra so they are commonly known as the cystic endometrial hyperplasia/pyometra complex (Agudelo, 2005; Schlafer and Gifford, 2008). The first stage of the disease is characterized by progesterone-induced cystic dilatation of the glandular endometrium. Evolution into pyometra is facilitated by the progesterone environment, but also depends on estrogen influences (Agudelo, 2005).

However, other causes besides cystic endometrial hyperplasia can induce pyometra making this disease a common feature in queens, and frequently the reason for uterus ablation. In a study of 139,075 female Swedish insured cats, 791 were diagnosed with pyometra (Hagman et al., 2014). In our experience, ovariohysterectomy surgical specimens clinically diagnosed with pyometra are rarely sent to the laboratory for morphological evaluation.

Regarding oncological diseases, adenocarcinomas of the endometrium are considered the most frequently diagnosed uterine neoplasia in this species (Argyle, 2008; Van Dijk et al., 2007). Compared with the amount of work performed on feline mammary diseases, few references are available concerning feline uterine pathology. The lack of knowledge in this area of veterinary medicine endorsed our commitment to the present work. We looked forward to describe and understand the morphology, histology and immunoprofile of feline endometrial adenocarcinomas (FEA), hopping that this will become the beginning of a journey that promises to be helpful to pathologists, surgeons and especially to cats. In the next chapter, we will review all the general aspects of FEA.

AIMS

The general aim of the present work was to understand the biopathology of FEA, including their epidemiologic aspects and histomorphological features.

Specific aims of our study included:

1) The characterization of the *state of the art* regarding FEA, recognizing epidemiologic data, clinical signs, diagnosis, available treatment and prognosis of the disease.

2) The description of the histological aspects of FEA of the papillary serous type, especially considering features generally related to prognosis.

3) The immunohistochemical characterization of the expression of several molecules in FEA of the papillary serous type, in comparison with normal endometrium in proliferative and luteal stages of the feline estrous cycle. Those included proteins extensively studied in the human endometrial neoplasms:

    a. Steroid hormones receptors;
    b. Cytokeratins 7 and 20;
    c. Ki-67;
    d. Cyclooxygenase-2;
    e. C-erbB-2.

    With the study of the patterns of expression of these proteins in feline normal and neoplastic endometrium, we intend to initiate the characterization of some of the molecular events underlying endometrial carcinogenesis in cats. Moreover, some of these proteins might be used as markers in the histological diagnosis of FEA.

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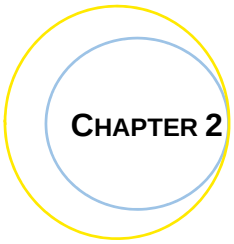

CHAPTER 2

FELINE ENDOMETRIAL ADENOCARCINOMAS

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Feline Endometrial Adenocarcinomas

Abstract

Endometrial adenocarcinomas are considered to be rare in domestic animals, in particular comparing to non-epithelial tumours of the uterus, such as leiomyoma, in part because they are likely underdiagnosed. In cats, though uterus was the most common site for genital tract tumours, endometrial adenocarcinomas were found to be rare in all available references.

In the study presented herein, forty feline primary endometrial adenocarcinomas, identified by a minimum of three pathologists on conventional haematoxylin and eosin-stained sections, obtained from the archives of four different laboratories, for a period of 13 years were used in a retrospective study on the subject.

In previous studies on feline endometrial adenocarcinomas (FEA) it has been found an increase incidence in purebred animals, which has been associated with a longer reproductive activity in those animals in comparison with domestic cats. However, this should be read with caution, as population ratios between purebred and domestic mongrel animals may change with the geographic location. Also the cultural adoption of gonadectomy as a contraceptive measure may interfere with the regional prevalence of the disease. In fact, in Portugal the number of intact domestic cats is higher than the purebreds, due to the tendency for late spaying age (most of them due to uterine or mammary diseases), which could influence the incidence of FEA in our country. Some of the specimens used in this study were obtained at ovariohysterectomy following a clinical diagnosis of pyometra. Unless metastasic disease exists, to which illness is often associated, most frequent signs for FEA are vague and unspecific, making difficult the early diagnosis of endometrial carcinoma.

The histological evaluation shows the existence of different tumour phenotypes, where clear cell are often found. The presence of nuclear atypia and the Ki67 expression seem not to be related to the malignancy of the tumour. Furthermore, one report refers the presence of oestrogen receptors, which could explain the low levels of metastization or invasiveness proposed for FEA, or the clinical outcome for the tumour. For most situations of primary FEA, in particular in early stages of the disease, myometrial invasion is limited and often absent. Also

vascular invasion is seldom observed even in tumours with moderate nuclear atypia. However, limited information exists on specimens from early FEA stages, which impair the presentation of a reliable prognosis for that animal. The major challenge seems to be the identification of valuable prognostic markers to achieve a definitive prognosis for animal life.

Feline endometrial adenocarcinomas are possibly more common than we might presume and the clinical impact of such tumours may be increasing with the increase in the ability to establish an early diagnosis allied to an extended duration of life in cats. A recent report defends that FEA are more common than smooth muscle tumours of the uterus. Furthermore, as it frequently co-exists with pyometra or mucometra, it is also possible that a large number of cases fail to reach the pathological evaluation and hence remain undiagnosed.

In this chapter we propose to discuss the clinical and morphological data of all the forty feline primary endometrial adenocarcinomas, supported by an extended review of the literature.

INTRODUCTION

Although the uterus is considered the most common site of feline reproductive tract tumours in one study, uterine tumours comprised only 0,29% of all feline neoplasms diagnosed during the study period (Miller et al., 2003). Adenocarcinomas of the endometrium are considered to be rare in all domestic species, with exception of cattle and rabbits (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976), with the common practice of ovariohysterectomy (OVH) in cats being referred as protective from uterine neoplasia (Miller et al., 2003; Taylor, 2010). Spaying of young queens for contraception is neither a worldwide procedure nor a common practice in purebred cats, and hence is may not always be the reason for the small percentage of uterine adenocarcinomas. Furthermore, there are also reports of adenocarcinoma in the uterine stump of neutered cats (Anderson and Pratschke, 2011; Miller et al., 2003). On the other hand, the rarity of these tumours in cats might be related to inadequate post-mortem examination of the genital tract, but also to lack of interest in anatomopathological evaluation of ovariohysterectomy surgical specimens. In fact, in the study by Miller et al. (2003),

from all the uterine tumours the endometrial adenocarcinoma was the most commonly found in cats.

According to clinical reports and our own experience, uterine carcinoma does not present specific signs. It rather shares some clinical signs with other uterine diseases and abortion, such as fluid accumulation with distension of the organ and increased thickness of the uterine walls, combined with emaciation or vaginal haemorrhagic discharge (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964). These features further strength the possibility that the situations may be underdiagnosed, as the excised genital segments at OVH are seldom sent to histopathological analysis. Endometrial adenocarcinoma occurs most frequently in middle age to old animals, and its putative association with progestagens-based contraception is controversial. Nevertheless, very young animals can develop the disease.

In other species, endometrial adenocarcinoma evolves as a malignant epithelial neoplasm. Also in felids, some reports exist on the presence of metastasis at the moment of diagnosis (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964); however, its occurrence seems sporadic, and predictive markers have not yet been established.

The major challenge seems to be the identification of diagnostic markers of feline endometrial adenocarcinomas, since there are few reports about these features, all of them in small case series. Because of the same reason, the achievement of prognosis of these lesions is difficult and requires further studies.

Different morphologies of feline endometrial adenocarcinomas can be defined, which included papillary serous carcinoma (with or without clear cells), clear cell carcinoma and in situ carcinoma according to cytological criteria of malignancy, cell morphology and invasion of adjacent tissues (Saraiva et al., 2011).

It is the intent of this paper to summarize what is known about the feline endometrial adenocarcinoma, supported by the findings of 40 histopathological situations. We will discuss the incidence of the disease, the main clinical signs as well as its diagnosis and the need for reliable prognostic markers. Further we will characterize the main morphological features of the three main types of feline endometrial adenocarcinoma (FEA) found in our casuistic.

Incidence / Epidemiology of FEA

Endometrial carcinoma/adenocarcinoma is more common in the cat than the dog (Morris and Dobson, 2001). Uterine adenocarcinoma is believed to be locally invasive, and the occurrence of metastasis are commonly reported. Severe illness is mainly associated with metastatic disease and is often at the origin of the presentation at the clinic.

Although no breed predisposition has been reported (Klein, 2007), in previous studies on feline endometrial adenocarcinomas (FEA) it has been found an increase incidence in purebred animals, which has been associated with a longer reproductive activity in those animals in comparison with domestic cats (Johnston et al., 2001). However, this should be read with caution, as population ratios between purebred and domestic crossbread animals may change with the geographic location. Uterine samples with FEA were more frequently found, within the cases used herein, in domestic crossbread animals than in purebred (with the registry of only two Siamese and one Persian queen). This might be explained by the fact that in Portugal the number of intact domestic cats is higher than the purebreds, due to the tendency for late spaying age (most of them due to uterine or mammary diseases), which could influence the incidence of FEA in our country.

From our database, in the last thirteen years 197 female genital tracts have been sent for analysis (almost 12% of all female feline analysis), from which 41.16% (81:197) did not show signs of uterine disease, 6.6% (13:197) were pregnant or with signs of abortion, 33.5% (66:197) showed inflammatory diseases of the uterus and 20.30% (37:197) were diagnosed with FEA. The relative frequency of FEA is quite high in our database, which could be associated to the fact that in the last three years, we performed the systematic evaluation of excised genitalia for a PhD project. The analysis of the frequencies for the other clinical situations also highlights the fact that most frequently the clinicians send to commercial laboratories for analysis the organs with macroscopic signs of disease. Consequently, it is possible that small lesions may be undetected during their first trial, hence not being submitted for diagnosis, or incorrectly assumed as having pyometra. FEA most frequently develops in older animals, with more than 9 years (Belter et al., 1968; Klein, 2007; McEntee, 1990; Morris and Dobson, 2001; Preiser, 1964), although sporadic reports on younger animals around 5 years old have been published.

Although clinical information was only possible for half the animals used in our studies, in our cases the age of the females diagnosed with FEA ranged from 2 to 15 years old. The age mean was 7 years and median for age was 6 years old.

Clinical Signs

Albeit several studies on feline endometrial carcinoma have been published, few reports detail the clinical aspects of the pathology. Furthermore, most descriptions refers to late stages of the disease (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964), as often FEA evolves as a silent disease, not detected clinically unless large lesions or metastases are present. Illness is usually related to metastatic disease, and the major presenting complaint.

The clinical signs of FEA vary with the size of the neoplasic lesions, but also with the age of the process and the existence and pattern of metastasis. It is possible that the morphological type of the tumour may also interfere with the most prevalent clinical sign. The size of the tumour may vary from less than one centimetre to greater than 10 cm (Johnston et al., 2001). Small-sized tumours are most frequently found incidentally at ovariohysterectomy or postmortem, whilst the large one usually gives more or less notorious clinical signs.

If metastases are not present, vague and unspecific signs of uterine disease may be found, since the presence of a mass within the uterus may induce in chronic inflammation causing a mucous, purulent or haemorrhagic vaginal discharge, or pyometra (Taylor, 2010). Vulvar discharge may be intermittent (Meier, 1956, cited by McEntee, 1990). As consequence of this inflammatory reaction signs such as vomiting, irregular appetite and polyuria/polydipsia are present, and together with the vaginal discharge are usually the main complaints at consultation. Rarely, abnormal oestrous cycles have been described in association with FEA. Enlargement of the uterus, abdominal distress and distension of the abdomen are also frequently found during transabdominal palpation or when larger tumours exist (Johnston et al., 2001; Taylor, 2010). Also, when the lesions grow large enough to compress adjacente viscera, other signs may develop (Klein, 2007), like constipation or dysuria. They are frequently found in published reports on metastatic FEA situations, but are not always associated with the metastasis itself. Whenever metastasis exists, ascites, anorexia and weight loss are commonly found clinical

signs, which may co-exist with anaemia, fever and cachexia (Meier, 1956, cited by McEntee, 1990; Johnston et al., 2001). FEA may metastasize regionally or at the distance, to lungs, brain, or eyes (Klein, 2007; McEntee, 1990; Taylor, 2010). Depending on the location of the metastasis, secondary symptoms may be found: cough and respiratory distress, if metastases are thoracic, blindness or motor incoordination if in the central nervous system, ascites if in the abdominal organs. According to Taylor (2010) most cats develop metastatic disease within 6 months of diagnosis, if uterine excision is not performed or lymphatic invasion already exist at diagnosis. However, this period should be considered as indicative, as for most published reports, the tumour was diagnosed in later stages of the disease.

Morphological Features of Primary Epithelial Tumours of the Uterus

Several studies had been published in small series of feline endometrial adenocarcinomas, mainly related with their immunoprofile (Espinosa de los Monteros et al., 1999; Gil da Costa et al., 2009; Martin de las Mulas et al., 1995; Miller et al., 2003), but there is lack of literature explaining the different morphological aspects of these tumours, even when case reports are described (Anderson and Pratschke, 2011; Belter et al., 1968; Sapierzynski et al., 2009).

Based in our experience, there are three different morphologies of primary endometrial adenocarcinomas in cats, which can be compared with women endometrial carcinoma (Horn et al., 2007): papillary serous carcinoma, clear cell carcinoma and a lesion restricted to the superficial endometrium that we might classified as "*in situ*" carcinoma.

Macroscopically, these three different morphologies appear to be indistinguishable, presenting as a diffuse thickening of the endometrium along both uterine horns and corpus or as multiple white nodules, projecting to the lumen (Figure 1). The uterine wall can be also thinner if an abundant purulent content is present within the organ. In invasive tumours, it is possible to detect tumoral infiltration through the myometrium, sometimes with umbilication in the serosa that could rupture, promoting in this cases peritonitis.

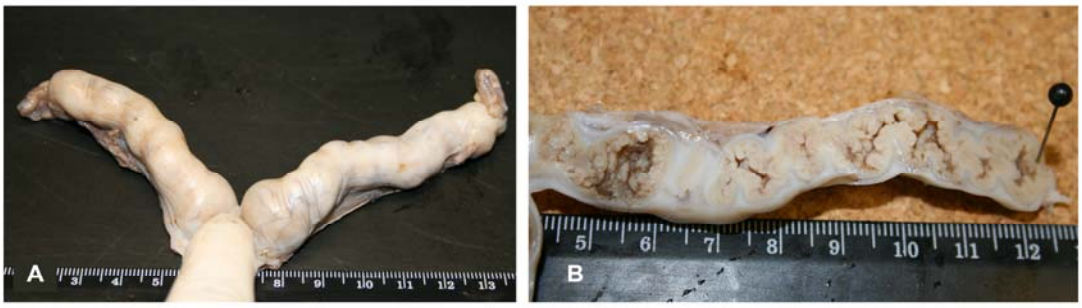

FIGURE 1: Feline endometrial adenocarcinoma. A. Gross evaluation shows the uterus enlarged in all the extension. B. Longitudinal section shows the endometrium diffusely thicker through the uterine horn, appearing as a proliferative white tissue.

PAPILLARY SEROUS CARCINOMA

The majority of feline endometrial carcinomas, as described in the references cited above, have a papillary growth pattern projected into the lumen (FIGURE 2 A). The papillae are supported by a thin fibrovascular stroma (that may be edematous or hyalinized) and lined by more than one layer of neoplastic cells. The cells are pleomorphic columnar shaped, with moderate amount of eosinophilic cytoplasm, sometimes with focal clearing. The nuclei are round to oval, vesicular or hyperchromatic, and the cells loose the normal polarity; large nucleoli are evident and sometimes are found intranuclear clear inclusions. Mitotic figures are frequent and often bizarre. There is a moderate degree of anisokaryosis and anisocytosis. Numerous multinucleated cells can be found within the tumour. In some tumours, is possible to see squamous metaplasia. Randomly distributed areas of necrosis are found within the neoplasm and occasionally psammoma bodies are present. The invasion of the myometrium, vessels and lymphatics is not a constant feature. Some tumours have a clear inflammatory component, with macrophages, plasma cells, lymphocytes, neutrophils and occasionally eosinophils. Although these tumours show a predominant papillary serous morphology, areas of solid growth and a glomerular pattern may be present, as well as various numbers of clear cells, which are described in the next section.

CLEAR CELL CARCINOMA

Clear cell carcinomas of the feline endometrium are almost entirely composed by large, round to polygonal cells, with foamy cytoplasm and eccentric

crenate or ovoid nucleus, with a prominent eosinoplilic nucleolus. The cells are arranged in papillae, sheets or solid nests surrounded by a fibrovascular stroma (Figure 2 B). As in papillary serous carcinoma, there is a moderate degree of anisokaryosis and anisocytosis but multinucleated cells are absent. It can also be found necrosis and inflammatory cells are rare. The invasion of the myometrium is not a constant feature. Vessel or lymphatic tumoral emboli were not found in the available cases.

"*In Situ*" Carcinoma

Morphologic aspects of "*in situ*" carcinoma are very similar to those described for the papillary serous carcinoma. The differences are related to the location of this tumour that is very superficial in the apical endometrium and to the lack of invasion of the stroma (Figure 2 C). As the name implies, these tumours are non invasive.

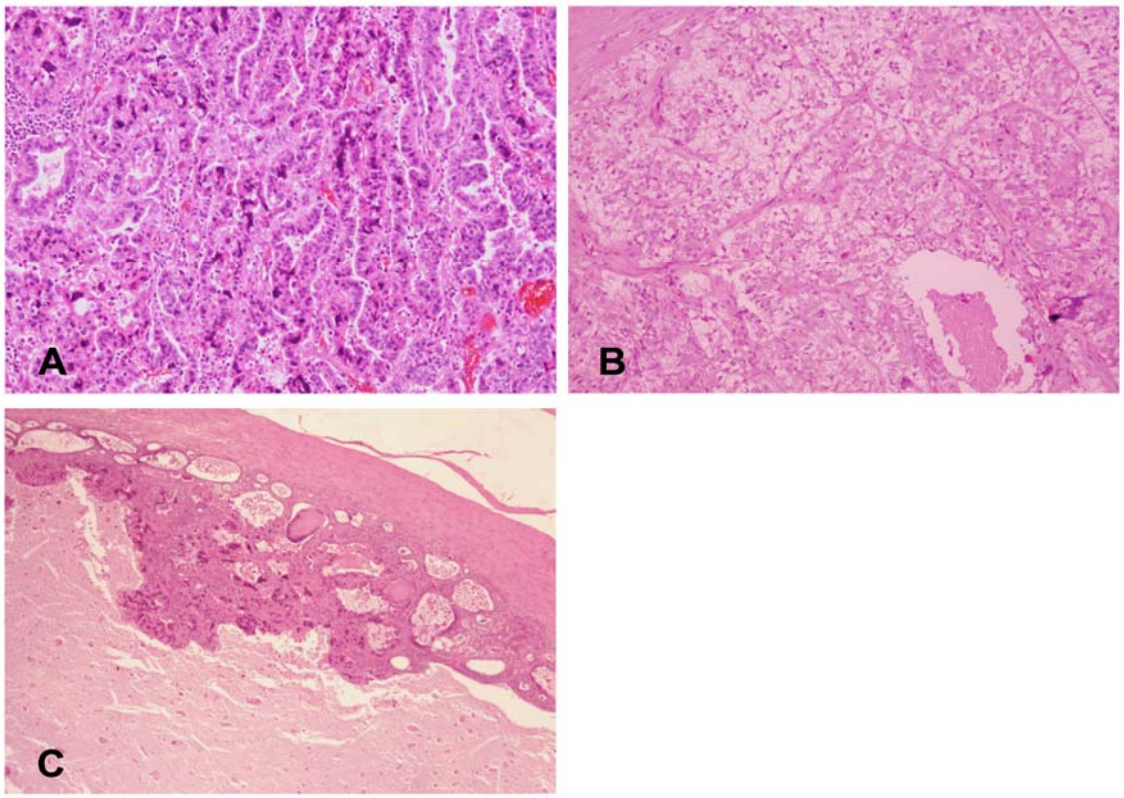

Figure 2: Morphological aspects of feline endometrial adenocarcinomas (HE). A. Papillary serous carcinoma (Obj. 10x). The papillae are surrounded by more than one layer of neoplastic cells and supported by scant fibrovascular stroma. It is possible to observe several multinucleated cells. B. Clear cell carcinoma (Obj. 10x). Nests of foamy cells, sometimes with central necrosis, surrounded by fibrovascular stroma. C. "*In situ*" carcinoma (Obj. 4x). Non invasive, superficial malignant proliferation of the endometrium.

Diagnostic Markers

Major available reports do not distinguish between the three abovementioned FEA phenotypes when studying the expression of molecular markers in uterine epithelial tumours. However, it is possible that their grow, invading behaviour and metastatic potential may differ between the tumour´ types. Only by searching for the differences we may be able to develop markers that can be use for treatment and prognosis.

Adenocarcinomas of the endometrium, like other epithelial tumours, tend to express broad-spectrum cytokeratins (CK), clones AE1/AE3 and MNF 116, but reactivity with AE1/AE3 is generally more intense and more frequent than with MNF 116 (Gil da Costa et al., 2009; Miller et al., 2003; Sapierzynski et al., 2009). Espinosa de los Monteros et al. (1999) described the coordinate expression of cytokeratins 7 and 20 in 67% of the feline endometrial carcinomas under study, in accordance with the results observed in the normal feline endometrium. Therefore, they proposed that coordinate expression of these two cytokeratins provides important information that can help in the diagnosis of these tumours (Espinosa de los Monteros et al., 1999). The tumours do not express CK14 (Gil da Costa et al., 2009), as well as intermediate filament cytokeratins RDK-102 and NCL-5D3 (Martín de las Mulas et al., 1995).

The great majority of feline endometrial carcinomas are negative for vimentin, but there are reports of tumours with expression of this molecule (Gil da Costa et al., 2009; Martín de las Mulas et al., 1995; Miller et al., 2003).

According to the literature, feline endometrial adenocarcinomas are generally negative for the estrogen receptor-α (ERα), but in some tumours, up to 50% of the neoplastic cells have nuclear staining for ERα (Gil da Costa et al., 2009; Miller et al., 2003; Sapierzynski et al., 2009). Miller et al. (2003) found a tendency for metastization by the tumours that are negative for Erα and they defend that the loss of expression of estrogen receptors in such tumours suggests estrogen independence and may indicate a worse prognosis.

The score of expression of progesterone receptor is very variable in endometrial adenocarcinomas, as well as the intensity of expression of this marker, but there is a tendency to a markedly reduced expression in these neoplasms (Gil da Costa et al., 2009; Sapierzynski et al., 2009). However, usually no information is

available on progesterone blood levels thus impairing the correct interpretaiton of the results.

There is a moderate to strong expression of cyclooxygenade-2 (COX-2) by up to 50% of the neoplastic cells and a loss of polarity compared with the normal endometrium (Gil da Costa et al., 2009).

Disperse information on other molecular markers, not explored from the biological point of view on respect to the tumour behaviour, are available in the literature. Endometrial adenocarcinomas of cats express E-cadherin and β- catenin, as the normal endometrium (Gil da Costa et al., 2009; Sapierzynski et al., 2009). In one case report, about 40% of tumoral cells of adenocarcinoma epithelium stained with Ki67 (Sapierzynski et al., 2009). The tumours are negative for smooth muscle actin, desmin, glial fibrillary acid protein and caveolin-1 (Gil da Costa et al., 2009; Martín de las Mulas et al., 1995; Miller et al., 2003; Sapierzynski et al., 2009).

Clinical Diagnosis

History, presenting complaint, and physical examination findings usually provide the basis for diagnostic. Vaginal bleeding or vaginal discharge may direct the differential diagnosis into abortion, uterine tumour or cystic endometrial hyperplasia/pyometra.

On early stages of the disease, FEA progress silently or giving discrete clinical signs. Most frequently, when signs of uterine disease are present, the animal is submitted to OVH under the suspicion of cystic endometrial disease or pyometra. Thus, the diagnosis is normally made at OVH or at necropsy. No consistent laboratory abnormalities have been noted, unless pyometra exist, and abdominal radiographs only confirm the presence of an abdominal or uterine mass. Hematological abnormalities might include regenerative anemia or neutrophilia (Klein, 2007; Morris and Dobson, 2001).

Whenever clinical signs for uterine disease are present, the diagnosis is based on the clinical signs, abdominal palpation and ultrasound or radiological exploration of the abdominal and thoracic cavities. A soft tissue mass in the mid or caudal abdomen may be detected on the palpation of abdomen, and its echogenicity may be study by plain abdominal radiography or ultrasonography (US). US findings may include hypoechoic masses in one or both uterine horns or lesions miming CEH

lesions. As frequently accompanying mucometra or pyometra are found, enlarged coils of uterus may be distinguishable, along with the presence of small amount of fluid in the uterus. Ultrasonography may further be useful to define the origin of the mass or to evaluate regional metastasis (Klein, 2007; Morris and Dobson, 2001). Sporadically, surgical exploration may be needed to achieve the diagnosis (Klein, 2007; Taylor, 2010). Radiographic findings can include soft tissue density masses that may displace the surrounding viscera. A definitive diagnosis is only obtained through histological examination of surgically excised specimens (Klein, 2007).

In FEA more advanced stages, clinical signs may occur as a result of metastatic disease even before any clinical indication of the primary tumour is detected. In such cases, the overall clinical signs may delude the origin of the carcinoma. If ascitis is detected, peritoneal fluid can be collected via abdominocentesis and sent for analysis.

Survival and outcome data are sparse for metastatic disease due to FEA (Klein, 2007; Taylor, 2010). The poor animal condition at diagnosis frequently limits the chances for successful treatment and often euthanasia is the human outcome for it. The differential diagnoses should contemplate other metastatic diseases associated to other carcinoma, as those at the mammary glands, liver, etc. In the absence of neurological signs, the presence of metastases on the lungs, eye, abdominal lymph nodes, liver and diaphragm should be ascertain, as it can influence both the treatment and the prognosis.

TREATMENT

In case of co-existing pyometra, supportive care should be provided. The use of antibiotics has to be pondered individually.

If metastatic disease is still not apparent, a complete OVH is recommended, and the uterus should be sent (the entire surgical piece) for histopathological analysis. Whenever the uterus shows abnormal macroscopic features, even if gross evidence of a tumour is not present, the histopathological analysis ought to be requested. During the surgical procedure, the abdominal cavity should be explored and any suspected mass biopsied for histopathology. Attempts should be made to remove all tumours and metastatic foci.

Chemotherapy and radiation therapy for treating uterine tumours are largely untested in veterinary cases. However, adjunctive chemotherapy may be considered if malignancy is detected upon histopathology (Morris and Dobson, 2001). Its use may be of survival benefit for patients, as it may prevent or delay the development of metastasis. However, the owner must be advertised that the benefits are yet unclear. For cats, the use of doxorubicin or carboplatin has been suggested. As limited information on the chemotherapy outcome for uterine tumours, health parameters should be closely monitored for undesirable side effects. Follow-up appointments should be schedule every three months to check for cancer spread and adjust the therapy as needed.

PROGNOSIS

In general, because clinical signs rarely are evident until late in the course of the disease, the prognosis should always be considered guarded, in particular because FEA have a documented metastatic potential. Metastatic disease warrants a severe prognosis.

Nevertheless, the quality of the studies made so far about this theme doesn't allow the accurate determination of prognosis of endometrial adenocarcinomas in cats, and its metastatic potential has been based on the number of clinical reports, but not on trustful clinical surveys. Only one study reveals that there might be a tendency to metastases by the tumours that are negative for ERα, which can indicate a worse prognosis (Miller et al., 2003). In case reports available in the literature, the tumours were presented in an advanced stage, resulting in a reserved prognosis and euthanasia (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964; Sapierzynski et al., 2009). According to our experience, if the tumour is detected precociously and the ovariohysterectomy is performed, the prognosis is favourable. Furthermore, "*in situ*" carcinomas, non invasive papillary serous carcinomas and non invasive clear cell carcinomas tend to have a better prognosis in the immediate future, but a longer period of time should be evaluated for definitive conclusions.

Conclusion

Feline endometrial adenocarcinomas are possibly more common than the literature describes and distinct morphologies are here proposed: papillary serous carcinoma, clear cell carcinoma and "*in situ*" carcinoma. Few studies are available and all on small case series of these tumours, which difficult the determination of several aspects of these neoplasms. Nevertheless, all of them describe the loss of expression of estrogen receptor by neoplastic cells, as being a consensual feature of feline endometrial adenocarcinomas, and possibly related to a worse prognosis. So far, from other authors' studies, this seems to be the best diagnostic marker associated with prognosis for feline endometrial adenocarcinomas. Another feature of feline endometrial adenocarcinomas is the coordinate expression of cytokeratins 7 and 20, which helps the diagnosis of these tumours. COX-2 may play an important role in the carcinogenesis of the endometrium, but more studies in larger case series are expected to extend the existent knowledge. New studies including the clinical data on the patients diagnosed with FEA in early stages of the disease are desired in order to establish incidence, common clinical profiles and survival rates for the disease.

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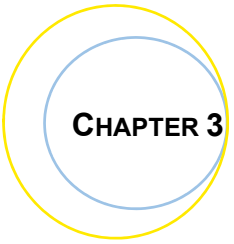

CHAPTER 3

AN IMMUNOHISTOCHEMICAL STUDY ON THE EXPRESSION OF SEX STEROID RECEPTORS, KI-67 AND CYTOKERATINS 7 AND 20 IN FELINE ENDOMETRIAL ADENOCARCINOMAS

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An immunohistochemical Study on the Expression of Sex Steroid Receptors, Ki-67 and Cytokeratins 7 and 20 in Feline Endometrial Adenocarcinomas

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Abstract

Background

Endometrial adenocarcinomas are a rare type of tumour in cats. Though different morphologies have been reported, the most frequent histological type of feline endometrial adenocarcinoma (FEA) is the papillary serous. Characterization of molecular markers expression in FEA may contribute to clarify the pathogenesis of these tumours and to assess the differences between normal endometrium and FEA regarding the expression pattern of several proteins. Therefore, this study aimed to evaluate the immunohistochemical profile of a wide panel of antibodies (specific for ER-α, PR, Ki-67, CK7 and CK20) in twenty-four cases of FEA. Comparisons were made between FEA and feline normal cyclic endometrium in follicular (n= 13) and luteal (n= 10) stages. Except for Ki-67, all other molecular markers were assessed independently for the intensity of immunolabeling and for the percentage of cells expressing the protein.

Results

This study showed that in FEA a loss of expression occurs, both in the proportion of labelled cells and in the intensity determined, both for ER ($P \leq 0.0001$) and less marked for PR ($P = 0.002$ and $P = 0.024$ for the percentage of positive cells and intensity of labelling, respectively). Proliferative activity, estimated via Ki-67 immunoreaction, significantly increased in FEA as compared to normal endometrium ($P \leq 0.0001$). Feline endometrial adenocarcinomas maintained the

CK7+/CK20+ status of normal endometrium. However, FEA showed decreased CK7 intensity of labelling compared to normal endometria ($P \leq 0.0001$) and lost CK20 expression, both in intensity ($P \leq 0.0001$) and in percentage of positive cells ($P = 0.01$), compared to normal tissues.

CONCLUSIONS

Data gathered in this study suggest that proliferation in FEA accompanies ER-α down-regulation, possibly following activation of pathways mediated by local growth factors. Moreover, FEA retains combined expression of CK7 and CK20, as evidenced in normal endometrial epithelia, although a decrease in CK7 expression was observed.

KEY-WORDS

Cat diseases; endometrium; adenocarcinoma; immunohistochemistry

BACKGROUND

Endometrial adenocarcinomas are a rare type of tumour in cats (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976). Uterine neoplasms account to 1 to 2% of the tumours affecting the queen's reproductive organs, representing 0.2 to 0.4% of all feline tumours (Johnston et al., 2001). Nevertheless, in recent years an increasing number of reports on feline endometrial adenocarcinomas (FEA) have been published (Anderson and Pratschke, 2011; Cho et al., 2011; Payan-Carreira et al., 2013; Saraiva et al., 2015; Sontas et al., 2013) suggesting that FEA may be more common than once believed.

Clinically, FEA are not distinguishable from other non-neoplastic diseases of the cat uterus, like pyometra, though they may have a completely different outcome, particularly in older females (Payan-Carreira et al., 2013; Pires et al., 2013).

Knowledge on FEA is very restricted and mostly originated from case descriptions, complemented with a few studies developed in a limited case series, supporting the need for additional studies in larger case series (Gil da Costa et al., 2009; Payan-Carreira et. al., 2013). Immunohistochemistry is an acknowledged well-established routine technique in anatomical pathology, very useful on account of its easiness, safety and inexpensiveness compared to other molecular techniques

(Millanta et al., 2005). Moreover, locating a protein in tissue sections may be helpful to study morphological characterization and potential behaviour of tumours.

The cyclic interchange of estrogens and progesterone secreted by the ovaries determines cyclic patterned changes in the mammalian endometrium – the endometrial cycle - with the ultimate goal of achieving a pregnancy. In the endometrium, major functions of circulating sex steroids are dependent on the estrogen and progesterone receptors (ER and PR). Particularly, these receptors mediate the continuous synchronized epithelial-stromal crosstalk that ultimately regulates the endometrial proliferation, differentiation and secretion and thereby promote embryo receptivity (Franco et al., 2012; Weihua et al., 2000). In general terms, estrogen stimulates the proliferation of both glandular and stromal cells, whereas progesterone inhibits the growth of glandular cells and stimulates the secretory activity in the endometrial glands. A disruption in the equilibrium of ER/PR (Olson et al., 2007), or mutations in the genes coding these molecules (Bender et al., 2011), may interfere with the normal proliferative or secretory patterns, and predispose to endometrial disease. Estrogen and progesterone receptors have been described in human endometrial carcinomas as independent prognostic factors (Trovik et al., 2013). Information on sex steroid receptors in feline endometrium is scarce. Furthermore, in FEA, available data concerning sex steroid receptors expression is limited and frequently opposed. In general, loss of ER-α has been reported, ranging from 50% (4/8 cases) (Miller et al., 2003) to 83.3% (5/6 cases) (Gil da Costa et al., 2009); one study also refers that PR are generally expressed in FEA (Gil da Costa et al., 2009). This may raise an important concern when sub-clinical FEA females are under progestagen contraceptive treatment, which could interfere with FEA progression and outcome. Moreover, the hormone receptors status of FEA may adjoin important information for medical management after ovariohysterectomy (OVH) (Gil da Costa et al., 2009), deserving additional studies.

In general, cancer development and progression is associated with deregulation of cell proliferation and of programmed cell death. The increased proliferative activity in a tumour is related to its growth rate, and may account for its malignancy and the clinical course of the disease. Thus, its assessment yields useful prognostic information related to survival of patients in various types of tumours (Kushi et al., 2002). Evaluation of the tumour proliferative activity is

frequently assessed by immunohistochemistry, using the expression of Ki-67 nuclear antigen (Dias Pereira et al., 2004), a nuclear non-histone protein present exclusively in proliferating cells, irrespectively of whether they are normal or neoplastic (Scholzen and Gerdes, 2000). Assessment of Ki-67 index has been applied to the normal endometrium, to characterize the cyclic changes in cell proliferation in mares (Aupperle et al., 2000), cows (Arai et al., 2013) and bitches (Van Cruchten et al., 2004). Furthermore, the immunohistochemical profile of Ki-67 has also been sporadically determined in feline endometrial lesions, including in two FEA case reports (Sontas et al., 2013) - in which Ki-67 varied from moderate to high - and in the description of multiple uterine lesions, which included an area of endometrial adenocarcinoma, that exhibited 40% of positive cells for Ki-67 (Sapierzynski et al., 2009).

Cytokeratins (CK) are the largest group of intermediary filaments proteins; they are essential in the development and differentiation of epithelial cells. They are also crucial for the normal structure and function of the epithelium, as CK are involved in signal transduction, cell polarity and gene regulation (Jasik, 2012); in addition, particular CK may also contribute to the epithelial innate defence mechanisms, through their antimicrobial properties (Tam et al., 2012). Cytokeratins are divided into two groups. Cytokeratin 20 is included in the type I CK, which are acidic, low molecular weight (40-56.5 kDa) proteins; whilst CK7 belongs to type II CK, that consist of basic, high molecular weight (52-67 kDa) proteins (Iwatsuki and Suda, 2010). Different types of epithelia show specific patterns of CK expression. In the uterus, CK are commonly found in the luminal and glandular epithelia (Jasik, 2012) and in the invading trophoblast (Maldonado-Estrada et al., 2004; Mühlhauser et al., 1995). Antibodies raised against CK are used as specific markers for epithelial cell differentiation and are largely used for tumour identification and classification (Thuróczy et al., 2009). The human uterine carcinoma presents a CK7+/CK20- phenotype (Jasik, 2012). Espinosa de los Monteros et al. (1999) strengthen the usefulness of the coordinate expression of CK7 and CK20 to distinguish different primary feline carcinomas and to ascertain its origin, in case of metastatic disease; they also described the normal pattern of these CK in feline normal endometrium. Cytokeratin 7+ / cytokeratin 20+ profiles were described in 2/3 (66.7%) FEA (Espinosa de los Monteros et al., 1999). In another study, 3/6 (50.0%) FEA

expressed CK7, whereas 4/6 (66.7%) FEA showed positive reaction for CK20 (Miller et al., 2003).

Most published case series studies on FEA used a small number of cases, ranging from three (Espinosa de los Monteros et al., 1999) to six (Gil da Costa et al., 2009) or eight (Miller et al., 2003), which might have contributed to the reported contrasting results.

Therefore, the objectives of this study were: 1) to evaluate the immunohistochemical expression of ER-α, PR, Ki-67, CK7 and CK20 in the papillary serous form of FEA using the largest case series reported so far; 2) to monitor the changes in the immunoexpression of these molecules as compared to the immunohistochemical profile of feline normal endometrium in two different stages of the estrous cycle; 3) to estimate putative associations between the molecular markers and the histopathological predictors of dedifferentiation; 4) to study the relationship between these markers.

Methods

Samples and Animals

The study was conducted in twenty-four samples of FEA retrieved from the archives of four different laboratories, during a period of 8 years. As controls, twenty-three archived samples of histologically normal feline uteri were selected (13 samples for the follicular stage – FS – and 10 samples for the luteal stage – LS). All samples were previously fixed in 4% neutral-buffered formalin and routinely processed for paraffin embedding.

Control uterine samples were obtained after elective ovariohysterectomy (OVH), from post-pubertal queens aged seven months to eight years of age (mean 1.5 years). Breed was unavailable in 65.3% of the records; on the other 34.7% records, represented breeds included Domestic Shorthaired cats (n= 7; 30.4%) and Persian (n= 1; 4.3%). For controls (normal endometria), only queens not submitted to contraceptive treatment were selected.

Feline endometrial adenocarcinomas were diagnosed in queens aged one year to 15 years of age (mean 7.9 years); breeds included Domestic Shorthaired cats (n= 17; 70.8%), Siamese (n= 2; 8.3%) and Persian (n= 1; 4.2%). Contraception was given in five (20.8%) animals and was denied in three (12.5%) FEA cases,

though the length of treatment was not mentioned in the form; no information existed in the request form for the remainder 16 cases (66.7%).

Regarding the clinical history of the animals diagnosed for FEA, data was collected from the histopathological request forms. The existence of clinical signs of uterine disease was mentioned in 11 (45.8%) cases, whilst in six (25.0%) other cases, the coexistence of pyometra and a concurrent mammary tumour were referred. FEA was diagnosed in two (8.3%) animals without acknowledge clinical symptoms, the lesions in the uterus being detected only during elective OVH as an enlarged organ with increased consistency. For all the other cases (n= 5; 20.8%), the reasons for OVH were not declared.

Morphological Evaluation

Feline endometrial adenocarcinomas diagnosis and the staging of the cycle stage in normal samples of healthy endometria (controls) were performed by light microscopy, on three-micrometres sections routinely stained with haematoxylin and eosin. The tumours were evaluated according to several criteria of malignancy described in the literature (Al Kushi et al., 2002; Goldschmidt et al., 2011; Horn et al., 2007), enabling the diagnosis of FEA of the papillary serous type (Saraiva et al., 2012). The histopathological features included: nuclear atypia, classified as low to moderate or high; mean number of mitoses per high power field (HPF), scored as lower than one, one to five and more than five; and the existence of myometrium, serosa or vascular / lymphatic invasion, evaluated as present or absent.

Normal uterine samples were staged as FS or LS based on the summative information gathered by the ovarian morphology (presence of follicles *vs.* corpora lutea), and the histological endometrial features (the epithelial cell height and the degree of development and coiling of endometrial glands). For patients diagnosed with FEA, determination of the stage of the estrous cycle was evaluated according to the presence of follicles in different stages of development – FS – or the presence of corpora lutea – LS – in the ovary.

For FEA cases, 11 cats (45.8%) were in FS and seven animals (25.0%) were in the LS of the estrous cycle; on the remaining cases (n = 7; 29.2%), the surgical specimen did not include the ovaries thus impairing the staging of the estrous cycle.

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Immunohistochemistry was performed in three-micrometre sections by the indirect avidin-biotin peroxidase complex technique. TABLE 1 summarizes the references of the antibodies used in this study, their dilution and incubating time. Antigen retrieval was performed in a steamer with slides immersed in boiling citrate buffer (pH 6.0; about 94 ºC) for 3 minutes. After cooling in phosphate buffered saline (PBS), the sections were immersed in 3% hydrogen peroxide during 20 minutes to block endogenous peroxidase activity. After the incubation with the normal serum for 5 minutes, the slides were incubated with the primary antibodies (TABLE 1) for an overnight period, in a humid chamber. Immunohistochemical labelling was achieved by using the products specified in TABLE 1, following the manufacturer's instructions. Colour was developed with 3,3-diaminobenzidine tetrahydrochloride and sections were counterstained with Gill's haematoxylin, dehydrated and mounted for evaluation on light microscopy.

TABLE 1: Primary antibodies used for immunohistochemistry.

ANTIBODY	CLONE	SOURCE	DILUTION	STAINING PATTERN	LABELLING
Monoclonal mouse anti-human estrogen receptor (ER)	ER-12	Cell Marque®, USA	1:40	Nuclear	Novolink® Polymer Detection System, (RE7280-K) Leica Biosystems®, UK
Monoclonal mouse anti-human progesterone receptor (PR)	1A6	Novocastra®, UK	1:30		
Monoclonal Mouse Anti-Human Ki-67 Antigen	MIB-1	Dako®, Denmark	1:50		
Clonal rabbit anti-human Cytokeratin 7 (CK7)	R17-S	DB Biotech®, Slovak Republic	1:100	Cytoplasm	Lab Vision® UltraVision® Large Volume Detection System, Thermo Fisher Scientific®, USA
Monoclonal mouse anti-human Keratin 20 (CK20)	Ks 20.8	Thermo Fisher Scientific®, USA	1:100		

Quantification of immunolabelling

In normal endometrium, the immunolabelling for ER-α, PR, Ki-67, CK7 and CK20 was evaluated independently in the surface epithelium (SE), superficial and deep glandular epithelium (SGE and DGE, respectively). In FEA the immunostaining was assessed in epithelial tumour cells. Stromal and myometrial labelling were evaluated independently for ER-α and PR in both normal and neoplastic epithelium. The intensity of ER-α and PR immunolabelling was graded as 0 = no staining, 1 = weak, 2 = moderate and 3 = strong. Regarding the percentage of cells expressing ER-α and PR, the negative cut-off was established at 5% (Ferrandina et al., 2005; Martín de las Mulas et al., 2000; Millanta et al., 2005; Mingzhu et al., 2014). Since the majority of the controls (n = 20/23) had more than 80% of positive cells, this was settled as the maximum cut-off. Therefore, the samples were further classified semiquantitatively according to the marks: 0 = negative (≤ 5 % positive nuclei); 1 = loss of expression (5 to 80% positive nuclei) and 2 = positive (≥ 80% positive nuclei). The evaluation of Ki-67 immunostaining was performed in 1000 cells in 10 HPF (x 400) and expressed as a percentage – proliferative index (Dias Pereira et al., 2004).

The immunoexpression for CK7 and 20 was semiquantitatively scored for both the percentage of labelled cells (1 to 33% = low; 34 to 66% = moderate; 67 to 100% = high) and the labelling intensity (1 = weak; 2 = moderate; 3 = strong) (Espinosa de los Monteros et al., 1999). This evaluation was performed for the entire endometrium section in controls and in representative microscopic fields for FEA. The labelling intensity was evaluated on the basis of the most frequently observed.

Statistical Analysis

Statistical comparisons were performed by using chi-square and Fisher exact tests in the IBM SPSS Statistics Base 20.0 software®. Ki-67 data were analysed using the ANOVA test, the post hoc paired comparisons were carried out using the Bonferroni correction. *P* values < 0.05 were regarded as statistically significant.

Results

Histopathological evaluation

In the present study, FEA were primarily characterized by the multi-layered proliferation of neoplastic endometrial epithelial cells on papillae into the lumen

supported by a thin fibrovascular stroma. Tubular and solid proliferation was scantly present. Therefore, tumours were histologically classified as FEA of the papillary serous type (FIGURE 1). Neoplastic cells were pleomorphic columnar shaped, with a moderate amount of eosinophilic cytoplasm and round-to-oval, vesicular or hyperchromatic nuclei that lost the normal polarity. Numerous multinucleated cells with darkened nuclei were present within and at the surface of the lesions. A variable number of clear cells - large, round to polygonal cells, with foamy cytoplasm and eccentric, crenated or ovoid nucleus – comprised less than 50% of the tumours' area. Nucleoli were evident; occasional intranuclear clear inclusions were also found. Randomly distributed areas of necrosis within the tumours were frequently present. A variable degree of atypia was found in FEA lesions (TABLE 2), with 54.2% (13/24) of the cases evidencing high atypia.

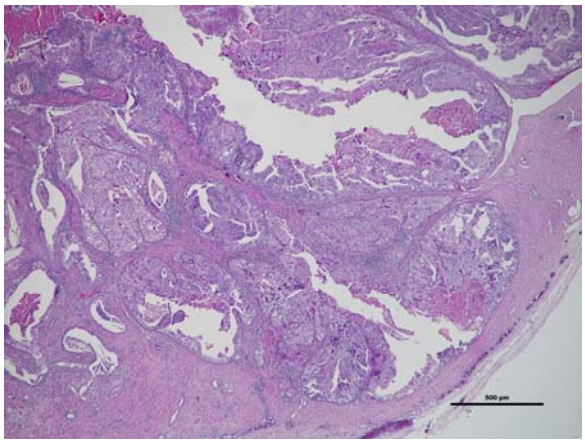

FIGURE 1: FEA. Papillary growth of epithelial tumour cells supported by thin fibrovascular stroma. Necrosis is observed at the lumen of neoplastic glands. At the right bottom, the tumour invades uterine myometrium. Haematoxylin and eosin. BAR = 500 µm.

The mean number of mitoses per HPF in FEA was higher, compared to normal epithelia ($P \leq 0.0001$). In the majority of FEA (n= 19; 79.2%), the mean number of mitoses per HPF was established between one and five, with very few cases (n= 2; 8.3%) presenting more than five mitoses per HPF (TABLE 2), in contrast with the recorded in the normal endometrium. The SE always presented less than one mitosis per HPF, both whether the FS and LS was considered (TABLE 2). The mean number of mitoses per HPF in the glandular epithelia was more variable, but the prevailing value was less than one for the SGE in 92.3% (12/13) to 100% (10/10) of the samples in FS and LS, respectively. The mean number of mitoses was similar

in the DGE in the FS (92.3%; 12/13), but slightly lower in the LS (70.0%; 7/10) (TABLE 2).

Myometrial invasion was observed in a large proportion of cases (66.7%; 16/24), while vascular invasion was observed in only 12.5% (3/24) of the cases; serosa impairment was only detected in 4.2% (1/24) FEA; though vascular and serosa invasion occurred independently in either situation myometrial invasion. In normal uterine samples, as expected, the anatomical integrity of myometrium and serosa layer was maintained.

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In the samples of normal endometrium, immunoreaction against ER-α and PR was consistently detected in all epithelial types, as well as in the stroma and myometrium (TABLE 3; FIGURE 2 A-B).

The intensity of labelling against ER-α was, in general, weaker in the SE than in the GE and the intensity scores were higher in the DGE than in SGE. However, the differences in the represented epithelia were significant for LS, but not for FS (P= 0.011 and P= 0.593, respectively; TABLE 3). The intensity of immunostaining for ER-α was slightly lower in the SE and SGE during LS in comparison to FS, but changes were more discrete regarding the DGE intensity of immunostaining. A moderate to strong intensity (scores 2 or 3) was evidenced in both the stroma and the epithelial elements in the normal endometrium for most samples (TABLE 3). A slight reduction was observed in the ER-α intensity for the endometrial stroma (TABLE 3). However, the stage of the cycle did not significantly affect the intensity of ER-α expression in stroma (P= 0.507). In the myometrium, the moderate intensity of ER-α staining prevailed in both stages, with no differences detected between the FS and LS (P= 0.363).

Regarding the percentage of positive cells for ER-α, all the represented endometrial epithelia expressed this marker during FS (score 2 for all the samples; TABLE 3). A slight reduction was observed in the percentage of labelled cells in the SE of LS, but not in the other endometrial epithelia (TABLE 3); still, the majority of samples retained a score 2 in the SE of LS, and the differences were non-significant. In FS, the number of cells ER-α positive in the endometrial stroma was more heterogeneous than in the endometrial epithelia, the scores ranging between 1 (5 to 80% of positive nuclei) and 2 (score 2 ≥ 80% of positive nuclei). The stromal

compartment in LS showed a reduction in the number of ER-α positive cells as compared to the FS, but these changes were not significant (*P*= 0.669). The myometrium was consistently positive to ER-α expression, independently of the stage of the cycle (TABLE 3).

In what regards the ER-α immunoreaction in FEA, a marked decrease in the intensity and the percentage of labelled cells was recorded for both the epithelium and the stromal compartments (FIGURE 2 C). In FEA, around 70% of the samples presented a score ≤ 1 for epithelial ER-α intensity, considerably lower than any epithelia in either the FS or LS normal endometrium (*P*≤ 0.0001; TABLE 3). Simultaneously, a marked decrease in the percentage of positive cells to ER-α in the neoplastic epithelium was observed as compared to normal endometrium epithelia (*P*≤ 0.0001): in FEA, half the samples showed positive nuclei in less than 5% of the cells, with 45.8% of the cases displaying ER-α positive nuclei in less than 80% of the cells (TABLE 3). Similarly, a loss in the overall expression of ER-α, both in intensity of the immunoreaction and the number of cells with positive nuclei, was observed in FEA stromal compartment compared to the normal endometrial stroma (*P*≤ 0.0001; TABLE 3). Tumours characterized by myometrial invasion were more likely to be negative for ER-α in the stromal compartment (*P*= 0.033). The percentage of ER-α positive smooth muscle cells in the FEA myometrium was considerably lower than in the normal endometrium (*P*≤ 0.0001); notwithstanding, the intensity of the ER-α immunoreaction did not change between normal endometrium and FEA (*P*= 0.153).

In general, the intensity of PR immunoreaction was similar between all the represented endometrial epithelia (TABLE 3). In the FS a moderate intensity prevailed over the strong intensity of immunolabeling, particularly in the SE and SGE (Table 3). The intensity of immunostaining showed a slight increase during the LS in all the represented epithelia, in particular in the DGE, but these changes were devoid of significance. The immunoreaction in the stromal compartment was more heterogeneous than the observed for ER-α. Higher scores were recorded in the FS; in LS it was observed a marked loss in the intensity of immunostaining (*P*= 0.010; Table 3). Contrasting, lower scores for PR intensity of immunostaining were observed in myometrium in FS compared to LS, but in the latter a wider variation of intensity scores was obtained. However, the differences among FS and LS were non-significant for this layer (*P*= 0.141).

TABLE 2: Main histological features of feline endometrial adenocarcinomas in comparison to the normal endometrium.

		Atypia n (%)			Number of mitoses HPF n (%)			Myometrial invasion n (%)	Serosa invasion n (%)	Vascular / lymphatic invasion n (%)
		Absent	Low to Moderate	High	≤ 1	1-5	≥ 5			
FS	SE	13 (100.0)	0	0	13 (100.0)	0	0	0	0	0
	SGE	13 (100.0)	0	0	12 (92.3)	1 (7.7)	0	0	0	0
	DGE	13 (100.0)	0	0	12 (92.3)	1 (7.7)	0	0	0	0
LS	SE	10 (100.0)	0	0	10 (100.0)	0	0	0	0	0
	SGE	10 (100.0)	0	0	10 (100.0)	0	0	0	0	0
	DGE	10 (100.0)	0	0	7 (70.0)	3 (30.0)	0	0	0	0
FEA		0	11 (45.8)	13 (54.2)	3 (12.5)	19 (79.2)	2 (8.3)	16 (66.7)	1 (4.2)	3 (12.5)

FS = follicular stage; LS = luteal stage; FEA = feline endometrial adenocarcinomas; SE = surface endometrium; SGE = superficial glandular epithelium; DGE = deep glandular epithelium; HPF = high power field.

TABLE 3: Results for the immunoexpression of the ER-α and PR in the normal feline uterus (at the FS and LS) and in the neoplastic epithelium in FEA.

	Scores	Intensity n (%)						Scores	Percentage of positive cells n (%)					
		ER-α			PR				ER-α			PR		
		FS	LS	FEA	FS	LS	FEA		FS	LS	FEA	FS	LS	FEA
SE	0	0	0		0	0		0	0	0		0	0	
	1	0	3 (30.0)		1 (7.7)	0		1	0	2 (20.0)		0	1 (10.0)	
	2	7 (53.8)	5 (50.0)		7 (53.8)	5 (50.0)		2	13 (100.0)	8 (80.0)		13 (100.0)	9 (90.0)	
	3	6 (46.2)	2 (20.0)		5 (38.5)	5 (50.0)								
SGE	0	0	0		0	0		0	0	0		0	0	
	1	0	0		1 (7.7)	0		1	0	0		0	1 (10.0)	
	2	3 (23.1)	6 (60.0)		7 (61.5)	5 (50.0)		2	13 (100.0)	10 (100.0)		13 (100.0)	9 (90.0)	
	3	10 (76.9)	4 (40.0)		5 (30.8)	5 (50.0)								
DGE	0	0	0		0	0		0	0	0		0	0	
	1	0	0		2 (15.4)	1 (10.0)		1	0	0		0	1 (10.0)	
	2	1 (7.7)	0		5 (38.5)	3 (30.0)		2	13 (100.0)	10 (100.0)		13 (100.0)	9 (90.0)	
	3	12 (92.3)	10 (100.0)		6 (46.2)	6 (60.0)								
FEA EPITHELIUM	0			12 (50.0)			0	0			12 (50.0)			0
	1			5 (20.8)			1 (4.2)	1			11 (45.8)			0
	2			6 (25.0)			19 (79.2)	2			1 (4.2)			13 (100.0)
	3			1 (4.2)			4 (16.7)							
STROMA	0	0	0	11 (45.8)	0	4 (40.0)	2 (8.3)	0	0	0	11 (45.8)	0	4 (40.0)	2 (8.3)
	1	0	1 (10.0)	2 (8.3)	1 (7.7)	1 (10.0)	1 (4.2)	1	7 (53.8)	7 (70.0)	13 (54.2)	10 (76.9)	6 (60.0)	21 (87.5)
	2	4 (30.8)	4 (40.0)	8 (33.3)	10 (76.9)	2 (20.0)	19 (79.2)	2	6 (46.2)	3 (30.0)	0	3 (23.1)	0	1 (4.2)
	3	9 (69.2)	5 (50.0)	3 (12.5)	2 (15.4)	3 (30.0)	2 (8.3)							
MYOMETRIUM	0	0	0	4 (16.7)	0	1 (10.0)	0	0	0	0	4 (16.7)	0	1 (10.0)	0
	1	0	1 (10.0)	0	5 (38.5)	4 (40.0)	4 (16.7)	1	0	0	9 (37.5)	0	2 (20.0)	6 (25.0)
	2	12 (92.3)	7 (70.0)	18 (75.0)	8 (61.5)	3 (30.0)	18 (75.0)	2	13 (100.0)	10 (100.0)	11 (45.8)	13 (100.0)	7 (70.0)	18 (75.0)
	3	1 (7.7)	2 (20.0)	2 (8.3)	0	2 (20.0)	2 (8.3)							

SE = surface endometrium; SGE = superficial glandular epithelium; DGE = deep glandular epithelium; FS = follicular stage; LS = luteal stage.). Intensity of immunolabelling: 1 = weak, 2 = moderate and 3 = strong. Percentage of positive nuclei: 0 = negative (≤ 5 % positive nuclei); 1 = loss of expression (5-80% positive nuclei) and 2 = positive (≥ 80% positive nuclei).

Progesterone receptors were consistently positive in all evaluated epithelia during the FS; in comparison, a slight non-significant decrease in the percentage of PR positive cells was observed in LS (*P*= 0.435; TABLE 3). The percentage of PR positive cells in the stromal compartment was higher in the FS than in the LS (*P*= 0.027; Table 3). However, score 1 (5 to 80% of positive nuclei) was the most prevalent in both stages. A small non-significant reduction in the percentage of PR positive cells was observed also in myometrium during the LS, compared to FS (*P*= 0.070; TABLE 3).

Compared to the normal endometrium, FEA displayed more discrete differences in what concerns PR expression than those presented for ER-α, both at the epithelial and the stroma compartment (TABLE 3; FIGURE 2 D). Feline endometrial adenocarcinomas epithelium showed a significant reduction in either the percentage of PR positive of cells (*P*= 0.002) and the intensity of immunolabeling (*P*= 0.024). Albeit a small decrease in the intensity and the percentage of cells with positive nuclei in both FEA stroma and myometrium compared to normal endometrium, the differences in PR expression in these compartments were devoid of significance (TABLE 3).

No association between ER-α and PR expression was found in the epithelial or stromal compartments of normal or neoplastic endometria. Also, we did not find a significant association between hormone receptor status and the stage of the estrous cycle in FEA.

The proliferative indexes, as estimated by Ki-67 counting, were similar between FS and LS (*P* > 0.05). In FS, the proliferative indexes were higher for SGE (16.7±5.4) as compared to SE and DGE (9.0±2.6 and 7.3±2.8, respectively). In LS, the proliferative indexes were higher for the glandular epithelia, particularly for the DGE (21.5±10.0 *vs.* 13.0±5.2 in SGE), than for the SE (7.1± 2.1). Considering the epithelia as a whole, the mean proliferative indexes were 11.0±2.3 and 13.9±3.8 in FS and LS, respectively. The proliferative index was considerably higher in the neoplastic epithelium (42.9±3.8) than in normal endometrial epithelia in FS (95% CI= 20.9 – 42.9) or LS (95% CI= 17.3 – 40.8) (FIGURE 3). Ki-67 expression was independent of the tested clinicopathological features analysed as an indication of tumour aggressiveness and of the hormonal receptor status.

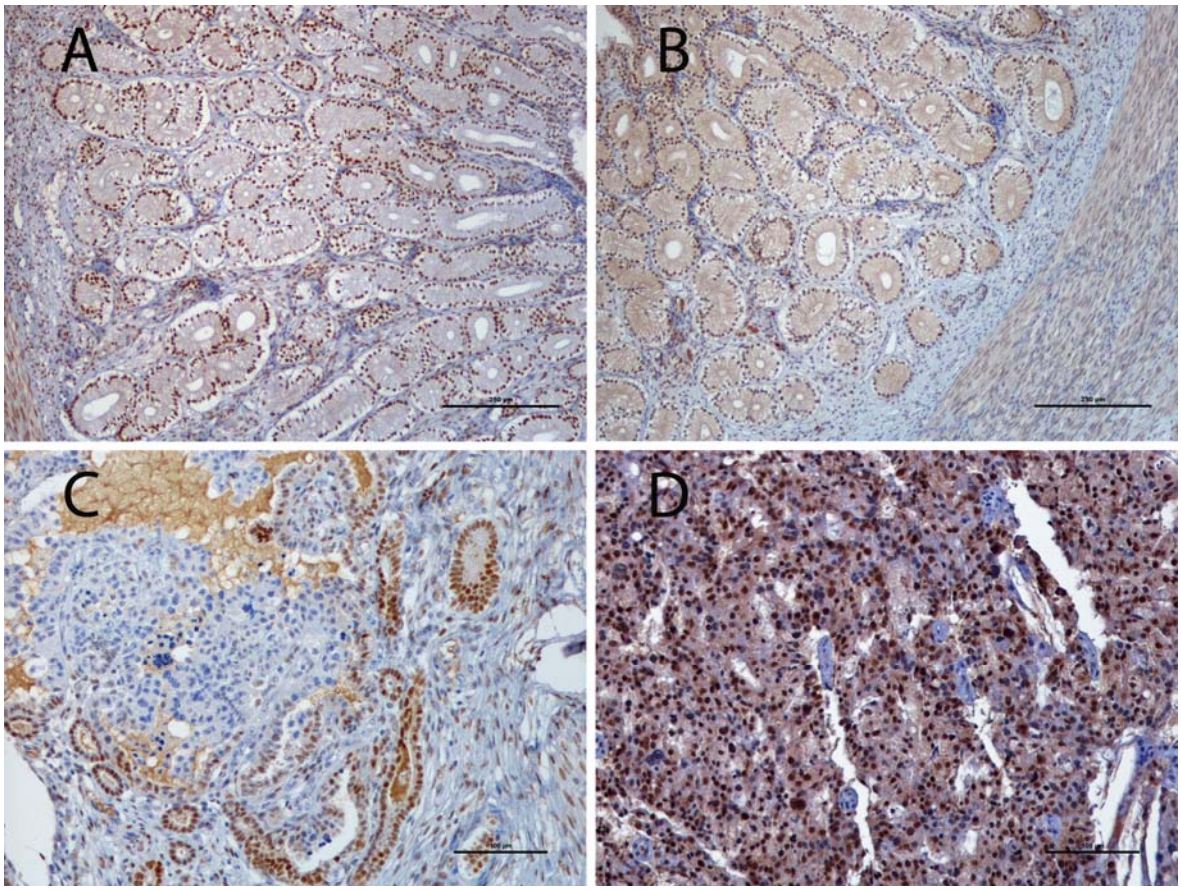

FIGURE 2: ER-α and PR immunohistochemical expression in feline normal and neoplastic endometrium. A. ER-α and B. PR in the LS of cyclic endometrium are expressed in all uterine layers. BAR = 250 µm C. ER-α expression is decreased in FEA. Transition between positive normal glands and negative neoplastic cells are notorious. BAR =100 µm. D. PR expression is maintained in FEA. BAR =100 µm. Counterstained with Gill´s haematoxylin.

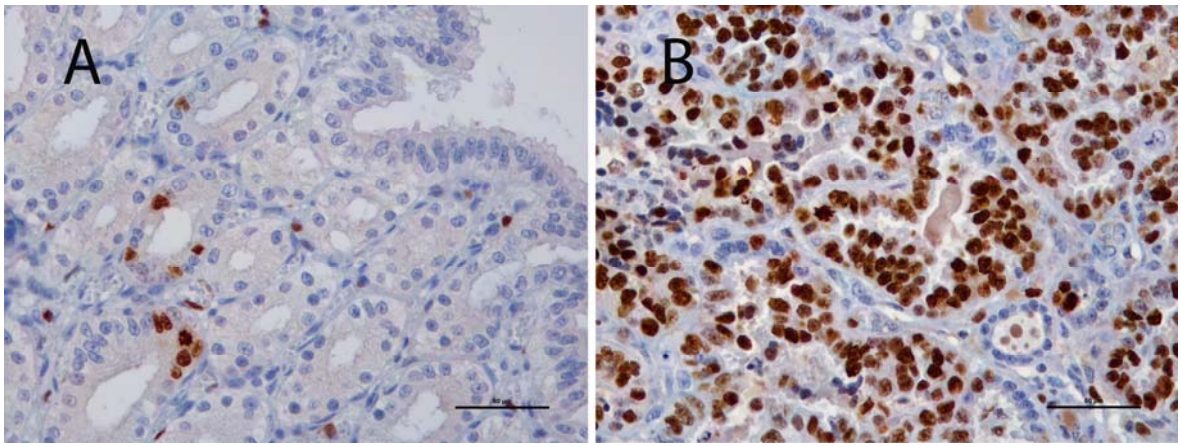

FIGURE 3: Ki-67 immunohistochemical expression in feline endometrium. A. Normal endometrium at the LS shows scarcely positive c ells. A positive cell in metaphase is positive to Ki-67 antigen at the lower bottom. B. FEA are largely positive to Ki-67 antigen. BAR = 50 µm. Counterstained with Gill´s haematoxylin.

Normal feline endometrium presented a CK7+/CK20+ immunoprofile (FIGURE 4 A-B). The SE presented a strong intensity of immunoreaction against CK7, which did not change with the stage of the estrous cycle (TABLE 4). The intensity of CK7 immunolabeling differed between the SGE and the DGE, according to the stage of the cycle. A strong intensity of immunolabelling prevailed in the SGE and in the DGE in FS, but a decrease in the labelling intensity for this molecule was observed in both epithelia during the LS (P= 0.04 and P= 0.039, respectively for SGE and DGE; TABLE 4), whereby the most prevalent intensity of labelling was the moderate. Cytokeratin 7 was consistently detected by all the epithelia represented in the endometrium, independently of the stage of estrous cycle (TABLE 4).

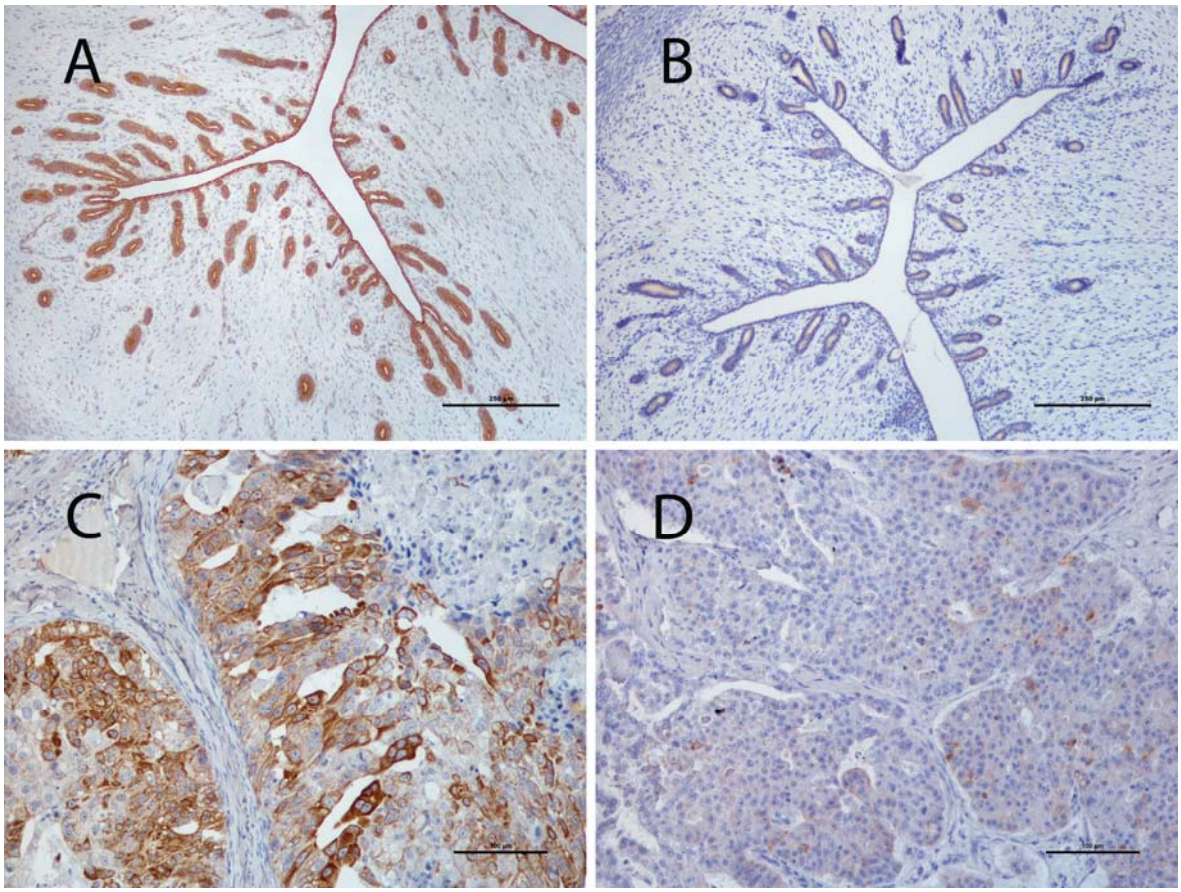

FIGURE 4: CK7 and CK20 immunohistochemical expression in feline endometrium. A. CK7 is strongly expressed in all epithelia of normal endometrium in FS. Contrasting, B. CK20 is expressed with a low intensity of labelling. BAR = 250 µm. C. FEA displays a maintenance of CK7+ expression although with a heterogeneous positivity D. CK20+ immunophenotype in FEA shows a decreased expression and a scarcely, heterogeneous positivity. BAR = 100 µm. Counterstained with Gill´s haematoxylin.

The stage of the cycle affected the intensity of immunoexpression for CK20. The intensity of immunostaining most often recorded in FS was the moderate, the DGE presenting a slightly increased immunostaining as compared with the SE and the SGE (TABLE 4). In LS, a shift towards stronger intensities was observed in the SE (P= 0.002) and in the SGE (P= 0.045), but not in the DGE (TABLE 4). As for the percentage of positive CK20 cells, similar scores were observed in FS and LS, despite the small non-significant decrease observed in the surface epithelium in the LS (P= 0.178; TABLE 4).

In FEA the CK7+/CK20+ epithelial immunoprofile was maintained (FIGURE 4 C-D). However, a heterogeneous, patchy immunolabelling was observed for both CK in the neoplastic epithelium. A loss in CK7 expression intensity was observed in FEA as compared to the normal endometrial epithelium ($P\leq$ 0.0001), whilst the percentage of CK7 positive cells remained practically unchanged (P= 0.065; TABLE 4). Similarly, CK20 was also lost in FEA as compared to the normal endometrium, both in terms of the percentage of positive cells and the intensity of labelling ($P\leq$ 0.0001 and P= 0.01, respectively; TABLE 4). No relation was established between CK7 and CK20 status in epithelial cells.

The comparison between the immunohistochemical results and the available clinicopathological data suggested that the myometrial invasion observed in FEA was associated with negative stromal ER-α status (P= 0.033 and P= 0.006, respectively for percentage of positive cells and intensity of immunolabelling) and with a higher percentage of CK20-positive cells (P= 0.033). In tumours, nuclear atypia was related to a lower intensity of CK7 labelling (P= 0.026). The loss of PR positive cells in the myometrium in FEA was related to a higher nuclear atypia in carcinoma cells (P= 0.016).

DISCUSSION

Despite the rarity of FEA described in the literature (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976), a recent increasing number of reports suggest that the prevalence of these tumours may be underestimated (Anderson and Pratschke, 2011; Cho et al., 2011; Payan-Carreira et al., 2013; Sontas et al., 2013).

TABLE 4: Results for the CK 7 and 20 immunolabelling in the epithelial cells from normal feline endometrium (at the FS and LS) and in FEA.

			SE SCORING N (%)			SGE SCORING N (%)			DGE SCORING N (%)		
			LOW	MODERATE	HIGH	LOW	MODERATE	HIGH	LOW	MODERATE	HIGH
INTENSITY	CK7	FS	0	0	13 (100.0)	0	3 (23.1)	10 (76.9)	0	2 (15.4)	11 (84.6)
		LS	0	0	10 (100.0)	0	7 (70.0)	3 (30.0)	0	6 (60.0)	4 (40.0)
		FEA	6 (25.0)	14 (58.3)	4 (16.7)						
	CK20	FS	4 (30.8)	9 (69.2)	0	2 (15.4)	11 (84.6)	0	1 (7.7)	10 (76.9)	2 (15.4)
		LS	0	0	10 (100.0)	1 (10.0)	5 (50.0)	4 (40.0)	3 (30.0)	7 (70.0)	0
		FEA	8 (33.3)	8 (33.3)	8 (33.3)						
			1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
PERCENTAGE OF POSITIVE CELLS	CK7	FS	0	0	13 (100.0)	0	0	13 (100.0)	0	0	13 (100.0)
		LS	0	0	10 (100.0)	0	0	10 (100.0)	0	0	10 (100.0)
		FEA	0	2 (8.3)	22 (91.7)						
	CK20	FS	0	0	13 (100.0)	0	0	13 (100.0)	0	0	13 (100.0)
		LS	0	2 (20.0)	8 (80.0)	0	0	10 (100.0)	0	0	10 (100.0)
		FEA	0	13 (54.2)	11 (45.8)						

SE = surface endometrium; SGE = superficial glandular epithelium; DGE = deep glandular epithelium; FS = follicular stage; LS = luteal stage. Intensity of immunolabelling: 1 = weak, 2 = moderate and 3 = strong.

Moreover, in one study on uterine tumours in domestic cats, FEA was the most commonly diagnosed neoplasm (Miller et al., 2003). The selection of cases diagnosed as FEA from the archives of four different laboratories allowed the use of a larger series than usual. Its architecture and the histological features of neoplasic epithelial cells classified the cases under papillary serous type, the most frequent type of FEA (Saraiva et al., 2012). Herein, we describe an immunohistochemical panel performed on FEA to gather helpful information regarding its diagnosis and management, as well as to drive upcoming areas for study on FEA.

Estrogen receptor alpha and PR expression was found in epithelial and stromal endometrial compartments and in myometrium of normal feline uteri. Information on ER-α and PR expression in normal healthy endometrium of this species is very limited, and mostly based on the work of Li et al. (1992) that mimicked the ovarian steroids effects on the feline uterus through the scheduled administration of exogenous estrogens and progesterone (Li et al., 1992). However, changes in the intensity of immunostaining of ER-α from FS to LS followed the expected physiological modifications, evidencing a small decrease in the LS suggestive of the suppressive effect of progesterone receptor activation. Changes were more notorious in the surface epithelium and the superficial glandular epithelia than in deep glandular epithelium; this may be related with to the increased branching of upper endometrial glands in FS and to the persistency of proliferation of basal glands during the LS, that is reflected in the increased coiling reported in this stage (Chatdarong et al., 2005), which was supported by data gathered by Ki-67 immunolabeling.

Progesterone receptor expression in epithelial cells from normal endometrium showed a small decrease from the FS to LS, following an expectable physiological pattern. The high heterogeneity of the intensity scores presented by the different samples might relate to individual differences in the moment of the LS or the blood levels of progesterone, which were not assessed in the present study. The unavailability of information concerning the normal PR expression in cat endometrium limits the interpretation of the decrease in the PR stromal expression during LS. However, it is possible that, in line with other species, stromal and epithelial compartments of the endometrium may respond differently to steroid hormones (Aupperle et al., 2000). In addition, since endometrial stroma and epithelium influence each other proliferation and differentiation (Pierro et al., 2001),

differences in either compartment responses to sex steroids may be necessary to the normal interplay through the uterine cycle.

Recently, a consensus was proposed on the standard guidelines for hormone receptor assessment using immunohistochemistry for canine mammary tumours (Peña et al., 2014). However, no guidelines exist for feline mammary or uterine neoplasms. Consequently, the cases used in the present study were evaluated on the basis of the results obtained in controls, using the negative cut-off established before for feline mammary tumours and human endometrial carcinomas (Ferrandina et al., 2005; Martín de las Mulas et al., 2000; Millanta et al., 2005; Mingzhu et al., 2014).

All FEA analysed herein lost ER-α immunoexpression in comparison to the normal endometrium. Moreover, the tumours were negative for epithelial and for stromal expression of ER-α (respectively in 50.0% and 45.8% of the samples).

The role of estrogen receptors in the regulation of mammalian endometrium, particularly the endometrial proliferation, remains unclear. Uterine proliferation seems to depend on ER mediated transcription, which may result from either the ligand ER-α activation (associated to estrogen stimulation) or a ligand-independent pathway (Hewitt and Korach, 2002). Activation of the ER-α drives the transactivation of numerous growth factors, which in turn activate their cognate receptors, leading to multiple signalling cascades controlling cellular proliferation. Estrogen receptor alpha may be induced in estrogen-driven tumours, and tumour growth is often limited by progesterone, once ER expression is down-regulated by activated PR (Leslie et al., 2013). However, proliferation in a tumour may occur driven by the constitutive activation of a parallel growth factor pathway. In that case, proliferation would not depend on the presence of estrogen and progesterone.

Down-regulation of ER-α expression in a variety of tissues has been associated to methylation and to loss of transcriptional activators (Hayashi et al., 2003; Pinzone et al., 2004) or to transdominance by ER-β which has an anti-proliferative role (Martinkovich et al., 2014; Weihua et al., 2000). These would explain the loss of ER expression and the acquisition of a hormone resistance status (Pinzone et al., 2004), often associated with high-grade adenocarcinoma developing in the uterus. Such independence from the sex steroid control is considered a negative indicator for the clinical outcome (Leslie et al., 2013).

It was shown that in the endometrium, the loss of ER-α compromises E2-induced vascular endothelial growth factor (VEGF) expression in epithelial cells, shifting VEGF production to stromal cells thereby inducing stroma-mediated epithelial cell proliferation (Koos, 2011). Data from the present study suggest that FEA may enter the category of tumours evolving in the absence or reduced expression of activated ER-α, thus highlighting the need to address in future studies the presence of local growth factors associated to proliferation, including insulin-like growth factor 1 (IGF-I) and VEGF. The proliferative index observed in neoplastic epithelium is considerably increased compared to normal, healthy endometrial epithelia, supporting the hypothesis that additional molecules other than the ER- β are involved in the regulation of the proliferative pathways in FEA. In the uterus ER-β has been described as a proliferation controller, while in other organs, such the mammary gland and the prostate, ER- β plays a pro-differentiating role (Morani et al., 2008).

As referred before, a different situation was found concerning PR. Although the tumours also lost PR expression, this was non-significant in both intensity and percentage of labelled cells for the stroma, contrary to the epithelium. Therefore, endometrial cells in FEA retain the ability to respond to progesterone stimulation, but show a reduced ability to respond to estrogens.

The expression of hormone receptors in FEA is still poorly understood, contrasting to the well-studied hormone status in human endometrial carcinomas. It has been recently proposed that the expression of PR changes during tumour progression in endometrial adenocarcinoma (Tangen et al., 2014). Several mechanisms for progesterone inhibition of endometrial proliferation have been proposed, including inhibition of proliferation through opposing the proliferative effects of estrogen in normal endometrium, which is generally associated with down-regulation of ER-α actions (Tangen et al., 2014) and up-regulation of ER-β, in a manner that is progesterone dose-related (Tessier et al., 2000).

Data on sex steroid receptors obtained in the present study share some resemblance to results from earlier studies in rabbit endometrial adenocarcinomas. Likewise cats, rabbits are an induced ovulation species, but, in contrast to cats, rabbits frequently develop endometrial adenocarcinomas, which present two main histological types: papillary adenocarcinoma and tubular/solid adenocarcinomas. In that species, papillary adenocarcinomas are negative for ER-α and PR (Asakawa et

al., 2008) and Vinci and collaborators (2010) concluded that PR expression was not directly involved in endometrial epithelial carcinogenesis and that such expression was not of prognostic value (Vinci et al., 2010). On the other hand, in women ER and PR have been established as prognostic markers for endometrial neoplasms (Tangen et al., 2014; Trovik et al., 2013). Also, loss of ER-α and PR is associated with markers of aggressiveness such as age, myometrial infiltration and lymph node status (Ferrandina et al., 2005; Tangen et al., 2014). Interestingly, in FEA, the negative status for ER-α expression in the stromal cells was associated with myometrial invasion. Our results suggest that loss of ER-α in FEA may be related to invasive characteristics of the tumour, and further strengthen the need for additional studies on the putative influence of growth factors acting over the proliferation pathways. In women, it was recently proposed that reduced expression of ER-α and PR-A, particularly in neoplastic stromal cells, may be of utmost importance in predicting invasiveness (Kreizman-Shefer et al., 2014). Additional studies are needed to ascertain this hypothesis in FEA.

Although in endometrial carcinomas of women, ER and PR show significant correlation (Ferrandina et al., 2005; Mingzhu et al., 2014), we did not fund such association in cats. Interestingly, we found that loss of positive cells for PR in the myometrium of FEA was related to a higher nuclear atypia in carcinoma cells. Recently, Tomica et al. (2014) observed lower levels of PR than ER in the myometrium of high-risk human endometrial carcinomas (Tomica et al., 2014). Also, a relation between cancer cells and the surrounding tissues has been proposed as a necessary event for endometrial normal functioning and carcinogenesis (Yang et al., 2011). Our results suggest that myometrial expression of PR may be related to tumour dedifferentiation and that myometrium may crosstalk with epithelial and stromal compartments during tumour progression.

The expression of hormonal receptors is usually used in humans to provide important information for adjuvant hormonal therapy in steroid-responsive tumours. In women, potential effectiveness of hormonal therapy is dependent upon the patient selection based on positive receptor status (Carlson et al., 2014). Thus, our study sheds light into whether or not the medical treatment would be of choice for most animals with endometrial carcinomas. Nevertheless, it would be of interest to rely on the expression of sex steroids receptors to predict metastasis development.

We strongly recommend that the hormonal receptor status of FEA should be determined by the time of histopathological diagnosis.

To the best of our knowledge, this is the first study on Ki-67 (clone MIB-1) expression in feline cyclic and neoplastic endometrium. In normal endometrium, proliferative indexes were higher in the SGE during the FS, while in the LS the higher proliferative indices were found in the DGE. These findings are in agreement with the morphological features that characterize the cycle of glandular development in the species (respectively the branching of the upper area of the glands in the FS and the coiling of the basal glandular area during LS) (Chatdarong et al., 2005). In FEA, the proliferative index was remarkably higher than in normal endometrial epithelia, alike the reported in humans (Kreizman-Shefer et al., 2014). Ki-67 is widely used to assess proliferative activity. In human endometrial carcinomas its expression correlates with the histological grade, depth of myometrium invasion and risk of carcinoma recurrence (Prat, 2004). Also in women, Ki-67 status is inversely related to hormonal receptor status, particularly in higher grade, ER-α negative, endometrial carcinomas (Ferrandina et al., 2005; Mingzhu et al., 2014; Tangen et al., 2014). In this particular feature, FEA may be included in the group of endometrial neoplasias with high proliferation indexes, but with reduced or null expression of ER-α. As discussed before, this is suggestive of the existence of alternative pathways controlling the proliferation in FEA, which needs to be explored in future studies.

Cytokeratins, the largest group of intermediary filament proteins, are an important partner in the renewal and repair of epithelia, to whom they provide rigidity and strength to cell cytoskeleton, by providing flexibility (Windoffer et al., 2011). Further, CK represent important differentiation markers for different types of epithelia and epithelial tumours (Thuróczy et al., 2009). Particularly, the coordinate expression of CK7 and CK20 defines unique subsets of carcinomas (Jasik, 2012). Different types of epithelia show specific patterns of CK expression; CK7 and CK20 are often named as “ductal-type” keratins (Moll et al., 2008). Limited knowledge exists on the normal pattern of CK7 and CK20 expression in domestic mammal endometrium. The present study showed that normal endometrial epithelia in cats present a profile CK7+/CK20+ that shows cyclic variation. For CK7, the intensity of immunolabeling remained unchanged for the surface epithelium, despite the decrease observed in the intensity of the glandular epithelia immunolabeling. As for

CK20, an increase in the intensity of immunostaining was observed in the LS. Moreover, the overall expression of CK7 was higher compared to that of CK20.

Immunohistochemical expression of CK7 and CK20 has been used in the differentiation of human primary and metastatic tumours of unknown origin (Chu et al., 2000; Moll et al., 2008; Shin et al., 2010). Cytokeratin 7 and CK20 are generally confined to epithelia and cell profile for those CK is largely conserved during malignant transformation (reviewed by Campbell and Herrington, 2001; Moll et al., 2008). Cytokeratin 7 and 20 are potentially potent epithelial differentiation and tumour markers (Moll et al., 2008) in human and domestic animals. The association CK7+/CK20- is used in humans to prove the endometrial origin of tumours (Chu et al., 2000; Moll et al., 2008).

Results from the present study also show that FEA retain a CK7+/CK20+ phenotype, despite the decrease observed in CK7 expression and a more heterogeneous intensity of labelling on regards to CK20. These findings corroborate previous reports in smaller case series (Espinosa de los Monteros et al., 1999). Notably, conflicting reports exist on the expression of CK7 and CK20 in FEA: Miller et al. 2003, described 3/6 and 4/6 positive FEA respectively for CK7 and CK20 (Miller et al., 2003). It has been suggested that CK20 may play a role in facilitating cytoskeleton breakdown and related keratin filament reorganization (Zhou et al., 2006). Furthermore, the loss of expression of CK20 has been associated to cell dedifferentiation (Moll et al., 2008). Myometrial invasion and atypia – histological features commonly associated to invasiveness - were related to a higher percentage of positive cells for CK20 and a lower intensity in CK7 labelling, respectively. Cytokeratins are also involved in multiple signalling pathways beyond their mechanical functions, among epithelial cells or between the epithelial and mesenchymal compartments (Moll et al., 2008). These widely complex mechanisms may be related to our findings, but currently we cannot conclude on the putative role of CK in FEA dedifferentiation and invasiveness. Our sample comprised six (25.0%) animals with clinical history of mammary gland tumour. One should be aware of the possibility of uterine metastasis of mammary gland adenocarcinomas. Moreover, morphological features of both tumours may be indistinguishable. Unlike primary FEA, feline mammary gland carcinomas are generally negative for CK20, maintaining the immunophenotype of the normal mammary gland in this species (Espinosa de los Monteros et al., 1999). Therefore, CK20 may be helpful to

distinguish between endometrial primary adenocarcinoma and a metastatic carcinoma in the uterus, as the pattern of expression of CK20 in different carcinomas is preserved in metastasis (Moll et al., 1992). Altogether, CK20 might be an important marker for FEA diagnosis in cases of concomitant mammary carcinomas, since CK7 was previously demonstrated in 50% of feline mammary gland (Espinosa de los Monteros et al., 1999). The results presented herein confirm the positivity of FEA for CK7 reported by Espinosa de los Monteros and co-workers (1999). Thus, CK7 profile does not seem valuable in differential diagnosis of FEA and a uterine metastasis of a mammary gland tumour.

Conclusions

Our results show that FEA have a self-hormonal status, different from that observed in normal endometrium. Their loss of expression of ER-α in all endometrial compartments (epithelium and stroma) as well as in myometrium, while retaining PR expression in stroma and myometrium suggests that epithelial proliferation may be determined by alternative pathways possibly involving local growth factors. As expected, proliferative index assessed by Ki-67 immunoreaction is higher in FEA than in normal endometrium.

Importantly, CK20 is regarded herein as a potentially powerful marker for the diagnosis of primary FEA, enabling to differentiate FEA from metastatic disease from mammary gland. Although other molecular studies are indicated to support our findings, determination of the immunohistochemical CK20 profile of uterine tumours in cats may be of utmost importance in the diagnostic routine.

In the present study, we highlight the importance of evaluation both epithelial, stromal and myometrial cells in neoplastic endometrium, comparing such results with normal controls. These compartments are likely to respond in a different way to overall hormonal environment and probably interact with each other. These mechanisms remain unclear and further studies must be performed to clarify these hypotheses.

With this study, we have unveiled some of the molecular events likely involved in feline endometrium carcinogenesis. This will certainly ascertain tumour morphological characterization. Future studies are needed in order to establish clinical outcome of FEA.

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AUTHOR CONTRIBUTIONS

ALS, RP-C, FG and MAP were involved in the acquisition of clinical and histopathological data, data analysis and interpretation, as well as the manuscript writing and the reviewing of the literature. MAP, ALS and MFC were responsible for the immunohistochemical analysis. MFC, AR, FF and LML were responsible for technical assistance. All authors read and approved the final manuscript.

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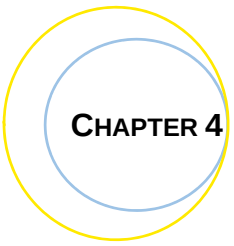

CHAPTER 4

IMMUNOHISTOCHEMICAL EXPRESSION OF CYCLOOXYGENASE-2 (COX-2) IN FELINE ENDOMETRIAL ADENOCARCINOMA AND IN NORMAL AND HYPERPLASTIC ENDOMETRIA

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Immunohistochemical Expression of Cyclooxygenase-2 (COX-2) in Feline Endometrial Adenocarcinoma and in Normal and Hyperplastic Endometria

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Abridged Title: COX-2 expression in feline endometrium

Contents

Cyclooxygenase-2 (COX-2) is overexpressed in several human and animal neoplasms, including the human endometrial carcinoma. It has been suggested as a prognostic marker and a potential therapeutic target. This study aimed to (1) clarify histological aspects of feline endometrial adenocarcinomas (FEA) of the papillary serous type and (2) characterize COX-2 immunohistochemical expression in normal, hyperplastic and neoplastic endometrium in this species. Archived paraffin embedded tissue samples of thirty-three FEA, eight cystic endometrial hyperplasia (CEH) and twenty-one samples of normal, healthy endometrium in the follicular (FS; n= 10) and luteal (LS; n= 11) stages were evaluated. Histological evaluation of haematoxylin and eosin stained sections of the FEA revealed a papillary proliferation of neoplastic cells of serous type, accompanied by clear and multinucleated cells. Other architectural arrangements mainly included solid and tubular growth. Randomly distributed areas of necrosis within the tumours were commonly observed. Invasion of the myometrium, of the serosa and of vascular and/or lymphatic vessels were not constant features. The mean number of mitoses was higher in FEA compared to non-neoplastic endometrium. COX-2 scores were lower in FEA (P= 0.003) and CEH (P= 0.05) when compared to normal epithelium (NE). The loss of the membrane apical reinforcement in epithelial cells was observed in FEA samples, which was accompanied by the dislocation of COX-2

labelling into the cytoplasm and the perinuclear area; contrasting, in epithelial cells in the healthy and hyperplastic endometria the immunoreaction showed the characteristic pattern of apical membrane reinforcement, suggestive of the membrane polarization. COX-2 epithelial scores were higher in FS than in LS. No differences were found in stromal COX-2 expression between normal, CEH and FEA groups but it was higher in LS than in FS. In summary, loss of COX-2 compartmentalization in neoplastic epithelial cells might be one of the molecular events underlying endometrial carcinogenesis.

INTRODUCTION

In contrast to the situation in women, endometrial adenocarcinomas are considered to be rare in cats (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976). The common practice of ovariohysterectomy (OVH) has been referred to as protective from uterine neoplasia (Miller et al. 2003; Taylor 2010). Still, there are reports of adenocarcinoma in the uterine stump of neutered cats (Anderson and Pratschke, 2011; Miller et al., 2003). The rarity of these tumours in cats has also been related to an inadequate *post mortem* examination of the genital tract (Schlafer and Miller, 2007), and to the infrequent routine histopathological analysis of OVH surgical specimens (Sontas et al., 2013). Therefore, feline endometrial adenocarcinomas (FEA) might be more common than described in the literature. The clinical impact of such tumours may increase with the ability to establish an early diagnosis and identify the molecular events that lead to endometrial carcinogenesis (Saraiva et al., 2012). Few studies are available on FEA (Gil da Costa et al., 2009; Martín de las Mulas et al., 1995; Miller et al., 2003). Previously, the authors have briefly described three different morphologies of FEA, named papillary serous adenocarcinomas, *in situ* adenocarcinomas and clear cell adenocarcinomas (Saraiva et al., 2012). One aim of this study was to clarify some histological features of FEA of papillary serous type.

Cystic endometrial hyperplasia is a phase of the CEH/pyometra complex (CEH-P), which culminates with bacterial colonization of the uterus and accumulation of exudates in the uterine cavity (Agudelo, 2005; Kempisty et al., 2013; Schlafer and Gifford, 2008). CEH-P occurrence has been proposed to be dependant of progesterone stimuli, although this is not the only hormonal factor

involved in the process (Agudelo, 2005). Along with specific characteristics of invading bacteria, changes within the endometrium are presumed to be involved in the pathogenesis of CEH-P syndrome (Schlafer and Gifford, 2008). Despite the high frequency of this lesion, little is known regarding the molecular events underlying CEH-P (Kempisty et al. 2013; Schlafer and Gifford, 2008).

Cyclooxygenase enzyme plays a key role in the conversion of arachidonic acid to prostaglandins (PG) and has two main isoforms: COX-1 and COX-2. COX-2 expression is induced by several agents such as cytokines, growth factors and mitogens, and is often associated with inflammation and cancer (Williams et al., 1999). Several animal and human neoplasms overexpress COX-2, including human endometrial carcinomas (Erkanli et al., 2007; Tong et al., 2000). A selective COX-2 inhibitor has been demonstrated as potentially beneficial for the treatment of COX-2 positive human endometrial carcinomas (Hasegawa et al., 2011). According to Doré (2011) COX-2 has also been demonstrated in several feline tumours, mainly in feline mammary invasive carcinomas. Considering that inhibition of COX-2 has been proposed as a potential strategy for prevention and treatment of some feline cancers (DiBernardi et al., 2007; Sayasith et al., 2009), studies on the expression of this molecule in feline reproductive disorders are needed. A second aim was to characterize the immunohistochemical expression of COX-2 in feline NE, CEH and in primary FEA of papillary serous type.

Materials and Methods

Source of Samples

Thirty-three samples of FEA were obtained from the archives of four different laboratories, during a period of sixteen years along with eight CEH and twenty-one archived samples of histologically normal feline uterus, which were selected as controls (10 samples for the follicular stage – FS - and 11 samples for the luteal stage - LS).

All samples were previously fixed in 4% neutral-buffered formalin and paraffin-embedded. FEA and CEH were submitted after OVH, which was performed routinely, or after clinical diagnosis of reproductive disease or mammary gland tumour. One case of FEA was diagnosed *post mortem*. Normal uterine samples were obtained after routine OVH, from post-pubertal animals. They were selected

from animals not treated with progestins; for the other samples, the use of progestin contraception was recorded where possible. Three of the eight animals diagnosed with CEH and 7 of the 33 cats diagnosed with FEA were under progestin based contraception. Patient signalment also included breed, age and the motive for surgery.

Histopathological Examination

Sections with 3µm were routinely stained with haematoxylin and eosin for diagnosis of FEA and CEH and staging of normal samples by light microscopy.

Normal uterine samples were classified as: (1) FS if presenting large antral follicles bulging at the ovarian surface and cuboidal epithelial cells in the endometrial epithelia, with glands scarcely distributed in the endometrium; (2) LS if presenting ovarian corpora lutea and columnar epithelial elements in the endometrial surface and glandular epithelia, together with glands highly coiled and developed.

Cystic endometrial hyperplasia was diagnosed according to the World Health Organization criteria (Kennedy et al., 1998). These included endometrial thickening by focal and diffuse cystic dilatation of the endometrial glands; cysts often lined by flattened epithelial cells; surface and glandular epithelia lined by cuboidal to low columnar cells; signals of stromal edema and inflammation were reported (Kennedy et al., 1998).

The tumours were evaluated according to several criteria of malignancy, described in the literature (Goldschmidt et al., 2011; Horn et al., 2007; Miller et al., 2003). The criteria included: significant nuclear and cellular pleomorphism, anisokaryosis, anisocytosis and nuclear atypia (considered as high or low to moderate); mean number of mitoses per high power field (categorized as lower than 1, 1 to 5 and more than 5); presence of randomly distributed areas of necrosis within the neoplasm and invasion of the myometrium, of the serosa or of lymphatic/vascular vessels. Neoplastic cells were evaluated according the following features: architectural arrangement; morphology; loss of polarity; presence of clear cells (considered present if more than 5% of neoplastic cells had abundant and foamy cytoplasm); and presence of multinucleated cells.

In CEH and FEA samples the co-existence of pyometra (presence of a purulent exudate within the uterine lumen) or endometritis (inflammatory cells in the supporting stroma) were recorded.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed using a monoclonal antibody against COX-2 (SP21; Neomarkers & LabVision Corporation®), previously tested in feline tissues, at 1:75 dilution (Gil da Costa et al., 2009). Immunohistochemical labelling was achieved by using the Novolink® Polymer Detection System (ref. RE7280-K, Novocastra®). Sections of human colonic carcinoma (as stated in the datasheet of antibody), feline corpora lutea and endometrial macrophages were used as positive controls. The primary antibody was omitted for negative controls.

QUANTIFICATION OF IMMUNOLABELLING

Epithelial and stromal immunolabelling for COX-2 were evaluated independently. The percentage of positive epithelial cells was assessed semiquantitatively, according to the marks: 0= < 5%, 1= 5-25%, 2= 26-50%, 3= 51-75%, and 4= >75%. When immunoreaction against COX-2 existed, its intensity was graded as 1= weak, 2= moderate and 3= strong. Overall scores were assessed by the product of percentage of positive cells and intensity of expression, being evaluated as follows: 1-4= low, 5-7= intermediate and 8-12= high (Sayasith et al., 2009). The intracellular staining patterns were evaluated as membrane (with or without polarity, i.e. in a diffuse membrane labelling or concentrated as apical reinforcement, respectively), cytoplasmic and perinuclear. Evaluation of the intracellular pattern did not contribute to the overall score. COX-2 expression in the stromal compartment was also evaluated. Since endometrial stromal compartment was not equally stained within the samples, it was graded according to the scale: 0= absent; 1= low; 2= intermediate and 3= high.

STATISTICAL ANALYSIS

Statistical comparisons were performed by using the IBM SPSS Statistics Base 20.0 software®. All statistical analyses were performed using the chi-square and Fisher exact tests. *P* values < 0.05 were regarded as statistically significant.

RESULTS

ANIMALS

Normal uterine samples were from post-pubertal animals aged 7 months to 8 years of age (mean 1.5 years); breeds included Domestic Shorthaired cats and Persian.

Cystic endometrial hyperplasia was diagnosed in queens 2 to 10 years of age (mean 6.1 years); the age of females diagnosed with FEA ranged from 1 to 15 years old (mean 7.3 years). For these two groups, breeds included Domestic Shorthaired cats, Persian and Siamese.

HISTOPATHOLOGICAL FEATURES

Feline endometrial adenocarcinomas of papillary serous type (FIGURE 1 A-B) were primarily characterized by the proliferation of endometrial epithelial cells on papillae into the lumen supported by a thin fibrovascular stroma and lined by multilayered neoplastic cells. Areas of tubular, solid and glomeruloid intracystic growth were also present. Neoplastic cells were pleomorphic columnar shaped, with moderate amount of eosinophilic cytoplasm and round to oval, vesicular or hyperchromatic nuclei that lost the normal polarity. Nucleoli were evident and occasionally intranuclear clear inclusions were found. There was a moderate to high degree of anisokaryosis and anisocytosis. Numerous multinucleated cells were present within and at the surface of the lesions. A moderate number of clear cells - large, round to polygonal cells, with foamy cytoplasm and eccentric crenate or ovoid nucleus - was identified. Randomly distributed areas of necrosis were frequently present. The mean number of mitoses per high power field was generally lower than 1 in non-neoplastic epithelia and lower than 5 in FEA. It was higher in tumour epithelium when compared to normal and hyperplastic endometrium ($P \leq 0.0001$). The invasion of the myometrium was not a constant feature, although FEA consistently invaded the deeper stromal layer and submucosa. Histopathological features of FEA are summarized in TABLE 1.

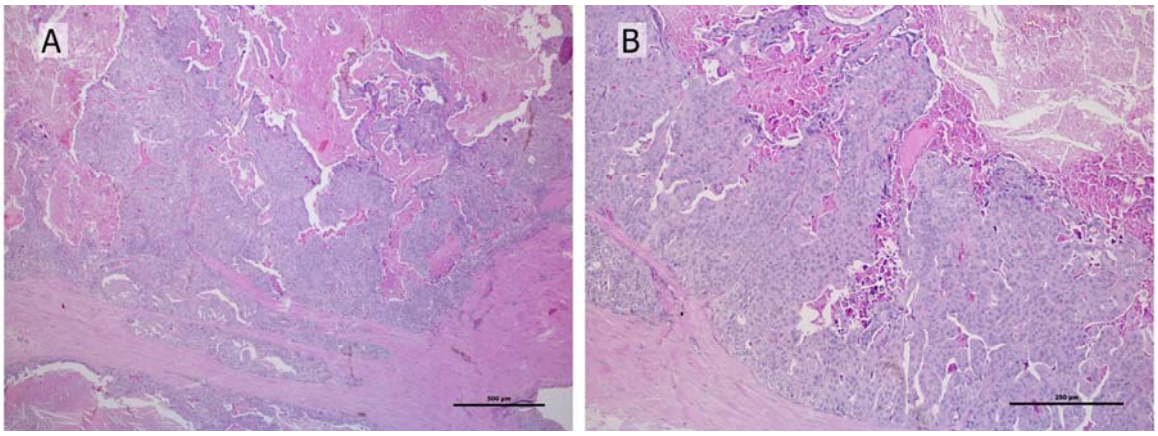

FIGURE 1: Feline endometrial adenocarcinomas of papillary serous type. A: Neoplastic cells projecting into the lumen and invading the myometrium. Focal areas of necrosis are seen within the tumour; bar = 500 µm. B: Multilayered papillary and solid growth of neoplastic cells. Images of myometrium invasion; bar = 250 µm. Haematoxylin and eosin stain.

TABLE 1: Histological features of feline endometrial adenocarcinomas.

		NUMBER OF CASES (N= 33)
ARCHITECTURAL REARRANGEMENT	SOLID	11
	TUBULAR	27
	GLOMERULOID	3
CLEAR CELLS (> 5%)		12
NUCLEAR ATYPIA	LOW TO MODERATE	14
	HIGH	19
PYOMETRA		10
ENDOMETRITIS		27
NECROSIS		27
MYOMETRIUM INVASION	ABSENT	12
	SUPERFICIAL	12
	DEEP	9
SEROSA INVASION		2
VASCULAR/LYMPHATIC INVASION		4
MEAN NUMBER OF MITOSES PER HIGH POWER FIELD	≤ 1	16
	1-5	14
	≥ 5	3

COX-2 IMMUNOHISTOCHEMISTRY

COX-2 expression was found in the surface and glandular epithelium of normal endometrium (FIGURE 2 A); in the surface, glandular and cystic lining epithelium of CEH (FIGURE 2 B); and also in neoplastic cells (FIGURE 2 C). Expressions of COX-2 score are shown in TABLE 2. COX-2 scores in NE did not differ amongst the epithelial type (surface *vs.* glandular; *P*= 0.741), but the overall scores were higher in the FS than in LS (*P*= 0.035). In CEH samples, no differences were found between the surface, glandular or cystic epithelia (*P*= 0.442). COX-2 expression was decreased in CEH and FEA epithelia compared to NE; however, no differences were found on what concerns the score between CEH and FEA (TABLE 2). COX-2 immunolabelling in CEH revealed a loss of homogeneity, particularly in cystic epithelium, and this was even more pronounced in FEA.

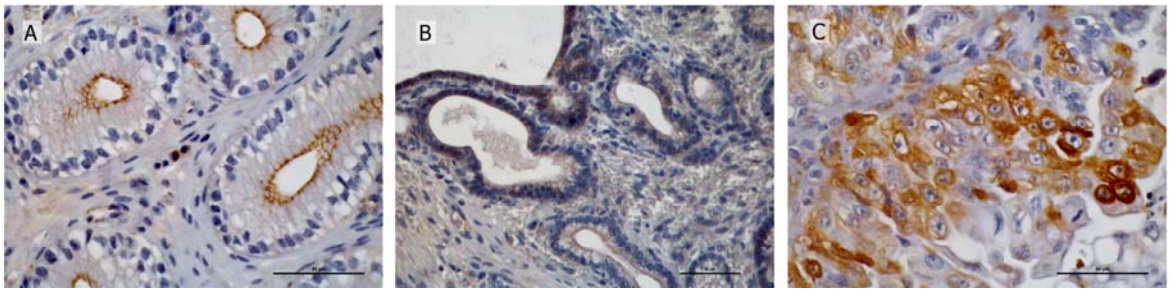

FIGURE 2: COX-2 immunoexpression in feline endometrium (bar = 50 µm). A: Normal endometrium (luteal stage). Membrane labelling with apical reinforcement is noticed in epithelial cells; stroma also exhibits COX-2 expression. B: Cystic endometrial hyperplasia. The cystic epithelium shows cytoplasmic staining while the glandular epithelium shows membrane labelling with apical reinforcement. C: Feline endometrial adenocarcinoma. Epithelial tumour cells show heterogeneous intensity in membrane and cytoplasm as well as perinuclear labelling. Avidin-biotin peroxidase technique counterstained with Gill's haematoxylin.

In NE, the dominant intracellular pattern for COX-2 expression was the membrane with apical reinforcement, with sporadic labelling in the cytoplasm and the perinuclear area (FIGURE 2 A). No difference was found regarding the intracellular pattern of immunostaining between surface and glandular epithelium of normal endometrium. However, when the two phases of oestrous cycle were compared, the perinuclear staining was only found in FS samples (*P*= 0.043). In CEH, an increase of cytoplasmic staining was observed in the surface and cystic epithelia, but not in glandular epithelium compared to NE; still, the COX-2 intracellular patterns were similar between NE and CEH (FIGURE 2 B). The

neoplastic epithelium had less membrane expression than NE; this was accompanied by a loss of the apical reinforcement along with the dislocation of the immunoreaction into the cytoplasm and the perinuclear area (FIGURE 2 C). The intracellular patterns of COX-2 staining in normal, hyperplastic and neoplastic epithelial cells are shown in FIGURE 3 and TABLE 3. COX-2 expression in the stromal compartment was higher in LS than in FS (*P*= 0.026), but the immunostaining expressed in NE stroma was not different from that observed in CEH and FEA. TABLE 4 summarizes COX-2 expression in the stromal compartment of normal, hyperplastic and neoplastic endometrium.

TABLE 2: COX-2 scores in the epithelial cells from normal and hyperplastic endometria and in FEA. The scores, assessed by the product of percentage of positive cells (using the marks: 0= < 5%, 1= 5-25%, 2= 26-50%, 3= 51-75%, and 4= >75%) and intensity of expression (1= weak, 2= moderate and 3= strong), were evaluated as absent (=0), low (1-4), intermediate (5-7) and high (8-12).

	GROUP			
COX-2 SCORE[a]	NE	CEH	FEA	***P* VALUE (BETWEEN 3 GROUPS)**
ABSENT	0.0 %	0.0 %	9.1 %	0.007
LOW	14.3 %	33.3 %	21.2 %	
INTERMEDIATE	7.1 %	16.7 %	27.3 %	
HIGH	78.6 %	50.0 %	42.4 %	

[a] NE *vs.* CEH, *P*= 0.050; NE *vs.* FEA, *P*= 0.003; CEH *vs.* FEA, *P*= 0.330.

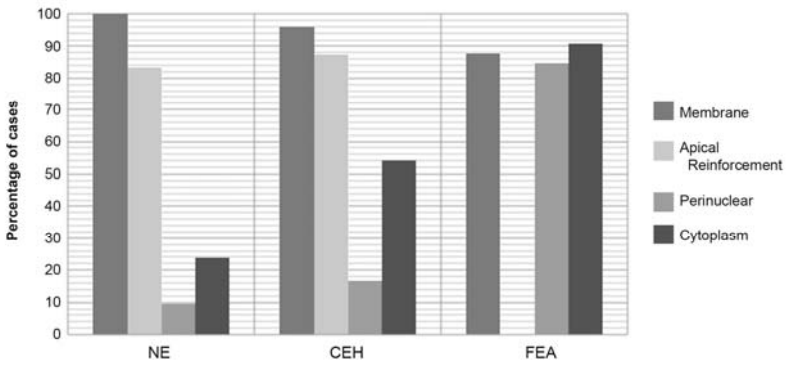

FIGURE 3: COX-2 intracellular staining patterns in the epithelial cells from normal and hyperplastic endometria and in FEA.

TABLE 3: COX-2 intracellular staining patterns in the epithelial cells from normal and hyperplastic endometria and in FEA.

	GROUP			
COX-2 STAINING PATTERN	NE	CEH	FEA	***P* VALUE (BETWEEN 3 GROUPS)**
MEMBRANE	100.0 % [a]	95.8 %	87.9 % [a]	0.041
APICAL REINFORCEMENT	83.3 % [b]	87.5 % [c]	0.0 % [b, c]	≤ 0.0001
PERINUCLEAR	9.5 % [d]	16.7 % [e]	84.8 % [d, e]	≤ 0.0001
CYTOPLASM	23.8 % [f, g]	54.2 % [g, h]	90.0 % [f, h]	≤ 0.0001

Different superscripts following percentages within a row indicate statistical significance at P< 0.05. [a] *P*= 0.034; [b-f] *P*≤ 0.0001; [g] *P*= 0.013; [h] *P*= 0.001.

TABLE 4: COX-2 expression in the stroma of normal and hyperplastic endometrium and FEA, graded according to the scale: 0= absent; 1= low; 2= intermediate and 3= high.

	STROMA GROUP					
	NE					
COX-2[a]	FS	LS	***P* VALUE (FS *VS* LS)**	CEH	FEA	***P* VALUE (BETWEEN 3 GROUPS)**
ABSENT	0.0%	18.2%	0.026	0.0 %	9.1 %	0.472
LOW	80.0%	18.2%		75.0 %	39.4 %	
INTERMEDIATE	10.0%	18.2%		25.0 %	30.3 %	
HIGH	10.0%	45.5%		0.0 %	22.2 %	

[a] NE *vs.* CEH, *P*= 0.275; NE *vs*. FEA, *P*= 0.605; CEH *vs.* FEA, *P*= 0.337.

DISCUSSION

The increasing number of reports on FEA (Anderson and Pratschke, 2011; Cho et al., 2011; Payan-Carreira et al., 2013; Sontas et al., 2013) alerts to the need of raising veterinarians awareness for endometrial tumours in cats. Early recognition of FEA along with the understanding of molecular events underlying carcinogenesis in feline endometrium is important for improving diagnosis, treatment and prognosis.

Definitive diagnosis of endometrial adenocarcinomas may only be accomplished by histopathological exam (Klein, 2007), which may be challenging because the described histological features are inconstant (Anderson and

Pratschke, 2011; Belter et al., 1968; Gil da Costa et al., 2009; Martín de las Mulas et al., 1995; McEntee and Nielsen, 1976; Miller et al., 2003; Preiser, 1964; Saraiva et al., 2012). In this study, 33 tumours were classified as primary FEA of papillary serous type, as proposed by Saraiva et al. (2012) and described by Payan-Carreira et al. (2013). In the present study, the mean number of mitoses supported previous findings reporting mitotic indexes below 5 mitoses per high power field (Anderson and Pratschke, 2011; Gil da Costa et al., 2009). This parameter does not seem to be correlated with tumour invasiveness or the outcome of the patient (Miller et al., 2003). However, in the present study, the mean number of mitosis per high power field was significantly higher in FEA than in non-neoplastic endometrium, confirming this morphological parameter as a tumour feature. Myometrial or vascular/lymphatic invasion were inconstant, as previously reported (Miller et al., 2003; Payan-Carreira et al., 2013; Saraiva et al., 2012). Miller et al. (2003) failed to correlate histologic appearance of FEA to the biological behaviour. Vascular invasion accompanied deep myometrial invasion and peritoneal carcinomatosis; however, metastases were also found in the absence of vascular permeation or invasion evidences and in a case of superficial myometrial invasion (Miller et al., 2003). Similar findings can be found in other FEA descriptions (Belter et al., 1968; Cho et al., 2011; Preiser, 1964). Inadequate vascularization during tumour growth, leads to ischaemia and hypoxia, which are the most common conditions for activation of necrosis (Proskuryakov and Gabai, 2010). In FEA, there were scattered areas of necrosis within 81.8% of the tumours, suggesting a rapid neoplastic growth that is not supported by adequate vascularization. This issue deserves to be addressed in future studies.

Constitutive expression of COX-2 was recognized in women reproductive tract (reviewed by Rouzer and Marnett, 2009). COX-2 expression has been described in the surface and glandular epithelium of cyclic feline endometrial samples (Gil da Costa et al., 2009), although that study did not conclude on recognised differences between COX-2 immunoreaction in the stages of oestrous cycle and did not assess COX-2 expression in endometrial stroma. However, reports on porcine and rat endometrium describe the existence of positive immunoreaction for COX-2 in the endometrial stroma (Blitek et al., 2006; St-Louis et al., 2010), as confirmed by the results presented in the present study. Higher COX-2 scores were found in epithelia in the FS than in LS, though no differences

were found between the surface and the glandular epithelia. In contrast, stromal expression of COX-2 was higher in LS than in FS. It is generally accepted that major uterine cell-types respond differently to progesterone and/or estrogen (Paria et al., 2000). The results suggest a progesterone influence on COX-2 expression, which might be associated with physiological tissue remodelling accompanying the glandular development. In sheep and pigs, COX-2 epithelial expression varies according to the stage of oestrous cycle, being higher during LS (Blitek et al., 2006; Kim et al., 2003). COX-2 was proposed as mediator of epithelial-stromal cell interactions (Kim et al., 2010), particularly important for the endometrial functioning. Proposed roles for COX-2 in normal tissues and in tumours include proliferation, angiogenesis, protection against apoptosis and cell adhesion (Kim et al., 2010; Sobolewski et al., 2010). COX-2 overexpression in tumours was associated with activation of membrane metalloproteinases, a system that has been also involved in the endometrial remodelling during the endometrial cycle (Kim et al., 2010; Plaisier et al., 2006).

Despite being a frequent condition in the dog and cat, CEH-P is still not much studied in cats (Kempisty et al., 2013). To the best of the authors' knowledge, this is the first study on COX-2 expression in feline hyperplastic endometrium.

An increase in TNFα production followed by elevated PG secretion by epithelial cells has been pointed out as an important pathway in innate resistance of the endometrium to infection. Recently, COX-2 was recognized as being involved in TNFα-induced PG secretion in cat endometrial cells, since a selective COX-2 inhibitor, nimesulide, abolished TNFα-induced PG synthesis, as well as basal PG synthesis (Jursza et al., 2014). In the present study, COX-2 expression was decreased in CEH, suggesting that disruption of this inflammatory pathway may contribute to the progression of the disease, enhancing bacterial infection. COX-2 inhibitors may thus not be indicated in the medical treatment of feline CEH.

In FEA, there was a clear change in COX-2 expression supporting previous findings (Gil da Costa et al., 2009), with loss of the cell polarity and a dislocation of the binding into the cytoplasm and the perinuclear area. Predominance of the cytoplasmic pattern has also been described in human bladder carcinomas and in chronic inflammatory conditions of the biliary epithelia, in organs that also express COX-2 in a constitutive way, and is envisaged as a poor prognostic marker associated with invasiveness (Kim et al., 2010).

Limited information on COX-2 expression in FEA exists, contrasting with the large number of studies existing for human endometrial carcinoma, in which COX-2 overexpression has been reported (Nasir et al., 2007; Tong et al., 2000), accompanying an intracellular cytoplasmic pattern of labelling in tumour cells (Ferrandina et al., 2005; Ferrandina et al., 2002; Lambropoulou et al., 2010; Nasir et al., 2007). The overall expression described for human endometrial carcinoma was not found in FEA in the current study. On the contrary, there was a decrease in COX-2 score of FEA, which may be explained by differences in the types of human and feline tumours, the uterine hormonal environment, the scoring systems or the antibodies used. To support these results, molecular quantification of COX-2 in tumour tissues is required.

COX-2 localization in the perinuclear area in FEA epithelial cells resembles the described in feline transitional cell carcinomas of the urinary bladder and mammary carcinomas (Beam et al., 2003; Sayasith et al., 2009), which has been proposed to be associated with PG pathways involved on cell growth, apoptosis or cell differentiation, including malignant transformation (Sobolewski et al., 2010; Vane et al., 1998).

Banu et al. (2008) showed that COX-2 inhibition decreases growth, migration, and invasion of immortalized human endometriotic epithelial and stromal cells. Their findings support the hypothesis that absence of an increased expression of COX-2 in FEA stroma and epithelia scores might be related to the apparently low aggressiveness of such tumours.

Interestingly, COX-2 scores and staining intracellular pattern were similar between normal, hyperplastic and neoplastic endometrium. Unlike the situation in the endometrium of women, where COX-2 was proposed to be overexpressed between endometrial hyperplasia and adenocarcinoma (Nasir et al., 2007), the results presented herein suggest that CEH might not be a precursor lesion of FEA. However, in one report of multiple uterine pathologies in a cat, the authors proposed that adenocarcinoma lesions might have been a consequence of long lasting inflammation or hyperplastic reaction of endometrial glands (Sapierzynski et al., 2009).

In conclusion, though COX-2 scores were decreased in FEA, evidence of loss of compartmentalization of COX-2 expression in neoplastic epithelium in FEA most likely reflects the involvement of this enzyme in feline endometrial

carcinogenesis. Additional studies are needed to fully understand the molecular background to these conditions.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

ALS, RP-C, FG and MAP were involved in the acquisition of clinical and histopathological data, data analysis and interpretation, as well as the manuscript writing and the reviewing of the literature. MAP and ALS were responsible for the immunohistochemical analysis. IS, AR and LML were responsible for technical assistance. All authors read and approved the final manuscript.

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CHAPTER 5

CHANGES IN C-ERBB-2 IMMUNOEXPRESSION IN FELINE ENDOMETRIAL ADENOCARCINOMAS

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CHANGES IN C-ERBB-2 IMMUNOEXPRESSION IN FELINE ENDOMETRIAL ADENOCARCINOMAS

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ABSTRACT

Human epidermal growth factor receptor type 2 (c-erbB-2) is overexpressed in several human and animal tumours. The expression of this oncoprotein in tumours was referred for potential prognostic marker and therapeutic target, but information regarding this molecule in feline tumours is scarce. This study aimed to assess the changes in the immunohistochemical expression of c-erbB-2 in feline endometrial adenocarcinomas (FEA) compared to normal endometrium. An immunohistochemistry assay using the antibody against c-erbB-2 was performed in FEA samples (n= 34) and in normal endometrium in the follicular (FS; n= 12) and luteal (LS; n= 11) stages. In FEA, the immunoexpression of c-erbB-2 was assessed in neoplastic epithelial cells, whilst in the normal endometrium, it was evaluated independently in the surface epithelium and the superficial and deep glandular epithelia (SE, SGE and DGE, respectively). In FS, all epithelia were consistently positive for c-erbB-2, with the exception of one sample (8.3%), negative for this molecule in the SE. In LS, the SE was positive for c-erbB-2 in 10 samples (90.9%) and negative in only one sample (9.1%); SGE was positive in all evaluated samples (100.0%), whereas DGE was positive in only 45.4% (n= 5). Twenty FEA (58.8%) were positive for c-erbB-2 immunolabelling. The c-erbB-2 expression was significantly higher in all the epithelia in the FS compared to FEA ($P \leq 0.0001$) or the LS ($P = 0.016$). In the LS, its expression was significantly higher in the SGE, compared to DGE ($P = 0.024$). Results suggest that c-erbB-2 expression in the endometrium reflects changes occurring during the estrous cycle and may also be

involved in feline endometrial carcinogenesis, though an open question remains on the importance of additional pathways of epithelial proliferation in the neoplastic changes in feline endometrium.

KEY-WORDS

Uterine diseases; endometrium; adenocarcinoma; c-erbB-2; cats

INTRODUCTION

Human epidermal growth factor receptor type 2 (HER2), also known as c-erbB-2 or HER2/neu, is a 185 kD membrane tyrosine kinase receptor, which has been associated to tumour cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis (Yarden and Sliwkowski, 2001; Selvarajan et al., 2004; Gutierrez and Schiff, 2011). Since c-erbB-2 does not have a ligand, when expressed at elevated levels to be activated it depends on heterodimerization with other HER-family receptors, among other molecules, or homodimerization (Yarden and Sliwkowski, 2001; Gutierrez and Schiff, 2011). Human epidermal growth factor receptor type 2 activates second messenger pathways and crosstalk with other membrane signaling pathways, including the sex steroid hormone. A negative correlation between c-erbB-2 and estrogen receptor (ER) and progesterone receptor (in a lesser extend) is described in human mammary carcinomas. Importantly, ER negative / c-erbB-2 positive neoplasms usually have a worst diagnosis. That immunophenotype concerning c-erbB-2 diminishes the response to anti-estrogenic therapy (Harari and Yarden, 2000; Yarden and Sliwkowski, 2001).

In women, c-erbB-2 is expressed in the female genital tract and thus seems to be a normal membrane constituent of uterine glandular epithelial cells (Wang et al., 1992). In the mouse uterus, c-erbB-2 is mainly expressed in epithelial cells, showing cyclic variations. Exogenous steroid hormone administration to ovariectomized mice resulted in up- or down-regulation of c-erbB-2 mRNA, respectively for estradiol and progesterone administrations, suggesting that ovarian hormones influence c-erbB-2 expression. c-erbB-2 is also expressed in the peri-implantation uterus, where it may be involved in epithelial cell proliferation and differentiation, since it expression is limited to the luminal and glandular epithelium (Lim et al., 1997). The studies of Mühlhauser and colleagues (1993) localized c-

erbB-2 protein along the apical membrane of the syncytiotrophoblast, in cells negative to Ki-67. In that work, the authors associate the c-erbB-2 protein to trophoblast differentiation, but not to the proliferative events occurring in the first-trimester human placenta (Mühlhauser et al., 1993).

Concerning the feline endometrium, the studies published so far are scarce. Misirlioglu and collaborators (2006) described for the first time, c-erbB-2 expression in normal and hyperplastic endometrium in 10 and 20 queens, respectively. They found that c-erbB-2 expression increased in surface and glandular epithelium in hyperplasia compared with the normal endometrium, concluding on the importance of c-erbB-2 activation in the pathogenesis of feline endometrial hyperplasia. This was supported by a second report on c-erbB-2 expression in two hyperplastic endometrial polyps (Misirlioglu et al., 2009).

Human epidermal growth factor receptor type 2 is overexpressed in several human cancers, including endometrial adenocarcinomas (Yarden and Sliwkowski, 2001), and its amplification is often associated with tumour invasiveness, particularly in breast cancer (Brix et al., 2014). In women, the reported rates of c-erbB-2 overexpression in endometrial carcinomas vary from 1% to 80% depending on the histological type of the tumours (reviewed by Buza et al., 2014). This very wide range of results is pointed as related to small sample sizes, different clinicopathological features considered and/or differences in the staining and the scoring systems used in immunohistochemistry evaluation of the tumours. For those reasons it is possible that c-erbB-2 overexpression and tumour prognosis and outcome may not always be correlated, in human endometrial carcinomas (Buza et al., 2014). On the other hand, an increased expression of c-erbB-2 is particularly well studied in women mammary carcinomas (Yarden and Sliwkowski, 2001). In c-erbB-2-overexpressing human breast carcinomas, targeted therapy against c-erbB-2 is proven to be effective. Therefore, standardization of c-erbB-2 assessment is well established in these tumours (Wolff et al., 2013), unlike in the endometrial cancer (Buza et al., 2014).

Scoring guidelines for c-erbB-2 evaluation by immunohistochemistry have been recently established for the canine mammary tumours (Peña et al., 2014), similar to those existing in humans. The evaluation of c-erbB-2 in feline mammary tumours has also been studied, though without similar guidelines (Millanta et al., 2005; Winston et al., 2005; Ordás et al., 2007; Rasotto et al., 2011). Several studies

suggest c-erbB-2 overexpression in feline mammary carcinomas as a marker of malignancy and a prognostic indicator (Millanta et al., 2005; Winston et al., 2005; Ordás et al., 2007), but this is controversial and Rasotto and collaborators (2011) defend that c-erbB-2 may not play a major role in mammary carcinogenesis and prognosis. Technical factors and subjective interpretation of c-erbB-2 immunolabelling may explain the disagreements in the incidence of c-erbB-2 expression between studies (Rasotto et al., 2011).

Increasing reports in feline endometrial adenocarcinomas (FEA) (Anderson and Pratschke, 2011; Cho et al., 2011; Gil da Costa et al., 2009; Payan-Carreira et al., 2013; Sontas et al., 2013; Saraiva et al., 2015a) suggest that the disease may be more common than previously described. The definitive diagnosis is usually accomplished by histopathological exam. Histopathological features of FEA, although not constant, include recognised criteria of malignancy such as nuclear atypia, bizarre mitosis, necrosis, and vascular, myometrium and/or serosa invasion (Anderson and Pratschke, 2011; Miller et al., 2003; Papparella et al., 1984; Saraiva et al., 2012). Despite the growing number of studies in FEA, little is known about the molecular events related to the carcinogenesis of the endometrium in queens. Moreover, immunohistochemical characterization of FEA is limited and performed in small case series (Gil da Costa et al., 2009; Miller et al., 2003; Espinosa de los Monteros et al., 1999). Nevertheless, the pathogenesis of FEA remains elusive.

Speculating that c-erbB-2 may play a role in feline endometrial carcinogenesis, the present study aimed to characterize the c-erbB-2 immunohistochemical pattern of expression in FEA compared to normal endometrium, and to explore putative roles of c-erbB-2 in feline cyclic and neoplastic endometrium

Material and Methods

Animals and Tissue Preparation

The samples of FEA (n= 34) and of normal postpubertal feline uterus (n= 23, 12 in the follicular stage and 11 in the luteal stage) used in the present work were collected from the archives of four different laboratories in Portugal, during a period of eight years (from 2003 to 2011). Animals' age, breed, and past or present progestin based contraception were assessed by the request forms that

accompanied surgical specimens for histopathological evaluation. For FEA, the clinical history that led to the submission of specimens to the laboratories was also recorded.

Tissues were fixed in 10% buffered formalin and paraffin embedded. Three-micrometer thick sections were cut, one slide routinely stained with haematoxylin and eosin for histopathological diagnosis. The tumours were evaluated according to several criteria of malignancy as described in Saraiva et al. (2015a). Briefly, the histopathological criteria used included potential indicators of tumours dedifferentiation, namely: nuclear atypia, considered as low to moderate or high; mean number of mitosis per high power field (HPF; 400x), valued as lower than 1 (<1), 1-5 and more than 5 (>5); and the myometrium, serosa and/or vascular invasion, evaluated as present or absent. Twenty-three normal uterine samples were staged as follicular (FS; n= 12) or luteal (LS; n= 11) based on the presence of follicles or corpora lutea in the ovaries and the epithelial cell height and the degree of development and coiling of endometrial glands (Saraiva et al., 2015a).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed using a polymer-based system (Novolink® Polymer Detection System, Product No: RE7280-K Leica Biosystems, Newcastke, UK), according to the manufacturer's instructions. The sections were deparaffinised with xylene and rehydrated through graded alcohols; antigen retrieval was performed in a pressure cooker (~ 96 ºC) in 10 mmol/L sodium citrate buffer (pH 6.0) for 3 minutes. The slides were cooled for 10 min at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 min. Endogenous peroxidase activity was blocked by treating the sections with the Peroxidase Block provided in the kit. Sections were incubated overnight at 4°C in a humid chamber with 1:40 Polyclonal Rabbit Anti-Human c-erbB-2 Oncoprotein (Code A0485 Dako, Denmark) that labels an intracellular domain of c-erbB2 oncoprotein. Colour was developed with 3,3'- diaminobenzidine (DAB), incubated at room temperature, and sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted. Sections of human colonic adenocarcinoma were used as positive control. For negative controls, the primary antibody was omitted.

Evaluation of immunolabeling

The immunoreactivity of c-erbB-2 was assessed semiquantitatively, in morphologically representative areas of the tumour at low power fields, based in the Hercep-Test scoring criteria, described in the American Society of Clinical Oncology (ASCO) guidelines (Wolff et al. 2013). Briefly, samples were classified according to the score: IHC 3+, when the circumferential membrane staining is complete, intense and within > 10% of tumour cells ("chicken-wire pattern"); IHC 2+, if the circumferential membrane staining is incomplete and or weak/moderate and within > 10 % of tumour cells or complete and circumferential membrane staining that is intense and within ≤ 10% of tumour cells; IHC 1+, if incomplete membrane staining is faint/barely perceptible and within > 10% of tumour cells; IHC 0, no staining is observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of tumour cells (Wolff et al. 2013). Scores of IHC 0 and 1+ were considered negative, whereas scores of IHC 2+ and 3+ were considered positive (Grushko et al., 2008; Rasotto et al. 2011; Togami et al., 2012). In the normal uterus, the analysis of immunoreaction against c-erbB-2 was assessed independently in the surface epithelium (SE), the superficial and deep glandular epithelia (SGE and DGE, respectively) and stromal compartment, using the same scoring system.

Statistics

Statistical analysis was performed using chi-square and Fisher exact tests in the IBM SPSS Statistics Base 20.0 software®. *P* values < 0.05 were regarded as statistically significant.

Results

The thirty-four FEA used in this study were diagnosed in queens with an aged range between one to 15 years (mean 7.8 years); this result respects to 26 queens, since this information was not declared for the remaining eight animals. Breeds included Domestic Shorthaired cats (n= 24; 70.6%), Siamese (n= 2; 5.9%) and Persian (n= 1; 42.9%); in 7 cases, breed was omitted. Contraception was confirmed in 9 (26.5%) animals, whilst it was denied in 3 (8.8%) cases. In the remaining 32 cases, data concerning contraception was not mentioned in the form. When such information existed, the length of the treatment was not declared. The reasons

leading to the request of histopathological analyses included clinical signs of uterine disease (n= 14; 41.2%), concurrent mammary tumour (n= 10; 29.4%) and uterine lesions detected during routine ovariohysterectomy (n= 4; 11.8%).

Normal uterine samples (n= 23) used as controls were obtained from post-pubertal animals submitted to routine ovariohysterectomy. Queens' age was declared for 16 controls and ranged between 7 months to 8 years of age (mean 1.4 years). Breeds included Domestic Shorthaired cats (n= 7; 30.4%) and Persian (n= 1; 4.3 %); this information was not specified for the remaining animals. In controls, none of the queens were submitted to contraceptive treatment.

The morphology of FEA was characterized by papillary proliferation, lined by multiple layers of neoplastic cells supported by a fibrovascular stroma. Other architectural patterns scarcely present within the tumours included solid, tubular and glomeruloid. These features supported the classification of the tumours as a papillary serous type. Neoplastic cells were pleomorphic columnar shaped, with a moderate amount of eosinophilic cytoplasm and round-to-oval, vesicular or hyperchromatic nuclei that lost the normal polarity. Numerous multinucleated cells were present within and at the surface of the lesions. A variable number of clear cells was also observed. Nucleoli were evident and occasionally intranuclear clear inclusions were also found. Randomly distributed areas of necrosis within the tumours were frequently present. Histopathological features presumably related to tumour dedifferentiation are summarized in TABLE 1. Seventeen tumours (50.0%) had a low to moderate degree of atypia, whereas the other half of the sample (n= 17; 50.0%) was characterized by high atypia. The majority of tumours (n= 24; 70.6%) showed a mean number of mitosis per HPF between 1 and 5, while in seven tumours (20.6%) it was <1 whilst in a minority of cases (n= 3; 8.8%) it was >5 mitosis. In normal samples, the SE in either FS or LS consistently presented <1 mitosis per HPF (n= 12 and n= 11, respectively; 100.0%). The mean number of mitosis per HPF in tumours was higher, compared to the normal epithelia ($P \leq 0.0001$) (TABLE 1). In the FS, the glandular epithelia (both the SGE and DGE) showed <1 mitoses per HPF. In the LS, the mean number of mitosis differed between the superficial and the deep glandular epithelia, though without statistical meaning: the SGE regularly presented a mean number of mitosis <1 (90.9%; n= 10) with only one sample showing a mean number of mitosis between 1 and 5

(9.1%), whilst the DGE showed a mean number of mitosis either <1 (n= 7; 63.6%) or between 1 to 5 mitosis (n= 4; 36.4%).

TABLE 1: Major Histopathological Features Assessed in FEA Cases.

HISTOPATHOLOGICAL FEATURES		NUMBER OF FEA IN 34 (%)
NUCLEAR ATYPIA	LOW TO MODERATE	17 (50.0)
	HIGH	17 (50.0)
MEAN NUMBER OF MITOSIS PER HPF	< 1	7 (20.6)
	1 - 5	24 (70.6)
	> 5	3 (8.8)
MYOMETRIUM INVASION	YES	22 (54.7)
	NO	12 (35.3)
SEROSA INVASION	YES	1 (2.9)
	NO	33 (97.1)
VASCULAR INVASION	YES	3 (8.8)
	NO	31 (91.2)

Myometrial invasion was observed in 22 tumours (64.7%). Vascular and serosa impairment were present in three (8.8%) and one (2.9%) tumours, respectively. These cases occurred independently but always co-existing with myometrial invasion.

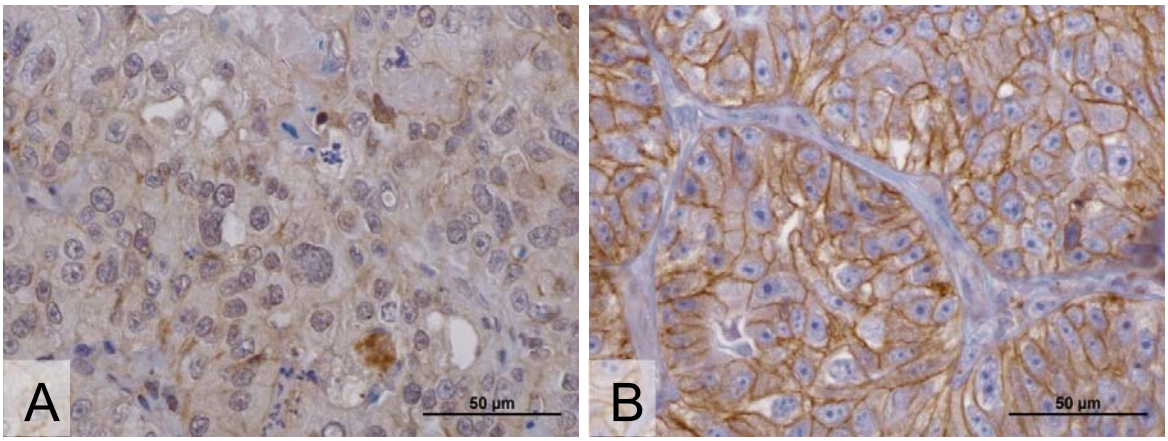

FIGURE 1: Immunohistochemical expression of c-erbB-2 in FEA, counterstained with Mayer's haematoxylin. Bar = 50 µm. (A) A negative scoring, with cells showing incomplete membrane staining that is faint/barely perceptible and within > 10% of tumour cells; (B) Positive FEA with cells displaying strong and complete membrane staining (referred as *chicken-wire pattern*) scored as 3+.

The immunoreaction against c-erbB-2 oncoprotein in FEA is summarized in TABLE 2. Of the 34 FEA cases, 14 (41.2%) were considered negative to c-erbB-2: four cases (11.8%) were classified as IHC0 and 10 cases (29.4%) as IHC1+.

The remainder 20 FEA samples (58.8%) were positive for c-erbB-2 (FIGURE 1), being 18 (52.9%) scored as IHC2+ and two (5.9%) as IHC3+. The immunoreaction of c-erbB-2 in both normal and FEA stroma was consistently negative.

TABLE 2: Immunohistochemical expression of c-erbB-2 oncoprotein in FEA and normal endometrium. Samples were classified according to the score: IHC 3+, circumferential membrane staining that is complete, intense and within > 10% of tumour cells ("chicken-wire pattern"); IHC 2+, circumferential membrane staining that is incomplete and or weak/moderate and within > 10 % of tumour cells or complete and circumferential membrane staining that is intense and within ≤ 10% of tumour cells; IHC 1+, incomplete membrane staining that is faint/barely perceptible and within > 10% of tumour cells; ICH 0, no staining is observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of tumour cells. Scores of 0 and 1+ were considered negative, whereas scores of 2+ and 3+ were considered positive and indicative of c-erbB-2 oncoprotein overexpression.

	c-erbB-2 IHC score *n* (%)					
	Negative			Positive		
	IHC 0	IHC 1+	TOTAL	IHC 2+	IHC 3+	TOTAL
Follicular Stage (n=12) [a, b]						
SE	1 (8.3)	0	1 (8.3)	9 (75.0)	2 (16.7)	11 (91.7)
SGE	0	0	0	4 (33.3)	8 (66.7)	12 (100.0)
DGE [c]	0	0	0	7 (58.3)	5 (41.7)	12 (100.0)
Luteal Stage (n=11) [a]						
SE	0	1 (9.1)	1 (9.1%)	6 (54.5)	4 (36.4)	10 (90.9)
SGE [d]	0	0	0	9 (81.8)	2 (18.2)	11 (100.0)
DGE [c, d]	2 (18.2)	4 (36.4)	6 (54.6)	5 (45.4)	0	5 (45.4)
FEA (n=34) [b]	4 (11.8)	10 (29.4)	14 (41.2)	18 (52.9)	2 (5.9)	20 (58.8)

IHC = immunohistochemical; FEA = feline endometrial adenocarcinomas; SE = surface epithelium; SGE = superficial glandular epithelium; DGE = deep glandular epithelium.

Different superscripts indicate a statistical significance: [a] P= 0.016; [b] P< 0.0001; [c] P= 0.004; [d] P= 0.024.

The immunoreaction against c-erbB-2 in the normal uterus differed according to the stage of the cycle. Concerning the overall epithelial expression of c-erbB-2 in FS and LS, controls of the FS were most commonly positive for c-erbB-2 (P= 0.016). In FS, the endometrial epithelia were generally positive for c-erbB-2 (TABLE 2; FIGURE 2), with only one case (8.3%) with negative scores for this oncoprotein in the SE. Scores varied between IHC2+ and IHC3+, accounting 75.0% (n= 9) and 16.7% (n= 2) in the SE, 33.3% (n= 4) and 66.7% (n= 8) in the SGE, and 58.3% (n= 7) and 41.7% (n= 5) in the DGE, respectively. In the LS, the SE was negative in only one sample (9.1%), being positive in 10 specimens

(90.9%), represented by six samples (54.5%) with a score IHC2+ and four (36.4%) with IHC3+. While the SGE of LS was considered positive in all the cases, being nine (81.8%) scored as IHC2+ and two (18.2%) scored as IHC3+, the DGE was mainly negative (n=6; %); two cases (18.2%) were scored as IHC0 and four (36.4%) samples had a score of IHC1+. Only four samples (36.4%) were found positive for c-erbB-2 with a score of IHC2+. In the LS, the SGE was more frequently positive for c-erbB-2 than the DGE (P= 0.024). Positivity for c-erbB-2 was more frequent in DGE of the FS than in the homologous counterpart of the LS (P= 0.004). Feline endometrial adenocarcinomas were more likely negative for c-erbB-2 expression than controls of FS (P≤ 0.0001). No significant differences were detected between FEA and LS.

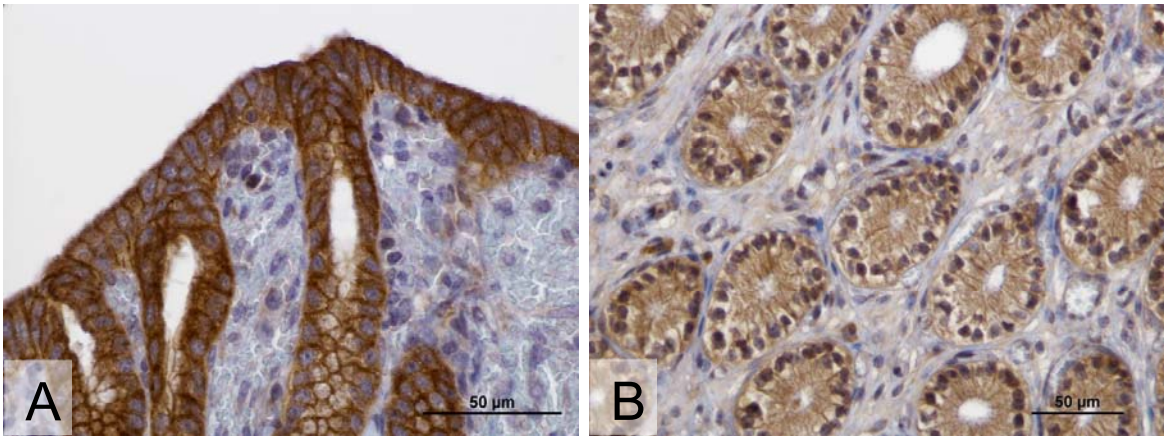

FIGURE 2: Immunohistochemical expression of c-erbB-2 in the normal feline endometrium, counterstained with Mayer's haematoxylin. Bar = 50 µm. (A) In the follicular stage, the superficial epithelium show positivity for c-erbB-2. (B) In the luteal stage, cells in the deep glandular epithelium are negative for c-erbB-2 immunostaining.

In tumours, c-erbB-2 expression was compared with nuclear atypia, number of mitosis per HPF and myometrium, serosa and vascular invasion, all features presumably indicative of tumour dedifferentiation. However, no statistically significant differences were recorded.

DISCUSSION

The present study proposed to establish the pattern of immunohistochemical expression for c-erbB-2 oncoprotein in feline endometrial adenocarcinomas (FEA), compared to the normal feline endometrium and to explore putative associations

between c-erbB-2 and the histopathological features of FEA that might be related to poor prognosis and worse outcome. To the best of our knowledge this is the first description of c-erbB-2 immunohistochemical expression in feline neoplastic endometrium.

Endometrial adenocarcinomas were usually considered to be rare tumours in all domestic species, with exception of cows and the rabbits (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976). The increasing number of reports on feline endometrial adenocarcinomas (FEA), however, questions the possibility that these tumors may be more common than the literature refers (Anderson and Pratschke, 2011; Cho et al., 2011; Gil da Costa et al., 2009; Payan-Carreira et al., 2013: Sontas et al., 2013). Therefore, additional studies addressing FEA characterization are required.

The 34 cases used herein were classified as FEA of the papillary serous type, using previously reported criteria (Preiser 1964; Belter et al. 1968; McEntee and Nielsen 1976; Martin de las Mulas et al., 1995; Miller et al., 2003; Gil da Costa et al., 2009; Anderson and Pratschke 2011; Saraiva et al., 2012; Saraiva et al., 2015a). Moreover, there were also evaluated specific histologic features of malignancy (Miller et al. 2003; Horn et al. 2007; Goldschmidt et al. 2011), namely nuclear atypia, mitotic activity and myometrial, vascular and/or serosa invasion.

Human epidermal growth factor receptor type 2 (c-erbB-2) was expressed in 58.8% of the cases under study. In women, the c-erbB-2 positive phenotype has been associated to high-grade tumours (Ferrandina et al., 2005; Mariani et al., 2005) and to early stages of the disease (Sugimoto et al., 2007). Data gathered in the present study failed to evidence an association between c-erbB-2 scoring and histopathological malignant features mentioned above, as it has been also referred regarding the women endometrial carcinomas (Ferrandina et al., 2005; Togami et al., 2012). Therefore, we can speculate that c-erbB-2 expression does not have a great impact in the progression or the prognosis of FEA.

In women endometrial carcinomas, myometrial invasion is correlated with proliferation, risk of recurrence and poor outcome (Prat, 2004). Mitotic indexes are used in routine histopathological diagnosis to assess proliferation and it is often a predictor of malignancy. Neoplastic cells are often compared to trophoblastic cells on respect to invasiveness. However, studies in humans showed that the cells of the cyto- and the syncytiotrophoblast seldom express c-erbB-2 protein (Mühlhauser

et al., 1993; Wang et al., 1992), suggesting that this molecule may not be directly associated with proliferation events, but mainly with differentiation phenomena. Thus, a positive expression of c-erbB-2 oncoprotein seems unrelated to poor clinical outcome of FEA. However, to assure this hypothesis, additional studies in follow-up of FEA and feline placenta are needed to further investigate the potential prognostic value of FEA histological features of malignancy.

The present study also localized the c-erbB-2 protein in the normal feline uterus in the follicular and luteal stages of the cat estrous cycle. Differences in the expression of c-erbB-2 between the normal feline endometrium and in endometrial hyperplasia had been determined (Misirlioglu et al., 2006). In that study, it was referred that the c-erbB-2 content in SE of normal feline uterus was lower than that of the glandular epithelium, contrasting to the findings in the present study. The differences may be explained by the fact that different antibody and scoring systems were used, as well as that the oestrous cycle was not staged, therefore impairing any inference on eventual cyclic changes in this molecule expression. Still, the results reported herein showed that expression for this protein was confined to epithelial compartment, as it has been reported regarding the human endometrium (Wang et al., 1992). Interestingly, in the present study, the expression of c-erbB-2 was significantly higher in the FS of feline cyclic endometrium than in LS. Van Dam and collaborators (1991) detected non-significantly higher levels of c-erbB-2 oncoprotein in the proliferative phase of premenopausal endometrium (grossly equivalent to the FS described herein), compared with the secretory phase (which would correspond to the LS in our work), alike to the results presented herein.

Estrogens increase the levels of epidermal growth receptor (EGFR) in the endometrium (Mukku and Stancel, 1985), and the cyclic interchange between estrogens and progesterone has been proved to have a greater effect on EGF synthesis (Imai et al., 1995). Steroids also determine the interplay between the epithelial and stromal elements of the endometrium, which seems to have a major role in the endometrial proliferation and differentiation (Pierro et al., 2001). Once c-erbB-2 shares functional and morphologic homologies with EGFRs, it could mediate estrogen-induced proliferation (Maia et al., 2002). However, our previous work showed that ER-α is underexpressed in FEA compared to normal endometrium, suggesting that the proliferation of FEA might be controlled by a different pathway, involving local growth factors, rather than by ER-α expression

(Saraiva et al., 2015b, submitted). In that work it was also reported that the proliferative indexes, estimated on the basis of Ki-67 (MIB-1) immunolabelling, were higher in FEA than in the normal endometrium, irrespectively of the oestrous stage considered (Saraiva et al., 2015b, submitted). A positive correlation between c-erbB-2 and MIB-1 is described in human endometrial carcinomas (Ioachim et al., 2001), which seems to contrast with results reported herein. In fact, c-erbB-2 expression in LS was higher in the SGE compared to DGE. However, the persistency of proliferation of the basal glands during LS, which is reflected by the increased coiling reported in this stage (Chatdarong et al., 2005), should accompanied an increase in c-erbB-2 expression if this pathway was involved in epithelial proliferation of feline endometrium. Watson and collaborators (1996) proposed that differentiation and decidualization in the LS is a progesterone-mediated event, regulated by EGF (Watson et al., 1996). Payan-Carreira et al. (2014) also suggested that progesterone is an important driver for differentiation events occurring in the canine endometrium during the LS. All the above agreed with the involvement of c-erbB-2 with differentiation phenomena occurring during trophoblast invasion, as suggested by Mühlhauser et al. (1993).

These could explain the absence of association between the c-erbB-2 protein expression and the histopathological malignant features expressed in FEA, such as mitotic index or the invasiveness. Therefore, data gathered in the current study regarding c-erbB-2 molecule sustain the hypothesis previously raised (Saraiva et al., 2015b, submitted) that other pathways controlling epithelial proliferation in the feline endometrium may be involved in FEA carcinogenesis. Therefore, further studies ought to address the importance of the loop between oncogenes, growth factors and steroid hormones in the determination of proliferative and differentiation phenomena in feline cyclic and diseased endometrium.

Conclusion

The immunohistochemical location and pattern of distribution of c-erbB-2 in FEA and the normal endometrium in FS and LS was described for the first time, to the best of our knowledge. The majority of the FEA cases under study were positive to c-erbB-2 immunolabeling; however, a decrease in the expression of this protein in FEA was observed when compared to the FS of normal endometrium. No

associations were established between c-erbB-2 expression and the histopathological features potentially related to FEA dedifferentiation and poor outcome. The expression of c-erbB-2 was significantly higher in the epithelia of the FS compared to LS, which suggests that this protein is influenced by functional changes, whether hormonal or non-hormonal, occurring during the feline estrous cycle. Additional studies are required to further investigate the role of c-erbB-2 in feline endometrial physiology and carcinogenesis.

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Conflicts of Interest: None.

Author Contributions

ALS, RP-C, FG and MAP were involved in the acquisition of clinical and histopathological data, data analysis and interpretation, as well as the manuscript writing and the reviewing of the literature. MAP and ALS were responsible for the immunohistochemical analysis. FF and LML were responsible for technical assistance. All authors read and approved the final manuscript.

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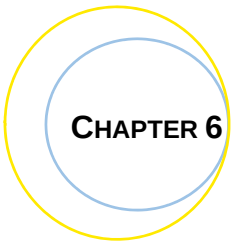

CHAPTER 6

GENERAL DISCUSSION

CONCLUSIONS AND FUTURE DIRECTIONS

General Discussion

Feline endometrial adenocarcinomas are considered rare neoplasms (Cotchin, 1964; McEntee and Nielsen, 1976; Kennedy et al., 1998). The common practice of spaying in early stages (Miller et al., 2003; Taylor, 2010), the inadequate *post mortem* examination of the genital tract (Schlafer and Miller, 2007) or the lack of interest in the anatomopathological evaluation of ovariohysterectomy surgical specimens (Sontas et al., 2013) were pointed as the major factors contribution to the limited number of diagnosed FEA. Therefore, FEA might be more common than described in the literature.

While a pathologist, that perception originated the interest in the study of FEA and the localization of FEA cases within the archives of four different laboratories in Portugal, allowing the identification of a total of thirty cases diagnosed as FEA during a period of fifteen years (1995-2010). The cases were revised and the diagnostic was confirmed on haematoxylin and eosin (H-E) stained sections.

In order to increase the series in this study, several private clinics and veterinary hospitals were contacted to collect as many surgical specimens of feline ovariohysterectomy as possible. These specimens would represent normal feline uterus at different stages of the estrous cycle, to be used as controls, as well as uterus displaying signs of disease. At the end of the first year of our work, we had collected additional nineteen samples of FEA. Staging of the estrous cycle and the diagnosis of uterine disease were performed in H-E, the later allowing to distinguish between primary FEA, delayed uterine involution and other inflammatory conditions of the uterus.

All the FEA were diagnosed taking in account several criteria of malignancy described in the literature (Miller et al., 2003; Horn et al., 2007; Goldschmidt et al., 2011), which included: significant nuclear and cellular pleomorphism, anisokaryosis, anisocytosis and nuclear atypia; mean number of mitoses per high-power field; the presence of randomly distributed areas of necrosis within the neoplasm; and invasion of the myometrium, of the serosa or of lymphatic/vascular vessels. Neoplastic cells were evaluated accordingly to the architectural arrangement, morphology, loss of polarity, presence of clear cells (considered present if more than 5% of neoplastic cells had abundant and foamy cytoplasm), and the presence of multinucleated cells.

The histological evaluation of those tumours revealed the existence of three different morphologies, as reported in CHAPTER 2: 1) FEA of the papillary serous type; 2) FEA *in situ* and 3) clear cell carcinomas/FEA (Saraiva et al., 2012).

In the course of the present study, an increasing number of FEA reports (Anderson and Pratschke, 2011; Cho et al., 2011; Sontas et al., 2013; Payan-Carreira et al., 2013), along with our aforementioned observations support the supposition that these tumours are seemly more common than previously established. However, limited knowledge on FEA still existed, in particular concerning their molecular characterization. These concerns triggered the present investigation.

Previous reports on FEA described several histological features (Preiser, 1964; Belter et al., 1968; McEntee and Nielsen, 1976; Martín de las Mulas et al., 1995; Miller et al. 2003; Gil da Costa et al., 2009; Anderson and Pratschke, 2011; Payan-Carreira et al., 2013). Earlier studies on FEA mainly refer to the papillary serous type, supporting the findings presented herein, in CHAPTER 2; the most prevalent tumour type found in that study was the papillary serous (71.4%), compared to FEA *in situ* (22.5%) and clear cell carcinomas (6.1%). In CHAPTER 2, the histopathological features related to FEA were also clarified. For the papillary serous type, these included a papillary growth pattern of neoplastic cells, with eosinophilic cytoplasm and atypical nucleus, projected into the lumen, with frequent and often bizarre mitosis. Other possible but less common arrangements included tubular, glomeruloid and solid. Interestingly, FEA were characterized by numerous multinucleated cells within or at the surface of the tumour. Randomly distributed areas of necrosis were found within the neoplasms. The invasion of the myometrium, vessels and lymphatics was not a constant feature.

Feline *in situ* adenocarcinomas were morphologically identical to the papillary serous FEA, however they were localized superficially in the endometrium, without invasion of surrounding tissues. Feline clear cell carcinomas were almost entirely composed by large, round to polygonal cells, with foamy cytoplasm and eccentric crenate or ovoid nucleus, with a prominent eosinoplilic nucleolus. There was a moderate degree of anisokaryosis and anisocytosis. The cells were arranged in papillae, sheets or solid nests surrounded by a fibrovascular stroma. Multinucleated cells were absent. Necrosis within the tumours was observed, but inflammatory cells

were rare. The invasion of the myometrium was not a constant feature. Vessel or lymphatic tumoural emboli were not found in available cases.

Moreover, the present study also characterized the morphology and immunoprofile of FEA of the papillary serous type, regarding a broad panel of antibodies, frequently used in routine diagnostic procedures. In fact, results presented herein will contribute to improve the histopathological diagnosis of FEA. Immunohistochemistry is a current tool to determine antigens in formalin-fixed, paraffin-embedded tissues, which principles and procedures apply across mammals (Ward and Regh, 2014). Furthermore, it is routinely performed in most laboratories of veterinary anatomical pathology to increase the diagnostic accuracy, because of its easiness, safeness and relative low cost (Millanta et al., 2005). The immunolocalization of cell structures and organelles is a fundamental attribute to morphology-based investigation (Leong et al., 2010). Another important trait of immunohistochemistry is the evaluation of over- or underexpression of several proteins (i.e. c-erbB-2, ER and PR) targeting cancer therapies (Leong et al., 2010). These features motivated the use of immunohistochemistry in the present work, aiming to provide additional markers for FEA histological diagnosis and for a better understanding of the feline endometrial cancer biology.

The use of controls and a critical detailed interpretation of the immunohistochemistry results are of the utmost importance to take full advantage of this technique (Leong et al., 2010; Ward and Regh, 2014). In the present work, all the used antibodies were also tested in positive controls, both external and internal. Moreover, normal uterine samples were also tested as controls (since for most markers the information on the normal pattern in the cat uterus was scarce) for comparison with the expression pattern of those markers in FEA tissues. This allowed to describe the immunohistochemical expression of a broad panel of antibodies in the normal cycling feline uterus and to determine the differences observed in diseased uteri.

As previously mentioned, the knowledge on FEA is rather limited. Some reports described the immunohistochemical expression of ER-α, PR, CK7, CK20 and COX-2 in small case series (Espinosa de los Monteros et al., 1999; Gil da Costa et al., 2009), often without a differential evaluation of the two endometrial compartments (epithelium and stroma) or of the myometrium. On the contrary, these molecules are largely studied in women endometrial carcinoma, with proved

importance as diagnostic and prognostic markers, together with Ki-67 and c-erbB-2 (Chu et al., 2000; Ferrandina et al., 2002; Prat, 2004). These evidences motivated the choice of antibodies used in the present study. To our knowledge herein the expression of Ki-67 and c-erbB-2 in FEA and in cyclic endometrium are described for the first time (CHAPTERS 3 TO 5).

CHAPTER 3 reports the expression of ER- α and PR in normal endometrium (epithelial and stromal compartments) and myometrium. It was shown a small decrease in the intensity of immunostaining of ER-α in the luteal stage compared to follicular stage, suggestive of the suppressive effect of progesterone receptor activation. Similarly, there was a small decrease in the percentage of positive epithelial and stromal cells to PR, from the follicular stage to the luteal stage. Our results indicate that stromal and epithelial compartments of the endometrium respond differently to steroid hormones, as it was suggested before in the mare (Aupperle et al., 2000); further, it is also currently accepted that a crosstalk exists between both compartments, influencing each other proliferation and differentiation (Pierro et al., 2001).

In FEA, a loss of immunoexpression of ER-α was observed, indicating that endometrial cells in FEA possess reduced ability to respond to estrogens. A slightly non-significant decrease was observed in the PR immunoexpression, suggestive that those cells retain the ability to respond to progesterone stimulation (CHAPTER 3); therefore, it can be speculated that, in animals with clinically undiagnosed FEA, putative progestagen contraception may interfere in the evolution of FEA. This issue deserves further investigation.

The reduced expression of ER-α and PR-A, particularly in neoplastic stromal cells, may be important in predicting invasiveness of women endometrial carcinomas (Kreizman-Shefer et al., 2014). Similarly, in the present study (CHAPTER 3) the negative status for ER-α expression in the stromal cells was associated with myometrial invasion, a feature usually related to malignancy. Also, lower myometrial PR levels compared to ER were found in high-risk human endometrial carcinomas (Tomica et al., 2014). Herein, a loss of positive cells for PR in the myometrium of FEA was also reported, in relation to higher nuclear atypia in carcinoma cells, suggesting that PR may be related to tumour dedifferentiation. Results presented in CHAPTER 3 also suggest an intimate interaction between the components of the endometrium - epithelium and stroma - as well as between these and the

myometrium, likewise the relation between cancer cells and the surrounding tissues described in human uterus as a required event for endometrial normal functioning and carcinogenesis (Yang et al., 2011).

In the normal endometrium, Ki-67 immunoexpression recapitulated the morphological features that characterize each stage in the cycling endometrium. In the follicular stage, the branching of the upper area of the glands was observed, whereas in the luteal stage, the basal glandular area is coiled (Chatdarong et al., 2005). Therefore, proliferative indexes were expectably higher in the superficial glandular epithelium during the follicular stage while in the luteal stage the higher proliferative indices were found in the deep glandular epithelium. However, no differences were established between the overall Ki-67 expression in the two evaluated phases of the estrous cycle (CHAPTER 3).

The proliferative index determined by Ki-67 immunoexpression was remarkably higher in FEA than in normal endometrial epithelia (CHAPTER 3). In women endometrial carcinomas, Ki-67 positively correlates with the histological grade, depth of myometrium invasion and risk of carcinoma recurrence (Prat, 2004), and is inversely related to hormonal receptor status, particularly in higher grade, ER-α negative, endometrial carcinomas (Ferrandina et al., 2005; Mingzhu et al., 2014; Tangen et al., 2014). However, those association between Ki-67 expression and histopathological features were not found in FEA, during the present studies.

Estrogen receptor alpha may be induced in estrogen-driven tumours, and tumour growth is often limited by progesterone, once ER expression is down-regulated by activated PR (Leslie et al., 2013). Herein, it was demonstrated that Ki-67 expression was higher in FEA than in normal endometrium similarly to the reported for women tumours (Kreizman-Shefer et al., 2014). Since epithelial cell proliferation may be stroma-mediated through VEGF production (Koos, 2011), future studies on the expression of this and other growth factors expression in FEA are needed. Vinci and collaborators (2010) speculated that PR expression is not directly involved in endometrial epithelial carcinogenesis (Vinci et al., 2010), a feature also hypothesised in the current study. Contrary to the described in women (Ferrandina et al., 2005; Mingzhu et al., 2014), no significant correlation was found between ER and PR in FEA.

Feline normal endometrium showed a CK7+/CK20+ immunoprofile, with a higher overall expression of CK7 (CHAPTER 3). Very limited knowledge exists

concerning cytokeratins' immunoprofile of feline normal endometrium. However, CK7 and CK20 expression is highly conserved during malignant transformation in human cancer (Moll et al., 2008), which could be important to the diagnostic procedure of primary epithelial neoplasms.

Feline endometrial adenocarcinomas under study conserved the CK7+/CK20+ phenotype observed in normal endometrium (CHAPTER 3). Our results are in accordance to those of Espinosa de los Monteros and collaborators (1999). Despite the different immunohistochemical profile (CK7+/CK20-) present in women endometrial carcinomas, conservation of the normal endometrium phenotype is an important tool for diagnosis (Moll et al., 2008). Importantly, the immunophenotype of FEA determined in the present study can be an important marker for endometrial origin, since the uterus is a possible metastatic site for tumours located elsewhere, including mammary neoplasms (Travassos, 2006), which are morphologically similar to primary FEA. However, contrary to feline primary endometrial neoplasms, feline mammary gland carcinomas are generally negative for CK20, maintaining the immunophenotype of the normal mammary gland in this species (Espinosa de los Monteros et al., 1999).

Cyclooxygenase-2 was constitutively expressed in feline normal uterus, as it was also reported for women (reviewed by Rouzer and Marnett, 2009). Our results support the descriptions by Gil da Costa and collaborators (2009), and further enhance that COX-2 is also expressed in feline endometrial stroma. In normal endometrial epithelial cells, we found an apical reinforcement of COX-2 immunolabelling. Epithelial expression of COX-2 was higher in follicular stage than in luteal stage. Conversely, the immunoexpression of this molecule was higher in the stroma in the luteal stage than in the follicular stage. Data presented in CHAPTER 4 suggest a progesterone influence on COX-2 expression, which might be associated with physiological tissue remodeling accompanying the glandular development (Saraiva et al., 2015).

In FEA, we observed a loss of cell polarity with a dislocation of COX-2 labelling to the cytoplasm and perinuclear area of epithelial cells (Saraiva et al., 2015). Loss of COX-2 compartmentalization in FEA supports earlier findings (Gil da Costa et al., 2009). In women endometrial carcinomas, COX-2 is overexpressed reported (Nasir et al., 2007; Tong et al., 2000). On the contrary, we found a decrease in COX-2 expression in FEA. The reasons proposed for this difference included:

variances in the types of human and feline tumours, the uterine hormonal environment, the scoring systems or the antibodies used (Saraiva et al., 2015).

Cytoplasmic pattern of expression of COX-2 is described in women endometrial carcinomas (Ferrandina et al., 2002, 2005; Lambropoulou et al., 2010; Nasir et al., 2007) and is often related with poor prognosis (Kim et al., 2010). However, COX-2 inhibition was proved to decrease growth, migration and invasion of immortalized human endometrioid epithelial and stromal cells (Banu et al., 2008). The results presented in the present study suggest that FEA are apparently low aggressive tumours, despite cytoplasmic expression of COX-2 (Saraiva et al., 2015). The perinuclear pattern of COX-2 immunolabelling was previously described in feline transitional cell carcinomas of the urinary bladder and mammary carcinomas (Beam et al., 2003; Sayasith et al., 2009). This feature may be related with prostaglandins pathways involved in cell growth, apoptosis or cell differentiation, including malignant transformation (Sobolewski et al., 2010; Vane et al., 1998).

The cyclic variations in the immunoexpression of the c-erbB-2 oncoprotein in feline normal uterus were demonstrated herein for the first time. As shown in CHAPTER 5, the expression of c-erbB-2 in the epithelial cells of the endometrium in the follicular stage was higher than in the luteal stage, especially concerning deep glandular epithelium. These results are not in accordance to the previously referred concerning Ki-67 status of normal feline endometrium, suggesting that in this tissue, proliferation might not be related to c-erbB-2 pathway. Van Dam and collaborators (1991) proposed that higher amounts of c-erbB-2 may increase proliferation for a given amount of released growth factor (Van Dam et al., 1991). Therefore, further studies on growth factors expression in feline endometrium may clarify this topic.

In FEA, c-erbB-2 was expressed in less extend than in the follicular stage of normal endometrium (CHAPTER 5). However, no differences were established between c-erbB-2 oncoprotein expression in FEA and the luteal stage. Currently it was not possible to clarify the role of c-erbB-2 in FEA, however, its decreased expression suggests some influence of this oncoprotein in feline endometrial carcinogenesis. Similarly to the results reported for women endometrial carcinomas (Ferrandina et al., 2005; Togami et al., 2012), no associations were found between histopathological data and c-erbB-2 status of FEA. Therefore, c-erbB-2 expression seems not to be related to poor clinical outcome of these tumours in queens, as it

has also being described for feline mammary neoplasia (Rasotto et al., 2011). Alike in the study of Rasotto and collaborators (2011), the results of the current study did not support the existence of an association between c-erbB-2 status and Ki-67 expression. In order to acknowledge the preliminary results on c-erbB-2 immunohistochemical expression presented herein, additional studies are required, namely for determination of gene amplification.

Conclusions and Future Directions

In conclusion, FEA despite being uncommon in proportion to the total of tumours diagnosed in cats, are more frequent than we initially thought. Concerning the uterine neoplasms of the queens, FEA are even the tumours most commonly diagnosed, the papillary serous type being the most frequently seen. These tumours are characterized by the papillary proliferation of neoplastic cells of serous type, accompanied by clear and multinucleated cells. Other architectural arrangements mainly included glomeruloide, solid and tubular growth. Randomly distributed areas of necrosis within the tumours are commonly observed. Invasion of the myometrium, of the serosa and of the vascular and/or lymphatic vessels are not constant features. The mean number of mitoses is higher in FEA compared to non-neoplastic endometrium. Whether these histopathological findings are related to poor prognosis in FEA is a subject of interest in future studies.

The results presented herein support that immunohistochemistry can be a helpful tool to the routine diagnosis of FEA. These neoplasms markedly lose the expression of ER-α compared to normal endometrium, though the loss of PR is not so remarkable. Additional studies are required to understand the prognostic and therapeutic implication of hormonal receptors status in FEA.

Proliferation indexes increase in FEA, compared to normal endometrium. The antibody against Ki-67 is routinely used to access proliferation status of tumours and can be related to a worse prognosis. Further investigation on FEA is important to additional information regarding this issue. Other markers of proliferation should be included (e.g. PCNA) and the follow-up of queens diagnosed with FEA is needed.

As in normal tissues, FEA maintain the CK7+/CK20+ profile, however they lose some expression of these filaments. This feature can be used as an important marker of primary endometrial origin, excluding uterine metastases of epithelial neoplasm with any other origin.

Interestingly, we assist to a different subcellular localization of COX-2 in FEA, compared to the apical membrane reinforcement in normal epithelium. Therefore, COX-2 is very likely involved in endometrial carcinogenesis. To determine at which level this occurs, additional studies are needed, namely in the quantification of the protein in normal and tumour tissues.

Finally, c-erbB-2 immunolabeling is seen in the majority of FEA, however a decrease in the expression of this protein is observed when compared to the follicular stage of normal endometrium and no differences are noticed between tumours and normal luteal stage. Although it is possible taht c-erbB-2 plays a role in feline endometrial carcinogenesis other than proliferation, however it remains unclear. Further studies using fluorescent *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH) or silver-enhanced *in situ* hybridization (SISH) to determine HER2 status should be used in complement to immunohistochemistry.

In the future, additional studies on molecular characterization of FEA can improve the knowledge of the mechanisms underlying the disease. Advanced molecular techniques are now available and we can benefit from its use to enhance our findings. Therefore, we hope that this work goes towards a profitable journey.

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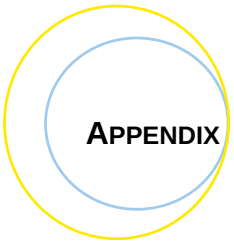

APPENDIX

FELINE ENDOMETRIAL ADENOCARCINOMA IN FEMALES <1 YEAR OLD: A DESCRIPTION OF FOUR CASES

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FELINE ENDOMETRIAL ADENOCARCINOMA IN FEMALES UNDER ONE YEAR-OLD: A DESCRIPTION OF FOUR CASES

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ABSTRACT

Uterine neoplasms of epithelial origin are rare in cats and most often are described in older females. Yet, in less than two years, 4 ovariohysterectomy specimens were submitted from different practices to the Laboratory of Histology and Anatomical Pathology, at UTAD (Vila Real, Portugal), that emitted a diagnosis of feline endometrial adenocarcinoma. Untypically, all the females were aged under one year old at the surgery. Access to the clinical files was requested to document the clinical features of the four cases, including any complementary data available, to construct the present case reports. The clinical situation developed with discrete signs, but vulvar discharge was present in 3 cases, ranging from bloody to brownish or colourless, and from purulent to mucous. The females were in dioestrus, though the estrus remained unperceived in most cases. In this study, the four clinical situations are described and discussed on the basis of available literature, highlighting the aspects that may impair an early diagnosis and that may favour the progression of the disease and also that age should not be an excluding criteria when analysing the differential diagnosis list.

KEY-WORDS

Feline endometrial adenocarcinoma; endometrial tumour; uterine tumour; epithelial tumour; female cat

INTRODUCTION

Inflammatory lesions in dog and cat endometrium are far more frequent than tumours. Among tumours, it is also believe that mesenchymal tumours are found more regularly than the epithelial ones (Kennedy et al., 1998; Papparella and Roperto 1984; Sapierzynski et al., 2009).

Feline endometrial carcinoma (FEA) has been considered a rare malignant condition that more frequently develops in the uterus of old females (Klein 2007; McEntee, 1990; Morris and Dobson, 2001). Dubious information exists concerning its association to contraceptive progesterone-based treatments (Miller et al., 2003). Yet, recently one case of feline endometrial adenocarcinoma has been described in a young, 2 years old, FeLV positive female cat (Cho et al., 2011). The routine ovariohysterectomy practiced in cats has been described as protective from uterine neoplasia (Miller et al., 2003), although this may not be completely true since FEA may develop from the uterine stump (Anderson and Pratschke, 2011). In most frequently described situations, uterine adenocarcinomas developed silently, the clinical signs for FEA being vague and unspecific, hampering the early diagnosis of endometrial carcinoma. The clinical condition becoming visible when metastatic disease originates the first clinical symptoms in older female cats (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964). Nevertheless, pyometra symptoms are often described in association to FEA. This feature raise the suspicion that FEA occurrence may be higher than the described (Sapierzynski et al., 2009; Saraiva et al. 2012a; Taylor, 2010), considering that insufficient histopathological evaluation of the surgical specimens may occurs whenever pyometra-like changes are observed in the excised genital tract.

The present report describes the clinical signs, the ultrasonographic images and the histopathological findings of feline uterine adenocarcinoma in four young domestic shorthaired queens, aged under one year old, which were never submitted to exogenous progestagens. They are an example of untypical occurrence of an endometrial epithelial tumour in very young animals, which only recently started their cyclic activity. The major clinical sign was the existence of a discontinuous vulvar

discharge that in one case was assumed to be a pyometra. In another situation, no discharge was ever reported. For all the four cases, the histopathological analysis excluded abortion and evidenced the existence of serous papillary adenocarcinoma of the endometrium.

Case Presentation

Clinical presentation

In a twelve months period (from May 2011 to May 2012) four cases of feline endometrial adenocarcinoma in female cats under one year of age were submitted to the Laboratory of Histology and Anatomical Pathology at UTAD (LHAP-UTAD), Vila Real. They all came from different practices in the north of Portugal that also provided the available clinical information. Clinical symptoms ranged from absent to a haemorrhagic or brownish discharge easily confused to pyometra or abortion. For most the situations, the owners were unaware of the existence of regular oestrous activity. Nevertheless, in all the cases, active corpora lutea were found in the ovaries, and the oestrous cycle was staged as dioestrus. A distended, firm uterus in such young individuals was the motive for submitting of the excised genitalia to the histopathology laboratory that emitted the final diagnosis of feline endometrial adenocarcinoma of the papillary type.

Table 1: Selected clinical details of the 4 cases of feline endometrial adenocarcinoma in animals under one year old.

Case number	Region	Breed	Age	Main clinical complaint at surgery	Last evaluation post-surgical
1	Vila Real	Domestic shorthair	8 months old	Intermittent bloody vulvar discharge	12 months – uneventfully
2	Águeda		7 months old	No clinical symptoms	8 months – uneventfully
3	Aveiro		10 months old	Dark vulvar discharge	15 months – after an 8-month period, an intermittent bloody vulvar discharge appeared
4	Vila Real		11 months old	Intermittent mucous vulvar discharge that sporadically turns reddish in colour	14 months - uneventfully

Case 1

A domestic shorthair female cat with 8 months old was presented to consultation at the veterinary practice #1 (Vila Real, Portugal), with complaint of episodic bloody vulvar discharge for the preceding two weeks. The animal had been recently adopted from a free-ranging rescue program, and was not adequately vaccinated or dewormed. The queen was found in good physical condition, alert and with normal appetite. The previous reproductive history for the animal was unclear. When inquired, the owner ignored if she had already passed her first oestrus. Besides the periodic bloody discharge, no other symptoms were observed.

At the physical examination the queen fur was dull, with symmetrical, bilateral areas of alopecia in the neck (suggestive of previous breeding), and in the perivulvar area the hair was stuck and stained in dark red. At the moment, no vulvar discharge was observed. The body temperature was 39 °C, the heart and respiratory rates were within normal limits and the capillary refill time under 2 seconds. There were no indicators of dehydration. Abdominal palpation showed a slightly increase of the uterine volume, but was not associated neither to an increase in the vulvar discharge nor to abdominal pain.

A basic complete blood count (CBC) and preliminary biochemistry analysis were performed (TABLE 2). Haematological changes included an increase in the leucocyte counts due to neutrophilia and eosinophilia. The haematocrit was near the lower reference value for the species (30.8%), and the biochemical parameters were within normal range values, with only an increase in ALT (204U/L; reference values = 10 to 75 U/L).

Abdominal ultrasonography showed a slightly enlarged, compact uterus. The uterine horns were faintly lobulated (FIGURE 1). Increased thickness of the uterine walls extended from the corpus to both cornua. In US images the thick walls nearly apposed, only separated by a very thin anechoic line corresponding to the uterine cavum, which was not distended by fluid (FIGURE 1).

Given the clinical signs and on ultrasound observations, together with the possibility of mating in its putative first oestrus, the differential diagnoses raised for the bloody discharge was abortion and endometrial adenocarcinoma. The occurrence of intermittent episodes of bloody discharge directed the diagnosis towards FEA, as for abortion it would be expected the vulvar discharge to be more frequent. It was further supported by the observation of uterine wall thickening in the

absence of fluid accumulation. The final tentative diagnosis was feline endometrial adenocarcinoma and ovariohysterectomy was advised.

Twelve months have past from the surgery, and no signs of relapses have been reported.

TABLE 2: Haematological and biochemical parameters at admittance for the queen in case 1.

PARAMETER (UNITS)	VALUE AT ADMITTANCE	LABORATORY REFERENCE VALUES
Haematocrit (%)	30.8	30–45
RBC (91012/l)	6.48	5–10
WBC (9109/l)	**25.12**	5.50–19.50
Neutrophils (x10^9/l)	**21.03**	2.5–12.5
Eosinophils (x10^9/l)	**1.47**	0.10–0.79
Lymphocytes (x10^9/l)	1.65	0.4–6.8
Monocytes (x10^9/l)	0.9	0.15–1.70
ALT (U/l)	**204**	10–75
BUN (mM)	13.03	10.71–21.42
Creatinine (μM)	72.49	74.26–180.34
Total proteins (g/l)	64.1	60–82
Albumin (g/l)	26.7	25–39

Values outside the reference values are in bold.

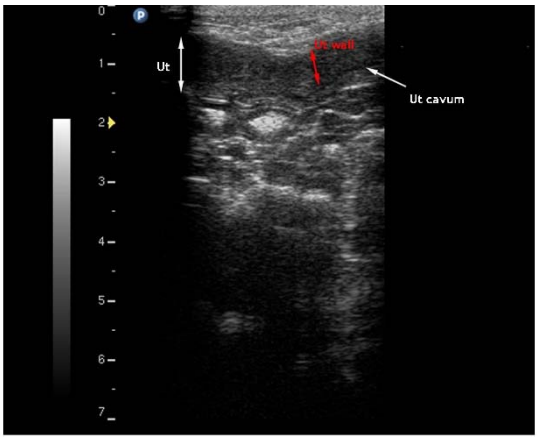

FIGURE 1: Abdominal ultrasound image at the day of admittance showed uterine (Ut) enlargement (white double-ended arrow), due to increased uterine wall thickness. In some scans, the anechoic uterine cavum (white arrow) presented an eccentric position as consequence of asymmetrical uterine wall width (red double-ended arrow).

Case 2

A 7 months old domestic shorthair female cat was presented to consultation

at the veterinary practice #2 (Águeda, Portugal), for routine OVH. Except for a respiratory disease episode and stromal keratitis associated to herpesvirus infection when she was a baby, this queen was healthy and never evidenced signs of uterine disease. Nevertheless, the growing rate was slower than the expected and she was a small queen for the age. According to the owner, no oestrus signs have been perceived previous to surgery. At admittance for surgery, the temperature was 38.3ºC and the heart and respiratory rates were within the normal parameters; the physical examination was declared normal, and no complementary analyses were required. At surgery, the uterus was found enlarged and slightly thickened and thus assumed to have a pyometra. Due to her age, the altered excised genital tract was sent for analysis.

From the surgery to this moment (10 months), the female health remains uneventfully. After the histopathology diagnosis, a set of complementary methods for detection of putative metastases was requested: blood analysis (Haematological and biochemical parameters), urinalysis, abdominal and thoracic radiology and ultrasonography. The results for the analysis were within normal values and imagiological exams devoid of evidences suggesting the presence of metastases.

Case 3

A 10 months old domestic shorthaired female cat was presented to consultation at the veterinary practice #3 (Aveiro, Portugal), with complaints of intermittent brownish vulvar discharge. The female was adopted while a baby. Previous medical history included episodic gastrointestinal problems and slow growing rate. The owner could not provide information on the occurrence of previous oestrus. The physical examination showed the perivulvar fur soiled in dark. The owner had noticed an intermittent dark vulvar discharge not associated to any clinical symptoms, but disregarded it. The temperature was within normal values for the species (38.0ºC), as well as the heart and respiratory rate and the capillary refill time. Due to economic constraints, complementary analyses were not performed, and ovariohysterectomy was decided under the suspicion of pyometra. At surgery, the uterus was found enlarged, firm and fluid-filled, and presented small nodules projecting towards the serosa. Due to its striking appearance the histopathological analysis was requested.

Surgery recovering was uneventfully, but eight months after the OVH the

female was presented once more due to recurrence of the intermittent bloody discharge. The haematology and biochemistry analysis were within the reference range for the species. At ultrasonography, no signs of intra-abdominal mass were found and the uterine stump was not visible. Vaginal cytology was requested but the material was insufficient for diagnosis. Afterwards, the owners elected against any additional intervention, even if maintaining the female under vigilance.

Case 4

An 11 months old domestic shorthaired female cat was presented for consultation at the veterinary practice #4 (Vila Real, Portugal), for routine OVH. The female exhibit her first heat three months prior to surgery. No additional oestrus was detected since. The main complaint was the existence of intermittent mucous vulvar discharge for the past 2 to 3 weeks, which occasionally was reddish in colour. Previous medical history is unknown. No contraceptive treatment was performed at home. Occurrence of male contact was possible, as the female had free access to a garden. At consultation, the heart and respiratory rates were within the normal parameters for the species, but the body temperature was 39.7ºC. Due to economic constraints, the complementary diagnostic assessments (blood analysis and ultrasound) were not requested. The preliminary tentative diagnoses were pyometra and abortion, and surgery was recommended. Fourteen months over the surgery the animal is in good fitness and no signs of genital disease were perceived.

HISTOPATHOLOGICAL EVALUATION

All the four surgical specimens were received fixed in 4% buffered formalin and analysed at the LHAP-UTAD, under routine haematoxylin-eosin staining. The uterus was longitudinally cut for better examination, and 4 to 7 blocks were prepared to cover a representative area of the altered morphology and neighbouring zone. None of the lesions showed a nodular pattern, but rather extended through both uterine horns. On TABLE 3, it is provided a brief summary of the content of histopathological records.

The major macroscopic finding was the uterine enlargement (FIGURES 2 A-D). In all the situations, a two to five-fold increase in the normal uterine horn diameter was present. Also, changes in the usual trajectory for the cat uterine horns were found, changing from slightly lobulated (cases 1 and 4; respectively FIGURES 2 A-D)

Table 3: Selected macroscopical details of the 4 cases of feline endometrial adenocarcinoma in queens under one year old.

Case number	Ovaries (cm)	Uterine size (cm)	Uterine content	Endometrium	Haemorrhage	Myometrium invasion	Rupture
1	0.9 x 0.6 x 0.5 3 regressing CL	5 x 1.8 5.5 x 2	No	Increased wall tickness; Some areas of hyperplasia	Sporadic foci, mainly cranially	Yes	No
2	1.0 x 0.5 x 0.4 1 regressing CL	10 x 1.1 8 x 1	Yes (purulent)	Increased wall tickness	No	No	No
3	1.2 x 0.6 x 0.4 4 mature CL	10 x 2 12 x 2	Yes (purulent)	Increased wall tickness; Some areas showing endometrial atrophy	No	Yes On umbilicated areas	Yes
4	1.0 x 0.6 x 0.5 3 mature CL	8 x 2.5 10 x 2	Yes (purulent)	Increased wall tickness; Atrophic endometrium in the apex areas	Yes Focal	No	No
Normal pattern *	0.8 x 0.7 x 0.5 (smaller) 1.3 x 0.8 x 1.0 (larger) 2 to 6 CL	4 X 0.5 (smaller) 6 x 0,7 (larger)	No	Marked coiling of the endometrial glands, leading to increased development of the deeper endometrial stratum	Non-existent		

*Values obtained from 5 queens in dioestrus, without ovarian or uterine disease, with similar age range, selected from the LHAP archives, and correspond to the minimum and the maximum recorded dimensions; for these control samples the 2 uterine horns were similarly sized. For the diseased specimens, only the dimensions for the largest ovary were given, whilst for the uterus the dimensions of the 2 uterine horns are presented. CL = Corpora lutea.

to moderately tortuous (cases 2 and 3; FIGURES 2 B-C, respectively). The uterine horns were firmer than the usual. The uterine wall was smooth (FIGURES 2 A-B and 2 D), except for case 3 that presented several umbilicated nodular areas spotting in both uterine horns (FIGURE 2 C). Limited collection of purulent fluid was also found for cases 2 and 3. In case 4, both uterine horns presented portions of vesicular distension of the uterus, filled of purulent content, intermingled with areas of increased density (FIGURE 2 D).

On transversal cuts of the uterine horns and body, an increased thickness of the endometrium was the major evidence (FIGURES 2 E-H). For most cases it extended through the entire wall. Restricted areas of uterine dilatation were present only in cases 2 and 4, which in the former was limited to the apex of one uterine horn.

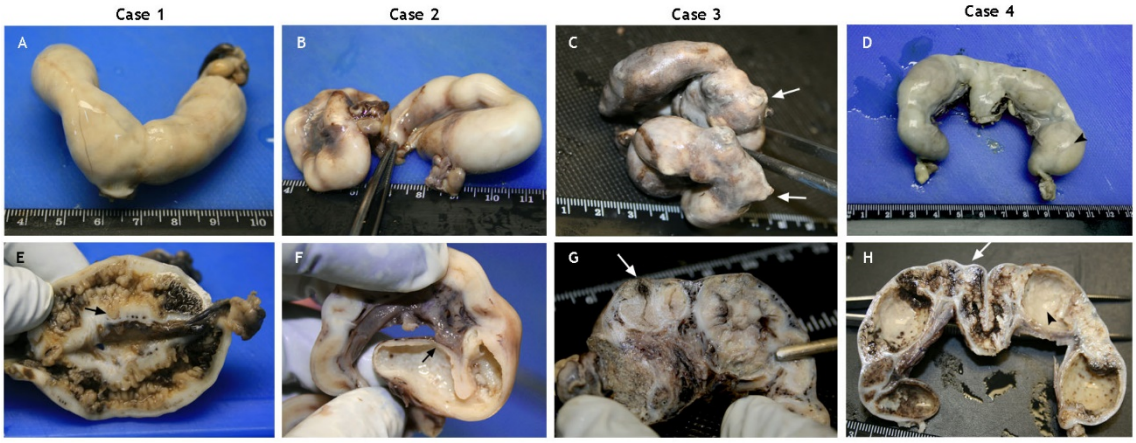

FIGURE 2: Macroscopic features of the Feline Endometrial Adenocarcinoma: A-D – Gross external morphology of the uterus in the cases described. A. Asymmetrical, nearly regular and bilateral enlargement of the uterine horns. B. Irregular enlargement of the uterine horns showing an increase in size in the cranial portion of the uterus; the ovary is laterally displayed. C. Enlarged uterine horns of irregular surface with exofitic prominences (arrows). D. Bilateral enlargement of both uterine horns showing 3 vesicular dilatations (arrowhead). E-H. Internal morphology of the uterus shown in sagittal sections. E. Uniform thickness of the endometrium, and necrotic/haemorrhagic areas (black tissues) were observed, along with endometrial areas invading the myometrium (arrow). F. In the dilated portions of the cranial uterine horn, it was observed the thinness of the wall (arrow) and nodular irregular proliferations, while in the other portions the uterus showed increased thickness. G. Increase thickness of the uterine wall showing haemorrhage and invasion of the myometrium up to near the serosa (arrow). H. Alternate disposition of the wall thinness matching the vesicular areas of dilatation (arrowhead) and thickness with exofitic endometrial proliferation (arrow) being more pronounced in the uterine body.

In areas of increased thickness, the uterine wall displayed yellowish proliferation of the endometrium, which seemed to deepen in some areas towards the myometrium (FIGURES 2 E and 2 G). In sections devoid of fluid, the uterine cavum was narrow, and opposing endometrium was closer than the normal. Further, it was perceived a papillary growth of the endometrium into the narrowed uterine lumen. Haemorrhage was sporadically found within both uterine horns for cases 1 and 4 (FIGURES 2 E and 2 H).

The ovaries presented a variable number of corpora lutea, attesting for the previous occurrence of an oestrus and possible mating.

Histologically, uterine sections evidenced an extensive branched papillary proliferation of the endometrium, which were displayed in an irregular arboriform papillae and a complex cystic patterns arising from both the superficial and deep glandular endometrial epithelia (FIGURES 3 A-D). The tumour cells were columnar and loosed their polarity towards a thin stromal axis and frequently display a stratified disposition (FIGURE 3 E). Only for case 2, the deep endometrial glandular layer remained closer to normal, even if presenting serous, proteinaceous content. For case 1 and 3, areas of glomeruloid differentiation (FIGURES 3 C and 3 G) were reported, with multinucleated and aberrant cells. The stroma adjacent to the adenocarcinoma lesions exhibited a desmoplastic reaction (FIGURE 3 A). Epithelial cells in the tumour showed anisocytosis, anisocariosis and intense cell pleomorphism (FIGURES 3 E-F), evidenced by severe atypia, irregular chromatin clumping, and aberrant, prominent nucleoli. Atypical mitoses were reported in cases 2, 3 and 4, even if mitotic activity was limited to 1 to 3 mitoses per 40x field.

The stromal axis and the endometrial stroma contained scattered inflammatory cells infiltrate, whose number was increased in areas with co-existing pyometra (cases 2 to 4). Pyometra with decreased thickness of the endometrium was limited to the cranial apex of one uterine horn in case 2, and to the dilated portions of the uterine horns in case 4. On the remainder portions of the uterus, as for the others cases, endometritis was referred in the histological report.

Myometrial invasion by the tumour was found in cases 1 and 3. Yet, in case 3, invasion of the myometrium was more pronounced, being particularly evident in the nodular areas where focal infiltration of the uterine wall by tumour cells reached the uterine serosa and was accompanied by granulation tissue proliferation. In none of the cases vascular permeation was mentioned.

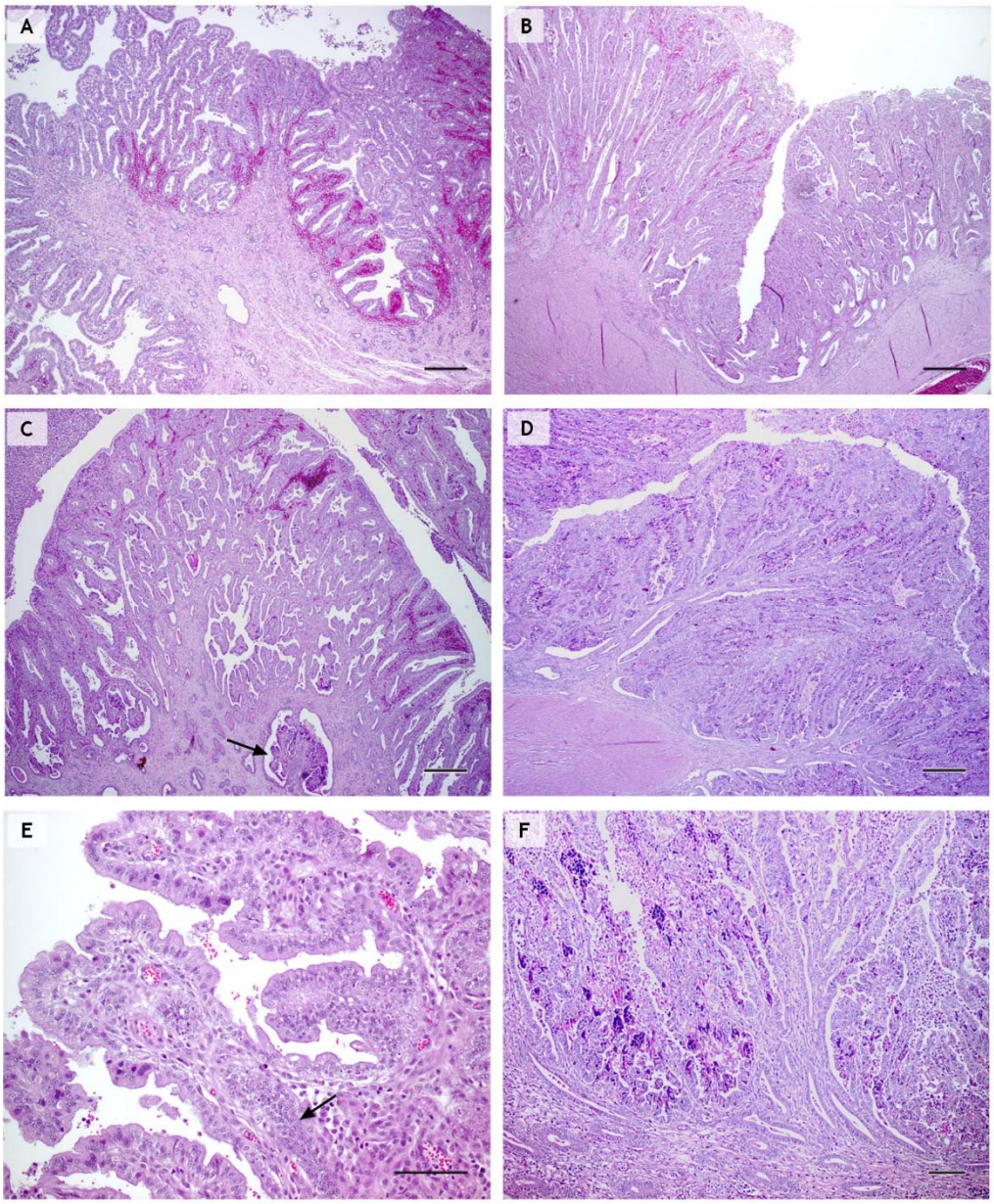

FIGURE 3: Microscopic features of Feline Endometrial Adenocarcinoma. [Hematoxilin and eosin; A-D: lower magnification – bar= 300µm; E,F: moderate magnification – bar= 100µm] A. Extensive and irregularly branched papillary proliferation of the endometrium, supported by a central conjunctive stroma; haemorrhages were observed in the superficial area (Case 1). B. Irregular, parallel papillary proliferation of the endometrium, with inward areas of solid and glomeruloid pattern (Case 2). C. Severe disarrangement of the endometrial glands that presented irregular papillary ingrowths with prevalent glomeruloid pattern; sub-surface haemorrhage was evident (Case 3). D. Proliferation of the endometrial glands into a solid and papillary pattern supported by a central fibrovascular stroma; the increased density of the cells lining the papilla is characteristic of anisocariosis. Mucous, cellular debris and neutrophils were found in the lumen (Case 4). E. The papillary ingrowths were often lined by epithelial cells displaying stratified disposition (arrow). F. Detail of D tumour showing anisocytosis, anisocariosis and cellular pleomorphism, severe atypia, and aberrant and multinucleated cells. Apoptosis and necrosis were present.

In addition, areas of haemorrhage were found in cases 1 and 4, as well as areas of papillary hyperplasia, peripherals to the tumour. The endometrial carcinoma extended throughout the uterus and was invading the oviducts in case 3. For all the four specimens, the histological diagnosis was primary feline endometrial adenocarcinoma of serous papillary type.

Discussion

Epithelial tumours of the feline uterus are considered rare (Cotchin, 1964; Kennedy et al., 1998; McEntee and Nielsen, 1976). Yet, incidence of feline endometrial adenocarcinomas may be underestimated due to the current practice of spaying young female cats. However, this procedure may not guarantee freedom from this disease, since some reports exist on adenocarcinoma in the uterine stump of spayed cats (Anderson and Pratschke 2011; Miller et al., 2003). Most frequently FEA are reported in older queens, over 9 years of age (Belter et al., 1968; Klein 2007; McEntee, 1990; Morris and Dobson, 2001; Preiser, 1964), and described clinical signs often correspond to the existence of metastasis (Anderson and Pratschke, 2011; Belter et al., 1968; Preiser, 1964). Nonetheless, in a recent report, a case of uterine adenocarcinoma has been described in a young 2 years old Persian queen with feline leukaemia virus infection (Cho et al., 2011); the authors raised the hypothesis that immunodepression associated to the viral infection would contribute to the process of tumorigenesis.

In the cases described herein, the animals were yearling females, with ages ranging from 7 to 12 months that recently acquired sexual maturity. All of them had access to the exterior and contact to other cats. None of the females was under contraceptive treatment. Unfortunately, at the time of surgery the animals were not tested for any of the most current feline virus (such as FeLV and PIF) and it was not possible to ascertain for a possible involvement of a viral infection on the carcinogenic process. Yet, in one of the cases described (Case 2), herpesvirus infection was referred earlier in the cat life.

If, in particular situations, some retrovirus have being implicated as trigger for proliferative diseases (Khan et al., 1993), it might also be needed that, in regard to the uterus, a specific endocrine environment must exit. In fact, in the report by Belter et al. (1968) and the one by Cho (2011) the diseased animals were in dioestrus, and

displayed corpora lutea in the ovaries, as it was the case for the 4 females from the present study. Also Sontas et al. (2013) refer to the existence of luteal cysts (that might be cavitary corpora lutea from recent ovulation) in one of the females with FEA. The work of Saraiva et al. (2012b) showed that all the fifty FEA analysed were positive for the progesterone receptor, supporting the earlier observations by Gil da Costa et al. (2009) that found progesterone receptors in 5 of 6 endometrial adenocarcinoma samples. In two of the cases described in previously neutered queens administration of exogenous progesterone for a variable period prior FEA diagnosis was reported (Anderson and Pratschke, 2011; Miller et al., 2003). Such findings raise the question on the eventual role for progesterone in the invasiveness and progression of the epithelial tumours of the cat endometrium, highlighting the fact that the species is an induced ovulator. Taken together, the above-mentioned information suggests that progesterone may favour the abnormal proliferation of the endometrial glands that, with an adequate stimulus (ex. a viral infection), might become uncontrolled. In the cases described herein, the number of oestrous cycle with a luteal stage was limited by the animal age, and no contraceptive treatment was previously used. So it is improbable that the endometrial carcinoma may be associated to prolonged progesterone overstimulation of the uterus. Still, cats are prone to present highly proliferative lesions in the mammary glands under progestagenic stimulation - mammary fibroepithelial hyperplasia (Johnston et al., 2001b) – suggesting that the species may be particularly sensible to progesterone influences, even if no evidences exist about those hyperplasic reactions (mammary or uterine) may acting as pre-neoplasic phenomena.

Despite being rarely reported, FEA are believed to be locally invasive, and the occurrence of metastasis are commonly reported and often associated to the development of FEA symptoms (Anderson and Pratschke, 2011; Belter et al. 1968; Preiser, 1964). In the absence of metastatic disease, FEA clinical signs are often discrete and the disease frequently develops silently. When analysing the available FEA descriptions (Anderson and Pratschke, 2011; Belter et al., 1968; Miller et al., 2003; Sapierzynski et al., 2009), it seems that clinical signs in this kind of tumours may vary with the size of the neoplasic lesions and the age of the process, which might determine the most prevalent clinical sign.

In the absence of metastases, vague and unspecific signs of uterine disease are often reported, such as infertility (Miller et al. 2003), weight loss and vaginal

discharge (Cotchin, 1964; Miller et al., 2003; Sapierzynski et al. 2009). In available reports, the vaginal discharge has been described as being mucous (Cotchin, 1964), malodorous (Miller et al., 2003) or haemorrhagic (Miller et al., 2003; Sapierzynski et al., 2009; Sontas et al., 2013), and may be constant (Miller et al., 2003; Sontas et al., 2013) or intermittent (Meier 1956, cited by McEntee, 1990; Sapierzynski et al., 2009). A vaginal discharge was noticed in three of our four cases, varying from mucous to bloody or purulent-hemorrhagic. In all the cases it was intermittent. All the females were considered to have an adequate body condition, except for the queens in cases 2 and 3, whose previous clinical history included reference to slow growing rate. However, in none of the cases described in the present work, inconspicuous signs such as lethargy and inhappetence were observed. Vomiting (Anderson and Pratschke, 2011; Cho et al., 2011), irregular appetite (Sapierzynski et al., 2009), polyuria/polydipsia, and lethargy are also possible to found in animals with FEA, but it often suggest the existence of a co-existing chronic inflammation in the uterus in moderately advanced situations. Frequently, these symptoms lead to a putative diagnosis of uterine disease, like pyometra, or abortion in queens that have been mated (Taylor, 2010).

Enlargement of the uterus, causing abdominal distension and compression of adjacent viscera with concurrent constipation or dysuria have been described (Johnston et al., 2001a; Klein, 2007; Taylor, 2010) whenever larger tumours exist. Enlargement of the uterus is easily found during abdominal palpation, and may elicit abdominal distress or pain during the procedure (Anderson and Pratschke, 2011). If metastases exist, depending on the location of the metastasis, ascites, cough and respiratory distress or blindness and motor incoordination may be found (Klein, 2007; Taylor, 2010). These symptoms are often present together with anorexia and important weight loss, fever, anaemia and dehydration (Belter et al., 1968; Johnston et al. 2001a; Meier 1956 quoted by McEntee, 1990; Miller et al., 2003). Seldom, recurrent oestrus has been reported (Anderson and Pratschke, 2011).

Amongst the cases reported in the present study, only the queen in case 3 developed signs compatible with possible persistency of the adenocarcinoma following OVH. Further, samples from this female also showed that FEA lesions deeply invaded the myometrium and reached the serosa, which increase the chance for subsequent abdominal metastization through the omentum or through the uterine stump, as it was described by Cho et al. (2011). If Taylor (2010) defends that most

cats develop metastatic disease within 6 months of diagnosis if OVH is not performed, one should be aware that this period would greatly vary with the stage of the disease at surgery and also with FEA aggressiveness, and thereby this period should be considered as indicative. For the two cases described by Sontas and colleagues (2013) 21 and 23 months after OVH the animals remained free of clinical uterine disease. For our four cases, only the case 3 showed reappearance of the intermittent bloody discharge, suggesting dissemination of the neoplastic lesions at the uterine stump. For all the other cases, the females remain asymptomatic.

The usefulness of the clinical blood analysis for diagnosing FEA is limited, particularly in early situations. On regards to clinical blood analysis in the cases reported herein, in part due to economic constraints. In case 1, besides an increased in white cells counting, due to increased values for neutrophils and eosinophils, the sole disturbed biochemical parameter was ALT (alanine aminotransferase), which almost triple the normal range values. Sapierzynski et al. (2009) found a similar rose in the case they described. Regenerative anaemia and neutrophilia are common findings in FEA situations (Klein, 2007; Morris and Dobson, 2001; Sontas et al., 2013).

The ultrasonography may be helpful for the initial assessment of the clinical situation, although the most often described ultrasound images are similar to those of pyometra. However, irregular coiling or increased thickness of uterine wall in the absence of limited collection of the fluid in the uterus or narrowing of the uterine cavum, co-existing with bloody vaginal discharge should lead to suspicion of endometrial adenocarcinoma. Ultrasonography may further be useful to define the origin of the mass or to evaluate regional metastasis in more advanced situations (Klein, 2007; Morris and Dobson, 2001).

Bloody or intermittent vulvar discharge whatever the queen´s age or the sexual status should always lead to include FEA in the differential diagnosis list.

Yet, a definitive diagnosis is only obtained through histological examination of surgically excised specimens (Klein, 2007). In FEA, the mitotic index (MI) seems not to be useful in distinguishing FEA with different invasiveness ability, as in general, MI below 5 mitoses per field have been referred (Anderson and Pratschke, 2011; Gil da Costa et al., 2009; Sapierzynski et al. 2009). According to Miller et al. (2003) in feline endometrial adenocarcinoma the mitotic index does correlated with invasiveness of the tumour or the outcome of the case. Loss of oestrogen and

progesterone receptors has been referred as sign of malignancy for feline endometrial carcinomas (Gil da Costa et al., 2009; Miller et al., 2003) and it may be important when searching for prognosis markers. Hormone-dependent FEAs may benefit from medical management of metastatic spread after ovariohysterectomy (Gil da Costa et al., 2009). Additional studies are needed to highlight the possible involvement of progesterone on the initial FEA development.

Intermittency of bloody vaginal discharge should be foreseen as a strong indicator of the initial stages of FEA, as it is seldom saw during other uterine diseases. Further, in case of abortion, despite the presence of haemorrhagic discharge, this tend to decrease with time and often corresponds to a dark bloody secretion containing cell debris corresponding to placental or embryonic necrotic material. Moreover the vaginal discharge, a disturbed appearance of the uterus on ultrasound images or at surgery should be deemed as indicators for histopathological evaluation of the excised specimen, independently of the female age. Recently, growing evidences points to the fact that, in cats, endometrial adenocarcinomas do not show a predisposition for a sexual status (intact *vs.* spayed) or age (Saraiva et al., 2012a)

Conclusions

In this report, four cases of endometrial carcinoma in yearling cats under without major clinical findings are reported. In all of them, there were uterine enlargement and increased uterine wall thickness; often uterine dilatation by fluid accumulation was recorded. For all the cases, corpora lutea were present within the ovaries. Underlying mechanism for FEA tumourigenesis remains unclear, and request additional studies in larger series of tumours. Also, due to its rarity, detailed reports on clinical cases would be needed to highlight the clinical course and outcome, as well as additional diagnosis approaches to FEA.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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