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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

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Abstract

The interest in small rodents as companion animals has been increasing, but their short longevity has been a difficult obstacle to overcome. One of the pathologies that most frequently affects rats are mammary tumors, but although this area is highly developed in laboratory animals (induced tumors), little is known about spontaneous mammary tumors in pet rats.

The rat has six pairs of mammary glands and females are most prone to develop mammary tumors than males, particularly fibroadenomas.

To better understand the pathophysiology of the mammary tumors, we decided to review the anatomy, histology and physiology of rats' mammary glands, as well as the most common clinical signs, means of diagnosis and differential diagnoses, treatments and prevention strategies, in order to systematize the knowledge about this common pathology.

We realize that surgical treatment is available, but as recurrences are very frequent its effectiveness is limited. As for pharmacological treatment, there are some options but their effectiveness lacks proof, so it is necessary to carry out further studies to test new drugs that can stop tumor evolution.

In this scenario, prevention remains the most effective weapon but has been neglected, so owners should be advised to timely spay their pets in order to reduce the probability of mammary tumor appearance.

ACKNOWLEDGMENTS

I want to thank my parents, Fernando and Paula, and my sister Joana for all the support they have given me during these 24 years, even though I am not the most studious person in the world. But I am a great daughter and sister, am I not? I love you so much!

Thanks to my family for giving me encouragement and for being the best family ever. I miss you Avô Tono.

To my best friend, Joana Baeta, who has been with me for 24 years and who has not had enough of me, and to all those who, on the way, I have known and remain by my side (Sara Aguiar, Vasco Torgal, Sandra Vicente, Diana Santa, Filipa Lento, Sara, Ana and Diogo Guerner).

To Pedro Laranjo, for raising my moral, being my daily support and never letting me give up of anything, I really love you.

Thank you to my mentor, Prof. Margarida, for the given support and trust throughout the process. Thanks for share the madness with me.

To Dr. Luísa Guardão, for giving me the bug of the exotic animal medicine since my first year of college and for giving me my two beautiful rats.

To Dr. Pedro Rodrigues, for always accepting me in his clinic and for making me feel always welcome, as well as Ana and Tomás, for all the laughter we gave, to not to cry.

Thank you to Dr. Joel Ferraz, Dr. Rute, Dr. Joana and others for making me fall (even more) in love with exotic animals.

Finally, I wanted to leave my symbolic thanks to all the animals I owned, with special affection for my dogs Cherie, Smeagol and Luna, as well as for my guinea pig Presunto and my two rats Bolota and Morcela. They will be always in my heart.

CONTENTS

ABBREVIATIONS

AB	Alveolar Buds
Bid	Twice a day
cAMP	Cyclic Adenosine Monophosphate
CL	Corpus Luteum
ER	Estrogen Receptor
EV	Endovenous
FSH	Follicle-stimulating Hormone
GAS	Gamma Interferon Activated Site
GnRH	Gonadotrophin-releasing Hormone
IM	Intramuscular
LH	Luteinizing Hormone
mRNA	Messenger RNA
PGF 2 α	Prostaglandin 2 alpha
PO	Oral
PIF	Prolactin Inhibition Factor
PR	Progesterone Receptor
PRF	Prolactin Releasing Factor
PRLR	Prolactin Receptor
SC	Subcutaneous
SID	Once a day
SP	Sprague-Dawley strain
TD	Terminal Ductules
TEB	Terminal End Buds
TIDA	Tuberoinfundibular Dopamine Receptors

INTRODUCTION

Over the decades, rats have been used as laboratory animals and serve as model for the study of the most varied human pathologies. However, in the last years, this species has conquered its place as a companion animal.

They are highly intelligent, affable and easily become the ideal pet for those who do not spend much time at home and appreciate rodents. Some of the main physiological parameters are described in table 1.

However, due to their short average life expectancy, the incidence of mammary tumors increases from 18 months. Other factors can potentiate its onset, like high levels of circulating prolactin and estrogen, as estrus cycle occurs each 4-5 days.

One pathology that elevates the level of prolactin is hypophysary adenoma, which is seen in many necropsies along with mammary tumor, although the relation between both has not been yet proven. Mammary tumor diagnosis is usually presumptive, since excised tumors are not often analyzed by histopathology and are most likely to be benign.

Cytology as diagnosis method is normally useless, and since mammary tumors tend to be small sized, histopathology is the gold standard method.

Treatments are limited, and veterinarians should encourage owners to focus on prevention, particularly in ovariohysterectomy in young rats. In addition, surgery, hormone implants, chemotherapy, and prolactin antagonists are available options, although more studies are needed.

Average Life Span (years)	2-3
Weight (g) Females and Males	250-1000+
Body Temperature (ºC)	37-38
Respiratory Rate (breaths/min)	70-150
Heart rate (beats/min)	300-450
Gestation (days)	19-21
Average number of pups	10-12
Age at weaning (days)	20-22
Sexual Maturity (weeks)	5-6

Table 1 - Physiological parameters of *Rattus norvegicus*
Source: Adapted from Exotic Animal Medicine: A quick reference guide (2009)

Rat mammary gland anatomy and histology

The adult rat (*Rattus norvegicus*) has six pairs of mammary glands: cervical, cranial thoracic, caudal thoracic, abdominal, cranial inguinal and caudal inguinal glands[1]. The cervical glands extend anteriorly to the salivary glands, and the caudal inguinal glands extend to the perianal region. Between the cervical and thoracic mammary glands is the muscle *pectoralis*, which is not present in the abdominal and inguinal mammary glands[2]. The thoracic, abdominal, and inguinal glands vary in their degree of development, with the caudal inguinal gland being the most differentiated, and the cervical glands being the least differentiated in the nulliparous rat[3,4].

In lactating rats, the glands can extend laterally to both sides of the body[1] (Figure 1).

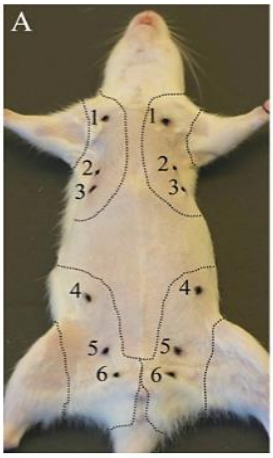

Figure 1 - Localization of the mammary glands: 1: Cervical; 2: Cranial thoracic; 3: Caudal thoracic; 4: Abdominal; 5: Cranial inguinal; 6: Caudal inguinal.
Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

Each mammary gland is composed of epithelium, adipose tissue and connective tissue stroma, which has blood vessels, nerves, smooth muscle fibers, lymphatic nodes and lymphatic vessels and near the nipple, keratinized epithelium and sebaceous and sweat glands[5].

Lymphatic vessels are lined by a luminal layer of simple squamous endothelium, have a large lumen relative to the thickness of the wall and are lined by a discontinuous basement membrane. Unlike blood vessels, lymphatics do not convey red blood cells and are difficult to distinguish in histological sections when the cross section contains lymphatic vessels with no leukocytes[5].

Mammary gland is a tree-like ductal system, with tubuloalveolar morphology in female and lobuloalveolar in males[2] (Figure 2).

Overall Structure	Female Rat	Male Rat
	Duct	
Name	Tubuloalveolar	Lobuloalveolar
Number	High	Low
Epithelium	Simple cuboidal	Pseudostratified or stratified cuboidal or short columnar
Epithelial cytoplasm	Scant, basophilic, nonvacuolated	Abundant, eosinophilic, vacuolated
Epithelial apoptosis	Rare	Frequent
Luminal space	Prominent	Not usually evident
	Acinus	
Number	Low, centered on ductules	High, contiguous lobules
Epithelium	Simple cuboidal	Pseudostratified or stratified cuboidal or short columnar
Epithelial cytoplasm	Scant, basophilic, nonvacuolated	Abundant, eosinophilic, vacuolated
Epithelial apoptosis	Rare	Frequent
Luminal space	Prominent	Not usually evident

Adapted from: Lucas et al: The rat mammary gland: morphologic changes as an indicator of systemic hormonal perturbations induced by xenobiotics. *Toxicol Pathol* 35(2):199–207, 2007. Review. Reprinted by Permission of SAGE Publications, Inc.

Figure 2 - Histologic differences in mammary gland of sexually mature rats.

Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

In this tree-like ductal system, all the alveoli, ductules and ducts merge into one primary collecting duct whose lumen is contiguous with the external surface of the nipple[1,2].

As described above, mammary glands are highly vascularized by branches of several arteries, such as superficial cervical, axillary, thoracic internal and external and external pudendal. Capillaries change dramatically during development, forming extensive capillary beds in the intralobular compartment during pregnancy and lactation[6,7,8].

Female gestation ranges 19-21 days, and the foetus mammary glands development begins approximately at 11 day of gestation. Cellular clamps proliferating from two longitudinal crests of ectoderm, parallel to the middle line, give origin to six pairs of mammary glands in the adult rat (Figure 3).

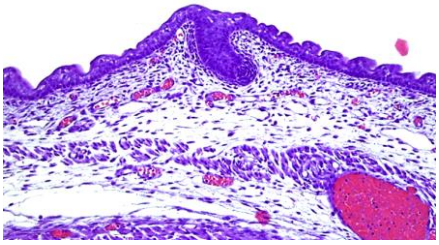

Figure 3 - Downgrowth of epithelium from the mammary streak in an 18-day-old fetal Wistar Han rat.

Source: Boorman's Pathology of Rat, 2nd edition.

The regulators of mammary gland morphogenesis include hormones, growth factors and receptors, cell cycle regulators and adhesion molecules[9]. The biggest growth occurs between the birth and puberty. The rat reaches puberty approximately at 5-6 weeks of age[1].

There is an accumulation of subcutaneous adipose tissue from the cervical region until the inguinal region, creating mammary fat pads and support for the mammary glands. The growth of the mammary gland starts from the nipple and progress into the fat pad[9]. These fat pads are composed by preadipocytes, adipocytes and fibroblasts and a thin layer of collagenous stroma separates it from the epithelial cells[1,10,11,12]. Most adipocytes in the rat mammary gland are unilocular.

Mammary gland can be divided into two compartments: the epithelial compartment and the stromal compartment[2,6]. The parenchymal compartment of the mammary gland is composed of

different epithelial structures with distinct morphologies, functional and proliferative activities, comprising the luminal epithelium of ducts, ductules, terminal end buds, alveolar buds, and alveoli, as well as the underlying myoepithelial layer[13]. This myoepithelial layer can be stained with cell markers such as smooth muscle actin and form a thick and continuous layer at the base of ducts[7,14].

Myoepithelial cells express higher levels of cell adhesion receptors and adhesion-associated molecules than the luminal epithelium and can be distinguished immunohistochemically from luminal epithelial cells by their expression of the intermediate filament proteins vimentin and cytokeratins 5 and 14, the basement membrane proteins collagen type IV and laminin, and the contractile proteins myosin and smooth muscle actin[15,16,17,18].

The luminal epithelium is the epithelium whose apical surface contacts the lumen of ducts, ductules and alveoli while the myoepithelium consists of the basal epithelial cells that surround the luminal epithelium in all those structures[14,19]. The luminal epithelium can also be defined immunohistochemically by its expression of cytokeratins 8, 18, and 19 in the rat[16,17] (Figure 4).

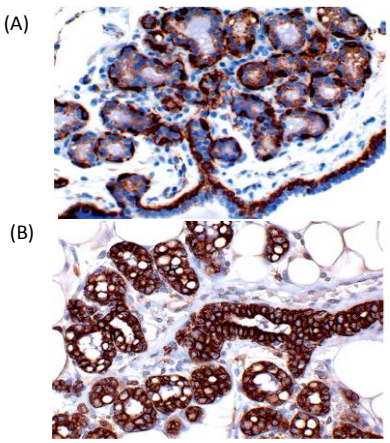

Figure 4 - Basal and luminal cell immunohistochemical markers in the mouse mammary gland. **(A)** Immunohistochemistry highlights the basal (brow labelling) and luminal (blue labelling) cell populations in these low-magnification images of a mouse pre-lactating lobular-acinar unit. **(B)** Cytokeratin 8/18 labelling of luminal epithelial cells (brow labelling).
Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

At the end of the first week, each mammary gland has one main lactiferous duct and three to five secondary ducts[1]. These ductal systems are surrounded by adipose tissue, poor in fibrous connective tissue. The duct of female has a more prominent lumina, lined by one or two layers of regular cuboidal cells or columnar epithelium underlain by myoepithelial cells, compared with the indistinct lumina and vacuolated pseudostratified epithelial cells of the lactiferous duct of the male[1,2]. Also, the primary duct is more regular in circumference than the lactiferous sinus but is likewise surrounded by a thick layer of connective tissue.

At the end of second week, secondary ducts branch into tertiary, quaternary and quinary ducts[1]. These ducts will end in terminal end buds (TEBs), which are the growing end of the mammary tree[2]. They are solid or semisolid bulbous clusters of immature epithelial cells at the ends of ducts[6,20].

Each TEB is composed of a central body surrounded by a single layer of pluripotent epithelial cells, and surrounding the TEB are host inflammatory cells, giving the appearance of “fuzzy halos” in histological preparations[2] (Figure 5). These structures are clinical important because they are believed to be the most susceptible targets for chemical carcinogens[3,21].

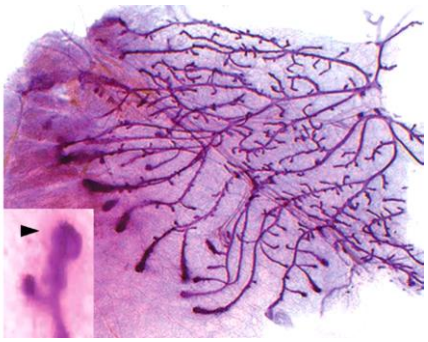

Figure 5 - Rat mammary extension of terminal end buds (TEBs) into the fat pad. Inset: Rat TEB with fuzzy halo (arrowhead), which contains inflammatory cells.
Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

The number of TEBs reaches a maximum and then, at 20 days of age, TEBs can cavitate to form terminal ductules (TDs) or give rise to alveolar buds (AB)[1,22,23].

Terminal ductules are composed of luminal epithelium and myoepithelium and therefore, lacks the multiple-layered epithelium associated with the TEB. When multiple TDs with small lumina are clustered in an island of connective tissue, they are referred to as an AB[20]. AB are composed of one or two layers of cells. The cells lining the lumina are cuboidal or columnar with a round or oval nucleus. Myoepithelial cells are present but vary in number and do not form a complete layer[1]. It is correct to say that TEBs and AB are the major sites of morphogenesis and cellular differentiation of the mammary glands. Hvid *et al.* observed that proliferation of the mammary epithelium in young rats was mainly in the TEBs whereas proliferating cells were more equally distributed in the different compartments of the mammary gland of mature animals[24] (Figure 6).

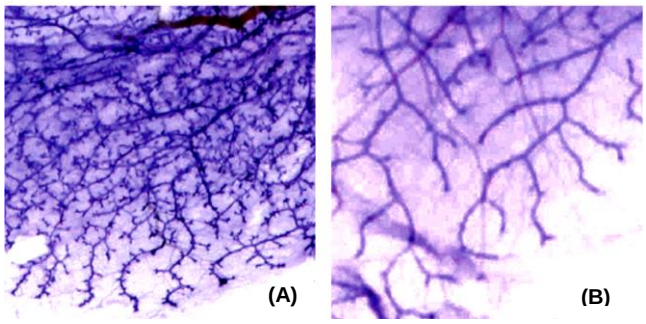

Figure 6 – Mouse mammary gland during the ovarian cycle. (**A**) - During estrus the mammary ducts are thicker, more abundant and have multiple side buds. (**B**) - During diestrus the glands regress to a simple branching structure with slender ducts with few side buds.
Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

Puberty, defined as the onset of estrus cycles, commences in the rat between days 35 and 42 of age. At that time, AB undergo differentiation into alveoli to form alveolar lobules. Each AB develops into 10-12 alveoli to form a lobule[1] (Figure 7). The shape of the luminal epithelial cell ranges from pyramidal (before secretion) to flattened (after secretion) in alveoli and this secretory epithelium is responsible for milk synthesis and secretion during lactation[7]. An outer discontinuous layer of fully differentiated myoepithelial cells surrounds the luminal epithelium[1]. After day 55 of

age, the number of alveolar lobules remains stable but can expand progressively over multiple estrus cycles[6,20,25]. Lobules are the ultimate milk-producing functional units.

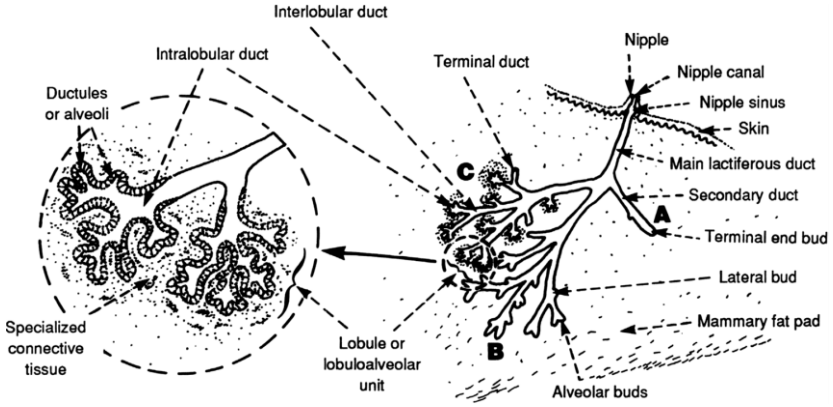

Figure 7 - Schematic representation of the mammary gland of rats. Source: Boorman's Pathology of Rat, 2nd edition.

During pregnancy, there is a rapid and continuous increase in the mammary gland epithelium during the approximately 19-21 days of pregnancy, with growth of both lobules and ducts occurring[26].

TEBs, if still present, rapidly divide to form alveolar buds, which give rise to alveoli and alveolar lobules. The alveolar lobules increase both in size and number until the space between ducts is almost filled with epithelium. Alveolar epithelium also shows hypertrophy, as the cells accumulate intracellular lipid and increase their rough endoplasmic reticulum and Golgi and secretory vesicles[27].

These lobules and mammary ducts contain myoepithelial and luminal epithelial cells, which are enclosed by a basement membrane that separates the epithelium from the surrounding connective tissue[28,29]. Myoepithelial cells form a continuous layer and secrete the continuous basement membrane[2,9]. Also, the myoepithelial cells surrounding alveoli during pregnancy and lactation form a discontinuous layer of stellate-shaped cells whose dendritic arms encircle the base of the alveolar epithelium and contract the alveoli during suckling[29]. The discontinuity of the myoepithelium can be seen by staining for myoepithelial cell markers as previously described[6,7,22].

During this time, there is a decrease in fat tissue and an increase of cellularity in the mammary gland stroma.

Later, alveolar epithelium becomes presecretory and begins to produce large fat vacuoles and Golgi vesicles containing milk components, like lactose, protein and water. These components are released by apocrine secretion. The milk passes from the alveoli to the collecting ducts and out though nipple during nursing[2].

During lactation, mammary gland lumina is full of basophilic secretions and casein micelles in association with lipid globules. The alveoli are heterogeneous in both their luminal distension and the thickness of the luminal epithelium lining them, both within and between lobules[30].

The alveolar epithelium becomes presecretory and has a spongy appearance due to intracellular lipid and osmotic swelling of lactose-containing secretory vesicles[14].

The luminal alveolar cells produce large fat vacuoles (Figure 8) and Golgi vesicles containing lactose, protein, and water.

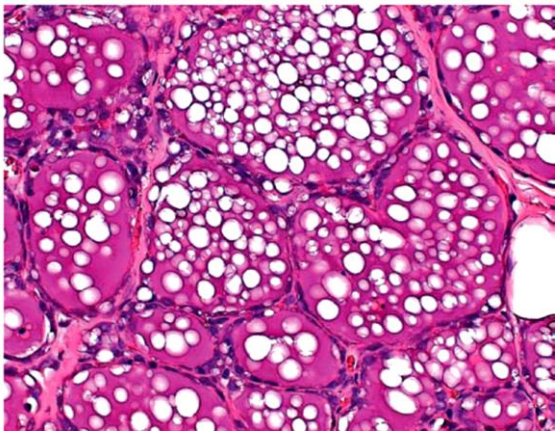

Figure 8 - Lactation in rat. Note the high volume of fat globules in the rat mammary gland. Images show secretory morphology comprised of acini with distended lumen that are filled with secreted milk and lined with flattened epithelial cells.

Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

The increased alveolar cell size causes the myoepithelial cell layer underlying the secretory alveolar epithelium to be even more discontinuous than in pregnancy[22].

The milk then passes from the lobules, to lactiferous duct (Figure 9). The lactiferous duct conveys milk through a constricted orifice from the lactiferous sinus to the nipple[5]. The lactiferous sinus has a distinctly convoluted lumen surrounded by two layers of cuboidal epithelium and a thick layer of eosinophilic stroma[6].

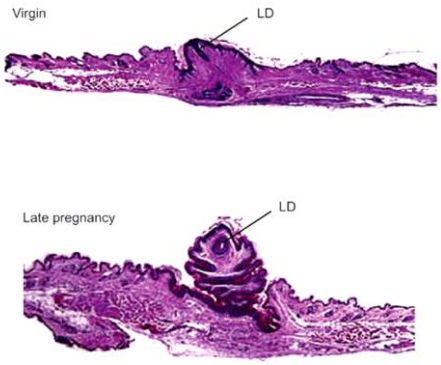

Figure 9 - The mouse nipple and collecting ducts. The mouse nipple becomes prominent during pregnancy. Note the single lactiferous duct (LD).

Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

The epithelium of the nipple is continuous with the keratinized stratified squamous epithelium of the epidermis, although the opening to the nipple may be sealed from the external environment by a plug of epithelial cells or keratin[6,31].

The transition from stratified squamous epithelium to two layers of cuboidal epithelium from the lactiferous sinus occurs in the lactiferous duct within the interior of the nipple[5]. The nipple also contains bands of smooth muscle that run parallel to the main lactiferous duct and circumferentially toward the base. The smooth muscle acts as erectile tissue and helps ejecting

milk[2]. During pregnancy the rodent nipples develop, protrude and the lactating mammary fat pad (MFP) becomes extensive (Figure 10).

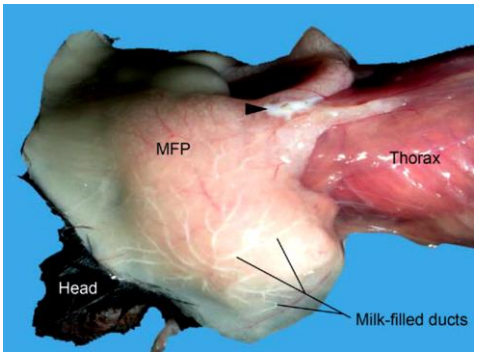

Figure 10 - Mouse necropsy dissection. The pelt was degloved and reflected over the head. The subcutaneous MFP removed with the skin. Milk-filled ducts are visible as white branching lines.
Source: Comparative Anatomy and Histology: a mouse, rat, and human atlas, 2nd edition.

After weaning, which is usually complete in 20-22 days, there is extensive epithelial cell apoptosis or autophagy with loss of alveoli[2]. Apoptosis of blood capillary endothelial cells speeds up the regression of lactating ducts and remaining epithelial cells become non-secretory[6,32,33,34]. Lobules involute and become smaller but the number remains higher and more differentiate than in virgin females of the same age. At the same time is also notable the increase in adipocyte areas[9].

In nulliparous rats, after a year the mammary glands begin to involute, lobules become smaller and TEBs become terminal ductules. In males, the growth of ducts ceases at eight weeks of age, resulting in fewer subdivisions of ducts. The lactiferous duct ends near the epidermis blindly, as male rats lack nipples[2].

Rat estrus cycle and mammary gland physiology

Female rats are poliestrics and spontaneous ovulators, showing successive estrus cycles, which are influenced by light, season of the year and age. With increasing age, female rats gradually develop irregular estrus cycles and eventually persistent anestrus[24].

Estrus cycles occur each 4-5 days and are divided in proestrus (12-18h), estrus (25-38h), metestrus (5-8h) and diestrus (47-58h)[35]. Westwood also considers the existence of an additional phase called anestrus, which is defined by ovarian inactivity, as observed when reproductive life is quiescent[36].

Every estrus cycle has temporal changes in the release of the three hypophysary hormones: Luteinizing Hormone (LH); Follicle-stimulating Hormone (FSH) and prolactin, as well as in the two ovarian hormones estrogen and progesterone. According to Robinson *et al*, the hypothalamus is responsible for the control of the release of FSH and LH from the adenohypophysis by the action of specific releasing and inhibitory substances[37]. These are secreted by the hypothalamic neurons

and are carried from the hypothalamus by the hypothalamic– hypophyseal portal system[37]. Both FSH and LH are located within the same gonadotrophic cell, and the exogenous administration of GnRH (Gonadotrophin-realising Hormone) stimulates the releasing of both FSH and LH[37,38]. However, it is known that exists differential secretion of FSH and LH from the adenohypophysis gland and that it changes in the different stages of the estrus cycle. It appears that GnRH is critical for the basal and pulsatile secretion of LH, which is particularly evident during the preovulatory LH surge. In contrast, the secretion of FSH secretion is more independent from direct GnRH receptor signalling[37,39].

The secretion of FSH and LH is controlled by two functionally separate systems with two hypothalamic centres involved in this control[37]. The tonic system is responsible for the continuous basal secretion of gonadotrophin that stimulates the growth of both germinal and endocrine components of the ovary[37]. This system predominates during the luteal phase, when LH pulses are described as high amplitude and low frequency. The second system is the surge system, which controls the short-lived massive secretion of gonadotrophins, particularly LH, and is responsible for ovulation[37].

Progesterone inhibits the tonic mode of LH secretion[40] by reducing LH pulse frequency. Thus, when the circulatory concentrations of progesterone fall, the release of LH from the anterior pituitary increase[37]. The increased frequency of LH pulses triggers the secretion of estradiol and stimulates the hypothalamic surge centre. Consequently, there is a surge of LH that will induce the final stages of follicular maturation, ovulation and the release of oocyte [41]. The preovulatory LH surge triggers a series of cellular and molecular changes in the follicle that culminate in ovulation[37] (Figure 11).

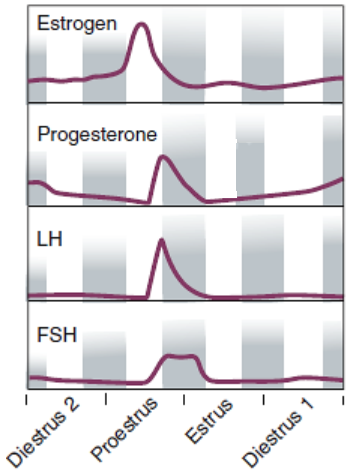

Figure 11 - Estrus cycle of the rat. The 4-day estrus cycle of the rat (gray bars indicate night, 6 p.m. to 6 a.m.), showing fluctuations in estrogen, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).
Source: A woman's prerogative, 2005.

Ovulation is a complex process**,** involving destruction of the follicle allowing the release of the oocyte and conversion of the follicle into the corpus luteum (CL). The fully formed CL consists of several different cell types: steroid-secreting large and small luteal cells, smooth muscle cells, pericytes, fibroblasts and endothelial cells[42]. The hormones that are most likely to be involved are prolactin and LH, with some evidence that they act in combination with each other. Prolactin plays

a critical role in maintaining the CL in rodents[37,43,44]. It can either be luteotrophic after mating or luteolytic in the absence of a mating stimulus. Prolactin acts as a luteotrophic hormone by maintaining the functional and structural integrity of the CL for 6 days after mating[45]. This action of prolactin, which has been described in the rat, is characterized by enhanced progesterone secretion[46,47]. Aside from its luteotrophic role, there is evidence in the rat that prolactin may be luteolytic as well[47,48] by inducing programmed cell death in the CL[49,50].

During pregnancy, the presence of a functional CL producing progesterone inhibits the return to estrus, by exerting a negative feedback effect upon the hypothalamus[37]. In nonpregnant rat, estrus and ovulation occur at equally regular intervals, and the main control of this cyclical activity would appear to be the CL[37].

Luteolysis of CL is controlled by endometrial-derived $PGF_{2\alpha}$, transported directly from the uterus to the ovary. The mechanisms whereby $PGF_{2\alpha}$ is transported to the ovary have not been conclusively shown in all species[37]. Luteal regression has two important parts: functional regression, in which the secretion of progesterone declines abruptly and structural regression, in which there is physical involution of the CL and regression of luteal tissue[37].

All these hormonal changes that occurs within the estrus cycle define a particular phase, that can be identified by vaginal smears, according with the cell types that are present: a) Proestrus is defined by the presence of round nucleated epithelial cells; b) Estrus is characterized by non-nucleated, cornified epithelial cells; c) Metestrus typically has a low cell number, often with a lot of cell debris and d) Diestrus II contains mostly lymphocytes. This epithelium changes during the estrus cycle are induced by estrogen and progesterone[51] (Figure 12).

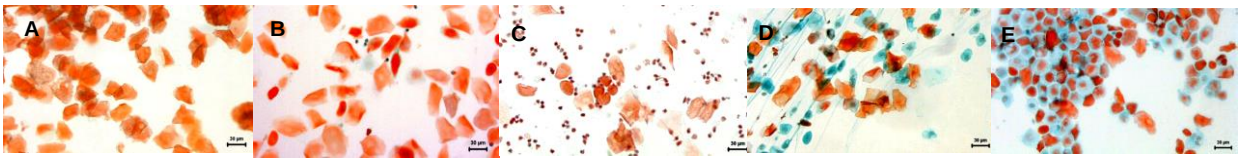

Figure 12 – Vaginal smears stained by the Shorr method. Left to right: **A**: Estrus; **B**: Metestrus; **C**: Late Metestrus; **D**: Diestrus and **E**: Proestrus.

A: Estrus - clumps of enucleated acidophilic cells are observed. **B**: Metestrus - leukocytes are also observed. **C**: Late Metestrus - high number of leukocytes. **D**: Diestrus - small (SBC) and large basophilic cell (LBC). **E**: Proestrus - basophilic cells and pre-acidophilic cells (PAC).

Source: The rat estrous cycle revisited: a quantitative and qualitative analysis, 2013

Through proestrus estrogen level increases and ovarian follicles grow fast[52,53]. During the night of estrus 10-12 h after the LH surge, ovulation occurs and, in the absence of mating at the time of ovulation, the CL are transiently functional and secrete a small amount of progesterone[54]. If a mate occurs, and ova are fertilized by sperm cells during the third hour after ovulation, CL life is extended throughout the first half of pregnancy[54]. During the second half, progesterone production takes place in the placenta[52,53,55].

Persistent estrus is caused by high levels of estradiol and low levels of progesterone, while pseudo-pregnancy is characterized by low levels of estradiol, elevated levels of progesterone and high levels of prolactin, which extends the lifespan of the CL from the normal 2 days to 10–14 days[24,56]. These estrus cycle irregularities and associated changes in endogenous concentrations of hormones are expected to influence on mammary gland proliferation.

Multiple hormones and growth factors are involved in mammary gland development, such as estrogen, progesterone, prolactin, oxytocin, growth hormone, insulin like growth factor and many others (reviewed by Kleinberg 2008), but in association to estrus cycle, the ovarian steroid hormones estrogen and progesterone are essential to its growth[57,58].

Henning Hvid concluded that, in both young and mature rats, the percentages of proliferating epithelial cells of the mammary gland were strongly dependent on the estrus cycle. Significantly increased proliferation was observed during metestrus and proliferation in diestrus was significantly higher compared to proestrus in young and mature female rats[24]. Although one study reported high proliferation during metestrus and early diestrus, two other studies reported high proliferation during proestrus and diestrus[24,59].

Besides estrogen and progesterone, prolactin plays an important role in secretory activity of CL in ovaries as well in morphologic and functional properties of the mammary gland. The best characterised role for prolactin is its activity on the CL, particularly in rodents. It is essential for CL function and the inhibition of prolactin secretion leads to luteal regression[44]. This hormone has an important role in spontaneous mammary tumours in rats and studies have suggested that prolactin may be involved in mammary tumorigenesis [60] by promoting cell proliferation and survival[61,62,63,64], increasing cell motility[65] and supporting tumour vascularization[60,66,67].

Prolactin is a polypeptidic hormone synthetized by lactotroph cells, which comprise 30–50% of rat adenohypophysis and is released by a calcium-dependent exocytosis. Unlike other anterior pituitary hormones, which require hypothalamic stimulation, it appears that prolactin secretion from lactotrophic cells of the adenohypophysis gland is spontaneous[37]. In general, slightly more than half the lactotrophs of female rats secrete prolactin in a continuous pattern, while those of males secrete it in a discontinuous or intermittent pattern[68].

Prolactin may act as an autocrine/paracrine factor within mammary tissue as this hormone is mainly expressed in hypophysis but also in lactating mammary gland[47,60,69,70,71]. There is ample chronobiological evidence that the temporal organization of prolactin secretion is controlled by circadian input in rats[47,72,73]. Plasma concentrations of prolactin are the highest during sleep and the lowest during the waking hours[47,74,75]. Prolactin can be detected in epithelial cells of the lactating mammary gland[43]. Apparently, prolactin reaches the milk by first crossing the mammary epithelial cell basement membrane, attaches to a specific prolactin binding protein within the mammary epithelial cell and is ultimately transported by exocytosis through the apical membrane

into the alveolar lumen[47,76,77]. In addition to uptake of prolactin from the blood, the mammary epithelial cells of lactating animals are capable of synthesizing prolactin. The presence of prolactin mRNA[78,69] as well as synthesis of immunoreactive prolactin by mammary epithelial cells of lactating rats was already described[47,79,80].

Serum prolactin levels are low during most of the estrus cycle, except in the afternoon of proestrus, when prolactin surge coincides with preovulatory LH surge[47,81]. LH surge is sharp and symmetrical, but the prolactin rise is triphasic, consisting of a sharp peak, a plateau, and a termination phase. In rodents, prolactin plays a significant role during the second half of pregnancy, replacing the suppressed hypophysary prolactin. Increased secretion of hypophysary prolactin during lactation causes inhibition of GnRH, LH and FSH, suppressing ovulation during lactation[47,82].

There are multiple physiological stimuli that regulate the secretion of prolactin, like nipple sucking during lactation, increased ovarian steroids, especially estrogen, both increasing prolactin release. These stimuli are sent to the hypothalamus, which produces Prolactin Releasing Factors (PRF) and Prolactin Inhibitory Factors (PIF) (Figure 13).

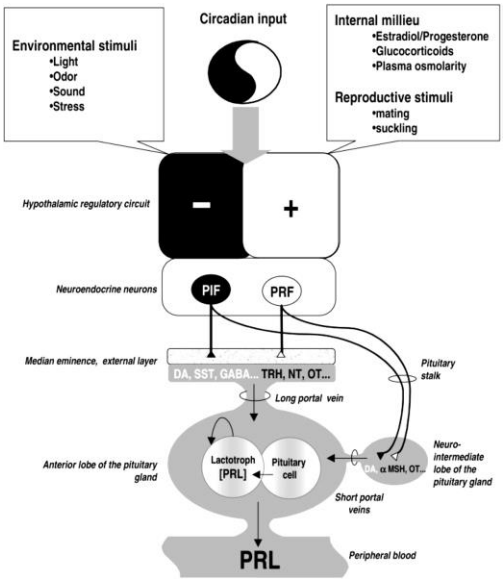

Figure 13 - Circadian input and control of prolactin (PRL) secretion.
Source: The Role of Prolactin in Men - Endocrinology & Metabolic Syndrome.

In mammals, hypothalamus exerts a mainly inhibitory effect on the synthesis and secretion of prolactin, which is also influenced by other factors that are released by lactotroph cells of the adenohypophysis.

Prolactin primarily acts through the prolactin receptor (PRLR), which belongs to the cytokine receptor superfamily[47,66]. The PRLR has multiple isoforms that are thought to arise by differential splicing. They have similar extracellular and transmembrane regions but have different intracellular domains[60,66]. The PRLR is detectable by immunohistochemical staining and its location staining also appears to differ in tumour cells in contrast with normal cells, such that

staining in normal cells is along the luminal cell border, while staining in malignant cells is primarily in the cytoplasm[66,83].

Jak-Stat signalling is the best characterized of the prolactin activated pathways. Jaks are nonreceptor tyrosine kinases, while Stats are latent cytoplasmic transcription factors composed of a modular structure of five domains. Phosphorylation of a tyrosine downstream of the SH2 domain is critical for Stat activation[84]. Jak2, which is constitutively associated with Box 1 of the PRLR, is rapidly activated after receptor dimerization and phosphorylates tyrosine residues on the PRLR[85]. Stat proteins, attached by SH2 domains to phosphotyrosine residues on the PRLR, are also targets of activated Jak2, with Stat 5a and Stat5b being the primary mediators of PRL action. After phosphorylation, Stat proteins uncouple from the PRLR, homo- or heterodimerize and translocate to the nucleus, where they bind to Gamma Interferon Activated Site (GAS) elements and promote transcription of target genes[86]. Termination of signalling is an important component of hormone action.

The PRLR may be associated with the estrogen (ER) and progesterone (PR) receptor expression and several *in vitro* studies have reported that long-term prolactin or estrogen exposure can increase both PRLR and ER expression[62,87]. Also, prolactin shares several characteristics with growth factors, including multiple extra-hypophysary sites of synthesis [88,89,90], wide distribution of receptors[91], homology of the PRLR to those of the cytokine/hematopoietic growth factor family[92], similarities in signal transduction pathways[93] and mitogenic and morphogenic actions[89,94]. For knowledge, growth factors are emerging as important mediators of carcinogenesis and have been implicated in malignant transformation, tumour cell proliferation, and tumour progression[95].

The main regulation of prolactin is provided in the form of tonic inhibition by dopamine, which is secreted by tuberoinfundibular dopamine neurons (TIDA) through the hypothalamus-pituitary towards the lactotroph cells of the adenohypophysis. Dopamine exerts its inhibitory function through dopaminergic receptors type 2 (D2) in the membrane of the lactotroph cells. The activation of D2 receptors in the membrane of the lactotroph cell leads to inhibitory G proteins that are associated with the D2 receptor. This inhibitory G proteins inhibit adenylate cyclase (cAMP) and, at the same time, excites potassium channels that coupled to G0 proteins and block voltage-sensitive calcium channels, inhibiting secretion of prolactin[16,47] (Figure 14).

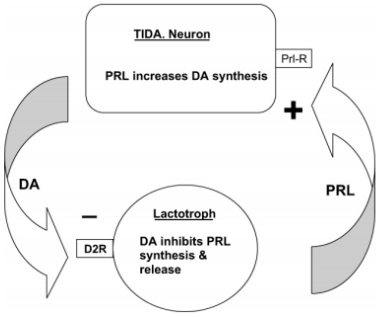

Figure 14 – Feedback control of prolactin regulation.
Source: Prolactin and dopamine: What is the connection? A Review Article, 2008

Neurotransmitters such as norepinephrine, epinephrine, histamine and acetylcholine also modulate the secretion of prolactin. Histamine acts through its H_1 and H_2 receptors. Activation of H_1 receptors has a stimulatory effect on prolactin secretion unlike H_2 receptors, which inhibit the secretion of prolactin[96,97]. Acetylcholine acts on cholinergic receptors stimulating TIDA neurons, thus favoring the secretion of dopamine and the consequent inhibition of the secretion of prolactin.

Other hormone that increases the secretion of prolactin is oxytocin. Oxytocin is synthesized in the paraventricular and supraoptic nuclei and is transported axonally to the neurohypophysis. The portal vessels that connect the neurohypophysis with the adenohypophysis allow its arrival in the lactotroph cells, where it promotes the release of prolactin. During pregnancy their concentration increases gradually until delivery, where there is a drastic decrease[98]. Nipple suction during lactation is the major prolactin secretory stimulus and overlaps over the control of its endogenous circadian secretion. The stimulation of the nipple causes a somatic response that is transmitted through the spinal cord and reaches the hypothalamus, where the release of serotonin and endorphin is increased. This causes a decrease in dopamine and an increase in the secretion of prolactin. The mammary stimulus also produces an increase in the release of oxytocin, which in the bloodstream reaches the breast where it induces the contraction of the myoepithelial cells, thus reducing the lumen of the milk ducts, promoting milk ejection[47,98].

Finally, neuropeptides also play an important role in prolactin secretion. Thyroid stimulating hormone (THR), in addition to its specific function on the thyroid gland, stimulates the secretion of prolactin by the lactotroph cells of the hypophysis. TRH receptors in lactotroph cells are coupled to G proteins that activate phospholipase C, catalysing the hydrolysis of phosphatidylinositol 4,5-bisphosphate generating inositol triphosphate and diacylglycerol. The inositol triphosphate (IP3) causes the release of calcium of non-mitochondrial origin, while the diacylglycerol (DAG) activates, through calcium, protein kinase C, which phosphorylates voltage-sensitive calcium channels by allowing its entry into the lactotroph cell, and thereby increases the secretion of prolactin[47,81].

Spontaneous mammary gland tumor frequency and incidence

Mammary gland fibroadenomas are the most common spontaneous subcutaneous tumor of domestic rats[99]. This type of tumor is associated with hyperprolactinemia in some strains of laboratory rats[100,101,102,103].

As referred previously, prolactin secretion increases due to a decrease in the secretion of dopamine from the hypophysary gland in geriatric rats[104] and persistent low concentrations of progesterone, constant estrus, or cessation of ovulation in female rats[105]. In some laboratory rat strains, including the Sprague-Dawley (SP)[104], Fisher[106] and Wistar[107,108] strains, hypophysary gland adenomas develop in more than 80% of rats with more than 24 months old. Some geriatric rats with mammary gland fibroadenomas develop concurrent prolactin-secreting hypophysary adenoma (chromophobic hypophysary adenoma)[105,104,107,108].

The incidence of spontaneous mammary gland tumors in rats is high, ranging from 30% to 67% in female SD rats[109]. Adenocarcinomas represent less than 10% of spontaneous mammary gland tumors in rats[105]. Authors Noble and Cutts found that, in a group of 150 female SP rats with an average life span of 2 years, mammary tumors accounted for 95% of the total tumours found in 54% of the animals[110]. Also, incidence rate of mammary gland tumors in female rats that are ovariohysterectomized at 90 days old was significantly smaller than older sexually intact female rats, and reproductive sterilization of rats between 5 and 7 months of age reduced the incidence rate of spontaneous subcutaneous (SC) tumors from 73.8% to 5.3%[111]. Concomitant abnormalities in companion rats with spontaneous mammary gland fibroadenomas are poorly described.

According to Poteracki's study, mammary fibroadenomas were the most common mammary tumor, occurring in 36.1% and 2.6% in females and males, respectively. On the other hand, mammary adenocarcinoma occurred in 6.7% of females. Mammary tumors accounted for 55% of neoplasms in the integument and 95% of these occurred in females[112].

Vergneau-Grosset *et al* described the prevalence and concomitant abnormalities of the various types of mammary gland tumors in companion rats. From the 105 SC masses initially detected in 100 rats, the most prevalent SC mass identified was mammary gland fibroadenoma, which comprised 53% of all masses, followed by mammary gland carcinoma (12%). Overall, 25% of the masses were malignant. At the time of the study, 37 subsequent SC masses appeared: 68% were benign and 32% were malignant. From this 68% of benign masses, 65% were mammary gland fibroadenoma, and from the 32% of malignant masses, 16% were mammary gland carcinoma. Fibroadenoma was the most common neoplasm identified in the axillary region[113].

Concomitant hypophysary tumor was identified in most rats with mammary gland fibroadenoma (75%) and other types of mammary gland tumors (59%). Also, 40% of the rats with mammary gland fibroadenoma had concomitant reproductive tract abnormalities[113].

Histologic diagnosis was also associated with sex. Mammary gland fibroadenoma is more likely to develop in sexually intact females and males than in neutered rats[113]. Additionally, eleven of the thirteen malignant mammary tumors were identified in sexually intact females. In sexually intact males, most initial SC masses were non-mammary gland tumours[113].

Curiously, of five spayed female rats present in one study, one had a mammary gland carcinoma, one had a hemangiosarcoma and the remaining had mammary gland fibroadenomas. The three spayed female rats that developed mammary gland fibroadenoma underwent ovariohysterectomy at 3.5, 5, and 12 months old, respectively[113]. This study also concluded that the prevalence of mammary gland carcinoma in the companion rats was 3 times greater than the 4% reported for sexually intact and ovariectomized SD rats in laboratory[105].

In rats that received no adjunctive treatment after excision of a mammary gland fibroadenoma, a second fibroadenoma was detected 1 to 8 months after initial excision, at a median of 4.5 months after surgery[113].

Harleman *et al* reviewed the incidence and coincidence of uterine and mammary tumors, suggesting a strong inverse relationship between uterine and mammary tumors in Wistar and SD rats. They settled that there were differences in background incidence data between the two strains. The Wistar rat had an evidently lower incidence of mammary tumors (24%) compared with SD rats (58%). In contrast, the Wistar rat had a markedly higher spontaneous incidence of uterine tumors (5%) compared with the SD rats (0.9%). In both strains, the occurrence of uterine tumors arises predominantly in animals without a mammary tumor, being more pronounced in Wistar rats. For this strain, there was a strong evidence of an inverse relationship between mammary and uterine tumors[114].

Once again, the driving factor for this response is likely to be prolactin. The increase in circulating prolactin is directly associated with an increase in the incidence of mammary tumors, as prolactin is the major promotional driver of mammary tumors in this species[114,115,116]. As previously stated, prolactin is luteotropic in rats and promotes progesterone production of the CL after ovulation and sustains gestation. During the luteal phase of the estrus cycle, the CL produces large quantities of progesterone, which antagonizes estrogenic stimulation of uterine growth[114,117]. Therefore, over a series of cycles, inhibition of prolactin results in an increased estradiol:progesterone ratio in rats. Estrogen has a trophic effect in the uterine endometrium, promoting an increased incidence of uterine tumors[114].

Dinse *et al* recognised that several types of tumors were common in both Fischer and SD female rats. Among eighteen studies, they found that mammary gland fibroadenomas ranged

48% in Fischer rats and 67% in SD rats and mammary gland carcinomas ranged 2% and 10% in Fischer and SD, respectively[109].

Spontaneous mammary gland tumor etiology

It is known that female intact rats are more commonly affected by spontaneous mammary tumors than male rats. Mammary tumors develop in 30%–90% of intact female rats (depending on the strain), and 0.5%–16% of intact males. Also, multiparous rats appear to have higher incidence of mammary tumors than nulliparous rats[118].

So genetic predisposition seems to be an important factor. Spontaneous mammary tumors develop in females of various strains of rats, such as August, Albany-Hooded, Copenhagen, Fisher, Lewis, Osborne-Mendel, Sprague-Dawley (SD), Wistar, and Wistar/ Furth[3]. The Fischer strain is generally less susceptible than the Sprague-Dawley and Wistar-derived strains[1].

Age is one of the main factors for the development of mammary tumors. They are uncommon in rats younger than 1 year of age and its incidence increase markedly after about 18 months of age[119]. Obesity and high-fat diets are associated with increased incidence of spontaneous and chemically-induced tumors.

Concomitant pathologies are common, with hypophysary adenoma being the principal disease. Literature data clearly demonstrate that most rat hypophysary adenomas are prolactin-positive[114,120]. They are functional prolactin-producing tumours and, because of the rapid discharge of the hormones they produce, they can be also called chromophobic hypophysary adenomas[114,121]. Studies in rats show that many animals with hypophysary adenomas have high levels of circulating prolactin and that this correlates with the size of mammary tumors[114,122].

Environmental conditions are also significant. Some authors suggest that chronic stress can influence the development of cancer by deregulation the corticosteroids levels. However, it can't be concluded that stress itself is a primary factor for the development of tumors in rats. One study was made to evaluate the effect of various light/dark regimens on the survival, life span and tumorigenesis in rats. Nocturnal rise of melatonin (produced by the pineal gland and released during the dark periods of the night) is abolished or substantially decreased in animals exposed to constant light, so they concluded that female rats exposed to constant illumination leaded to disturbances in estrus function and spontaneous tumor development. This study was the first to show that the exposure of male rats to constant illumination accelerated the development spontaneous tumors[123,124].

Other review reported that suppression of pineal function, either by pinealectomy or with appropriate light exposure, enhanced tumor incidence, number, and size and reduced tumor latency. These antitumoral actions of the pineal gland may be related to its ability, via the secretion

of melatonin, of downregulate some of the hypophysary and gonadal hormones, which control mammary gland development and are also responsible for the growth of hormone-dependent mammary tumors[124].

In a recent study, authors proposed that infant temperament predicted life span in female rats that developed spontaneous tumors[125]. They found that, for females that developed either mammary or pituitary tumors, and were reluctant to explore a new environment in infancy died approximately 6 months earlier than their exploratory sisters. Also, they have shown that female temperament may have the greatest effect on when tumors first develop, as opposed to the rate at which tumors proceed following tumorigenesis[125].

Clinical signs of mammary tumors

When owners come to the clinic, they may describe that the animal is lethargic, has increased appetite without gaining weight, or has decreased appetite and lost some weight. The weight loss despite good appetite may occur because nutrients are supporting the growth of the mass.

Others notice a small subcutaneous mass, although rats can reach the clinic with ulcerated masses that can be up to one-third of the animal body weight, causing a decreased mobility. (Figure 15). Complications of large ulcerated mammary tumors are potential risk for blood loss, anaemia, necrosis and secondary infection, which can lead to septicaemia, organ failure, and possible death[126].

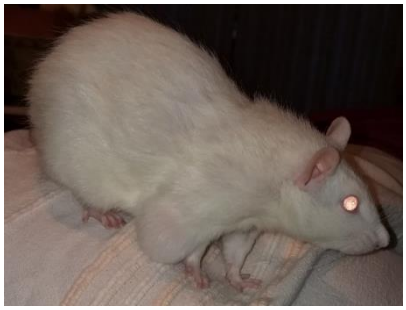

Figure 15 - Mammary tumor in a non-neutered female Wistar rat. *Original picture*

Because mammary tissue is so extensive, tumors can develop anywhere from the cervical region to the base of the tail. These tumors grow rapidly, becoming very large within weeks.

Normally, after clinical examination of the animal and palpation of the mammary gland chain, it can be felt single or multiple, small or large, spherical, soft to firm subcutaneous painless masses that usually are not attached to deeper structures. Less spherical masses adherents to underlying musculature may be suggestive of malignancy. Sometimes, mammary gland infection or abscesses can also be present[127].

Diagnosis of mammary tumor and differential diagnosis

Usually, when a mass appears along the mammary gland chain, a fine-needle aspiration should be performed. However, in rats this method is considered unreliable, since rat's tumors have poor exfoliation properties and necrotic areas that contain inflammatory cells may mask the neoplastic element of interest[128]. The gold standard method is histopathology, so the veterinary who performs the excisional biopsy of the mass, should kept the tumor in formaldehyde for later analysis[129]. If the veterinary suspects of malignant mammary tumor, a thoracic radiography should be performed, although metastasis of these tumors are considered rare[126,130].

Regarding differential diagnosis, one should consider SC abscess (that can be secondary to cage mate aggression or trauma), hematoma, mastitis (in which the affected glands could be warm, swollen and/or with a haemorrhagic discharge) and cyst or hyperplasia of the mammary gland. It is important to diagnose mammary hyperplasia, since it can progress to mammary tumor. Considering mammary tumors, besides fibroadenoma the clinician should consider the possibility of being a lipoma, sarcoma, squamous cell carcinoma, mast cell tumor or lymphoma[127].

Histopathology evaluation is essential to characterize mammary tumors in rats, since they are classified according to epithelial components and stroma involvement. Fibroadenomas are the most common benign neoplasm of the mammary gland of the rat. They are round to irregularly oval masses, generally greater than 5 mm in diameter, reaching up to 8 cm in diameter[1]. They are well-defined and may be encapsulated. On cut section, tumors are lobulated with regions of highly fibrous to glandular tissue. Histologically, alveoli and ductules are surrounded by prominent mature connective tissue stroma. The alveoli within the lobules are usually well formed and lined by cuboidal epithelial cells, frequently with prominent vacuoles in the cytoplasm. Smaller fibroadenomas often have a lobular growth pattern. This pattern is not so evident in larger neoplasms, where widely separated ductules are surrounded by concentric layers of dense connective tissue[1]. Fibroadenomas are composed of glandular epithelium (ducts, ductules, and/or alveoli) and fibrous connective tissue, with varying proportions of epithelial and fibrous components, which can vary the texture and consistency of the mass. Generally, epithelium is uniform without atypia or stratification, but if it occurs is only focally. Mitotic figures are rare and atypical cells do not constitute an expanding mass. Areas of necrosis may be present in the centre of the mass[1].

The second most common tumors are malignant adenocarcinomas, round or irregular masses, greater than 5 mm in diameter with some grade of local invasion, that rarely metastasize. They are relatively poorly demarcated and may invade adjacent tissues, like muscle or skin[1]. These tumors where previously classified as "cribriform", "tubular", "anaplastic adenocarcinoma", "papillary" and "comedo carcinoma", depending of its shape. They can arise from focal

hyperplasia with atypia in ducts, ductules or alveoli and foci of atypia in adenomas or fibroadenomas. It consists of epithelium arranged in alveolar, ductular, papillary or solid structures or frequently combinations of these structures[1]. The epithelial cells form single or multiple layers and small compact nests or nodules of neoplastic cells are sometimes present.

The more malignant neoplasms often have solid sheets and nodular masses of neoplastic cells. The neoplastic epithelium exhibits cellular atypia, consisting of altered size or shape of cells, chromatin content of nucleus, prominent nucleoli, altered nuclear/cytoplasmic ratio and altered staining quality of cytoplasm[1]. Pleomorphism or diffuse stratification is also present and areas of necrosis, skin ulceration and haemorrhage may also occur[1]. When present or associated with adenoma or fibroadenoma, the malignant component constitutes an expanding mass that compresses and displaces the benign components.

Other forms of mammary tumors are described in figure 16, since histologic diagnosis of SC mammary masses is important to establish the treatment protocol, prevent mass recurrence and predict the prognosis.

I. Epithelial neoplasms
- A. Benign lesions
  - 1. Intraductal papilloma
  - 2. Papillary cystadenoma
  - 3. Adenoma
    - (a) Tubular
    - (b) Lactating
- B. Precancerous lesions
  - Intraductal proliferation (IDP)
- C. Malignant lesions
  - 1. Noninvasive–*in situ*—carcinoma
    - (a) Ductal papillary
    - (b) Ductal solid and cribriform
    - (c) Ductal comedo
  - 2. Invasive carcinoma
    - (a) Papillary
    - (b) Cribriform
    - (c) Comedo
    - (d) Tubular
    - (e) Adenoid cystic

II. Stromal neoplasms
- A. Benign: fibroma
- B. Malignant: fibrosarcoma

III. Epithelial–stromal neoplasms
- A. Benign: fibroadenoma
- B. Malignant: carcinosarcoma

IV. Nonneoplastic lesions
- Cystic changes
  - (a) Ductal
  - (b) Lobular|

Figure 16 – Mammary tumor classification in domestic rats.

Source: Significance of Rat Mammary Tumors for Human Risk Assessment, 2015.

Treatment, prognosis and prevention

There are two types of treatment that can be applied in rat mammary tumor: medical and surgical treatment. Regarding medical treatment, it comprises dopamine agonists, hormonal implants and chemotherapy. Knowing that prolactin influences the incidence of mammary tumors via a direct trophic effect[114] and that that dopamine decreases prolactine release throught the activation of dopamine receptors type 2 (D_2), the use of dopamine agonists is a predictable therapeutic option[131]. Cabergoline is one of this drugs approved for veterinary use in Portugal. Nagasawa concluded that the administration of cabergoline prevented the development of spontaneous mammary gland fibroadenoma in some strains of laboratory rats[100]. However, in another study, tumors recurred at the same location within 1 to 9 weeks after the administration

of the dopamine agonists was discontinued[132]. Cabergoline is prefered to bromocriptine, due to its longer duration of action. Results of a study indicate that administration of 0.6 mg of bromocriptine/kg, PO to laboratory rats suppressed serum prolactin concentration for only 6 hours, whereas administration of the same dose of cabergoline suppressed serum prolactin concentration for up to 6 days[133]. Dopamine agonists are also associated with a reduction in the incidence of hypophysary adenomas and hyperplasia, but unfortunatly also increase the incidence of uterine tumors[134,135,136].

Other treatment to prevent and stabilize the growth of mammary tumors is the application of deslorelin implant. This implant (Suprelorin® Virbac) is usually applied subcutaneously in the interscapular zone (Figure 17). Deslorelin is a GnRH agonist and exert its mechanism of action through the desensitization and downregulation of adenohypophysis GnRH receptors.

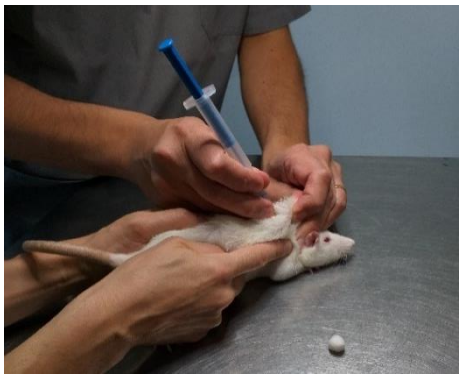

Figure 17 – Appling a subcutaneous deslorelin implant to a rat.
Original picture

In male rats, administration of deslorelin results in internalization of GnRH receptors and downregulation, inhibiting secretion of FSH subunit β by hypophysary gonadotrophs[137]. It is assumed that there is a transient increase in the secretion of LH and FSH that lasts for approximately 2 weeks following the administration of a deslorelin implant in rats[138]. However, its use as an adjunctive treatment for mammary gland fibroadenoma in rats has not been described. Instead, it has been used as a contraceptive in male and female rats since 2011, although its reversibility remains unidentified[138,139].

According to Alkis, after the implantation of a 4.7-mg deslorelin implant in rats, serum progesterone and estradiol 17β concentrations are suppressed for up to 12 months [139]. However, there are some type of tumors that have a more aggressive phenotype, and its growth can become independent from hormone influence[140].

In a recent study, a 12-month-old sexually intact female rat with mammary gland fibroadenoma received a deslorelin implant (4.7 mg, SC) five weeks after surgical excision of the tumor. No subsequent SC masses were detected during the 12 months after administration of the implant. In the same study, another rat received a second deslorelin implant 15 months after the first, because of the emergence of a new SC mass. The second implant stabilized growth tumor for

only 5 months, after which the mass began to increase in size[113]. Authors suggested that the increase of the mass may have happened due to premature loss of efficacy by the implant, a decrease in the responsiveness of the tumor following the introduction of the second implant, or evolution of the tumor receptors toward an aggressive phenotype that could not be regulated by reproductive hormones[113].

There are few studies on the efficacy of chemotherapy in the treatment of spontaneous rat mammary tumors. It is known that in dogs and cats, chemotherapy is administered based in a protocol treatment for mammary tumors. One of the protocols is to administer orally a tyrosine kinase inhibitor, like toceranib, together with a non-steroidal anti-inflammatory drug, such as meloxicam. The dosages for rats are not standardized and are usually extrapolated from other species' recommendations[130].

Cisplatin, a platinum-based chemotherapeutic agent, has been used for treatment of induced mammary tumors in rats, and the results showed a decrease in the development of these tumors. In other study one rat received intralesional cisplatin (1.6 mg, each week). However, that rat experienced two excisional biopsies and had recurrence of the mammary gland carcinoma in the inguinal area twice. The last recurrence was only 4 days after the last intralesional cisplatin treatment, and that rat was humanly euthanized. The authors could not draw any conclusions regarding the effectiveness of cisplatin[113].

Besides medical treatment, in most cases it is necessary to perform surgery. Surgical treatment implies the excision of the mass or masses, and some authors suggest performing an ovariohysterectomy together with the nodulectomy in order to minimize tumor recurrence[105]. This last procedure is recommended when excision of the entire mammary chain is not possible due to the extension of mammary tissue. The anaesthetic risk must be taken into consideration, as well as the physical condition of the patient. The removal of mammary tumors must be done as soon as it is diagnosed, and surgery time must be minimized.

In the peri-operatory time rats should receive supportive treatment, both pharmacological and nutritional. Pharmacologically, analgesic, antibiotic and anti-inflammatory drugs should be administered to rats, but before considering surgery the patient must be stabilized, so subcutaneous (SC) hydration and a good plane of nutrition are necessary. If the rat does not eat by itself, it is necessary to force feed. Atropine can be administered to prevent bradycardia and secretions, but the analgesic should be administered before, during and after surgery and EV or SC fluids should be provided. Patient temperature must be checked regularly, so a heating mat can be used to produce heat, as hypothermia is one of the main causes of death during surgery. Preanesthetic medication should be given, but during the surgery anaesthesia should be maintained with volatile anaesthetics. Previously to incision, the surgeon must decide if considers the mass as a benign or a malignant tumor, because in benign masses the skin is preserved,

unlike ulcerated large masses, or suspected malignant masses, in which the removal of the tumor is via an elliptical incision with wide margins[127]. As the majority of the tumors are fibroadenomas, we are going to discuss the approach of a benign mass further.

The animal must be positioned in dorsal recumbency and the surgical area must be prepared aseptically. Then, the surgeon can block local nerves to prevent pain in the site of incision. The incision is performed and the mass should be debrided. If the tumor is well irrigate, ligating vessels prevents bleeding and helps haemostasis. The skin must be closed with an absorbable suture yarn, with a discontinuous pattern or intradermal continuous pattern, since visible skin sutures will probably be chewed by the patient. Tissue glue is also a good option[126]. The excised mass should be kept in formaldehyde for further analysis.

Prospects of recovery are favourable if the animal does not have any concomitant pathology (e.g. hypophysary adenoma), but still suture dehiscence and infection can occur. Mammary tumors frequently reoccur within months, so veterinary should advise the rats owners to monitor for mammary masses in patients and to have regular check-ups[127].

To prevent mammary tumors, early ovariohysterectomy or ovariectomy is indicated, as mammary tumors develop in 30%–90% of intact female rats. If the patient has a mass but the owner does not want perform surgery, the veterinary should suggest a deslorelin implant to slow down the growth of the mass.

Owners should provide low-fat nutritious diet and prevent obesity. Food *ad libitum* should not be given. Some vegetables and fruits actively prevent formation of tumors, such apples, broccoli and brussels sprouts, because of the phytochemicals they possess. These phytochemicals have antioxidant activity against free radicals and act on the regulation of gene expression in cell proliferation, cell differentiation, oncogenes and tumor suppressor genes. They also induce cell cycle arrest and apoptosis[141].

Finally, owners should be able to select tumor free parents, and choose male over female rats. Rats must be kept in pairs to reduce environmental stress and provide exercise and environmental enrichment. Further studies are necessary to investigate adjunct treatment protocols, including the use of chemotherapy and GnRH agonists for companion rats with mammary gland fibroadenoma or other SC masses[113].

CLINICAL CASE

Sirius was a standard *Rattus norvegicus*, mink, self-dumbo, intact female, two years old and 503g in weight (Figure 18).

Figure 18 - Sirius, a *Rattus norvegicus* with a clear mammary mass

The reason for consultation was the growth of a previous right cranial thoracic mammary nodule. Sirius lived with more rats in a cage and had access to the outside, always supervised, with ad libitum access to food and water.

Regarding Sirius medical history, five months ago she had been given a hormonal implant of deslorelin because she had a growing nodule in the right cranial thoracic mammary gland. The growth of the nodule was stabilized for 5 months after the implant.

Examining the general state, one can say that she had normal mental state and balanced temperament. The degree of dehydration was <5%, body condition 6/9, respiratory movements with frequency within the parameters.

At physical examination, Sirius demonstrated difficulty in locomotion and the owner reported that she had difficulty raising the cage to feed. At palpation, the mass was mobile, painless, with fibrous consistency and well-defined limits, 5cm x 5cm in diameter. No further analysis was performed and Sirius was indicated for nodulectomy.

The list of problems was cranial thoracic mammary nodule. The differential diagnosis was mammary hyperplasia. No additional tests were performed, so the final diagnosis was not achieved because the owner did not want the mass to be analyzed histopathologically.

Nevertheless, the treatment was surgical. Since the incidence of mammary tumors is high in intact females and most are fibroadenomas, Sirius was subjected to nodulectomy. The patient was hospitalized and medicated with: tramadol (Tramal® Grünenthal inj. 50mg/ml) 0,2ml/kg SC bid as painkiller, meloxicam (Acticam® Ecuphar 5mg/ml) 0,1 ml/kg SC sid as anti-inflammatory and enrofloxacin (Baytril® Bayer 5%) 0,2 ml/kg SC bid as antibiotic, in appropriate doses.

Before surgery, the patient was stabilized with SC hydration and a nutrition plane. Analgesia with an opioid was provided before, during and post-surgery, as well as hydration, via intravenous

(IV) catheter or SC[126]. Finally, atropine 0,5mg/kg was given intramuscular (IM) in a dose of 0,8ml/kg.

The pre-anaesthesia was given to Sirius: Tramadol (0,2 ml/kg) and Medetomidine (Domitor® Pfizer 1mg/ml) 50 µg/kg via IM plus Ketamine (Imalgene® Merial 100mg/ml), 2–4 mg/kg via IM. During anaesthesia the patient was on oxygen mask linked to volatile anaesthesia (Isoflurane) and oxygen (Figure 18).

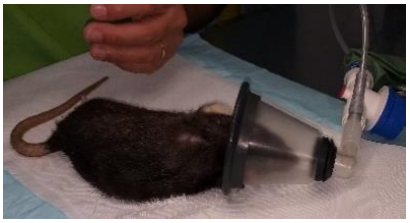

Figure 18 – Sirius pre-anaesthesia with oxygen mask.

The animal was positioned in dorsal recumbency and the surgical area was prepared aseptically with alcohol and chlorhexidine (Figure 19).

Figure 19 - Shaving the mass to further cleaning.

Previously to the incision the surgeon decided to treat the mass as a benign tumor and performed local blocks with lidocaine 5% SC along the incision line[126] (Figure 20). Then, a single incision was performed and debridement of the mass was made with a blunt tipped scissor (Figure 21). The mass was extirpated and the skin was closed with an absorbable suture yarn, in an intradermal continuous pattern (Figure 22).

Figure 20 - Infiltration of the incision site with lidocaine.

Figure 21 – Blunt dissection of the tumor.

Figure 22 – Closure of the skin with an intradermal patter.

At the end of surgery, atipamezole (Antisedan® Zoetis 5 mg/ml) was administered IM at the same volume of medetomidine, to revert its effects. The patient continued with supportive treatment during the days it was hospitalized.

When Sirius went home she showed some signs of recovery but did not feed properly. The tutors reported that unfortunately she continued to refuse food, and eventually died fifteen days after the surgery.

CONCLUSION

This work aimed to systematize the existing knowledge about a pathology that frequently affects domestic rats - mammary tumors. Their incidence ranges from 30% to 67% in female SD rats and the most frequent tumors are fibroadenomas. Numerous factors potentiate its onset, but high levels of circulating prolactin and estrogen are generally implicated.

Cytology is not a reliable method to diagnosis these masses, but since most owners do not want to send excisional biopsy to analysis, these mammary tumors diagnosis is usually presumptive.

Treatments are limited, so it is necessary to carry out further studies to test new drugs that can stop tumor evolution. There is the need to conduct more studies to test the influence of deslorelin implants in mammary tumor, as well as test chemotherapy that could help increase the average life expectancy.

Prevention remains the most effective weapon but has been neglected, so owners should be advised to timely spay their pets in order to reduce the probability of mammary tumor appearance.

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