UNIVERSITI PUTRA MALAYSIA

CLASSIFICATION AND GRADING OF FELINE AND CANINE MAMMARY GLAND TUMOUR (MGT) PRESENTED TO HISTOPATHOLOGY LABORATORY, VLSU, UPM FROM 2020-2022

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AIN UMAIRAH BINTI RAHMAT

A student project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

DECEMBER 2022

CERTIFICATION

It is hereby certified that we have read this project paper entitled **"Classification and Grading of Feline and Canine Mammary Gland Tumours (MGT) Presented to Histopathology Laboratory, VLSU, UPM from 2020 - 2022"** by Ain Umairah binti Rahmat and in our opinion, it is satisfactory in term of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

Praise to the One Almighty God, who has made all things possible,

To my incredible supervisor and fantastic co-supervisor

To my supportive family and friends

To my pets especially Baby

To myself

ACKNOWLEDGEMENT

I want to take this opportunity to extend my thanks and appreciation to everyone who assisted me in completing my project paper at any point along the way.

First and foremost, I would like to extend my gratitude to my family members, most notably my parents, for providing me with unwavering support and encouragement throughout the course of my five years of study in Veterinary Medicine. This has been a major source of inspiration for me to continue pushing myself and not giving up.

Second, I would like to use this opportunity to convey my unending gratitude to my cherished advisor, Dr Nurul Izzati Uda Zahli, for the invaluable wisdom, knowledge, and experience that she has shared with me, which led to the creation of this satisfying final year project paper.

Thirdly, no words can adequately explain how grateful I am to Assoc. Prof. Dr. Gayathri a/p Thevi Selvarajah for the unwavering direction, commentary, and support she provided during the entire project paper.

Lastly, I would want to express my gratitude to my friends (Cili Kering) and DVM23 for all the assistance and encouragement they have provided me with while I focus on finishing the thesis.

I would like to extend my gratitude in particular to Gfriend, Westlife, James, Mia, Piss, and Baby for spending the evenings with me while I worked on the thesis. Despite the fact that it was a tough journey and sometimes felt like a never-ending upward spiral, I was truly enjoying all the sweat and tears thanks to all of them.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperkuan kursus VPD4999 – Projek Tahun Akhir.

KLASIFIKASI DAN PENGGREDAN TUMOR KELENJAR MAMA (MGT) KUCING DAN ANJING YANG DIBENTANGKAN KE MAKMAL HISTOPATHOLOGI, VLSU, UPM DARI 2020-2022

Oleh

Ain Umairah binti Rahmat

2022

Penyelia: Dr. Nurul Izzati Uda Zahli

Penyelia bersama: Prof. Madya Dr. Gayathri Thevi Selvarajah

Klasifikasi dan penggredan histologi adalah faktor prognostik yang ditetapkan untuk tumor kelenjar mama (MGT) kucing dan anjing. Tambahan pula, kiraan limfosit penyusupan tumor (TIL) telah menunjukkan potensi sebagai penanda prognostic dalam kanser payudara manusia, walaupun terdapat sedikit bukti dengan karsinoma kelenjar mama kucing dan anjing. Kajian ini bertujuan untuk 1) menerangkan kepelbagaian klasifikasi dan gred histologi untuk MGT kucing dan anjing; 2) menentukan perkaitan antara gred dan klasifikasi histologi; dan 3) menyiasat hubungan antara TIL dengan klasifikasi dan gred histologi. Slaid histopatologi sampel kelenjar mama yang diarkibkan telah dikelaskan dan digredkan sebelum TIL dikira

dan dinyatakan dalam peratusan mengikut kriteria kiraan TIL kanser payudara manusia. Keputusan kajian menunjukkan karsinoma tiub adalah subjenis yang paling biasa dalam kedua-dua spesies (anjing, 20%; kucing, 60%). MGT anjing mempamerkan klasifikasi histologi yang lebih pelbagai [karsinoma ringkas, 47.3% (n=26); karsinoma bukan ringkas, 29.1% (n=16); karsinoma berkaitan duktus, 18.2% (n=10); karsinoma jenis khas, 1.8% (n=1) dan sarkoma, 3.6% (n=2)] berbanding dengan MGT kucing [karsinoma ringkas, 95.0% (n=38) dan karsinoma berkaitan duktus, 5.0% (n=2)]. Terdapat perkaitan antara gred histologi dan klasifikasi dalam MGT kedua-dua spesies (anjing $p=0.026$; kucing $p<0.001$ dan $p=0.037$). Kehadiran TIL dalam MGT anjing berkait rapat dengan klasifikasi histologi ($p=0.026$), gred ($p=0.021$), dan kiraan mitosis ($p=0.025$). Sementara itu, kiraan TIL dalam MGT kucing didapati berkait dengan pleomorfisme selular ($p=0.048$) dan kiraan mitosis ($p=0.042$). Secara keseluruhannya, penemuan ini memberikan maklumat yang bernas untuk kajian masa depan mengenai epidemiologi karsinoma kelenjar mama kucing dan anjing dan limfosit boleh menyumbang kepada keagresifan MGT anjing.

Kata kunci: anjing; klasifikasi dan penggredan histologi; kucing; tumor kelenjar mama; limfosit penyusupan tumor.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

CLASSIFICATION AND GRADING OF FELINE AND CANINE MAMMARY GLAND TUMOUR (MGT) PRESENTED TO HISTOPATHOLOGY LABORATORY, VLSU, UPM FROM 2020-2022

by

Ain Umairah binti Rahmat

2022

Supervisor: Dr. Nurul Izzati Uda Zahli

Co-supervisor: Prof. Madya Dr. Gayathri Thevi Selvarajah

Histological classification and grading are established prognostic factors for feline and canine mammary gland tumours (MGT). Furthermore, tumour infiltrating lymphocytes (TIL) counts have displayed potential as a prognostic marker in human breast cancer, in contrast to feline and canine mammary carcinoma. The study aims to 1) describe the diversity of histological classification and grade of feline and canine MGT; 2) determine the association between histological grade and classification; and 3) investigate the relationship between TIL with histological classification and grade. Histopathology slides of archived mammary gland samples were classified and graded before TIL were counted and expressed in percentage according to human breast cancer TIL count criteria. Subsequently, tubular carcinoma was the most common

subtype in both species (canine, 20%; feline, 60%). Canine MGT exhibiting a more diverse histological classification [simple carcinoma, 47.3% (n=26); non-simple carcinoma, 29.1% (n=16); ductal-associated carcinoma, 18.2% (n=10); special-type carcinoma, 1.8% (n=1) and sarcoma, 3.6% (n=2)] than feline MGT [simple carcinoma, 95.0% (n=38) and ductal-associated carcinoma, 5.0% (n=2)]. There was an association between histological grade and classification in both species (canine $p=0.026$; feline $p<0.001$ and $p=0.037$) MGT. The presence of TIL in canine MGT was highly associated with histological classification ($p=0.026$), grade ($p=0.021$), and mitotic count ($p=0.025$). Meanwhile, TIL count in feline MGT was found to be associated with cellular pleomorphism ($p=0.048$) and mitotic count ($p=0.042$). Overall, the findings imply insightful information for future studies on the epidemiology of feline and canine mammary carcinoma and that lymphocytes may contribute to the malignancy of canine MGT.

Keywords: canine; histological classification and grading; feline; mammary gland tumour; tumour infiltrating lymphocytes.

CHAPTER 1.0

INTRODUCTION

1.1 INTRODUCTION

Mammary gland tumour (MGT) is the most common neoplasm among female intact dogs whereas MGT is the second most common cancer in female cats (Bergman, 2017). Due to MGTs' heterogeneous nature, which comprises the benign and malignant types that differ greatly in appearance and biological behaviour, it is tough to predict how the individual tumour will progress. This is seen in the pathological aspect of MGTs in felines and canines that show substantial differences, where cats have up to 90% malignant form (Zappuli *et al.,* 2015) while in dogs, it varies with almost 50% in a malignant form (Bergman, 2017).

Histological categorization and grading were found to be one of the prognostic factors for MGT in both feline and canine. The combination of both factors helps to foresee the biological behaviour of mammary cancer, which in turn helps to facilitate histological analysis and provides a specific guideline for pathologists to follow (Goldschmidt *et al.,* 2006, p.734). In addition, tumour infiltrating lymphocytes (TIL) are lymphocytes that directly oppose or surround the tumour cells. The degree of TIL is determined by the extent and density of the infiltration. TIL is one of the valuable prognostic factor in human breast cancer as it helps to predict the response of tumours toward neoadjuvant chemotherapy, specifically in triple-negative breast cancer (TNBC) and HER2-positive breast cancer (Denkert *et al.,* 2010; Savas *et al.,* 2016).

Few studies conclude that higher TIL linked with better a response towards neoadjuvant chemotherapy in all breast cancer subtypes with longer survival rate for TNBC and HER2-positive patients (Dishyanthen *et al.,* 2015; Denkert *et al.,* 2017; Ruan *et al.,* 2018). However, there were not much evidence for TIL to be a prognostic factor in feline and canine MGT.

Therefore, this study is conducted to evaluate the most recent distribution of histological classification and grade as well as the roles of TIL in feline and canine MGT. In exchange, this retrospective study will aid future research on the prognosis, survival rate and recurrence rate as well as appropriate treatment and potential development of molecular markers for feline and canine MGT.

1.2 OBJECTIVE

The objectives of this study were:

1. To describe the diversity of histological classification and grade of feline and canine MGT.
2. To determine the association between the histological grade of feline and canine malignant MGT with age, neuter status, and histological classification.
3. To determine the association between TIL with the histological classification and grade.

1.3 HYPOTHESIS

The hypotheses for this study were:

1. a) H_0 = There is no association between the histological grade with age, neuter status, and histological classification.

   b) H_a = There is an association between the histological grade with age, neuter status, and histological classification.
2. a) H_0 = There is no association between TIL with histological classification and grade.

   b) H_a = There is an association between TIL with histological classification and grade.

CHAPTER 2.0

LITERATURE REVIEW

2.1 FELINE MAMMARY GLAND TUMOUR

Mammary gland tumour (MGT) is one of the highest prevalent diseases in cats, particularly females and those in the middle to older age range, with an average age of 10 to 12 years (Withrow *et al.,* 2000) while the likelihood of being affected by MGT increased with age up to the age of 16 (Beth *et al.,* 2008). The study by Manuali *et al.* (2020) ranked mammary neoplasm as the third highest among European Shorthair cats in Italy while a study by Graf *et al.* (2016) found MGT as the most common neoplasm among feline in Switzerland.

Other reported risk factors include breed, in which Withrow *et al.* (2001) and Hayes *et al.* (1981) suggest that the mammary tumour is breed-associated with the Siamese breed has twice the risk of getting MGT as compared to other breeds. Not only that, Siamese and Domestic Shorthairs, especially European Shorthairs, are frequently observed breeds in studies of feline MGTs (Beth, *et. al.,* 2005). Moreover, a study in Japan (Ito *et al.,* 1996) found breed predisposition in Japanese cats while Oriental Shorthair, Somali and Abyssinian are more likely affected by MGT in Switzerland (Graf *et al.,* 2016).

Also, the MGT occurrence is highly related to the hormonal exposure and the reproductive status of affected feline, as Overley *et al.* (2005) discovered that female cats spayed before 6 months of age have a 91% reduced risk of getting MGT and an 86% reduced risk if spayed before 1 year of age compared to intact cats. These findings

proved that the duration of exposure to estrogen and progesterone is also important in the development of feline MGT. However, the exact mechanism of the hormone on the development of feline mammary carcinoma remains obscured.

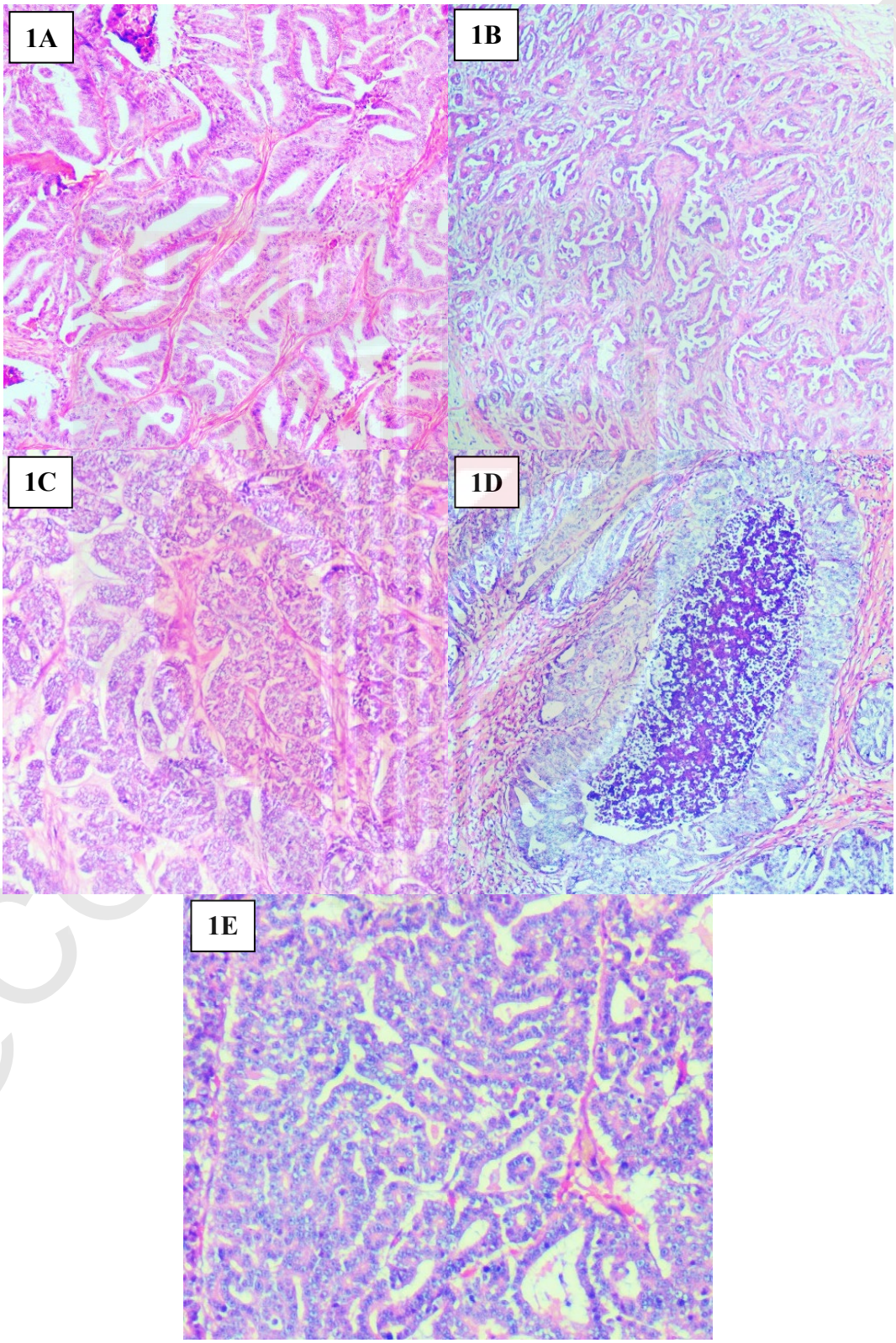
2.1.1 Histological classification of feline mammary tumour

Originally, the first histological classification for domestic animals MGT was published in 1974 by The World Health Organization (WHO) in which descriptive morphology for feline MGT has been included (Hampe & Misdorp, 1974). In 1999, a revised histological classification system was published including a few new histological subtypes (Misdorp *et al.,* 1999). As myoepithelial mammary neoplasm is rarely observed in feline MGT, its histological classification is limited to epithelial cells. Table 1 shows the current histological classification system used for feline mammary carcinoma.

Table 1 Histological classification in feline MGT based on International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019.

NO.	CLASSIFICATION	TYPE	SUBTYPE
1.	Hyperplasia / dysplasia	1.1 Duct ectasia	NA
		1.2 Lobular hyperplasia (adenosis)	1.2.1 Regular 1.2.2 With secretory activity 1.2.3 With fibrosis 1.2.4 With atypia
		1.3 Epitheliosis	NA
		1.4 Papillomatosis	NA
		1.5 Fibroadenomatous change	NA
2.	Benign epithelial neoplasm	2.1 Simple benign tumour	2.1.1 Adenoma – simple
		2.2 Non-simple benign tumour	NA
		2.3 Ductal-associated benign tumour	2.3.1 Ductal adenoma 2.3.2 Intraductal papillary adenoma
3.	Malignant epithelial neoplasm	3.1 Carcinoma in situ	NA
		3.2 Simple carcinoma	3.2.1 Tubular carcinoma 3.2.2 Tubulopapillary carcinoma 3.2.3 Solid carcinoma 3.2.4 Invasive micropapillary carcinoma 3.2.5 Comedocarcinoma 3.2.6 Anaplastic carcinoma
		3.3 Non-simple carcinoma	NA
		3.4 Ductal-associated carcinoma	3.4.1 Ductal carcinoma 3.4.2 Intraductal papillary carcinoma
4.	Malignant epithelial neoplasm – special type	4.1 Squamous cell carcinoma	NA
		4.2 Adenosquamous carcinoma	NA
		4.3 Mucinous carcinoma	NA
		4.4 Lipid-rich (secretory) carcinoma	NA
		4.5 Spindle cell carcinoma	NA
		4.6 Inflammatory mammary carcinoma	NA
5.	Malignant mesenchymal neoplasm of mammary gland	5.1 Osteosarcoma	NA
		5.2 Chondrosarcoma	NA
		5.3 Fibrosarcoma	NA
		5.4 Hemangiosarcoma	NA
		5.5 Other sarcomas	NA
6.	Carcinosarcoma	NA	NA
7.	Hyperplasia / dysplasia of the teat	7.1 Hyperplasia of the teat	NA
8.	Neoplasm of the teat	8.1 Benign ductal-associated neoplasm	8.1.1 Ductal adenoma 8.1.2 Intraductal papillary adenoma
		8.2 Malignant ductal-associated neoplasm	8.2.1 Ductal carcinoma 8.2.2 Intraductal papillary carcinoma
		8.3 Carcinoma with epidermal infiltration (Paget-like disease)	NA

Figure 1 The histological classification of feline MGT. 1A: Tubular carcinoma. 1B: Tubulopapillary carcinoma. 1C: Solid carcinoma. 1D: Comedocarcinoma. 1E: Intraductal papillary carcinoma.



2.1.2 Histological grading of feline mammary tumour

Initially, Elston and Ellis's (E&E, 1991) grading system of feline MGT has been introduced and modified following the Nottingham Grade System (NGS) for human breast cancer. The parameters evaluated were tubular formation, cellular pleomorphism and mitotic count in the form of 3 scoring levels for each parameter, in which the total score obtained by the tumour will determine the grade (Table 2).

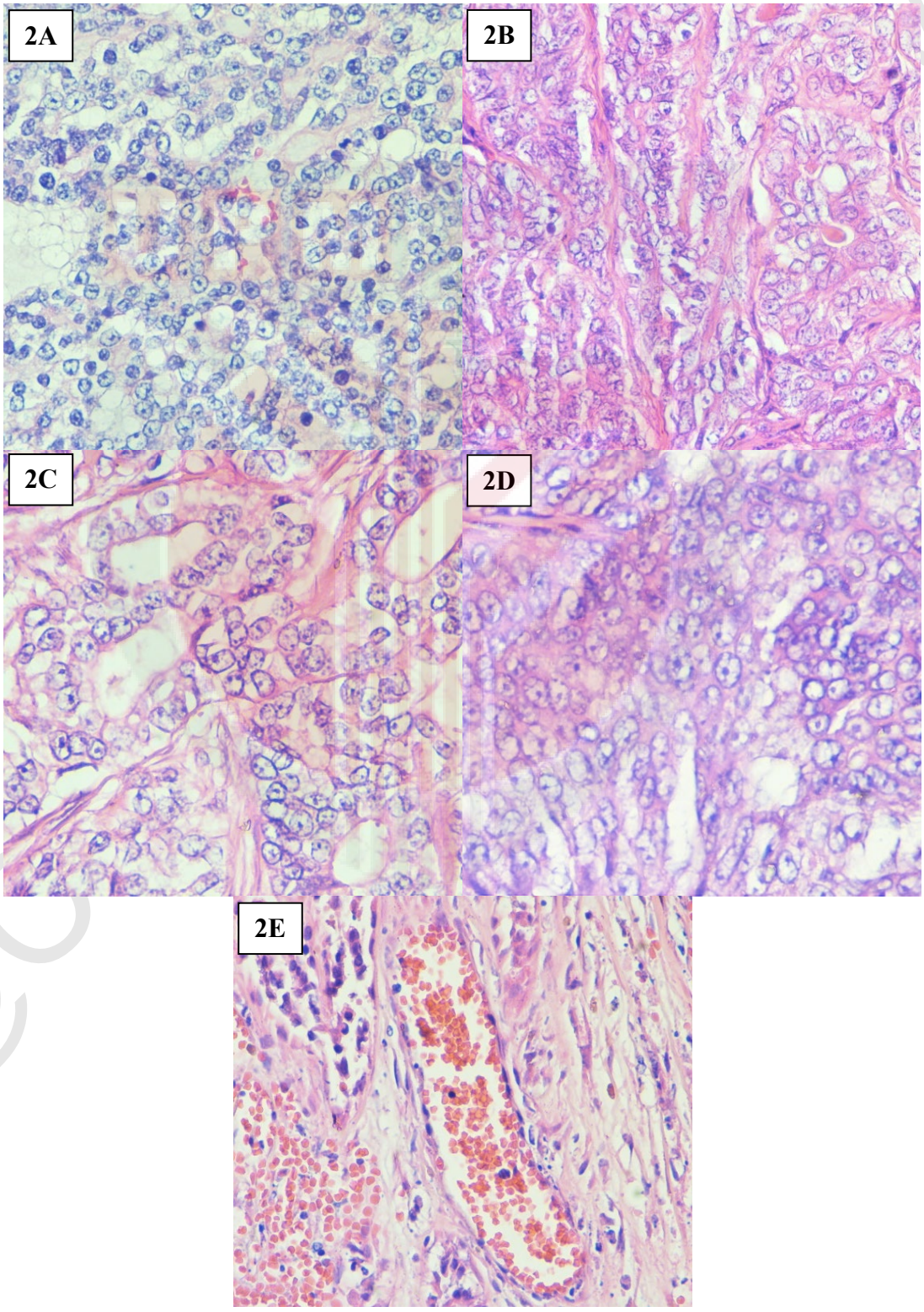
In 2015, a new grading system for feline MGT have been published by Mills and co-author in which the lymphovascular invasion, nuclear form and mitotic count were assessed (Figure 2). Each parameter has 2 scoring levels and similarly, the tumour was scored to determine the grade as shown in Table 2.

Table 2 Criteria for histological grading of feline MGT based on International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019.

ELSTON & ELLIS, 1991 GRADING SYSTEM (E&E, 1991)	
Features	**Points**
A. Tubules formation	
• Tubules comprise >75% of the tumour	1
• Tubules comprise 10-75% of the tumour (moderate formation of tubular arrangements admixed with areas of non-tubular patterns)	2
• Tubules comprise <10% (minimal or no tubule formation)	3
B. Cellular pleomorphism	
• Uniform, regular, small nuclei with occasional small nucleoli	1
• Moderate degree of variation in nuclear size and shape, hyperchromatic nucleus, presence of nucleoli (some of which can be prominent)	2
• Marked variation in nuclear size, hyperchromatic nucleus, often with parachromatin clearing and one or more prominent nucleoli	3
C. Mitotic count (mitoses per 10 HPF) – 0.59mm (0.274mm^2)	
• 0-9	1
• 10-19	2
• >19	3
Histological grade (Total scoring: A+B+C)	**Total**
I (low, well-differentiated)	3-5
II (intermediate, moderately differentiated)	6-7
III (high, poorly differentiated)	8-9
MILLS *et al.*, 2015 GRADING SYSTEM (Mills *et al.*, 2015)	
Features	**Points**
A. Lymphovascular invasion	
• Absent	0
• Present	1
B. Nuclear form	
• ≤5% abnormal	0
• >5% abnormal	1
C. Mitotic count (mitoses per HPF)	
• ≤62	0
• >62	1
Histological grade (Total scoring: A+B+C)	**Total**
I (low grade)	0
II (intermediate grade)	1
III (high grade)	2-3

HPF (high-power field).

Figure 2 The parameters of histological grade based on the E&E, 1991 grading system (2A-2D). The mitotic count: 2A = Score 3. 2B = Score 1. The cellular pleomorphism: 2C = Score 3. 2D = Score 1. The lymphovascular invasion: 2E = Score 1 based on the Mills et al., 2015 grading system.



2.1.3 Tumour infiltrating lymphocytes count in feline MGT

When compared to canine MGT, the evaluation of TIL in feline MGT is something that is quite rarely explored. According to a study conducted by Nascimento *et al.* (2022) on the tumour microenvironment in feline MGT, there is a significant potential for certain subsets of immune cells to influence the clinical outcome of feline affected by mammary carcinoma. However, additional research is required to prove the importance of TIL in feline MGT.

It is also worth to note that to this date, there is no standardized guideline for TIL evaluation in feline MGT. In contrast, human breast cancer has an established TIL evaluation guideline by the International TIL Working Group (Salgado *et al.,* 2015; Hendry *et al.,* 2017; Dieci *et al.,* 2018), and it is proven to be useful for TIL count in domestic animals (Schöniger *et al.,* 2020).

2.2 CANINE MAMMARY GLAND TUMOUR

MGT is most frequently diagnosed in canines (Sorenmo *et al.,* 2011; Rodriguez *et al.,* 2022) as compared to felines and it is also not uncommon among the females. Older dogs aged between 8 and 11 years old are more likely to be affected by MGTs, especially the malignant form (Seronmo *et. al,* 2010).

According to a study conducted in Malaysia, purebred dogs like German Shepherds, Dobermans, Dachshunds, and Boxers have a higher risk of developing MGT than mixed or local breeds (Sahabi *et al.,* 2015). Meanwhile in Spain, a higher incidence of malignant epithelial MGT was found in breeds of Samoyed, Schnauzer, Poodle, German Pinscher, Cocker Spaniel, Dobermann, West Highland White Terrier, Dalmatian, Dachshund, Yorkshire Terrier, and Boxer (Rodriguez *et al.*, 2022).

A stronger association was seen between hormonal exposure and reproductive status with the risk of getting MGT as a study by Schneider *et al.* (1969) discovered that female dogs spayed before the first estrus cycle had a 0.05% occurrence rate of MGT and female dogs spayed after first and second heat had 8 % and 26% risk of being affected by MGT respectively (as cited in Burrai *et al.,* 2020).

2.2.1 Histological classification of canine MGT

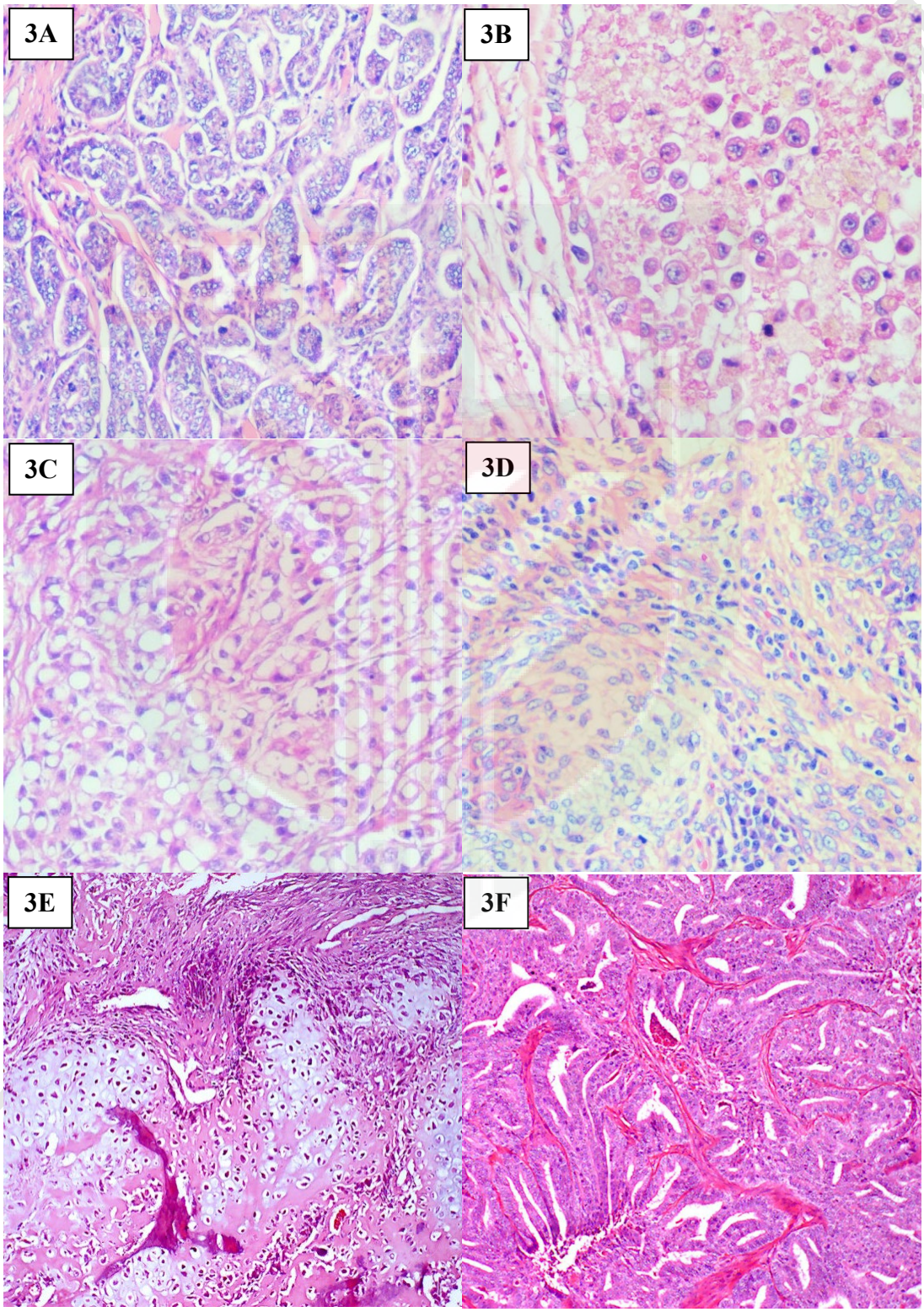
The first histological classification system of canine MGT was published in 1974 followed by a modified system in 1999 by WHO. Currently, the histological classification introduced by Goldschmidt and co-author in 2011 is widely used together with Peña *et al.* (2013) grading system to evaluate the biological behaviour of canine MGT (Table 3). The system highlights the architectural arrangement of the neoplastic cells and the involvement of myoepithelial cells in the neoplastic process.

Table 3 Histological classification in canine MGT based on International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019.

NO.	CLASSIFICATION	TYPE	SUBTYPE
1.	Hyperplasia / dysplasia	1.1 Duct ectasia	NA
		1.2 Lobular hyperplasia (adenosis)	1.2.1 Regular 1.2.2 With secretory activity 1.2.3 With fibrosis 1.2.4 With atypia
		1.3 Epitheliosis	NA
		1.4 Papillomatosis	NA
		1.5 Fibroadenomatous change	NA
		1.6 Gynecomastia	NA
2.	Benign epithelial neoplasm	2.1 Simple benign tumour	2.1.1 Adenoma – simple 2.1.2 Myoepithelioma
		2.2 Non-simple benign tumour	2.2.1 Adenomyoepithelioma 2.2.2 Benign mixed tumour 2.2.3 Fibroadenoma
		2.3 Ductal-associated benign tumour	2.3.1 Ductal adenoma 2.3.2 Intraductal papillary adenoma
3.	Malignant epithelial neoplasm	3.1 Carcinoma in situ	NA
		3.2 Simple carcinoma	3.2.1 Tubular carcinoma 3.2.2 Tubulopapillary carcinoma 3.2.3 Solid carcinoma 3.2.4 Invasive micropapillary carcinoma 3.2.5 Comedocarcinoma 3.2.6 Anaplastic carcinoma
		3.3 Non-simple carcinoma	3.3.1 Carcinoma arise in complex adenoma / benign mixed tumour 3.3.2 Complex carcinoma 3.3.3 Carcinoma and malignant myoepithelioma 3.3.4 Mixed carcinoma
		3.4 Ductal-associated carcinoma	3.4.1 Ductal carcinoma 3.4.2 Intraductal papillary carcinoma
4.	Malignant epithelial neoplasm – special type	4.1 Squamous cell carcinoma	NA
		4.2 Adenosquamous carcinoma	NA
		4.3 Mucinous carcinoma	NA
		4.4 Lipid-rich (secretory carcinoma)	NA
		4.5 Spindle cell carcinoma	NA
		4.6 Malignant myoepithelioma	NA
		4.7 Inflammatory mammary carcinoma	NA

5.	Malignant mesenchymal neoplasm of mammary gland	5.1 Osteosarcoma	NA
		5.2 Chondrosarcoma	NA
		5.3 Fibrosarcoma	NA
		5.4 Hemangiosarcoma	NA
		5.5 Other sarcomas	NA
6.	Carcinosarcoma	NA	NA
7.	Hyperplasia / dysplasia of teat	7.1 Melanosis of skin of teat	NA
		7.2 Hyperplasia of teat	NA
8.	Neoplasm of the teat	8.1 Benign ductal-associated neoplasm	8.1.1 Ductal adenoma 8.1.2 Intraductal papillary adenoma
		8.2 Malignant ductal-associated neoplasm	8.2.1 Ductal carcinoma 8.2.2 Intraductal papillary carcinoma
		8.3 Carcinoma with epidermal infiltration (Paget-like disease)	NA

Figure 3 Histological classification of canine MGT. 3A: Invasive micropapillary carcinoma. 3B: Anaplastic carcinoma. 3C: Lipid-rich carcinoma. 3D: Carcinoma arises in benign mixed tumours. 3E: Carcinosarcoma. 3F: Tubular carcinoma.



2.2.2 Histological grading of canine MGT

Initially, canine MGT was histologically graded based on E&E, 1991 grading system, however the wide variation of canine MGT in terms of tumour heterogeneity leads to a modified grading system published by Peña and co-author in 2013 (Avallone *et al.,* 2020). Three parameters: tubules formation, cellular pleomorphism and mitotic count were evaluated, in which tumour was scored to obtain a grade (Table 4).

Table 4 Criteria for histological grading of canine MGT based on International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019.

PEÑA *et al.*, 2013 GRADING SYSTEM (PEÑA *et al.*, 2013)	
Features	**Points**
A. Tubules formation	
• Tubules comprise >75% of the tumour	1
• Tubules comprise 10-75% of the tumour (moderate formation of tubular arrangements admixed with areas of non-tubular patterns)	2
• Tubules comprise <10% (minimal or no tubule formation)	3
B. Cellular pleomorphism	
• Uniform, regular, small nuclei with occasional small nucleoli	1
• Moderate degree of variation in nuclear size and shape, hyperchromatic nucleus, presence of nucleoli (some of which can be prominent)	2
• Marked variation in nuclear size, hyperchromatic nucleus, often with parachromatin clearing and one or more prominent nucleoli	3
C. Mitotic count (mitoses per 10 HPF) – 0.59mm (0.274mm^2)	
• 0-9	1
• 10-19	2
• >19	3
Histological grade (Total scoring: A+B+C)	**Total**
I (low, well-differentiated)	3-5
II (intermediate, moderately differentiated)	6-7
III (high, poorly differentiated)	8-9

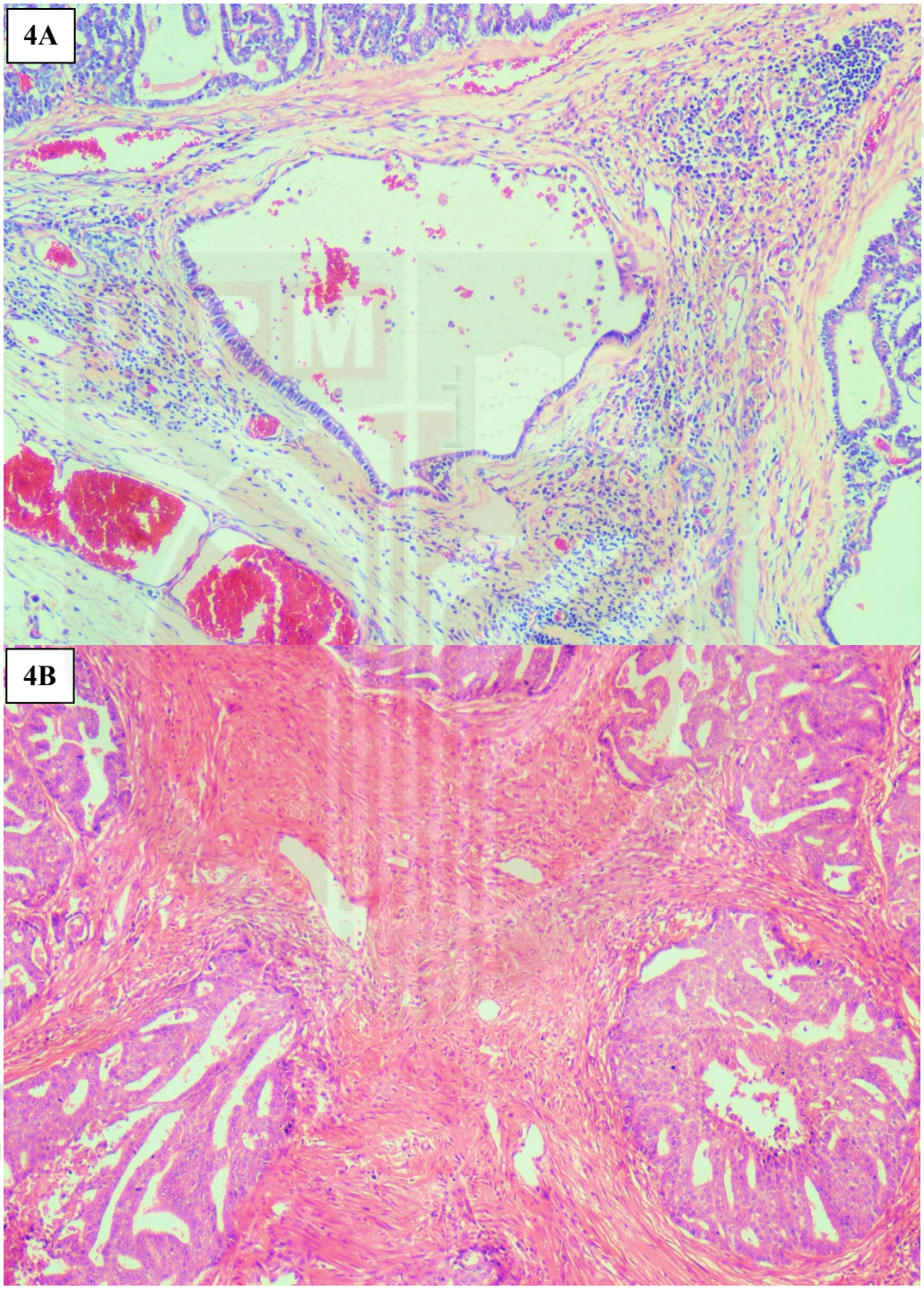
HPF (high-power field).

2.2.3 Tumour-infiltrating lymphocytes count in canine MGT

Studies on TIL evaluation in canine MGT, such as those by Saeki *et al.* (2012), Kim *et al.* (2013), and Carvalho *et al.* (2014), reveal that lymphocytes may have a significant role in the greater malignancy of canine mammary neoplasms.

To this date, there is no universal guideline for TIL evaluation in canine MGT as compared to human breast cancer. Muscatello *et al.* (2022) conclude that TIL evaluation for human breast cancer by the International TILS Working Group is beneficial to be adapted in TIL count of canine MGT.

Figure 4 Tumour infiltrating lymphocytes (TIL) count in canine MGT. 4A: High stromal TIL count (50-100%). 4B: Low stromal TIL count (10-40%).



CHAPTER 3.0

MATERIALS AND METHODS

3.1 DATA RETRIEVAL

Data collected from the Histopathological Laboratory, Veterinary Laboratory Service Unit (VLSU), Universiti Putra Malaysia (UPM) from August 2020 until July 2022 were reviewed retrospectively. A total number of mammary gland tissue samples submitted to the laboratory, including relevant information such as age, breed and neuter status were also recorded and reviewed retrospectively.

Next, the histology slides of the respective mammary gland cases stained with haematoxylin & eosin (H&E) were retrieved, examined under a light microscope, and classified into malignant and benign according to their histological classification based on the International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019.

Tumour grading was performed on the malignant mammary gland neoplasm cases based on the International Histologic Classification of Mammary Tumors of Domestic Animals (3rd Series), 2019. The tumours were graded according to respective grading systems, E&E, 1991 and Mills *et al.,* 2015 grading system for feline MGT while Peña *et al.,* 2013 grading system for canine MGT.

In addition, TIL was assessed for malignant cases based on the guideline provided by the International TIL working group on TIL count in human breast cancer. It was then categorized into two categories: low (0-40%) and high (50-100%) for statistical analysis.

3.2 INCLUSION CRITERIA

All mammary gland samples submitted at Histopathology Laboratory from August 2020 until July 2022 were included in the data collection, in which additional information on the age, breed and neuter status were also recorded. This was followed by the retrieval of H&E stained histological slides in which only mammary gland cases with slides available were viewed under microscope and classified into MGT, other neoplasm and other diagnoses. The MGT slides were then categorized into the malignant and benign group where malignant mammary neoplasm were further classified according to their histology classification and graded. Lastly, TIL was assessed for further statistical analysis.

3.3 EXCLUSION CRITERIA

There were a few exclusion criteria established in the study:

1. Mammary gland cases with no slides retrieved.
2. Necrotic samples of mammary gland slides.
3. Mammary gland slides that require IHC for further confirmation.

3.4 STATISTICAL ANALYSIS

All data collected were recorded in Google Sheet systematically while statistical analysis was conducted using IBM SPSS software version 27.0. Differences in histological grade and TIL count with other categorical variables were evaluated using Fisher Exact test (Fisher-Freeman-Halton test). Statistical significance was established at $p < 0.05$.

CHAPTER 4.0

RESULTS

4.1 FELINE MGT

4.1.1 Analysis of feline mammary gland samples

A total of 167 mammary gland samples were submitted at Histopathology Laboratory, VLSU, UPM from August 2020 until July 2022 and 49 (29.3%) out of 167 were feline mammary gland samples. 46 (93.9%) out of the 49 samples were categorized as mammary gland neoplasm cases while the rest 3 (6.1%) were classified as other cases (inflammation and cyst).

All 46 slides of feline mammary gland samples were managed to be retrieved, comprising 42 (91.3%) malignant mammary gland neoplasm and 4 (8.7%) hyperplasia mammary gland cases.

As in Table 5, feline diagnosed with malignant MGT were all (100.0%) female, aged between 2 to 18 years old [mode 10y; mean 10.67 with ± standard deviation 3.7y; median 10.5y] with 6 (14.3%) aged between 1 to 7-year-old and 36 (85.7%) aged 8-year-old and above. From 42 samples of feline malignant MGT, there were 30 (71.4%) Domestic Short Hair, 4 (9.5%) Persian, 2 (4.8%) Domestic Long Hair, and 1 from each of Siamese (2.4%) and Egyptian Mau (2.4%) while another 4 (9.5%) cat breeds were unidentified. In highlighting the neuter status of the feline with malignant MGT, 23 (54.8%) were intact while the rest 19 (45.2%) were neutered.

Figure 5 Summary flowchart of feline mammary gland cases.

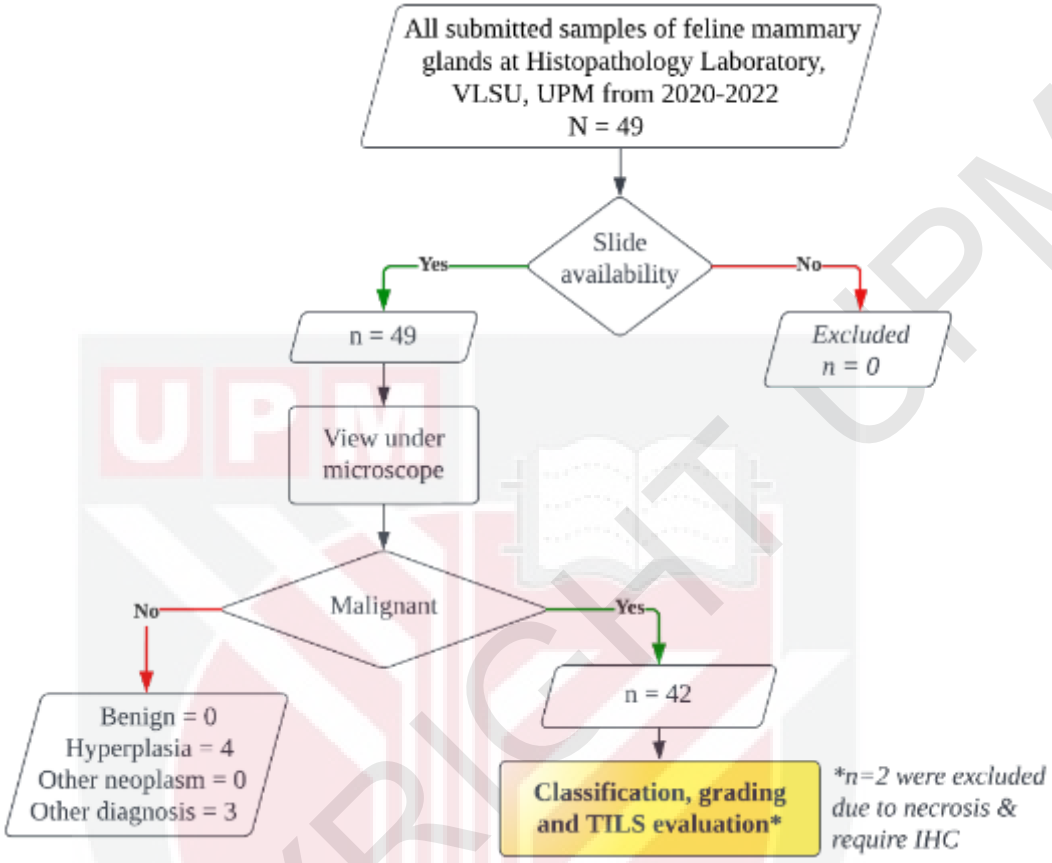


Table 5 Result on the feline malignant mammary gland samples.

VARIABLE (n=42)	TOTAL
Age	
1 to 7-year-old	14.3%; 6
8-year-old above	85.7%; 36
Breed	
Egyptian Mau	2.4%; 1
Domestic Short Hair	71.4%; 30
Domestic Long Hair	9.6%; 4
Persian	4.8%; 2
Siamese	2.4%; 1
Neuter status	
Intact	54.8%; 23
Neuter	45.2%; 19

4.1.2 Analysis of histological classification, grade, and TIL evaluation

A total of 44 slides of feline mammary gland samples were examined and categorized into malignant and hyperplasia as 2 were excluded due to necrosis and required IHC for confirmation.

For histological classification as shown in Table 6, feline MGT had 2 types of mammary carcinoma with 5 subtypes, and the most represented subtype was tubular carcinoma (60.0%; 24) whereas for hyperplasia feline MGT, there were 2 major types recorded which were duct ectasia (50.0%; 2) and fibroadenomatous hyperplasia (50.0%; 2).

Table 6 Result on histological classification of feline MGT.

HISTOLOGICAL CLASSIFICATION (N=44)	TOTAL
Malignant (n=40)	
a) Simple carcinoma (n=38)	**95.0%**
i. Tubular carcinoma	60.0%; 24
ii. Tubulopapillary carcinoma	12.5%; 5
iii. Solid carcinoma	17.5%; 7
iv. Comedocarcinoma	5.0%; 2
b) Ductal-associated carcinoma (n=2)	**5.0%**
i. Intraductal papillary carcinoma	5.0%; 2
Hyperplasia (n=4)	
a) Duct ectasia	50.0%; 2
b) Fibroadenomatous hyperplasia	50.0%; 2

For histological grading in Table 7, both grading systems E&E, 1991 and Mills *et al.,* 2015 had grade II the highest with 62.5%; 25 and 55.5%; 22 respectively, followed by Grade III (22.5%; 9) and Grade I (15.0%; 6) for E&E, 1991 whereas in Mills *et al.,* 2015, both Grade I and Grade III had a similar number of cases with 22.5%; 9 each.

Table 7 Result on histological grade of feline MGT based on E&E, 1991 and Mills et al.,2013 grading system.

HISTOLOGICAL GRADE (n=40)	**E&E, 1991**	**MILLS *et al.*, 2013**
Grade I	15.0%; 6	22.5%; 9
Grade II	62.5%; 25	55.5%; 22
Grade III	22.5%; 9	22.5%; 9

For TIL evaluation in feline mammary carcinoma, 60.0%; 24 were categorized as low TILs (10-40%) count while 40.0%; 16 had high TIL (50-100%) count as shown in Table 8.

Table 8 Result on TILs evaluation of feline MGT.

TIL CATEGORY (n=40)	**TOTAL**
Low (10-40%)	60.0%; 24
High (50-100%)	40.0%; 16

4.1.3 Statistical analysis

For statistical analysis, comedocarcinoma and intraductal papillary carcinoma were excluded due to insufficient samples from these histological classification.

Table 9 shows that there was no relationship between histological grade of grading system E&E, 1991 with age (p=1.00) and neuter status (p=0.901), however there was statistically significant between histological grade and histological classification (p=0.026).

Table 9 Relationship between histological grade of feline malignant MGT (E&E, 1991) with age, neuter status, and histological classification.

VARIABLE (n=40)	**HISTOLOGICAL GRADE BASED ON ELSTON & ELLIS, 1991**			**p-VALUE**
	I *(n=6)*	**II *(n=25)***	**III *(n=9)***	
Age				
1 to 7-year-old *(n=4)*	-	3 (75.0%)	25.0%; 1	NS 1.00[a]
8-year-old and above *(n=36)*	6 (16.7%)	22 (61.1%)	22.2%; 8	
Neuter status				
Intact *(n=22)*	4 (18.2%)	13 (59.1%)	22.7%; 5	NS 0.901[a]
Neuter *(n=18)*	2 (11.1%)	12 (66.7%)	22.4%; 4	
Histological classification *				
Solid carcinoma *(n=7)*	-	14.3%; 1	85.7%; 6	<0.001[a]
Tubular carcinoma *(n=24)*	16.7%; 4	75.0%; 18	8.3%; 2	
Tubulopapillary carcinoma *(n=5)*	40.0%; 2	60.0%; 3	-	

***** Comedocarcinoma and intraductal papillary carcinoma were excluded.

[a] Done with Fisher-Freeman-Halton exact test.

Based on Table 10, negative correlation was seen between the histological grade of Mills *et al.,* 2015 with age (p=1.00) and neuter status (p=0.168), however, there was a correlation between histological grade and histological classification (p=0.037).

Table 10 Relationship between histological grade of feline malignant MGT (Mills et al., 2015) with age, neuter status, and histological classification.

VARIABLE (n=40)	**HISTOLOGICAL GRADE BASED ON MILLS, *et al.*, 2015:**			**P-VALUE**
	I *(n=9)*	**II *(n=22)***	**III *(n=9)***	
Age				NS 1.00[a]
1 to 7 year old *(n=4)*	25.0%; 1	50.0%; 2	25.0%; 1	
8-year-old and above *(n=36)*	22.2%; 8	55.6%; 20	22.2%; 8	
Neuter status				NS 0.168[a]
Intact *(n=22)*	18.2%; 4	68.2%; 15	13.6%; 3	
Neuter *(n=18)*	27.8%; 5	38.9%; 7	33.3%; 6	
Histological classification *				0.037[a]
Solid carcinoma *(n=7)*	-	42.9%; 3	57.1%; 4	
Tubular carcinoma *(n=24)*	29.2%; 7	62.5%; 15	8.3%; 2	
Tubulopapillary carcinoma *(n=5)*	20.0%; 1	40.0%; 2	40.0%; 2	

* Comedocarcinoma and intraductal papillary carcinoma were excluded.

[a] Done with Fisher-Freeman-Halton exact test.

Table 11 shows that there was a positive relationship between TIL evaluation with histological classification (p=0.026), histological grade (p=0.021) and one of the parameters of the E&E, 1991 grading system used which was cellular pleomorphism (p=0.048).

Table 11 Relationship between TIL count with histological classification, grade and parameters of E&E, 1991 grading system.

VARIABLE (n=40)	TUMOUR INFILTRATING LYMPHOCYTES (TIL) COUNT:		p-VALUE
	LOW (10-40%) *(n=24)*	HIGH (50-100%) *(n=16)*	
Histological classification *			
Solid carcinoma *(n=7)*	57.1%; 4	42.9%; 3	NS
Tubular carcinoma *(n=24)*	62.5%; 15	37.5%; 9	1.000[a]
Tubulopapillary carcinoma *(N = 5)*	60.0%; 3	40.0%; 2	
Histological grade based on E&E, 1991			
I *(n=6)*	66.7%; 4	33.3%; 2	NS
II *(n=25)*	60.0%; 15	40.0%; 10	1.000[a]
III *(n=9)*	55.6%; 5	44.4%; 4	
Histological parameters of E&E, 1991			
a) Tubular formation			
Score 1 *(n=28)*	57.1%; 16	42.9%; 12	NS
Score 2 *(n=8)*	75.0%; 6	25.0%; 2	0.593[a]
Score 3 *(n=4)*	50.0%; 2	50.0%; 2	
b) Cellular pleomorphism			
Score 1 *(n=5)*	60.0%; 3	40.0%; 2	0.048[a]
Score 2 *(n=16)*	81.3%; 13	18.8%; 3	
Score 3 *(n=19)*	42.1%; 8	57.9%; 11	
c) Mitotic count			
Score 1 *(n=0)*	-	-	NS
Score 2 *(n=9)*	55.6%; 5	44.4%; 4	1.000[a]
Score 3 *(n=31)*	61.3%; 19	38.7%; 12	

***** Comedocarcinoma and intraductal papillary carcinoma were excluded.
[a] Done with Fisher-Freeman-Halton exact test. Not significant (NS).

Table 12 shows that TIL evaluation had a strong linked with the histological classification (p=0.026), histological grade (p=0.021) and one of the parameters of the Mills *et al.,* 2015 grading system which was the mitotic count (p=0.025).

Table 12 Relationship between TIL count with histological classification, grade, and parameters of Mills et al., 2015 grading system.

VARIABLE (n=40)	TUMOUR INFILTRATING LYMPHOCYTES (TIL) COUNT:		p-VALUE
	LOW (10-40%) *(n=24)*	HIGH (50-100%) *(n=16)*	
Histological classification *			
Solid carcinoma *(n=7)*	57.1%; 4	42.9%; 3	NS
Tubular carcinoma *(n=24)*	62.5%; 15	37.5%; 9	1.000[a]
Tubulopapillary carcinoma *(n=5)*	60.0%; 3	40.0%; 2	
Histological grade based on Mills et al., 2015			
I *(n=9)*	88.9%; 8	11.1%; 1	NS
II *(n=22)*	59.1%; 13	40.9%; 9	0.062[a]
III *(n= 9)*	33.3%; 3	66.7%; 6	
Histological parameters of Mills et al., 2015			
a) Lymphovascular invasion			
Absent *(n=24)*	66.7%; 16	33.3%; 8	NS
Present *(n=16)*	50.0%; 8	50.0%; 8	0.339[a]
b) Nuclear form			
≤5% *(n=23)*	73.9%; 17	26.1%; 6	NS
>5% *(n=17)*	41.2%; 7	58.8%; 10	0.053[a]
c) Mitotic count			
≤62 *(n=26)*	73.1%; 19	26.9%; 7	0.041[a]
>62 *(n=14)*	35.7%; 5	64.3%; 9	

***** Comedocarcinoma and intraductal papillary carcinoma were excluded.

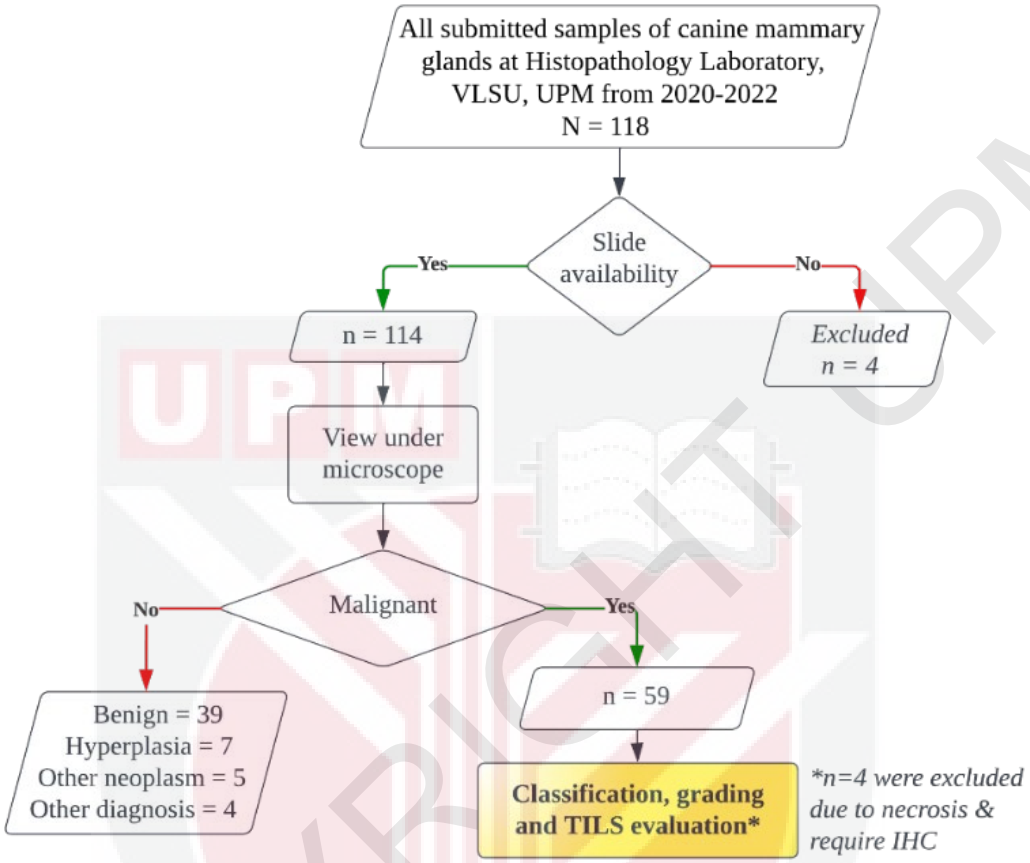
[a] Done with Fisher exact test. Not significant (NS).

4.2 CANINE MGT

4.2.1 Analysis of mammary gland samples

A total of 167 mammary gland samples were submitted at Histopathology Laboratory, VLSU, UPM from August 2020 until July 2022 with 118 (70.7%) out of 167 being canine mammary gland samples. 114 H&E histology slides of canine mammary gland samples were successfully retrieved, comprised of 59 (51.6%) malignant MGT, 38 (33.3%) benign MGT, 8 (7.0%) hyperplasia mammary gland cases, 5 (4.4%) were diagnosed at other neoplasms (1 hemangiosarcoma; 1 lipoma; 1 lymphoma; 1 melanoma; 1 soft tissue sarcoma) and 4 (3.5%) were other diagnosis (2 inflammation; 1 inconclusive; 1 pyogranulomatous mastitis).

Figure 6 Summary flowchart of canine mammary gland cases.



From 59 malignant canine MGT samples, 57 (97.0%) were female and 2 (3.0%) were male, aged between 4 to 16 years old [mode 10y; mean 10.23 with ± standard deviation 2.5y; median 10.0y] with 6 (21.0%) aged 7-year-old and below, 47 (79.0%) aged 8-year-old and above while the rest 6 (10.9%) were not recorded.

Based on Table 13, purebreds made up 36 (61.0%) of the total malignant MGT cases, which have 9 Shih Tzus, 6 Poodles, 4 Rottweilers, 2 each of Golden Retriever, Shepherd, Spaniel, and Schnauzer while Beagle, Corgi, Terrier, Maltese, and Pinscher each has 1 representative. 11 (18.6%) were local breeds, followed by 3 (5.0%) mix breed diagnosed with malignant MGT whereas the remaining 9 (15.25%) dogs breed were not known. Breed size of canines having malignant MGT was categorized into two (small and large) according to their breed characteristics in which 23 (63.9%) were small-sized breeds (toy, small and medium) and 13 (36.1%) were large-sized dogs (large and giant) while remaining 23 made up of local, mix and the unknown breed were excluded. Concerning the neuter status of the female dogs diagnosed with MGT, 49 (83.0%) were intact and 10 (17.0%) were neutered.

Table 13 Result on the canine malignant mammary gland samples.

VARIABLE (n=59)	TOTAL
Age	*
1 to 7-year-old	21.0%; 6
8-year-old above	79.0%; 47
Breed	*
Shih Tzu	15.25%; 9
Poodle	10.17%; 6
Rottweiler	6.8%; 4
Golden Retriever	3.4%; 2
Spaniel	3.4%; 2
Schnauzer	3.4%; 2
Shepherd	3.4%; 2
Beagle	1.7%; 1
Corgi	1.7%; 1
Terrier	1.7%; 1
Maltese	1.7%; 1
Pinscher	1.7%; 1
Local / Mongrel	18.6%; 11
Mix	5.0%; 3
Breed size	**
Small-sized (toy, small and medium)	63.9%; 23
Large-sized (large and giant)	36.1%; 13
Neuter status	**
Intact	83.0%; 49
Neuter	17.0%; 10

*Missing data were excluded. **Only purebred size was included.

4.2.2 Analysis of histological classification, grade, and TIL evaluation

4 out of 59 malignant canine MGT slides were excluded from further classification, grading and TIL evaluation due to necrotic samples, and some require IHC for confirmation. A total of 101 slides of canine mammary gland samples were then undergone histological classification.

Table 14 shows that there were 5 types of canine mammary carcinoma with 13 subtypes, and the most seen subtype was tubular carcinoma (20.0%; 11), while canine mammary benign tumour had 3 types and 5 subtypes recorded with benign mixed tumour (47.4%; 18) as the highest benign subtype seen. For hyperplasia, there were 3 major types seen and lobular hyperplasia (75.0%; 6) is the most common hyperplasia seen.

Table 14 Result on the histological classification of canine MGT.

HISTOLOGICAL CLASSIFICATION (N=101)	TOTAL
Malignant (n=55)	
c) Simple carcinoma (n=26)	**47.3%**
i. Tubular carcinoma	20.0%; 11
ii. Tubulopapillary carcinoma	5.5%; 3
iii. Solid carcinoma	7.3%; 4
iv. Invasive micropapillary carcinoma	5.5%; 3
v. Comedocarcinoma	7.3%; 4
d) Non-simple carcinoma (n=16)	**29.1%**
i. Anaplastic carcinoma	1.8%; 1
ii. Carcinoma arise in benign mixed tumour	5.5%; 3
iii. Carcinoma and malignant myoepithelioma	14.5%; 8
iv. Complex carcinoma	3.6%; 2
v. Mixed carcinoma	5.5%; 3
e) Ductal-associated carcinoma (n=10)	**18.2%**
i. Ductal carcinoma	3.6%; 2
ii. Intraductal papillary carcinoma	14.5%; 8
f) Special type carcinoma (n=1)	**1.8%**
i. Lipid-rich carcinoma	1.8%; 1
g) Carcinosarcoma (n=2)	**3.6%**
Benign (n=38)	
a) Simple benign tumour (n=1)	**2.6%**
i. Simple adenoma	2.6%; 1
b) Non-simple benign tumour (n=35)	**92.1%**
i. Complex adenoma	36.8%; 14
ii. Benign mixed tumour	47.4%; 18
iii. Fibroadenoma	7.9%; 3
c) Ductal-associated benign tumour (n=2)	**5.3%**
i. Intraductal papillary adenoma	5.3%; 2
Hyperplasia (n=8)	
a) Duct ectasia	12.5%; 1
b) Lobular hyperplasia	75.0%; 6
c) Fibroepithelial polyp	12.5%; 1

For histological grading, carcinosarcoma (n=2) was excluded as sarcoma is a special type that does not require grade (Peña *et al.,* 2013) in which no relationship can be seen between grade and TIL count for this type, therefore a total number of canine MGT graded and undergone TIL evaluation was 53.

Based on Table 15, Grade II (56.6%; 30) had a higher occurrence rate than the other two grades, with grade I (20.8%; 11) and III (22.6%; 12) having almost similar number of cases.

Table 15 Result on histological grading of canine malignant MGT.

HISTOLOGICAL GRADE (n=53) *	**TOTAL**
Grade I	20.8%; 11
Grade II	56.6%; 30
Grade III	22.6%; 12

* Carcinosarcoma were excluded.

For TIL evaluation in canine mammary carcinoma, low TIL (10-40%) count was seen the most with 71.7%; 38 whereas high TIL (50-100%) count was only 28.3%; 15 as shown in Table 16.

Table 16 Result on TIL evaluation of canine malignant MGT.

TIL CATEGORY (n=53) *	**TOTAL**
Low (10-40%)	71.7%; 38
High (50-100%)	28.3%; 15

* Carcinosarcoma were excluded.

4.2.3 Statistical analysis

In contrast to feline statistical analysis, no histological classification was excluded as there was a trend seen with histological grade. Table 17 shows that there was no relationship between the histological grading of canine MGT with age (p=0.325) and neuter status (p=0.646), however, there was a statistically significant between the histological grade of Pena *et al.,* 2013 and the histological classification (p=0.026).

Table 17 Relationship between histological grade of canine malignant MGT (Pena et al., 2013) with age, neuter status, and histological classification.

VARIABLE (n=53)	**HISTOLOGICAL GRADE BASED ON PENA *et al.*, 2013:**			**p-VALUE**
	I *(n = 11)*	**II *(n = 30)***	**III *(n = 12)***	
Age *				NS 0.325[a]
1 to 7-year-old *(n= 6)*	33.3%; 2	33.3%; 3	33.3%; 2	
8-year-old and above *(n=41)*	14.6%; 6	63.4%; 26	22.0%; 9	
Neuter status				NS 0.646[a]
Intact *(n=43)*	23.3%; 10	55.8%; 24	20.9%; 9	
Neuter *(n=10)*	10.0%; 1	60.0%; 6	30.0%; 3	
Histological classification				0.026[a]
Tubular carcinoma *(n=11)*	36.4%; 4	54.5%; 6	9.1%; 1	
Tubulopapillary carcinoma *(n=3)*	25.0%; 1	75.0%; 3	-	
Solid carcinoma *(n=4)*	-	50.0%; 2	50.0%; 2	
Invasive micropapillary carcinoma *(n=3)*	-	33.3%; 1	66.7%; 2	
Comedocarcinoma *(n=4)*	-	25.0%; 1	75.0%; 3	
Anaplastic carcinoma *(n=1)*	-	-	100.0%; 1	
Carcinoma arise in benign mixed tumour *(n=3)*	66.7%; 2	33.3%; 1	-	
Carcinoma and malignant myoepithelioma *(n=8)*	-	100.0%; 8	-	
Complex carcinoma *(n=2)*	50.0%; 1	50.0%; 1	-	
Mixed carcinoma *(n=3)*	-	66.7%; 2	33.3%; 1	
Ductal carcinoma *(n=2)*	-	100.0%; 2	-	
Intraductal papillary carcinoma *(n=8)*	37.5%; 3	50.0%; 4	12.5%; 1	
Lipid-rich carcinoma *(n=1)*	-	-	100.0%; 1	
Carcinosarcoma (n=2)	*NOT GRADED*			

*Missing data are excluded.

[a] Done with Fisher-Freeman-Halton exact test. Not significant (NS).

There was a positive association seen between TIL evaluation with the histological classification (p=0.026), histological grade (p=0.021) and one of the parameters of Pena *et al.,* 2013 grading system which was the mitotic count (p=0.025) based on Table 18.

Table 18 Relationship between TILs count with histological classification, histological grade, and parameters of Pena et al., 2013 grading system.

VARIABLE (n=53)	**TUMOUR-INFILTRATING LYMPHOCYTES (TIL) COUNT:**		**p-VALUE**
	LOW (10-40%) *(n=38)*	**HIGH (50-100%)** *(n=15)*	
Classification			
Tubular carcinoma *(n=11)*	81.8%; 9	18.2%; 2	
Tubulopapillary carcinoma *(n=3)*	66.7%; 2	33.3%; 1	
Solid carcinoma *(n=4)*	75.0%; 3	25.0%; 1	
Invasive micropapillary carcinoma *(n=3)*	33.3%; 1	66.7%; 2	
Comedocarcinoma *(n=4)*	25.0%; 1	75.0%; 3	
Anaplastic carcinoma *(n=1)*	-	100.0%; 1	0.026[a]
Carcinoma arise in benign mixed tumour *(n=3)*	100.0%; 3	-	
Carcinoma and malignant myoepithelioma *(n=8)*	87.5%; 7	12.5%; 1	
Complex carcinoma *(n=2)*	100.0%; 2	-	
Mixed carcinoma *(n=3)*	100.0%; 3	-	
Ductal carcinoma *(n=2)*	-	100.0%; 2	
Intraductal papillary carcinoma *(n=8)*	87.5%; 7	12.5%; 1	
Lipid-rich carcinoma *(n=1)*	-	100.0%; 1	
Histological grade based on Pena, 2013			
I *(n=11)*	100.0%; 11	-	0.021[a]
II *(n=30)*	70.0%; 21	30.0%; 9	
III *(n=12)*	50.0%; 6	50.0%; 6	
Histological parameters of Pena, 2013			
a) Tubular formation			
Score 1 *(n=18)*	83.3%; 15	16.7%; 6	NS
Score 2 *(n=26)*	65.4%; 17	34.6%; 9	0.397[a]
Score 3 *(n=9)*	66.7%; 6	33.3%; 3	
b) Cellular pleomorphism			
Score 1 *(n=5)*	80.0%; 4	20.0%; 1	NS
Score 2 *(n=29)*	82.8%; 24	17.2%; 5	0.073[a]
Score 3 *(n=19)*	52.6%; 10	47.4%; 9	
c) Mitotic count			
Score 1 *(n=8)*	100.0%; 8	-	0.025[a]
Score 2 *(n=14)*	85.7%; 12	14.3%; 2	
Score 3 *(n=31)*	58.1%; 18	41.9%; 13	

[a] Done with Fisher-Freeman-Halton exact test. Not significant (NS).

CHAPTER 5.0

DISCUSSION

5.1 RISK FACTORS OF FELINE AND CANINE MGT

Both species presented a high number of malignant cases of MGT (Table 6 and Table 14). Bergman (2017) stated that the pathology of MGT in feline and canine were significantly different with the cat having more than 90% malignant form whereas the dog varies with up to 50% of malignant form.

For age distribution in both species, older cats and dogs aged 8-year-old and above were the most affected by MGT (Table 5 and Table 13) with age 10-year-old in both species had the highest cases of MGT. Several studies reported dogs aged 8-11 years old were highly likely to get MGT (Pastor *et al.,* 2018; Ežerskytė *et al.,* 2011) while a study by Nascimento *et al.* (2022) on feline MGT found that cats aged 8-year-old and above was diagnosed with MGT the most compared to age 7 and below. There was however no correlation found between the histological grade of all three grading systems with the age of both species (Table 9, Table 10 and Table 17).

For breed distribution, DSH and Siamese were the most mentioned breed in previous studies on feline MGT (Graf *et al.,* 2016; Hayer *et al.,* 1983) supporting our findings (Table 5). In addition, DSH is also the most common breed of feline in Malaysia (Yusof *et al.,* 2022) which may explain the high number of DSH breed diagnosed with MGT in this study. However, our study only encountered one Siamese breed (2.0%) which may be due to Siamese breed not being a popular breed in Malaysia. Unfortunately, there were no further study to support the reasoning. In dog,

purebred had the highest incidence of mammary carcinoma (Table 13) supported by Gunnes *et al.,* (2017), Zheng *et al.* (2022) and Seung *et al.,* (2021). This could be due to low genetic variation in purebred dogs than in local or mixed breeds (Sahabi *et al.,* 2015). A study conducted by Thumser-Henner *et al.* (2020) state that there were possibilities for canine MGT caused by genetic component specifically canine BRCA2 gene alterations. However, since MGT in feline and canine are hormonal-dependent, there might be not many possibilities for genetic to be one of the risk factors of MGT.

From the aspect of the breed size among purebred dogs as shown in Table 13, the study discovered that small-sized dogs (toy, small and medium size) especially Shih Tzu had a greater predisposition to mammary carcinoma than large-sized dogs (large and giant size). In contrast, the study by Pastor *et al.* (2018) found that large-breed dogs had a higher risk of developing malignant MGT with no correlation found between breed size and the likelihood to be affected by mammary carcinoma. In this study, Mongrel and mix breed were excluded from the size category because they featured a great variation of size and weight.

Next, for gender distribution in MGT cases, few literatures mentioned MGT occurrence in males Zheng *et al.* (2022) which aligned with our findings (Table 13). In this study, an intact male dog was diagnosed with ductal-associated MGT whereas a neutered male dog had non-simple carcinoma. However, there was a pattern seen among intact female dogs in which 50% (22/44) had simple carcinoma whereas among neutered female dogs, no obvious pattern was seen as simple and ductal-associated carcinoma was diagnosed the most with 44.4% (4/9) in both types. In the meantime, the odds of female cats developing mammary carcinoma were higher than male cats (Graf *et al.,* 2016) which explained the findings in feline MGTs (Table 5).

In term of neuter status, both species showed high intact status with MGT (Table 5 and Table 13). Spaying at early age does reduce the risk of getting MGT in both species due to lower duration of exposure towards hormone (Overley *et al.,* 2005; Schneider *et al.,* 1969). However, there was no correlation found between gender and neuter status of both species in this study, therefore we could not conclude that the high occurrence of feline and canine MGT in Malaysia among intact female was attributable to extended hormonal exposure. There was also no correlation between histological grade and neuter status (Table 9, Table 10, and Table 17).

5.2 FELINE MGT

5.2.1 Diversity of histological classification and grade of feline MGT

In agreement with Seixas *et al*., (2011), Caliari *et al.,* (2014) and Carvalho *et al.,* (2017), this study also found that histological classification for feline MGT was limited to epithelial neoplasms (Table 6). There was also a study that reported a diversity of histological classification of feline MGT (De Campos *et al.,* 2016). This lack of variation in terms of histological classification of feline MGT was also the reason on why E&E, 1991 grading system is still relevant for feline malignant carcinomas as it mainly focused on epithelial neoplasm (Avallone *et al.,* 2021).

Based on Table 7, Grade II was seen as the highest compared to Grade I and II for both grading systems with more Grade I detected using the Mills *et al.,* 2015 grading system. A study found that the tumours heavily leaned towards grade II or III (58.3% and 39.8%) leading to a lack of presentation of grade I (1.9%) in E&E, 1991 grading system, whereas the modified grading system were claimed to be able to detect grade I much better (Mills *et al.* (2015).

Avallone *et al.* (2021) also stated that there was no agreement on which grading system of feline MGT (E&E, 1991 and Mills *et al.,* 2015) should be utilized. Due to this reason, both grading systems were used in this study. Most studies conducted using E&E, 1991 grading system found that grade I has a better survival rate and grade III with the worst survival rate, however, there was no conclusive outcome for Grade II (Avalone *et al.,* 2021; Castagnaro *et al.,* 1998). Therefore, Mills and co-author introduced a new grading system for feline MGT in 2015, and it is proven to be statistically significant between histological grade and median overall survival rate (Avalone *et al.,* 2021). Dagher *et al.* (2019) state that this may be due to

the high mitotic count in feline mammary carcinoma as compared to human breast cancer, which leads to a new cut-off at more than 62 (Table 2). As there was no survival analysis done, our study could not determine the significance of these two grading systems with overall survival rates.

5.2.2 Relationship between histological grade with age, neuter status, and histological classification of feline MGT

The histological grade of both grading system was not associated with the age and neuter status of the affected feline and no distinct pattern was seen.

However, there was a positive association seen between the histological classification and grade in both grading systems (Table 8 and Table 9). While certain histological classifications have variable histological grades and different overall survival rates, others have specific histological classifications that tend to have certain histological grades (Louis *et al.,* 2019) which is in parallel as our findings especially the tubular carcinoma and solid carcinoma (Table 8 and Table 9).

5.2.3 Relationship between TIL evaluation with the histological classification and grade of feline MGT

Our result shows that there were no meaningful findings between TIL evaluation with histological classification and grade of feline MGT (Table 10 and Table 11). One study, however, discovered the exact opposite in terms of TIL with histological grade. Specific immune cells have a major roles in feline mammary carcinoma such as high $CD8^+$ T cells was linked with good survival rate (Nascimento *et al.,* 2022). The location of TIL evaluated may also play an important part, as Nascimento *et al.* (2022) found that the intratumoral $CD3^+$ and $CD4^+$ cells were correlated with poor clinicopathological outcome. In our study, only stromal TILs were evaluated and based on the guideline used, the intratumoral TILs were excluded due to insufficient evidence of its benefits in human breast cancer (Salgado *et al.,* 2015; Hendry *et al.,* 2017; Dieci *et al.,* 2018).

In between TIL evaluation and parameters of the grading system used, there was an association found with mitotic count of Mills *et al.,* 2015 grading systemin which low TIL count was seen the most in low mitotic count (Table 12). Correlation between TIL count and cellular pleomorphism of E&E, 1991 was also observed, but no visible trend was seen as both low and high TIL count dominated the higher score of cellular pleomorphism (Table 11). Other studies in feline melanocytic tumours also discovered an association between higher TIL count with cellular pleomorphism and mitotic count (Porcellato *et al.,* 2021). However, additional research on TIL evaluation and feline MGT is needed to draw a firm conclusion.

5.3 CANINE MGT

5.3.1 Diversity of histological classification and grade in canine MGT

Data presented in Table 14 showed that canine MGT has a variety of histological classifications as there was epithelial neoplasm and myoepithelial neoplasm discovered, in agreement with the findings from these epidemiological studies in Spain (Pastor *et al.,* 2018; Rodriguez *et al.,* 2022). Also, the most common subtype seen in the canine MGT was tubular carcinoma (Table 14) which supports those previous studies that mentioned the most common subtype of canine MGT was simple carcinoma (Ežerskytė *et al.,* 2011; Tavasoly *et al.,* 2013).

For the histological grade as displayed in Table 15, Grade II was also seen the most and the similar pattern was reported in a study conducted by Zheng *et al.,* (2022) (Grade II 40.2%, 98/244); nevertheless, another study had tumour grade I the most with 44.6%; 29/65 (Peña *et al.,* 2013). As canine MGT shown a diversified histological classification, a species-specific histological grading system for canine mammary carcinoma has been introduced by Peña and co-author to suit the huge diversity shown (Avallone *et al.,* 2021). The grading system has been used simultaneously with the Goldschmidt *et al.* (2011) histological classification system in Volume 2 (Mammary Tumors) of the series Surgical Pathology of Domestic Animals of the CL Davis Foundation C. Even though the Peña *et al.* (2013) grading system shown an accurate prediction of survival rates among Grade I and Grade II tumour (Canadas *et al.,* 2019), there were uncertainty raised between grade II and the survival rates. However, Santos *et al.* (2015) conclude that the grading system with 3 scoring levels is better at predicting the prognosis of the affected animal than 2 scoring levels.

5.3.2 Relationship between histological grade with age, neuter status, and histological classification of canine MGT

There was no correlation found between histological grade with the age and neuter status of the affected canine (Table 17). However, older dogs aged between 8-year-old and above had a higher likelihood of having a higher grade of MGT, which was also observed in intact female dogs. This is similar as findings from the previous research on canine MGT (Sahabi *et al.*, 2015; Seronmo *et al.*, 2010; Pastor *et al.*, 2018).

Histological grade was found to be closely linked with histological classification as certain histological classifications showed a tendency towards specific histological grade (Table 17). Comedocarcinoma, lipid-rich carcinoma and anaplastic carcinoma are usually classified as grade III while complex carcinoma is rarely seen with Grade III (Louis *et al.,* 2019), in which comparable as what was reported by our study (Table 17). Despite that, the lack of samples from these histological subtypes may affect the judgement made.

5.3.3 Relationship between TIL evaluation with the histological classification and grade of canine MGT

There was a trend between TIL evaluation and histological grade of canine found in this study, in which higher TIL count was strongly associated with the higher grade and the higher mitotic count (Table 18). These findings were supported by Saeki *et al.* (2012) and Muscatello *et al.* (2022) who also seen a higher TIL count in higher malignancy of canine MGT and Kim *et al.* (2013) discovered a higher lymphocytes infiltration in aggressive tumour behaviour of canine MGT, specifically with higher histological grade and presence of lymphatic invasion.

It is worth to mention that most of the previous research explored deeper towards the significance of specific subpopulation of TIL in canine MGT towards survival rates such as Treg cells, $CD3^+$ T-cells, $CD4^+$ T-cells and $CD8^+$ T-cells (Franzoni *et al.,* 2019; Carvalho *et al.,* 2016; Kim *et al.,* 2012; Estrela-Lima *et al.,* 2010). In canine MGT, a higher $CD4^+$ cells was correlated with the greater malignancy of MGT with a poorer prognosis whereas $CD8^+$ cells was seen more in the non-metastatic MGT (Estrela-Lima *et al.,* 2010). The study by Kim *et al.* (2013) found a higher $CD3^+$ T cells and $CD79\propto^+$ B cells in higher histologic grade with lymphatic invasion. While the exact role of TIL in canine MGT remains unknown, Kim *et al.* (2010) suggests that the expression of TIL, cytokine, and mutation of BRCA1 may play an important part in the tumour growth.

Additionally, the positive correlation between TIL count and histological subtype showed that the tumour types with a preference for higher grades (Louis *et al.,* 2019), such as comedocarcinoma, lipid-rich carcinoma, and anaplastic carcinoma exhibit a higher TIL count (Table 18). The conclusion drawn, however, might be

impacted by the scarcity of samples from these histological subtypes. This preliminary study suggests that lymphocytes may contribute towards malignancy and aggressiveness of canine mammary carcinoma.

5.4 EVALUATION OF TIL ON FELINE AND CANINE MGT ACCORDING TO THE TIL CRITERIA FOR HUMAN BREAST CANCER

To the best of the author's knowledge, there are currently no defined criteria for assessing infiltrating lymphocytes in mammary carcinomas of different animal species, and there are few published studies on TILs in feline and canine MGT. Study on feline MGT, for instance by Nascimento *et al.* (2022) counted the immune cell populations on three randomly selected individual fields at 200x magnification (total area = 1.15mm2) while in the study of canine MGT, Estrela-Lima *et al.* (2010) assessed lymphocytes in eight selected "hot spots" histological area and Kim *et al.* (2012) classified the degree of TILs based on their distribution and intensity.

Recent research conducted by Schöniger *et al.* (2020) on pet rabbit mammary carcinoma demonstrates that the TIL guideline for human breast cancer (Salgado *et al.,* 2015; Hendry *et al.,* 2017; Dieci *et al.,* 2018) applies to mammary carcinoma in rabbit as well as other domestic animals including feline and canine. A thorough search of relevant literature garnered only one reported study that use the similar guideline for TIL evaluation in canine MGT and it was proven to be practical (Muscatello *et al.,* 2022), but there are no published studies for TIL count on feline MGT that utilizes the TIL criteria for human breast cancer.

Therefore, making use of the TIL guideline that has been published by the International TIL Working Group on feline and canine MGT in this study will enable standardized criteria to be established across a variety of animal species along with human beings.

5.5 LIMITATION AND RECOMMENDATION

1. The overall survival rate of affected animals was not recorded, therefore it is recommended to include survival analysis in a future study to strongly support the previous findings that state classification and grading associated with mean overall survival rate.
2. As the study did not specify on treatments administered, the findings may have been affected if the study includes the affected animals that received chemotherapy session. In conclusion, it is advisable to include treatment given towards affected animals in future studies.
3. The exact spay age was not included in the study, therefore the risk factors involving hormonal exposure with feline and canine MGT could not be studied properly. Therefore, recording the exact spay age may benefits the future epidemiological study on feline and canine MGT.
4. There was an insufficient number of mammary gland samples for canine MGT as there were necrotic samples and some require IHC leading to several histological classifications being underrepresented. Thus, it is recommended to have a bigger sample size for the study on canine MGT with at least 90 samples to obtain more variation in histological classification whereas for the study on feline MGT, the sample size is sufficient (n=40).

CHAPTER 6.0

CONCLUSION

To conclude, there is a variation of histological classification and grading of feline and canine MGT with canine MGT displaying a much diversified histological classification and grade as compared to feline MGT. The malignant mammary carcinoma was also higher than benign tumour in both species.

Also, there is an association between histological grade and histological classification in the MGT of both feline and canine.

Lastly, the findings in this study concur with the earlier studies in which higher TIL count is strongly linked with higher histological grade and higher mitotic count in canine mammary carcinoma.

CHAPTER 7.0

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