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Ik ga akkoord,

CREEMERS, Anneleen

Datum: 14.12.2009

Prognostication of canine MCTs

A comparison of proliferation markers Ki67 and MCM7

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Eindverhandeling voorgedragen tot het bekomen van de graad master in de biomedische wetenschappen klinische moleculaire wetenschappen

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Abbreviations

-	AD	Distilled water
-	AgNOR	Argyrophilic nucleolar organizer region
-	CDC6	Cell division cycle 6
-	CDK	Cyclin-dependent kinase
-	CDT1	Chromatin licensing and DNA replication factor 1
-	DAB	Diaminobenzidine
-	DDK	DBF4-dependent kinase
-	DNA	Deoxyribonucleic acid
-	E-NPP3	Ecto-nucleotide pyrophosphatase/phosphodiesterase
-	FCS	Fetal calf serum
-	FNA	Fine needle aspirate
-	G0	Quiescent phase
-	G1	Increase of cell size
-	G2	Cell growth
-	HE	Hematoxylin and eosin stain
-	Hpf	High power field
-	IgE	Immunoglobulin E
-	ITD	Internal tandem duplication
-	M	Mitosis phase
-	MACS	Magnetic activated cell sorting
-	MCTs	Mast cell tumours
-	MI	Mitotic index
-	NAD	Nicotinamide adenine dinucleotide
-	ORC	Origin replication complex
-	OsO_4	Osmiumtetroxide
-	PCNA	Proliferating cell nuclear antigen
-	Pre-RC	Pre-replication complex
-	P/S	Penicillin/Streptamycin
-	PBS	Phosphate buffered saline
-	RT	Room temperature
-	RTK	Receptor tyrosine kinase
-	S	DNA replication
-	SCF	Stem cell factor

Acknowledgements

During my education, I have learned to appreciate all the of the human body.. even the kidneys thanks to Prof. Paul Steels! So first of all, I would like to thank all of the professors and their assistants for sharing their knowledge. Throughout these five years, I have also had the chance to experience the workflow and team spirit at several research groups.

I still remember the day my first internship started, nervous tension and little experience within the laboratory. But, it made me realize my true passion.. lab work! There were moments however, I have wished the polymerase chain reaction was never developed or the moments when I lost my patience while the FACS apparatus took it's time to process urgent results. Then, there also were those moments when the PCR song could be heard across the hall, as we all were singing along with it or when we were laughing with each other's silly laboratory bloopers. The latter made us forget all of the stress and the time consuming processes!

When I had to choose the topic for my senior internship, I was immediately attracted by the histological study of canine mast cell tumours. So, a very big thanks goes to Prof. Ivo Lambrichts and veterinarian Tom Hendrickx for giving me the opportunity to work at the histology department of the Hasselt University in corporation with veterinary practice St.- Katherina. I appreciate their help in the lab, their advice whenever I encountered a problem and their critical eye while correcting this thesis. Moreover, I value the help and advice of Marjan Moreels, she let me in on all of the secrets to maintain a healthy cell culture.

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Samenvatting

Inleiding: Mastcel tumoren (MCTen) behoren tot de meest voorkomende neoplastische aandoeningen bij honden, ze representeren 7 à 21% van alle huidtumoren. MCTen worden gekenmerkt door hun variërende biologische gedrag, waardoor er geen standaard behandelingsprotocol kan worden aangereikt.

De Patnaik classificatie is momenteel de meest frequente methode om MCTen prognostisch te graderen en steunt voornamelijk op de aanwezigheid van bepaalde histologische karakteristieken, die gecorreleerd kunnen worden aan een algemene overlevingstijd. Doch is dit gradeersysteem niet perfect, aangezien er sprake is van variatie tussen de verschillende pathologen onderling. Met behulp van dit onderzoek trachten we betere histopathologische methodes te ontwikkelen, bruikbaar om de verschillende graden MCTen van elkaar te kunnen onderscheiden. We stellen dat MCM7 een nauwkeuriger beeld geeft van de proliferatie in MCTen en dat de drie differentiatiegraden onderling ultrastructurele verschillen vertonen.

Materiaal en methode: De MCT stalen werden geanalyseerd met behulp van immunohistochemie. Hiervoor gebruikte men enkele proliferatie markers, namelijk Ki67 en MCM7 alsook het proto-oncogen *c-kit*. Bovendien vond er een morfologische studie van enkele MCTen plaats, waarbij er getracht werd de individuele mastcellen te visualiseren alsook hun intracellulaire componenten.

Resultaten: Immunohistochemische analyse toont aan dat MCM7 significant verschilt van Ki67 betreffende het identificeren van proliferatie in mastcel tumoren. De evaluatie van het KIT proteïne duidt op een overheersend cytoplasmatisch patroon. Ultrastructurele analyse verduidelijkt de morfologische en morfometrische verschillen tussen de verschillende differentiatiegraden van MCTen.

Conclusie: Sommige morfologische en morfometrische eigenschappen van de drie differentiatiegraden vertonen onderling verschillen. Bovendien, blijkt MCM7 een betere marker dan Ki67 wat betreft het aantonen van proliferatie in MCTen. Deze resultaten kunnen tesamen met de KIT analyse en de huidige classificatie volgens Patnaik bijdragen aan de ontwikkeling van een nieuw, universeel gradeersysteem.

Abstract

Introduction: Mast cell tumours (MCTs) are frequently occurring canine cutaneous neoplasms, representing 7-21% of all skin tumours in dogs. They show a wide variety of biologic behaviour (e.g. benign – malignant), making it difficult for clinicians to make treatment decisions. Currently, prognostic grading of MCTs is achieved by using the Patnaik histological grading system. This procedure is largely based on histological characteristics, which correlate to an overall survival period. However, this course of action is subject to interpathologist variation, making it in part a subjective technique. So, the aim of this study is to develop better histopathological procedures useful in the differentiation of the various grades of MCTs. We hypothesize that MCM7 is a better marker of proliferation than Ki67 and that the various grades defined by Patnaik, present with ultrastructural differences.

Materials and methods: MCTs were immunohistochemically analyzed by use of proliferation markers Ki67and MCM7 as well as the proto-oncogene c-KIT. The results of both markers were compared with regards to their prognostic significance. Some tumour samples were also included in an ultrastructural study in order to visualize the individual mast cells and their intracellular structures more in particular.

Results: The proliferation marker MCM7 significantly differs from Ki67 regarding its capacity to indicate proliferating cells. The KIT protein is primarily located in the cytoplasm, which results in the typical diffuse cytoplasmic stippling. Grade I, Grade II and Grade III MCTs present with morphological differences (e.g. the collagen surface area, the number of type 1 cells and the presence of eosinophils). What's more, the morphometric analysis of Grade I MCTs differs from Grade III MCTs (e.g. cell diameter, the presence of granules and the diameter of mitochondria).

Conclusion: We conclude that the ultrastructural characteristics of canine MCTs could be of great importance regarding the prediction of prognosis. What's more, immunohistochemistry using MCM7 is more accurate than the use of classical proliferation markers. These new markers of cellular proliferation are promising and when related to histopathology, MCMs can contribute to the right treatment choice for each affected dog.

1 Introduction

Mast cell tumours (MCTs) are the most common skin neoplasms in dogs, representing 7-21% of all canine cutaneous tumours (Figure 1). These malignant growths are characterized by a very diverse biological behaviour, which is best predicted by evaluating factors such as the histological grade, proliferative markers (e.g. Ki67, AgNOR, PCNA ...) and oncogenes. What's more, MCTs present with various clinical appearances[1].

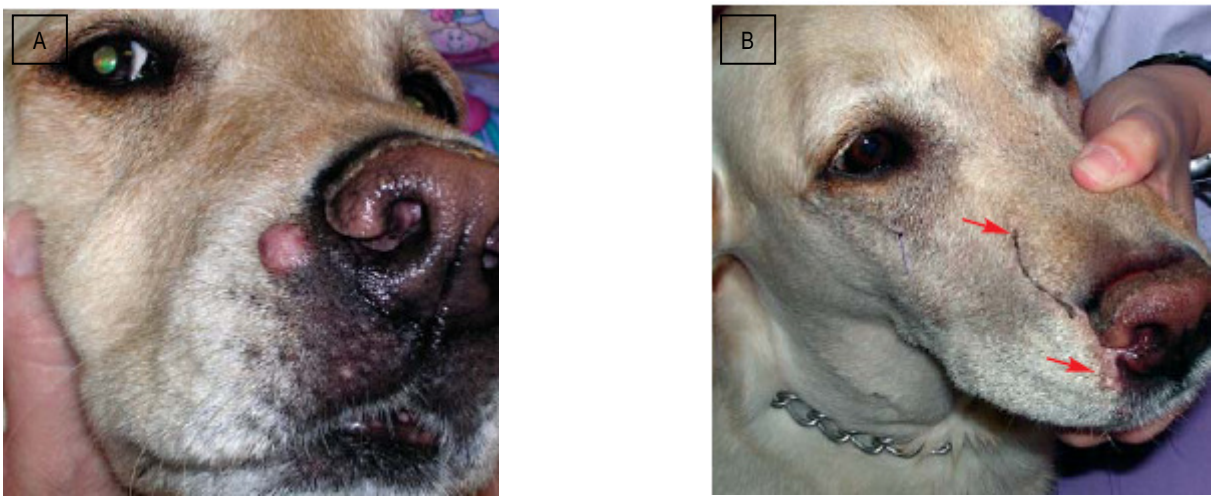

Figure 1: Mast cell tumour on the muzzle of a Labrador Retriever. A: Before surgery, MCTs on the muzzle have a more aggressive behaviour; B: After surgery, the red arrows indicate the ends of the surgical incision[2].

The aetiology of canine MCTs is still unknown but, as with most forms of cancer, is most likely multi-factorial. Recent work has implicated mutations in *c-kit* in the development of the tumours. This proto-oncogene encodes for KIT, which is a growth factor receptor that is normally expressed on the surface of mast cells. Activated KIT binds and phosphorylates a range of intracellular substrate proteins, which initiate a signalling cascade that results in a broad range of biologic activities including proliferation, migration, maturation and survival of mast cells[3].

The well-documented breed predispositions however, can indicate an underlying genetic component. Breeds found to be subject to the development of MCTs include Staffordshire Terriers, Boxers, Labrador Retrievers, Pugs, English Bulldogs...[4]

1.1 Biology of mast cells

Mast cells play a central role in inflammatory and immediate allergic reactions of the immune system. Arising from precursor cells of the myelomonocytic lineage ($CD34^+$ hematopoietic cells) in the bone marrow, they are distributed over lymphoid organs and highly vascularised tissues throughout the body (e.g. lungs, liver, skin and gastrointestinal tract). Mature mast cells contain cytoplasmic granules which store several mediators of inflammation such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid[5]. Extracellular release of these mediators may be precipitated by physical or chemical means as well as immune mechanisms (e.g. antigen-specific binding of immunoglobulin E to IgE receptors).

Mast cells constitutively express IgE receptors on their cell surface, which are highly specific to the Fc part of Immunoglobulin E (IgE). Allergens induce the production of IgE, when IgE is bound by those antigens the receptors crosslink, resulting in the activation of mast cells. As a consequence, preformed cytoplasmic granules are released and the synthesis and secretion of prostaglandins, leukotriens and cytokines is initiated[6].

1.2 Morphology of mast cells

Normal mast cells are characterized by a round or ovoid shape and a nonsegmented, centrally situated nucleus. The cytoplasm contains granules, a scarce rough endoplasmatic reticulum (RER), free ribosomes, a Golgi apparatus, mitochondria, vacuoles and glycogen[7, 8]. The cell membrane has distinct microvilli, which promote cell contact.

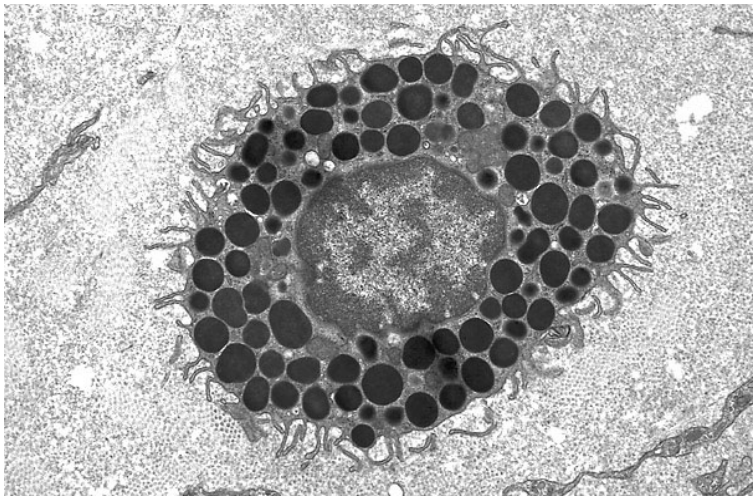

Figure 2: Mast cells have a diameter of 12 – 15 µm; numerous granules of irregular size are present in the ample cytoplasm.

1.2.1 Clinical presentation of MCTs

The gross appearance of MCTs varies widely, these tumours can mimic other skin tumours and non-neoplastic conditions. MCTs often appear as cysts, fatty tumours or other common benign skin and subcutaneous lesions. As a result, a prediction of their malignancy can not be made based on clinical presentation alone. Most canine MCTs present as intracutaneous nodules of 2 to 3 cm in diameter, tumours arising from other sites however are also common. Some MCTs cause ulceration of the overlying epidermis, while other tumour growths present as single or multiple elevated, erythematous nodules resulting in alopecia of the overlying skin. Rapidly growing MCTs frequently extend into the subcutaneous tissue and musculature.

The vast majority of cutaneous MCTs can be found on the trunk, the extremities and the head and neck. The scrotum, back and tail are less commonly affected[9]. In most dogs, tumours are solitary, but approximately 6% of all dogs develop more than one MCT[10].

1.3 Diagnostics

The first diagnosis is usually based on cytology from a fine needle aspirate (FNA) of the tumour, this determines the type and extent of surgical intervention (biopsy). Such a presurgical aspiration provides a cytology sample characterized by well differentiated round cells that have large cytoplasmic granules or on the other hand, cells that look more anaplastic with small granules[10]. What's more, eosinophils are often present because of eosinophil chemotaxis after histamine release[5].

Depending on the location and size of the mast cell tumour, the veterinarian can perform a complete surgical excision (excisional biopsy). If obtaining tumour free margins however is difficult, an incisional biopsy is taken[2]. Later, the tumour samples can be examined by a pathologist to study the histopathology and determine the grade.

1.4 Grading versus staging

Currently, most canine MCTs are prognostically graded by means of the Patnaik classification. This grading method divides mast cell tumours into three categories based on their histological appearance and invasiveness. Staging, on the other hand, depends on the clinical extent of the tumour[11]. So, the clinical stage is a determination of the degree of local and systemic involvement of a tumour (Table 1).

Table 1: World Health Organization clinical staging system for canine mast cell tumours.

I	One tumour confined to dermis, no nodal involvement
II	One tumour confined to dermis with local or regional lymph node involvement
III	Multiple dermal tumours or large infiltrating tumour with or without regional lymph node involvement
IV	Any tumour with distant metastases

As mentioned before, the Patnaik histological grading system is largely based on histological characteristics, which correlate to the overall survival period (Table 2). Grade I MCTs are benign and well-differentiated. The cell differentiation of grade III MCTs however, is poor and these tumours are likely to metastasize. Grade II MCTs, which are intermediately differentiated, can have a benign clinical outcome or a malignant clinical course[12]. Thus, the Patnaik histological grading system describes the differentiation state of the tumours. However, this course of action is subject to interpathologist variation, making it in part a subjective technique[13, 14]. Since there are no markers of cell differentiation, this process can only be assessed in an indirect way by use of proliferation markers. Much proliferation refers to little cell differentiation and a highly malignant tumour.

Table 2: Summary of the Patnaik morphologic grading classifications for canine cutaneous mast cell tumours.

	I	II	III
Location	Dermis & interfollicular spaces	Lower dermal & subcutaneous tissues	Subcutaneous & deep tissues
Cell morphology	Round & monomorphic, medium-sized granules	Round - oval & moderately pleomorphic, fine - large granules	Round - oval - spindle shaped & pleomorphic, fine granules
Nuclear morphology	Round & condensed chromatin	Round - indented & scattered chromatin, some with double nuclei	Indented - round vesiculated, binucleated cells
Architecture, stromal reaction	Rows - small groups & separation by collagen fibers of dermis	Groups & thin, fibrovascular stroma some areas of hyalinization	Closely packed sheets & fibrovascular - fibrocollagenous stroma + hyalinization
Mitotic figures	None	Rare	Frequent
Edema, necrosis	Minimal	Diffuse edema & necrosis	Edema, hemorrhage & necrosis

1.5 Prognostic markers in MCTs

The grading of malignancy and prognosis of a tumour based on clinical factors, the Patnaik classification and histopathological characteristics such as size and location of the tumour, presence of regional lymph node metastases and distant metastases is often not sufficient to make treatment decisions. Therefore, some molecular markers can be applied to assist in the prediction of prognosis in canine MCTs[15].

1.5.1 Mitotic index

The mitotic index (MI) is an indirect measure of cell proliferation based on the quantification of mitotic figures in histopathological specimens. Here, the fraction of mitosis is expressed as a percentage of the total number of cells. The validity of the MI however, remains controversial as it is not a standardized method[16].

1.5.2 Proliferating cell nuclear antigen

The proliferating cell nuclear antigen (PCNA) helps to coordinate the DNA replicating protein complex at the replication fork. PCNA belongs to the family of the DNA sliding clamp family of proteins, which are essential components of the DNA replication system. This nuclear protein is present in all active phases of the cell cycle (G1, S and G2). It's expression though, can be induced by DNA damaging agents[17]. Moreover, PCNA is not a standardized marker.

1.5.3 Ki67

Ki67 is a large (395 kDa) non-histone nuclear protein, of which the function is not fully known. During interphase, the Ki67 antigen can be exclusively detected within the nucleus, whereas in mitosis, most of the protein is relocated to the surface of the chromosomes[17]. In addition, the Ki67 protein is present during all active phases of the cell cycle (G1, S and G2). Nevertheless, this proliferative marker is undetectable during the early G1 phase[18].

1.5.4 Argyrophilic nucleolar organizer region proteins

Argyrophilic nucleolar organizer region (AgNOR) proteins are a collection of nuclear proteins. When cells proliferate, the amount of interphase AgNOR proteins augments from early G1 phase. At the end of S phase, a maximum value is reached that remains constant during late G2 phase. The quantity of interphase AgNOR proteins are strictly related to the proliferation rate[15]. This marker of proliferation however, is poorly standardized.

1.5.5 c-KIT

c-kit is a proto-oncogene that encodes the type III receptor tyrosine kinase KIT, which consists of an extracellular ligand binding domain composed of five immunoglobulin-like loops, a transmembrane domain, a negative regulatory juxtamembrane domain and a split cytoplasmic kinase domain. In healthy dogs, this gene is expressed by various cell types (e.g. mast cells, germ cells, melanocytes and hematopoetic precursor cells)[19].

As mentioned before, the interaction of KIT and its ligand stem cell factor (SCF), can result in cell survival, proliferation, differentiation, chemotaxis, degranulation and fibronectin adhesion[20]. When the cytokine SCF binds to the cell surface it cross links two KIT receptors, resulting in the activation of the tyrosine kinase enzymatic activity. As a consequence, ATP binds the phosphotransferase enzymatic pocket and several tyrosine residues autophosphorylate. The latter then act as docking sites for SH_2-containing adapter and signal transduction molecules. This results in the initiation of a downstream signalling cascade.

Deletions and point mutations, but most of all internal tandem duplications (ITD) have been identified in the juxtamembrane domain of c-KIT in canine cutaneous MCTs. Tandem duplication mutations in the juxtamembrane coding region (exon 11, exon 12 and intron 11) constitutively activate the KIT receptor, despite the absence of the SCF ligand. Previous studies have shown that these mutations in c-KIT may play a role in the neoplastic transformation of canine mast cells[21, 22].

Earlier IHC studies showed that c-KIT expression in healthy mast cells differs from cancerous mast cells. Normal mast cells express c-KIT on their cell membrane. While in neoplastic mast cells, c-KIT accumulates in the cytoplasm. This results in three possible expression patterns, namely a perimembrane KIT localization, a diffuse cytoplasmic stippling and a perinuclear KIT localization. Moreover, the expression pattern correlates with the differentiation of the mast cell tumour. Well-differentiated neoplasms have a relative low amount of c-KIT expression, whereas MCTs, which are poorly differentiated, have a considerably high c-KIT accumulation. Increased cytoplasmic localization of KIT in canine MCTs is significantly associated with a decreased survival duration and disease-free interval as compared to MCTs with perimembrane KIT localization[23].

1.6 Minichromosome maintenance proteins

Minichromosome maintenance (MCMs) proteins of which MCM2-7 are the best known, play a crucial role in the replication of DNA (Figure 3). They belong to a family of conserved proteins, which are the most important components of the replication initiation complex[24].

In order to prevent the occurrence of mutations to the genome, it is important that chromosomal DNA is precisely duplicated during the S phase of the cell cycle. The activity of thousands of replication forks scattered throughout the genome must be carefully regulated to guarantee accurate chromosome duplication. If too few replication origins are active, the chromosomal DNA could not be completely replicated during S phase. As a consequence, DNA strand breaks and gross chromosomal rearrangements could occur in surviving daughter cells. It is however, equally important to ensure that every replication origin initiates only once in each cell cycle[25]. Correct regulation of the licensing system of replication is responsible for ensuring the proper regulation of replication origins during cell cycle progression.

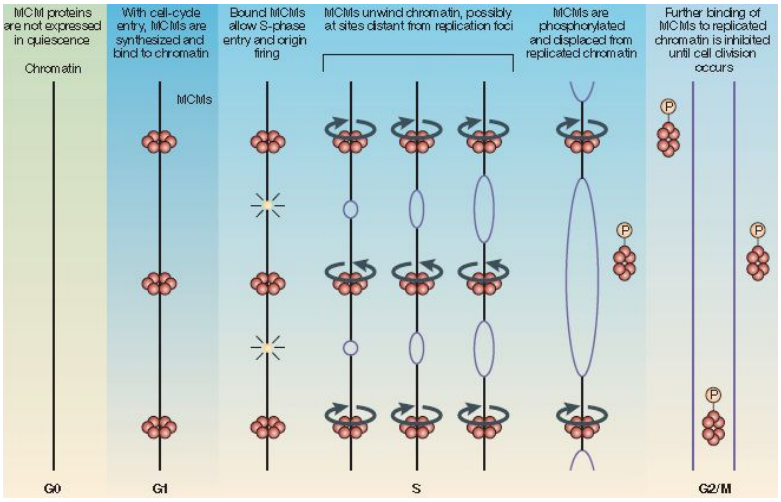

Figure 3: Control of DNA replication.

These biomarkers of cell cycle state display a weak helicase activity and form a ring structure with a central pore that can accommodate double-stranded DNA[26]. MCMs are expressed during all phases of cell division, except for the G0 phase. The assembly of origin replication complexes (ORC), MCMs and CDC6 at the replication origins makes the chromatin competent for all replication activities. The ORC is bound to chromatin throughout the whole cell cycle and forms a landing pad for CDC6 and CDT1, which are MCM loading factors[27]. Their binding results in the formation of the pre-replication complex (pre-RC). Next, the MCM proteins are loaded onto the DNA at replication origins and they start unwinding the chromatin. An increase in cyclin A-CDK2 levels and the activity of DDK then lead to origin firing and the initiation of S phase. As a consequence, the MCM proteins gradually dissociate from chromatin as they become phosphorylated, which ensures that replication occurs only once per cell cycle[28, 29].

Many studies have shown that MCMs offer better results in predicting cell cycle state and proliferation in several human cancers, in comparison to the standard proliferation markers[18, 30, 31]. A pilot study performed by our lab has shown that the immunoreactivity of MCM7 is significantly higher than the immunoreactivity of Ki67, in grade III MCTs.

1.7 Therapeutics

Surgery is the treatment of choice for the majority of mast cells tumours. Here, an attempt should be made to obtain tumour-free margins. Typically, three cm lateral margins and one fascial plane below the tumour is considered acceptable, increasing the probability of a microscopically tumour-free margins[10]. When the location or size of the tumour precludes complete surgical excision, radiation therapy offers a very effective modality of treatment. This kind of therapy is also recommended for patients with inoperable mast cell tumours[11]. Another possibility is chemotherapy (e.g. Vinblastine and Prednisone). This is mainly applied on patients with simultaneously occurring multiple tumours, high grade tumours, intermediate grade tumours with high grade KIT staining patterns and/or Ki67, and patients with regional lymph node involvement or systemic disease[32, 33].

What's more, recent studies have identified RTK-inhibitors Masitinib (Masivet®, AB Science) and Toceranib (Palladia®, Pfizer) as a potent and selective inhibitor of KIT in dogs with nonresectable or recurrent grade II or III nonmetastatic MCTs. This specific treatment blocks the growth of mast cell tumours that are characterized by mutations in the juxtamembrane domain as well as mutations in the the 5th immunoglobulin-like domain[34].

1.8 Goal of the study

Canine cutaneous MCTs provide a challenge to veterinary surgeons, the correct grading is very important for the planning of the right treatment. As mentioned before, with the Patnaik classification, there is a possibility of interpathologist variation. Combined with a large majority of grade II MCTs which have a biological behaviour that is hard to predict, many immunohistochemical studies (IHC) using c-KIT and a set of proliferation markers (e.g. Ki67, PCNA and AgNOR) have been performed to try and objectify the prognostic grading of MCTs.

The aim of this study is to analyze the cellular morphology of MCTs as well as the new proliferation marker MCM, in comparison with Ki67 and c-KIT. We hypothesize that MCM7 is a more sensitive marker of proliferation than Ki67 and that the grades of MCTs characterized by Patnaik present with ultrastructural differences in their cellular morphology. Previous studies by our own lab have shown that grade I MCTs consist of two different mast cell types. These cell types differ in the colour of their cytoplasm, the shape of their nuclei as well as the location of their granules. What's more, as an important indicator of malignancy, the expression pattern of c-KIT in MCTs will also be analyzed.

2 Materials and methods

2.1 Tumour samples

Canine MCTs were gathered in corporation with the St.-Katharina Veterinary Practice in Hasselt. A total of 40 dogs (Table 3) with one or more MCTs were included in this study, approximately 74 tumour samples were selected according to some premised inclusion criteria. These were the availability of sufficient tissue to perform all immunohistochemical analyses and the surgical removal of the MCT as the sole treatment. The selected canine MCTs were graded first by board-certified veterinary pathologist H. De Cock according to the Patnaik histological grading system with incorporation of the mitotic index, the rate of invasiveness, haemorrhage, necrosis and other neoplastic characteristics.

2.2 Histopathology

The obtained tumour samples were processed for cellular analysis using immunohistochemistry and HE staining.

2.1.1 Sample fixation and embedding

In order to analyze the tumour fragments by light microscopy, the samples were to be fixed in Unifix™ (Klinipath, Belgium). Then, the tissue was dehydrated in rising concentrations of alcohol followed by butanol and paraffin, this process was performed in a semi-enclosed tissue processor Leica TP 1020 (GMI, USA). During a next step, these dehydrated samples were embedded in paraffin and sections (6-8 µm) were made by use of a microtome Mod.1140/ Autocut (Reichert-Jung, Germany). Finally, the paraffin sections were placed on slides coated with poly-L-lysine which resulted in a better attachment of the tissue. Part of the samples were already fixed and embedded before arrival to the lab, these were stained in the same manner as the other slides.

2.2.2 Hematoxylin & eosin stain

The combination of hematoxylin and eosin, is the most widely used staining technique in histology. Basophilic cellular structures contain nucleic acids and are stained blue-purple (hematoxylin), while the eosinophilic components of cells are generally composed of intracellular or extracellular protein and colour bright pink (eosin).

Firstly, the tumour sections were placed in the Robot-Stainer HMS 740 with various containers filled with Clear Rite™, hematoxylin, isopropanol and eosin (Supplement 1). Clear Rite™ is a mixture of isoparaffinic aliphatic hydrocarbons which is designed specifically for tissue processing and staining. This clearing agent allows for excellent lipid extraction during tissue processing and renders complete deparaffinization and clearing during the staining process. Secondly, the tissue was rehydrated and the tumour sections were placed in a container filled with hematoxylin. After several rinse steps, the slides were stained with eosin. Finally, the tissue was rinsed once more and placed in various containers filled with isopropanol and Clear Rite™.

Table 3: Overview of the overall information of the 40 dogs incorporated in the histological study.

Name	Sex	Breed	Date of birth	Date of diagnosis	Location MCT
Xilio Claesen	M	French bulldog	25/03/2006	3/07/2006	Knee
Beau Froyen	M	Golden Retriever	20/05/2003	18/04/2006	Thin flank
Jimmy Joris	M	Belgian Malinois	1/02/1994	27/06/2006	Abdominal
Zakkie Foets	M	Labrador Retriever	26/05/1993	7/07/2006	Thin flank
Kim Lecocq	F	Springer Spaniel	1/01/1992	27/02/2004	Thin flank
Uzi Vleminckx	M	Hovawart	1/07/1996	8/04/2005	Chest
Bo Frijns	M	Bordeau Dog	13/05/2000		Tow
Lady Derwael	F	Labrador Retriever	22/02/1996	27/12/2006	Groin
Senna Huybrighs	F	Berner Sennen	1/04/1994	18/06/2002	Tibia
Quizan Tielens	M	Labrador Retriever	11/05/1992	3/07/2002	Scrotum
Pruts Poleyn	F	West Highland White Terrier	28/07/1991	10/08/2002	Knee
Jimmy Jacobs	M	English Setter	15/11/1997	2/05/2003	Buttock
Marieke Van der Auwera	F	St. Bernard	28/07/2000	2/06/2003	Groin
Yack Tits	M	Briard	1/12/1992	17/10/2003	Thin flank
Max Lambrichts	M	Labrador Retriever	5/12/1993	8/09/2004	Buttock
Sandy Ory	F	Malteser	24/11/1993	1/10/2004	Front leg
Ysha Saro	F	Pug	1/01/1999	17/12/2004	Knee
Tara Grizell	F	Labrador	2/12/2002		Cheek
Boss Beks	M	Bouvier	15/04/2001		Hind leg
Sus Vanbilsen	F	West Highland White Terrier	4/09/1991	31/08/2005	Knee
Dipsy Milissen	F	Miniature Pinscher	6/03/1999	10/09/2005	Hind leg
Sam TNC	M	Rottweiler	1/05/1995	23/09/2005	Tail
Dino Torfs	M	Irish Setter	1/06/1991	24/11/2005	Elbow
Amie Strick	F	Boxer	26/07/2001	6/12/2005	Tarsus
Shiba Maex	M	Canis Vulgaris	24/09/1995	14/01/2006	Chest

Dieuwke Pipeleers	F	Golden Retriever	17/05/2004	29/08/2006	Auricle
Axana Keyen	F	Lhasa Apso	27/04/2001	2/10/2006	Chest
Uksi Menten	M	Labrador Retriever	23/06/1996	23/10/2006	Hind leg
Toby Jacobs	M	Teckel (long hair)	9/09/1993	22/01/2007	Armpit
Snoepie Punie	F	Fox (mongrel)	7/05/1997	15/04/2005	Scapula
Fien Vanhorenbeek	F	Jack Russell Terrier	22/03/1998	14/11/2006	Back
Cartouche Driesen	M	Teckel (hard hair)			
Zita Samyn	F	American Staffordshire Terrier			
Arthur Willekes	M	Boxer			
Yani Van Leemput	M	Dogo Argentino			
Rufo Tutds	M	Labrador Retriever			
Senna Debaer	F	Jack Russell Terrier			
Elvis Mullens	M	English Bulldog			
Jack Van Herle	M	Teckel (short hair)			

2.2.3 Immunohistochemistry

Before staining, the sections were warmed first in an oven and rehydrated in a Robot-Stainer HMS 740 (Microm International GmbH, Germany) with various containers filled with Clear Rite™ (Thermo Scientific, Sweden) and isopropanol.

After rehydration, the accessibility of the antigens in the tissue samples was first optimized (antigen retrieval) by heating the tumour sections for 30 minutes in citrate buffer 10X pH 6.0 (Dako, USA). Secondly, the containers were filled with citrate buffer and the sections were placed on the bench to cool down for 15 minutes. Next, the slides were washed with wash buffer 10X (Dako, USA) for 5 minutes. Then, all samples were placed into the BD SMS 3600™ Molecular Stainer (BD TriPath Imaging, USA; Supplement 2). Here, the tumour sections were first treated with Dual Endogenous Enzyme Block (DakoCytomation, USA) to prevent background staining, before incubation with the primary antibody (Table 4). During a next step, the Envision™+ Dual Link System-HRP reagent (Dako, USA) was applied as a secondary antibody. Finally, all samples were treated with the peroxidase substrate diaminobenzidine (DAB) which resulted in the visualization of the immunoreactivity of the proliferation markers (Ki67, MCM7), the apoptosis marker caspase-3 and c-KIT.

Table 4: Overview of the primary antibodies which diluted with Antibody Diluent with Background Reducing Components (Dako, USA).

Primary Antibody	**Species**	**Diluent Factor**	**Source**
Ki67	Rabbit mAb	1/200	Thermo Scientific
c - KIT	Rabbit pAb	1/300	Thermo Scientific
MCM7	Mouse mAb	1/100	Abcam

Later, the tissue was counterstained with 'Mayer's Haematoxylin'. Next, the stained sections were placed both in isopropanol and toluene for 15 minutes, to dehydrate. As a final step, coverslips were placed on top of the sections by use of a Shandon ClearVue Coverslipper (Thermo Scientific, Sweden).

2.2.4 Analysis

For all the immunohistochemical staining evaluations (Figure 4), areas with the highest proportion of immunopositive neoplastic cells were identified at 10X magnification using an Leica DMLS light microscope (Leica Microsystems Wetzlar GmbH, Germany). Upon identification of highly proliferative areas, the number of Ki67, MCM7 and caspase-3 immunopositive cells present in a 10 X 10 mm area was counted using a 1 cm^2 10 x 10 grid at 40X magnification. The amount of immunopositive cells per grid area was evaluated over 3 high power fields and subsequently averaged.

KIT staining was assessed by studying the pattern of KIT protein localization. Each MCT was classified on the basis of the highest staining pattern present in at least 10% (estimated on the basis of 100 neoplastic cells in a high power field) of the neoplastic cell population. The immunopositive cells present in the margins of tissue sections however, were not considered due to possible artifactual staining.

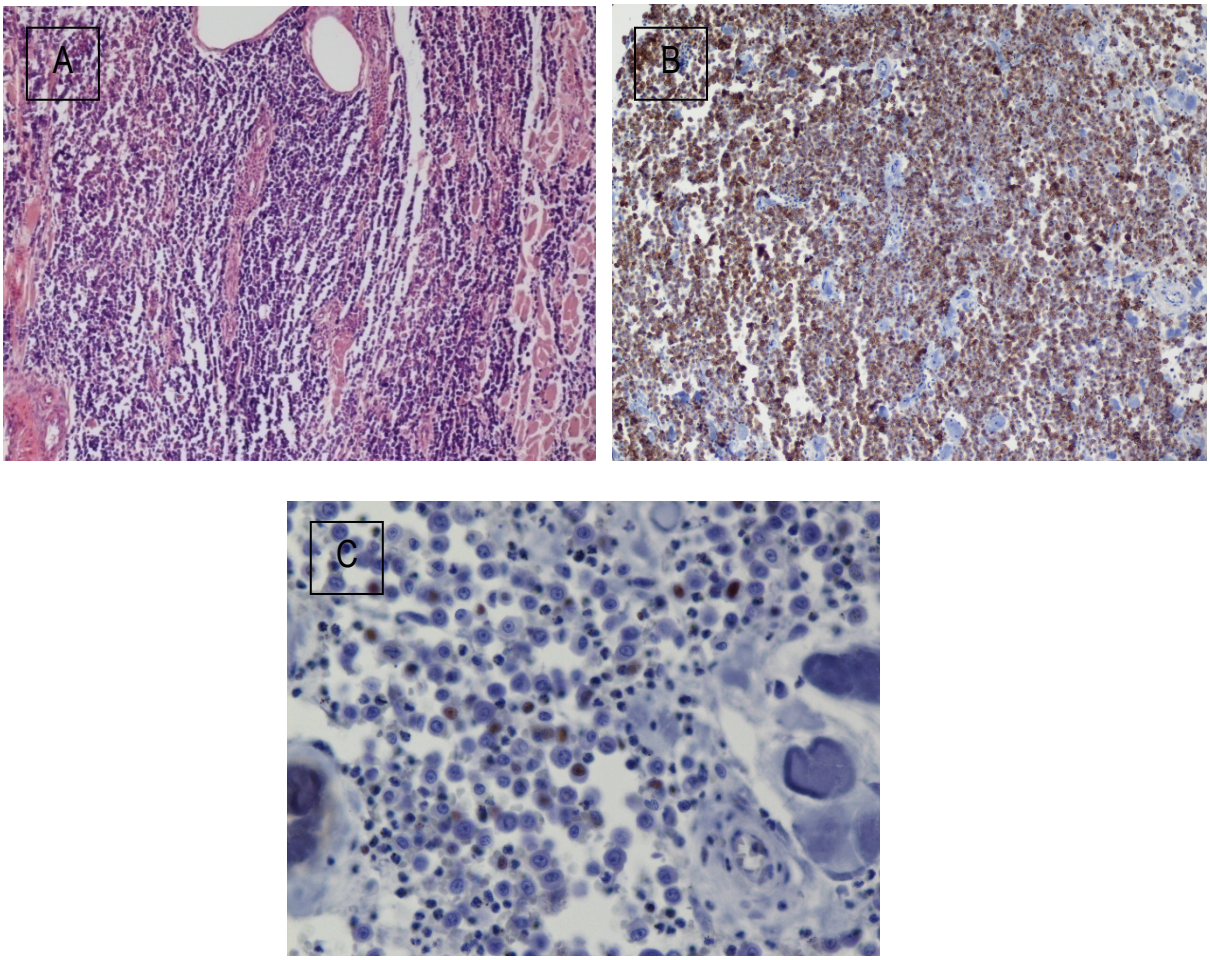

Figure 4: Examples of HE and immunohistochemical stainings. A: A HE staining at 10X magnification; B: An immunohistochemical staining using c-KIT at 10X maginification; C: An immunohistochemical staining using MCM7 or Ki67 at 40X maginification.

Positive controls were used to be sure that the relevant immunohistochemical staining (Ki67, MCM7 and c-KIT) worked appropriately. These were tissue slides from testis, tonsil and lymph node, respectively. Another control taken into account was the staining of the epithelium (skin) or hair follicles, which was present in most tumour samples as an internal control.

2.3 Electron microscopy

Some tumour samples were processed for ultrastructural analysis by use of fixation, dehydration and embedding.

2.3.1 Sample fixation

After resection of the tumour, the tissue samples were cut (1 mm^3) and kept in 2% glutaraldehyde (Laborimpex, Brussels). To preserve the ultrastructures adequately, the tumour fragments had to be obtained and fixed as soon as possible after interruption of the blood supply. Then, the tissue was allowed to fix at 4°C.

The standard fixation method comprises of a primary treatment with buffered aldehyde followed by a buffer wash and a secondary fixation step with osmium tetroxide (OsO_4; Aurion, the Netherlands). Glutaraldehyde fixes biological tissue through the extensive cross-linking of proteins. While treatment with osmium tetroxide results in the fixation of fat which prevents them from dissolving during dehydration (Supplement 3). Both fixatives are buffered with cacodylate buffer (Aurion, the Netherlands) to maintain pH, which is necessary to overcome the effects of impurities in reagents and to maintain the physiological acid-base balance.

2.3.2 Dehydration and embedding

Before infiltration with an embedding medium, it is necessary to replace the water present in the fixed tissue with a solvent miscible with the resin monomers.

Removal of water was carried out by transferring the tissue through increasing concentrations of aceton. Following dehydration, the tumour samples were infiltrated with Araldite® (Aurion, the Netherlands; Supplement 4). This epoxy resin is polymerized by heat to produce a solid block suitable for ultramicrotomy. Embedding in plastic media results in the preservation of the specimen so that ultrastructural information is retained. Another advantage is the stabilization of the tissue against the conditions of high vacuum and electron bombardment which exist in the electron microscope.

Later, sections in the order of 80 nm or less were cut by use of an Ultracut E ultramicrotome (Reichert-Jung, Germany). These ultrathin sections were then placed onto copper specimen grids (Aurion, the Netherlands) and contrasted with uranium and lead salts. A Philips EM 208 S transmission electron microscope was used to analyze the tumour samples (Supplement 5).

2.4 Cell culture

The tumour fragments were transported to the laboratory in sterile tubes containing 0,01M phosphate buffered saline (PBS; pH 7,2) supplemented with 1% penicillin/streptamycin (P/S; Gibco, UK) and 0,2% amphotericin (Fungizone®; Gibco, UK). Within 48 hours, the tissue samples were processed according to the explant culture method.

2.4.1 Explant technique

First, the grade III tumour was washed in 1X PBS to remove residual blood and to cut off skin if necessary. Then, the tissue was transferred to a petri dish filled with 1X PBS. Here, the tumour fragment was cut into small pieces (1-2 mm^3). During a next step, these fragments were transferred to 6 well plates (Nunc, Denmark) containing DMEM 1X with low L-glutamine, low pyruvate, 1g/L D-glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany), 1% Penicillin (10000 units/ml) /Streptamicin (10000 µg/ml) and 0,2% amphotericin (250 µg/ml) for culture expansion. The culture was maintained at 37°C in a humified atmosphere containing 5% CO_2 and monitored with a phase contrast microscope. When attachment of explants and outgrowth of cells was observed, the medium was changed every 3 days (Supplement 6).

2.4.2 Trypsinisation

At 80% confluence, the cultures were rinsed with 1X PBS and harvested with 0,05% trypsin-EDTA (Gibco, UK) after 5 minutes of incubation at 37°C. The cell suspension was then pelleted at 1500 rpm for 5 minutes and resuspended in fresh culture medium (Supplement 7). Only a small sample was used for counting with a Fuchs-Rosenthal counting chamber by means of trypan blue exclusion. Further expansion was achieved by seeding the mast cells into culture flasks (Nunc, Denmark) containing fresh culture medium. In order to identify the mast cells by use of immunohistochemistry, small amounts of the cell suspension were seeded onto glass coverslips.

2.4.3 Immunohistochemistry

When the coverslips were overgrown with cells, immunohistochemical stainings with antibodies against MCM7, c-KIT and CD203c were performed at different time periods (Supplement 8). Later, the morphology and proliferation of the cells was assessed by use of a Nikon Eclipse TS100 Phase Contrast Microscope equipped with a digital camera (Nikon, Japan) even as the various immunohistochemical stainings. The amount of immunopositive cells was evaluated over 2 different areas of the relevant coverslip and subsequently averaged.

2.5 Statistical analysis

Firstly, the mean value of all obtained measurements was calculated. Then, the relevant mean values were compared with each other by use of the student t-test.

3 Results

Fourthy (eighteen female and twenty-two male) dogs were included in this study, several dog breeds were represented such as Labrador Retrievers, English Bulldogs, Dachshunds and Boxers. Ten of the seventy-four MCTs were histologic grade I (13.5%), fourty-one MCTs were histologic grade II (55%) and ten MCTs (13.5%) were histologic grade III (Table 5). Some of the remaining thirteen samples were lymph nodes or testis, but there were also samples of which the grade could not be determined.

Table 5: Overview of all the tumour samples and their characteristics. 0: No eosinophils; 1: Scarce eosinophils; 2: A small amount of eosinophils; 3: A moderate number of eosinophils; 4: Numerous eosinophils.

Slide	Patnaik	Eosinophils	Mitotis	Characteristics
600-1	Grade 1	1	0	Small cells, good granulation
600-2	Grade 2	4	0	Large cells, moderate granulation & FF
600-3	Grade 3	3	>5	Large cells, little granulation
600-4-1	Grade 2	1	1	Moderate sized cells, very good granulation
600-4-2	Grade 2	1	0	Moderate sized cells, very good granulation
600-4-3	Grade 2	1	0	Moderate sized cells, very good granulation
601-1-2	Grade 2	3	0	Large cells, moderate granulation
601-2	Grade 2	3	0	Small cells, moderate granulation
601-3	Grade 2	1	0	Small cells, good granulation
630	Grade 2	3	1	Large cells, moderate granulation
639-1-2				Lymph node
639-2	Grade 3	1	>5	Small cells, little granulation
639-3	Grade 3	3	2	Small cells, little granulation
639-4-2	Grade 2	4	0	Moderate sized cells, good granulation & FF
639-5-1	Grade 2	4	0	Moderate sized cells, moderate granulation
639-5-2	Grade 3	4	0	Moderate sized cells, moderate granulation
639-6	Grade 1	2	0	Large cells, good granulation
639-7-1	Grade 2	1	0	Small cells, good granulation
639-8-2	Grade 2	2	0	Large cells, good granulation
639-9	Grade 3	4	0	Large cells, little granulation
639-10	Grade 2	3	0	Small cells, good granulation
639-11	Grade 2	2	0	Small cells, good granulation
639-12-1	Grade 2	3	0	Moderate sized cells, moderate granulation
639-12-2	Grade 2	4	0	Large cells, good granulation & FF
639-12-3	Grade 2	3	0	Moderate sized cells, moderate granulation
639-13	Grade 2	2	0	Small cells, good granulation
639-14-2	Grade 2	4	0	Small cells, litlle granulation
639-15-1	Grade 3	3	>5	Large cells, little granulation
639-15-2				Lymph node
639-16	Grade 2	4	0	Large cells, good granulation
639-17	Grade 2	1	1	Moderate sized cells, little granulation
639-18	Grade 1	1	0	Small cells, good granulation
639-19	Grade 1	3	0	Small cells, good granulation

639-20-1				Testis
639-20-2				Testis
639-20-3				Histiocytair carcinoma
639-21-2	Grade 2	3	0	Small cells, good granulation
639-24-1	Grade 2	4	0	Moderate sized cells, good granulation
639-24-2	Grade 3	4	1	Moderate sized cells, good granulation & FF
639-24-3	Grade 3	4	0	Moderate sized, good granulation
640-1	Grade 2	2	1	Moderate sized cells, moderate granulation
640-2-1	Grade 1	2	0	Large cells, very good granulation
640-2-2	Grade 2	4	1	Large cells, moderate granulation
640-3-1	Grade 2	4	0	Large cells, little granulation
669a	Grade 2	2	0	Moderate sized cells, moderate granulation
669b	Grade 3	3	1	Moderate sized cells, little granulation
670	Grade 2	3	1	Small cells, moderate granulation
671a	Grade 1	1	0	Moderate sized cells, good granulation
671b	Grade 1	1	0	Moderate sized cells, good granulation
672	Grade 2	0	0	Small cells, good granulation
673a	Grade 2	3	0	Small cells, good granulation
673b	Grade 2	2	0	Small cells, good granulation
674	Grade 2	2	0	Large cells, moderate granulation
675a	Grade 2	3	1	Small cells, good granulation
675b	Grade 2	3	0	Small cells, good granulation
676a	Grade 2	1	0	Small cells, good granulation
676b	Grade 2	1	0	Small cells, good granulation
677a	Grade 2	1	0	Small cells, good granulation
677b	Grade 2	1	0	Small cells, good granulation
678a	Grade 1	0	0	Small cells, good granulation
678b	Grade 1	1	0	Moderate sized cells, good granulation
679a	Grade 2	3	0	Small cells, good granulation
679b	Grade 2	3	0	Small cells, good granulation
681a	Grade 1	3	2	Large cells, moderate granulation & FF
681b	Grade 3	4	2	Large cells, moderate granulation & FF

Figure 5 shows two special features frequently present in canine MCTs, namely flame follicles and dilated sweat glands. A flame follicle can be described as a region of collagen surrounded by numerous eosinophils. These eosinophils degranulate around the collagen bundles, which results in the typical flame figure[35].

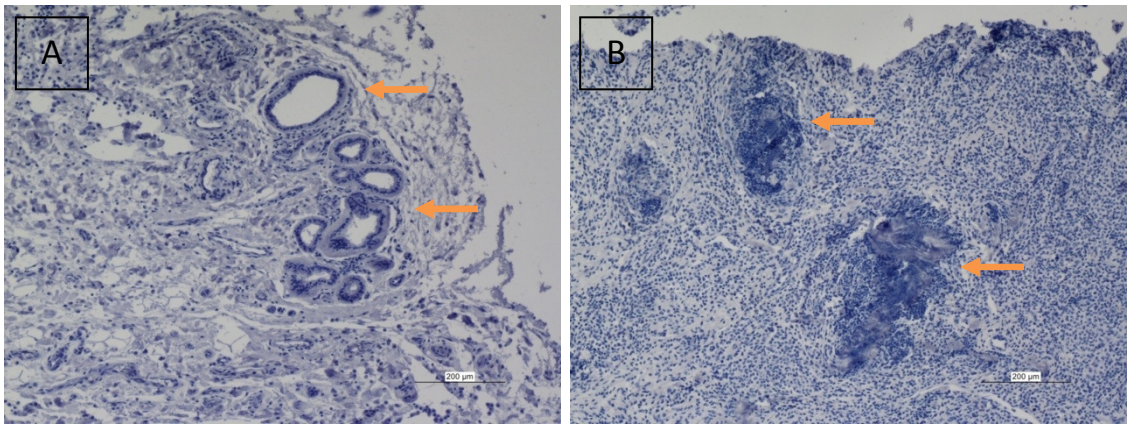

Figure 5: Morphological characteristics of canine MCTs at 10X magnification.
A: Dilated sweat glands; B: Flame follicles.

3.1 Comparison of proliferation markers Ki67 and MCM7

In order to determine the capacity of MCM7 as a proliferative marker, 74 tumour samples were immunohistochemically stained for both Ki67 and MCM7. Later, each of these MCTs was analyzed by use of a light microscope and the number of immunopositive cells in both stains were evaluated (Figure 6).

Ki67 counts range from 0 to 81 positive cells/grid area with an average of 5.78 positive cells/grid area (SD ± 13.71). MCM7 counts, on the other hand, range from 0 to 847 positive cells/grid area with an average of 81.05 positive cells/grid area (SD ± 130.86). The amount of Ki67 positive stained cells differs significantly from the amount of MCM7 positive stained cells ($p = 0.000$).

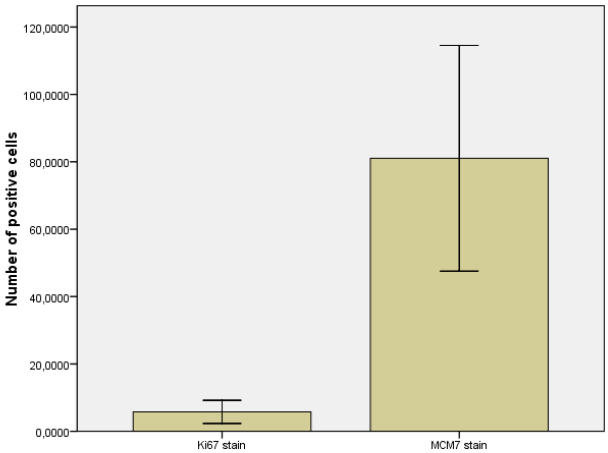

Figure 6: Comparison of the proportion Ki67 and MCM7 immunopositive cells in a canine population diagnosed with MCTs.

3.2 c-KIT expression pattern

All MCT samples were also stained with KIT antibodies in order to assess the pattern of KIT protein localization. Hereto, each MCT was classified on the basis of the highest staining pattern present in at least 10% (estimated on the basis of 100 neoplastic cells in a high power field) of the neoplastic cell population (Table 6).

Table 6: Overview of all the tumour samples and their KIT expression pattern in percentages.

Slide	Perimembrane expression pattern	Diffuse cytoplasmic stippling	Perinuclear expression pattern
600-1	7	88	5
600-2	10	86	4
600-4-1	15	73	12
600-4-2	10	87	3
600-4-3	0	56	44
601-2	1	82	17
630	19	77	4
639-3	17	83	0
639-5-1	8	75	17
639-5-2	2	74	24
639-8-2	4	86	10
639-9	0	77	23
639-11	45	54	1
639-13	62	38	0
639-15-1	10	64	26
639-15-2	2	70	28
639-16	3	92	5
639-19	48	51	1
639-20-2	0	92	8
639-20-3	25	75	0
639-21-2	1	96	3
639-24-1	1	77	22
639-24-3	8	84	8
640-1	4	62	34
640-2-2	2	88	10
640-3-1	10	42	48
669a	2	90	8
669b	0	57	43
670	1	65	34
671a	2	89	9
671b	14	80	6
672	1	71	28
673a	19	81	0
673b	20	74	6
674	10	90	0
675a	13	82	5

675b	13	80	7
676a	7	83	10
676b	13	83	4
677a	27	69	4
677b	14	82	4
679a	4	96	0
679b	2	94	4
681a	16	71	13
681b	6	65	29

The vast majority of MCTs presents with a diffuse cytoplasmic stippling in combination with a perimembrane KIT localization and/or a perinuclear KIT expression pattern. Every possible KIT localization that is characterized by a presence of 20% or more, is considered important for the prognosis of the relevant MCT.

The results indicate that most of the canine MCTs in our study (27/45 MCTs = 60%) are characterized by diffuse cytoplasmic stippling. There are however, tissue samples that have both a considerable amount of diffuse cytoplasmic stippling and a lot of perinuclear KIT staining (12/45 MCTs = 27%). Only a few tCT samples (6/45 MCTs = 13%) have a large percentage of perimembrane KIT localization.

3.3 Ultrastructural study of canine MCTs

Some tumour samples were morphologically analyzed after fixation with glutaraldehyde and OsO_4, dehydration in aceton and embedding with the epoxy resin Araldite®.

3.3.1 Morphology of MCTs

The morphology of the three grades of MCTs was studied and several characteristics (e.g. the amount of collagen, the number of eosinophils and the amount of Type 1 and 2 cells) were analyzed.

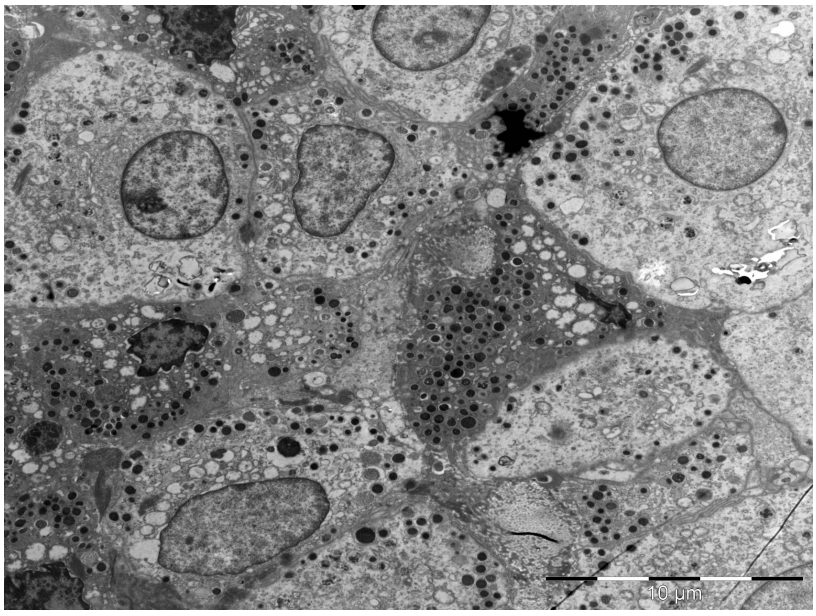

Figure 7: Ultrastructure of a mast cell tumour at 3500X magnification.

As mentioned before, previous studies performed by our lab have shown that grade I MCTs consist of 2 different mast cell types (Figure 7). Here, we tried to study their presence in Grade II and Grade III MCTs as well. Six images of each grade were made by use of an electron microscope. Later, the number of Type 1 and 2 cells, the amount of collagen and the number of eosinophils present in Grade I, Grade II and Grade III MCTs were measured by use of the ITEM FEI software.

Type 1 cells have a bright cytoplasm that contains a lot of granules (Figure 8A). Mostly, these granules are situated at one side of the mast cell. The nucleus of type 1 cells is round to ovoid. Type 2 cells on the other hand, are characterized by a darker cytoplasm. These cells count many granules as well, though they are spread throughout the whole cell. These cells have a irregularly shaped nucleus which can be identified by its condensed chromatin near the nuclear membrane (Figure 8B).

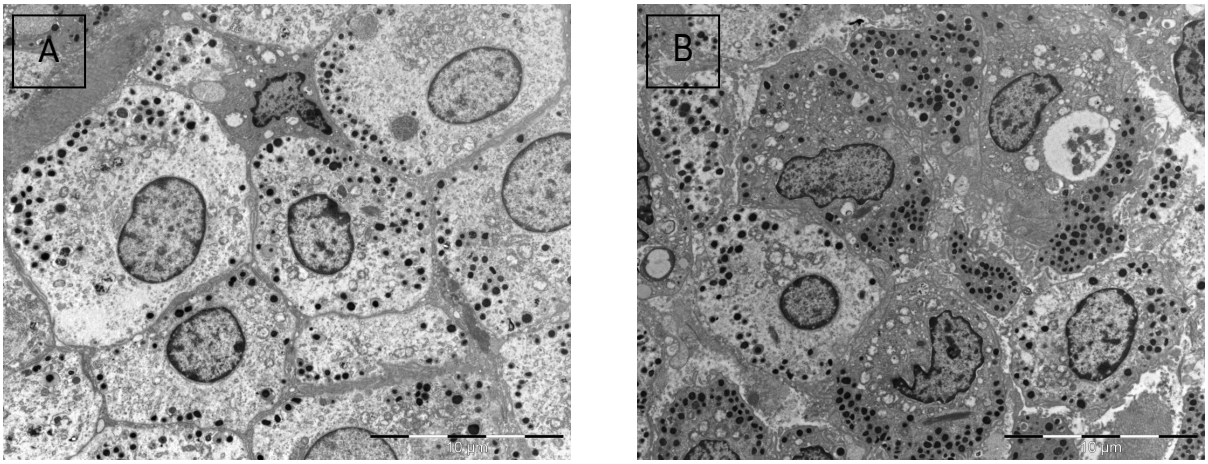

Figure 8: Two mast cell types present in canine MCTs at 3500X magnification.

Firstly, the surface area of collagen was evaluated, but there was no significant difference between Grade I and Grade II MCTs ($p = 0.152$) or Grade I and Grade III MCTs ($p = 0.225$) or Grade II and Grade III MCTs ($p = 0.845$). Grade I MCTs have a mean collagen surface area of $8.231*10^7$ nm² (SD ± $7.579*10^7$), while Grade II MCTs have a mean collagen surface area of $2.891*10^7$ nm² (SD ± $3.712*10^7$) and Grade III MCTs have a mean collagen surface area of $3.407*10^7$ nm² (SD ± $5.103*10^7$). Secondly, the presence of eosinophils was studied. Here, a significant difference was found only between Grade I and Grade III neoplasms ($p = 0.007$). The p-values regarding the number of eosinophils present in Grade I versus Grade II MCTs and Grade II versus Grade III MCTs are 0.078 and 0.117, respectively. Grade I MCTs have a mean number of eosinophils of 1.00 (SD ± 1.265), Grade II MCTs have a mean number of eosinophils of 2.50 (SD ± 1.378) and Grade III MCTs have a mean number eosinophils of 4.17 (SD ± 1.941). Thirdly, the presence of mast cell type 1 (Figure 3A) was assessed between Grade I and Grade II MCTs, Grade I and Grade III MCTs and Grade II and Grade III MCTs. No significant difference was found, p-values were 0.701, 0.676 and 1.000, respectively. The mean number of Type 1 cells in Grade I MCTs is 1.33 (SD ± 1.751), Grade II MCTs have a mean number of Type 1 cells of 1.00 (SD ± 1.095) and Grade III MCTs present with a mean number of Type 1 cells of 1.00 (SD ± 0.632). Next, the mean number of Type 2 cells (Figure 3B) was evaluated in all three grades, 5.00 (SD ± 2.098) for Grade I MCTs, 1.17 (SD ± 0.753) for Grade II MCTs and 0.67 Type 2 cells (SD ± 1.033) were present in Grade III

MCTs. A significant difference was found between Grade I and Grade II MCTs (p = 0.005) and between Grade I and Grade III MCTs (p = 0.001). The p-value concerning the presence of Type 2 cells in Grade II versus Grade III MCTs on the other hand, was 0.670.

3.2.2 The morphometric analysis of Grade I and Grade III MCTs

In addition, the morphometric characteristics of Grade I and Grade III of the Patnaik classification were evaluated. Twenty cells of each grade were photographed and analyzed by use of the ITEM FEI software. The diameter of the each cell and its nucleus was determined. The presence of granules and mitochondria was also studied, a mean value was defined regarding their diameters by analyzing five structures at random.

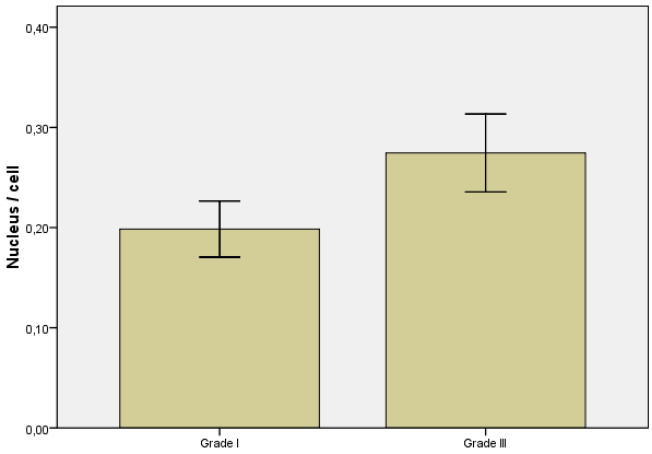

Figure 9: Graphic presentation of the difference in nucleus to cell ratio between Grade I and Grade III MCTs.

Firstly, the diameters of the cell and the nucleus were evaluated. There is a significant difference in cell and nucleus diameter between Grade I and Grade III MCTs (Figure 10B), the mean values are 12009.813 nm (SD ± 1816.763) and 10635.337 nm (SD ± 1832.119) for the cell diameter (p = 0.022), and 5035.777 nm (SD ± 968.630) and 5804.429 nm (SD ± 1246.516) for the diameter of the nucleus (p = 0.035). Moreover, the nucleus to cell ratio differs significantly with a p-value of 0.003. The mean ratio is 0.199 (SD ± 0.0625) and 0.275 (SD ± 0.0870) for Grade I and Grade III MCTs, respectively (Figure 9).

Next, the presence of granules was defined for both grades. A significant difference is found (p = 0.000) for the mean amount of granules with mean values of 62.900 (SD ± 28.182) in Grade I MCTs and 5.900 (SD ± 3.972) in Grade III MCTs (Figure 10C). Figure 10B shows a significant difference for the mean diameter of those granules (p = 0.000). Grade I MCTs have a mean granule diameter of 438.108 nm (SD ± 82.472), while Grade III MCTs are characterized by a mean granule diameter of 732.844 nm (SD ± 300.269).

Finally, the mitochondria present in the MCTs were counted and their diameter was also evaluated. Here, no significant difference in the mean number of mitochondria is found (p = 0.455). Figure 10C represents the mean number of mitochondrion, Grade I and Grade III MCTs have a mean value of 14.800 (SD ± 6.685) and 13.350 (SD ± 5.393), respectively.

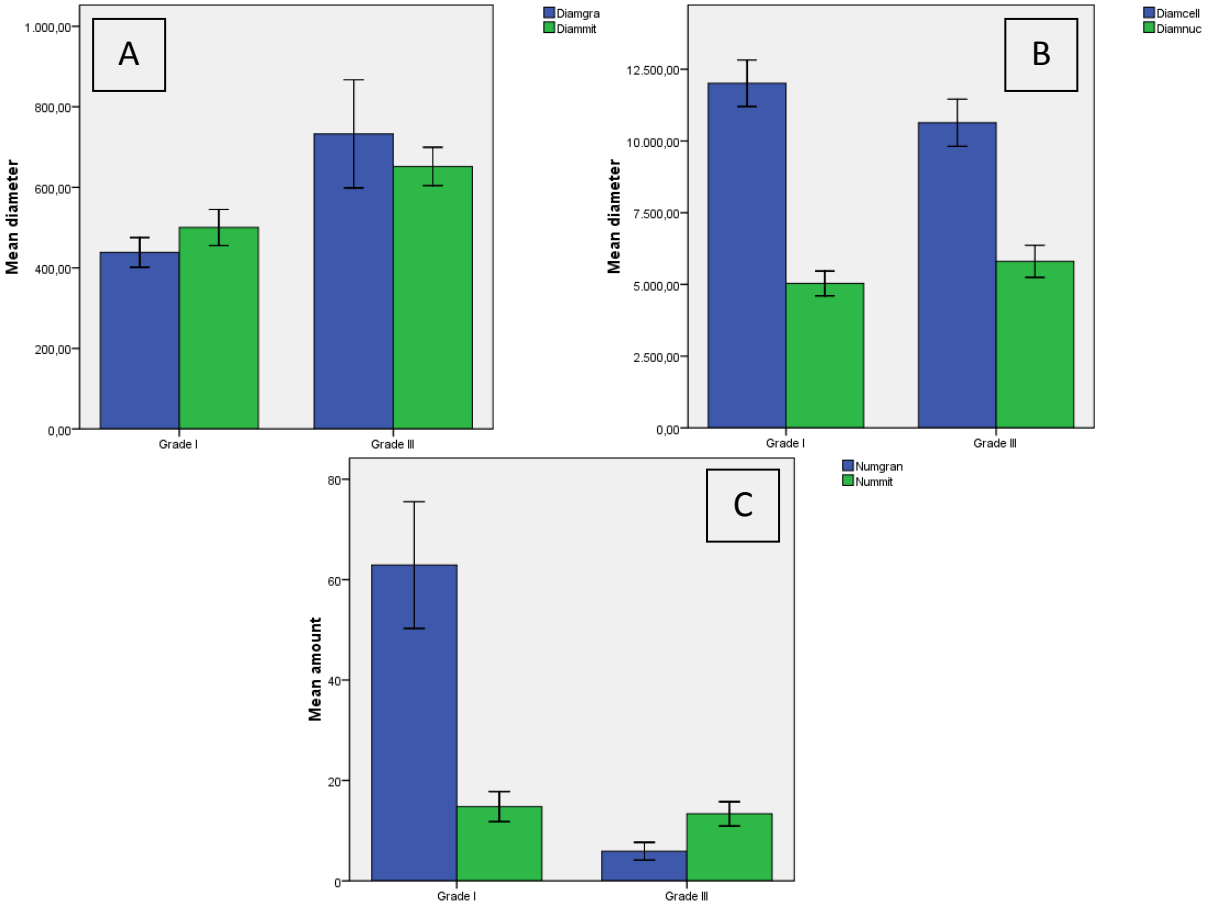

Figure 10: Comparison of morphometric characteristics in Grade I and Grade III MCTs.

The mitochondrion diameter however, differs significantly between Grade I and Grade III MCTs (p = 0.000) (Figure 10B). Grade I MCTs have a mean mitochondrion diameter of 500.224 nm (SD ± 100.423), the mean mitochondrion diameter for Grade III MCTs is 652.011 nm (SD ± 106.187).

3.3 Cell culture of mast cells derived from canine MCTs

One grade III tumour was brought into culture, which was maintained at 37°C in a humified atmosphere containing 5% CO_2. At 80% confluence, the cell culture was harvested and pelleted. Later, the cell suspension was resuspended and seeded onto coverslips in fresh culture medium.

3.3.1 IHC identification of cultured mast cells

To distinguish the cultured mast cells from fibroblasts, several immunohistochemical stainings were performed. First of all, an antibody against MCM7 was applied on coverslips overgrown with cells from a MCT. Figure 6A and 6B represent the proportion of MCM7 immunopositive cells after a culture period of nineteen days in DMEM 1X with low L-glutamine, low pyruvate, 1g/L D-glucose, antibacterial and antifungal agents.

The MCM7 count is 45 on a total of 400 cells in figure 11A and 30 on a total of 385 cells in figure 11B. By consequence, the resulting percentages of immunopositive cells are 11.25% and 7.79%, respectively. The average number of MCM7 positive cells is 9.50%. Figure 12 represents the blanco coverslip, which contains no immunopositive cells or background staining.

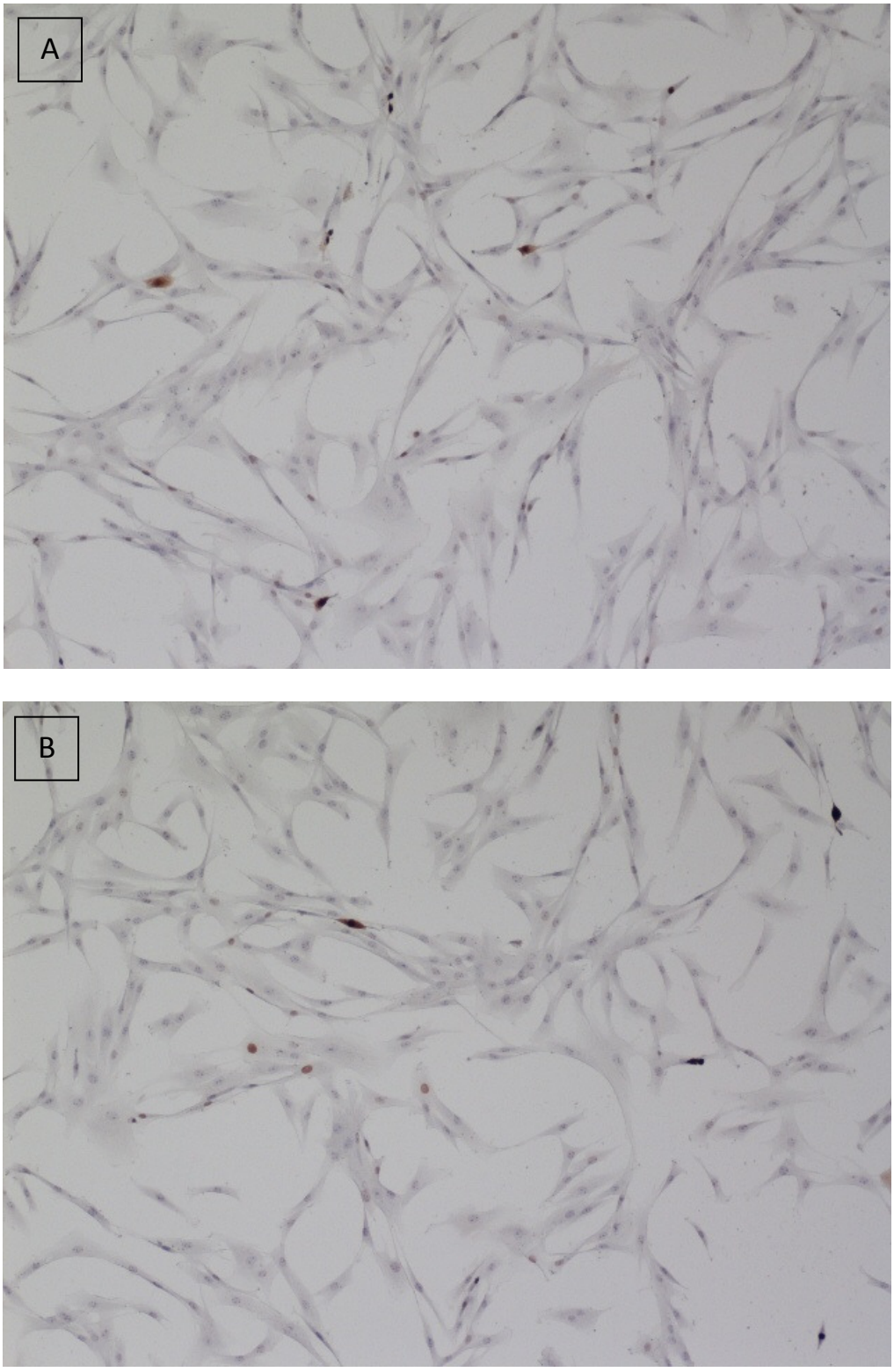

Figure 11: The relative number of MCM7 (dilution 1:50) positive cells at 10X magnification after a mast cell culture of 19 days.

Later, a second IHC stain using an antibody against c-KIT was performed, and the MCM7 stain was repeated to verify the previous results. Both results however, are not presented here since the analysis of the relevant coverslips revealed a lack of cell growth.

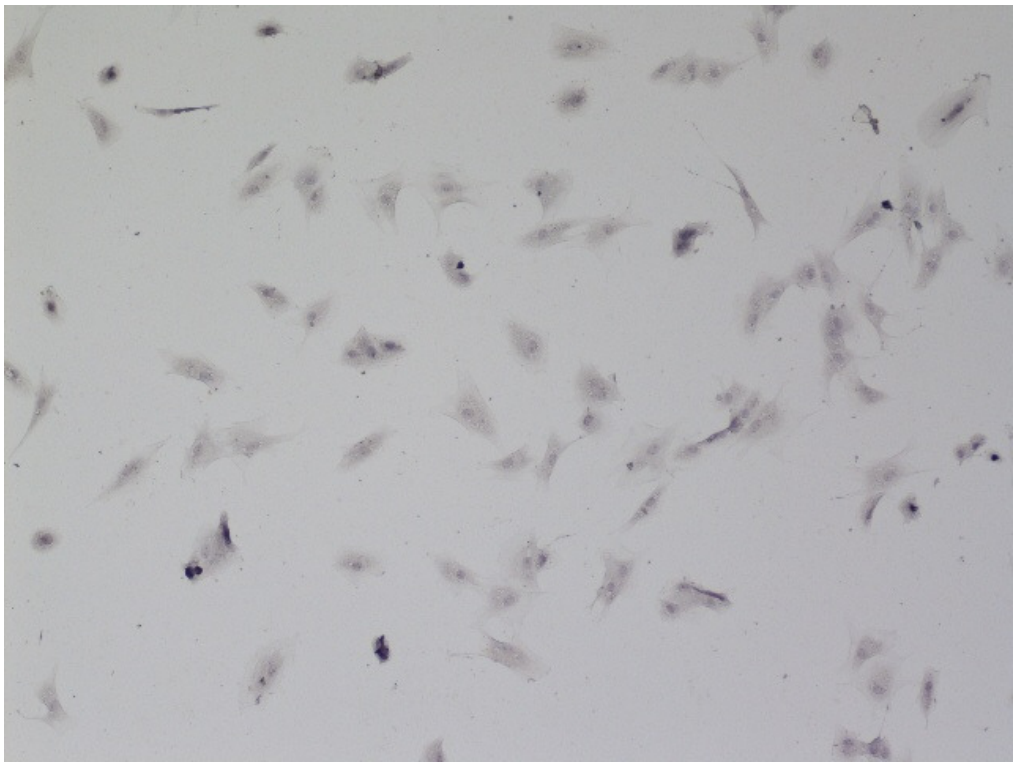

Figure 12: The blanco coverslip at 10X magnification on which only the secondary antibody was applied during the IHC stain.

A third IHC stain was performed using an antibody against CD203c. This antigen is a glycosylated type II transmembrane molecule and belongs to the family of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP3) enzymes that catalyze the hydrolysis of oligonucleotides, nucleoside phosphates, and NAD. Among hematopoietic cells, expression of CD203c is restricted to basophils, mast cells and their precursors, and has been described as a specific marker for this lineage[36].

Figure 13A represents the results of the CD203c IHC stain, notably a lot of background staining is present. No immunopositive cells can be identified on the relevant coverslip. When compared with the blanco coverslip (Figure 13B), there is no difference visible. Both coverslips are characterized by the presence of brown stippling.

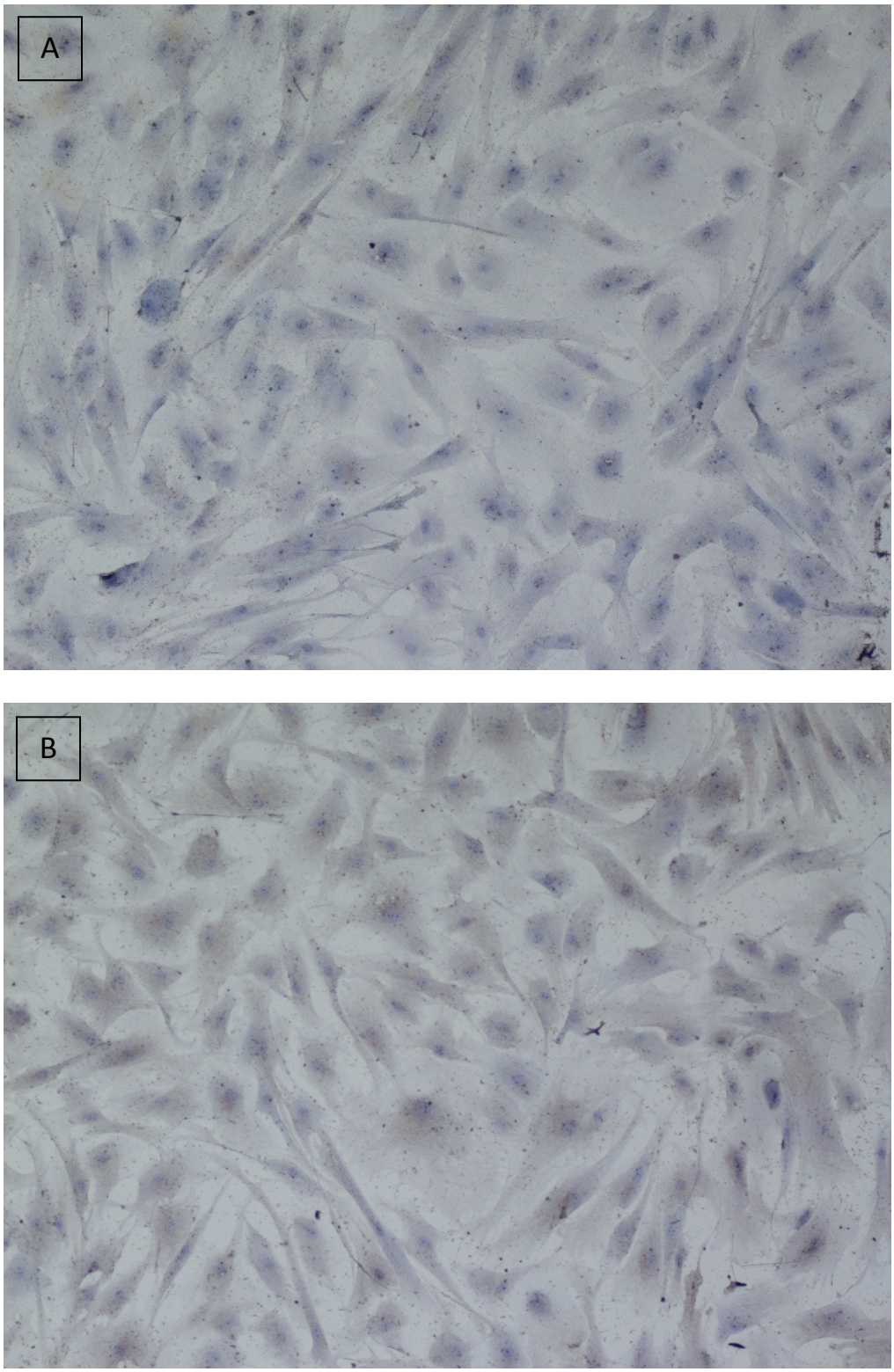

Figure 13: The relative number of CD203c (dilution 1:200) positive cells at 10X magnification.

3.3.2 Ultrastructural identification of mast cells

As the previous IHC results were not very conclusive, some cells were also seeded onto Thermanox coverslips. So, an ultrastructural analysis of the cell culture could be performed. Figure 9 represents the findings of this morphological study.

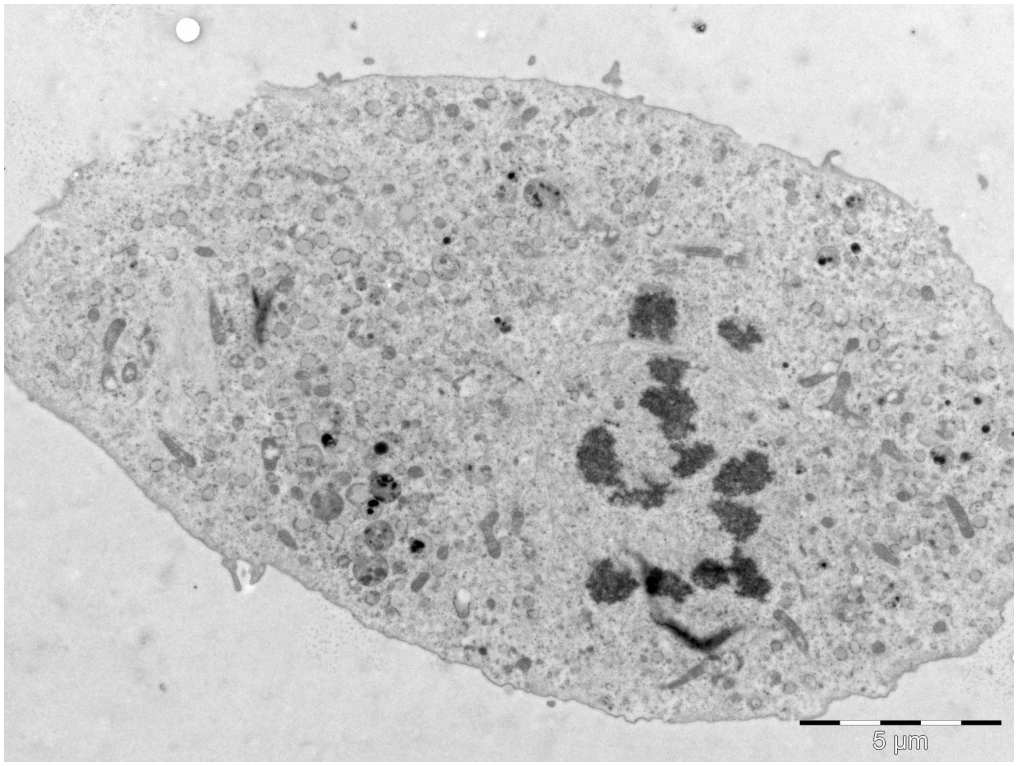

Figure 14: The ultrastructure of a mast cell at 4400X magnification present in the cell culture.

The morphological study of the cell culture revealed the presence of cells with distinct mast cell characteristics. These cells present with an ample cytoplasm and a centrally located nucleus. What's more, they contain numerous granules of different sizes. Their cell membrane has some microvilli, which promote cell communication and cell contact.

4 Discussion

This study centralized several aspects of canine mast cell tumours, which could be of importance to the stating of a correct prognosis. As mentioned before, MCTs are characterized by a diverse biologic behaviour. These neoplasms also present with various clinical appearances. This however, is not conducive to the choice of the right treatment.

The characterization of a number of morphological, morphometrical and immunohistochemical properties of canine MCTs was the main objective. In addition, an attempt was made to correlate those characteristics with the tumour grade, Webster et al. (2007) have shown this to be relevant. Grade II MCTs however, can have a benign clinical outcome or a malignant clinical course (e.g. metastases), which complicates the linking with histopathology. What's more, the use of a histopathologic grading system is subject to interpathologist variation.

The percentage of Grade I, Grade II and Grade III MCTs in this study is 13.5%, 55% and 13.5%, respectively. These percentages differ from those described by Patnaik et al. (1984). Their research group has found that 37% of all MCTs are Grade I, 42% of all MCTs are Grade II, and 20% of all MCTs are Grade III. A possible explanation for this difference could be our small population of canine MCTs and the subjectivity of grading. A solution for this problem could be to grade the MCT samples by more than 1 pathologist and study their opinions about the relevant MCTs. Such a procedure could result in a more accurate grading.

Uncontrolled cellular proliferation is a hallmark of cancer. As a consequence, various measures to analyze this have been extensively used trying to predict behaviour in cancerous tissue. An immunohistochemical analysis of the canine MCTs was performed as the amount of proliferation within an MCT is of importance to prognosis. Proliferation was assessed using two markers, namely Ki67 and MCM7. Ki67 is frequently used to evaluate proliferation as it is present in the G1, M, G2 and S phase. Several studies (e.g. Abadie et al, 1999) have shown Ki67 to be an good marker of proliferation, because a high Ki67 expression has been shown to be associated with an increased mortality, the rate of local recurrence and the presence of metastases[37]. Webster et al. (2007) have found that MCTs with a Ki67 index larger than 23 are associated with an increased rate of local recurrence, MCT occurrence at distant sites and an increased rate of MCT-related mortality. This nuclear protein however, does not detect cells that enter the early G1 phase.

MCM7 on the other hand, is expressed during all active phases of the cell division (G1, M, G2 and S). Because of this, this new marker could be better in predicting prognosis than the classical proliferation marker Ki67. In human cancers, numerous studies have been performed comparing MCMs and Ki67, and/or other proliferation markers. Burger et al. (2007) have shown that MCM2 provides a more accurate assessment of proliferation. Despite these promising results, these markers of proliferation have not yet been applied in veterinary medicine. So, that is why the expression of both Ki67 and MCM7 was analyzed in this study.

The expression of both Ki67 and MCM7 in MCTs was evaluated by the determination of the number of immunopositive cells, which is a rather subjective method. This kind of quantification though, is often used in IHC studies. Our results show a significant difference in the staining of proliferating neoplastic mast cells. Ki67 counts in a canine population containing all three grades described by Patnaik, range from 0 to 81 positive cells/grid area with an average of 5.78 positive cells/grid area. Whereas, MCM7 counts range from 0 to 847 positive cells/grid area with an average of 81.05 positive cells/grid area.

Notably, not all MCT samples could be included in this IHC study as some of the tissues were washed away during the process of antigen retrieval. Another possibility that needs to be considered is the time when the preparation (e.g. fixation, embedding and slicing) took place. Some samples have been embedded more than 2 years ago, whilst others have been recently embedded in paraffin. This time period could influence the antigenicity of the MCTs. We also noticed that the IHC staining of some samples was not excellent, whilst other MCTs stained very well. This could be due to the thickness of the slides. We assume thin tissue slides to be light-coloured after IHC staining and their counterstain using HE. Here, immunopositive cells coloured brown by DAB are easier to distinguish from the other, HE stained cells.

As mentioned before, the type III receptor tyrosine kinase KIT plays an important role in all sorts of mast cell processes. Therefore, a second IHC analysis was performed to study the expression pattern of c-KIT. The results indicate that most of the canine MCTs in our study (60%) have a large percentage of diffuse cytoplasmic stippling. There are however, MCTs that are characterized by both the presence of a considerable amount of diffuse cytoplasmic stippling and a lot of perinuclear KIT staining (27%). Only a few tissue samples (13%) have a large percentage of perimembrane KIT localization. Normal mast cells though, are characterized by KIT localization at their cell membrane. Our findings correspond to the results of Kiupel et al. (2004), who have found that cancerous mast cells have a predominant cytoplasmic KIT expression pattern.

There are however, other cellular processes that need to be considered in relation to prognosis. For example, two other hallmarks of cancer, namely apoptosis and angiogenesis.

Tumours depend on the rate and amount of proliferation, and the rate of cell death. A tumour characterized by a high proliferation and little apoptosis can differ in malignancy from a tumour characterized by little cellular proliferation and a lot of cell death. Carcinogenesis depends on the inhibition of apoptosis, because mutations in cells are recognized by the DNA repair systems (Whitfield et al, 2006). As a consequence, irreversible damage to cells is resolved by the induction of cell death. These findings resulted in the use of markers of apoptosis or molecules related to cell death (e.g. caspase-3, bcl-2).

Angiogenesis is the formation of new blood vessels as well as the remodelling of damaged arteries or veins. This process has a large value in the growth and metastases of tumours, as neoplasms can not survive without sufficient vascularisation. Tumours need the continuous supply of oxygen and nutrients to proliferate.

The ultrastructural analysis was split up into two different studies. Firstly, the morphological characteristics of all three tumour grades were evaluated and compared with each other. As previously described, two mast cell types are present in Grade I MCTs. Here, we also checked their presence in Grade II and Grade III MCTs.

No significant difference was found between the three possible grades regarding the number of Type 1 cells. The mean number of Type 1 cells in Grade I MCTs is 1.33, Grade II MCTs have a mean amount of 1.00 and Grade III MCTs also present with a mean number of 1.00. Analysis of Type 2 cells however, resulted in a significant difference between Grade I and Grade II MCTs and between Grade I and Grade III MCTs. The amount of these cells in Grade II versus Grade III MCTs on the other hand, was not significantly different. Well differentiated MCTs are characterized by the presence of more Type 2 cells than intermediate and poorly differentiated mast cell tumours. The mean number of Type 2 cells is 5.00 for Grade I MCTs, 1.17 for Grade II MCTs and 0.67 for Grade III MCTs. Next, the surface area of collagen was evaluated, but there was no significant difference was found. Grade I MCTs have a mean collagen surface area of $8.231*10^7$ nm², while Grade II MCTs have a mean collagen surface area of $2.891*10^7$ nm² and Grade III MCTs have a mean collagen surface area of $3.407*10^7$ nm². The presence of eosinophils was studied studies as well. Here, a significant difference was found only between Grade I and Grade III neoplasms. Grade I MCTs have a mean number of eosinophils of 1.00, whilst Grade II MCTs have a mean number of 2.50 and Grade III MCTs present with a mean number of 4.17. These eosinophils are attracted to the MCT site by release of eosinophil chemotactic factor (ECF) from the cancerous mast cells. As a result, the eosinophils leave the systemic blood vessels. This however, does not clarify how they aggregate around collagen bundles, which results in the formation of flame follicles. All of these results though, are not representative for all canine MCTs, because only two samples of each grade were evaluated.

Secondly, a few morphometric properties of Grade I and Grade III MCTs were analyzed. A Grade II MCT was not included in this study due to a lack of time. The diameters of the cell, the nucleus, granules and mitochondria were evaluated. In addition, the amount of granules and mitochondria were counted. There is a significant difference in cell and nucleus diameter between Grade I and Grade III MCTs, the mean values are 12009.813 nm and 10635.337 nm for the cell diameter, and 5035.777 nm and 5804.429 nm for the diameter of the nucleus. Moreover, the nucleus to cell ratio differs significantly. The mean ratio is 0.199 and 0.275 for Grade I and Grade III MCTs, respectively. These findings confirm the results from Madewell et al. (1984) who have shown that the diameter of the nucleus and the nucleus to cell ratio is larger in poorly differentiated mast cell tumours. Their research group has also described a significant difference in cell diameter between Grade I and Grade III MCTs, this is yet again confirmed in our study.

The presence of granules was defined for Grade I and Grade III MCTs. We found that well differentiated MCTs contain more granules than poorly differentiated MCTs, the mean values are 62.900 in Grade I MCTs and 5.900 in Grade III MCTs. The analysis of granule diameter revealed that Grade III MCTs are characterized by the presence of large granules, whilst well-differentiated MCTs present with smaller granules. Grade I MCTs have a mean granule diameter of 438.108 nm, while Grade III MCTs are characterized by a mean granule diameter of 732.844 nm. These findings correspond with a study from Dean et al. (1988)[38].

Another significant difference was found regarding the diameter of the mitochondria. Well differentiated MCTs contain large mitochondria, which is a grade-dependent difference. Grade III MCTs on the other hand, are characterized by the presence of small mitochondria. The mean mitochondrion diameter is 500.224 nm and 652.011 nm for Grade I and Grade III MCTs, respectively. This correlation has not been found by Madewell et al. (1984). The mitochondria present in the MCTs were also counted, but no significant difference was found. Madewell et al. (1984) however discovered a relation between the number of mitochondria and the differentiation status of the tumour.

Finally, we set up a cell culture of mast cells starting from a Grade III MCT. Here, the objective was to culture cancerous mast cells and study their behaviour and proliferation by use of IHC staining. Several IHC stains were applied, MCM7 was used to detect proliferation amongst the cell culture that did not only consist of mast cells. Fibroblast-like cells were also present. The analysis of the MCM7 stain revealed little proliferation, which could be due to the large amount of fibroblast-like cells in the culture. As these cells are known for their possible overgrow in cell cultures, it could have been that the mast cells did not have enough nutrients or space to proliferate. To confirm these results another IHC stain using MCM7 was performed, this time however no cells could be visualized. The coverslips were not overgrown with cells, although they were fixated after 80% confluence. A possible explanation could be that the fixative did not work or that the time span between fixation and immunohistochemistry was too long.

Then, we searched specific markers of mast cells and came up with CD203c. As mentioned before, this is a glycosylated type II transmembrane molecule that belongs to the family of E-NPP3 enzymes. Expression of CD203c is restricted to basophils, mast cells and their precursors. The results however, were not very conclusive as both the sample and the blanco coverslip were characterized by brown stippling. Maybe, this background staining could be due to the contamination of the cell culture with fungi. Another possibility could be the nonspecific binding of the secondary antibody to molecules present in our cell culture.

To identify the mast cells in our culture with certainty, we seeded some cells on Thermanox coverslips after trypsinization. This allowed the ultrastructural analysis of the cell culture. After thorough inspection with the electron microscope, several mast cell-like cells were identified in the presence of numerous fibroblasts. This confirms our first thought, that the fibroblasts take up all the nutrients. As a consequence, the mast cells could not sufficiently proliferate and little of them survived.

5 Conclusion

We can conclude that several characteristics (morphological and morphometrical) of canine MCTs could be of great importance regarding the prediction of prognosis. What's more, immunohistochemistry using MCM7 is a more accurate marker of proliferation than Ki67. This new marker of cellular proliferation is promising and when related to histopathology, it can contribute to the right treatment choice for each affected dog. Nevertheless, this study should be repeated in a larger canine MCT population to verify these findings.

In order to relate the KIT localization to the tumour grade, an experiment with a large amount of MCT samples should be conducted. What's more, if it is possible to follow the canine population throughout their life and obtain information about their health status, life span and more specifically local or distant recurrence, the c-KIT expression pattern can be correlated with survival. This could then also contribute to the prediction of prognosis and the treatment options.

As mentioned before, the results from the morphological study of canine MCTs are not representative. Therefore, another study with more tumour samples should be performed. The morphometrical part of this study however, shows promising results. It would be a good idea to perform the same study in a larger population of canine MCTs to verify the obtained results. Another future perspective could be the development of a new classification system that incorporates the characteristics of Patnaik et al. (1984), the cellular morphology of the MCT and histopathology (e.g. MI, presence of eosinophils, c-KIT staining pattern).

The set up of a cell culture could also be repeated, but a fibroblast-inhibiting factor or mast cell stimulation are strongly recommended. Otherwise, the fibroblasts will overgrow the mast cells and take up all of their nutrients. Another possibility is the use of mast cell specific medium, which is adapted to the needs of the cancerous mast cells (e.g. a higher FCS concentration). A third solution could be the isolation of mast cells from an MCT using techniques like cell lysis and magnetic activated cell sorting (MACS).

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Supplements

Supplement 1 – Thermo Scientific Microm HMS 740 Robot Stainer

Protocol HE staining:

- Entry
- Treat slides 2 minutes with Clear Rite™
- Treat slides 2 minutes with Clear Rite™
- Treat slides 2 minutes with Clear Rite™
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Rinse slides 2 minutes
- Treat slides 1minute with Haematoxiline
- Rinse slides 1minute
- Treat slides 2 minutes with Clarifier
- Rinse slides 1 minute
- Bluing slides 1/2 minute
- Rinse slides 1 minute
- Treat slides 1minute with eosine
- Rinse slides 1minute
- Treat slides 2 minutes with isopropanol
- Treat slides 2 minutes with isopropanol
- Treat slides 2 minutes with isopropanol
- Treat slides 2 minutes with Clearifier
- Toluene Exit

Protocol rehydration:

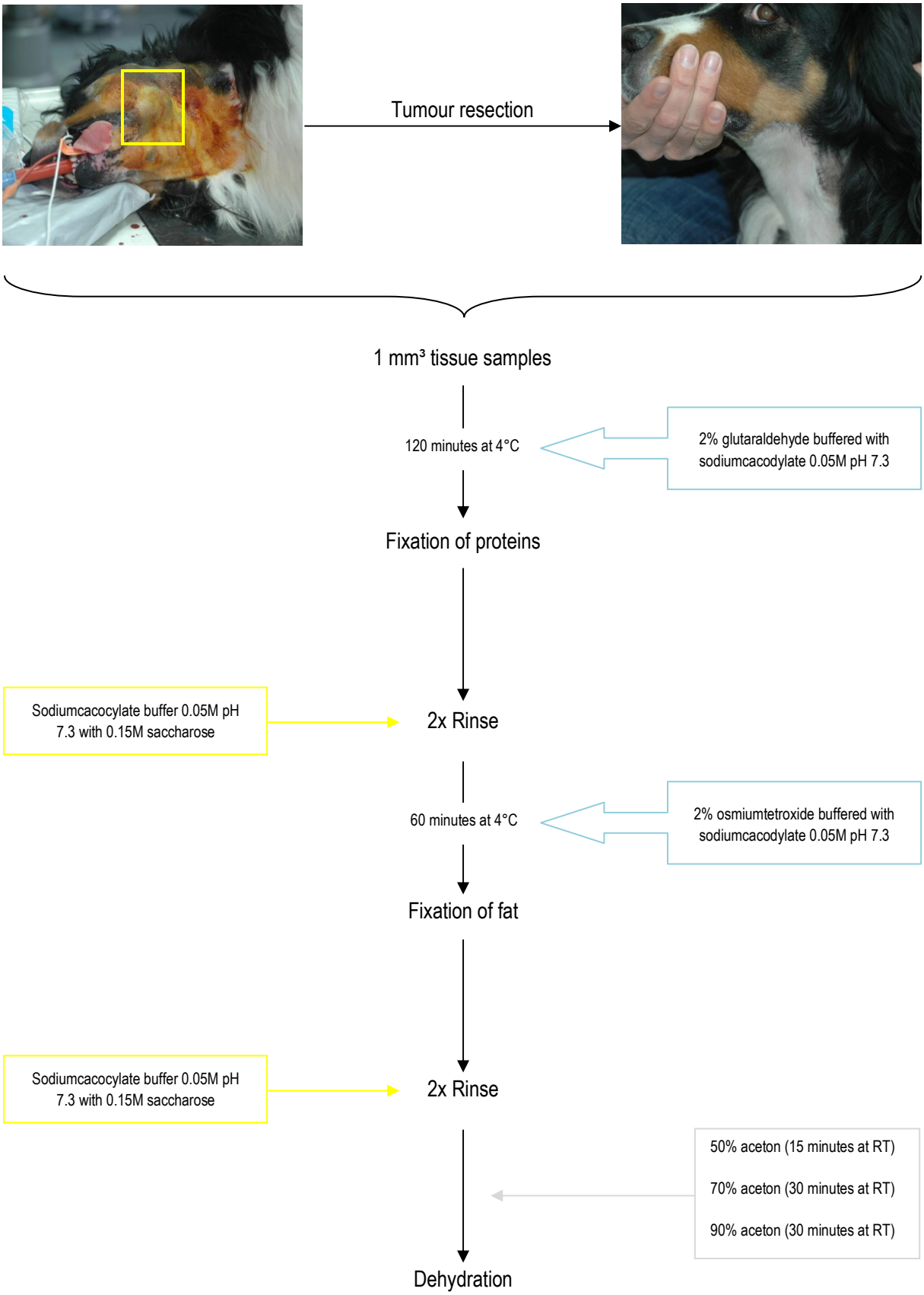
- Entry
- Treat slides 2 minutes with Clear Rite™
- Treat slides 2 minutes with Clear Rite™
- Treat slides 2 minutes with Clear Rite™
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Treat slides 1 minute with isopropanol
- Rinse slides 2 minutes
- H_2O Exit

Supplement 2 – BD SMS 3600™ Molecular Stainer

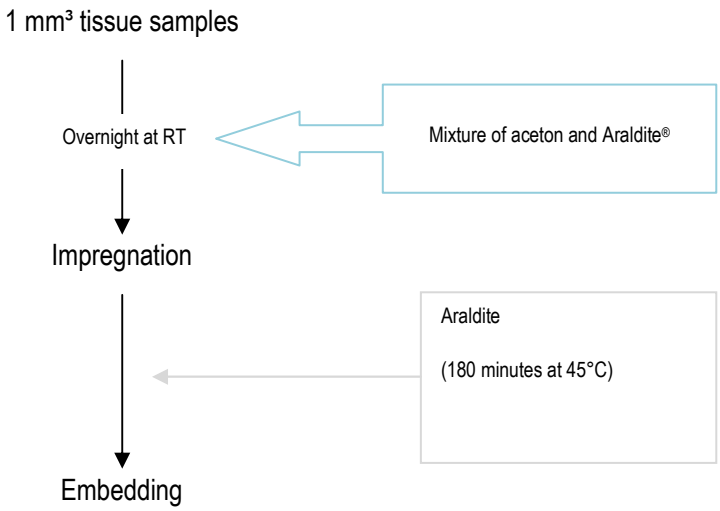
Protocol:

- Rinse with wash buffer 10X
- Treat slides 5 minutes with 200 µl Dual Endogenous Enzyme Block
- Rinse with wash buffer 10X
- Treat slides 30 minutes with 200 µl primary antibody
- Rinse with wash buffer 10X
- Treat slides 30 minutes with 200 µl Envision™+ Dual Link System-HRP reagent
- Rinse with wash buffer 10X
- Rinse with wash buffer 10X
- Treat slides 10 minutes with 200 µl DAB+ Substrate Chromogen System (Dako, USA)
- Rinse with wash buffer 10X
- Treat slides 4 minutes with 200 µl 'Mayer's Haematoxylin'
- Rinse with wash buffer 10X
- Rinse with A.D.

Supplement 3 – Electron microscopy: Fixation and dehydration



Supplement 4 – Electron microscopy: Embedding



Supplement 5 – Electron microscopy: Principle

An electron microscope utilizes radiation of shorter wavelength in the form of an electron beam. These electrons are produced by a tungsten filament at the top of a cylindrical column. Air must first be pumped out of this column to create a vacuum, because electrons are scattered by collisions with air molecules. Then, the electrons are accelerated from the filament by use of a anode and they are allowed to pass through a tiny hole. As a result, an electron beam is formed that travels down the column, this beam is focused by electromagnets placed along the column.

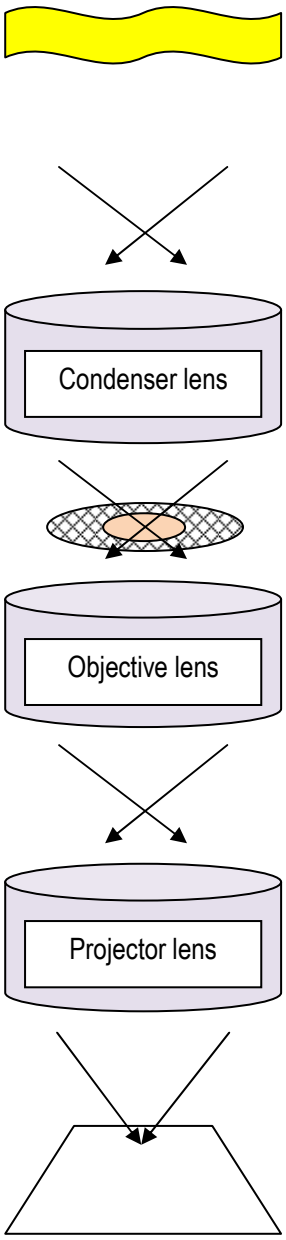
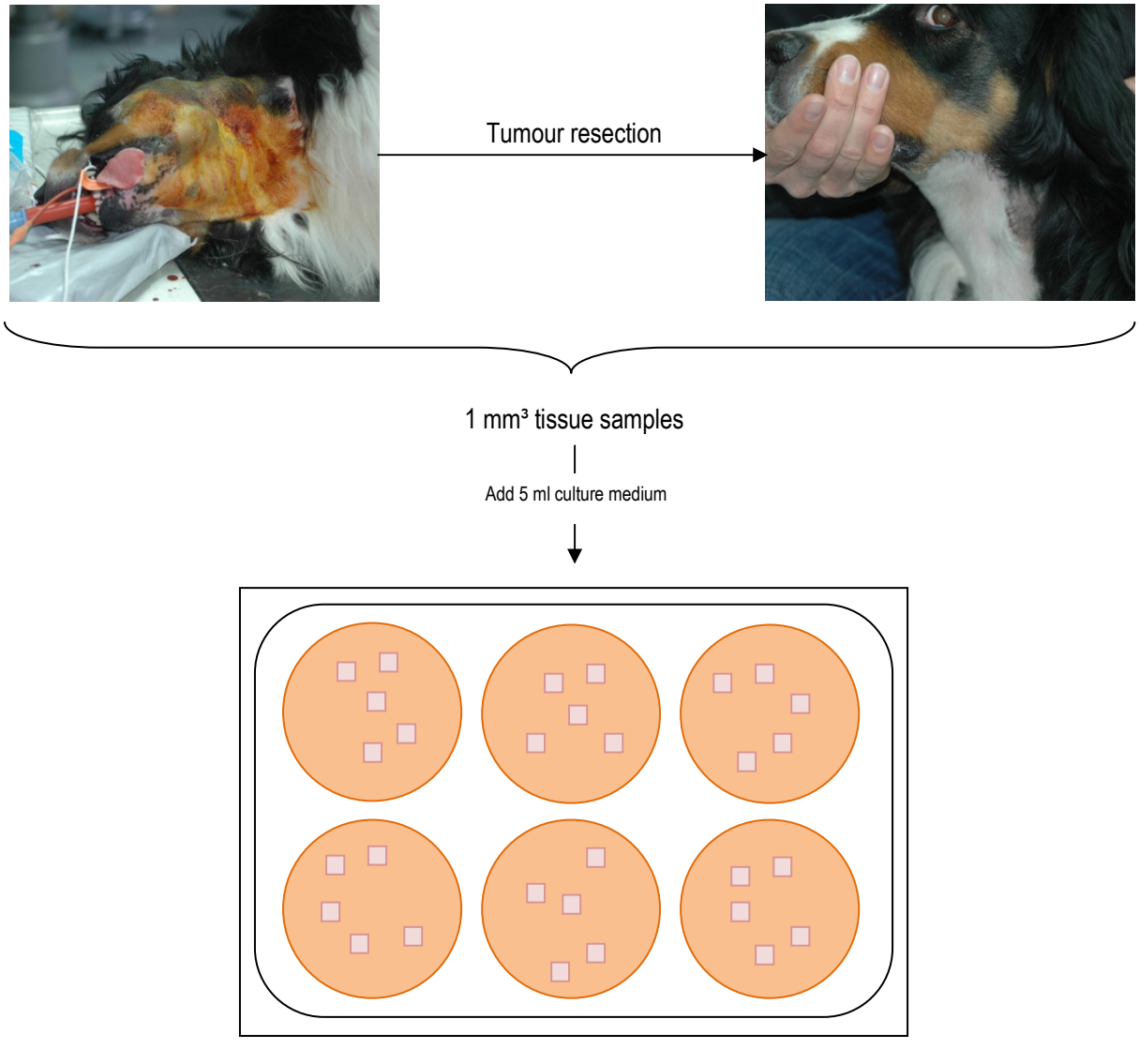


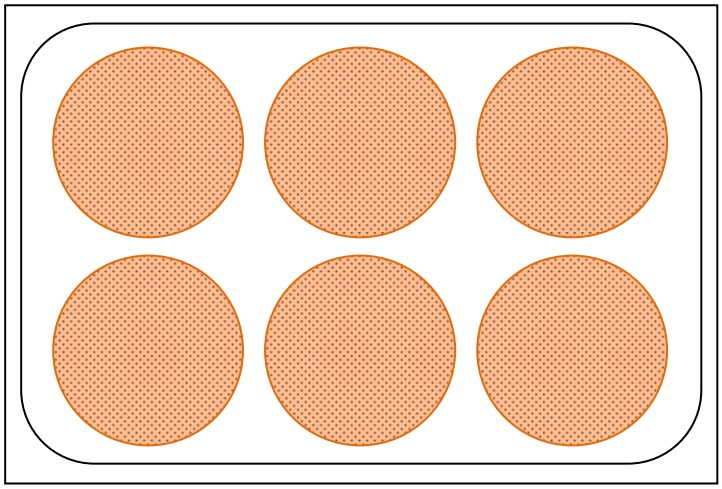
Figure 1: The principal features of a transmission electron microscope.

The specimen, which is stained with electron-dense material, is placed into the path of the electron beam. Some of the electrons passing through the tissue sample are scattered by the stained structures, the remainder are focused to produce an image on a fluorescent screen (Figure 1). The loss of electrons by scattering result in areas of reduced electron flux, as a consequence those specimen regions are darkened in the formed image.

Supplement 6 – Cell culture: Explant technique



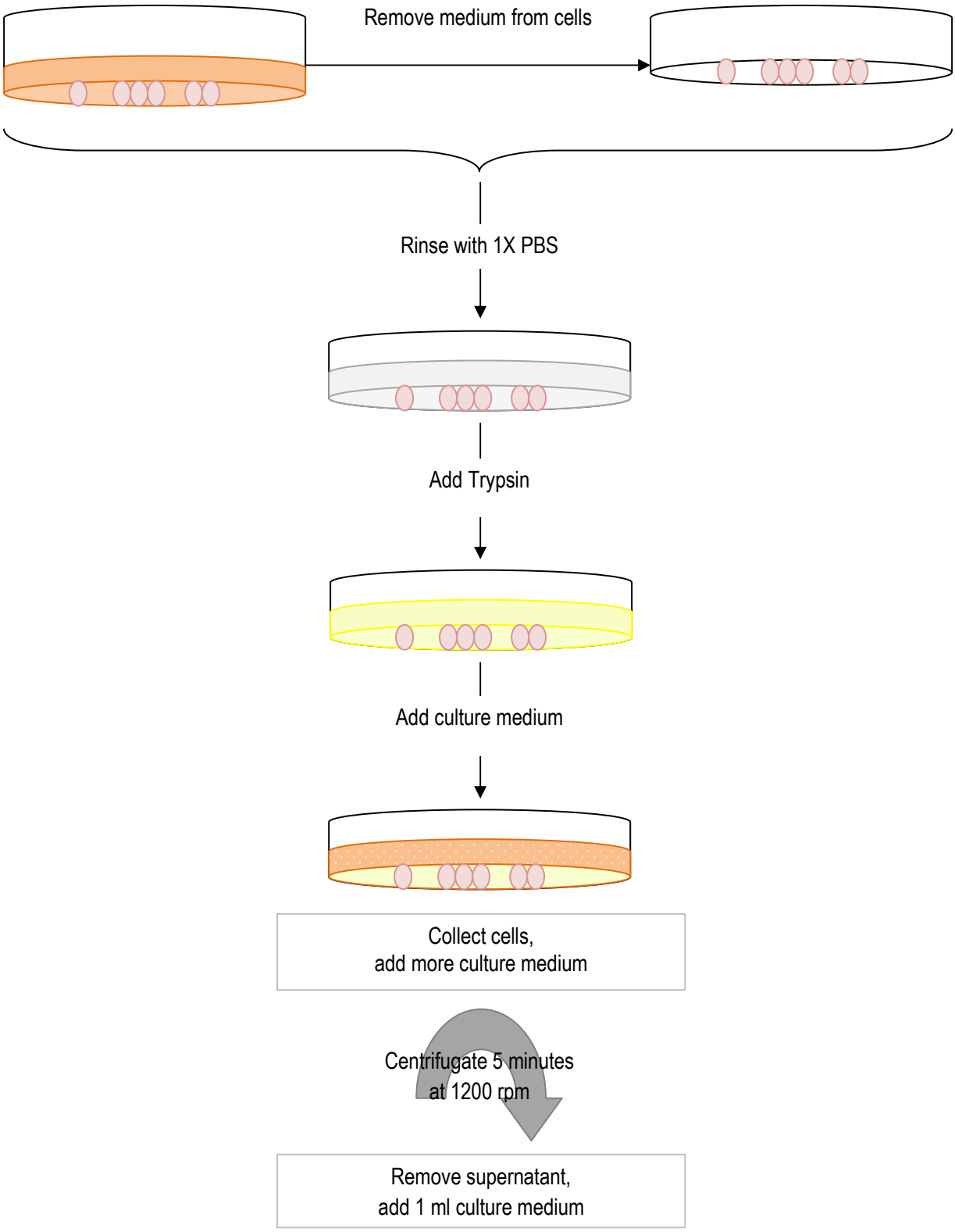
Remove tumour samples



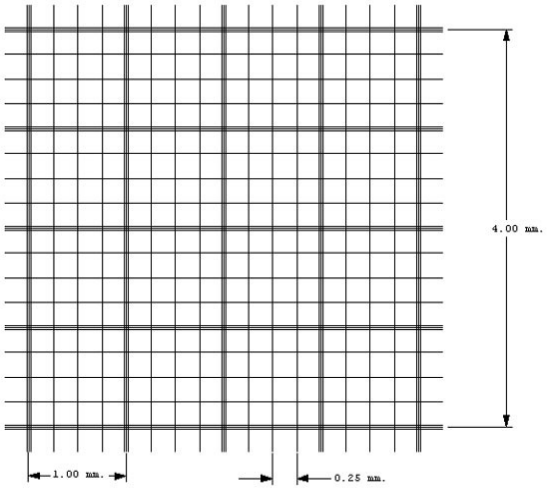
↑↑ cell growth (80% confluence)

Trypsinisation

Supplement 7 – Cell culture: Trypsinisation



20 µl cell suspension + 20 µl Trypan Blue



$$\frac{\text{cells} * 10\,000}{3}$$

Distribute cells over flasks, Thermanox™ and coverslips

Supplement 8 – Cell culture: Immunohistochemistry

- Fill the wells with 1X PBS
- Add 1 coverslip to each well
- Rinse the coverslips with 1X PBS
- Membrane permeabilisation
    - Add 200 µl 0.05% Triton 10X
    - 30 minutes at 4°C
- Rinse the coverslips 4x with 1X PBS
- Block aspecific binding
    - Add 250 µl serum specific for secondary antibody
    - 20 minutes at RT
- Rinse the coverslips 4x with 1X PBS
- Primary antibody binding
    - Add 250 µl primary antibody
    - 60 minutes at RT
- Rinse the coverslips 4x with 1X PBS
- Secondary antibody binding
    - Add 5 droplets/coverslip
    - 30 minutes at RT
- Rinse the coverslips 4x with 1X PBS
- Detection
    - Add 5 droplets/coverslip DAB
    - 5 minutes at RT
- Rinse the coverslips 4x with AD
- Counterstaining
    - Add Mayer’s Hematoxylin
    - 6 minutes at RT
- Rinse the coverslips 4x with water
- Place the coverslips on slides