FACULTY OF VETERINARY MEDICINE
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State of the art overview of organoids in canine veterinary medicine

Word count: 18387

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Veterinary Medicine

Academic year: 2022 – 2023

GHENT UNIVERSITY

Preface

The literature study of this thesis highlights the importance of canine organoids for both veterinary and human medicine. To illustrate this, a state of the art overview was established of all reported canine organoids. My attention was drawn to this specific subject, because I wanted to establish something that would benefit both humans and animals. We are connected in our daily life and I believe that this could also be applied to research. Given the benefits that canine organoids can have for both canine and human patients, I decided to write my thesis about the use of canine organoids for the translation to human disease, and more specifically cancer.

During the process of writing this thesis, my fascination for organoids grew. In addition, I was surprised to learn how little people, including veterinarians, know about the existence of organoids. The target audience for this thesis would be anyone who would like to learn more about this interesting new research tool.

I want to thank my supervisors, Dr. Jonas Steenbrugge and Julie Bellemans, for the time they invested in the project and to allow me to give my own interpretation to the subject, to guide me through the writing process and to provide any helpful feedback.

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List of abbreviations

A83-01	tumour growth factor β type 1 receptor inhibitor
ASCs	adult stem cells
BC	bladder cancer
CDCs	cardiosphere-derived cells
cFTC	canine follicular thyroid carcinoma
CHF	chronic heart failure
CKD	chronic kidney disease
CMTs	canine mammary tumours
COMMD1	copper metabolism domain containing 1
COPD	chronic obstructive pulmonary disorder
CSCs	cancer stem cells
DCM	dilated cardiomyopathy
DMEM	Dulbecco's modified eagle medium
DMEM/F12	Dulbecco's modified eagle medium/Ham's F-12
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ESCs	embryonic stem cells
ESRD	end-stage renal disease
FBS	foetal bovine serum
FGF	fibroblast growth factor
Gpr49	G-protein-coupled receptor 49
GSK3β	glycogen synthase kinase 3β
HBC	human breast cancer
HEPES	N-2-hydroxyethyl piperazine-N2-ethane sulfonic acid
HFs	hair follicles
HGF	hepatocyte growth factor
IBD	inflammatory bowel disease
IFE	intrafollicular epidermis
IMDM	Iscove's modified Dulbecco's medium
iPSCs	induced pluripotent stem cells
Lgr5	leucine-rich-repeat containing G-protein-coupled receptor 5
MEM	minimal essential medium
MM	malignant mesothelioma
MTC	medullary thyroid cancer
NAC	N-acetylcysteine
NHL	non-Hodgkin's lymphoma
NIS	sodium iodide symporter
NPCs	neural progenitor cells
NSCs	neural stem cells
OE	oviductal epithelium
OSA	osteosarcoma
PBS	phosphate buffered saline
PC	prostate cancer
PDOs	patient-derived organoids
PLE	protein loosing enteropathy
PS	penicillin/streptomycin
PSS	portosystemic shunt
PSC	pluripotent stem cell
RPMI	Roswell Park memorial institute
Rspo1	R-spondin-1
Rspo3	R-spondin-3
SB202190	P38-inhibitor
TME	tumour microenvironment
TMS	trimethoprim sulphate
TPO	thyroid peroxidase
TSHR	thyrotropin receptor
Y-27632	rho-associated coiled-coil containing protein kinase inhibitor
Zo-1	zonuline-1

Abstract

Organoids are a recent development that has already shown great promise within human medicine. However, in veterinary medicine the applications of organoids are vastly underestimated and reports are far more limited. Besides the potential benefits of canine organoids in veterinary medicine, the concept of 'One Health' implies that scientific and clinical results are potentially exchangeable between humans and animals. Therefore, also humans can benefit from these pet-derived organoids. Previous reports already discussed the use of canine organoids to investigate human disease, such as cancer. Nonetheless, a complete review on all available canine organoids is currently lacking. Moreover, studies that compare the culture media between different types of canine organoids cannot be found. This literature review critically reviews the use of canine organoids for translation to human medicine. Canine organoids show great resemblance to their human counterparts and they overcome many ethical limitations that are related to animal models and human tissue availability. A state of the art overview of all reported canine organoids and tumouroids was also assembled, with a detailed description of their culture conditions. Interestingly, we here show that there are clear correlations in the culture media between the many types of canine organoids and tumouroids that have already been established. As a future perspective, it is necessary to perform more research and develop standardized organoid culture conditions. Increasing of the organoid applicability, reproducibility, efficiency and safety is crucial for canine organoids to have an impact in both veterinary and human medicine.

Organoïden zijn een recente ontwikkeling die in de humane geneeskunde al veelbelovend is gebleken. In de diergeneeskunde worden de toepassingen van organoïden echter sterk onderschat en is het aantal studies veel beperkter. Naast de potentiële voordelen van caniene organoïden in de diergeneeskunde impliceert het concept van 'One Health' dat wetenschappelijke en klinische resultaten tussen mens en dier kunnen worden uitgewisseld. Hierdoor kan ook de mens voordelen halen uit het gebruik van deze huisdier-afgeleide organoïden. Eerdere publicaties bespraken al het gebruik van caniene organoïden voor onderzoek naar ziekten bij de mens, zoals kanker. Echter, een compleet overzicht van alle beschikbare caniene organoïden ontbreekt op dit moment. Er zijn verder ook geen studies beschikbaar die de kweekmedia tussen verschillende types caniene organoïden vergelijken. In dit literatuuronderzoek wordt het gebruik van caniene organoïden voor translatie naar de humane geneeskunde kritisch bekeken. Caniene organoïden vertonen een grote gelijkenis met hun humane tegenhangers en ze vermijden veel ethische beperkingen die samenhangen met diermodellen en de beschikbaarheid van menselijk weefsel. Er wordt ook een 'state of the art' overzicht gegeven van alle gerapporteerde beschikbare caniene organoïden en tumoroïden, met een gedetailleerde beschrijving van hun kweekcondities. Opvallend is dat duidelijk correlaties waargenomen kunnen worden tussen de verschillende kweekmedia van de vele soorten caniene organoïden en tumoroïden die reeds ontwikkeld zijn. Met het oog op de toekomst is het nodig om meer onderzoek te doen en om gestandaardiseerde kweekomstandigheden voor organoïden te ontwikkelen. Het vergroten van de toepasbaarheid, reproduceerbaarheid, efficiëntie en veiligheid van organoïden is cruciaal voor caniene organoïden om een blijvende impact te hebben op zowel de humane als de veterinaire geneeskunde.

1. Introduction

Organoids are a recent concept that has gained popularity over the past decades, both within human and veterinary medicine. Already in the years 1965-1985, an increase in the number of publications reporting the term 'organoid' can be observed[1]. However, the origins of organoid technology can be found in stem cell research[2]. In conjunction with studies in regenerative medicine, it was shown that stem cells can be used to create three dimensional (3D) miniature organs in vitro, so called 'organoids'[2]. This is possible because of the unique quality of stem cells being "self-renewing and their ability to generate differentiated cells, allowing them to create a variety of tissues within a developing embryo"[3]. Therefore, an organoid is defined as a "stem cell-based and self-organizing 3D culture that mimics the cell type composition, architecture and, to a certain extent, functionality of differentiated tissues"[3]. Organoids provide a bridge between two dimensional (2D) cell line cultures and animal models, and they provide a much better representation of the healthy or pathological situations in the living organism[2,3,4,5,6,7]. Moreover, they allow a connection between fundamental in vitro research and patient treatment[2-6]. Organoids also have ethical benefits, providing solutions for ethical restrictions in human medicine and contributing to the "replacement, refinement, and reduction" of animal models, the so called '3R-concept'[2,7,8,9,10].

The applications for organoids can be divided into four main groups: for studying organogenesis and homeostasis (1), for use in regenerative medicine (2), for disease modelling (3) and for therapeutic and pharmacological applications (4)[2,5,].

1. Organogenesis and homeostasis

Organoids are able to mimic certain principles of the original organ biology[2,11]. Therefore, they provide information in organogenesis and homeostasis[2,11]. Moreover, organoids can be used to study mechanisms involved in self-organization, research which is not accessible to human experimentation[2,11]. Studies already showed that for example optic cup or intestinal organoids can be used to observe the underlying developmental mechanisms for these tissues[12,13].

2. Regenerative medicine

Organoids offer a potential solution for donor-tissue shortages and for organ rejection by the patient's immune system[2,14,15,16]. Therefore, they create a promising alternative to organ transplantation[2,14-16]. Transplanted dissociated cells are limited by their lack of physiological context[2,17]. In contrast, organoids are able to provide cells that can reconstruct organ function and create a niche to protect the graft from hostile pathological environment[2,17]. 'Proof of concept' transplantation experiments for regenerative medicine in animal models have already been performed, such as intestinal organoids of mice being able to regenerate damaged colon[2,14,18].

3. Disease modelling

Organoids can be used to determine the onset and progression of a disease at the organ level and to identify novel biomarkers for disease severity[2,5,16]. This advantage cannot be observed in traditional cell cultures that exist of a single cell type[2,5,16]. Organoid biobanking is defined as the "systemic collection, processing, storage and distribution" of organoids and their associated data[2,19,20]. Therefore, organoid biobanks contribute to global research and clinical and translational applications[2,19,20]. Organoids can be used to for example model genetic diseases and to study host-pathogen interactions[2,21,22,23]. In addition, patient derived organoids (PDOs) can be used to define genetic causes for complex pathologies with a high interpatient variability[2,21-23]. The oncological applications of organoids are also a very important part of disease modelling[2]. More specifically, organoids are able to incorporate the tumour microenvironment (TME), mimicking the histological structure of the tumour[2,24,25].

4. Therapeutic and pharmacological applications

Organoids, especially derived from adult stem cells (ASCs), can be used for drug testing and screening[7,11]. In addition, PDOs can be used for personalized medicine[7,11]. By testing medication on both healthy and pathological PDOs, more effective and personalized treatment can be designed with minimal side effects and potential therapeutic resistance can be determined[7,15,].

Many applications have been developed for organoids in human medicine, including using veterinary organoids for studying human disease[9]. However, few studies are focussing on the applications in veterinary medicine[9]. The concept of 'One Health', also known as 'One Medicine' or 'Cross-Health', argues that no distinction should be made between human and veterinary medicine and that it is possible to translate research findings to other species[10,26,27].

The purpose of this literature study is to create a state of the art overview of organoids in canine veterinary medicine. The current available canine tissues from which organoids can be derived will be discussed, with a special focus on oncology, and their culture media will be compared. Moreover, a connection will be made to human medicine in view of the 'One Health' concept. The focus on canine veterinary medicine was chosen, because of the strong interest from Ghent University in this concept and their proven translational applications in human medicine.

2. The evolution of organoid research

2.1 From 2D cell lines to 3D structured organoids over time

In vitro cell cultures are essential to study the development of disease in an organism[7]. 2D cell line cultures are frequently used, because they are easy to maintain, rapidly grown, highly reproducible, and they have a low cost[28,29,30]. However, most 2D monolayer cell lines do not resemble the cells in vivo, which are composed of a 3D architecture and are surrounded by extracellular components[7,11,25,31]. This makes 2D cell lines limited translation models[7,11,25,31]. Indeed, the surface topography and stiffness of the 2D cell cultures is significantly different from the tissues they are derived from, which also alters the cell function [6,7,11,25,31]. In addition, for long-term growth of 2D cell line cultures, malignant degeneration needs to be induced[7]. Thanks to many developments within stem cell research, the idea of using stem cell technology for the creation of organoids emerged[2,11,19]. Organoids consist of 3D cell cultures, in which an environment is created where cells "grow and interact with their environment in three dimensions" and where prolonged growth can occur without changing any genetic or phenotypic features[4,,7]. The evolutionary change in the number of publications that can be found in PubMed, considering some specific search terms on the concept of organoids, is illustrated in *figure 1*.

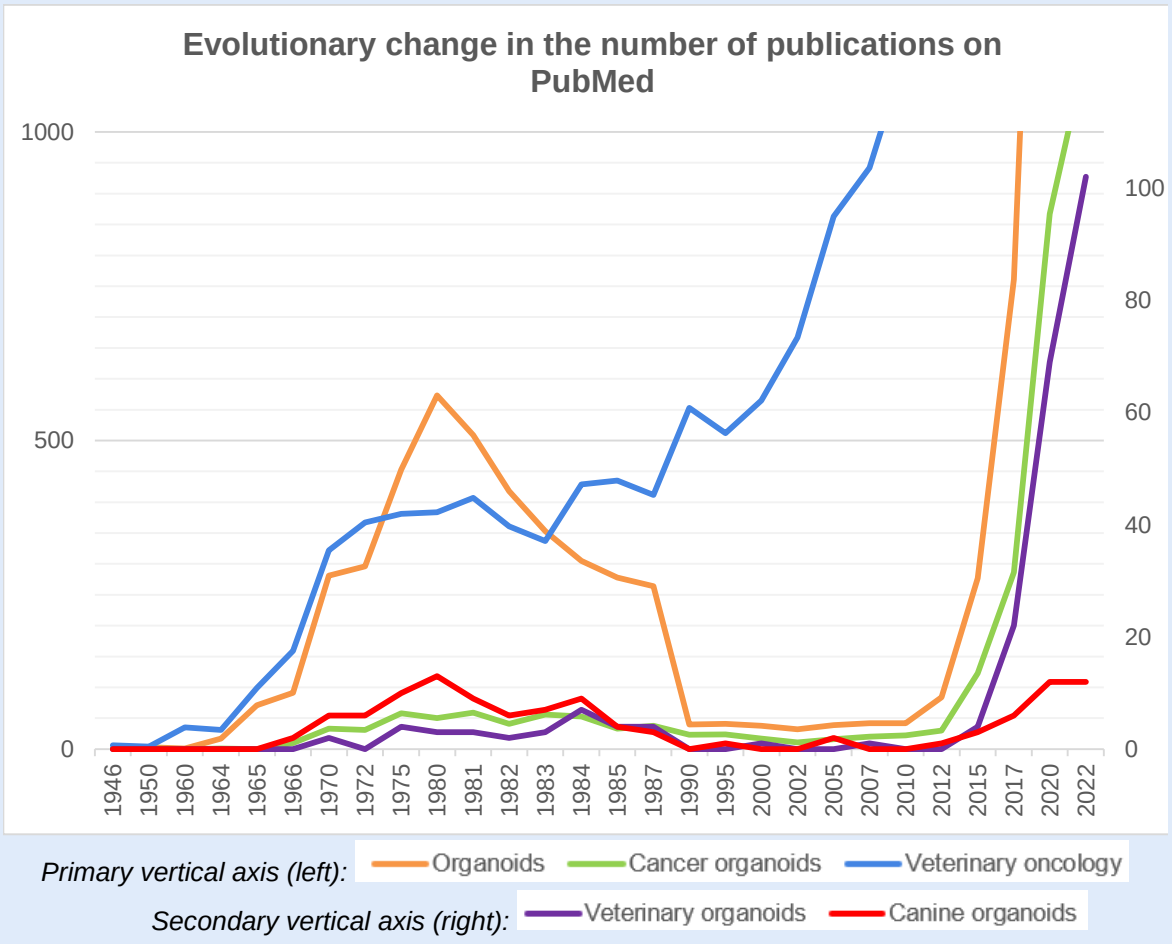


Figure 1: The evolutionary change in the number of publications based on a PubMed search performed on Friday December 2nd. The results are divided over two vertical axes, due to the range in article numbers for the individual results.

In vitro research on organ regeneration can be traced back to 1907[31,32,33,34]. In this study, Wilson's experiment showed that dissociated sponges could organize themselves into perfect new sponges when kept under the right conditions[31-34]. However, the term 'organoid' first appears in PubMed in 1946[1] in a case report on a cystic organoid teratoma in a two month old infant, and is referred to as 'being similar to an organ'[2,35]. In 1989, Barcellos-Hoff et al. succeeded in the formation of 3D structured alveoli by cultivating primary mammary cells on a reconstituted basement membrane[2,160]. This can be seen as one of the first examples of an engineered organoid[2]. Nevertheless, Hans Clevers *(1957- present)* and Yoshiki Sasai *(1962-2014)* could be considered the true founders of the idea of the in vivo organ resemblance of organoids, due to their research on specific stem cell subtypes[2,36]. Organoids derived from mouse or human tissues are already widely described[16]. However, research studies that are focussing on organoids from large domesticated animals, such as pigs, cattle, poultry, sheep, horses cats and dogs, are far more limited[9]. The different developments that have contributed to organoid research, more specifically on canine organoids, are illustrated in ***figure 2***[2,16].

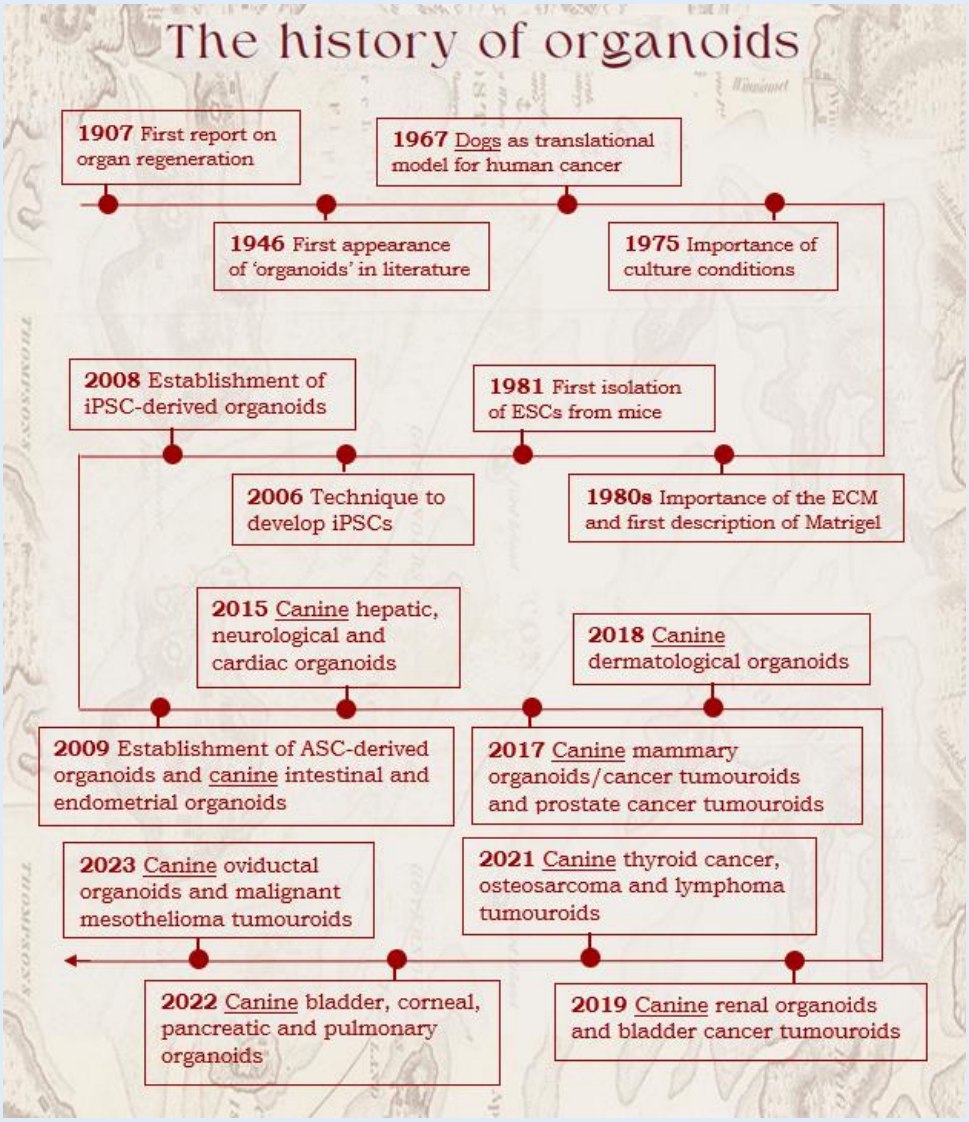


Figure 2*:* Timeline of the developments in the organoid field and establishments of canine non-malignant tissue derived organoids and tumour derived tumouroids, based on scientific literature. Abbreviations: ECM (extracellular matrix), ESCs (embryonic stem cells), iPSCs (induced pluripotent stem cells), ASC (adult stem cell).

[1] Retrievable at: https://pubmed.ncbi.nlm.nih.gov/?term=organoid&sort=pubdate&sort_order=asc (last consulted on 20-01-2023).

2.2 The three main features of organoid development

The exponential use and characterization of organoids has called for a clear definition of factors that influence efficient organoid growth and establishment. To this end, three main features that affect the self-organization and specificities of the final organoid have recently been defined: the physical characteristics of the culture environment (1), endogenous and exogenous signals provided to the culture (2), and the starting cell type (3)[2].

1. Physical characteristics of the culture environment

In 1975, Rheinwald and Green co-cultured human keratinocytes and 3T3-fibroblasts to create a stratified squamous epithelium colony in a monolayer[37]. This was the first realisation of the need for special conditions necessary for this cultivation[37]. However, due to the focus of this study on cultivating cells on a 2D surface, the used conditions did not fully apply to the cultivation of cells into a 3D organization[2]. The cells of which a tissue is composed are not simply attached to each other, but are woven into a complex network[2]. This network, called the extracellular matrix (ECM), retains a level of water and the homeostatic balance of the tissue and it provides structural support[2].

To establish the 3D culturing of cells and create an organoid that recapitulates the structure and environment of the tissue in vivo, the ECM needs to be included, with all positional information[2,33,38]. Purification of natural ECM produced by Engelbreth-Holm-Swarm tumours in mice, leads to the formation of a gelatinous protein mixture that is commercialized as Matrigel®[2,7,12,30,39,40,41,42]. Matrigel® was first described in the mid 1980s and it is now the most widely used solid ECM for organoid development[2,7,12,30,39-42]. However, Matrigel® is not a well-defined matrix, due to its complex structure and variable composition[2,38,41]. As a result, it is difficult to fully control the culture environment, which can lead to a lower reproducibility of the organoids[2,38,41]. Matrigel® also contains animal-derived products, which provides a risk of immunogen and pathogen transfer when used for clinical applications[2,30]. Because of these limitations, different culturing strategies have been adopted, as illustrated in ***figure 3***[2,30,38]. These matrices consist of synthetic ECM-analogues that are composed of natural or non-natural polymers[2,30,38]. At this point, the choice of used ECM components mostly depends on experience and previous observations[2].

Matrix	Composition	Derivation	Applications
Matrigel®	collagen type IV, laminin, heparan sulfate proteoglycans, entactin/nidogen, and a number of growth factors	murine Englebreth-Holm-Swarm sarcoma tumors	3D cell culture
Collagen type I	collagen type I	rat tail	3D cell culture
HA hydrogel	hyaluronic acid	synthetic	3D cell culture; clinical applications
PEG hydrogel	polyethylene glycol (PEG) hydrogel supplemented with an RGD (Arg-Gly-Asp) peptide	synthetic	3D cell culture; clinical applications
PEG-4MAL hydrogel	PEG with maleimide groups at each terminus	synthetic	3D cell culture; clinical applications
Fibrin/laminin hydrogel	fibrin, laminin	synthetic	3D cell culture; clinical applications

***Figure 3*:** Most commonly used matrices to develop organoids. Matrigel® is the most often used matrix, but has also limitations, which is why other strategies have been developed in the form of synthetic matrices.
From: *Fiorini et al., 2020*

[2] Retrievable at: https://biologydictionary.net/extracellular-matrix/ (last consulted on 22-01-2023).

2. Endogenous and exogenous signals

To successfully develop and survive, the majority of the organoids needs both endogenous and exogenous signals to be present, including growth factors[2,7,30]. Yet, some organoids seem to rely only on endogenous signals and derive their necessary exogenous signals from the Matrigel® that is used in the culture[2]. Overall, different organoid types have their own unique culture media and alternation of this medium will automatically influence the organoid function[30].

3. Starting cell type

The self-organization of organoids depends on the starting cell type and the initial system conditions of the cell population[2]. To this end, starting cell types have been identified for organoid formation as illustrated in *figure 4*: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adult stem cells (ASCs) and cancer stem cells (CSCs)[2,7,43].

The first ESCs were discovered in mice in 1981[44]. However, the use of ESCs as starting cell type can be regarded as controversial[2,7,44]. ESCs need to be derived directly from blastocysts, which is associated with several ethical issues[2,7,44]. Moreover, it has been difficult to derive ESCs from large domesticated animal species, such as dogs[44].

As an alternative approach, Hans Clevers and his colleagues reported the use of ASCs as starting population for organoid development[12]. They established in 2009 the derivation of intestinal crypt-villus like organoids by using ASCs of the small intestine and colon from mice[12]. Moreover, the use of specific culture conditions allowed long-term culturing of these organoids for more than 8 months[12]. This is also the first report of ASCs that are marked by the Lgr5-gene (leucine-rich-repeat containing G-protein-coupled receptor 5)[12]. Lgr5, also known as Gpr49 (G-protein-coupled receptor 49), was later discovered to be a more general marker of stem cells and Lgr5-expressing stem cells proved to have a high capacity to generate organoids[12].

CSCs, a term often used interchangeably with tumour-initiating cells, represent a small subpopulation of stem cells that drive tumour growth and metastasis in vivo[7,33,45,46]. CSCs can be directly used from tumour resection or biopsies[7,33,45,46]. In addition, tumour cells can be created in vitro when inducing oncogenic mutations in healthy organoids by genetic modification[7,30].

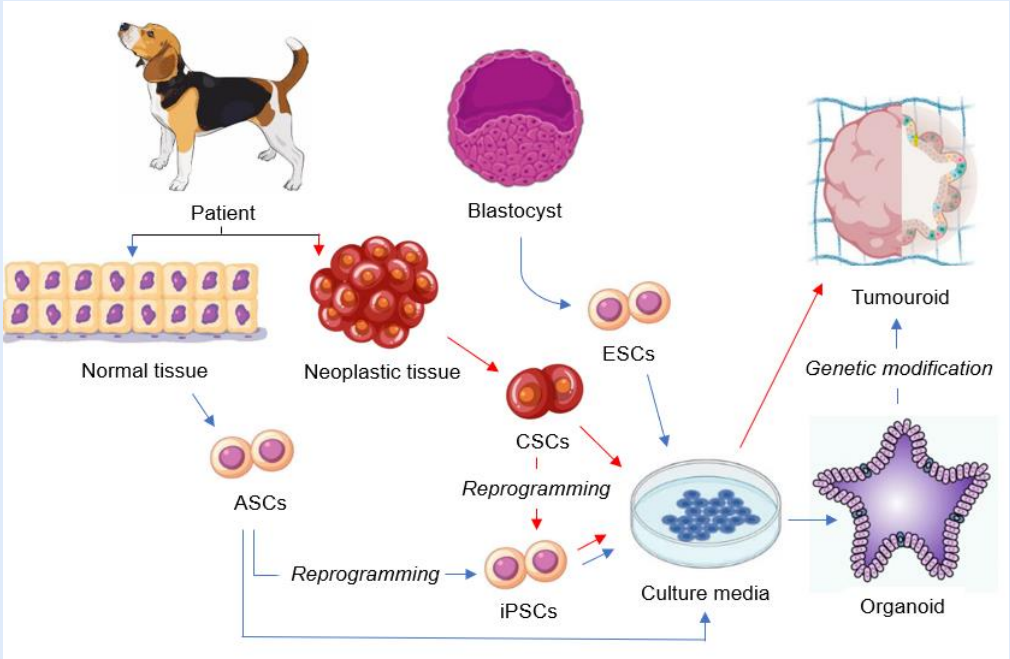


***Figure 4*:** Generation of organoids derived from different starting cell types. Organoids can be derived from adult stem cells (ASCs), induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), indicated by the blue arrows. Tumouroids are typically generated by using cancer stem cells (CSCs), or iPSCs from reprogrammed CSCs, indicated by the red arrows. Genetic modification of organoids can also induce oncogenic mutations, generating tumour cells in vitro.

Reprogramming ASCs or CSCs can further provide a more pluripotent stem cell type with a broader differentiation range, so called 'induced pluripotent stem cells' (iPSCs)[7,47]. Important transcription factors for this reprogramming include c-Myc, KLF4, Oct4 and Sox2[7,47].The first organoids created by iPSCs were organoids of the cortical tissues and the optic cup, shown in research performed by Sasai and his colleagues in respectively 2008 and 2011[13,48]. The technique to develop iPSCs was discovered in 2006 by S. Yamanaka and K. Takahashi in mice and one year later in human cells[7,47,49,50]. iPSCs can be seen as a ground breaking discovery as they provide an unlimited source of pluripotent stem cells that can be used for scientific purposes[44,47,49- 51]. Besides, they solve ethical difficulties by avoiding the endangering of the life of an embryo in the process of obtaining ESCs and they will not elicit an immune response due to their autologous nature[44,47,49-51].

The different starting cell types have also repercussions on the maturation characteristics of the final organoid, as shown in ***figure 5***[2]. ASC-derived organoids are useful for the study of adult tissue biology, because they phenocopy the homeostatic or regenerative conditions that can also be seen in the original tissue[1,2]. Pluripotent stem cell (PSC)-derived organoids are mainly used to study organogenesis and tissue formation[2,48,52,53]. However, they rarely reach the adult tissue stage due to the missing of crucial environmental factors in vitro[2,48,52,53]. Because of the limited reconstruction of organogenesis with classic PSC-derived organoids, self-organized PSC-derived structures creating embryonic organoid systems can be used, called 'embryoids'[2]. Embryoids consist of cells from multiple germ layers, as can be seen in real embryos[2]. Tumour cells or CSCs can be used in a similar way to generate tumour organoids, also called tumouroids[2,24,54,55,56,57]. Tumouroids are highly similar to the original tumour, considering that they have similar histological features and 74% of the proteins occur in both tumouroids and the original tumour[38,58]. By co-culturing the tumouroid with a variety of cell lines, such as immune cells, interactions between tumour cells and stromal cells can be observed[38,58].

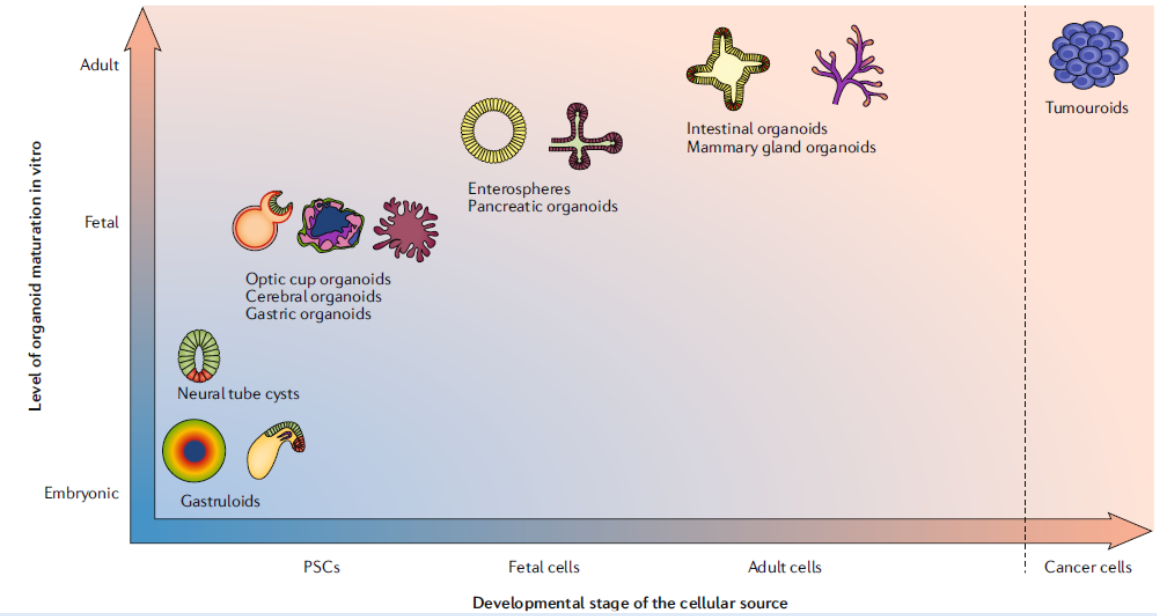


***Figure 5*:** The starting cell type determines the organoid maturation characteristics. The graphs show the level of organoid maturation in vitro (vertical axis) depending on the developmental stage of the starting cell type (horizontal axis), which are either PSCs (pluripotent stem cells), foetal cells, adult cells or cancer cells.
***From**: Rossi et al., (2018)*

3. Canine organoids for modelling human disease

3.1 Pros and cons of dog models

Before new drugs are allowed to be tested in human clinical trials, preclinical animal studies are required to test the safety and efficiency of the drug[59]. More than 95% of all used animal models are mice, due to their easy maintenance, financial accessibility, rapid reproduction, and standardization by age, sex, history and genetic predisposition[20,59,60,61]. However, mouse models also have disadvantages in translating results to humans, due to differences in their anatomy, physiology, environment and pathogenesis of diseases[2,20,60,62,63]. Dogs (*Canis lupus familiaris*) share similarities with humans in genetic, anatomical and physiological features, making them more appropriate translational models for human medicine[20,64,65,66]. Since their domestication 15.000-100.000 years ago, dogs have lived alongside humans[16,67]. This results in dogs and humans being exposed to analogous environmental and nutritional factors, which often makes them susceptible to similar diseases[20,68,69,70]. Companion dogs also benefit from high-quality medical care due to the increased appreciation of pets and the improved financial situation after the end of World War II, which increases the available data[16,68,70]. As a result of improved vaccination protocols, nutrition, diagnostic techniques and treatment options, dogs have an increased lifespan that ranges from 5.5-14.5 years, depending on their body mass[64,66,69,71]. This is also highly relevant for humans[64,66,69,71]. Moreover, dogs age seven to eight times faster than humans, which results in more efficient clinical trials and follow-up studies[20,29,66,69-71]. For example, in cancer research is the latency period between exposure to a carcinogenic substance and tumour development in dogs a lot shorter than in humans[20,29,66,69-71]. The canine genome has been sequenced in 2005[72]. Canine genetics can be used in research on genetic factors in diseases that are also common in humans[72]. The selective breeding of dogs has led to more genetically homogenous subpopulations, which are predisposed to certain diseases[16,68,70,72,73]. In addition, stem cell markers and their expression can also be translated to dogs[74,75,76,77,78].

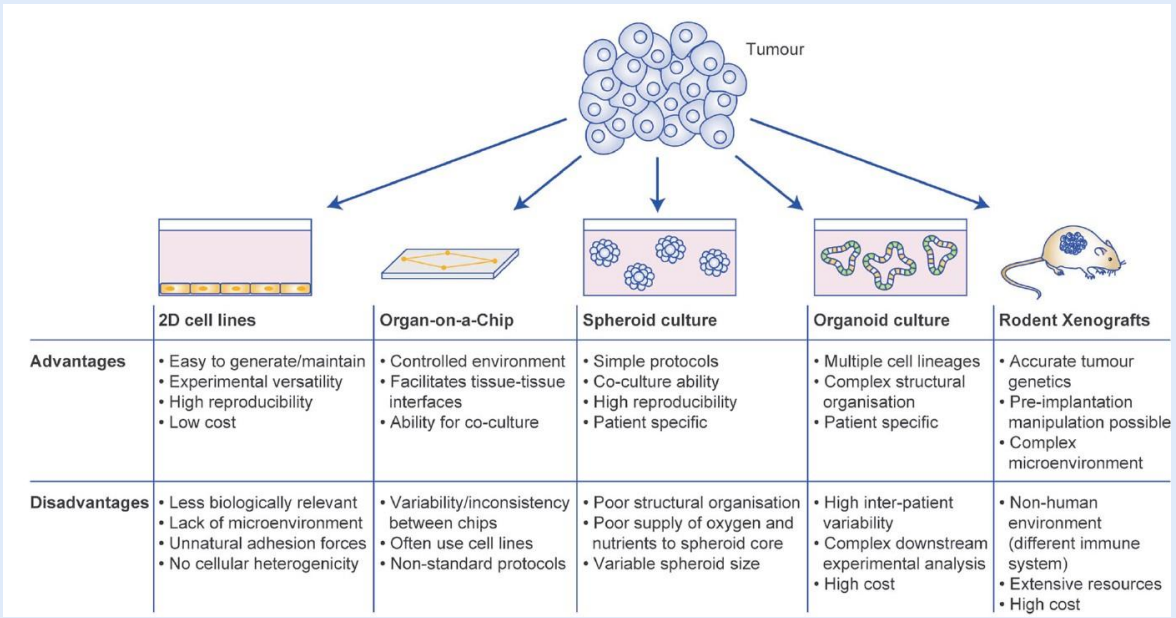
Cancer remains one of the most challenging and lethal diseases worldwide, in both human and veterinary medicine[70,72,79]. This is mainly because we do not yet fully understand the cellular and molecular mechanisms underlying the heterogeneity of cancer, including the acquired resistance, recurrence and metastasis[73,79,80,81]. Each year, about 6 million dogs are diagnosed with some type of cancer[72,82]. Moreover, more than 50% of dogs older than 10 years of age will develop cancer in their life[72,82]. Nowadays, owners are highly motivated to contribute to research for new treatment options for their pets, opening perspectives for further studies and also translation to human medicine[70,82]. One of the first studies of canine models as a translational model for human cancer, was performed in 1967[82,83]. In this study, companion dogs with non-Hodgkin's lymphoma (NHL) were treated by bone-marrow transplantation[82,83].

Despite the advantages, there are also limitations in using dog models, especially when they are considered pets[66,72]. Firstly, there are some ethical challenges, due to research requiring either invasive methods, genetic modification or sacrifice of the animal[66,84]. Secondly, clinical trials in dogs take longer than in mice and because dogs are larger in size, a higher dose of the drugs is necessary[72,82]. This will increase the costs and pharmaceutical companies may not be willing to sponsor clinical trials for these reasons[72,82]. Lastly, translation from an epidemiological point of view is limited, due to the differences in lifestyle[69,70].

3.2 Canine tumouroids as alternative for other model systems

One of the main obstacles in anti-cancer drug development and cancer research is the lack of appropriate preclinical model systems[30,58]. This results in only 4% of the discovered anti-cancer drugs reaching the clinical trial phase[30,58]. The model systems that are currently used in cancer research are illustrated in *figure 6*[30]. Recent literature highlights that the limitations of working with living dogs as disease models can be overcome through organoids[72]. In addition, high tissue availability and fewer ethical constraints are most noteworthy advantages of canine organoids compared to human tissue

biopsies[58]. This is especially of relevance in the field of oncology, where canine tumoroids come into place[58].



	2D cell lines	Organ-on-a-Chip	Spheroid culture	Organoid culture	Rodent Xenografts
Advantages	• Easy to generate/maintain • Experimental versatility • High reproducibility • Low cost	• Controlled environment • Facilitates tissue-tissue interfaces • Ability for co-culture	• Simple protocols • Co-culture ability • High reproducibility • Patient specific	• Multiple cell lineages • Complex structural organisation • Patient specific	• Accurate tumour genetics • Pre-implantation manipulation possible • Complex microenvironment
Disadvantages	• Less biologically relevant • Lack of microenvironment • Unnatural adhesion forces • No cellular heterogenicity	• Variability/inconsistency between chips • Often use cell lines • Non-standard protocols	• Poor structural organisation • Poor supply of oxygen and nutrients to spheroid core • Variable spheroid size	• High inter-patient variability • Complex downstream experimental analysis • High cost	• Non-human environment (different immune system) • Extensive resources • High cost

Figure 6: Overview of current model systems used in cancer research: 2D cell lines, organ-on-a-chip, spheroids, organoids and animal models, with their advantages and disadvantages.
From: *Porter et al., (2020)*

Overall, two major outcomes can be envisaged with canine tumouroids.

1. **Studying the biology of cancer: the emergence, growth, behaviour, metastasizing, reoccurrence and prognostic consequences of tumours**

Tumouroids are a valuable resource for the identification of cancer-associated genes and for studying tumour biology and progression[82]. Moreover, canine tumouroids can provide insights in factors that are responsible for tumour heterogeneity and for the loss of tissue organization that can be observed in carcinogenesis[7,30,85]. In fact, the heterogeneity of tumours is the main cause for differences in sensitivity to cancer therapies, making it an important aspect for therapeutic resistance[7,58,82,85]. Investigating tumour heterogeneity has been greatly facilitated by the possibility to establish canine tumouroids from cryopreserved cancer tissue[58]. These frozen tissue-derived tumouroids show more similarities to fresh tumouroids compared to cryopreserved tumouroids[58]. Tumouroids can also be used as a suitable model to investigate the cell-to-cell and cell-to-TME communications that contribute to tumour development[38]. Even when used in combination with an organ-on-a-chip technology, organoid cultures can be used to study metastasis and associated diagnostic markers, without the need for animal models[30,86].

2. **Enabling new treatment for cancer: the development and in vitro testing of anti-cancer therapy**

Current canine cancer options include surgery, chemotherapy, radiation therapy, hyperthermia, photodynamic therapy and immunotherapy[82]. At this point, no chemotherapeutic agents have been approved for cancer in dogs[82]. As a result, many human chemotherapy protocols are used off-label in veterinary medicine[82]. When developing anti-cancer drugs, a three-step process is followed[59,62,63.72,87]. This process consists of laboratory studies, preclinical trials on transplanted or generated tumours in mice, and finally human clinical trials that consist of three phases themselves[59,62,63,72,87]. Canine tumouroids can be used to test the safety, efficiency and pharmacokinetics of novel anti-cancer drugs[58,73,82]. Moreover, they can be used to evaluate whether a specific tumour type is sensitive to an already existing anti-cancer drug, before introducing the drugs in canine or human clinical trials[58,73,82]. Previous studies have already shown that cryopreservation of tumouroids in biobanks does not have an impact on the drug response[58,88]. Therefore, it provides options to compare several different anti-cancer

drugs at the same time[58,88]. Tumouroids can even be used in research concerning the patient response to immunotherapy, by co-culturing of the tumouroids with lymphocytes[30]. Organoids can also have a post-therapeutic effect in cancer, which is illustrated by studies from Schwartz et al., (2017) and Lenti et al., (2019), in which organoids are used to re-epithelialize areas that are affected by radiation therapy[89,90]. Similarly, transplantation of lympho-organoids after radiation therapy or lymph node dissection can be used to restore lymphatic drainage and reduce the risk of long term complications, such as lymphoedema[30].

Apart from their use to study cancer biology and to test novel anti-cancer treatments that can benefit both dogs and humans from a 'One Health' perspective, patient-derived canine tumouroids can be used to predict the clinical response to a specific drug[7,79]. Indeed, this approach contributes to the so-called 'personalized medicine' and allows to test different therapies at the same time to ascertain which treatment will be the most successful[7,30,79,91]. It is especially useful for tumours without any available biomarkers, or in rare or metastatic cancers that lack robust data[7,30].

4. Different organoid tissues in canine veterinary medicine

As shown in *figure 7*, both non-malignant tissue-derived organoids and tumour-derived tumouroids can be distinguished from published literature. In the following section, an overview will be provided for each canine organoid type. In addition, relevant publications will be described about the applied technique and culture conditions for organoid development, as well as downstream application.

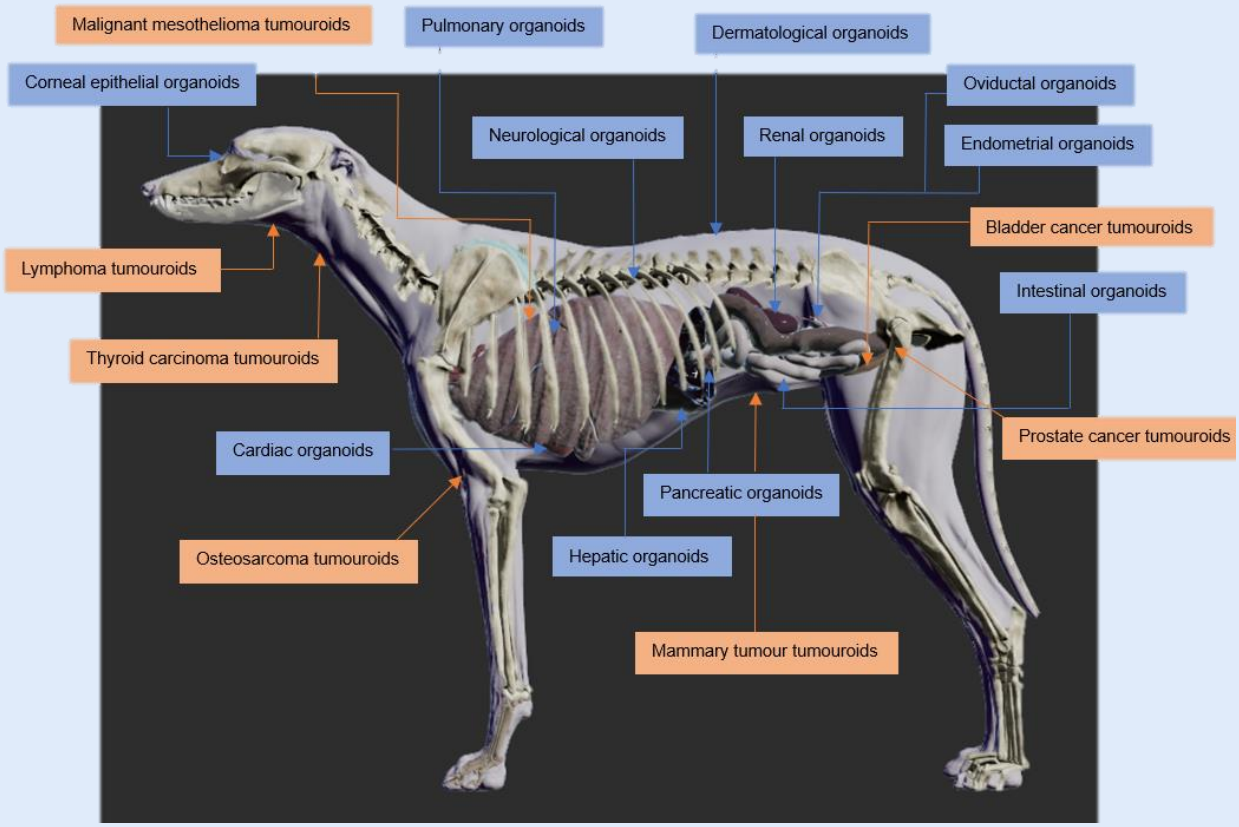


Figure 7: Schematic, fictional overview of the different canine organoids derived from non-malignant tissues, indicated by the blue text boxes, and canine tumouroids derived from tumour tissue, indicated by the orange text boxes.

For the generation of all these different organoids, the same three steps can be followed, as illustrated in *figure 8*: the tissue collection, the tissue preparation and cell isolation, and the culturation[86]. Tissue can be collected from living dogs, that undergo surgery or biopsy, or from euthanized dogs, preferably not for reasons related to the study of the organoid itself[92]. Both fresh and frozen tissues can be used[58]. The next step is microdissection and enzymatical digestion of the obtained tissue samples to isolate the desired cells[92, 93]. For the 3D cell culture, the cells are plated into a matrix and specific culture media and supplements are introduced for the different tissues[86]. Incubation of the culture occurs at 37°C and 5%CO_2 is used in order to maintain a stable physiological pH[94].

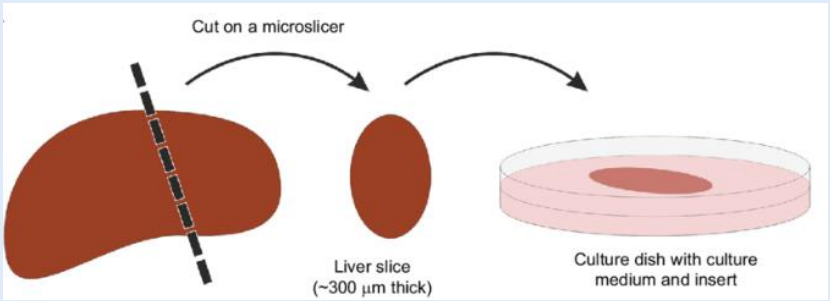


Figure 8: The three steps of organoid generation: tissue collection, tissue preparation and cell isolaton, and the culturation
From: *Kloker et al., (2018)*[158]

4.1 Canine organoids derived from non-malignant tissues

4.1.1 Canine dermatological organoids

The skin is the largest organ and it regulates the body's temperature[16,60,92,95]. As it is the body's first protective barrier, the skin is constantly exposed to external factors and susceptible to trauma[60]. Skin repair in mammals occurs through a variation of intra- and extracellular repair mechanisms, which will recover the homeostasis of the skin[60]. However, due to scar formation, full regeneration of skin is not possible in adult mammalian organisms[16,60,96]. The repair mechanisms and scar formation that occur in skin repair are very similar to those of the tissue repair that can be observed in myocardial infarction or spinal cord injuries[60]. To this end, the skin could be used as a translational model for studying tissue repair and the development of new techniques in regenerative medicine[60].

Keratinocytes are the main epidermal cell type[60,97]. They are responsible for generating epidermal compartments, including the intrafollicular epidermis (IFE) and adnexa, such as hair follicles (HFs) and sebaceous glands[60,97]. Hair loss (alopecia) is a common disease in dogs, which is likely caused by problems in the HF-stem cells[97].

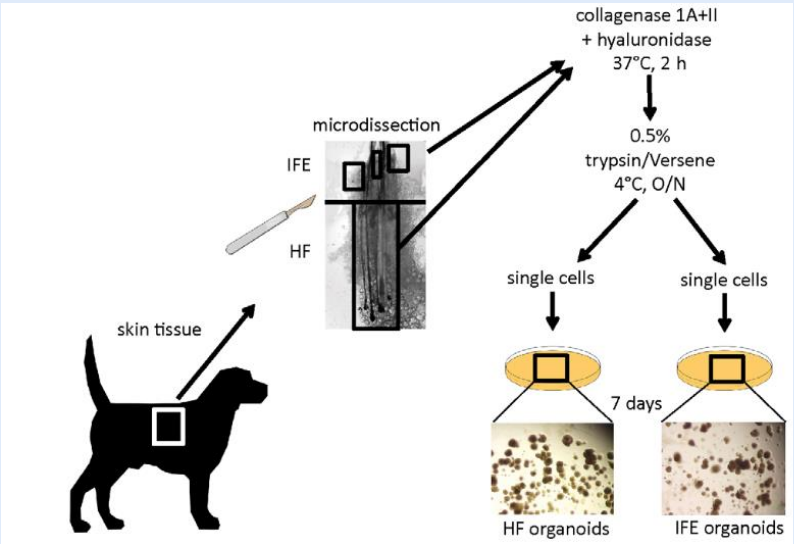


Figure 9: Schematic overview of the generation of keratinocyte organoids, including hair follicle (HF) organoids and intrafollicular epidermis (IFE) organoids. Canine skin tissue was first microdisected into HF and IFE cells and then enzymatically digested. The single cells were then seeded into Matrigel® and cultured for 7 days.
From: *Wiener et al., (2018)*

Representative in vitro models, such as canine dermatological organoids, are crucial to study the pathogenesis of disorders, such as alopecia or skin cancer[97]. Canine keratinocyte organoids were established in 2018 and can be used to evaluate the function, dysfunction and structure of the epidermal layer of the skin, as illustrated in *figure 9*[92]. Two studies on canine keratinocyte organoids are described in more detail in *table 1*. However, due to the lack of connective tissues, blood vessels and immune cells that are normally present, dermatological organoid systems still need further optimization to allow their clinical applications[95].

Table 1 : Culture conditions used in specific studies to generate canine dermatological organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Wiener et al., (2018)	Canine keratinocyte organoids as a reliable in vitro model to study defects in the IFE and HF morphogenesis, reconstitution and differentiation that leads to alopecia	Skin biopsies were taken from freshly euthanized dogs of 1-2 years old without skin abnormalities on their backs. The dogs were euthanized for other reasons than this study. Tissue was microdissected and enzymatically digested for 2 hours at 37℃. The	**Advanced Dulbecco's Modified Eagle Medium/ Ham's F-12 (DMEM/F12)**	Basic medium: • **Penicillin/streptomycin (PS)** (1%) • **Primocin** (5mM) • **N-2-hydroxyethyl piperazine-N2-ethane sulfonic acid (HEPES)** (10mmol/l) • **GlutaMax** (1%) • **B-27 supplement** (1x) • **N-acetylcysteine (NAC)** (1mM)

	and epidermal disease.	cells were then seeded into Matrigel®. Cells from IFE and HFs were cultured into keratinocyte organoids.		• **Noggin** (25%) • **R-spondin-1 (Rspo1)** (25%) • **Rho-associated coiled-coil containing protein kinase inhibitor (Y-27632)** (10µM) HF-organoid: • **Fibroblast growth factor (FGF)-2** (50µg/ml) • **FGF-10** (100µg/ml) • **Heparin solution** (0,2%) IFE-organoid: • **Epidermal growth factor (EGF)** (50ng/ml) • **Forskolin** (10µM) • **Tumour growth factor β type 1 receptor inhibitor (A83-01)** (5µM)
Wiener et al., (2021)	Optimization of the canine keratinocyte organoid system by checking mRNA expression of selected epidermal markers. The results showed expression differences with the use of expansion versus differentiation medium.	Skin tissue was obtained from the backs of recently euthanized dogs with no skin abnormalities. The dogs were euthanized for other reasons than this study. After tissue preparation, cells were plated into Matrigel® and cultured at 37°C. The cells were cultured by using expansion and differentiation medium to compare the effect of the culture medium on the generated keratinocyte organoids.	**Advanced DMEM/F12**	• **PS** (1%) • **Primocin** (5mM) • **HEPES** (10mmol/l) • **GlutaMax** (1%) • **B-27 supplement** (1x) • **NAC** (1mM) • **Noggin** (25%) • **Rspo1** (25%) • **Y-27632** (10µM)

4.1.2 Canine corneal epithelial organoids

Figure 10: Representative immages of canine corneal epithelial organoids at day 6, 10 and 25 after culturing of the cells. Scale bar: 100µm.
From: *Bedos et al., (2022)*

The corneal epithelium is the clear outermost layer of the cornea of the eye[98]. It consists of a stratified squamous non-keratinized epithelium[98]. The cells of the corneal epithelium have a high turn-over rate and new epithelial cells are constantly originating from limbal epithelial stem cells[98]. There are many corneal diseases, such as corneal blindness and keratitis that occur both in humans and dogs[16,99]. Corneal blindness is caused by a group of eye disorders that will change the transparency of the cornea[16,99]. In humans, it is mostly caused by infectious diseases and predisposed factors, such as trauma, the use of contact lenses or steroid medications[99]. One of the treatment options for corneal blindness is cornea transplantation, which comes with many ethical factors[99]. The first human corneal epithelial organoids, derived from iPSCs and ASCs, were respectively developed in 2017 and 2020[98,100,101,102]. At this point, there is only one publication about the generation of corneal epithelial organoids in dogs, established in 2022[16,98]. These canine corneal epithelial organoids are illustrated in *figure 10* and described in more detail in *table 2*.

Table 2: Culture conditions used in specific studies to generate canine corneal epithelial organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Bedos et al., (2022)	Isolation, culturing and characterization of canine corneal organoids. The expression of key markers of corneal epithelium and stromal cells was evaluated.	Corneal samples were derived from healthy corneal limbal epithelium. This tissue was obtained by performing bilateral transconjunctival enucleation that occurred after euthanasia, for reasons not related to this study. The prepared tissue cells were seeded into Matrigel® and the organoids were cultured at 37 ℃ with 5% CO_2. The medium was changed every other day.	**Advanced DMEM/F12**	• **Foetal bovine serum (FBS)** (8%) • **GlutaMax** (2mM) • **HEPES** (10mM) • **Primocin** (100µg/ml) • **B-27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **Murine EGF** (50ng/ml) • **Murine Noggin** (100ng/ml) • **Human Rspo1** (500ng/ml) • **Murine Wnt-3a** (100ng/ml) • **[Leu^{15}]-Gastrin I human** (10nM) • **Nicotinamide** (10mM) • **A83-01** (500nM) • **P38-inhibitor (SB202190)** (50µM) • **Trimethoprim sulphate (TMS)** (10µg/ml) • **FGF-2, FGF-7, FGF-10** • **Y-27632** • **Glycogen synthase kinase 3β (GSK3β)-inhibitor**

4.1.3 Canine intestinal organoids

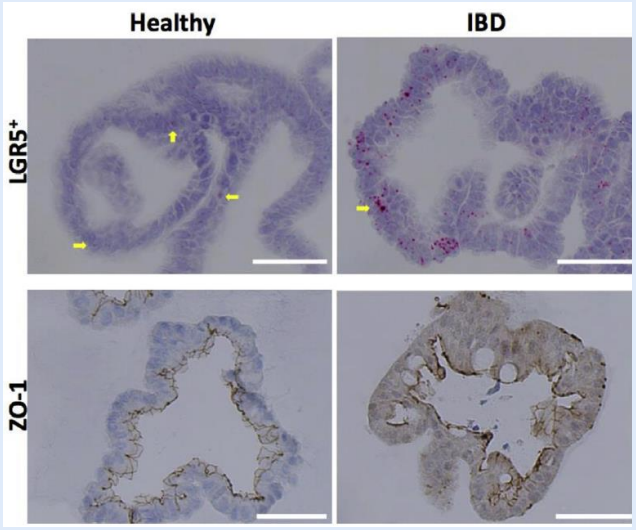


Figure 11: Organoids from dogs with IBD with staining of stem cells and tight-junctial proteins. RNA in situ hybridization is used to stain mRNA of intestinal stem cells (Lgr5+) and immunohistochemistry to stain tight-junctional zonulin protein (Zo-1). Increased Lgr5-expression and upregulation of Zo-1 can be observed in the PLE-organoids compared to the healty tissue.
***From**: Allenspach and Iennarella-Servantez, (2021).*

Canine models can be used to study the normal biology of the gastro-intestinal (GI)-tract[10,65,103,104]. In addition, they can be used for research concerning multiple GI-diseases, such as inflammatory bowel disease (IBD) and protein loosing enteropathy (PLE), as well as diseases caused by enteric pathogens and intestinal carcinomas[10,65,103,104]. The intestinal epithelium is the most rapidly self-renewing tissue, which results in an increased susceptibility to tumour development[12, 105]. Canine GI-models can also be used for translational purposes to human medicine, such as toxicology studies and host-pathogen interactions[65]. In terms of organoids, both human and canine intestinal organoids were established in 2009[12, 106] . The first canine intestinal organoids were derived from distal ileum and foetal intestines[106]. After 4 weeks, the canine intestinal organoids were allo- and autotransplanted to determine if intestinal stem cell organoid transplantation could be used to generated neomucosa in dogs[106].

Canine IBD organoids have also been described to further understand the complexity of the disease and to screen therapeutic drugs before they are used in clinical trials[107]. In fact, a study by Allenspach and Iennarella-Servantez (2021) found that an increased amount of Lgr5-expressing (Lgr5+) stem cells and increased tight-junctional expression of zonuline-1 (Zo-1) is characteristic for canine IBD organoids. As illustrated in *figure 11*, this increase cannot be observed in healthy epithelium, making these cells potential therapeutic targets[104]. Importantly, the tissue required for these organoids can be obtained minimal invasively by endoscopic biopsies, which improves the welfare of animals and improves experimental efficiency[107]. Many reports have already been published on canine intestinal organoids. Four of these reports are described in more detail in *table 3.*

Table 3: Culture conditions used in specific studies to generate canine intestinal organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Agopian et al., (2009)	First report of successful generation of intestinal neomucosa after the transplantation of intestinal stem cell organoids in a dog.	In a first group, tissue was obtained from the ileum of 2-months old Beagles and after generation, the intestinal organoids were autotransplanted. In a second group, the tissue was harvested from Beagle foetuses and the generated intestinal organoids were allotransplanted into 10-months old mother animals.	**DMEM**	• **D-sorbitol** (2%) • **FBS** (2.5%) • **PS** (penicillin 100IU/ml + streptomycin 100 IU/ml)
Chandra et al., (2019)	Canine GI organoids were derived from healthy tissue from the small intestine and colon, as well as from patients with IBD and intestinal carcinoma to study intestinal diseases in both humans and dogs.	ASCs isolated from canine intestine crypts from the small intestine and colon were obtained from healthy dogs and dogs with IBD. They also collected full-thickness biopsies of the proximal jejunum of healthy dogs 30 min after euthanasia. The cells were seeded into Matrigel® on a 24-well plate. The organoid culture conditions on the right were used. The plate was incubated at 37℃.	**Complete medium with intestinal stem cell growth factors (CMGF)**	• **B-27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **EGF** (50ng/ml) • **Noggin** (100ng/ml) • **Rspo1** (500ng/ml) • **Wnt-3a** (100ng/ml) • **Gastrin** (10nM) • **Nicotinamide** (10mM) • **A-8301** (5mM) • **SB202190** (50µM) • **FBS** (8%) • **Y-27632** (10µM) • **GSK3β-inhibitor** (2.5µM)
Ambrosini et al., (2020)[158]	Canine colon organoids are used to generate an intestinal epithelial monolayer. This monolayer could be used for investigating nutrient and drug absorption, for studying host-microbiome crosstalk and for toxicity testing.	Canine colon cells were obtained by colonoscopic biopsy. The tissue samples were cut into smaller pieces and incubated with a complete chelating solution and ethylenediaminetetraacetic acid (EDTA) at 4℃ for 60 min. The cells were then seeded into Matrigel® and culture medium (a complete medium with supplements shown on the right) on a 24-well plate. The culture was incubated at 37℃ and the culture medium was changed after two days with complete medium.	**Advanced DMEM/F12** (20%)	Culture medium: • **Y-27632** (10µM) • **GSK3β-inhibitor** (2,5µM) Complete medium: • **HEPES** (10mM) • **GlutaMax** (1x) • **PS** (100 units/ml penicillin + 100µg/ml streptomycin) • **Wnt-3a** (50%) • **Rspo1** (20%) • **Noggin** (10%) • **Murine EGF** (50ng/ml) • **SB202190** (30µM) • **A83-01** (500nM) • **Gastrin** (10nM) • **NAC** (1mM)

				• **Nicotinamide** (10mM) • **N2 supplement** (1x) • **B-27 supplement** (1x)
Kramer et al., (2020)[159]	Generating long-living and differentiating canine intestinal organoids derived from the duodenum, jejunum and colon to resemble the cellular diversity of the intestine.	Tissue samples were obtained from three dogs that were euthanized for other reasons than this study. Duodenal biopsies were taken from a dog that underwent gastroduodenal endoscopy. The tissue was incubated with 5mM EDTA and washed two times with phosphate buffered saline (PBS) and Advanced DMEM/F12. The cells were resuspended in Matrigel® and seeded on a 24-well plate. Culturing conditions for the expansion medium on the right were performed. The medium was changed every two-three days.	**Advanced DMEM/F12**	• **PS** • **HEPES** (10mM) • **GlutaMax** (2mM) • **B27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **Gastrin** (10nM) • **Nicotinamide** (10mM) • **EGF** (50ng/ml) • **A83-01** (500nM) • **SB202190** (10µM) • **Hepatocyte growth factor (HGF)** (50ng/ml) • **Human Noggin** (100ng/ml) • **Y-27632** • **Rspo1** (10%) • **Wnt-3a** (43%)

4.1.4 Canine hepatic organoids

Canine hepatic organoids can be used to study the biology and diseases of the liver, such as portosystemic shunts (PSS) and copper storage disease[108]. The first canine hepatic organoids were developed in a study of Nantasanti et al. in 2015 from dogs with Copper Metabolism Domain Containing 1 (COMMD1)-deficiency. The genetic defect that leads to this deficiency and subsequent hepatic copper accumulation could be restored by incorporating the missing gene through lentiviral transduction[108].

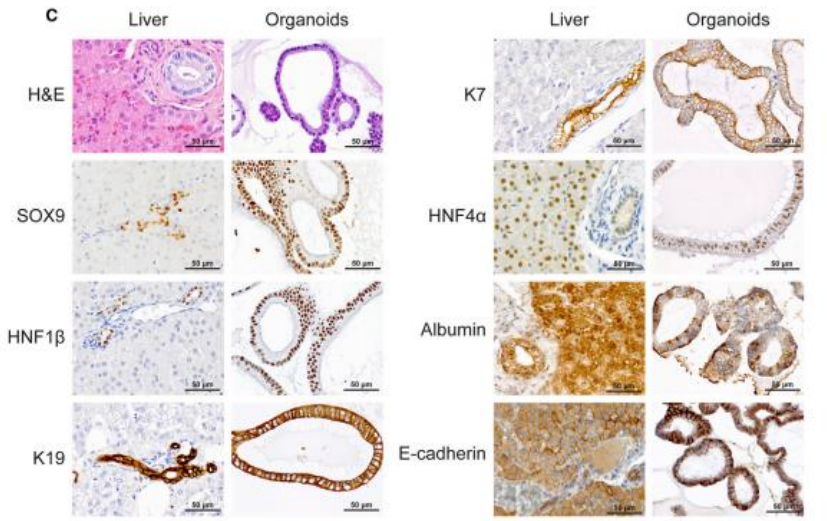


Figure 12: Canine hepatic organoids are compared to normal liver tissue. Immunohistochemical or cytochemical staining was used and showed that organoids were able to express cholangiocyte markers (SOX9, HNF1β, K19 and K7), hepatocyte markers (HNF4α and albumin), and epithelial markers (E-cadherin).
From: *Nantasanti et al., (2015)*

To evaluate the representativeness of canine hepatic organoids, immunohistochemical and cytochemical staining techniques were used on both canine hepatic organoids and liver tissue[108]. As a result, it was proven that canine hepatic organoids are able to express a spectrum of cellular markers that are also present in the liver, as indicated in *figure 12*[108]. These markers include cholangiocyte markers (SOX9, HNF1β, K19 and K7), hepatocyte markers (HNF4α and albumin) and epithelial markers (E-cadherin).

A second study focussed organoids derived from dogs with COMMD1-deficiency as a model for copper storage disease in humans, also called 'Wilson's disease'[108]. The shortage in donor organs associated with liver transplantation limits current treatments in human liver disease[109]. Therefore, the use of liver cell transplantation could provide a less invasive alternative [109]. The study showed that after generating hepatic organoids from dogs with COMMD1-deficiency, the genetic defect could be corrected and the cells could safely be transplanted back into the dog, as illustrated in *figure 13*[109].

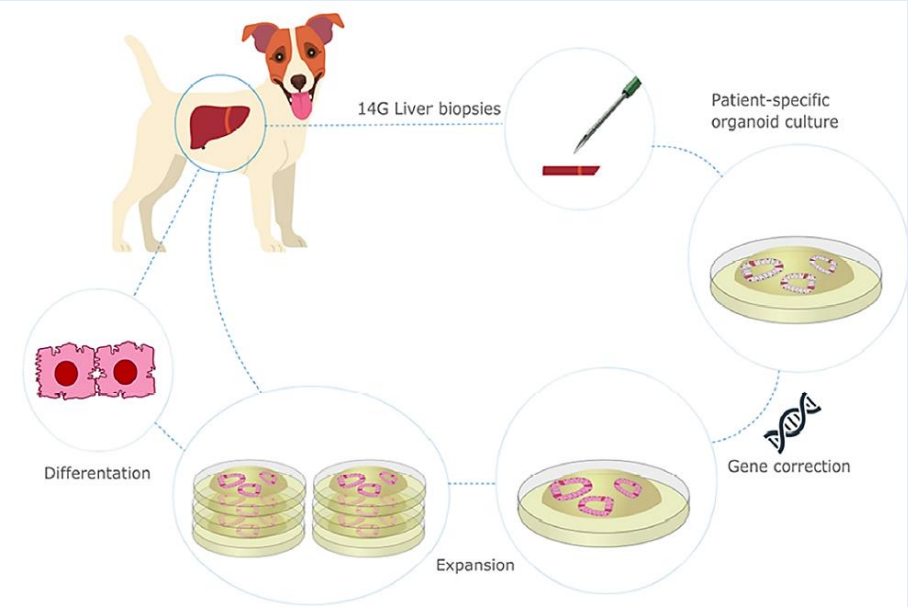


Figure 13: Schematic overview of the establishment, genetic correction and expansion of canine hepatic organoids from dogs with copper metabolism domain containing 1 (COMMD1)-deficiency that were then used for autologous transplantation via the portal vein.
From: *Kruitwagen et al., (2020)*

A third study by Van den Bossche et al., (2017) describes canine hepatic organoids of dogs with PSS to further understand hepatic lipid accumulation[110]. This could provide insight in non-alcoholic fatty liver disease that is the most common liver disorder in humans[110].

Four reports on canine hepatic organoids and their culture conditions are described in *table 4*.

Table 4: Culture conditions used in specific studies to generate canine hepatic organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Nantasanti et al., (2015)	First time canine hepatic organoids were developed. It is demonstrated that gene supplementations in these organoids from COMMD1-deficient dogs, restores the hepatocyte function. This opens possibilities to cure copper storage disease.	Canine hepatic organoids are derived from COMMD1-deficient dogs. Cultures can be initiated from fresh and frozen liver tissues using Tru-cut or fine needle biopsies. After mechanical dissection, the tissue was enzymatically digested at 37℃ for 3-5 hours. The cells were mixed with Matrigel® and cultured.	**Advanced DMEM/F12**	• **B-27 supplement** (1%) • **N2 supplement** (1%) • **NAC** (1.25 mM) • **Gastrin** (10nM) • **EGF** (200ng/ml) • **Rspo1** (5%) • **FGF-10** (100ng/ml) • **Nicotinamide** (10mM) • **HGF** (25ng/ml) • **Noggin** (100ng/ml) • **Wnt-3a** (30%) • **Y-27632** (10µM) • **A-8301** (0,5µM)
Van den Bossche et al., (2017)	Comparing the lipid accumulation in dogs with extrahepatic and intrahepatic PSS to healthy control dogs, using LD540. This gained insight into	Liver tissues were obtained from fresh wedge liver biopsies of pet dogs with PSS. Liver tissue from healthy dogs, that were euthanized for other reasons than this study, was used as	**Advanced DMEM/F12**	• **B-27 supplement** (2%) • **N2 supplement** (1%) • **NAC** (1,25µM) • **Gastrin** (10nM) • **EGF** (200ng/ml) • **Rspo1** (5%)

	the pathogenesis of steatosis that can be observed in dogs with PSS. The study showed that steatosis probably occurs secondary to the PSS.	a control group. Liver tissue was dissected and enzymatically digested in DMEM medium with 1% FBS at 37℃ for 3-5 hours. The tissue was then mixed with Matrigel® and culture medium was added, described on the right. The organoids were grown at 37℃ with 5% CO_2.		• **FGF-10** (100ng/ml) • **Nicotinamide** (10mM) • **HGF** (25ng/ml) • **Noggin** (100ng/ml) • **Wnt-3a** (30%) • **Y-27632** (10µM) • **A-8301** (0,5µM)
Wu et al., (2019)	Canine hepatic organoids of dogs with COMMD1-deficiency can serve as a model for Wilson's disease. There is a reduced activity of Farnesoid X nuclear receptor (FXR) in dogs with COMMD1-deficiency resulting in reduced FXR-target gene expression.	Canine hepatic organoids are developed from COMMD1-deficient dogs. Cultures can be initiated from fresh and frozen liver tissues using Tru-cut or fine needle biopsies. After mechanical dissection of the tissues, the tissue was enzymatically digested in DMEM with 1%FBS at 37℃ for 3-5 hours. The cells were mixed with Matrigel® and the culture medium described on the right was added.	**Advanced DMEM/F12**	• **B-27 supplement** (1%) • **N2 supplement** (1%) • **NAC** (1.25 mM) • **Gastrin** (10nM) • **EGF** (200ng/ml) • **Rspo1** (5%) • **FGF-10** (100ng/ml) • **Nicotinamide** (10mM) • **HGF** (25ng/ml) • **Noggin** (100ng/ml) • **Wnt-3a** (30%) • **Y-27632** (10µM) • **A-8301** (0,5µM)
Kruitwagen et al., (2020)	Transplantation of genetic restored canine hepatic organoids, originally derived from dogs with COMMD1-deficency.	The hepatic progenitors of five dogs with COMMD1-deficiency were isolated after Tru-cut or fine needle biopsy. After mechanical dissection of the tissues, the tissue was enzymatically digested in DMEM with 1%FBS at 37℃ for 3-5 hours. The cells were mixed with Matrigel® and the culture medium described on the right was added. The genetic defect was corrected by using a lentivirus. The cells were then transplanted back into the liver of the dog via the portal vein or via intrahepatic injection.	**Advanced DMEM/F12**	• **B-27 supplement** (1%) • **N2 supplement** (1%) • **NAC** (1.25 mM) • **Gastrin** (10nM) • **EGF** (200ng/ml) • **Rspo1** (5%) • **FGF-10** (100ng/ml) • **Nicotinamide** (10mM) • **HGF** (25ng/ml) • **Noggin** (100ng/ml) • **Wnt-3a** (30%) • **Y-27632** (10µM) • **A-8301** (0,5µM)

4.1.5 Canine pancreatic organoids

Canine pancreatic organoids can be used to study pancreatitis, the most common disease of the exocrine pancreas of dogs, and for the disease modelling of pancreatic cancer, which is one of the most lethal cancers in humans[84,111]. In human medicine, pancreatic organoids have already been established from healthy and diseased tissue parts[112]. However, adult human pancreatic organoids are difficult to establish and culture[111]. At this point, there has only been one report of canine pancreatic organoids, described by a study of Zdyrski et al. in 2022, as illustrated in *table 5*.

Table 5 : Culture conditions used in specific studies to generate canine pancreatic organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Zdyrski et al., (2022)[84]	First report of canine pancreatic organoids. There was upregulation of multiple genes, including Dual oxidase 2, Cadherine 17 and Early Growth Response 1.	6 organs, including the pancreas, were obtained from 2 female dogs of 4 weeks old that were euthanized. Biopsies were washed three times with 10ml Complete Chelating Solution and then transferred to 6ml of Advanced DMEM/F12. The cells were plated into Matrigel®. The growth media on the right was used. Isolation, expansion and differentiation of the different organoids took between 17 and 31 days.	**Advanced DMEM/F12**	• **FBS** (8%) • **GlutaMax** (2mM) • **HEPES** (10mM) • **Primocin** (100ng/ml) • **B-27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **Murine EGF** (50ng/ml) • **Murine Noggin** (100ng/ml) • **Human Rspo1** (500ng/ml) • **Murine Wnt-3a** (100ng/ml) • **[Leu15]-Gastrin I human** (10nM) • **Nicotinamide** (10nM) • **A83-01** (500nM) • **SB202190** (10uM) • **SMZ-TMP DS** (10µg/ml) Additional: • **Y-27632** (10uM) • **GSK3β-inhibitor** (2.5uM)

4.1.6 Canine renal organoids

Chronic kidney disease (CKD), which means the presence of functional or structural changes in one or both kidneys for a longer period than three months, is an irreversible and progressive disease that will eventually lead to end-stage renal disease (ESRD)[112]. CKD is the most common kidney disease in small animals and the incidence of humans with ESRD is globally increasing[94,112]. Current therapies for human patients with ESRD include dialysis and kidney transplantation[94]. However, these therapies come with shortcomings, such as low quality of life and the shortage of organ donors[94]. Treatment of CKD in dogs is mostly based on diet changes to reduce serum phosphorus concentrations, monitoring, and drugs, such as angiotensin-converting enzyme inhibitors[113]. Kidney organoids could be used for further drug screening and even for regenerative medicine[114]. At this point, there is only one publication on canine renal organoids by Chen et al. in 2019, where adult kidney epithelial stem cells were cultured and gave rise to dome-forming tubular cells in vitro, as illustrated in *table 6*[16].

Table 6: Culture conditions used in specific studies to generate canine renal organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Chen et al., (2019)	Canine adult kidney epithelial cells give rise to dome-forming tubular organoids. The canine kidney epithelial cells expressed stem cell markers and they were capable of multilineage differentiating	Excision of a whole canine kidney was performed in dogs that were euthanized for different reasons than this study. It was washed with PBS, minced into smaller pieces, washed again with PBS and then plated into Matrigel® on 0,5ml/plates. The medium was daily or every other day refreshed, depending on the stage of the culturing.	**K-NAC medium (0,5 ml)**	• **Recombinant epithelial growth factor** (5ng/ml) • **Bovine pituitary extract** (50µg/ml) • **Insulin** (5µg/ml) • **Hydrocortisone** (74ng/ml) • **T_3** (6.7ng/ml) • **Nicotinamide** (5mM) • **FBS** (10%)

4.1.7 Canine pulmonary organoids

The lungs are a vulnerable organ, because they are continuously exposed to environmental factors[115]. Dogs can be used as a model for respiratory disorders, including asthma, bronchitis, pneumonia, pulmonary fibrosis, lung cancer and chronic obstructive pulmonary disease (COPD)[116]. COPD is a progressive chronic airway inflammation with a high morbidity and mortality in humans and there is no effective treatment[116,117]. Human pulmonary organoids are already established[118]. However, there has been only one report on canine pulmonary organoids by Zdyrski et al., (2022) that consisted of alveolar type-2 cells and bronchial epithelial cells, as illustrated in *table 7*[84].

Table 7: Culture conditions used in specific studies to generate canine pulmonary organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Zdyrski et al., (2022)	First establishment of canine lung organoids. There was upregulation of the lung marker NK2 homeobox 1 and expression of Surfactant Protein B and C genes.	6 organs, including the lungs, were obtained from 2 female dogs of 4 weeks old that were euthanized. Biopsies were washed three times with 10ml Complete Chelating Solution and then transferred to 6ml of Advanced DMEM/F12. The cell were plated into Matrigel®. The growth media on the right was used. Isolation, expansion and differentiation of the different organoids took between 17 and 31 days.	**Advanced DMEM/F12**	• **FBS** (8%) • **GlutaMax** (2mM) • **HEPES** (10mM) • **Primocin** (100ng/ml) • **B-27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **Murine EGF** (50ng/ml) • **Murine Noggin** (100ng/ml) • **Human Rspo1** (500ng/ml) • **Murine Wnt-3a** (100ng/ml) • **[Leu15]-Gastrin I human** (10nM) • **Nicotinamide** (10nM) • **A83-01** (500nM) • **SB202190** (10uM) • **SMZ-TMP DS** (10µg/ml) • **Y-27632** (10uM) • **GSK3β-inhibitor** (2.5uM)

4.1.8 Canine cardiac organoids

The heart is the main organ of the circulatory system[119]. The heart has numerous functions, such as providing a sufficient blood pressure to transport blood to all parts of the body, supplying them with oxygen and nutrients and removing metabolites[119]. Cardiac development, structure and diseases are complex, indicating the difficulty to study the heart and the need for new research strategies[119]. Cardiac diseases, such as myocardial infarction, congestive heart failure (CHF), genetic heart diseases and arrhythmias, occur in both human and veterinary medicine[16,93,120,121]. Little treatment options are available apart from heart transplantation, which is often compromised by donor shortage[120]. Cardiovascular diseases are currently the main cause of death in humans[119]. In dogs, more than 50% of Doberman Pinchers in Europe are diagnosed with dilated cardiomyopathy (DCM) and once CHF occurs, only palliative and symptomatic treatment is available[93,122]. The development of cardiac organoids is quite recent[120,123]. The first notification of a human cardiac organoid mimicking the early heart development is described by a study of 2015[119,123]. However, heart-forming human cardiac organoids from ESCs or iPSCs have just been developed since 2021 and are still immature[119,124]. At this point, all available organoid models are based on humans[119,124]. Canine cardiac organoids are only mentioned in using cardiosphere-derived cells (CDCs) that were cultured as 3D organoids, creating a heterogenous population of cardiac cells, as described in *table 8*[120]. In a study by Hensley et al., (2015), acute treatment with canine CDCs that were intravenously injected in mice with doxorubicin-induced DCM showed reduced fibrous tissue formation and cardiac angiogenetic improvement[16,93].

Table 8: Culture conditions used in specific studies to generate canine cardiac organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Hensley et al., (2015)	Canine CDCs are used to be cultured as 3D organoids. Canine CDCs were then injected intravenously in mouse models with DCM to demonstrate the regenerative potential of CDCs	CDCs were obtained from the myocardial tissue of healthy heart dog donors. The tissue samples were washed with PBS, microdissected and washed again with PBS for 3 times. The process was followed by enzymatic digestion at 37 °C in collagenase solution for 5 minutes. IMDM was added with 20% FBS to inactivate the collagenase. The tissue sample was further minced in pieces of 0,5x0,5 mm before plating on fibronectin coated plates into Matrigel® with the culture condition described on the right. For the maintenance of the culture, the media had to be changed every other day.	**Iscove's Modified Dulbecco's Medium (IMDM*)***	• **FBS** (20%)

4.1.9 Canine neurological organoids

The brain is a highly complex organ and phenocopying brain development remains a major challenge in neuroscience[125]. At this point, cerebral organoids can only be created from primate and rodent species[125]. Because brain biopsies are not performed routinely, most brain organoids are based on iPSCs that are then differentiated into mini brain-structured organoids[16]. Cerebral organoids derived from tissues of large domestic animals, such as dogs, could provide new insights in both veterinary and human research[125]. Indeed, dogs are also susceptible for brain diseases, such as cognitive deficits that show similarities to Alzheimer disease in humans, and dogs can also develop brain tumours[125]. Because the initial cellular and molecular mechanisms in brain development are similar in different mammalian species, it could be hypothesized that the protocols to develop human cerebral organoids, can also be used for the generation of canine cerebral organoids[125].

In veterinary medicine, the use of canine neurological organoids can be traced back to two publications that are both focussing on neurogenesis[16]. More specifically, the publication by Lowe et al., (2015) showed that organoids could be derived from adult canine hippocampal neural precursors[126]. These organoids provided evidence that hippocampal neurogenesis is depending on dorsal or ventral location[126]. The publication of Santos et al., (2021) described a protocol to isolate, culture and characterise neural stem cells (NSCs) and neural progenitor cells (NPCs) from the foetal canine spinal cord[127]. Both reports on these canine neurological organoids are described in *table 9.*

Table 9: Culture conditions used in specific studies to generate canine neurological organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Lowe et al., (2015)	First evidence that the amount of adult canine hippocampal neurogenesis is depending on the dorsal or ventral location. The dorsal hippocampus of dogs has a markedly higher rate of adult neurogenesis than the ventral part.	Canine hippocampal neural precursors were obtained from five dogs four hours post-mortem. The tissue was digested with 0,1% Trypsin EDTA, which was then halted by DMEM with 10% FBS. The cells were plated into collagen. The organoid cultures were incubated at 37 ℃ with 5% CO_2	**NeuroCult NSC basal medium (rat)**	• **Rat NSC proliferation supplements** • **FBS** (2%) • **Heparin** (2µg/ml) • **EGF** (20ng/ml) • **FGF** (10ng/ml)

Santos et al., (2021)	A protocol was established for the isolation, culture and characterization of NSCs and NPCs that are derived from the spinal cord of canine foetuses.	NSCs and NPCs were isolated from spinal cord fragments of canine foetuses at approximately 40d of gestation. The tissue samples were washed with PBS and supplemented with 2% PS and 1% amphotericin B. Enzymatic digestion was performed with 0,25% TrypLE Express, which was halted with DMEM supplemented with 15% FBS. The organoids were cultured at 37℃ with 5% CO_2. The medium was changed every 4 days.	**DMEM/F12**	• **B-27 supplement** (2%) • **L-glutamine** (1%) • **PS** (1%) • **Non-essential amino acids** (1%) • **EGF** (20ng/ml) • **FGF-2** (20ng/ml)

4.1.10 Canine endometrial organoids

The endometrium is important in canine veterinary medicine, because it is frequently affected by diseases, including pyometra and cystic endometrial hyperplasia[128,129]. Dogs can also be used as a model for endometriosis in humans, a chronic disease in which tissue similar to the lining of the uterus grows outside the uterus[3,130]. The first canine endometrial organoids were described as models of the uterine glands by a study of Stadler et al. in 2009. A second study was performed by Zdyrski et al. in 2022. Both reports on canine endometrial organoids are described in *table 10.*

Table 10 : Culture conditions used in specific studies to generate canine endometrial organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Stadler et al., (2009)	Establishment of the first canine endometrial organoids that model the uterine glands.	Tissue was obtained during routine ovariohysterectomy. Samples were taken from three different areas of the uterus. The tissue was microdissected and washed three times with PBS. The cells were plated into different ECMs such as Matrigel® and incubated in collagenase type A at 37℃ containing 5% CO_2	**DMEM**	• **FBS** (10%) • **PS** (10.000U/ml) • **Nystatin** (15ml/l) • **L-glutamine** (2ml/l)
Zdyrski et al., (2022)	Canine endometrial organoids show upregulation of multiple genes, including Dual oxidase 2, Cadherine 17 and Early Growth Response 1.	Six organs, including the endometrium, were obtained from 2 female dogs of 4 weeks old that were euthanized. Biopsies were washed 3x with 10ml Complete Chelating Solution and then transferred to 6ml of Advanced DMEM/F12. The cells were plated into	**Advanced DMEM/F12**	• **FBS** (8%) • **GlutaMax** (2mM) • **HEPES** (10mM) • **Primocin** (100ng/ml) • **B-27 supplement** (1x) • **N2 supplement** (1x) • **NAC** (1mM) • **Murine EGF** (50ng/ml) • **Murine Noggin** (100ng/ml)

[3] Retrievable at: https://www.who.int/news-room/fact-sheets/detail/endometriosis (last consulted on 12-5-2022).

		Matrigel®. The growth media on the right was used. Isolation, expansion and differentiation of the different organoids took between 17 and 31 days.		• **Human Rspo1** (500ng/ml) • **Murine Wnt-3a** (100ng/ml) • **[Leu15]-Gastrin I human** (10nM) • **Nicotinamide** (10nM) • **A83-01** (500nM) • **SB202190** (10uM) • **SMZ-TMP DS** (10µg/ml) Additional: • **Y-27632** (10uM) • **GSK3β-inhibitor** (2.5uM)

4.1.11 Canine oviductal organoids

The oviduct is an essential and complex component of the reproductive system that connects the ovary to the uterus[131]. Oviductal epithelium (OE) organoids can be used for personalized medicine and to investigate fertilization and pathologies[131]. However, because there are many inter-species differences between the physiology of the oviduct, the applicability of OE organoids from one species to another is limited[131]. In a study by Lawson et al. in 2023 canine OE organoids were established, in comparison to other domesticated animals, as described in *table 11*. This study resulted in porcine, equine and bovine oviduct-derived organoids showing more resemblance to human oviducts than the OE organoids of dogs, cats and mice[131].

Table 11 : Culture conditions used in specific studies to generate canine oviductal organoids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Lawson et al., (2023)	OE organoids were established from five domesticated species, including dogs, to compare the differences in organoid development.	Complete oviducts were obtained from dogs that underwent hysterectomy. After collecting the tissues, it was washed with PBS and 1%PS. Multiple fringes were dissected from the infundibulum and enzymatically digested. After epithelial cell enrichment, the cells were plated into Matrigel®. The OE organoid culture was used and the medium was changed every 3 days. After 21 days the organoids were harvested.	**DMEM/F12**	• **Glutamax** (1%) • **HEPES** (1%) • **PS** (1%) • **Wnt3a, Rspo3, Noggin conditioned medium** • **Human EGF** (12ng/ml) • **B-27 supplement** (2%) • **Y-27632** (5mM) • **NAC** (1.25mM/ml) • **Nicotinamide** (10mM/ml) • **Primocin** (0,2%) • **A83-01** (0,5mM)

4.2 Canine tumouroids

4.2.1 Canine mammary tumour tumouroids

Canine mammary tumours (CMTs) are the most diagnosed type of neoplasia in female dogs, accounting for around 50% of all cancers and 82% of all reproductive occurring tumours[29,58,132,133,134,135,136,137]. In comparison, human breast cancer (HBC) is the most frequently occurring and deadly cancer in women worldwide, accounting for 25% of all cancers[137]. Older female intact dogs, around 6-7 years old, are mostly affected and 25% of unspayed female dogs will develop CMTs in their lifetime[133,137]. CMTs can be used as a translational model for oncological research for HBC, due to many similarities in the age at onset, predominance of carcinomas, environmental risk factors, histological features, prognostic

factors, the metastatic pattern, and molecular and genetic characteristics[29,135136-137]. Both CMTs and HBC are also often hormone dependent and express oestrogen (ER+), progesterone and HER2 receptors[58,132]. Currently available genetically engineered mouse models lack to mimic ER+ HBC, which is why ER+ CMTs represent a valuable model[137]. Besides, CMTs occur spontaneously in dogs and it is possible to derive tumouroid lines from different epithelial origins from the same dog, which is not possible in humans[137]. One limitation of the use of CMTs in translational medicine is the decreased incidence of CMTs over the years due to early preventive ovariectomies in dogs[72,133,135]. One major difference between CMTs and HBC is that chemotherapy is not commonly used in the treatment of CMTs[58]. However, some studies have shown that CMTs can be sensitive to certain similar chemotherapeutics as in HBC, such as paclitaxel[58]. The first CMT tumouroids were established in 2017[138]. Current research focusses on further development of these tumouroids that show even more resemblance to the primary tumour, by including stromal components, such as immune cells, as illustrated in *table 12*[58,138]. Expression of specific biomarkers, such as CK18 and CK14 confirm that these tumouroids have indeed a similar phenotype to CMTs in vivo[16].

Table 12: Culture conditions used in specific studies to generate canine mammary tumour tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Cocola et al., (2017)	Using different culture conditions, FGF-2 and EGF were found to have an response on CSCs to regulate organoid formation	CMT tissues were obtained freshly after mastectomy from female dogs with spontaneous mammary tumours. Also, non-tumoural tissues were isolated from the same dog. After mechanical dissection and enzymatic digestion with 0,25% trypsin and 0,53mM EDTA for 15 min at 37℃, the cells were grown for 7-10 days. The cells were plated in Growth Factor Reduced Matrigel® on 48-well plates. Complete medium was added. Half of the medium was changed every 2,5 days. After 10-15 days, the cultures were microscopically examined.	**DMEM/F12**	• **GlutaMax** • **FBS** (5%) • **Serum replacement (SR)** (10%) • **Minimal Essential Medium (MEM)** (1%) • **Hydrocortisone** (0,5µg/ml) • **Insulin** (1µg/ml) • **FGF-2** (20ng/ml) • **EGF** (20ng/ml) • **PS** (1%)
Inglebert et al., (2022)	Organoids derived from CMTs as a model for translational carcinogenesis and in vitro drug screening. They can also be used to investigate whether specific tumour mutations predict therapy outcomes by developing a biobank of CMT-organoids.	Spontaneous CMT tissues and normal mammary tissue was obtained from client-owned dogs that underwent surgery. The tissue was then dissected into 1-2 mm^3 pieces and cryopreserved in a freezing medium. The cells were then plated in pre-warmed 24-well plates into Cultrex PathClear Reduced Growth Factor Basement Membrane Extract Type 2.	**Advanced DMEM/F12**	• **GlutaMax** (1x) • **HEPES** (10mM) • **PS** (50 U/ml) • **Primocin** (50mg/ml) • **R-Spondin-3 (Rspo3)** (10%) • **Noggin** (2%) • **Neuregulin-1** (5nM) • **FGF-7** (5ng/ml) • **FGF-10** (20ng/ml) • **EGF** (0,5ng/ml) • **A83-01** (500nM) • **Y-27632** (5mM) • **SB202190** (1mM) • **B-27 supplement** (1x) • **NAC** (1,25mM) • **Nicotinamide** (10mM)

Raffo-Romero et al., (2023)	First study in which tumouroids are created from heterogeneous CMT tissues to develop a tumouroid biobank. The histological and molecular impact of cryopreservation is compared.	CMTs were obtained in dogs with primary mammary tumours that underwent surgery. Fresh tissue was mechanically and enzymatically dissociated for 2 hours at 37℃ and plated in Matrigel® on 24-well plates, overlaid with optimized mammary tumouroids culture medium.	**Advanced DMEM**	• **GlutaMax** (1x) • **HEPES** (10mM) • **PS** (1x) • **Amphotericin** (1x) • **Primocin** (50µg/ml) • **B-27 supplement (**1x) • **Nicotinamide** (5mM) • **NAC** (1,25mM) • **Rspo1** (250ng/ml) • **Heregulinβ-1** (5nM) • **Noggin** (100ng/ml) • **FGF-10** (20ng/ml) • **FGF-7** (5ng//ml) • **EGF** (5ng/ml) • **A83-01**(500nM) • **SB202190** (500nM) • **Y-27632** (5µM)

4.2.2 Canine lymphoma tumouroids

Malignant lymphoma is the most common hematopoietic cancer in dogs and despite aggressive treatment, relapse frequently occurs and lymphoma is in the top five of cancer related deaths, similar to human lymphoma[68,69,139]. Within the different types of lymphoma that can be observed in dogs, canine non-Hodgkin's lymphoma (cNHL) occurs in 83% of the cases[69]. cNHL mostly affects middle-aged and old dogs of all breeds, but it can also be observed in very young dogs less than one year old[132]. There are many similar features in the morphology, behaviour, genome instability, chemotherapy response and driver molecular abnormalities between cNHL and human NHL (hNHL)[68,69,73,132]. Yet, the incidence in dogs is higher, which is why research on cNHL has a dual purpose for both human and canine veterinary medicine[68]. Although high-grade diffuse large B-cell lymphoma is the most common subtype of cNHL and hNHL, the other subtypes that are commonly observed in dogs, are quite rare in humans[68]. Both cNHL and hNHL are sensitive for chemotherapy in the form of a multi-agent protocol, called 'CHOP'[68,69,132]. 'CHOP' is an acronym for a combination of chemotherapeutics that consists of cyclophosphamide, hydroxydaunorubicin (also known as doxorubicin), oncovin (also known as vincristine) and prednisolone[68,69,133]. Radiation therapy can be used in hNHL, but it requires more research to determine the efficiency of this treatment in cNHL[69]. cNHL tumouroids could provide new solutions for investigating both cNHL and hCHL[139]. There is only one report on cNHL tumouroids, described in a study by An et al. in 2021, as illustrated in *table 13*.

***Table 13**: Culture conditions used in specific studies to generate canine lymphoma tumouroids*

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
An et al., (2021)	Co-culturing of both lymphoma tumour cells and stromal cells in different ratios to investigate the relationship between tumour cells and the TME.	Stromal cells were obtained from submandibular lymph nodes from 3 healthy Beagles via needle biopsy. Lymphoma cell lines were mixed with stromal cells to create hybrid tumouroids and plated on StemFIT 3D cell culture 25-well arrays.	**Roswell Park Memorial Institute (RPMI) - 1640 medium**	• **PS** (1%) • **FBS** (20%)

4.2.3 Canine osteosarcoma tumouroids

Osteosarcoma (OSA) is the most diagnosed type of primary malignant bone cancer in both humans and dogs[70,140]. OSA has a higher incidence in dogs than in humans, where OSA is a rare disease[70,88]. Both canine OSA (cOSA) and human OSA (hOSA) seem to be bimodal and similarities can be observed in their clinical presentation, progression, molecular factor and risk factors[70,88]. However, hOSA is mostly seen in children and adolescents[88]. In contrast, cOSA mostly occurs in older dogs[88]. cOSA is mostly observed in male individuals and localised in long, weight-bearing bones, which means mostly in the front legs[70,140]. Rapidly growing large dog breeds and tall humans are predisposed[70,140]. The incidence rate is also affected by different families or populations in humans or by different dog breeds, such as Irish Wolfhounds or Great Danes[70,72]. Treatment in cOSA and hOSA is identical and includes surgery, often in the form of amputation of the affected limb, combined with adjuvant radiation therapy or chemotherapy[70,88,132]. cOSA and hOSA highly metastatic tumours that often migrate to the lungs and other bones[70,132,140]. Without adjuvant chemotherapy treatment after amputation of the limb with the primary tumour, metastasis occurs within 1 and 2 years in respectively cOSA and hOSA[88,132]. The survival rate in case of metastasis or recurrence is poor[88,141]. This is often the result of resistance to standard chemotherapy, presumably due to the high intratumoural heterogeneity of OSA[88,141]. Current research has highlighted the efficiency of generating OSA tumouroids in reproducing the TME[140]. The first cOSA tumouroids were established in 2021, as illustrated in ***figure 14***[141]. Another study on cOSA tumouroids was performed in 2022[142]. Both reports on cOSA tumouroids are described in more detail in *table 14*.

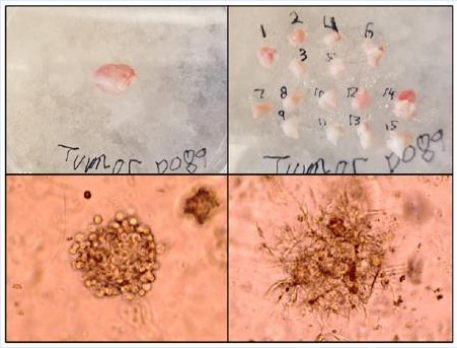


Figure 14: Generation of canine osteosarcoma (cOSA) tumouroids. The tumour sample, in this case number D089, can be observed on the illustrations above. The tumouroids that were derived from these samples are shown on the ilustrations below, using light microscopy at 20x magnification.
From: *Flamant et al., 2021.*

Table 14 : Culture conditions used in specific studies to generate canine osteosarcoma tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Flamant et al., (2021)	cOSA tumouroids are used to investigate the functional consequences of tumour heterogeneity on treatment response.	cOSA samples were received fresh on-ice from veterinary partners, sectioned into pieces. They were plated into Matrigel® and cultured into tumouroids.	**DMEM** (30%)	• **FBS** (10%)
Graves et al., (2022)	cOSA tumouroids are used for comparative oncology. They can be used to model the heterogeneity in drug response and to pinpoint collateral sensitivities in chemotherapy-resistant tumours. This heterogeneity can be used in chemotherapy resistance as a predictive biomarker.	cOSA samples were obtained from collaborating veterinary partners, divided into sections. They were plated into a 3D scaffold matrix and cultured into tumouroids within 24 hours of tissue biopsy.	/	/

4.2.4 Canine bladder cancer tumouroids

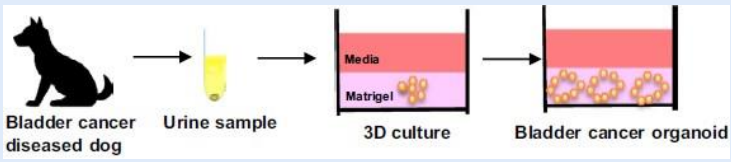


Figure 15: Schematic overview of the generation of canine bladder cancer (cBC) tumouroids derived from a canine urine sample of a dog with BC.
From: *Elbadawy et al., (2019)*

Bladder cancer (BC) is the most common cancer of the urinary tract in both dogs and humans and it compromises 1-2% of all naturally occurring tumours[86, 143 , 144] . More than 90% of BC in dogs (cBC) is classified as intermediate or high grade muscle invasive urothelial carcinoma, also known as transitional cell carcinoma[86,143,144145]. In addition, diagnosis often happens at a late stage of the disease[86,143,144146]. As a result, the prognosis of cBC is very reserved[86,143,144147]. cBC tumouroids could provide an important model for the determination of suitable chemotherapy in canine patients[86]. However, spontaneously occurring cBC also resembles muscle-invasive human bladder cancer (hBC)[86,143,144]. Therefore, cBC tumouroids also function as a translational model for muscle invasive hBC[86].

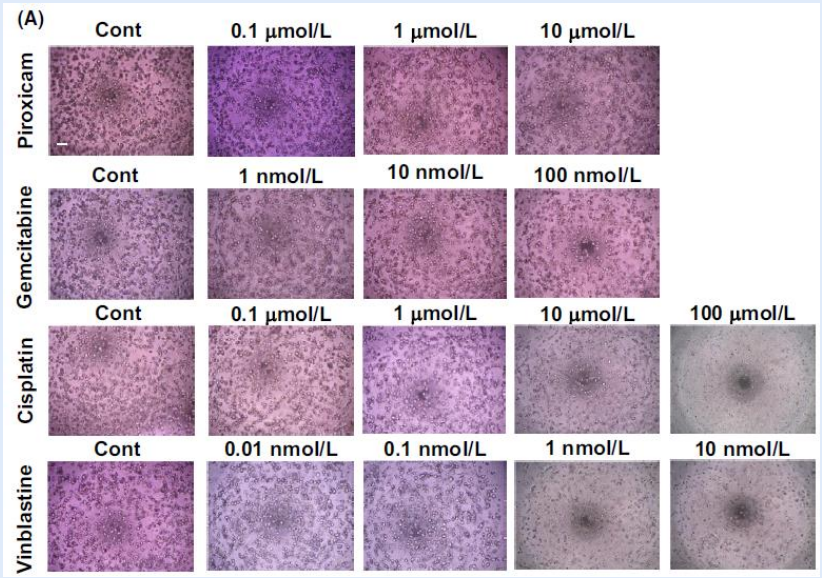


Figure 16: Different chemotherapeutics (piroxicam, gemcitabine, cisplatin and vinblastine) tested in different doses to determine the viability of the cells of the canine bladder cancer (*cBC)* tumouroid. A control group (Cont) has also been taken into account, as shown on the far left.
From: *Elbadawy et al., (2019)*

cBC tumouroids were first described in a study of 2019 by Elbadawy et al., as illustrated in *figure 15*. The tumouroids were successfully generated, with an efficiency of 70% and the tumouroids showed tumourigenesis after transplantation in vivo[86]. In addition, certain urothelial biomarkers were expressed or upregulated compared to normal bladder tissue or 2D cell lines of cBC[86]. Different chemotherapeutics were tested on the cBC tumouroids, as illustrated in *figure 16*[86]. Other reports on cBC tumouroids were published in 2020 and 2021[143,144]. However, it was only in 2022 that organoids were established from the normal canine bladder, which could also be useful to investigate the pathogenesis of BC[147]. The different reports on cBC tumouroids are described in *table 15*.

Table 15: Culture conditions used in specific studies to generate canine bladder cancer tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Elbadawy et al., (2019)	First establishment of cBC tumouroids with the identification of several specific organoid genes. It proved that cBC	cBC tumouroids were created by using urine samples from dogs with BC. They were plated into Matrigel® and cultured.	**Advanced DMEM**	• **Wnt-3a** • **Noggin** • **Rspo1** • **GlutaMax** • **Primocin** (100µg/ml) • **B-27 supplement**

	tumouroids can be used to analyse tumour development and to determine the sensitivity of anti-cancer drugs.			• **NAC** (1mM) • **Nicotinamide** (10mM) • **Mouse-EGF** (50ng/ml) • **A83-01** (500nM) • **SB202190** (3µM) • **Y-27632** (10µM)
Abugomaa et al., (2020)	Using 3D organoids of cBC to develop 2.5D organoids and to perform a comparative analysis.	Culture conditions for the cBC organoid derived from urine samples of dogs with BC were similar to those of prostate cancer (PC). For the 2.5D-organoid, a special culture medium is required that uses FBS instead of Wnt, Noggin and R-spondin. For the rest, culture conditions are very similar.	**Advanced DMEM/F12**	• **HEPES** (10mM) • **GlutaMax** (1%) • **Nicotinamide** (10mM) • **NAC** (1mM) • **A83-01** (0,5µM) • **PS** (1%) • **FBS** (5%)
Elbadawy et al., (2021)	cBC tumouroids are used to investigate the in vitro and in vivo effect of the chemotherapeutic trametinib. This study provided a promising new treatment for BC.	cBC tumouroids were created by using urine samples from dogs with BC.	**Advanced DMEM**	• **Wnt-3a** • **Noggin** • **Rspo1** • **GlutaMax** (1%) • **Primocin** (100µg/ml) • **B-27 supplement** • **NAC** (1mM) • **Nicotinamide** (10mM) • **Mouse-EGF** (50ng/ml) • **A83-01** (500nM) • **SB202190** (3µM) • **Y-27632** (10µM)
Elbadawy et al., (2022)	Normal bladder organoids from dogs are used to further understand the pathogenesis of cBC and muscle invasive hBC.	Bladder mucosa cells were collected by scratching the cells of the bladder wall from healthy dogs. The cells were plated on 24-well plates into Matrigel® and cultured.	**Advanced DMEM**	• **Wnt-3a** • **Noggin** • **Rspo1** • **GlutaMax** (1%) • **Primocin** (100µg/ml) • **B-27 supplement** • **NAC** (1mM) • **Nicotinamide** (10mM) • **Mouse-EGF** (50ng/ml) • **A83-01** (500nM) • **SB202190** (3µM) • **Y-27632** (10µM)

4.2.5 Canine prostate cancer tumouroids

Prostate cancer (PC) is the most common type of cancer in men worldwide[148,149]. Dogs are the only other large mammals that commonly develop PC spontaneously, which makes canine PC (cPC) a suitable translational model for the diagnosis and disease of human PC (hPC)[16,148,149,150]. Metastases often occur in the pelvis and/or lumbar spine in both humans and dogs[150]. However, there are also differences[87,148,150]. In dogs, the incidence of PC is lower than in humans and it presents itself in a very aggressive form, whereas in hPC, the cancer is more low grade and slowly progressing[16,148-150]. cPC and hPC also show differences in hormone-responsiveness and prostate-specific antigen can be easily found in the blood of humans, but not in dogs[148-150]. It is possible to generate tumouroids from cPC by using canine urine samples[16,148]. Tumouroids can be used to clarify the biology of PC and to identify CSCs as well as to select the most effective treatment for a specific patient[149,150]. The first cPC tumouroids were developed in 2017 and are described in *table 16*[148].

Table 16: Culture conditions used in specific studies to generate canine prostate cancer tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Usui et al., (2017)	First establishment of cPC tumouroids using CSCs in urine samples from dogs with PC. An urine-derived organoid culture was used. These cPC tumouroids recapitulated the TME and showed tumorigenesis in vivo.	Urine samples from dogs with PC were plated into Matrigel® and cultured with modified culture media.	**Advanced DMEM**	• **Wnt-3a** • **Noggin** • **Rspo1** • **GlutaMax** • **Primocin** (100µg/ml) • **B-27 supplement** • **NAC** (1mM) • **Nicotinamide** (10mM) • **Mouse-EGF** (50ng/ml) • **A83-01** (500nM) • **SB202190** (3µM) • **Y-27632** (10µM)

4.2.6 Canine thyroid cancer tumouroids

Canine thyroid cancer occurs in 1-2% of all neoplasias, 90% being malignant[151]. From all thyroid carcinomas in dogs, 70% can be classified as canine follicular thyroid carcinoma (cFTC) and 30% as canine medullary thyroid carcinoma (cMTC)[151]. Predisposed breeds for thyroid carcinoma are Boxers, Beagles, Golden Retrievers and Siberian Huskies and it mostly occurs in older dogs with a median age of 9-10 years[151]. cFTC and its metastases can be effectively treated with radioactive iodine, specifically radioiodine-131 (^{131}I) [50]. This treatment increases the survival time from 3 months in untreated dogs to a median survival time of 27-30 months[151]. However, the efficacy of the treatment depends on the uptake of ^{131}I by the cFTC and the metastases[151]. Moreover, to improve treatment, there is need for models that help understand the mechanism of the ^{131}I uptake, such as the expression of proteins that are involved[151]. Thyrotropin receptor (TSHR), sodium iodide symporter (NIS) and thyroid peroxidase (TPO) are essential for the uptake of iodine by follicular thyroid cells[151]. Research on the expression of these proteins could lead to improvement of the therapy in cFTC[151]. By generating tumouroids that are derived from cFTC, a model can be created to investigate the iodine uptake by immunohistochemistry, with new possibilities for the treatment of cFTC[151].

cMTC is less invasive and metastasis occurs at a slower rate than cFTC, but the prognosis after thyroidectomy is similar for both tumours[152]. In 2022, the first tumouroids of cMTC were successfully established[152]. The generation of cFTC and cMTC tumouroids are described in more detail in *table 17*.

Table 17: Culture conditions used in specific studies to generate canine thyroid cancer tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Jankovic et al., (2021)	Using cFTC tumouroids to establish immuno-histochemistry protocols. The uptake of iodine in cFTC with the expression of TSHR, NIS and TPO was studied.	cFTC samples were obtained during thyroidectomy. They were frozen in dimethyl sulfoxide (DMSO)-containing freezing medium within 24 hours. Cells were plated on 24-well plates into Cultrex Basement Membrane Extract and cultured at 37℃ and 5% CO_2 atmosphere and the medium was changed twice a week.	**Advanced DMEM/F12**	• **L-glutamine** • **HEPES** • **PS** • **NAC** • **B-27 supplement** • **Epithelial growth factor** • **Noggin** • **Rspo1** • **Y-27632**

Scheemaeker et al., (2022)	Establishment of cMTC tumouroids to study the effect of anti-cancer drugs.	Tissues of cMTCs were obtained from the biobank of canine thyroid tumour tissue. The tissue was originally obtained from client-owned dogs that underwent thyroidectomy. The tissue was washed in PBS and microdissected and then stored in freezing medium. The frozen tissues were then washed, centrifuged and enzymatically digested. They were then cultured using basic growth medium that was mixed with Cultrex® basement membrane extract. Incubation occurred at 37℃ and 5% CO_2.	**Advanced DMEM/F12**	• **HEPES** (10mM) • **L-glutamine** (2mM) • **PS** (50U/ml) • **Rspo1** (10%) • **Noggin** (10%) • **B-27 supplement** (2%) • **Bovine thyroid stimulating hormone (TSH)** (8mIU/ml)

4.2.7 Canine malignant mesothelioma tumouroids

The mesothelium is the serous epithelium that is covering the body cavities and the surface of organs and it is composed of mesothelial cells[153]. Canine malignant mesothelioma (cMM) is a rare and drug-resistant tumour that is very similar to human MM (hMM)[153]. cMM arises mainly from the mesothelial lining of the pleura, peritoneum and pericardium[153]. In humans, pleural mesothelioma is the most common form[153]. cMM tumouroids could become a potential in vitro tool for developing new treatment options for both cMM and hMM[153]. In 2023, the study of Sato et al. established cMM tumouroids for the first time, using pleural effusion samples, as illustrated in *table 18*. The cMM tumouroids exhibited the characteristics of MM and expressed mesothelial cell markers[153].

Table 18: Culture conditions used in specific studies to generate canine malignant mesothelioma tumouroids

Study (year)	Significance of the study	Technique	Growth medium	Supplementation
Sato et al., (2023)	First establishment of cMM tumouroids and screening of different anti-cancer drugs	cMM cells were obtained from pleural effusion samples. They were washed three times with PBS and plated into Matrigel® and seeded on 24-well plates. They were cultured at 37℃ for 30 minutes. The culture medium was changed three times per week.	**Advanced DMEM**	• **Wnt** • **Noggin** • **Rspo1** • **Glutamax** • **Primocin** (100µg/ml) • **NAC** (1mM) • **Nicotinamide** (10mM) • **A83-01** (500nM) • **EGF** (50ng/ml) • **FGF-2** (5ng/ml) • **FGF-7** (5ng/ml) • **Insulin-like growth factor (IGF)** (100ng/ml)

Discussion

Organoids are able to phenocopy the cellular composition, architecture, and functionality of differentiated tissues in vivo[3]. This has resulted in several applications, including in the field of oncology in the form of tumouroids[5,16]. However, the resemblance of organoids to the original tissue only applies to a certain extent[2]. Therefore, the main challenge will be to develop organoids that function as a real substitute for native organs. Furthermore, the co-culturing of organoids with non-epithelial cells, such as immune cells, also comes with its own limitations[2,5,7,58]. In addition, the total lifespan of organoids is limited, due to insufficient vascularization[2,7,11]. At this point, the efficiency of generating tumouroids varies between 30-90%, which could also be explained by the amount of tissue that is used and the tumour type the tumouroid is derived from[7,154]. Although this also has benefits as it reflects the inter-patient variability, it results in greater sample sizes that are needed to create reliable tumouroids[30]. Overall, organoids and tumouroids are more costly than 2D cell lines, because of the requirement for a matrix, specific incubation conditions, and a culture medium with added supplements that needs to be changed multiple times[7,11]. Therefore, organoids and tumouroids could be too expensive for most health care systems to be used for personalized medicine[30]. A second thing to consider when using organoids for therapeutic applications, is their prolonged growth rate compared to 2D cell lines[7]. Even so, organoids have numerous benefits over 2D cell lines and by creating organoid and tumouroid biobanks, information from tissues and tumours could be used more generally to make the technique more accessible[2,5,30]. Organoids offer a potential solution for ethical problems by contributing to the 3R-concept, i.e. reducing the amount of required animal models[19]. However, organoids do not incorporate the full body of the patient they are derived from, which is why animal models will continue to be necessary for preclinical trials[28,30,61,63.70,155]. In addition, organoids also have ethical difficulties of their own, especially with regard to human organoids[19]. When using human organoids, there are ethical challenges around human tissue availability when considering the moral and legal status of organoids, the lacking of adequate consent procedures for the use of PDOs, and the global distribution of organoid biobanks[19,20].

Dogs are useful animal models for human disease, due to their many similarities[16]. However, differences can be observed between laboratory and companion dogs[66,67]. Laboratory dogs are exposed to a different environment, receive different nutrients, exhibit different behaviour patterns, have a shorter lifespan, and their population consists of little genetic variation, because only a few breeds (e.g. Beagles) are used for scientific purposes[66,67]. This is why companion dogs could be considered to be more reliable and representative models. Nevertheless, ethical and financial challenges limit the use of living companion dogs[69,70]. Therefore, canine organoids could provide a more accessible translational research tool.

Literature using the term ‘organoids’ dates back to 1946[35]. A literature search in PubMed on organoids also shows a similar trend in the amount of published reports, with a spike in publications between 1965-1985[1]. This spike could be explained by the rising interest in organ development and regeneration, as the definition of an organoid at that time was reported as ‘being similar to an organ’[35]. Interestingly, between 1985-2010, almost no publications can be found on organoids. Several explanations could account for this reduction, such as the 3R-principle that arose in 1959, as well as economical events, including the Fall of the Berlin Wall in 1989, which induced an increase in interest rates and less available money for new scientific research strategies[10,4]. It was only since 2010 that the popularity of organoids started to really grow and it has been rising ever since. This increasing trend can also be linked back to the first reports on canine organoids in 2009, and to the numerous benefits of organoids compared to earlier strategies[2,5,106,128].

In this thesis, a thorough literature study was performed to list the currently available canine organoids and tumouroids and to compare their different culture conditions. Performing a search in PubMed publications on the term ‘canine organoids’ resulted in a total number of 216 publications. Yet, this includes all the results starting from 1966 and, considering the first “real” canine organoids were generated in 2009, the amount of publications could actually be reduced to only 70[128]. In the end, 41 publications were used to investigate the different organoids and tumouroids in canine veterinary

[4] Retrievable at: https://www.cbs.nl/nl-nl/cijfers/detail/7336SHFO# (last consulted on 17-5-2023).

medicine, from which 33 are described in more detail. When comparing the different culture conditions throughout the published studies, a few observations are noticeable. Firstly, Matrigel® is the most common used ECM for canine organoids. However, the limitations of Matrigel® have been discussed and it could limit their applications[2,38,41]. Secondly, most of the reported canine organoid cultures use DMEM, advanced DMEM or advanced DMEM/F12 as growth medium. Compared to Minimal Essential Medium (MEM), DMEM contains four times the amino acid and vitamin concentration, which is why DMEM is a "widely used basal medium for supporting the growth of many mammalian cells"[5,5,6]. DMEM is used to culture adherent cells, which is the case for most of the reported organoids[5,5,6]. Advanced DMEM is "enriched with normal-serum constituents and therefore requires 50-90% less FBS supplementation", which reduces the variability of the cell culture and lowers the costs of the culture medium[5]. Advanced DMEM/F12 also requires less FBS supplementation as it has added ingredients that allow serum reduction[7]. There are also reports on other types of media, such as RPMI-1640 medium that is used for lymphoma organoids and IMDM that is used for cardiac organoids[93,139]. RPMI-1640 is used on suspension cultures for the culture of for example lymphoblasts, as is the case for canine lymphoma organoids, and it contains "glutathione and high concentrations of vitamins, including vitamins not found in MEM and DMEM[8,9]. IMDM is a modification of DMEM that can also be used for adherent cell cultures, with added "selenium and additional amino acids, vitamins and inorganic salts"[10,11]. These extra nutrients are essential for the growth of specific cell types, that could grow less effectively in other growth media, such as CDCs of cardiac organoids and myeloblasts[93,12]. A last observation on the comparison of the different culture media are the often similar supplementations that are used. However, there are still some differences noticeable in the exact amount of supplementation and the use of tissue-specific growth factors, for example HGF[108-110]. HGF is essential to liver development as it stimulates "epithelial cell proliferation, motility, morphogenesis and angiogenesis" and "supplementation of HGF produces therapeutic outcomes under pathological conditions"[108 -110,156]. Therefore, the use of HGF can be observed in all reported canine hepatic organoid cultures.

All these observations suggest that it might be possible to use almost the exact same culture conditions for different types of organoids. Indeed, using similar culture conditions could improve the reproducibility and thus the efficiency of generating canine organoids and their translational purposes. The study of Zdyrski et al., (2022) is the first report on using similar culture conditions for different organoid types. However, it should be mentioned that this study is a preprint that requires further peer review. Considering the different canine tissues and tumours for translational purposes, not all of them are yet available to generate organoids or tumouroids. There have been no reports on for example, melanoma, squamous cell carcinoma, lung carcinoma and mast cell tumouroids, although living dogs are already used as animal models for these types of cancers[82,132]. Nevertheless, some additional canine tumour types have been investigated in the form of spheroids. Both spheroids and organoids are composed of a 3D structure, but they are certainly no synonyms[30]. Spheroids are simpler than organoids, because they are homogenous, have no matrix component, and they present themselves as free-floating aggregates of cells, with a less representative architecture[30]. When comparing the different canine organoids for bladder cancer (BC), Abugomaa et al., (2020) also describes the establishment of so called '2.5D organoids', using strains from 3D organoids and specific culture media. Despite their name, 2.5D organoids are not consisting of a 2.5D structure, but they include the benefits of both 2D cell lines and 3D organoids[143,157]. Indeed, 2.5D organoids show similar histopathological characteristics and sensitivity to anti-cancer drugs as 3D organoids, which could make them a potential cheaper and less

[5] Retrievable at: https://www.thermofisher.com/nl/en/home/life-science/cell-culture/mammalian-cell-culture/cell-culture-media/dmem.html#:~:text=Dulbecco's%20Modified%20Eagle%20Medium%20(DMEM)%20is%20a%20widely%20used%20basal,7%2C%20and%20PC%2D12 (last consulted on 17-5-2023).
[6] Retrievable at: https://www.thermofisher.com/nl/en/home/life-science/cell-culture/mammalian-cell-culture/cell-culture-media/mem.html (last consulted on 17-5-2023).
[7] Retrievable at: https://www.thermofisher.com/order/catalog/product/12634010 (last consulted on 17-5-2023).
[8] Retrievable at: https://www.thermofisher.com/nl/en/home/life-science/cell-culture/mammalian-cell-culture/cell-culture-media/rpmi.html?ef_id=821ead1076711d3314a9c974d43628f4:G:s&s_kwcid=AL!3652!10!76691109315655!76691086026799&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000_0se_bng_bt_pur_con (last consulted on 17-5-2023).
[9] Retrievable at: https://www.thermofisher.com/nl/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/maintaining-cultured-cells/recommended-media-types-for-common-cells.html (last consulted on 17-5-2023).
[10] Retrievable at: https://www.labome.com/method/Cell-Culture-Media-A-Review.html#:~:text=IMDM%20is%20a%20modification%20of,contains%20HEPES%20and%20sodium%20pyruvate. (last consulted on 15-5-2023).
[11] Retrievable at: https://www.thermofisher.com/order/catalog/product/12440053 (last consulted on 17-5-2023).
[12] Retrievable at: https://www.thermofisher.com/nl/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/maintaining-cultured-cells/recommended-media-types-for-common-cells.html (last consulted on 17-5-2023).

time consuming model system[143,157]. However, 2.5D organoids have been very recently established and they are not yet fully applicable. Indeed, to include all potential benefits of 2.5D organoids, they should be generated directly from tissues. At this point, there has been only one study in 2022 that established direct generation of 2.5D organoids using cancer tissues of companion animals[157].

To conclude, canine organoids could be a great potential research tool for both veterinary and human medicine, especially for cancer research. Many canine organoids and tumouroids have already been established and there are similarities that can be observed between their culture conditions. However, this literature study shows that the amount of reports is limited. Besides, there are still tissues and tumours that have not yet been investigated. These results indicate that more research is required to introduce new organoid types and to improve current organoids to establish more standardized culture protocols.

References

[1] Clevers H. Modeling Development and Disease with Organoids. *Cell* 2016; **165**: 1586-97.

[2] Rossi G, Manfrin A, Lutolf MP. Progress and Potential in Organoid Research. *Nat Rev Genet* 2018; **19**: 671-87.

[3] Artegiani B, Clevers H. Use and application of 3D-organoid technology. *Hum Mol Genet* 2018; **27**: R99-107.

[4] Antoni D, Burckel H, Josset E, Noel G. Three-Dimensional Cell Culture: A Breakthrough in Vivo. *Int J Mol Sci* 2015; **16**: 5517-27.

[5] Huch M, Knoblich JA, Lutolf MP, Martinez-Ariaz A. The hope and the hype of organoid research. *Development* 2017; **144**: 938-41.

[6] Zhou J, Su J, Fu X, Zheng L, Yin Z. Microfluidic device for primary tumor spheroid isolation. *Exp Hematol Oncol* 2017; **6**: 22.

[7] Driehuis E, Clevers H. Organoïden als in-vitro model: mini-organen in het laboratorium. *Nederlands Tijdschrift voor Oncologie* 2018; **15**: 244-51.

[8] Rusche B. The 3Rs and animal welfare – conflict or the way forward? *ALTEX* 2008; **20**: 63-76.

[9] Kar SK, Wells JM, Ellen ED, Te Pas MFW, Madsen O, Groenen MAM, Woelders H. Organoids: a promising new in vitro platform in livestock and veterinary research. *Vet Res* 2021; **52**: 43.

[10] Csukovich G, Pratscher B, Burgener IW. The World of Organoids: Gastrointestinal Disease Modelling in the Age of 3R and One Health with Specific Relevance to Dogs and Cats. *Animals (Basel)* 2022; **12**: 2461.

[11] Qian X, Nguyen HN, Song MM, et al. Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell* 2016; **165**: 1238-54.

[12] Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009; **459**: 262-5.

[13] Eiraku M, Takata, N, Ishibashi H, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 2011; **472**: 51-6.

[14] Yui S, Nakamura, T, Sato Y, et al. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5^{+} stem cell. *Nat Med* 2012; **18**: 618-23.

[15] Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. *Nat Cell Biol* 2016; **18**: 246-54.

[16] Penning LC, Van den Boom R. Companion animal organoid technology to advance veterinary regenerative medicine. *Front Vet Sci* 2023; **10**: 1032835.

[17] Assawachananont J, Mandai M, Okamoto S, et al. Transplantation of embryonic and induced pluripotent stem cell-derived 3D retinal sheets into retinal degenerative mice. *Stem Cell Reports* 2014; **2**: 662-74.

[18] Fordham RP, Yui S, Hannan NRF, et al. Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 2013; **13**: 734-44.

[19] Boers SN, Van Delden JJ, Clevers H, Bredenoord AL. Organoid biobanking: identifying the ethics: Organoids revive old and raise new ethical challenges for basic research and therapeutic use. *EMBO Rep* 2016; **17**: 938-41.

[20] LaLonde-Paul D, Mouttham L, Dog Aging Project Consortium, Promislow DEL, Castelhano MG. Banking on a new understanding: translational opportunities from veterinary biobanks. *Geroscience* 2023; online ahead of print.

[21] Dekkers JF, Wiegerinck CL, De Jonge HR, et al. A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* 2013; **19**: 939-45.

[22] Mariani J, Coppola G, Zhang P, et al. FOXG1-Dependent Dysregulation of GABA/Glutamate Neuron Differentiation in Autism Spectrum Disorders. *Cell* 2015; **162**: 375-390.

[23] Vermeire B, Gonzalez LM, Jansens RJJ, Cox E, Devriendt B. Porcine small intestinal organoids as a model to explore ETEC-host interactions in the gut. *Vet Res* 2021; **52**: 94.

[24] Drost J, Karthaus WR, Gao D, Driehuis E, Sawyers CL, Chen Y, Clevers H. Organoid culture systems for prostate epithelial and cancer tissue. Nat Protoc 2016; 11: 347-58.

[25] Soker S, Skardal A. Introduction. In: Tumor Organoids, First edition. Humana Press, Totowa, NJ, USA, 2018 pp. 13.

[26] Schultz M. Rudolf Virchow. *Emerging Infectious Diseases* 2008; **14**: 1480-1.

[27] Gyles C. One Medicine, One Health, One World. *Can Vet J* 2016; **57**: 345-6.

[28] Cree IA, Glaysher S, Harvey AL. Efficacy of anti-cancer agents in cell lines versus human primary tumour tissue. *Curr Opin Pharmacol* 2010; **10**: 375-9.

[29] Abdelmegeed SM, Mohammed S. Canine mammary tumors as a model for human disease. *Oncol Lett* 2018; **15**: 8195-205.

[30] Porter RJ, Murray GI, McLean MH. Current concepts in tumour-derived organoids. *Br J Cancer* 2020; **123**: 1209-18.

[31] Corrò C, Novellasdemunt L, Li VSW. A brief history of organoids. *Am J Physiol Cell Physiol* 2020; **319**: C151-65.

[32] Wilson HV. A new method by which sponges may be artificially reared. *Science* 1907; **25**: 912-5.

[33] Armstrong L, Lako M, Buckley N, et al. Editorial: Our top 10 developments in stem cell biology over the last 30 years. *Stem Cells*; **30**: 2-9.

[34] Wang X. Stem cells in tissues, organoids, and cancers. *Cell Mol Life Sci* 2019; **76**: 4043-70.

[35] Smith E, Cochrane WJ. Cystic organoid teratoma; report of a case. *Can Med Assoc J* 1946; **55**: 151.

[36] Alvarez-Buylla A. Yoshiki Sasai (1962-2014). *Nature* 2014; **513**: 34.

[37] Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; **6**: 331-43.

[38] Fiorini E, Veghini L, Corbo V. Modeling Cell Communication in Cancer With Organoids: Making the Complex Simple. *Front Cell Dev Biol* 2020; **8**: 166.

[39] Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, Swarm R. A murine tumor producing a matrix of basement membrane. *J Exp Med* 1977; **145**: 204-20.

[40] Stingl J, Eaves CJ, Zandieh I, Emerman JT. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. *Breast Cancer Res Treat* 2001; **67**: 93-109.

[41] Hughes CS, Postovit LM, Lajoie GA. Matrigel: a complex protein mixture required for optimal growth of cell culture. *Proteomics* 2010; **10**: 1886-90.

[42] Passaniti A, Kleinman HK, Martin GR. Matrigel: history/background, uses, and future applications. *J Cell Commun Signal* 2022; **16**: 621-6.

[43] Zhao Z, Chen X, Dowbay AM, et al. Organoids. *Nature Reviews Methods Primers* 2022; **2**: 94.

[44] Cibelli J, Emborg ME, Prockop DJ, et al. Strategies for improving animal models for regenerative medicine. *Cell Stem Cell* 2013; **12**: 271-4.

[45] Crespo M, Vilar E, Tsai S, et al. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. *Nat Med* 2017; **23**: 878-84.

[46] Bongiovanni L, Brachelente C, Moreno E, Welle MM. Canine Epithelial Skin Tumours: Expression of the Stem Cell Markers Lgr5, Lgr6 and Sox9 in Light of New Cancer Stem Cell Theories. *Vet Sci* 2020; **7**: 62.

[47] Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. *Stem Cell Res Ther* 2019; **10**: 68.

[48] Eiraku M, Watanabe K, Matsuo-Takasaki M, et al. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell 2008; 3: 519-32.

[49] Shi D, Lu F, Wei Y, Cui K, Yang S, Wei J, Liu Q. Buffalos (Bubalus bubalis) cloned by nuclear transfer of somatic cells. *Biol Reprod* 2007; **77**: 285-91.

[50] Sommer CA, Mostoslavsky G. Experimental approaches for the generation of induced pluripotent stem cells. *Stem Cell Res Ther* 2010; **1**: 26.

[51] Sidhu KS. New approaches for the generation of induced pluripotent stem cells. *Expert Opin Biol Ther* 2011; **11**: 569-79.

[52] Xinaris C, Brizi V, Remuzzi G. Organoid Models and Applications in Biomedical Research. *Neprhon* 2015; **130**: 191-9.

[53] McCauley HA, Wells JM. Pluripotent stem cell-derived organoids: using principles of developmental biology to grow human tissues in a dish. *Development* 2017; **144**: 958-62.

[54] Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011; **141**: 1762-72.

[55] Gao D, Vela I, Sboner A, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell* 2014; **159**: 176-187.

[56] Boj SF, Hwang C, Baker LA. Organoid models of human and mouse ductal pancreatic cancer. *Cell* 2015; **160**: 324-38.

[57] Hubert CG, Rivera M, Spangler LC, et al. A Three-Dimensional Organoid Culture System Derived from Human Glioblastomas Recapitulates the Hypoxic Gradients and Cancer Stem Cell Heterogeneity of Tumors Found In Vivo. *Cancer Res* 2016; **76**: 2456-77.

[58] Raffo-Romero A, Aboulouard S, Bouchaert E. Establishment and characterization of canine mammary tumoroids for translational research. *BMC Biol* 2023; **21**: 23.

[59] Vandamme TF. Use of rodents as models of human diseases. *J Pharm Bioallied Sci* 2014; **6**: 2-9.

[60] Zomer HD, Trentin AG. Skin wound healing in humans and mice: Challenges in translational research. *J Dermatol Sci* 2018; **90**: 3-12.

[61] Brown SDM. Advances in mouse genetics for the study of human disease. *Hum Mol Genet* 2021; **30**: R274-84.

[62] Perel P, Roberts I, Sena E, et al. Comparison of treatment effects between animal experiments and clinical trials: systematic review. *BMJ* 2007; **334**: 197.

[63] Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res* 2014; **6**: 114-8.

[64] Jimenez AG. Physiological underpinnings in life-history trade-offs in man's most popular selection experiment: the dog. *J Comp Physiol B* 2016; **186**: 813-27.

[65] Chandra L, Borcherding DC, Kingsbury D, et al. Derivation of adult canine intestinal organoids for translational research in gastroenterology. *BMC Biol* 2019; **17**: 33.

[66] Sándor S, Kubinyi E. Genetic Pathways of Aging and Their Relevance in the Dog as a Natural Model of Human Aging. *Front Genet* 2019; **10**: 948.

[67] Kaeberlein M. The Biology of Aging: Citizen Scientists and Their Pets as a Bridge Between Research on Model Organisms and Human Subjects. *Vet Pathol* 2016; **53**: 291-8.

[68] Ito D, Frantz AM, Modiano JF. Canine lymphoma as a comparative model for human non-Hodgkin lymphoma: recent progress and applications. *Vet Immunol Immunopathol* 2014; **159**: 192-201.

[69] Marconato L, Gelain ME, Comazzi S. The dog as a possible animal model for human non-Hodgkin lymphoma: a review. *Hematol Oncol* 2013; **31**: 1-9.

[70] Simpson S, Rizvanov AA, Jeyapalan JN, De Brot S, Rutland CS. Canine osteosarcoma in comparative oncology: Molecular mechanisms through to treatment discovery. *Front Vet Sci* 2022;**9**: 965391.

[71] O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Longevity and mortality of owned dogs in England. *Vet J* 2013; **198**: 638-43.

[72] Jacob JA. Researchers Turn to Canine Clinical Trials to Advance Cancer Therapies. *JAMA* 2016; **315**: 1550-2.

[73] Paoloni MC, Khanna C. Comparative oncology today. *Vet Clin North Am Small Anim Pract* 2007; **37**: 1023-32.

[74] Mercati F, Pascucci L, Gargiulo AM, Dall''Anglio C, Ceccarelli P. Immunohistochemical evaluation of intermediate filament nestin in dog hair follicles. *Histol Histopathol* 2008; **23**: 1035-41.

[75] Kobayashi T, Shimizu A, Nishifuji K, Amagai M, Iwasaki T, Ohyama M. Canine hair-follicle keratinocytes enriched with bulge cells have the highly proliferative characteristic of stem cells. *Vet Dermatol* 2009; **20**: 338-46.

[76] Brachelente C, Porcellato, Sforna M, Lepri E, Mechelli L, Bongiovanni L. The contribution of stem cells to epidermal and hair follicle tumours in the dog. *Vet Dermatol* 2013; **24**: 188-94.

[77] Purba TS, Haslam IS, Pbolet E, Jiménez F, Gandarillas A, Izeta A, Paus R. Human epithelial hair follicle stem cells and their progeny: current state of knowledge, the widening gap in translational research and future challenges. *Bioessays* 2014; **36**: 513-25.

[78] Gerhards NM, Sayar BS, Origgi FC, Galichet A, Müller EJ, Welle MM, Wiener DJ. Stem Cell-Associated Marker Expression in Canine Hair Follicles. *J Histochm Cytochem* 2016; **64**: 190-204.

[79] Xu L, Lin W, Wen L, Li G. Lgr5 in cancer biology: functional identification of Lgr5 in cancer progression and potential opportunities for novel therapy. *Stem Cell Res Ther* 2019; **10**: 219.

[80] Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002; **2**: 563-72.

[81] Linde N, Fluegen G, Aguirre-Ghiso JA. The Relationship Between Dormant Cancer Cells and Their Microenvironment. *Adv Cancer Res* 2016; **132**: 45-71.

[82] Paoloni M, Khanna C. Translation of new cancer treatments from pet dogs to humans. *Nat Rev Cancer* 2008; **8**: 147-56.

[83] Storb R, Epstein RB, Ragde H, Bryant J, Thomas ED. Marrow engraftment by allogeneic leukocytes in lethally irradiated dogs. *Blood* 1967; **30**: 805-11.

[84] Zdyrski C, Gabriel V, Ospina O, et al. Establishment and Characterization of Novel Canine Organoids with Organ-Specific Physiological Similarity. BioRxiv Preprint 2022.

[85] Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol* 2018; **15**: 81-94.

[86] Elbadawy M, Usui T, Mori T, et al. Establishment of a novel experimental model for muscle-invasive bladder cancer using a dog bladder cancer organoid culture. *Cancer Sci* 2019; **110**: 2806-21.

[87] Hansen K, Khanna C. Spontaneous and genetically engineered animal models; use in preclinical cancer drug development. *Eur J Cancer* 2004; **40**: 858-80.

[88] Somarelli JA, Rupprecht G, Altunel E, et al. A Comparative Oncology Drug Discovery Pipeline to Identify and Validate New Treatments for Osteosarcoma. *Cancers (Basel)* 2020; **12**: 3335.

[89] Schwartz DM, Pehlivaner Kara MO, Goldstein AM, Ott HC, Erkenseair AK. Spray Delivery of Intestinal Organoids to Reconstitute Epithelium on Decellularized Native Extracellular Matrix. *Tissue Eng Part C Methods* 2017; **23**: 565-73.

[90] Lenti E, Bianchessi S, Proulx ST, et al. Therapeutic Regeneration of Lymphatic and Immune Cell Functions upon Lympho-organoid Transplantation. *Stem Cell Reports* 2019; **12**: 1260-8.

[91] Van de Wetering M, Francies HE, Francis JM, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 2015; **161**; 933-45.

[92] Wiener DJ, Studer IC, Brunner MAT, et al. Characterization of canine epidermal organoid cultures by immunohistochemical analysis and quantitative PCR. *Vet Dermatol* 2021; **32**: 179.

[93] Hensley MT, Andrade JD, Keene B, et al. Cardiac regenerative potential of cardiosphere-derived cells from adult dog hearts. *J Cell Mol Med* 2015; **19**: 1805-13.

[94] Chen T, Neupane M, Chien S, Chuang F, Crawford RB, Kaminski NE, Chang C. Characterization of Adult Canine Kidney Epithelial Stem Cells That Give Rise to Dome-Forming Tubular Cells. *Stem Cells Dev* 2019; **28**: 1424-33.

[95] Sun H, Zhang Y, Li Y. Generation of Skin Organoids: Potential Opportunities and Challenges. *Front Cell Dev Biol* 2021; **9**: 709824.

[96] Kishi K, Okabe K, Shimizu R, Kubota Y. Fetal skin possesses the ability to regenerate completely: complete regeneration of skin. *Keio J Med* 2012; **61**: 101-8.

[97] Wiener DJ, Basak O, Asra P, Boonekamp KE, Kretzschmar K, Papaspyropoulos A, Clevers H. Establishment and characterization of a canine keratinocyte organoid culture system. *Vet Dermatol* 2018; **29**: 375.

[98] Bedos L, Wickham H, Gabriel V, et al. Culture and characterization of canine and feline corneal epithelial organoids: A new tool for the study and treatment of corneal diseases. *Front Vet Sci* 2022; **9**: 1050467.

[99] Tidke SC, Tidake P. A Review of Corneal Blindness: Causes and Management. *Cureus* 2022; **14**: e30097.

[100] Foster JW, Wahlin K, Adams SM, Birk DE, Zack DJ, Chakravarti S. Cornea organoids from human induced pluripotent stem cells. *Sci Rep* 2017; **7**: 41286.

[101] Susaimanickam PJ, Maddileti S, Pulimamidi VK, et al. Generating minicorneal organoids from human induced pluripotent stem cells. *Development* 2017; **144**: 2338-51.

[102] Higa K, Higuchi J, Kimoto R, Miyashita H, Shimazaki J, Tsubota K, Shimmura S. Human corneal limbal organoids maintaining limbal stem cell niche function. *Stem Cell Res* 2020; **49**: 102021.

[103] Ambrosini YM, Park Y, Jergens AE, et al. Recapitulation of the accessible interface of biopsy-derived canine intestinal organoids to study epithelial-luminal interactions. *PloS One* 2020; **15**: e0231423.

[104] Allenspach K, Iennarella-Servantez C. Canine Protein Losing Enteropathies and Systemic Complications. *Vet Clin North Am Small Anim Pract* 2021; **51**: 111-22.

[105] Barker N, Van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-7.

[106] Agopian VG, Chen DC, Avansino JR, Stelzner M. Intestinal stem cell organoid transplantation generates neomucosa in dogs. *J Gastrointest Surg* 2009; **13**: 971-82.

[107] Kopper JJ, Ienarella-Servantez C, jergens AE, et al. Harnessing the Biology of Canine Intestinal Organoids to Heighten Understanding of Inflammatory Bowel Disease Pathogenesis and Accelerate Drug Discovery: A One Health Approach. *Front Toxicol* 2021; **3**: 773953.

[108] Wu X, Chien H, Van Wolferen ME, Kruitwagen HS, Oosterhoff LA, Penning LC. Reduced FXR Target Gene Expression in Copper-Laden Livers of COMMD1-Deficient Dogs. *Vet Sci* 2019; **6**: 78.

[109] Kruitwagen HS, Oosterhoff LA, Van Wolferen ME, et al. Long-Term Survival of Transplanted Autologous Canine Liver Organoids in a COMMD1-Deficient Dog Model of Metabolic Liver Disease. *Cells* 2020; **9**: 410.

[110] Van den Bossche L, Schoonenberg VAC, Burgener IA, et al. Aberrant hepatic lipid storage and metabolism in canine portosystemic shunts. *PloS One* 2017; **12**: e0186491.

[111] Wiedenmann S, Breunig M, Merkle J, et al. Single-cell-resolved differentiation of human induced pluripotent stem cells into pancreatic duct-like organoids on a microwell chip. *Nat Biomed Eng* 2021; **5**: 897-913.

[112] Dunaevich A, Chen H, Musseri D, Kuzi S, Mazaki-Tovi M, Aroch I, Segev G. Acute on chronic kidney disease in dogs: Etiology, clinical and clinicopathologic findings, prognostic markers, and survival. *J Vet Intern Med* 2020; **34**: 2507-15.

[113] Grauer GF. Treatment Guidelines for Chronic Kidney Disease in Dogs and Cats. *Today's Veterinary Practice* 2017; **7**: 41-8.

[114] Nishinakamura R. Human kidney organoids: progress and remaining challenges. *Nat Rev Nephrol* 2019; **15**: 613-24.

[115] Choi J, Park J, Tsagkogeorga G, Yanagita M, Koo B, Han N, Lee J. Inflammatory Signals Induce AT2 Cell-Derived Damage-Associated Transient Progenitors that Mediate Alveolar Regeneration. *Cell Stem Cell* 2020; **27**: 366-82.

[116] Ghorani V, Boskabady MH, Khazdair MR, Kianmeher M. Experimental animal models for COPD: a methodological review. *Tob Induc Dis* 2017; **15**: 25.

[117] Williams K, Roman J. Studying human respiratory disease in animals--role of induced and naturally occurring models. *J Pathol* 2016; **238**: 220-32.

[118] Matkovic Leko I, Schneider RT, Thimraj TA, et al. A distal lung organoid model to study interstitial lung disease, viral infection and human lung development. *Nat Protoc* 2023; online ahead of print.

[119] Li J, Yang J, Zhao D, Lei W, Hu S. Promises and challenges of cardiac organoids. *Mamm Genome* 2023; online ahead of print.

[120] Kasai-Brunswick TH, Da Costa AR, Barbosa RAQ, et a. Cardiosphere-derived cells do not improve cardiac function in rats with cardiac failure. *Stem Cell Res Ther* 2017; **8**: 36.

[121] Zhao D, Lei W, Hu S. Cardiac organoid - a promising perspective of preclinical model. *Stem Cell Res Ther* 2021; **12**: 272.

[122] Wess G, Schulze A, Butz V. Prevalence of dilated cardiomyopathy in Doberman Pinschers in various age groups. *J Vet Intern Med* 2010; **24**: 533-8.

[123] Ma Z, Wang J, Loskill P, et al. Self-organizing human cardiac microchambers mediated by geometric confinement. *Nat Commun* 2015; **6**: 7413.

[124] Zhu L, Liu K, Feng Q, Liao Y. Cardiac Organoids: A 3D Technology for Modeling Heart Development and Disease. *Stem Cell Rev Rep* 2022; **18**: 2593-2605.

[125] Pain B, Baquerre C, Coulpier M. Cerebral organoids and their potential for studies of brain diseases in domestic animals. *Vet Res* 2021; **52**: 65.

[126] Lowe A, Dalton M, Sidhu K, Sachdev P, Reynolds B, Valenzuela M. Neurogenesis and precursor cell differences in the dorsal and ventral adult canine hippocampus. *Neurosci Lett* 2015; **593**: 107-13.

[127] Santos SIP, De Oliveira VC, Pieri NCG, Bressan FF, Ambrósio CE, Feitosa MLT. Isolation and characterization of neural stem cells from fetal canine spinal cord. *Neurosci Lett* 2021; **765**: 136293.

[128] Stadler K, Handler J, Schoenkypl S, Walter I. A three-dimensional culture model of canine uterine glands. *In Vitro Cell Dev Biol Anim* 2009; **45**: 35-43.

[129] Hagman R. Diagnostic and prognostic markers for uterine diseases in dogs. *Reprod Domest Anim* 2014; **49**: 16-20.

[130] Varughese EE, Adams GP, Singh J. Development of a domestic animal model for endometriosis: surgical induction in the dog, pigs, and sheep. *Journal of Endometriosis and Pelvic Pain Disorders* 2018; **10**: 2.

[131] Lawson EF, Ghosh A, Blanch V, et al. Establishment and characterisation of oviductal organoids from farm and companion animals. *Biol Reprod* 2023; online ahead of print.

[132] MacEwen EG. Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. *Cancer Metastasis Rev* 1990; **9**: 125-36.

[133] Doré M, Lanthier I, Sirois J. Cyclooxygenase-2 expression in canine mammary tumors. *Vet Pathol* 2003; **40**: 207-12.

[134] Saba CF, Rogers KS, Newman SJ, Mauldin GE, Vail DM. Mammary gland tumors in male dogs. *J Vet Intern Med* 2007; **21**: 1056-9.

[135] Nguyen F, Pena L, Ibisch C, et al. Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. *Breast Cancer Res Treat* 2018; **167**: 635-48.

[136] Garcia APV, Reis LA, Nunes FC, Longford FGJ, Frey JG, De Paula AM, Cassali GD. Canine mammary cancer tumour behaviour and patient survival time are associated with collagen fibre characteristics. *Sci Rep* 2021; **11**: 5668.

[137] Inglebert M, Dettwiler M, Hahn K, et al. A living biobank of canine mammary tumor organoids as a comparative model for human breast cancer. *Sci Rep* 2022; **12**: 18051.

[138] Cocola C, Molgora S, Piscitelli E, et al. FGF2 and EGF Are Required for Self-Renewal and Organoid Formation of Canine Normal and Tumor Breast Stem Cells. *J Cell Biochem* 2017; **118**: 570-84.

[139] An JH, Song WJ, Li Q, Bhang DH, Youn HY. 3D-culture models as drug-testing platforms in canine lymphoma and their cross talk with lymph node-derived stromal cells. *J Vet Sci* 2021; **22**: 25.

[140] De Luca A, Raimondi L, Salamanna F, et al. Relevance of 3d culture systems to study osteosarcoma environment. *J Exp Clin Cancer Res* 2018; **37**: 2.

[141] Flamant E, Cullen MM, Hoskinson SM, et al. A Cross-Species Osteosarcoma Organoid System for Profiling Functional Intratumoral Heterogeneity in Proliferation and Therapy Response. AAOS event September 2 2021, category/classification Musculoskeletal Oncology, Po686.

[142] Graves L, Flamant EM, Plumlee S, et al. Canine-derived Osteosarcoma Organoids Model Tumour Heterogeneity and Chemotherapy Resistance. CTOS annual meeting November 16-19 2022, P171

[143] Abugomaa A, Elbadawy M, Yamanaka M, et al. Establishment of 2.5D organoid culture model using 3D bladder cancer organoid culture. *Sci Rep* 2020; **10**: 9393.

[144] Elbadawy M, Sato Y, Mori T, et al. Anti-tumor effect of trametinib in bladder cancer organoid and the underlying mechanism. *Cancer Biol Ther* 2021; **22**; 357-71.

[145] Elbadawy M, Fujisaka K, Yamamoto H, et al. Establishment of an experimental model of normal dog bladder organoid using a three-dimensional culture method. *Biomed Pharmacother* 2022; **151**; 113105.

[146] Elbadawy M, Fujisaka K, Yamamoto H, et al. Establishment of an experimental model of normal dog bladder organoid using a three-dimensional culture method. *Biomed Pharmacother* 2022; **151**; 113105.

[147] Elbadawy M, Fujisaka K, Yamamoto H, et al. Establishment of an experimental model of normal dog bladder organoid using a three-dimensional culture method. *Biomed Pharmacother* 2022; **151**; 113105.

[148] Usui T, Sakurai M, Nishikawa S, et al. Establishment of a dog primary prostate cancer organoid using the urine cancer stem cells. *Cancer Sci* 2017; **108**: 2383-92.

[149] Elbadawy M, Abugomaa A, Yamawaki H, Usui T, Sasaki K. Development of Prostate Cancer Organoid Culture Models in Basic Medicine and Translational Research. *Cancers (Basel)*; **12**: 777.

[150] Leroy BE, Northrup N. Prostate cancer in dogs: comparative and clinical aspects. *Vet J* 2009; **180**: 149-62.

[151] Jankovic J, Dettwiler M, Fernández MG, et al. Validation of Immunohistochemistry for Canine Proteins Involved in Thyroid Iodine Uptake and Their Expression in Canine Follicular Cell Thyroid Carcinomas (FTCs) and FTC-Derived Organoids. *Vet Pathol* 2021; **58**: 1172-80.

[152] Scheemaeker S, Inglebert M, Daminet S, et al. Organoids of patient-derived medullary thyroid carcinoma: The first milestone towards a new in vitro model in dogs. *Vet Comp Oncol* 2023; **21**: 111-22.

[153] Sato Y, Elbadawy M, Suzuki K, et al. Establishment of an experimental model of canine malignant mesothelioma organoid culture using a three-dimensional culture method. *Biomed Pharmacother* 2023; **162**: 114651.

[154] Seino T, Kawasaki S, Shimokawa M. Human Pancreatic Tumor Organoids Reveal Loss of Stem Cell Niche Factor Dependence during Disease Progression. *Cell Stem Cell* 2018; **22**: 454-67.

[155] Becher OJ, Holland EC. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res* 2006; **66**: 3355-8.

[156] Nakamura T, Mizuno S. The discovery of Hepatocyte Growth Factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci* 2010; **86**: 588-610.

[157] Abugomaa A, Elbadawy M, Yamamoto H, et al. Establishment of a direct 2.5D organoid culture model using companion animal cancer tissues. *Biomed Pharmacother* 2022; **154**: 113597.

[158] Kloker LD, Yurttas C, Lauer UM. Three-dimensional tumor cells cultured employed in virotherapy research. *Oncolytic Virotherapy* 2018; **7**: 79-93.

[159] Kramer N, Pratscher B, Meneses AMC, et al. Generation of Differentiating and Long-Living Intestinal Organoids Reflecting the Cellular Diversity of Canine Intestine. *Cells* 2020; **9**: 822.

[160] Barcellos-Hoff MH, Aggeler J, Ram TG, Bissell MJ. Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* 1989; **105**: 223-35.