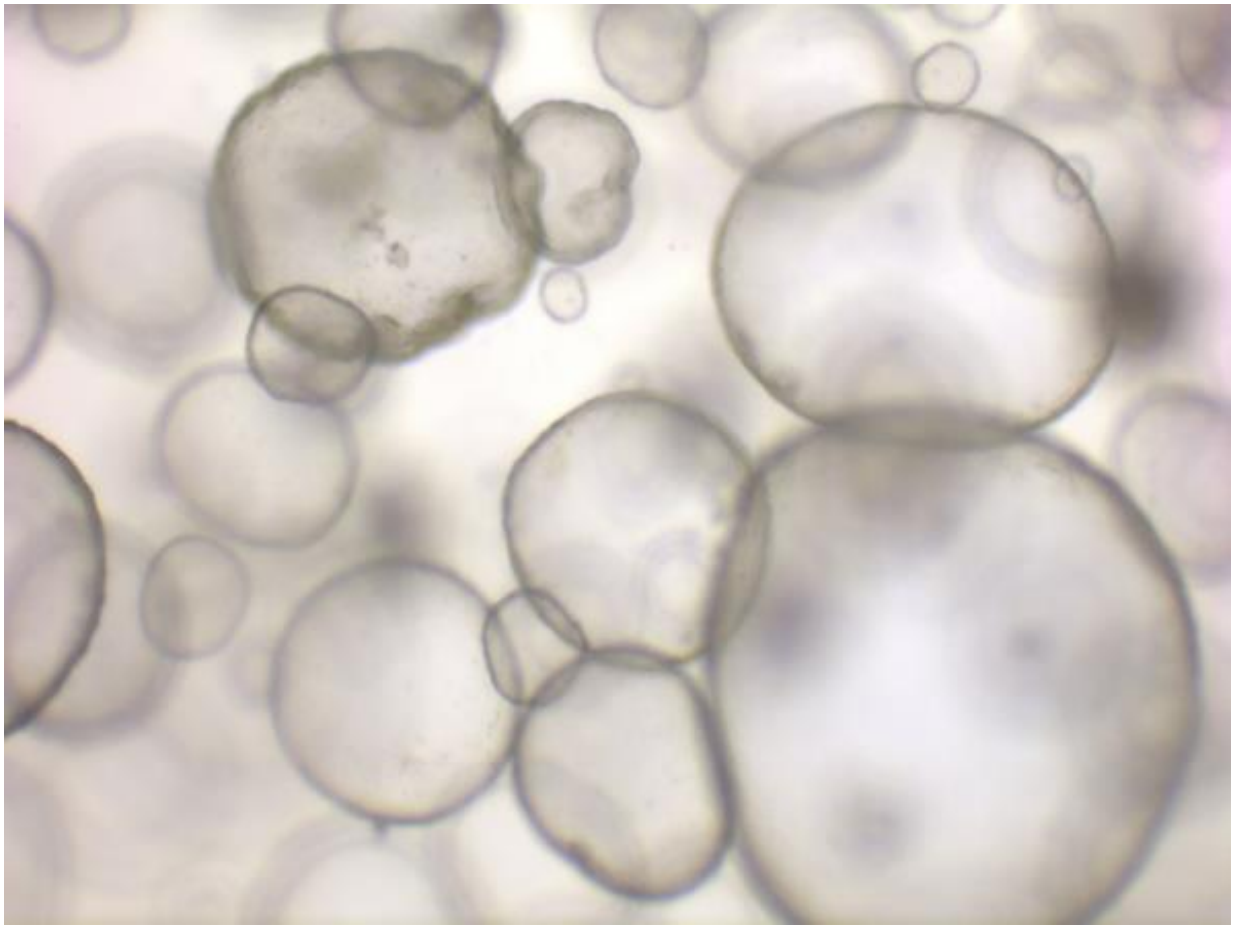
August, 2015

Master Honours Program



Canine liver organoids for disease modeling of copper toxicosis and transplantation

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Abstract

A new culture method for culturing hepatic stem cells in three dimensional cultures is called liver organoids. In this culture method the genomic integrity of the stem cells is maintained and also the differentiation potential of these cells is retained. Liver organoids can be used in disease modeling or cell transplantation. This research is focused on copper toxicosis, a liver disease often seen in Bedlington terriers. This disease has many similarities with Wilson's disease in humans. In the case of copper toxicosis, a mutation in het COMMD1 gene causes copper accumulation in the hepatocyte, which eventually leads to chronic hepatitis and cirrhosis. Current treatment with D-penicillamin has several limitations. The development of a disease model could give more understanding of the underlying mechanisms leading to the copper accumulations, but also new drugs could be tested on this model. The first part of this research was aimed at the development of a model for canine copper toxicosis. Wild type liver organoids, COMMD1 deficient liver organoids and COMMD1 deficient organoids transduced with a vector containing the correct COMMD1 gene were cultured and tested for their ability to take up and accumulate copper. Intracellular copper was measured using a copper sensor (CS1) that becomes red fluorescent when binding intracellular copper. After lysis of the cells the fluorescence intensity was measured and an estimation of the intracellular copper concentration was made. Even though, lots of variations of the disease model have been tested, we didn't succeed to find a disease model that is repeatable and mimics the disease *in vitro.*

The second part of this project focused on using liver organoids for transplantation. Several liver diseases in humans eventually lead to chronic hepatitis and liver cirrhosis, which can only be treated with liver transplantation. However, shortage in liver donors restricts this treatment option. A lot of research has been done on the possibility to transplant cells instead of a complete new liver. Liver organoids are very promising for this use as well. In this study, hepatic progenitor cells were isolated from liver biopsies of dogs with COMMD1 deficiency. They were cultured as liver organoids and genetically corrected by a viral transduction. These transduced cells were then expanded and differentiated towards hepatocyte-like cells. These differentiated organoids were then injected into the portal vein using a port-a-cath. Our hypothesis was that these organoids will engraft in the liver tissue and have a growth advantage in comparison to the dog's own hepatocytes. The liver tissue will eventually be (partly) repopulated with the new cells and a new functional liver will form. When this alternative to liver transplantation is successful in dogs, the next step could be to test this in humans with for example Wilson's disease. We succeeded to transplant two COMMD1 deficient dogs with these patient specific liver organoids. For now, the results of the COMMD1 staining on liver biopsies taken after transplantation look promising. However, more dogs remain to be transplanted and more biopsies from several time-points need to confirm the presence of COMMD1 positive cells to confirm our hypothesis.

General introduction

The liver

The liver is a very important organ in body homeostasis. It plays a central role in a lot of metabolic processes. When the body is being exposed to toxins, the liver is the first organ to get in contact with these toxins, and therefore the liver is often the main site of cell damage[1]. The histology of the liver is quite complex. Parenchymal cells of the liver consist mostly of hepatocytes and cholangiocytes (biliary epithelium). Hepatocytes are the primary functional cells of the liver and make up 75-80% of the total liver volume[2]. They are polarized cells, the apical side aligns the bile canaliculi and the basolateral side aligns the space of Disse, which is separated from the blood sinuses by the endothelium and hepatic stellate cells[3].

The liver tissue consists of lobules (Figure 1), which are hexagonal and contain a portal triad at every corner. The portal triad consists of the vena porta, arteria hepatica and a bile duct. In the center of the hexagonal lobule, there is the central vein which carries the blood to the hepatic vein[1,3].

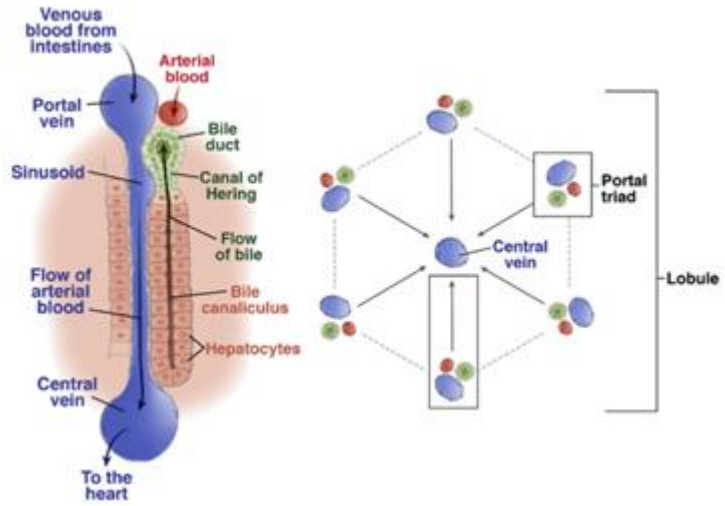

Figure 1. Liver lobule. Schematic view of the hexagonal liver lobule with at each corner the portal triad and the central vein in the middle.
Duncan et al. Gastroenterology 2009.

Due to the anatomy of the liver tissue, the parenchyma can be divided in three zones[1,3] (Figure 2). The cells in zone 1 are closest to the supplying arteria hepatica and vena porta. They will therefore retrieve the most oxygen and this zone will be less sensitive for ischemic injury. On the other hand, in zone 1 the cells are the first to be exposed to certain toxins. So, this zone is more sensitive to toxins carried in the blood[1,3]. Copper accumulation occurs first in the centrolobular region of the liver lobule[1].

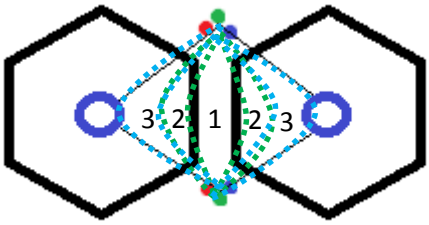

Figure 2. Liver zonation. Schematic view of the three different zones within a liver lobule. Zone 1 is closest to supplying a. hepatica and v. porta and therefore less sensitive for ischemic injury, but more sensitive for intoxication. Copper accumulation starts at the centrolobular region.

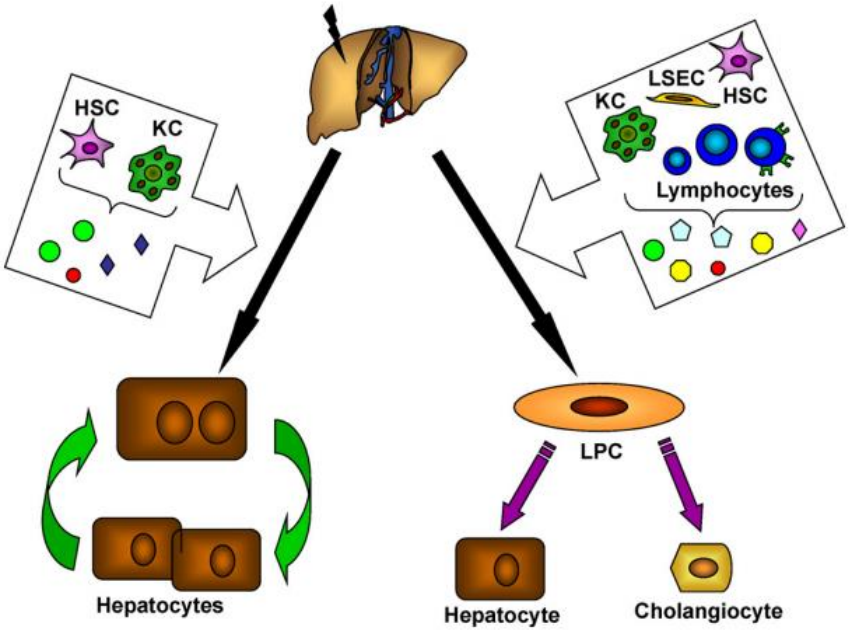

Figure 3. Liver regeneration. When the liver is exposed to acute injury, the adult hepatocytes will proliferate in order to restore the original liver mass. This process is triggered by the production of cytokines by non-parenchymal cells like Kupffer cells (KC) and Hepatic stellate cells (HSC). In case of chronic injury of the liver, the hepatic proliferation is impaired or exhausted. In this case, the hepatic progenitor cells (LPCs) will proliferate and differentiate towards hepatocytes or cholangiocytes. This process is regulated by the cytokines produced as a result of the interaction between inflammatory cells and non-parenchymal cells. LSEC: Liver sinusoid endothelial cells.
Viebahn et al. The International Journal of Biochemistry & Cell Biology 2008

One of the unique qualities of the liver is the ability to regenerate itself[1]. There are two physiological forms of regeneration in the liver: proliferation of mature hepatocytes and regeneration due to the activation of the liver progenitor cell population[4]. Hepatocytes normally have a very low turnover rate[2,5]. In response to acute injury the turnover rate will be strongly increased. When the proliferation of the hepatocytes is impaired or exhausted, due to progressive chronic liver disease for example, the regenerative capacity of the liver is impaired. When this happens, the hepatic progenitor cells will be activated[2,4,5]. Histologically, the activation of progenitor cells is called the ductular reaction[2]. These hepatic progenitor cells reside in the smallest branches of the biliary tree, known as the Canals of Hering[6]. They can proliferate and form new hepatic progenitor cells, but they can also differentiate towards hepatocytes or cholangiocytes[2,4,5]. They are considered to be an adult stem cell compartment of the liver.

Copper associated hepatitis

Copper is an essential element for several biological processes. However, an excess of copper can be extremely toxic. Therefore, copper uptake and excretion is tightly regulated[1]. The hepatocytes also contain a mechanism to excrete excess of copper. A schematic view of this pathway is presented in Figure 4. Copper is transported into the hepatocytes by CTR1[7]. In the cell, the copper can be located to several destinations. The copper can be bound to intracellular glutathione or metallothionein[7]. Next to this, there are several copper chaperones who will shuttle the copper to destination molecules. CCS, COX17 and ATOX1 are examples of these chaperones who shuttle copper to respectively SOD1, cytochrome C oxidase and ATP7B[7]. ATP7B plays a role in the formation of ceruloplasmin, which is then secreted into the blood. Furthermore, ATP7B, together with COMMD1, facilitates the excretion of copper into the bile[7].
Mutations in genes encoding one or more of these proteins can cause accumulation of copper in the hepatocyte, which can have disastrous consequences for the function of the liver. For example, the autosomal recessive disorder Wilson's disease in humans is caused by a mutation in ATP7B[8], which causes copper accumulation in the liver, brain and cornea. In dogs a very similar disease is seen, called

copper toxicosis. Copper toxicosis is an autosomal recessive copper storage disease, which has an high incidence in Bedlington terriers[9]. Genetic mapping of the copper toxicosis gene has shown that a complete deletion of exon 2 of the COMMD1 gene is responsible for this disease in the Bedlington terrier[10]. This deletion results in a complete loss of function of the COMMD1 protein, which underlies the pathology of copper toxicosis[11]. In these dogs the biliary excretion of copper is dramatically reduced[12] resulting in copper accumulation in the lysosomes of the hepatocyte. This excess of copper eventually leads to chronic hepatitis and liver cirrhosis[13]. There are also other copper associated liver diseases with a more complicated genetic background in both humans and dogs[7,14,15].
The first signs of hepatic damage is a minor decrease in serum albumin and an increase in alanine aminotransferase (ALT)[16]. When the disease progresses, the serum albumin will further decrease and ALT, alkaline phosphatase (AP), bile acids, and ammonia will show an increase[16]. However these clinical signs do not distinguish copper toxicosis from other liver diseases. For the Bedlington terrier a DNA test is available, for other dog breeds the diagnosis is confirmed by histological evaluation of a liver biopsy[16]. Evaluation of these biopsies consists of haematoxylin and eosin[17], reticulin[18] and copper staining[19]. These stainings show the inflammatory activity, staging of fibrosis and can be used for the semiquantitative determination of the copper levels[16]. Characteristic for canine primary copper toxicosis is the localization of copper in the centrolobular hepatocytes[1]. Quantitative determination of the copper levels is done by instrumental neutron activation analysis[20] or spectrophotometric methods on a separate biopsy.

In this manuscript, we will focus on hepatic copper accumulation caused by the COMMD1 mutation in Bedlington terriers. Although a lot of functions and interactions of the COMMD1 protein have already been identified in previous studies[21–24], the exact mechanisms leading to copper accumulation due to COMMD1 deficiency are still unknown. Since copper toxicosis in the Bedlington terrier shows great similarity with human Wilson's disease[7,12], new insights in this disease could lead to a better understanding of Wilson's disease in humans. Research on copper toxicosis could also lead to new insights or new therapeutic options for these diseases.

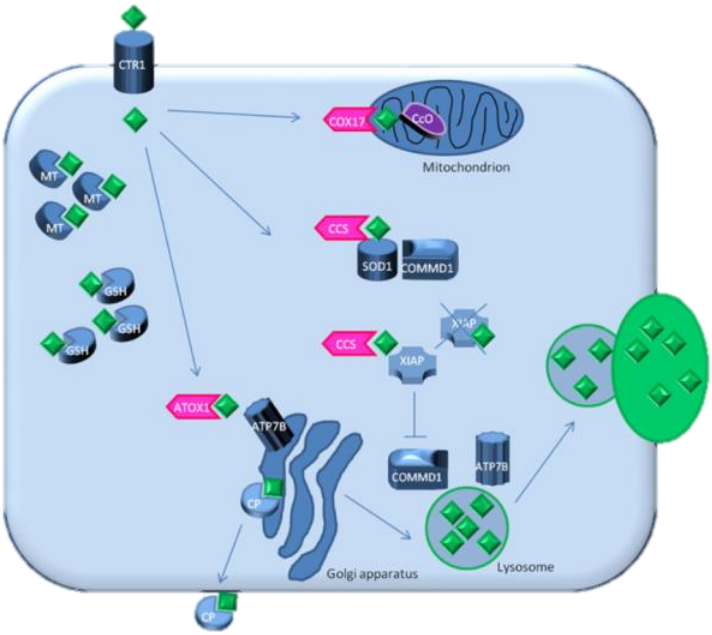

Figure 4. Copper excretion pathway in the hepatocyte. Copper enters the cell through CTR1. Copper can then be bound to metallothionin (MT) or glutathione (GSH). Several chaperones (ATOX1, CCS, COX17) bring copper to their destination protein. ATP7B is involved in the excretion of copper via ceruloplamin (CP) and via the bile. COMMD1 collaborates with ATP7B to excrete copper into the bile.
Fieten et al. Mamm. Genome 2012.

Therapy for copper associated hepatitis

The aim of the therapy nowadays for copper associated hepatitis in both men and dogs is to create a negative copper balance. This is achieved by the copper chelator D-penicillamin, which promotes the urinary excretion of copper[25]. A life-long treatment is often indicated for the Bedlington terrier. D-penicillamin treatment in other breeds with copper associated liver disease is also effective, but there is a risk of zinc and copper deficiency. Other side effects include gastrointestinal signs like vomiting and anorexia[16,26]. In humans more serious side effects occur: nephropathy, bone marrow depression and drug-induced systemic lupus erythromatosis[16]. Dietary intake of low copper levels and high zinc levels has also shown to have a positive effect on the hepatic copper levels in the Labrador Retriever[27]. Monitoring of the treatment effects still relies on evaluation of repeated liver biopsies. Since D-penicillamin often has to be administered life-long and is associated with many side effects, research on new therapeutic options for the treatment of copper-associated hepatitis is required.

In both humans and dogs when chronic hepatitis eventually leads to liver cirrhosis and liver failure, the only therapeutic option is allogeneic liver transplantation[28]. Although, due to the shortage of donor livers this is often not possible[28]. Next to this, allogeneic liver transplantation is a very invasive technique with high risk of surgical complications. Also life-long immunosuppression is required, which is associated with many side effects[28]. In the veterinary clinics, allogeneic liver transplantation is usually not performed. Using mature hepatocytes or stem cells for transplantation would be a good alternative. These cells could also be used as a disease model for studying liver diseases.

Culture systems for cell transplantation and disease modeling

Embryonic stem cells (ESCs) are derived from the inner cell mass of the mammalian blastocysts. These cells can proliferate indefinitely while maintaining the ability to differentiate into cells of all the three germ layers[29]. ESCs can be differentiated towards hepatocyte-like cells by administration of specific growth factors[30]. These hepatocyte-like cells express many hepatocyte related genes and perform hepatic functions. There are a few shortcomings to this method: (1) Protocols for differentiation of ESCs towards hepatocyte-like cells, do not generate fully functional and mature hepatocytes as can be isolated from the liver[5], (2) these cells are allogeneic in nature, so immunosuppression is necessary when using these cells for transplantation, (3) there are some ethical concerns for using these cells, since the embryo dies during isolation of the ESCs[31] and (4) the level of liver repopulation obtained after transplantation of the differentiated ESCs is very low[32].

Induced pluripotent stem cells (iPS) can be created by transduction of fully differentiated adult cells with specific transcription factors[33]. These cells are then able to differentiate towards all cell types of the body. This technique is potentially a good alternative for embryonic stem cells, since you circumvent the ethical concerns of using ESCs and no immunosuppression is needed for iPS cell transplantation. iPS cells also hold potential for disease modeling purposes. A disadvantage of this technique is that the reprogramming process and culturing of iPS cells can cause genetic and epigenetic abnormalities, which can cause neoplastic development of the cells derived from the iPS cells[34–38].

The culturing of primary hepatocytes has shown to be very difficult, since these cells have a limited life span in culture and also undergo rapid dedifferentiation[39]. Several adjustments to the culture conditions have been developed to enhance hepatocyte proliferation and contribute to the retardation of the dedifferentiation process. Although, the expression of liver specific markers is lost regardless of the culture conditions[39].

An *in vitro* model of the liver, which is characterized by long term expression of all liver specific functions and could be used for long term *in vitro* studies and transplantation, does not yet exist.

Liver organoids

Recently, a new culture method for culturing stem cells have been developed. Pluripotent stem cells or progenitor cells isolated from a specific organ can be cultured in a three-dimensional culture system[40]. These cells differentiate to form an organ-like tissue that exhibits multiple organ-specific cell types. These structures show many similarities with the organ *in vivo*, and therefore are called organoids[40]. This culture method has been developed for many organs like gut[41], brain[42,43], kidney[44,45], retina[46] and liver[47,48]. Liver organoids, which consist of hepatic progenitor cells (HPCs), were successfully established for mouse[48], human[47] and dog (*Nantasanti et al.* 2015. Unpublished data). The HPCs reside in the smallest branches of the biliary tree[6], and therefore they can be isolated by isolating ducts from a liver biopsy. When the ducts fragments are embedded in Matrigel and cultured with a defined culture medium, liver organoids will form[47,48].

Like mentioned before, the culture conditions for stem cells are very important to make sure that the cells keep proliferating and maintain their undifferentiated state. Liver organoids can be successfully cultured in Matrigel. Matrigel is considered the optimal matrix for culture of stem cells[49]. When organoids are cultured in Matrigel, they maintain their self-renewal capacity and undifferentiated state[49]. Matrigel consists of a mixture of extracelullar matrix proteins derived from a Englebreth-Holm-Swarm tumor in mice[49,50]. This mixture primarily consists of laminin, collagen IV and enactin. Also, Matrigel contains several growth factors[51]. Not all components of Matrigel have been identified, and also these components are variable. For this reason there can be variability in experimental results, when using Matrigel for culturing stem cells.

Culturing HPCs in this three-dimensional culture system has several advantages: (1) the hepatic progenitor cells retain their differentiation potential over time, (2) the genomic integrity of mouse, dog and human organoids is maintained for over months, (3) the cultured cells in the organoid system are directly obtained from adult tissue without the need for genetic modifications or reprogramming factors, (4) no malignant transformation has been observed after transplantation of mouse or human organoids in mice[47,48,52]. The liver organoid cultures therefore hold promise as a safe clinical source of hepatocytes for transplantation in both humans and animals, as they can be differentiated towards hepatocyte-like cells. *Huch et al.* managed to culture mouse and human liver organoids and also successfully transplant these organoids back into mice[47,48]. Next to this purpose, (differentiated) liver organoids can also be used for modeling liver diseases *in vitro* (Figure 5)[52]. Since liver organoids can be cultured from patient specific liver biopsies, several liver disorders could be modeled *in vitro* using liver organoids. This model can be used to study the development of the disease, but also for testing new therapeutic compounds (personalized medicine).

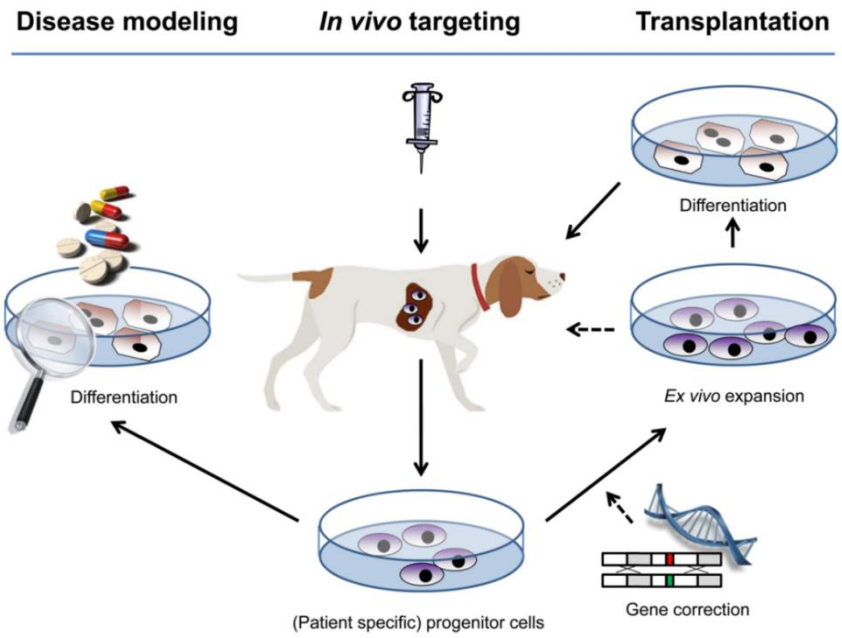

Figure 5. Possible purposes for liver organoids. Patient-specific hepatic progenitor cells can be cultured as liver organoids. These liver organoids can be used for disease modeling and drug testing, but also for transplantation. In case of a genetic disorder of the patient, the hepatic progenitor cells can be genetically corrected and expanded before transplanting the cells back into the patient.
Schotanus et al. Veterinary Quarterly 2014.

Position in faculty program

The faculty of Veterinary Medicine has several research programs including the research program Regenerative medicine, stem cells and cancer. Within this research field, there are several groups with their own expertise. My research project was part of the research in the liver group. This group mainly focuses on copper associated hepatitis, liver regeneration and translational medicine.

Part A: Disease modeling

Abstract

Copper toxicosis is an autosomal recessive disorder often seen in the Bedlington terrier. This disease is caused by a mutation in the COMMD1 gene, which leads to complete loss of the protein. This protein is involved in the copper excretion pathway in the hepatocyte. Copper toxicosis is therefore characterized by copper accumulation in the hepatocytes, which will eventually lead to chronic hepatitis and liver cirrhosis. The therapy nowadays is aimed at creating a negative copper balance in the body, using D-penicillamin. Since this is a life-long treatment and it is associated with many side effects, more research is required on alternative treatment options. To achieve this, we aimed to develop a disease model for copper toxicosis using canine liver organoids. Although, many variations in the model were tested, we did not succeed to create a model that is repeatable and mimics the disease *in vitro*.

Introduction

Copper toxicosis is an genetic disorder often seen in the Bedlington terrier[9]. It is associated with hepatic copper accumulation, and it eventually leads to chronic hepatitis and cirrhosis[12,13]. In other dog breeds, the same phenotype is seen associated with a more complicated genetic background[7]. Copper toxicosis also has many similarities with Wilson's disease in humans[7,12]. The current treatment for all these copper associated liver diseases is D-penicillamin, which promotes the urinary excretion of copper[25]. This eventually leads to a decrease of hepatic copper levels[26], although the exact mechanism of D-penicillamin is still unclear. D-penicillamin is a life-long treatment, associated with several side effects[16,26]. To find new therapeutic options for these diseases and discover the mechanisms behind D-penicillamin treatment, we aim to develop a disease model for copper toxicosis. A disease model is an *in vitro* model that mimics the events happening in a disease *in vivo*. An *in vitro* disease model can be used to study the exact pathways and mechanisms occurring during the disease, but also several drugs can be tested on this model. An important advantage for using an *in vitro* model is that there is no need for animal experiments.

The model for copper toxicosis will be developed using canine liver organoids, established by *Nantasanti et al*. 2015 (Unpublished data). Liver organoids consist of hepatic progenitor cells (HPCs) and can be cultured from patient specific liver biopsies by isolating ducts[40]. These ducts are embedded in Matrigel and treated with a defined culture medium, which will lead to the formation of spherical structures. The addition of a specific differentiation medium (*Nantasanti et al.* 2015. Unpublished data) will lead to differentiation of liver organoids to hepatocyte-like cells. Several studies have investigated the use of organoids for disease modeling purposes. Intestinal organoids have been used for disease modeling of cystic fibrosis[53,54]. These organoids demonstrated the defective chloride channel and the therapeutic effect of corrector compounds could be tested on this model[53]. Human liver organoids retrieved from a liver biopsy of an Alagille syndrome patient, also demonstrated the structural duct defects present in these patients[47]. Since these organoids exhibit the disease occurring in a specific patient, drugs can be tested to find the most effective drug for that patient (personalized medicine). We hypothesize that isolation of ducts from a liver biopsy of a COMMD1 deficient dog, will lead to liver organoids that exhibit the phenotype of copper toxicosis. In this study, differentiated liver organoids retrieved from COMMD1 deficient dogs and wild type dogs will be used. The intracellular copper concentration in these hepatocyte-like cells will be measured using a copper sensor (CS1), developed in previous studies[55]. This copper sensor gives a fluorescent staining when bound to intracellular copper. The fluorescence of the cells will then be measured and the amount of intracellular copper can be estimated. We hypothesize that the COMMD1 deficient organoids will show higher copper sensor concentration in comparison to wild type organoids when they are both treated with copper. We also aim to test several drugs on this disease model to see their effect *in vitro*.

Material and methods

Liver samples

The use of liver tissues from dogs was approved by the Utrecht University's ethical committee, as required under Dutch legislation (ID 2007.III.06.080). Liver samples from healthy dogs (from now on will be called 'wild type') were obtained from surplus material from dogs used in non-liver related research projects. COMMD1 deficient dog liver samples were obtained from Paul, a COMMD1 deficient dog involved in the transplantation project (DEC approval: 2014.III.12.112), Sylvia, Viola and Paula, who were involved in a previous study on COMM1 deficiency of *Favier et al*[56].
Human liver tissue for the culture of human organoids was obtained from Dr. Luc van der Laan (Erasmus MC, Rotterdam, The Netherlands). Human Wilson's disease liver tissue was provided by Dr. Hans Zischka (Institute of Molecular Toxicology and Pharmacology, München, Germany).

Duct isolation and Organoid culture

Dog liver organoids were derived from fresh or frozen liver tissue collected by biopsies. Duct isolation of this tissue was done using 0,3 mg/ml collagenase and 0,3 mg/ml dispase (Life technologies) in DMEM Glutamax containing 1% FCS (Life technologies) and 1% Penicillin/streptomicin (Pen/Strep, Life technologies) at 37 °C. Every twenty minutes the supernatant was checked for ductal structures. When ductal structures appeared, the supernatant was collected and centrifuged at 80 g for 5 min. The pellet was then resuspended in Matrigel (BD Biosciences) and cultured using 250 ml of medium per well in 48 wells plate (Greiner Bio One).
For *in vitro* expansion of the dog liver organoids expansion medium (EM) was used. The expansion medium was based on advanced DMEM/F12 (Life technologies) supplemented with 1% Pen/Strep, 1% Glutamax (Life technologies), 1% HEPES buffer (Life technologies), 30% Wnt conditioned medium (Hubrechts Institute), 1.25 μM N-acetylcysteine (NAC, Sigma-Aldrich), 1% N2 (Life technologies), 2% B27 (Life technologies), 200 ng/ml Epidermal Growth Factor (EGF, Peprotech), 100 ng/ml Fibroblast Growth Factor 10 (FGF10, Peprotech), 10 nM gastrin (GAS, Sigma-Aldrich), 100 ng/ml Noggin (NOG, Peprotech), 25 ng/ml Hepatocyte Growth Factor (HGF, Peprotech), 10 mM nicotinamide (NIC, Sigma-Aldrich), 5% R-spondin-1 conditioned medium (Hubrechts Institute), 10 μM Rho kinase inhibitor (ROCKi, Sigma-Aldrich), 0.5 μM A83-01 (Tocris Bioscience). For the differentiation of the organoids towards hepatocyte-like cells a differentiation protocol (Figure 6) was used, which consists of different media compositions for different time-points during differentiation. Organoids intended for differentiation were seeded on day -2 and fed for two days with EM. On day 0 of the differentiation process, the organoids were fed with Weaning Medium (WM), where Wnt conditioned medium, noggin and Rock inhibitor were withdrawn from the medium. WM was supplemented to the organoids to prevent that the transition between EM and the differentiation medium has a deleterious impact on the viability of the cells. On day 2 the organoids were fed with Differentiation Medium (DM) in which also gastrin, HGF, NIC and R-spondin were withdrawn and 10 nM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma-Aldrich) was added to the medium. From day 6, 30 μM Dexamethasone (Sigma-Aldrich) was added to the differentiation medium. For an overview of the specific contents of these different media see table 1.
The organoids were cultured in a humidified atmosphere at 37 °C with 5% CO_2 in air. The culture media was replaced every Monday, Wednesday and Friday. The organoids were passaged when the Matrigel was completely filled with organoids, which was about once a week (usually split 1:8). For the copper experiments the organoids were plated on a 48-wells plate. In some copper experiments viral transduced organoids were also involved. For the methods of the viral transduction of organoids, see part B (p. 44 and 45). For culture of human organoids the culture methods published by Huch *et al.* were used[47].

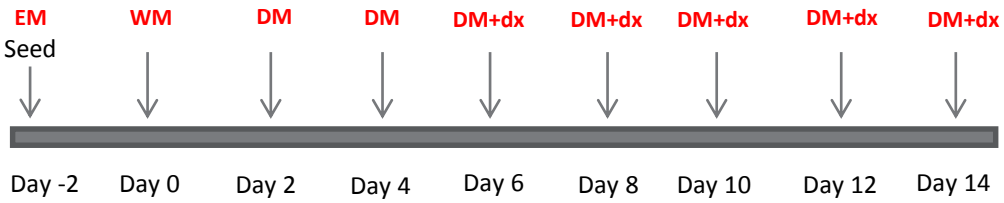

Figure 6. Differentiation protocol. Schematic view of the differentiation protocol. The organoids were seeded on day -2 and treated with expansion medium (EM). On day 0 the organoids got weaning medium (WM). The organoids obtained differentiation medium (DM) on day 2 and 4. From day 6, the organoids were treated with DM supplemented with 30 µM dexamethasone.

Cell lines

For the drug testing pilot, two type of cell lines were used: human hepatoma cell line (Huh7) and human hepatic progenitor cell line (HepaRG). Huh7 cells are hepatocyte-like cells and were purchased at JCRB Cell Bank (JCRB0403). HepaRG cells are hepatic progenitor-like cells and were obtained from BioPredic International (Rennes, France). Huh7 cells were cultured in advanced DMEM/F12, 10% FCS and 1% Pen/strep. The culture medium for HepaRG cells consists of Williams Medium E (Life Technologies) with 10% FCS, 5 µg-ml insulin (Sigma-Aldrich), 50 µM hydrocortisone hemisuccinate (Sigma-Aldrich), and 1% Pen/strep. Cells were cultured in a T75 culture flask (Greiner Bio One) and passaged when fully confluent. Medium was changed once a week. For the drug testing pilot, 500 µl of a cell suspension containing 100,000 cells/ml was added to each well of a 24 wells plate (Greiner Bio One) one day prior to the experiment.

Quantitative real-time PCR

RNA isolation of the liver organoids and liver tissue was performed using RNeasy micro kit (Qiagen). After RNA isolation the RNA was converted to cDNA using the iScript kit (BioRad). The BioRad CFX 384 real time system/C1000 Touch thermo cycler (BioRad) was used for qPCR. The detection of PCR products was enabled by a fluorescent reporter molecule (SYBR green).

Four reference genes were used for normalization, based on their stable expression in liver: Ribosomal protein S5 (RPS5), Hypoxanthine-guanine phosphoribosyl-transferase (HPRT), Ribosomal protein L8 (RPL8), Ribosomal protein S19 (RPS19). The genes of interests were selected for their role in the copper excretion pathway of the hepatocyte: Adenosine triphosphatase 7A (ATP7A), X-linked inhibitor of apoptosis protein (XIAP), Glutamate-cysteine ligase catalytic subunit (GCLC), Cytochrome c oxidase 17 (COX17), Metallothionein-like protein type 2 (MT2), Glutathione synthetase (GSS), Metallothionein-1E (MT1E), Glutathionreductase (GSR), Adenosine triphosphatase 7B (ATP7B), Glutathione peroxidase 1 (GPX1), Ceruloplasmin (CP), Copper transport protein 1 (CTR1), Copper metabolism gene MURR1-containing domain 1 (COMMD1), Methionine adenosyltransferase 1 alpha (MAT1A), Superoxide dismutase 1 (SOD1), Copper chaperone for superoxide dismutase (CCS), Antioxidant 1 copper chaperone (ATOX1), Heme oxygenase (HO), Mathionine adenosyltransferase 2 alpha (MAT2A).

As a differentiation readout, the expression of several hepatic and stem cell markers were measured after the first differentiation experiment. SRY (Sex Determining Region Y)-Box 9 (SOX9) is a stem cell marker, highly expressed in stem cells. On the other hand, Cytochrome P450 3a4 (Cyp3a4), Hepatocyte nuclear factor 4 alpha (HNF4α) and Glucose-6-Phosphatase catalytic subunit (G6PC) are hepatic markers. Primers were developed using Perl Primer v1.1.14 and ordered at Eurogentec.

In table 2 you can see an overview of the primers and PCR conditions.

	Company	Expansion medium (EM)	Differentiation medium day 0 (Weaning medium, WM)	Differentiation medium day 2 – 4 (DM)	Differentiation medium day 6-14 (DM + dx)
Advanced DMEM/F12	Life technologies	+	+	+	+
Penicillin/streptomycin	Life technologies	+	+	+	+
Glutamax	Life technologies	+	+	+	+
HEPES buffer	Life technologies	+	+	+	+
Wnt conditioned medium	Hubrechts Institute	+	-	-	-
N-acetylcysteine (NAC)	Sigma-Aldrich	+	+	+	+
N2	Life technologies	+	+	+	+
B27	Life technologies	+	+	+	+
Epidermal Growth factor (EGF)	Peprotech	+	+	+	+
Fibroblast growth factor 10 (FGF10)	Peprotech	+	+	+	+
Gastrin (GAS)	Sigma-Aldrich	+	+	-	-
Noggin (NOG)	Peprotech	+	-	-	-
Hepatocyte growth factor (HGF)	Peprotech	+	+	-	-
Nicotinamide (NIC)	Sigma-Aldrich	+	+	-	-
R-spondin	Hubrecht institute, the Netherlands	+	+	-	-
Rho kinase inhibitor (Rocki)	Sigma-Aldrich	+	-	-	-
A8301	Tocris Bioscience	+	+	+	+
DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester)	Sigma-Aldrich	-	-	+	+
Dexamethasone	Sigma-Aldrich	-	-	-	+

Table 1. Different media compositions. An overview of the different media types and their compositions.

Gene	Primer sequences	Tm (°C)	Product size (base pairs)
ATP7A	F: CTACTGTCTGATAAACGGTCCCTAAA	50	99
	R: TGTGGTGTCATCATCTTCCCTGTA		
XIAP	F: ACTATGTATCACTTGAGGCTCTGGTTTC	55	80
	R: AGTCTGGCTTGATTCATCTTGTGTATG		
RPL8	F: CCATGAATCCTGTGGAGC	55	64
	R: GTAGAGGGTTTGCCGATG		
GCLC	F: CAAACCCAAACCATCCTACC	59	129
	R: GGCGTTTCCTCATGTTGTC		
HPRT	F: AGCTTGCTGGTGAAAAGGAC	59	104
	R: TTATAGTCAAGGGCATATCC		
COX17	F: ATCATTGAGAAAGGAGAGGAGCAC	60	127
	R: TTCATTCTTCAAGGATTATTCATTTACA		
MT2	F: GATGTGGGGGAGAGCCTATT	60	96
	R: TTGGACCCAAAACAAAAAGC		
GSS	F: CTCCTCTGATGTGGTAAGC	61	110
	R: CCACCAGCATGTTGAAGTC		
MT1E	F: AGCTGCTGTGCCTGATGTG	61	130
	R: TATACAAACGGGAATGTAGAAAAC		
GSR	F: TTCAACCACCTTTACCCCAATGTATC	61	103
	R: GATCCCAACCACCTTTTCCTCCA		
ATP7B	F: ATCCTGAAATCATCGGTCC	61	131
	R: CTTCTTCCACTGCTTTATTTCC		
GPX1	F: GCAACCAGTTCGGGCATCAG	62	123
	R: CGTTCACCTCGCACTTCTCAAAA		
CP	F: AATTCTCCCTTCTGTTTTTGGTT	62	97
	R: TTGTTTACTTTCTCAGGGTGGTTA		
CTR1	F: GGATCGAATCCAAACACAAGTC	62	94
	R: GTCTGCAGCCCTTTATCC		
COMMD1	F: CCTGAGTTGGAGAGTTGAC	62	114
	R: AGAAATTCTGATTCCTGTCCAC		

Gene	Primer sequences	Tm (°C)	Product size (base pairs)
MAT1A	F: CACTGTCCATTTCCATCTTCACCT	63	128
	R: GGGCTTCTTCAAATCCAAATCC		
B2MG	F: TCCTCATCCTCCTCGCT	63	85
	R: TTCTCTGCTGGGTGTCG		
RPS19	F: CCTTCCTCAAAAAGTCTGGG	63	95
	R: GTTCTCATCGTAGGGAGCAAG		
RPS5	F: TCACTGGTGAGAACCCCCT	63	141
	R: CCTGATTCACACGGCGTAG		
SOD-1	F: TGGTGGTCCACGAGAAACGAGATG	64	99
	R: CAATGACACCACAAGCCAAACGACT		
CCS	F: TGTGGCATCATCGCACGCTCTG	64	96
	R: GGGCCGGCCTCGCTCCTC		
ATOX1	F: ACGCGGTCAGTCGGGTGCTC	67	137
	R: AACGGCCTTTCCTGTTTTCTCCAG		
HO	F: GGGCGTCGACTTCTTCACCTT	67	99
	R: ACCTCGGGCGTCATCTCCA		
MAT2A	F: TGCTTTTGGCGGGGAGGAG	67	121
	R: TTTAAAAGCTGCCATCTGAGGTGA		
CYP3a4	F: AGTATGGAGATGTGTTGGTG	58	151
	R: TCTTGTGGGTTGTTGAGG		
HNF4α	F: CATGGTGTTCAAGGATGTTCTG	64	144
	R: TTGTCATCGATCTGTAGCTCC		
G6PC	F: CACATCTACCCTTTCTATCTTTCGG	63	91
	R: AGCCCAGAATCCCAACCA		
SOX9	F: CGCTCGCAGTACGACTACAC	63	105
	R: GGGGTTCATGTAGGTGAAGG		

Table 2. Primer sequences and PCR conditions of genes of interest. An overview of the genes of interest and their primer sequences and PCR conditions.

Copper incubation experiments

For the copper incubation experiments $CuCl_2.2H_2O$ (Fluka Chemika) was used. $CuCl_2$ was diluted in culture media for organoids (EM or DM) to retrieve end-concentrations of 100, 250, 500 or 1000 µM $CuCl_2$. The organoids were incubated with the copper medium for 3, 6 or 24 hours in the culture stove at 37°C. The copper concentrations and incubation times were based on pilot experiments (data not shown). In the 'long term' experiments the organoids were incubated with the copper medium for 8 or 20 days. The organoids were either still in the Matrigel when incubated with copper medium or the organoids were first harvested with cold Advanced DMEM/F12 in a 15 ml tube, fragmented using mechanical dissociation and then incubated with copper medium.

Measuring intracellular copper

Copper sensor 1 (CS1) was kindly provided by Jeff Chan (Howard Hughes Medical Institute, University of California). After copper incubation, the organoids were washed and incubated with 5 µM of CS1 in Hank's balanced salt solution (HBSS, Life technologies) containing 0.25% dimethylsulfoxide (DMSO) for 20 minutes at 37°C. After the copper sensor incubation organoids were lysed with RIPA (Tris-HCl: 50 mM, pH 7.4: NP-40 1%, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, PMSF 1 mM, Aprotinin, leupeptin, pepstatin 1ug/ml each, Na3VO4 1 mM, NaF 1 mM) on ice on an orbital shaker for 10-30 minutes. After lysis the lysate was transferred to a 48 or 96 wells plate (Greiner Bio One). Fluorescence was measured by a TECAN plate reader (Infinite M200) with excitation wavelength 488 nm, emission wavelength 550 nm, excitation bandwidth 9 nm, emission bandwidth 20 nm and the gain set at optimal.

Protein assay

To correct the copper sensor values, the protein levels were measured using a Lowry assay (DC protein assay, Biorad Laboratories). The protein standard was made using BSA (Bovine serum albumin) protein from 0,25 mg/ml to 1,5 mg/ml in RIPA. Absorbance was measured at 650 nm in a TECAN plate reader (Infinite M200) or with a photospectrometer (Beckman Coulter, DTX880 multimode detector).

Viability read-out

To measure viability, an Alamar blue assay was performed. Organoids were incubated with 5% Alamar blue (Invitrogen) in advanced DMEM/F12 without phenol red (Life technologies) for 40-120 minutes at 37°C. Fluorescence was measured by a TECAN plate reader (Infinite M200) with excitation wavelength 540 nm, emission wavelength 590 nm and the gain set at 100.

Liver perfusion for retrieving primary hepatocytes

For the copper experiments on primary hepatocytes, hepatocytes were isolated from the liver using a slightly modified 3-step protocol for liver perfusion, published by *Arends et al*[57]. The liver was first perfused with perfusion buffer containing 0,5 mM EGTA (0,14 M NaCl, 6,7mM KCl and 10mM HEPES, pH: 7,4) for 15 minutes (30 ml/min). Second, the liver was perfused with perfusion buffer without EGTA for 15 minutes (30 ml/min). Finally, the liver was perfused with liberase solution for 20 minutes (25 ml/min), containing 4,8 mM $NaCl_2 2H_2O$ and 0,14 wunsch units/ml of liberase (Roche). The released liver homogenate was put through a filter, and the cell suspension was put through a 70 µm nylon mesh filter (BD Biosciences). Percoll (Amersham Biosciences) was then added to the cell suspension to a final concentration of 55%, and the tubes were spun at 50 g for 10 minutes at 4°C. The cell pellet contained the fresh hepatocytes and could then be seeded for culturing using a density of $3.5*10^5$ cells/ml. These cells were cultured on a 24 wells plate coated with collagen I (Sigma-Aldrich), or on a 24 wells Primaria plate (Corning). The media for culturing hepatocytes consisted of Hepatozyme (Life technologies), 10% FCS and 1% Pen/strep.

Drug testing on disease model

D-penicillamin (DP) was obtained at the pharmacy of Clinical Sciences of Companion Animals in Utrecht, The Netherlands. DP and bathocuproinedisulfonic acid (BCS, Sigma-Aldrich) were diluted in regular medium for organoids (EM or DM) or medium for cell lines (Huh7 or HepaRG) to achieve final concentrations of 1000 µM, 5000 µM, 8 mM, 15 mM and 25 mM (DP), and 50 µM, 125 µM, 250 µM, 500 µM, 1000 µM, 2500 µM and 5000 µM (BCS). The drugs were either incubated after the copper incubation or were added in the medium together with the $CuCl_2$. The incubation time of the drugs was 3 hours or overnight. The concentrations and incubation times of these drugs were based on pilot experiments and literature[11,58–63]. After drug incubation intracellular copper was measured using CS1 as described before.

Statistical analysis

For statistical analysis IBM SPSS Statistics version 22 was used. Non-parametric Kruskall Wallis and Mann-Whitney U tests were done on the data, since none of the data was normally distributed. Differences were stated significant when $P \leq 0,05$.

Results and discussion

Gene expression levels of copper pathways associated genes in canine liver organoids

Undifferentiated (EM) canine liver organoids were compared with differentiated (DM) canine liver organoids, to see which of these is better suitable in the disease model. Figure 7 shows the relative expression of the copper pathway associated genes, with the left hepatocyte representing undifferentiated organoids and the right hepatocyte representing differentiated organoids. For GCLC, SOD1, CCS, ATOX1, XIAP, MAT2A, CTR1, MT2, COX17, CP, GPX1, MT1E and ATP7B the gene expression was significantly higher in DM organoids compared to EM organoids. Appendix 1 contains the gene expression graphs of all the copper pathway associated genes in Sylvia (COMMD1 deficient) organoids. The same experiment was done on wild type organoids, and the same pattern was seen here (data not shown).

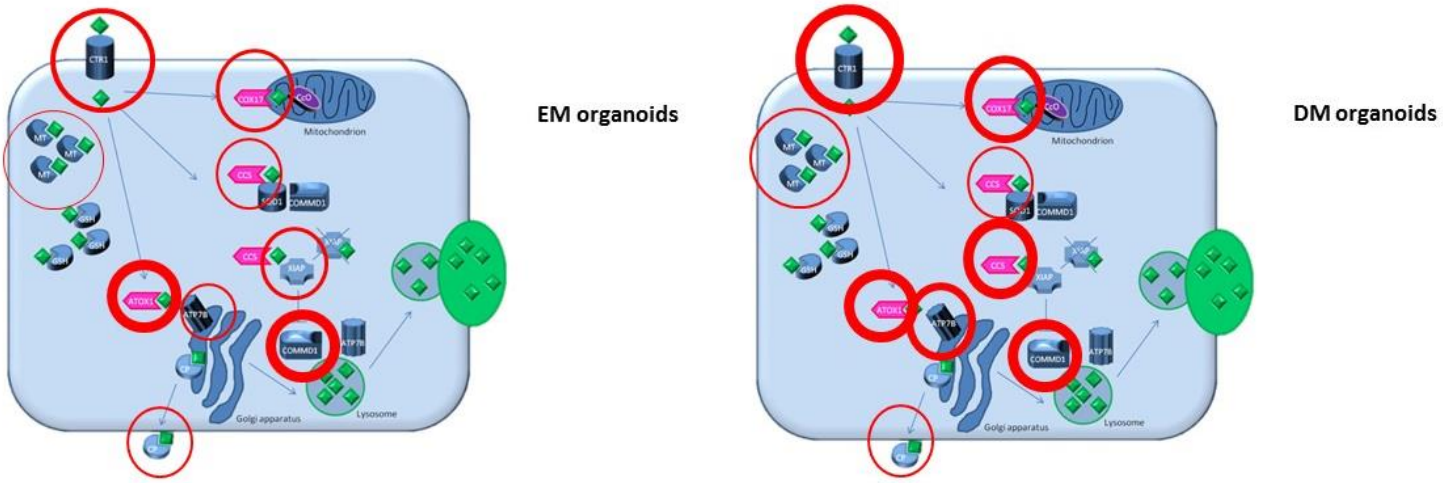

Figure 7. Gene expression of copper pathway associated proteins. Schematic representation of the relative expression of copper pathway associated genes of undifferentiated organoids (EM) in comparison to differentiated organoids (DM). The differentiated organoids show higher (thicker red circle) expression of most of the copper pathway associated genes.

Fluorescence measurement upon copper sensor treatment

For estimation of the intracellular copper concentration we obtained a copper sensor (CS1) that becomes red fluorescent when bound to copper. By measuring the intensity of the fluorescence, the amount of intracellular copper can be quantified. To verify the working mechanism of the copper sensor, organoid fragments were treated with 500 µM $CuCl_2$, the copper sensor was added and the fluorescence was observed using the fluorescence microscope (Figure 8). The negative control consists of cells without copper sensor treatment, and do not show any fluorescence (Figure 8, A). The cells treated with copper sensor become red fluorescent (Figure 8, B).

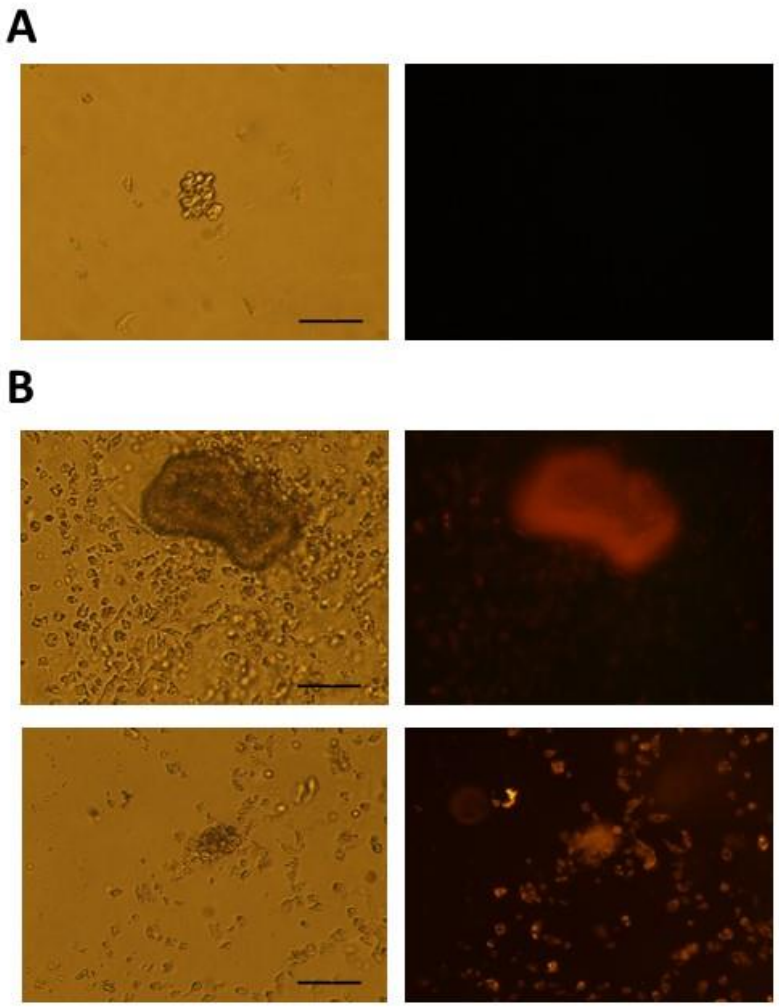

Figure 8. First copper experiment. On the left pictures in bright field. A: negative control (no copper sensor added). B: cells treated with 500 µM $CuCl_2$ for 3 hours.
All scale bars represent 500 µm.

Copper accumulation in canine liver organoids

Differentiated wild type organoids were compared with COMMD1 deficient organoids and Viola transduced organoids. These organoids were retrieved from Matrigel prior to the copper incubation of 3 hours with 0, 500 and 1000 µM $CuCl_2$. All organoid types showed a significant increase in fluorescent units after copper treatment. Also, COMMD1 deficient organoids showed significantly higher levels of copper sensor concentration than wild type and transduced organoids at 0, 500 and 1000 µM $CuCl_2$. Wild type organoids had a significant higher level of copper sensor concentration than transduced organoids (Figure 9, B).

Even though the transduced and wild type organoids should have the appropriate pathways for excretion of copper, these organoids showed an increase in fluorescent units after treatment with copper. A possible explanation is that the cells need time to set a new equilibrium of the amount of copper in and outside the cell, before they can start with the excretion of the excess of copper. Next to this, the administered copper concentration could be too much for wild type and transduced organoids to excrete as well. The transduced organoids might have lower levels of copper sensor concentration than wild type organoids, because the transduced organoids have an overexpression of the COMMD1 protein. The transduction of the organoids with the lentivirus can lead to integration of more than one copy into the genome of the organoids, which will lead to more expression of the protein than seen in wild type.

To confirm differentiation, gene expression of several hepatic markers and stem cell markers was measured (Figure 10). Gene expression of hepatocyte markers HNF4a and G6PC was increased upon differentiation but did not reach the levels that were measured in normal liver. Expression of the stem cell marker SOX9 was significantly decreased when the organoids were differentiated. Also for SOX9, the expression level of differentiated organoids was significantly different from normal liver. To conclude, the differentiated organoids show more expression of hepatic markers and less expression of stem cell markers, which indicates a differentiation towards hepatocyte-like cells.

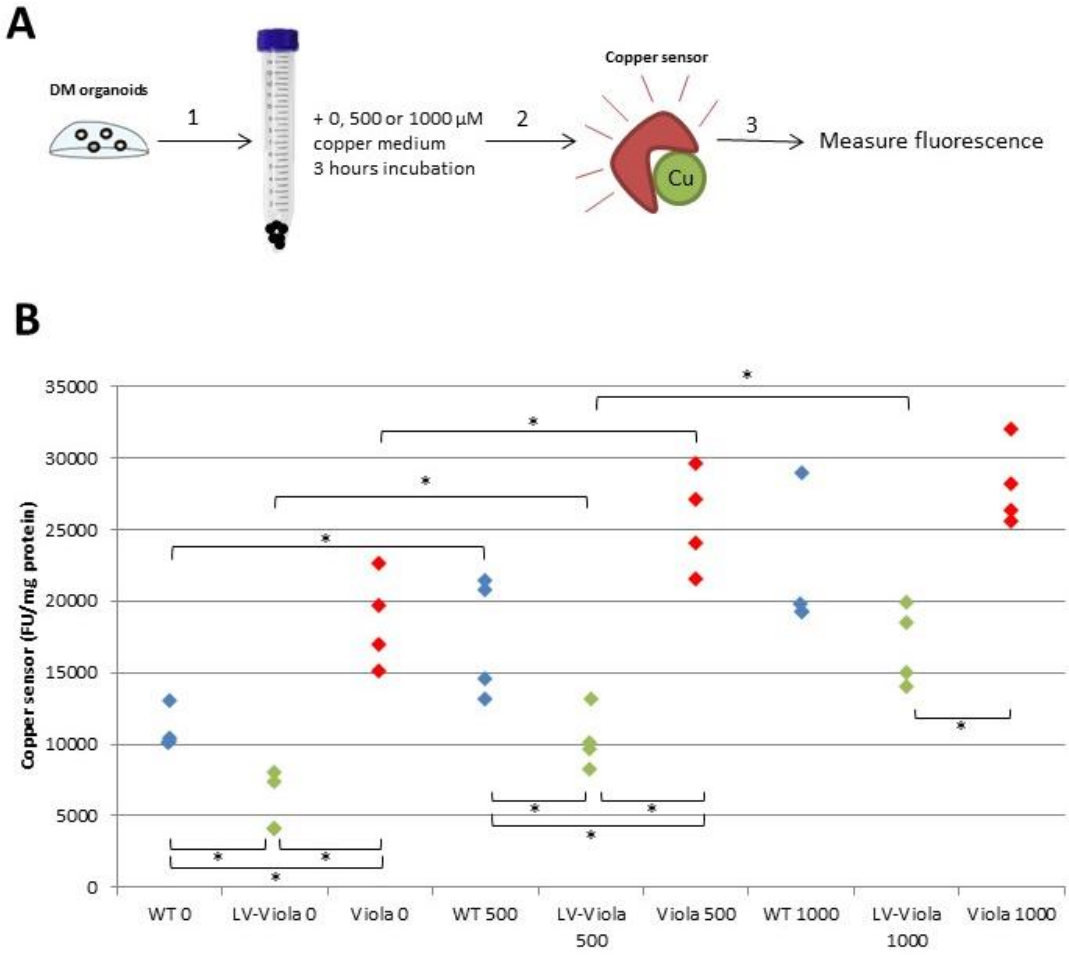

Figure 9. Copper experiment 1. A: schematic view of experimental design, DM organoids were retrieved from the Matrigel (1) and incubated with copper medium in the tube. The cells were washed with HBSS (2) and the copper sensor was added. The cells were then washed, lysed and fluorescence was measured (3). B: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment.
WT: wild type liver organoids, LV-Viola: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene. Viola: COMMD1 deficient liver organoids. 0, 500 and 1000: 0, 500 or 1000 µM $CuCl_2$.

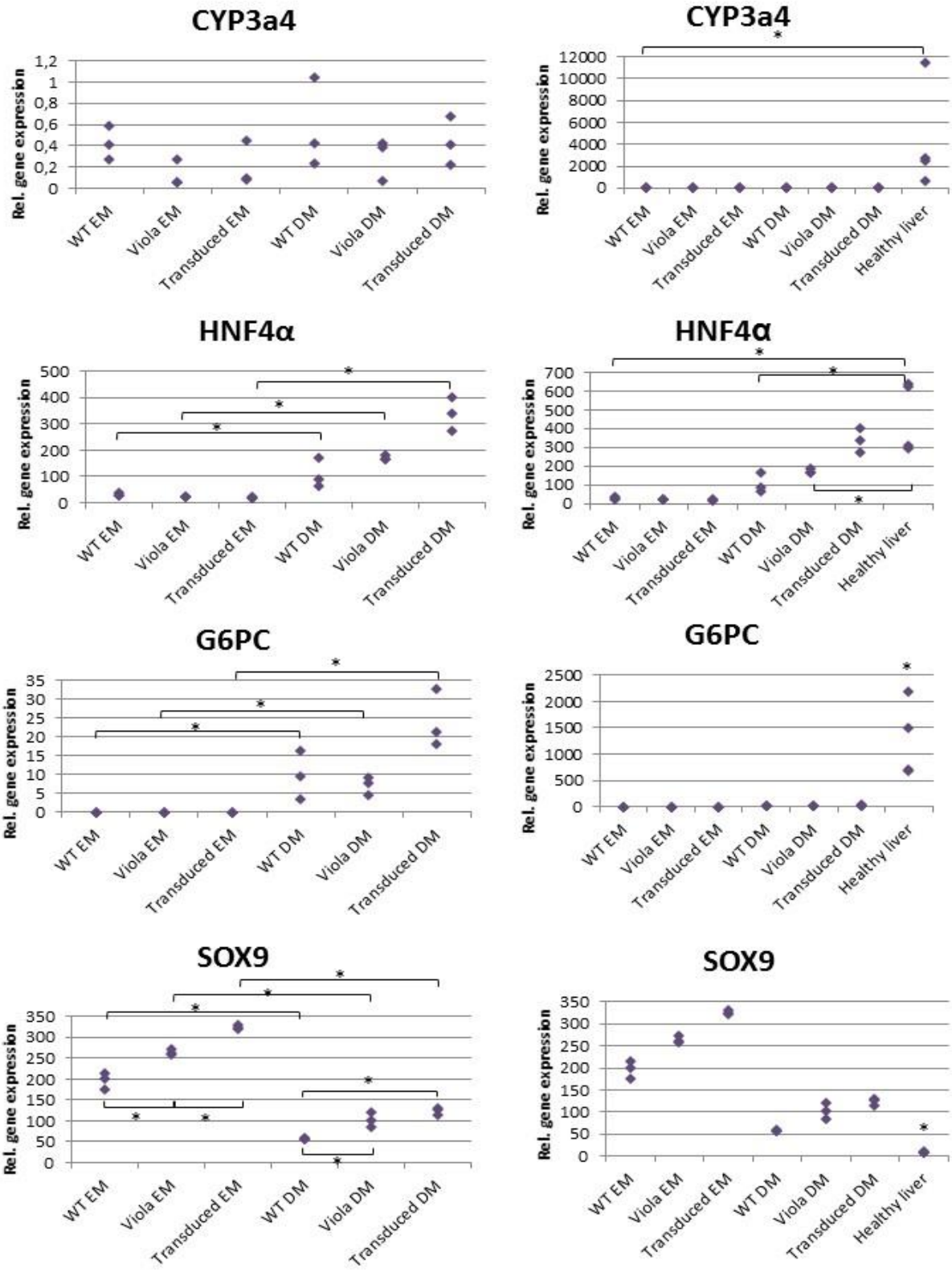

Figure 10. Gene expression. Gene expression levels of three hepatic markers (Cyp3a4, HNF4α and G6PC) and one stem cell marker (SOX9). Differentiation of liver organoids leads to an significant increase of the expression of HNF4α and G6PC. The expression of SOX9 is significantly decreased.
WT: wild type liver organoids, Viola: COMMD1 deficient liver organoids, transduced: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene, DM: differentiated organoids, EM: undifferentiated organoids.

Optimizing experimental procedure: influence of Matrigel

We tested if we could also keep the organoids in the Matrigel during the copper incubation in copper experiment 2(Figure 11, A). The protocol for retrieving the organoids out of the Matrigel prior to the copper incubation is time consuming and involves many steps where organoids can be lost. This protocol therefore induces more variability. Copper experiment 2 resulted in the same outcome as seen in copper experiment 1 (Figure 9). COMMD1 deficient organoids showed higher levels of copper sensor concentration than wild type organoids at 0 and 500 µM $CuCl_2$. Also, the organoids had significantly higher copper sensor concentrations when treated with $CuCl_2$. These organoids are undifferentiated contrary to the previous experiments where the organoids were differentiated. We concluded from experiment 2 that organoids in Matrigel will accumulate copper when this is provided in the medium. Matrigel is permeable enough for copper during a three hour incubation. Since this protocol was less time consuming and induced less variability, we decided to continue using this method.

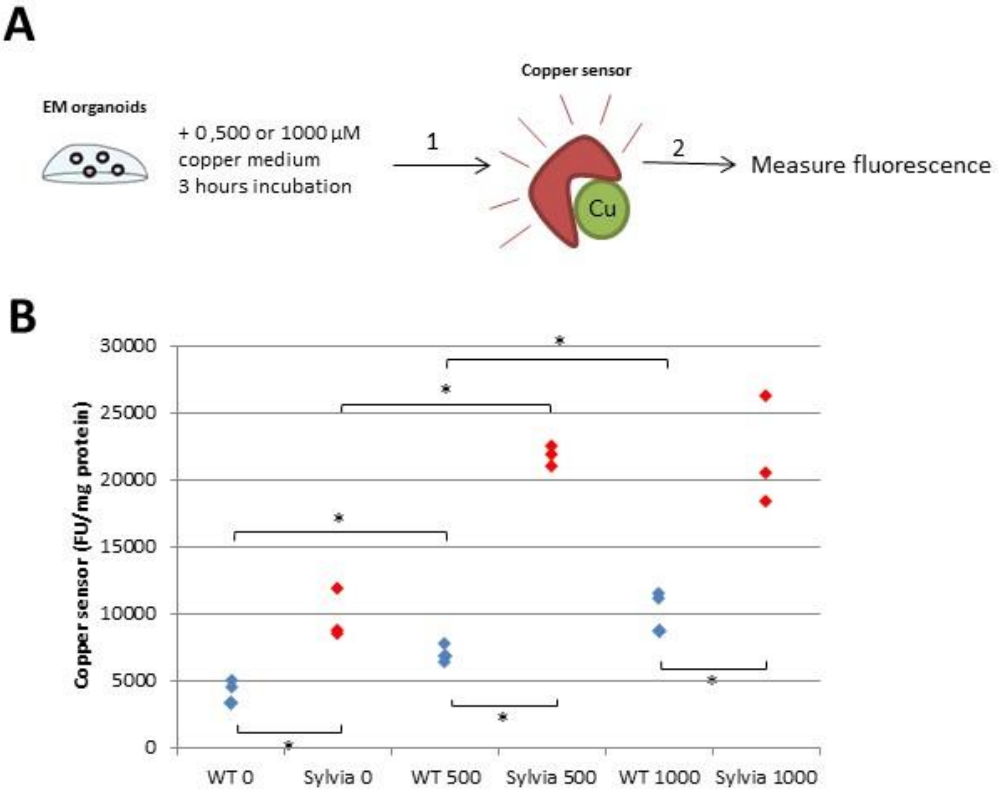

Figure 11. Copper experiment 2. A: schematic view of experimental design. Undifferentiated organoids remained in Matrigel and were incubated with 0, 500 or 1000 µM $CuCl_2$. The cells were washed with HBSS (1) and the copper sensor was added. The cells were then washed, harvested, and lysed (2), after which the fluorescence was measured. B: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment. WT: wild type liver organoids, Sylvia: COMMD1 deficient liver organoids. 0, 500 and 1000: 0, 500 or 1000 µM $CuCl_2$.

Chronic disease modeling

Our next hypothesis was that a more 'chronic' model would mimic copper toxicosis better *in vitro*, since copper toxicosis is a chronically developing disease[13]. In the 'chronic' model the organoids were exposed to lower $CuCl_2$ concentrations for a longer period of time. Two 'long term' experiments were performed, one on undifferentiated organoids (Copper experiment 3, Figure 12) and one on differentiated organoids (Copper experiment 4, Figure 13). In both experiments the copper sensor concentration was not significantly different between COMMD1 deficient organoids and wild type organoids (Figure 12, B and Figure 13, C). Also the growth curves showed no difference between COMMD1 deficient organoids and wild type organoids treated with 0 or 250 µM $CuCl_2$ (Figure 12, C and Figure 13, B). From these results was concluded that the 'long term' experiments do not mimic copper toxicosis better than copper experiment 1 and 2. Copper experiment 3 was performed on undifferentiated organoids, which have less expression of copper pathway associated genes. This could explain why no difference was found between COMMD1 and wild type organoids. There could be other explanations for the results, that also applies to the experiment on differentiated organoids: (1) the copper concentrations could be too high or the incubation time could be too long for wild type organoids as well, which causes them to accumulate copper and show the same phenotype as COMMD1 deficient organoids. An argument that is contradictory with this, is that the organoids do not show a significant decrease in viability during the 'long term' copper treatment. (2) The differentiation process could have been less successful, which causes the excretion pathways to be less expressed.

In case of copper experiment 4, the circumstances for the experiment were not ideal. There were little COMMD1 deficient organoids in comparison to wild type organoids, which is also shown in the pictures (Figure 14). This caused problems with the viability assay. Alamar Blue incubation lasted for 40 minutes, but at day 5 the values of the COMMD1 deficient organoid wells came very close to the background. The incubation time had to be expanded to 2 hours. Therefore, the growth curve from day 1 till day 5 cannot be compared with the growth curve of day 7 till day 12. Figure 14 also shows that the treatment of copper does not have an effect on the morphology of the organoids. The pictures of the wild type organoids show that the organoids become darker and some lose their spherical structure upon differentiation.

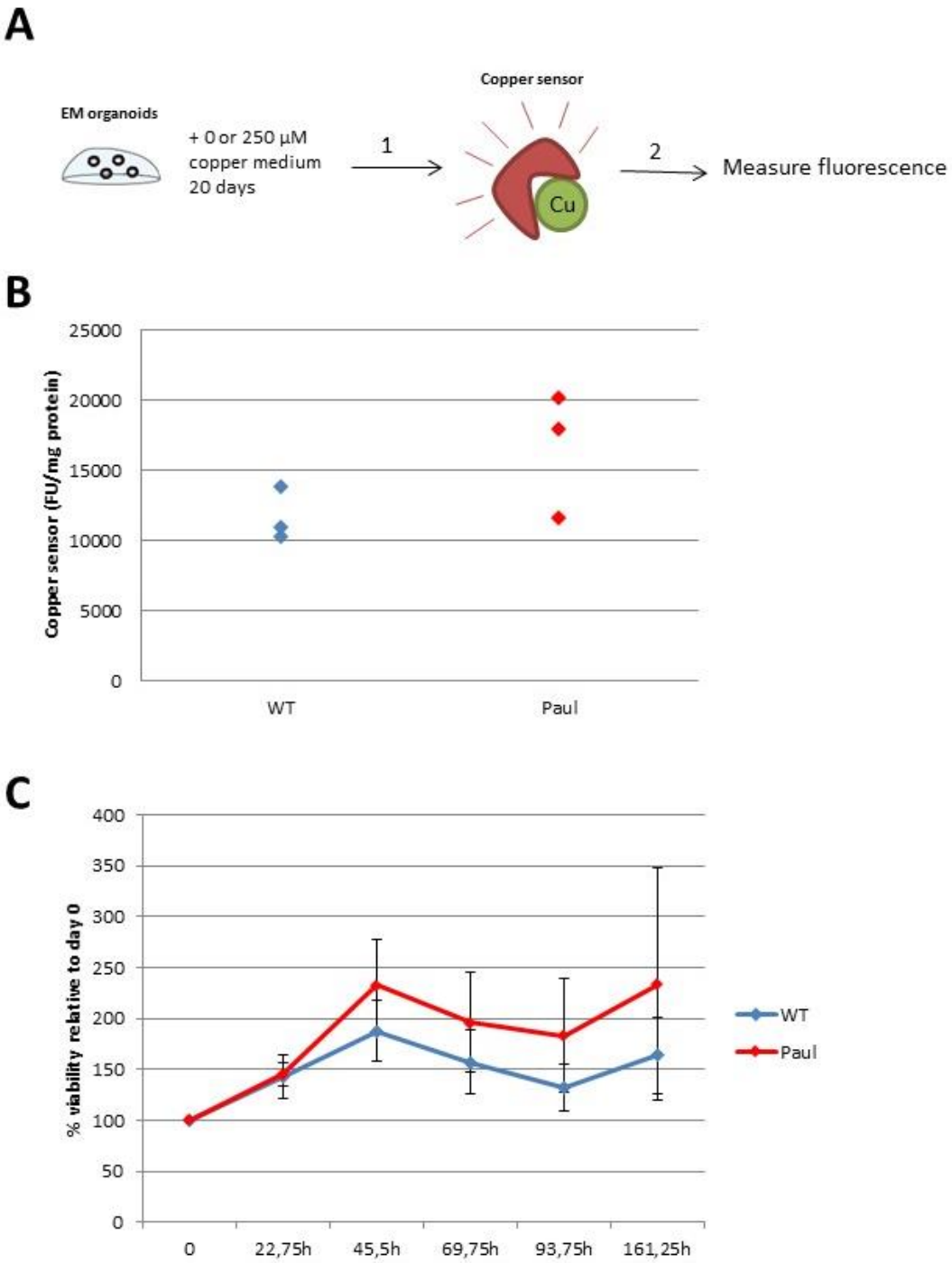

Figure 12. Copper experiment 3, 'Long term' experiment on undifferentiated organoids. A: schematic view of experimental design. Undifferentiated organoids were treated with 0 or 250 µM $CuCl_2$ for 20 days. Cells were washed with HBSS (1) and the copper sensor was added. The cells were then washed, harvested, lysed and the fluorescence was measured (2). B: Number of fluorescent units (FU) per mg protein of the different liver organoids. WT: wild type liver organoids, Sylvia: COMMD1 deficient liver organoids. 0 and 250: 0 and 250 µM $CuCl_2$. C: Percentage of viability compared to time point 0 (set at 100%).

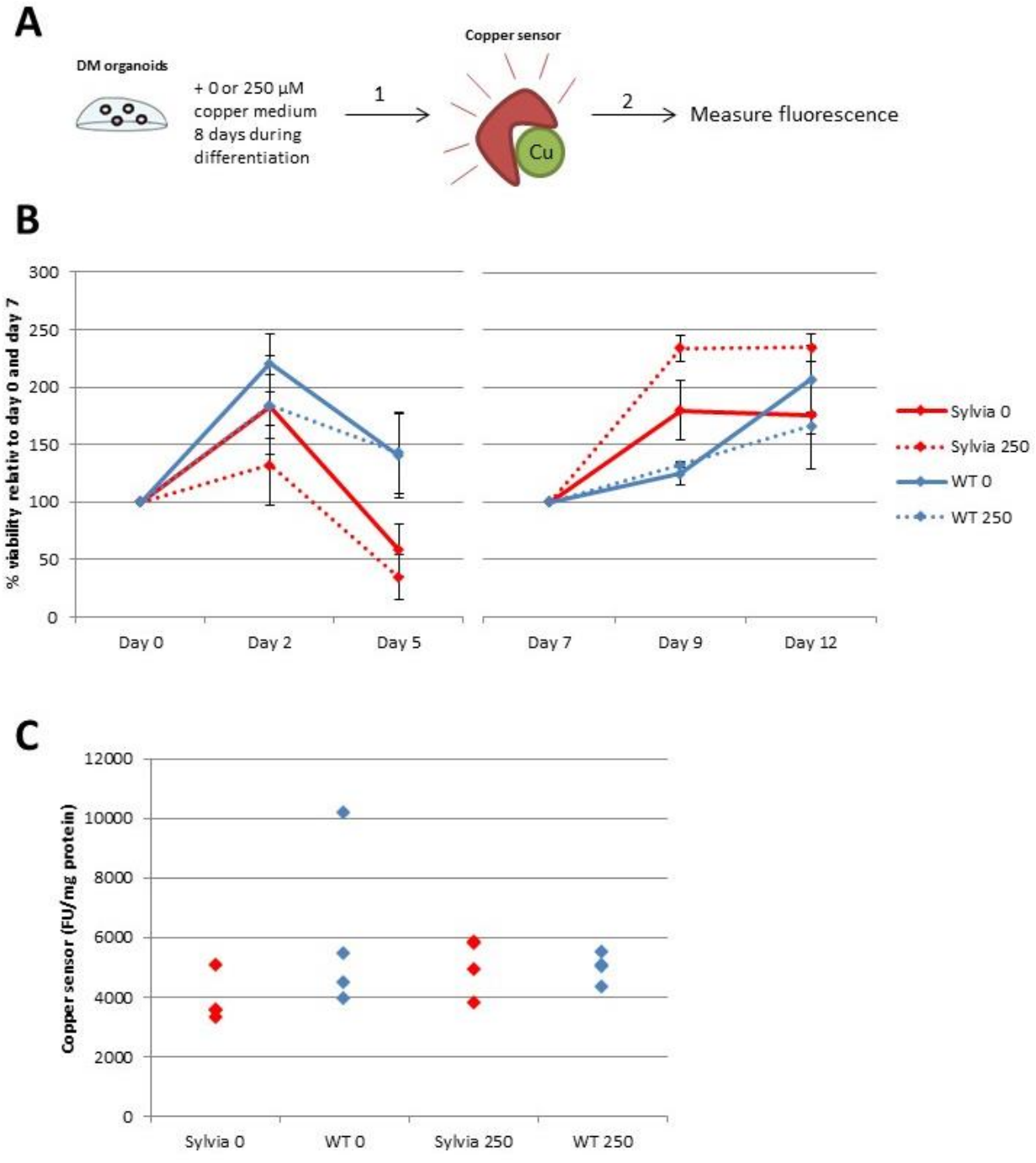

Figure 13. Copper experiment 4, 'Long term' experiment on differentiated organoids. A: schematic view of experimental design. Differentiated organoids were treated with 250 µM $CuCl_2$ for 8 days during the differentiation process. After the differentiation the cells were washed with HBSS (1) and the copper sensor was added. The cells were then washed, harvested, lysed and the fluorescence was measured (2).
B: Percentage of viability compared to day 0 or 7 (set at 100%). C: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment. WT: wild type liver organoids, Sylvia: COMMD1 deficient liver organoids. 0 and 250: 0 and 250 µM $CuCl_2$.

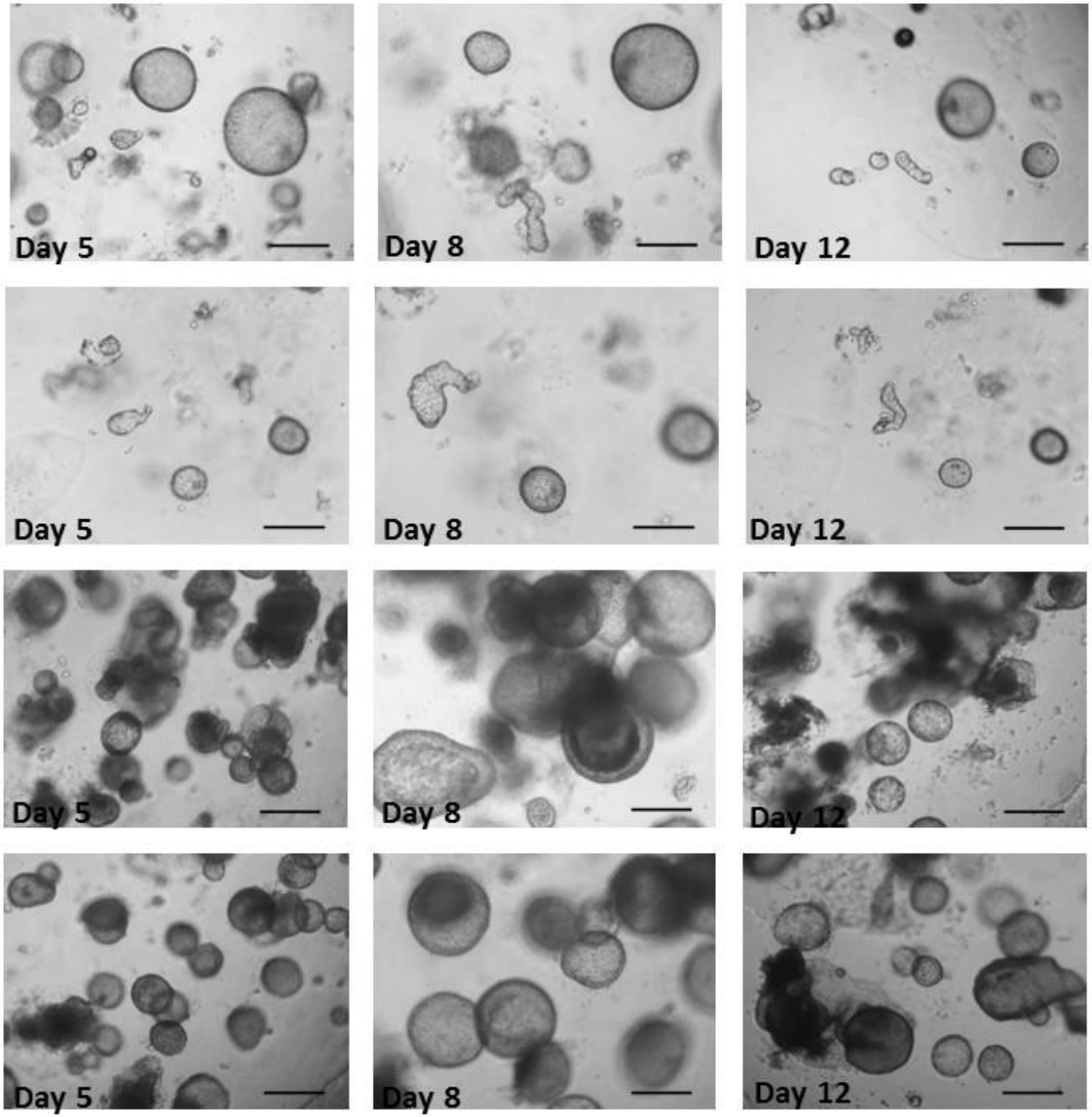

Figure 14. Pictures copper experiment 4, 'long term' experiment on differentiated organoids.
The copper treatment was started on day 5. All scale bars represent 500 µm.
A: Pictures on several time points of COMMD1 deficient organoids treated with 0 µM $CuCl_2$. B: Pictures on several time points of COMMD1 deficient organoids treated with 250 µM $CuCl_2$. C: Pictures on several time points of wild type organoids treated with 0 µM $CuCl_2$. D: Pictures on several time points of wild type organoids treated with 250 µM $CuCl_2$.

Influence of long term culture on copper accumulation in COMMD1 deficient organoids

Copper experiment 1 and 2 (Figure 9 and 11) showed that there was a difference in copper sensor concentrations between COMMD1 deficient organoids and wild type organoids treated with 0 µM $CuCl_2$. We hypothesized that the COMMD1 deficient organoids slowly accumulate copper during the passages. Wild type and transduced organoids are capable of excreting the small amount of copper always present in the media (5,2 nM). Therefore, there will already be a difference between COMMD1 deficient organoids and wild type organoids without exposure to extra copper. To test this hypothesis, a low and high passage of COMMD1 deficient organoids were compared by measuring the intracellular copper level (Figure 15). The morphology of the two passages was slightly different. The higher passage organoids had more irregular edges and more single cells were seen (Figure 15, A). After seeding, it also took the passage 10 organoids fragments longer to form complete spherical organoids. The copper sensor measurement showed that the organoids of passage 3 have a significantly higher copper sensor concentration than the organoids of passage 3. This does not confirm our hypothesis.

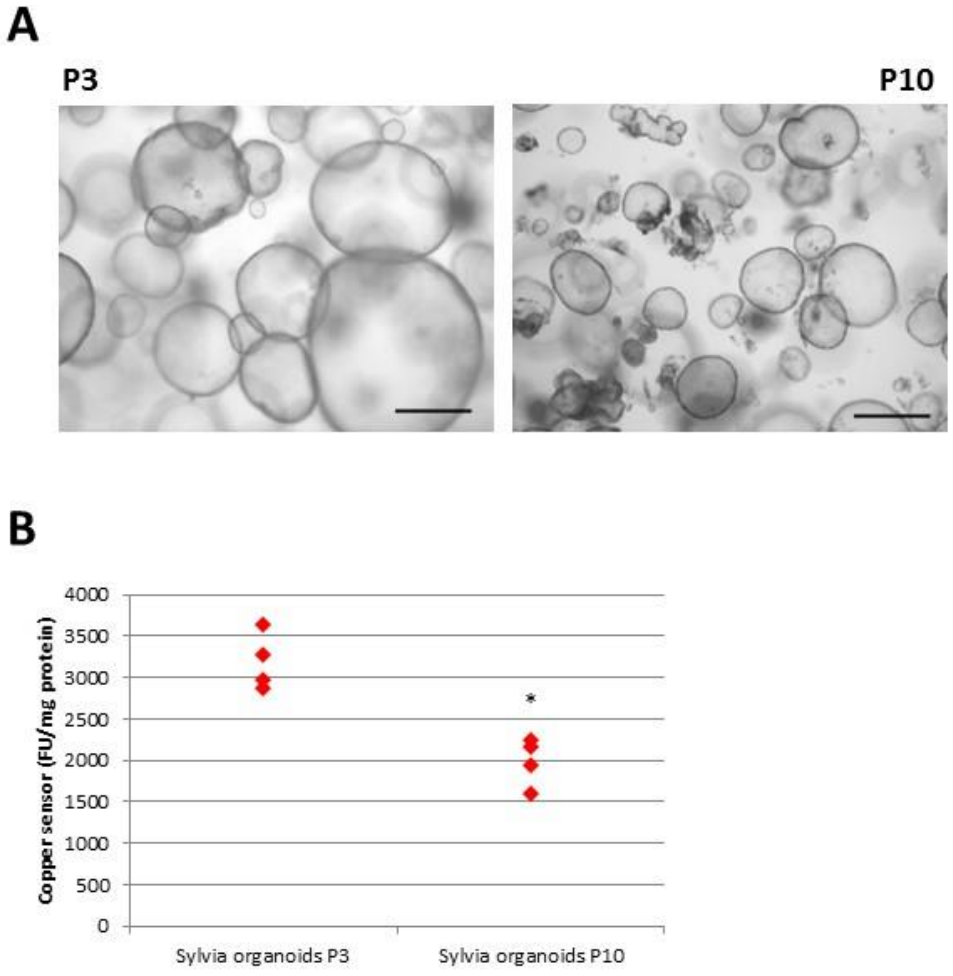

Figure 15. Comparing different passages of COMMD1 deficient organoids. A: The morphology of the COMMD1 deficient organoids, passage 3 compared to passage 10. All scale bars represent 500 µm. B: Number of fluorescent units (FU) per mg protein of the different passages of COMMD1 deficient liver organoids.

Drug testing on liver organoids in Matrigel

The 'long term' experiments did not show a better outcome than copper experiment 1 and 2. The protocol of copper experiment 2 was repeated with extra conditions for drug testing in copper experiment 5 (Figure 16). We hypothesized that treatment with BCS and D-pen after copper treatment would decrease the intracellular copper concentration of COMMD1 deficient organoids. Differentiated organoids were treated for 3 hours with 0 or 500 µM $CuCl_2$. After the copper incubation the organoids were treated with 50, 125 or 250 µM BCS, 8, 15, or 25 mM D-penicillamin or vehicle (no drug) overnight. Before and after the copper incubation a viability assay was performed, and also the next day after drug treatment. Kruskall Wallis test on this data showed that there was no significant differences between any of the groups (p = 0,206). The drug treatment didn't have an effect of the copper sensor concentration. The results showed no difference between COMMD1 and wild type organoids and also no difference between organoids treated with 0 or 500 µM $CuCl_2$. It is unclear why the organoids did not show a higher copper sensor concentration when treated with 500 µM $CuCl_2$ in copper experiment 5, and why the wild type organoids show the same phenotype as the COMMD1 deficient organoids. Copper experiment 2 indicated that keeping the organoids in the Matrigel during the copper treatment, still leads to an increase in copper sensor concentration. Also, 3 hours of copper incubation was successful in copper experiment 1 and 2.

We hypothesized to see a decrease in copper sensor concentration when the organoids were treated with a drug after the copper incubation. This was not found in copper experiment 5. There are several factors that could explain this: (1) Even though the concentrations were based on other publications, there were no publications where the drugs were tested on organoids. It is possible that other concentrations are necessary for this type of cells, (2) the drugs had an effect on the intracellular copper level, but this was too small to be detected by the copper sensor or (3) the drugs are unable to diffuse through Matrigel and reach the cells.

We hypothesized that the viability of COMMD1 organoids would decrease significantly more than the viability of wild type organoids during copper treatment. The viability results showed that the wild type organoids decreased significantly more than the COMMD1 deficient organoids during copper treatment (Figure 16, C), which is in contradiction with our hypothesis. Overnight, all the organoids show an increase in viability. Although, there is no significant difference between any of the drug treatment groups. (Figure 16, D).

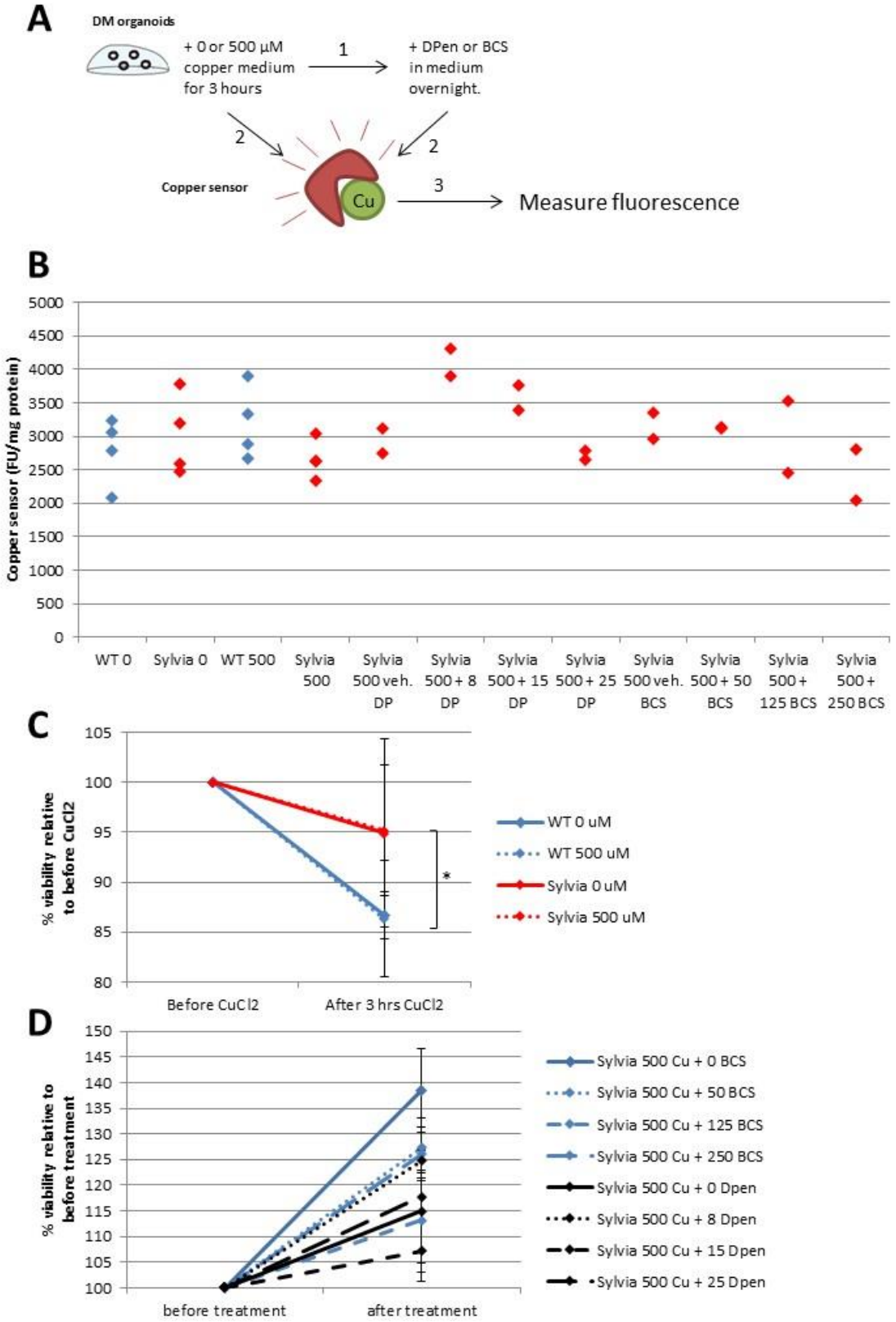

Figure 16. Copper experiment 5. A: Schematic view of experimental design. The cells were treated with 0 or 500 µM $CuCl_2$ for 3 hours. Cells were washed (1) and were treated overnight with several concentrations of BCS, D-penicillamin or vehicle. Cells were thenwashed (2) and the copper sensor was added. After this, the cells were washed, harvested, lysed and the fluorescence was measured (3). B: Number of fluorescent units (FU) per mg protein for the different copper and drug treatment groups. C and D: Percentage of viability compared to before $CuCl_2$ or drug treatment (set at 100%). DP: D-penicillamin (in mM), 0 or 500: 0 or 500 µM $CuCl_2$, BCS: Bathocuproinedisulfonic acid disodium salt (in µM), veh.: vehicle.

Verification of original disease model

Only in copper experiment 2 a difference between WT and COMMD1 deficient organoids was seen when the organoids were kept in the Matrigel (Figure 11). After this experiment we never saw this difference again, and all of our hypothesis were not confirmed. Experiment 2 was performed on undifferentiated organoids, which is less reliable than the results seen in differentiated organoids because of the differences in gene expression. We decided to take a few steps back and return to the protocol used in copper experiment 1 (Figure 9). We hypothesize that retrieving the organoids from the Matrigel leads to more exposure to copper, which leads to a greater and detectable difference between COMMD1 deficient and wild type organoids. The protocol of copper experiment 1 was repeated in copper experiment 6 and 7. Copper experiment 6 was performed on undifferentiated organoids, and copper experiment 7 on differentiated organoids. In copper experiment 6 extra conditions treated with copper medium for 24 hours were added. We hypothesize that 24 hours of copper treatment leads to a significantly higher decrease in viability of COMMD1 deficient organoids when compared to wild type organoids.

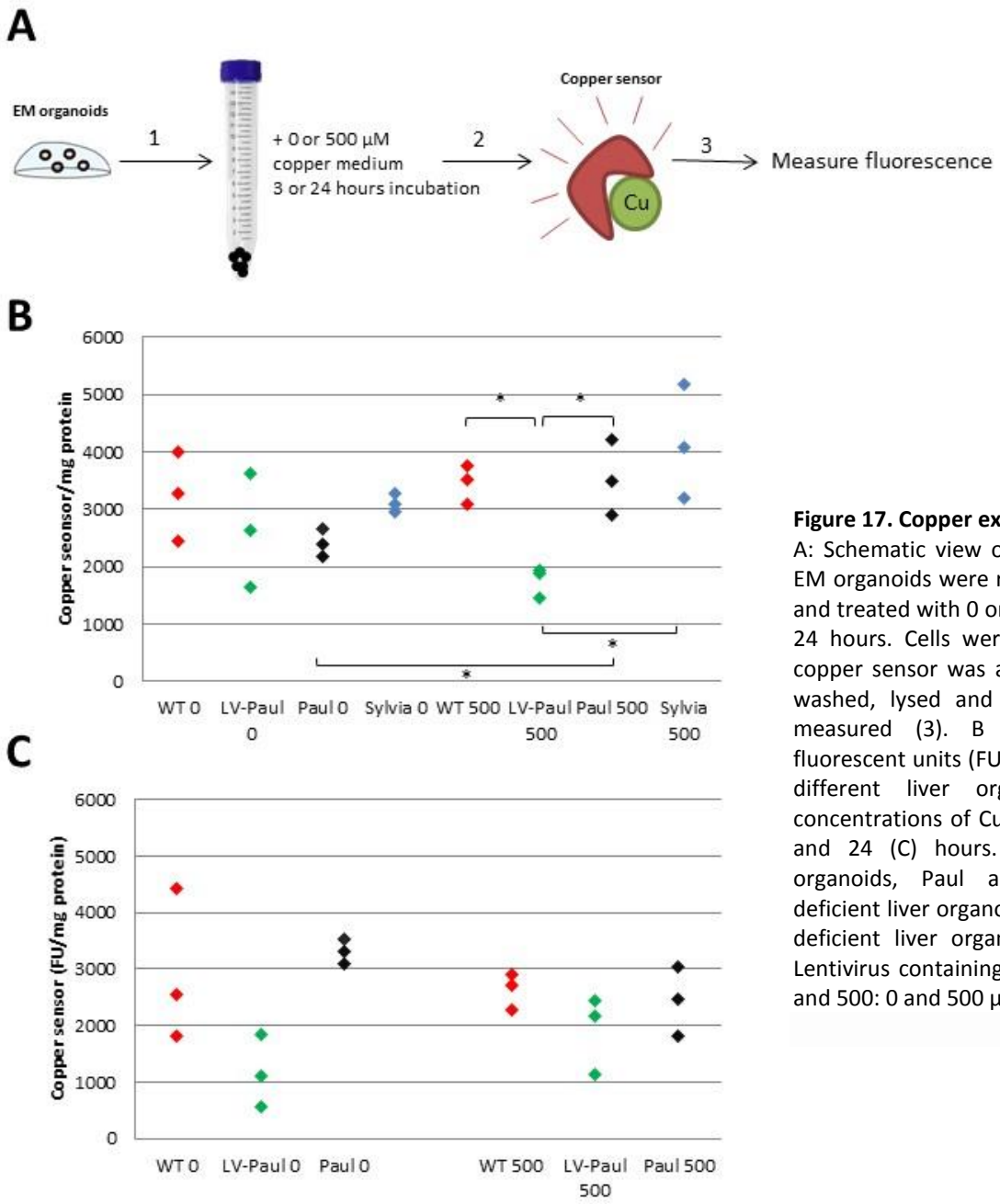

Figure 17. Copper experiment 6.
A: Schematic view of experimental design. EM organoids were retrieved from Matrigel and treated with 0 or 500 µM $CuCl_2$ for 3 or 24 hours. Cells were washed (2) and the copper sensor was added. Cells were then washed, lysed and the fluorescence was measured (3). B and C: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment for 3 (B) and 24 (C) hours. WT: wild type liver organoids, Paul and Sylvia: COMMD1 deficient liver organoids, LV-Paul: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene. 0 and 500: 0 and 500 µM $CuCl_2$.

The results of copper experiment 6 showed no significant difference between COMMD1 deficient organoids and wild type organoids when treated with 0 or 500 µM $CuCl_2$ (Figure 17, B). The transduced Paul organoids did have a significant lower copper sensor concentration in comparison to COMMD1 deficient organoids and wild type organoids when treated with 500 µM $CuCl_2$. Only the COMMD1 deficient organoids showed a significant increase of copper sensor concentration when treated with 500 µM $CuCl_2$ in comparison to treatment with 0 µM $CuCl_2$.
In copper experiment 6, cells were also treated for 24 hours with copper (Figure 17, C). These results are in contradiction with the findings after 3 hours of copper incubation. None of the organoids showed an increase in copper sensor concentration when they were treated with copper. No differences were measured between COMMD1 deficient organoids and wild type or transduced organoids. Again the same explanations as before can be named here. The 24 hours treatment with copper could be too much for the wild type and transduced organoids as well, which makes them also show the phenotype of accumulating copper. Furthermore, in copper experiment 6 the organoid were undifferentiated, which could also explain that there is no difference between COMMD1 deficient and wild type organoids. However, this does not explain why there is no increase in copper concentration in this experiment.
The protocol of copper experiment 1 was repeated on differentiated organoids (Figure 18, copper experiment 7), making it an exact repetition of copper experiment 1. Results of experiment 7 were similar to the results of experiment 1. All the organoids showed a significant increase in copper sensor concentration after treatment for 3 hours with 500 µM $CuCl_2$. At 0 and 500 µM $CuCl_2$ the COMMD1 deficient organoids had the highest level of copper sensor concentration, which was significantly different from the levels in wild type and transduced organoids. Possibly, a reason why the disease model worked in experiment 7 and not in copper experiment 6 could be because it was on differentiated organoids in experiment 7. The differentiated organoids have higher expression of the copper excretion associated genes, and therefore will mimic the disease better.

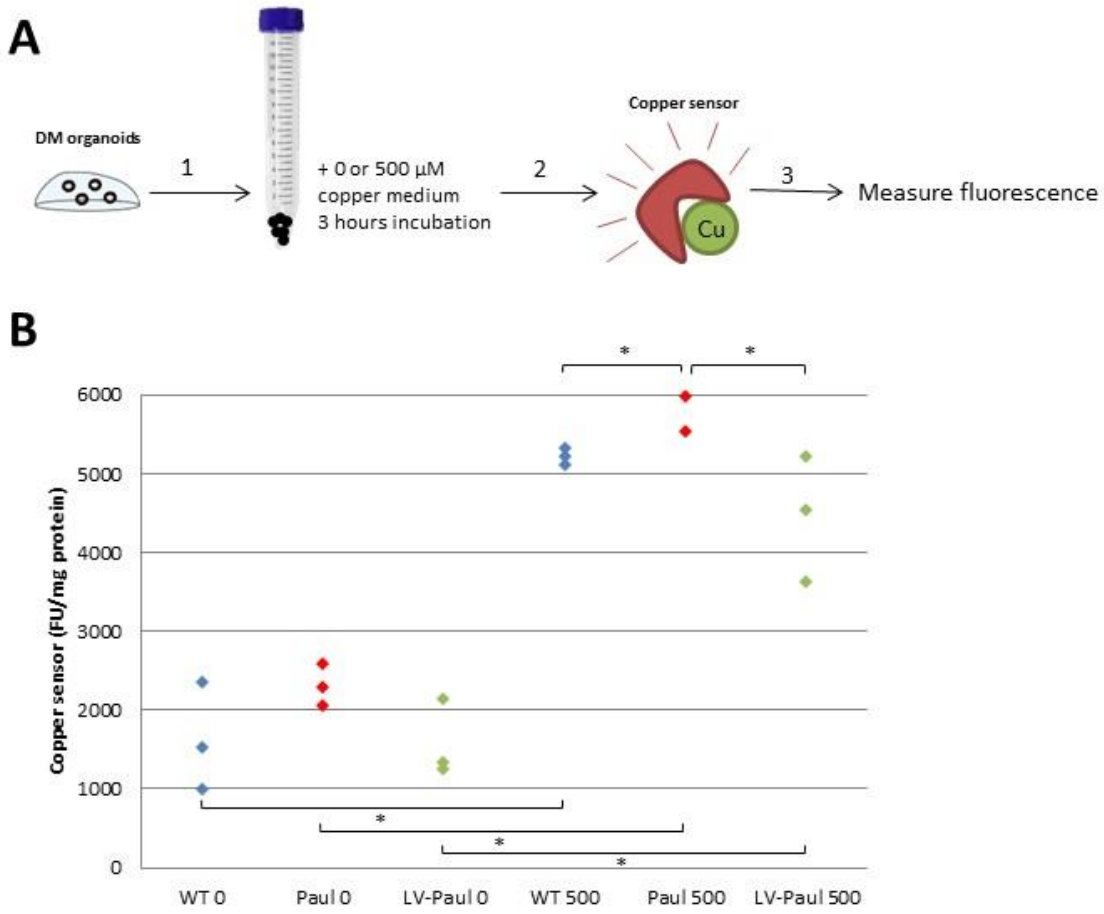

Figure 18. Copper experiment 7. A: Schematic view of experimental design. Cells were retrieved from Matrigel and treated with 0 or 500 µM $CuCl_2$ for 3 hours. Cells were washed (2) and the copper sensor was added. Cells were then washed, lysed and fluorescence was measured (3).
B: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment. WT: wild type liver organoids, Paul: COMMD1 deficient liver organoids, LV-Paul: COMMD1 deficient liver organoids transduced with lentivirus containing the corrected gene. 0 and 500: 0 and 500 µM $CuCl_2$.

Influence of copper treatment on viability of canine liver organoids

In copper experiment 6 the viability was measured before and after the copper treatment of the undifferentiated organoids. The viability results showed that the COMMD1 deficient organoids, the wild type organoids and the transduced organoids treated with 500 µM $CuCl_2$ for 3 hours decreased in viability (Figure 19, A). Only the transduced organoids treated with 0 µM $CuCl_2$ increased in viability. No significant differences between any of the groups was found.

When the organoids were treated with 500 µM $CuCl_2$ for 24 hours, significant differences were measured (Figure 19, B). Wild type and the COMMD1 deficient organoids treated with 500 µM $CuCl_2$ have significant lower viability scores than wild type and COMMD1 deficient organoids treated with 0 µM $CuCl_2$. No significant difference was found between transduced organoids treated with 0 or 500 µM $CuCl_2$. The wild type and transduced organoids have a significantly higher viability when compared to the COMMD1 deficient organoids when treated with 500 µM $CuCl_2$. Also, the transduced organoids have a significantly higher viability than the wild type organoids when treated with 500 µM $CuCl_2$. The results presented in Figure 19 (B) confirms our hypothesis. When the organoids are treated with copper, their viability decreases significantly, and this had the largest impact on the COMMD1 deficient organoids.

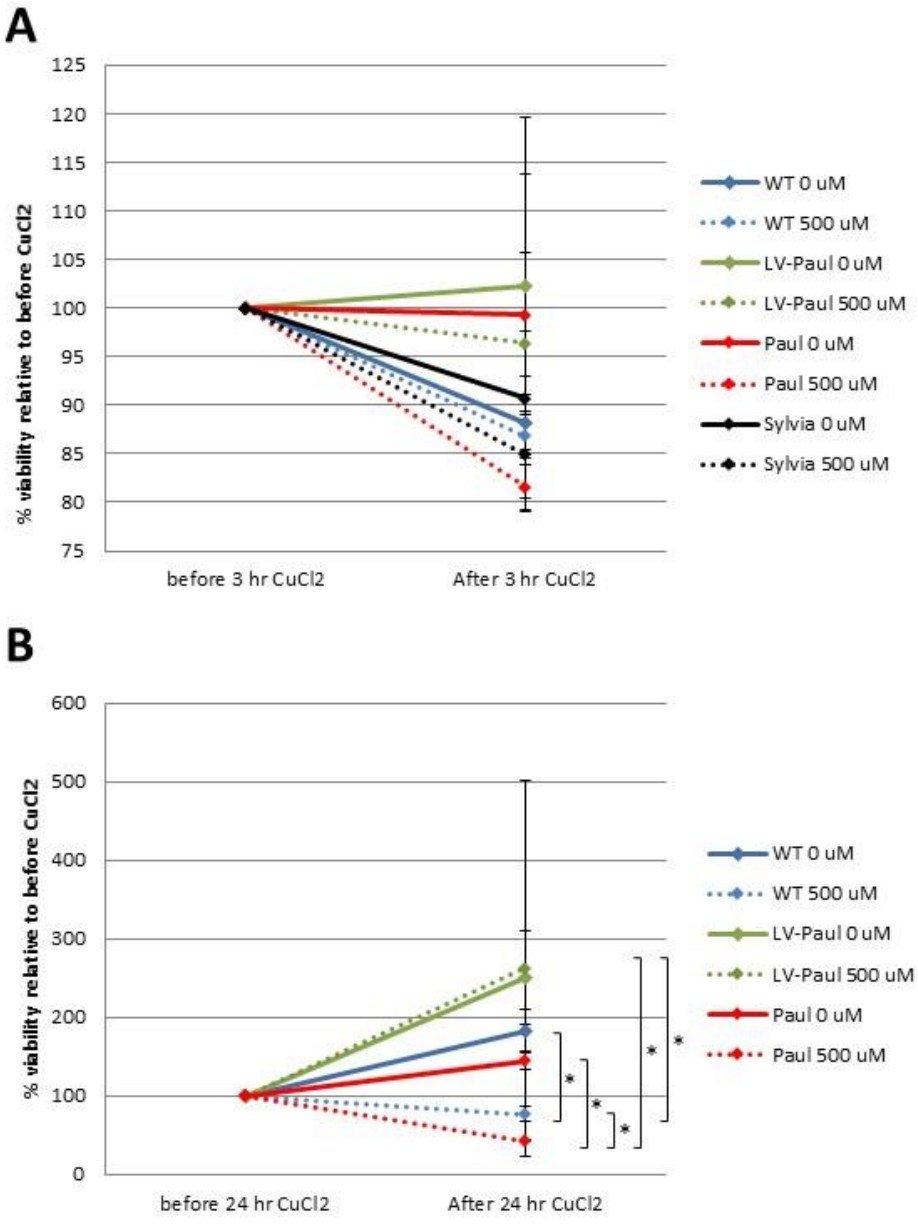

Figure 19. Viability assays. A and B: Percentage of viability compared to before $CuCl_2$ treatment of 3 or 24 hours (set at 100%) on undifferentiated organoids. WT: wild type liver organoids, Paul and Sylvia: COMMD1 deficient liver organoids, LV-Paul: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene. 0 and 500: 0 and 500 µM $CuCl_2$.

Viability testing after 24 hours of copper incubation was repeated on differentiated organoids in two independent experiments (Figure 20). The results of the first repetitive experiment were in contradiction with the findings of the viability testing on undifferentiated organoids (Figure 19, B). The wild type and COMMD1 deficient organoids treated with 0 µM $CuCl_2$ decreased significantly more in viability than the same type of organoids treated with 500 µM $CuCl_2$. There were no significant differences in viability between the wild type, COMMD1 deficient or transduced organoids. The results of the second repetitive experiment were in accordance with the findings of the viability testing on undifferentiated organoids. The organoids treated with 0 µM $CuCl_2$ increased in viability during the 24 hours, and the organoids treated with 500 µM $CuCl_2$ decreased in viability, which was a significant difference for wild type, COMMD1 deficient and transduced organoids. COMMD1 deficient organoids treated with 500 µM $CuCl_2$ had significantly lower viability when compared to wild type organoids treated with 500 µM $CuCl_2$. No significant difference was found between the transduced organoids and the COMMD1 deficient organoids, or wild type organoids.

Hepatocytes normally have a very low turn-over rate[2,5]. When differentiating organoids, they become hepatocyte-like cells. We therefore hypothesized that differentiated organoids don't proliferate and do not increase in viability when they are kept in culture overnight. Our first repetitive viability experiment is in line with this hypothesis, although it is unclear why the cells treated with 0 µM $CuCl_2$ decrease significantly more in viability than the cells treated with 500 µM $CuCl_2$. The organoids treated with 0 µM $CuCl_2$ in the second repetitive experiment showed an increase in viability overnight, which indicates an incomplete differentiation of the organoids.

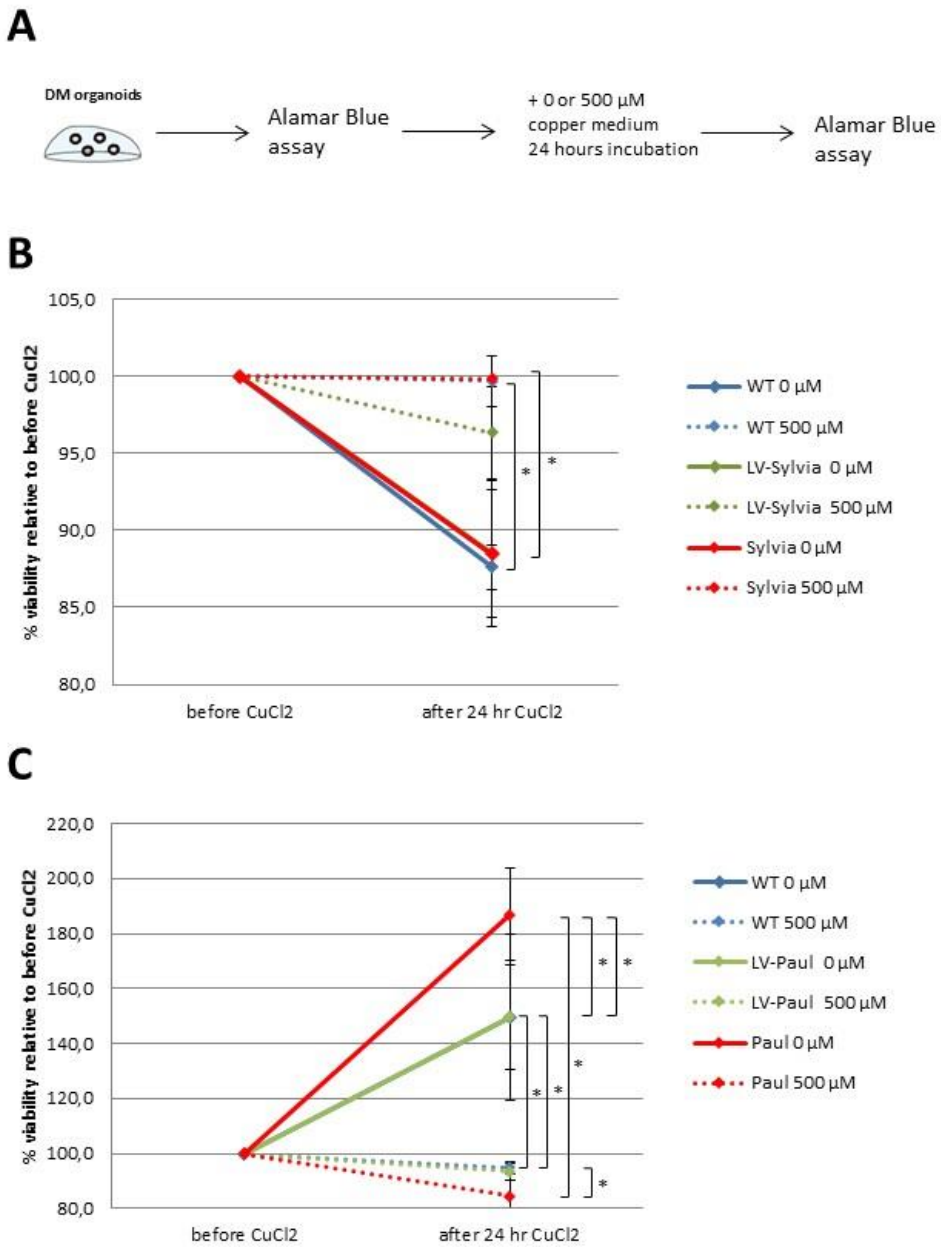

Figure 20. Viability assays. A: Schematic view of experimental design. Viability of differentiated organoids was measured using Alamar blue assay before the copper treatment. The cells were treated with 0 or 500 µM $CuCl_2$ for 24 hours. After this, the viability was measured again.
B and C: results of two independent experiments. Percentage of viability compared to before $CuCl_2$ treatment of 24 hours (set at 100%). WT: wild type liver organoids, Paul and Sylvia: COMMD1 deficient liver organoids, LV-Paul and LV-Sylvia: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene. 0 and 500: 0 and 500 µM $CuCl_2$.

The effect of copper treatment on fresh primary canine hepatocytes

Primary hepatocytes can function as a positive control, since we try to differentiate our organoids towards hepatocytes. Our research question was how freshly isolated canine hepatocytes respond to the copper experiment, and if BCS could deplete cells of copper. Hepatocytes were incubated with 0 or 500 µM $CuCl_2$ for 3 hours, after which wells received 3 hours of 250 or 500 µM BCS treatment or vehicle (Figure 21, A). The primary hepatocytes were obtained using a liver perfusion technique and the cell were seeded on a 24 wells plate. The next day the hepatocytes were attached to the plate (Figure 21, B). Copper incubation of primary hepatocytes did not increase the intracellular copper concentration. Addition of BCS or vehicle increased the intracellular copper concentration significantly. The only difference between the group treated with 500 µM $CuCl_2$ and the drug vehicle group, was that the latter had 3 hours of extra incubation with medium without any copper or drugs. We didn't expect this group to show other results than the group treated with 500 µM $CuCl_2$ alone. The hepatocytes in the vehicle group could have had extra time to excrete the copper, which would result in a lower copper sensor concentration than the group only treated with 500 µM $CuCl_2$. However, these results showed the opposite outcome. The BCS treatment didn't decrease the intracellular copper concentration of the hepatocytes. Possibly, the used concentrations of BCS are insufficient to extract copper from the cells, or the incubation time to achieve this is not long enough.

Drug testing pilot

To further study which protocol should be used in drug testing experiments a pilot experiment including various BCS concentrations and incubation times was performed. Huh7 and HepaRG cell lines were used. Huh7 cells were incubated with 0 or 500 µM $CuCl_2$ for 3 hours, after which wells were treated with 250, 500 or 1000 µM of BCS or vehicle for 3 hours. Cells were then treated with the copper sensor and the cells were lysed (Figure 22, A). HepaRG cells were treated with BCS or vehicle overnight (Figure 22, C). Incubation with 500 µM $CuCl_2$ for 3 hours on Huh7 cells resulted in significantly higher levels of copper sensor concentrations. However, no difference was measured between the drug treatment groups. We expected to see a decrease of copper sensor concentration when more BCS was added, but this wasn't measured in neither the group treated with 0 µM $CuCl_2$ nor the group treated with 500 µM $CuCl_2$ in advance (Figure 22, B).

HepaRG cells also showed a significant higher level of copper sensor concentration when treated with 500 µM $CuCl_2$ when compared to treatment with 0 µM $CuCl_2$. For both the cells treated with 0 µM $CuCl_2$ and the cells treated with 500 µM $CuCl_2$, the copper sensor concentrations increased when higher concentrations of BCS are added. However, the cells treated with the highest concentration of BCS (1000 µM) showed lower values of copper sensor concentration than the cells treated with 500 µM BCS. So, the conclusion that addition of more BCS gives an increase in copper sensor concentrations, is also not correct.

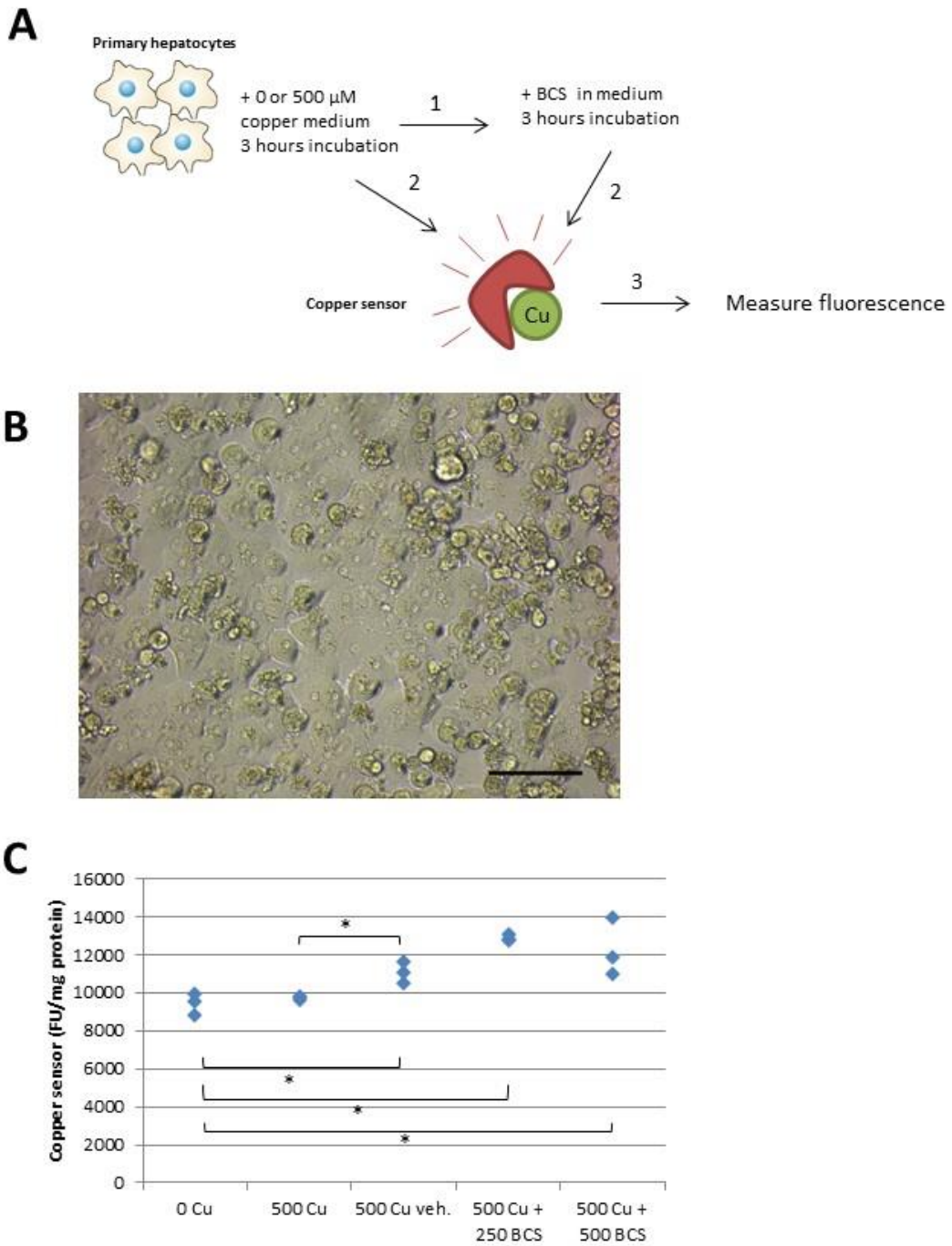

Figure 21. Copper experiment on fresh primary canine hepatocytes. A: Schematic view of experimental design. Cells were treated with 500 µM $CuCl_2$ for 3 hours. Cells were washed (1) and treated for 3 hours with several concentrations of BCS or vehicle. After drug treatment the cells were washed (2) and the copper sensor was added. Cells were then lysed and fluorescence was measured (3). B: Fresh hepatocytes attached to the plate. Scale bar represents 50 µm. C: Number of fluorescent units (FU) per mg protein for the different copper and drug treatment groups. 0 or 500 Cu: 0 or 500 µM $CuCl_2$, BCS: Bathocuproinedisulfonic acid disodium salt (in µM), veh.: vehicle.

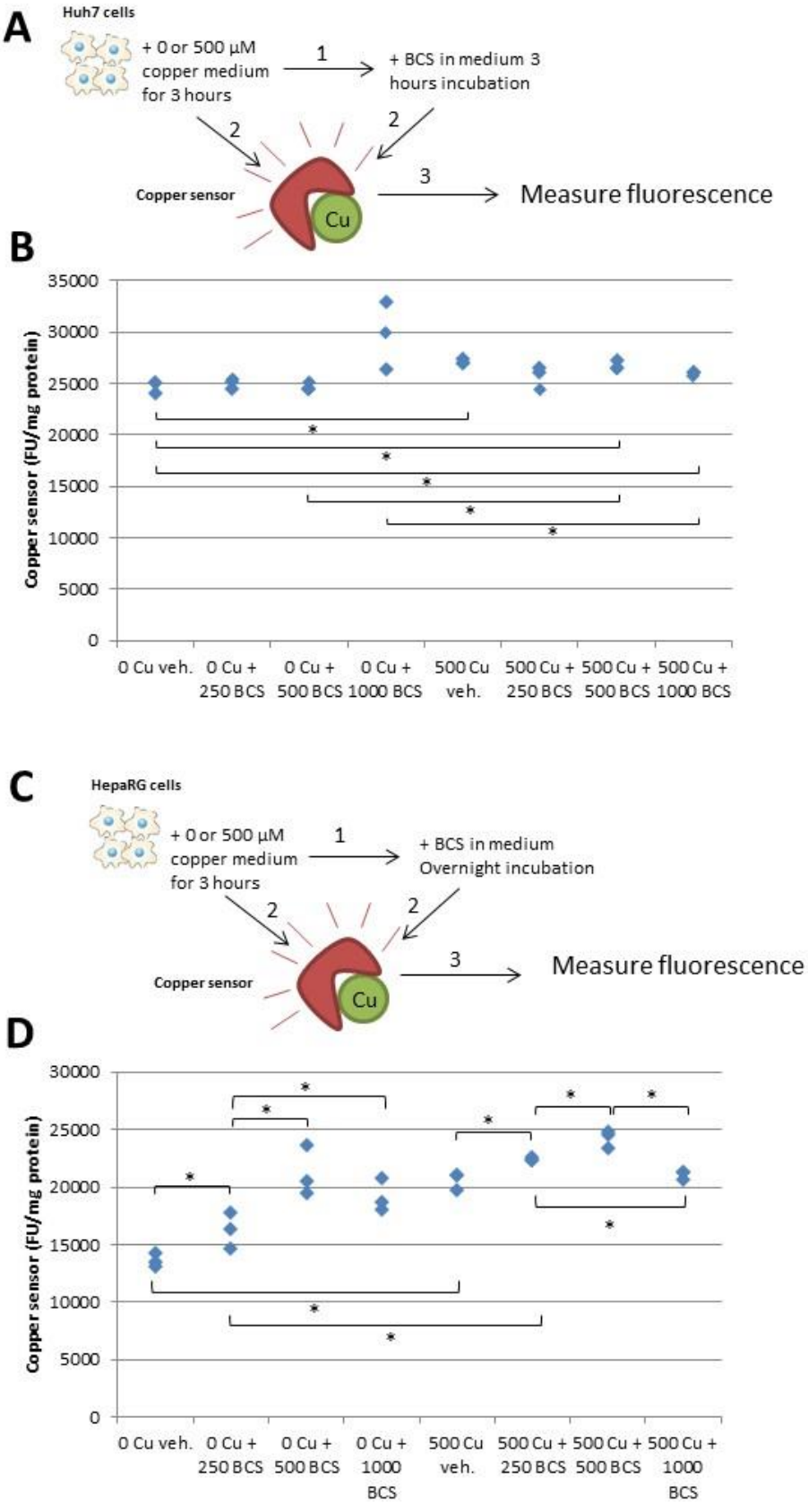

Figure 22. Drug testing pilot. A: Schematic view of experimental design of pilot with Huh7 cells. Cells were treated with 500 µM $CuCl_2$ for 3 hours. Cells were washed (1) and treated for 3 hours with several concentrations of BCS or vehicle. Cells were then washed (2) and the copper sensor was added. Cells were then lysed and fluorescence was measured (3). C: Schematic view of experimental design of pilot with HepaRG cells. The same design as the pilot on Huh7 cells, only now with the drug treatment overnight. B and D: Number of fluorescent units (FU) per mg protein for the different copper and drug treatment groups (in µM).
BCS: Bathocuproinedisulfonic acid disodium salt (in µM), 0 or 500 Cu: 0 or 500 µM $CuCl_2$, veh.: vehicle

Drug testing on liver organoids out of Matrigel

Based on the results of the drug testing pilot, we considered a new approach for the drug testing experiments. In previous experiments, the drugs were incubated after the $CuCl_2$ incubation. Both BCS and D-penicillamin were unable to extract copper from the cells. We decided to change the protocol for drug testing to adding the drug together with the copper to the cells. We asked if D-penicillamin and BCS would be able to chelate excess of copper in the medium, thereby preventing cellular uptake. Also, the same concentrations for D-penicillamin and BCS were used, so we can compare both drugs.
In copper experiment 8 differentiated organoids were taken out of the Matrigel and were treated for 3 hours with 0 or 500 µM $CuCl_2$. Extra conditions of COMMD1 deficient organoids were treated with 500 µM $CuCl_2$ with the addition of either 1000 µM BCS, 1000 µM D-penicillamin or a vehicle. When adding BCS to the copper medium, a color change of the medium occurred. After the 3 hours of incubation, copper sensor was added, cells were lysed and fluorescence was measured (Figure 23, A). Copper experiment 8 showed again that the protocol used in experiment 1, 6 and 7 to achieve the disease model, is not repeatable (Figure 23, B). The results of copper experiment 8 showed that all types of organoids significantly increased in copper sensor concentration when treated with 500 µM $CuCl_2$. In both 0 and 500 µM $CuCl_2$ the COMMD1 deficient organoids have the lowest concentrations of copper sensor. This is in contradiction with the results of copper experiment 1 and 7, where the same protocol was used. The drug treatment with either BCS or D-penicillamin didn't have a significant effect on the intracellular copper concentration.
In copper experiment 9 the same protocol was used as in experiment 8, only higher concentrations of BCS and D-penicillamin were tested. The Kruskall Wallis test on this data gives a p-value of 0,318, which means that there is no significant difference between any of the groups. No increase in copper sensor concentration was seen when 500 µM $CuCl_2$ was added, and no differences between the different types of organoids. These results are also not in line with copper experiment 1 and 7, so this protocol doesn't give repeatable results. The General Discussion of part A describes possible explanations for this.

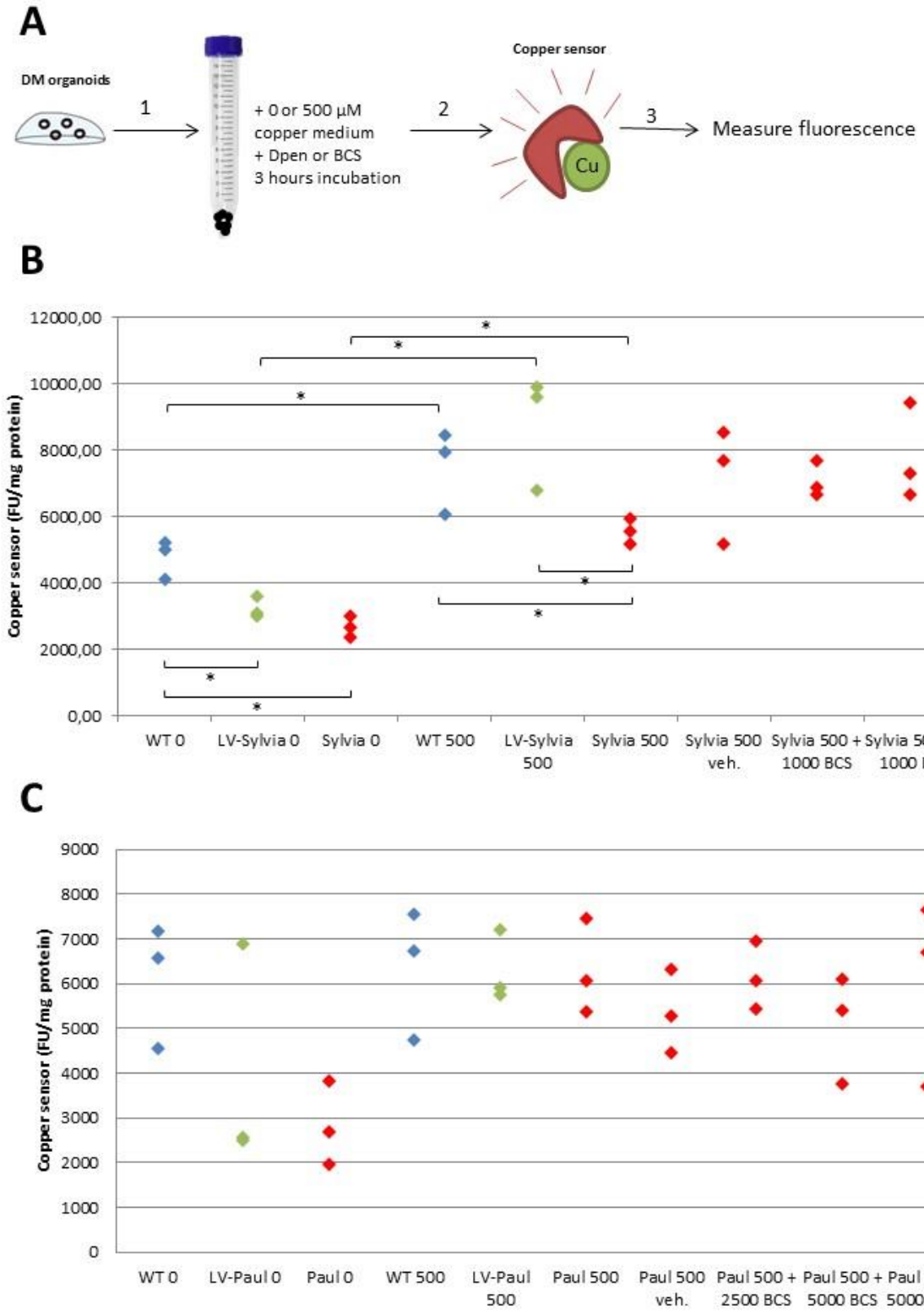

Figure 23. Copper experiment 8 and 9 with drug testing. A: Schematic view of experimental design. Differentiated organoids were retrieved from Matrigel (1) and were treated for 3 hours with 0 or 500 µM $CuCl_2$ plus different concentrations of BCS, D-penicillamin or vehicle. Cells were washed (2) and the copper sensor was added. Cells were then washed, lysed and the fluorescence was measured (3). B: Copper experiment 8, number of fluorescent units (FU) per mg protein for the different copper and drug treatment groups. C: Copper experiment 9, number of fluorescent units (FU) per mg protein for the different copper and drug treatment groups. WT: wild type liver organoids, Paul: COMMD1 deficient liver organoids, LV-Paul: COMMD1 deficient liver organoids transduced with Lentivirus containing the corrected gene, DPen: D-penicillamin (in µM), BCS: Bathocuproinedisulfonic acid disodium salt (in µM), 0 or 500: 0 or 500 µM $CuCl_2$, veh.: vehicle

Effect of copper treatment on human liver organoids

Copper toxicosis in dogs has many similarities with Wilson's disease in humans[7,12]. We received a liver sample of a Wilson's disease patient through a collaboration with Dr. Hans Zischka (Institute of Molecular Toxicology and Pharmacology, München, Germany). The organoids isolated from the Wilson's disease sample, were compared to human wild type liver organoids. We asked how human liver organoids would respond to our disease model protocol for copper toxicosis in dogs. Human liver organoids were differentiated using a protocol published by *Huch et al*[47]. After differentiation, the organoids were retrieved from Matrigel and were incubated for 6 hours with 0, 100, 250 or 500 µM $CuCl_2$. After this, the cells were washed and the copper sensor was added. The fluorescence was measured after lysis of the cells (Figure 24, A).

No significant differences were found between any of the groups. No increase was seen when the organoids were treated with $CuCl_2$, and also no difference was seen between wild type organoids and Wilson's disease organoids (Figure 24, B).

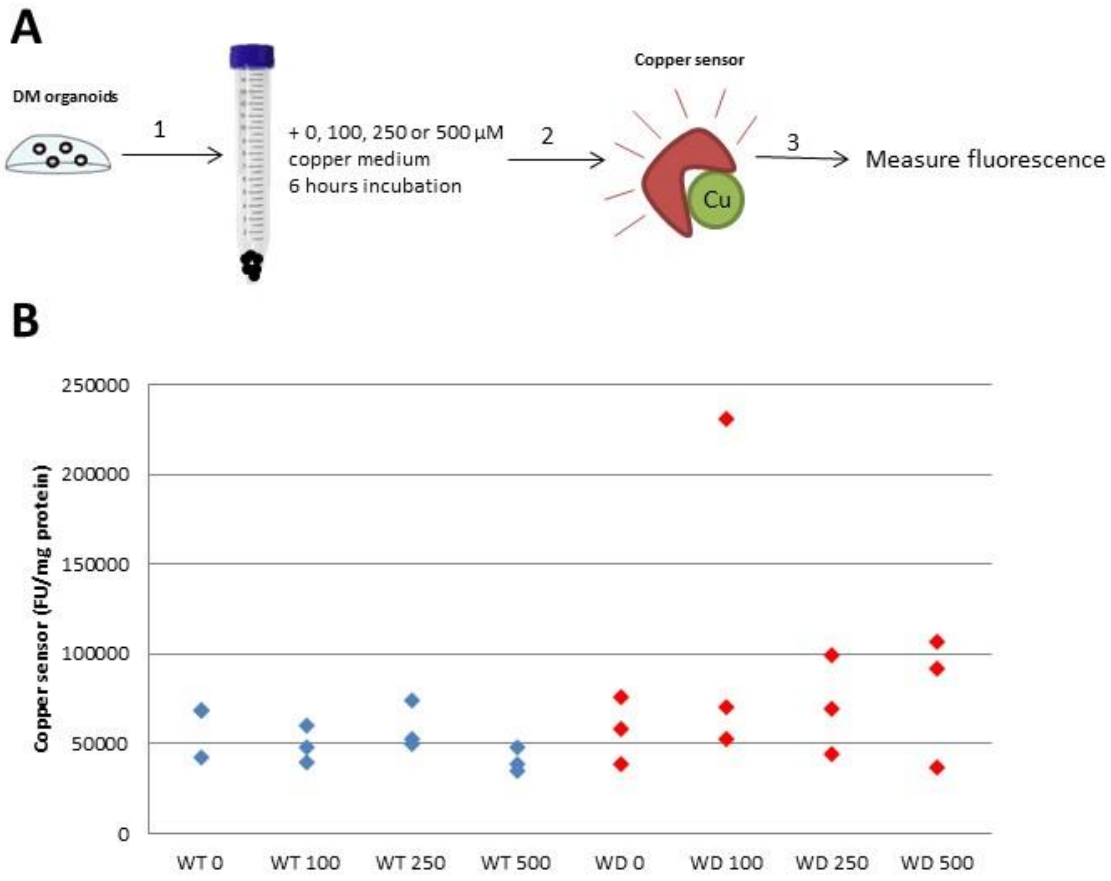

Figure 24. Copper experiment 10 on human liver organoids. A: Schematic view of experimental design. Differentiated human liver organoids were retrieved from Matrigel and treated with 0, 100, 250 or 500 µM $CuCl_2$ for 6 hours. Cells were washed (2) and the copper sensor was added. Cells were then washed, lysed and fluorescence was measured (3). B: Number of fluorescent units (FU) per mg protein of the different liver organoids at different concentrations of $CuCl_2$ treatment. WT: wild type liver organoids, WD: wilson's disease liver organoids, 0, 100, 250 and 500: 0, 100, 250 and 500 µM $CuCl_2$.

General discussion part A

To develop a disease model, one should first know what characteristics the model should have. Is it important that every aspect of the disease is reflected in the model or only the main features? Copper toxicosis is a disease that develops over many years[13], although copper accumulation is already visible at 6 months of age[56,64]. We can't mimic this in the model, since we can't keep the liver organoids in culture for that long and also this would not be an efficient way of research. We want to use the disease model for drug testing and to investigate the pathways leading to the disease. To this goal, it is important that the cells show the main feature of the disease, which is accumulation of copper in the hepatocyte. At first, we aimed to mimic the disease as much as possible. We decided to try a more 'long term' mimicking of the disease by increasing the incubation time and lowering the amount of copper exposure. One could speculate about how this mimics the long term development of the disease better, since we extended the model to about a week instead of the actual disease that develops over months. The 'long term' experiments did not show a better outcome. During these experiments, we still thought that keeping the organoids in the Matrigel during copper treatment, was not a problem. Later in the developing process, we decided to retrieve them from Matrigel before copper treatment. The reason why the long term experiments didn't work, could be because the organoids were kept in the Matrigel. Other reasons could be that the long term treatment was too much copper for the wild type organoids as well, which also caused them to accumulate copper. However, we did not see much effect on the viability of the organoids, which contradicts with previous statement. The 'long term' experiment might come closer to reality in theory, but in practice this was not the case. It is less important how the accumulation is achieved in the disease model, as long as the COMMD1 deficient organoids show the disease's phenotype and the wild type organoids don't.

The disease model developed in this study, which consisted of 3 hours of $CuCl_2$ incubation on liver organoids retrieved from Matrigel, was not repeatable and showed high variability. There could be several reasons for this. First, we question the sensibility of the copper sensor. In most of the copper experiments the copper concentration increased significantly when organoids or cells were treated with copper. The copper sensor is sensitive enough to measure this difference in amount of copper. However, within the same condition the variability of the values was very high. The model was repeated many times, and every time different groups were significantly different and it was not repeatable. Possibly, the copper sensor is not sensitive enough to pick up the differences between the groups. Also, the effect of BCS and D-penicillamin could be too little to be detected by the sensor. Part of this could also be because the sensor lost it sensitivity due to exposure to light before use. Next to this, we don't know the exact qualities of the copper sensor. The copper sensor is capable of binding intracellular copper, but it is unclear how efficiently it binds. It could be that it binds so strongly to copper that it takes the copper from intracellular proteins like glutathione or metallothionein. In that case we would detect a much higher level of intracellular copper than was actually freely present in the cytosol. The copper sensor CS1 is originally designed to make copper visible under the fluorescent microscope, not for estimating the amount of copper by quantifying the fluorescence intensity[55,65]. It would be a good idea to study for other copper sensors that might be more sensitive for the quantification of the copper concentration.

Second, it is hard to standardize the conditions to culture organoids. Each well can't be seeded with the same amount of cells/organoids. The density of the wells has an effect of the growth speed of the organoids but probably also on differentiation, since cell-cell interaction is very important for the differentiation towards hepatocytes[39]. For this reason, there will be variation between the same type of wells. Next to this, Matrigel is a complex protein mixture, which is also variable and certain components are still unknown[49–51]. This could explain variability between experiment where different batches of Matrigel have been used for culturing.

Third, our differentiation protocol can be quite variable. The differentiation of cells towards hepatocytes is not only dependent on the components present in the medium. Cell-cell interaction and cell-matrix interaction are also very important for the differentiation towards hepatocytes[39]. Since it is hard to standardize the amount of organoids in each well, the success of differentiation may be

different when density of the wells differ. Next to this, the exact components of Matrigel (which function as the extracellular matrix) may differ even between each well, which can cause differences in the differentiation status of the cells.
Pilot experiments were mostly performed on undifferentiated organoids, because the results were obtained rather fast. Only when a sophisticated experiment with high expectations based on pilot results was performed, we used differentiated organoids, since these experiments take at least two weeks and therefore are more expensive as well.
Hepatocytes are highly polarized cells. They contain two membrane domains which are strictly separated by tight junctions. The apical membrane domain faces the bile duct and is involved in the secretion of bile acids and detoxification products. The basolateral domain faces the space of Disse and is involved in exchange of metabolites with the blood[3]. Cell-cell and cell-matrix interaction are very important for the cells to gain and retain their polarity[66,67]. The copper excretion pathway is a process that depends on this polarity, since the copper has to be excreted via the apical membrane into the bile ducts. So, for this disease model to work properly, the cells in the liver organoids have to be polarized. The question remains whether the liver organoids are polarized enough to have a functional copper excretion pathway. The study of *Nantasanti et al (2015.* Unpublished data*)* shows for example that the tight junction marker ZO-1 is not expressed in undifferentiated organoids, but is expressed in most of the differentiated organoids. A tight junction separates the cell membrane into a apical and basolateral part[68]. Expression of ZO-1 suggests that there is some polarization in the differentiated organoids. However, whether this polarization also results in a functional apical membrane where bile acids and copper can be excreted, is unclear.
In theory liver organoids have a lot of advantages when compared to other stem cell culturing methods. Also for the purpose of disease modeling they are very promising. Since organoids are patient specific, the disease occurring in a specific patient can be modeled and treatment options for each patient (personalized medicine) can be investigated[52]. In comparison, using iPS cells for disease modeling is harder to standardize. To create iPS cells, the cells are exposed to several reprogramming factors. These reprogramming factors can have an influence on the phenotype of the disease, which may vary between patients[52]. When culturing the cells directly from the damaged liver, this should result in a more standardized and truthful model. To make the disease model work, the complete differentiation of the liver organoids towards hepatocytes is important. The problem with using fresh hepatocytes for a disease model, is the fact that they dedifferentiate very quickly after isolation[39]. Next to this, the liver perfusion technique destroys gap and tight junctions between hepatocytes and results in a loss of cell polarity[39]. This also raises the question if we can use these cells as a positive control, as we did in the copper experiments. The fresh hepatocytes were probably already partly dedifferentiated and they no longer contained a polarized membrane when the experiment was performed.
For the drug testing experiments, BCS and D-penicillamin were used. BCS is often used for depleting cells of copper[11,24,61–63,69]. We wanted to use this drug as a 'golden standard' to compare other drugs to. However, we failed to measure an effect of BCS on the intracellular copper concentration. BCS is an membrane-impermeable copper chelator[62,63], which explains why it didn't work in our first experimental design where BCS was added after the $CuCl_2$ incubation. The only way it would have worked then, is when BCS could actively transport copper out of the cells. Since, it is an membrane-impermeable chelator, this is not expected. However, other studies showed that BCS can deplete cells of copper when present in the medium. In many studies cells are incubated with medium containing copper or BCS, to create cells with high levels of intracellular copper and low levels of intracellular copper[11,21,24,61]. In the study by *Dodani et al*. cells were cultured with medium containing BCS to create copper-depleted conditions to test their new copper sensor (CS3)[62]. In these studies, BCS has never been used in combination with copper treatment. In our study, we expected BCS to decrease the intracellular copper levels as well in both the groups treated with 0 or 500 µM $CuCl_2$ in advance. However, we did not measure an effect of BCS. When we added BCS simultaneously with $CuCl_2$ to the cells, we expected the BCS to bind copper and prevent it from getting into the cells. When adding BCS

to the $CuCl_2$ medium we noticed a change in color, which suggests that there is a chemical reaction. However, we did not measure any decrease in copper sensor concentration. Possibly BCS did have an effect, but the copper sensor was not sensitive enough to detect this decrease.
Currently, D-penicillamin is the most used therapy. It has an effect on kidney level and promotes more urinary excretion of copper[25]. However, it exact mechanism is still unclear. To investigate the mechanism of D-penicillamin, we tested the effect of D-penicillamin on the disease model in organoids. If it only has an effect on the kidney level, we probably won't see an effect on the intracellular copper concentration of the cells. However, D-penicillamin is a chelator and can bind copper, so maybe it could have an effect on the cells. In our experiments we didn't measure an effect of D-penicillamin.
There were several limitations to our experimental design. As differentiation readout we only measured gene expression of stem cell and hepatic markers. It would be better to also have a functional readout to test if the cells have hepatic functions, like albumin secretion. We tried to measure albumin secretion in the medium at every copper experiment, only these results were very variable. We tried to figure out what was the best negative control to measure this correctly. However, even freshly prepared organoid medium gave extremely high values of albumin. We didn't find a good way to measure the albumin production of the organoids yet, and so we do not have a functional readout for our differentiation process.
Not all transduced organoids we used in the copper experiments were generated with exactly the same method. The transduced organoids of the COMMD1 deficient dog Viola, were produced with a virus dilution 1:2000, while the other transduced organoids were produced with a virus dilution of 1:20000. It is better to standardize this for all the transduced organoids, because a difference in virus dilution can cause a difference in the amount of vectors getting into the cells. The transduced organoids of Viola could therefore have more overexpression of COMMD1 than the other transduced organoids. This could be assessed by measuring the gene expression level of COMMD1 by real-time PCR in these organoids or by estimating the amount of COMMD1 protein by Western Blot analysis.
After adding the copper sensor, the cells were washed with HBSS. This should wash away the copper sensor molecules that are still outside of the cells. However, we saw that after washing and centrifugation, the pellets were covered with a pink layer. Even when washing again, this pink layer remained. It could be that the copper sensor molecules stick to the outside of the cells, which could give a falsely elevated measurement of the copper sensor concentration.
The lysis of the organoids with RIPA buffer also presented some problems. We expected the RIPA to fully 'dissolve' the organoid fragments. However, this was only the case when very little organoids were present. In the rest of the cases, fragments always remained in the tubes. It is unclear whether these fragments are cell debris or that there are still intact cells present. Higher values of protein and copper sensor were measured when taking the fragments along in the measurement. This could be because the copper sensor also sticks to the outside of the cells, like mentioned before. In that case we also measured copper sensor that never came into the cells and bound copper, but that stuck to the outside of the cells. Another problem with taking the fragments into the measurement was that when doing the Lowry assay, the triplicate had higher standard deviations, which makes the protein values less reliable.
When dissolving $CuCl_2$ in Advanced DMEM/F12, the solution obtained a green color, but the $CuCl_2$ powder never completely dissolved. After filter sterilization the solution became completely transparent. Some of the $CuCl_2$ is probably lost when you filter sterilize the solution, since it doesn't completely dissolve. Probably the concentrations of $CuCl_2$ are lower than we want them to be.
There are several suggestions for future research. A major problem in our experimental design is the sensibility of the copper sensor. We would therefore suggest to test a new copper sensor, which is more suitable for the quantification of the intracellular copper concentration. A new candidate could be copper sensor 3 (CS3) used in the paper of *Dodani et al*[62]. Next to this, research on the standardization of the culturing conditions for liver organoids and further optimization of the differentiation protocol to obtain fully functional copper excretion pathways should be done.

Furthermore, the polarization of liver organoids is necessary for fully functional excretion pathways, and therefore research should be done on the polarization status of these cells and on methods to increase the polarization. Determining the position of more proteins, for instance gap junctions, that are normally restricted to a specific part of the membrane, can give more insight in the polarization of the cells within an organoid. To evaluate if the copper excretion or bile acid excretion occurs, life cell imaging could be used. Bile acids or copper could be labeled using a fluorescent sensor and the localization of these molecules could be studied. For studying the copper excretion pathway, perhaps the cells could be imaged from the moment of administration of copper until the excretion. Sensors for the life cell imaging of copper have already been developed by *Miller et al.*[55] and *Dodani et al*[62]*.*
A very interesting candidate for further drug testing research has been described by *Summer at al*[70]*.* Methanobactin is a small copper chelating molecule with an extremely high affinity for copper. This molecule is normally produced by Methanotrophic bacteria.[71] In the study of *Summer et al.* the therapeutic effect of methanobactin in a rat model for Wilson's disease has been determined. This treatment resulted in a significant reduction of liver copper levels and increased excretion of copper, in the form of a methanobactin-copper complex, into the bile.[70] Therefore, methanobactin could be an interesting alternative in the treatment of copper toxicosis in Bedlington terriers. Also, since methanobactin actively excretes copper into the bile, it could be an interesting alternative drug for all forms of copper associated hepatitis, including Wilson's disease in humans.

Conclusion

This study shows that the disease model for copper toxicosis when using canine liver organoids and CS1, does not give repeatable results. This copper sensor might not be sensitive enough to detect the differences between the COMMD1 deficient organoids and wild type organoids. The current method for culturing organoids is not well standardized, which may give variability in the results. Next to this, the differentiation protocol still needs improvement to achieve fully differentiated hepatocytes for an optimal disease model. When this optimal disease model is achieved, the exact mechanism of D-penicillamin can be studied *in vitro* and also new promising drugs can be tested, like methanobactin.

Part B: Transplantation study

Abstract

Donor liver shortage restricts the use of liver transplantation for the treatment of end-stage liver diseases in humans. A lot of research has already been done to find alternatives for this treatment. Very promising techniques are the transplantation of stem cells or primary hepatocytes. In this study we investigated the possibility to use canine liver organoids for transplantation in COMMD1 deficient dogs. Hepatic progenitor cells were isolated from COMMD1 deficient dogs and cultured as three dimensional structures in Matrigel. We then genetically corrected them by transducing them with a viral construct. The organoids were then expanded and differentiated towards hepatocyte-like cells. Two COMMD1 deficient dogs have been transplanted with these hepatocyte-like cells for 3 successive days using a port-a-cath connected to the portal vein. One COMMD1 deficient dog served as a negative control and received vehicle instead of cells. Prior to the first injection, a liver lobe was resected to stimulate liver regeneration. COMMD1 staining on liver biopsies taken post transplantation showed COMMD1 positive cells that could be derived from the transplanted organoids. This study is still running, and 3 more COMMD1 deficient dogs will be transplanted with these organoids. Also, more liver biopsies of later time-points need to confirm the presence of COMMD1 positive cells in the transplanted dogs.

Introduction

The only therapeutic option for end-stage liver diseases in humans is allogeneic liver transplantation[28]. This is an invasive procedure with high risk of surgical complications and life-long immunosuppression must be administered to the patient in order to prevent graft rejection[28]. Next to this, there is a shortage in liver donors[28]. For this reason, a lot of research has been done on the regeneration of the liver and the possibilities for cell transplantation.

Transplantation of primary hepatocytes has been proposed for the treatment of liver diseases. This approach is less invasive, since the cells can be injected into the spleen or portal vein[72]. Also, hepatocytes for transplantation of more patients can be retrieved from one donor liver and the hepatocytes can be cryopreserved until needed[72]. Several animal models have shown that the transplanted hepatocytes have normal hepatic function and could remain functional for a long time in the recipient[72]. However, transplantation with donor hepatocytes still requires life-long immunosuppression. In many clinical cases, the hepatocyte transplantation only led to partial correction of the disease, and the graft was lost after several months to years[72], so there are still a few problems to overcome.

Recently, a new culture method called liver organoids has been developed[47,48]. This is a very promising technique that can culture patient specific hepatic progenitor cells for over months[47,52]. These liver organoids can be isolated from a liver biopsy and expanded for a long period of time. Using a specific differentiation protocol, these organoids can be differentiated towards hepatocyte-like cells before transplanting them back into the patient. Since these cells are derived from the patient's own liver, there is no need for immunosuppression[52]. Also, in case of a genetic disorder of the patient, a genetic correction of the progenitor cells can be performed before expansion and differentiation. *Huch et al.* showed that mouse and human organoids can be successfully transplanted in mice[47,48]. The injected organoids were detected as islets of cells distributed through the liver parenchyma and they were shown to contribute to the liver function *in vivo*[47,48].

The aim of this study is to successfully transplant transduced canine liver organoids in COMMD1 deficient dogs. These COMMD1 deficient dogs function as a model for other copper associated liver diseases occurring in other dog breeds and in humans. Patient specific liver organoids will be cultured after isolating hepatic progenitor cells from a liver biopsy of the dog. These organoids will then be transduced by a lentivirus, containing a vector. This vector contains the correct COMMD1 gene and a red fluorescence gene. The organoids will then be differentiated towards hepatocyte-like cells, using a defined differentiation medium. Our hypothesis is that these differentiated liver organoids will

engraft in the liver tissue of the patient after injection into the vena porta. The organoids will have a growth advantage compared to the patient's own cells, due to the gene correction. The transplanted cells won't be restricted to proliferate by the high intracellular copper concentration, unlike the patient's own cells. These organoids will eventually repopulate the liver, which will result in a new functional liver as an alternative for liver transplantation. In this study we also optimized stainings to trace the transplanted cells in the liver biopsies taken after transplantation. We hypothesize that we can detect the transplanted cells using a staining for dsRed and/or COMMD1.
The transplantation study is a very big project. This research year I've been involved in several aspects of this project. In this manuscript the main aspects of this research will be discussed with a more detailed description of the parts I've been involved in. The focus in this manuscript will be on the immunohistochemical stainings developed in the project.

Materials and methods

Liver samples

The use of liver tissues from dogs was approved by the Utrecht University's ethical committee, as required under Dutch legislation (ID 2007.III.06.080). Wild type liver samples were obtained from healthy dogs used in non-liver related research projects. COMMD1 deficient liver samples were obtained from Paul, a COMMD1 deficient dog involved in the transplantation project (DEC approval: 2014.III.12.112). Liver biopsies were taken using a 14G Tru-cut biopsy needle. For immunohistochemistry, tissue samples were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in ethanol, embedded in paraffin wax and sectioned at 4 µm.

Duct isolation and organoids culture

Duct isolation as described in part A was performed on the liver biopsies of a COMMD1 deficient dog. For the culture methods of canine liver organoids, see the description in part A.

Production of viral construct containing canine COMMD1

HEK293T cells were cultured in a 15-cm dish and used for the production of lentivirus. The cells were incubated for 5 minutes at RT with 3 ml of Advanced DMEM plus 315 µl of Polyethylenimine (Polysciences). Next, 2 ml of DNA solution was added in each dish containing Advanced DMEM plus 3.6 µg HDM-Hgpm2, 3.6 µg RC-CMV-Rev1b, 3.6 µg HDM-tat1b, 7.2 µg HDM-VSV-G and 45 µg Backbone DNA. This solution was incubated for 10-20 minutes at RT. The 5 ml of transfection mix was then added to 20 ml complete MEF media (cultured in DMEM with 10% FCS) on the HEK293T cells and incubated overnight. The next day, the media was refreshed with 9 ml of complete MEF media. On day 2 and 3, respectively 12 and 14 ml of fresh MEF media was added to the cells. The media containing virus of day 2, 3 and 4 was filter sterilized and transferred to a 50 ml Falcon tube and stored at 4 °C. The tubes with harvested media was centrifuged on day 4 at 72,000 g for 1,5-2 hours at 4 °C. The pellet was then resuspended in 150 µl sterile PBS + 1% BSA and aliquots were made and stored at -80 °C.
The vector (Figure 25) contains several important genes including a puromycin resistance gene, a dsRed gene and the canine COMMD1 gene. The puromycin resistance gene makes the transfected organoids resistant to the otherwise toxic puromycin. The transfected organoids can then easily be distinguished from the wild type organoids by puromycin treatment. The dsRed gene encodes for a red fluorescent protein, which can be detected under the fluorescence microscope. This way we could easily evaluate the transduction efficiency under the fluorescence microscope. The wild type COMMD1 gene is also in this vector to genetically correct the COMMD1 deficient organoids.

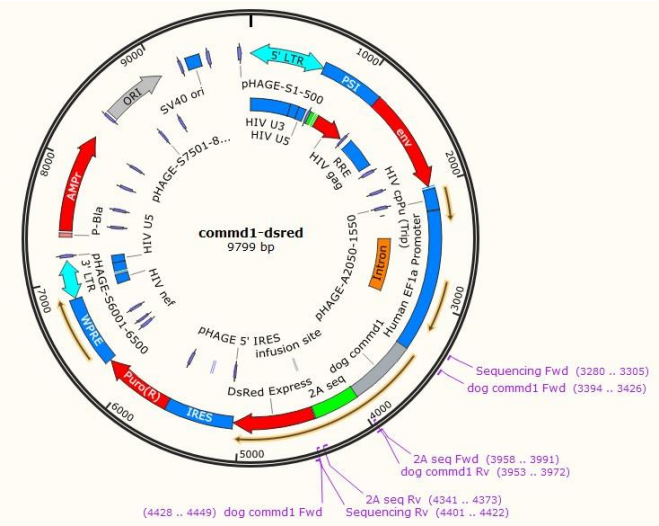

Figure 25. The developed vector. The viral construct containing the canine COMMD1 gene, Puromycin resistance gene and dsRed gene.

Transduction of the organoids

Transduction of the lentivirus was accomplished by spinoculation. The organoids were harvested using ice cold Advanced DMEM/F12 (Life Technologies), and were fragmented using a 200 µl pipette tip. Fragments were then incubated with TrypLE (Life Technologies) to obtain smaller clusters of cells. Trypsinisation was stopped using Advanced DMEM/F12 + 10% FCS. After centrifugation at 100g for 5 minutes, the cell pellet was resuspended with 500 µl of viral suspension containing 8 µg/ml polybrene (Hubrecht Institute) and different concentrations of virus: 1:2000, 1:10,000 and 1:20,0000. Suspensions were transferred to a Costart ultra-low attachment 24-wells plate (Corning, Tewksbury). Spinoculation of the cells with virus and polybrene occurred for 60 minutes at 32 °C at 600 g. After spinoculation the organoids were incubated in the culture cabinet at 37°C with 5% CO_2 in air in a humidified atmosphere for 6 hours. The organoids were then transferred to a 1,5 ml Eppendorf tube and were centrifuged at 250 g for 5 minutes. The pellet was then resuspended in Matrigel and plated out in a 48-wells plate. After two days of culturing, 2 µg ml-1 puromycin (Life Technologies) was added to the medium to select for the transduced cells. A standard of 0,5 µg ml-1 puromycin was used in the culture medium from 3 days post-transduction.

To evaluate transduction efficiency, the amount of red fluorescent cells was determined by looking under the fluorescence microscope

Expansion and differentiation of transduced organoids

For the expansion and differentiation of the transduced liver organoids, the same methods were used as described in part A.

Transplantation days

Transplantation of the organoids took place on three successive days. On each day 108 wells of a 12-wells plate with transduced differentiated organoids were harvested and prepared for transplantation. The organoids were harvested using cold Advanced DMEM/F12. The cell suspension was put on ice for 30 minutes before centrifugation for 5 minutes at 100 g, with intermediate shaking of the tubes. The pellet was then resuspended in warm TrypLE 10x (Life Technologies) and incubated in the water bath at 37°C. Trypsinisation was stopped using Advanced DMEM/F12 plus 10% FCS. This cell suspension was then put through a 70 µm cell strainer (Greiner Bio One) and centrifuged at 100 g for 5 minutes. Cell counts and viability were performed using a hemocytometer (Bürker-Türk chamber, Incyto) and trypan blue (Sigma-Aldrich). Cells were resuspended in 20 ml of sterofundin ISO (B. Braun).

During the preparation of the cells at the lab, on the first day, a liver lobectomy was performed by the surgeon at the Veterinary Clinic of Utrecht to stimulate liver regeneration. A permanent catheter was placed into the portal vein (port-a-cath, Smiths Medical, Rosmalen). The cells were injected using the subcutaneous port of the port-a-cath and reached the liver through the portal vein. The negative control received a vehicle instead of cells, but the surgical procedure was the same. Before and after the injection the portal pressure was measured manually by using a fluid column in connection with the port-a-cath.

Long term follow up and readouts

One week prior to the transplantation, a copper excretion study was performed. The dog received a radio-active copper[64] injection and after 6 hours bile was aspirated from the gallbladder. Urine samples were collected after 6, 24 and 48 hours. Feces samples were collected 24 and 48 hours after injection. This copper excretion study was repeated 3 months, 6 months and 12 months after transplantation.

After transplantation biopsies were taken at several time points: 3 days, 7 days, 1 month, 3 months, 6 months, 9 months, 12 months, 18 months and 24 months post transplantation. For immunohistochemistry, biopsy samples were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in ethanol, embedded in paraffin wax and sectioned at 4 µm.

Fixation organoids for immunohistochemistry

Organoids were collected with cold Advanced DMEM/F12 (Life Technologies), and centrifuged at 50 g to remove Matrigel. Organoids were then fixed with 4% Paraformaldehyde (PFA) + 1% eosin for 30 minutes at room temperature (RT). After this the organoids were dehydrated with 25%, 50% and 70% ethanol for 15 minutes each. The organoids were then incubated with 96% Ethanol + 1% eosin for 30 minutes. After this, dehydration was continued using 100% ethanol (3 times for 20 minutes each) and n-butanol (3 times for 20 minutes each). Then they were immersed with hot paraffin (65°C, Klinipath) three times for 20 min, embedded and sectioned at 4 µm.

Immunohistochemistry

Immunohistochemistry was performed on sections of formalin-fixed, paraffin-embedded samples of liver tissue or organoids. Antibody specifications used for the stainings are described in table 4.

Slides were routinely dewaxed and rehydrated followed by antigen retrieval in 10 mM hot citrate buffer (pH 6.0), Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) or proteinase K (Dako). Endogenous peroxidase activity was blocked using dual endogenous enzyme-blocking reagent (Dako) for 10 minutes at RT. Unspecific binding was blocked using 10% normal goat serum for 30 minutes. Primary antibodies were diluted in antibody diluent (Dako) and incubated overnight (4 °C) or for 60 minutes at RT. The secondary antibody (Envision, Dako, HRP-labeled polymer, anti-mouse or anti-rabbit) was incubated for 45 min at RT. The staining was visualized with diaminobenzidine (DAB, Dako), and counter-stained with haematoxylin quickstain (Vector Laboratories) for 10 seconds. The slides were covered with Vectamount (Vector laboratories). Different washing buffers were used for the different stainings: PBS buffer (pH: 7.4), PBS buffer plus 0.1% Tween 20 (PBS/T, pH: 7.4) and TBS buffer plus 0.1% Tween 20 (TBS/T, pH: 7.4).

Using a rubeanic acid staining on liver biopsies of the COMMD1 deficient dog, the hepatic copper concentration was semiquantitatively determined by a grading system developed in a previous study[73]. Table 4 presents an overview of the antibody and staining specification used in this study.

Primary antibody	Antibody target	Source	Company	Antigen retrieval	Buffer	Dilution	Primary antibody
CF504900	COMMD1	Mouse	Origene	10 mM hot citrate buffer pH 6.0	PBS/T	1:100, 1:150, 1:200 and 1:300	O/N at 4°C
Orb2388	COMMD1	Rabbit	Biorbyt	10 mM hot citrate buffer pH 6.0	PBS/T	1:50, 1:100, 1: 150 and 1:200	O/N at 4°C
NBP2-03755	COMMD1	Mouse	Novusbio	Tris-EDTA buffer	PBS	1:150	60 min RT
Sc-390909	dsRed	Mouse	Santa Cruz	10 mM hot citrate buffer pH 6.0	PBS/T	1:50	O/N at 4°C
Ab62341	dsRed	Rabbit	Abcam	10 mM hot citrate buffer pH 6.0	PBS/T	1:200, 1:600 and 1:1000	60 min RT
NCL-CK19	K19	Mouse	Novocastra	Proteinase K	TBS/T	1:100	O/N at 4°C

Table 4. Antibody and staining specifications. An overview of the specifications of the primary antibodies used in different stainings.COMMD1: Copper metabolism gene MURR1-containing domain 1, dsRed: Red fluorescence protein, K19: Keratin 19, PBS/T: PBS buffer containing 0.1% Tween 20, TBS/T: TBS buffer containing 0.1% Tween 20, O/N: overnight, RT: room temperature.

Results and discussion

Histological evaluation liver biopsies

Before transplantation, liver biopsies were taken from the COMMD1 deficient dog Paul to determine copper accumulation status. The quantitative copper values of the liver biopsy was 3211 mg/kg (ref: 50-400 mg/kg dry weight). Histopathological evaluation showed a mild chronic inflammation, which was located primarily in the centrolobular region. The hepatocytes were filled with copper granula, and the copper score was 4+. Figure 26 shows rubeanic acid staining done on the biopsies.

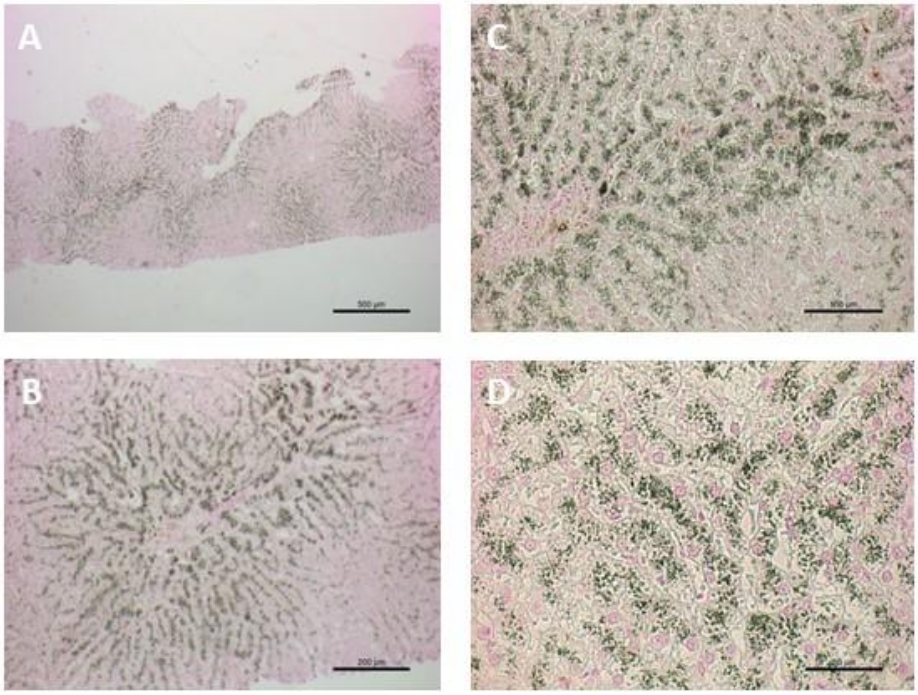

Figure 26. Rubeanic acid staining. A-D: Liver biopsies of Paul (COMMD1 deficient dog) before his transplantation. The hepatocytes are filled with copper granula. The semi quantitative copper concentration score was 4+.

Viral transduction of liver organoids

Puromycin treatment was started two days after the viral transduction to select for the organoids that had been transduced with the virus. Within one day, the organoids that were not transduced, gained a darker appearance. After 9 days, these organoids lost their spherical structure. The number of dsRed positive organoids was very small at the beginning. However, after 5 passages 95-100% of the organoids were dsRed positive (Figure 27)

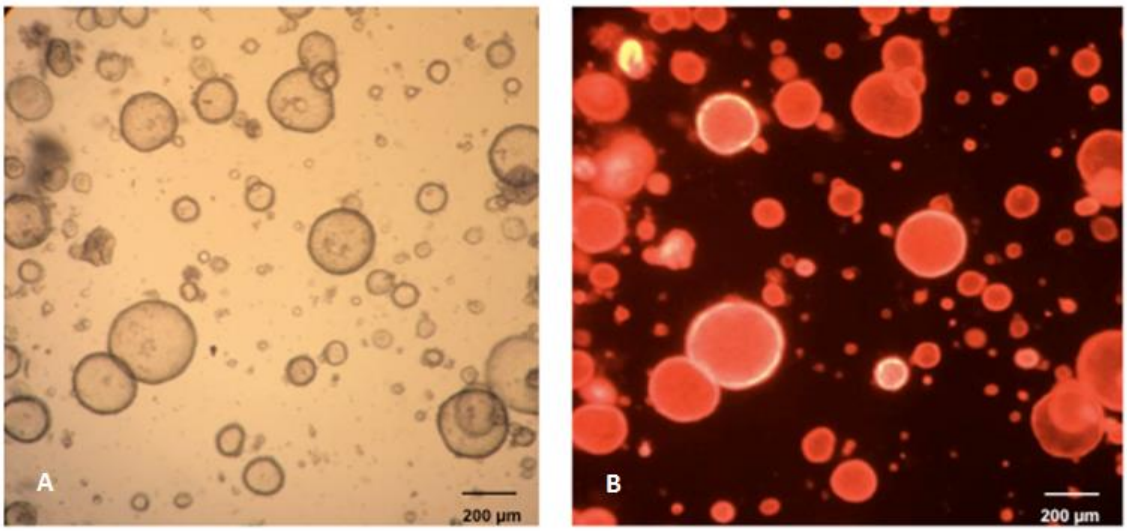

Figure 27. dsRed positive organoids after viral transduction. A. In bright field, the transduced organoids were morphologically similar to the wild type organoids. B. After 5 passages, almost all organoids were dsRed positive.

The transplantation days

Three COMMD1 deficient dogs were transplanted during my research year. A catheter was placed into the portal vein, which was connected to a port-a-cath (Figure 28). The cells were injected using the subcutaneous port of the port-a-cath (Figure 29). One of the COMMD1 deficient dogs served as a negative control and didn't receive liver organoids, but only vehicle. The rest of the transplantation procedures were performed the same way on the negative control. The first two dogs suffered from a complication after surgery. Both dogs had postoperative bleeding at the stump of the liver lobe that had been resected. They went into a hypovolemic shock and had to go back to the operating room where the bleeding was stopped. For the third dog the surgical approach for liver lobe resection was modified to a finger fracture technique[74]. In this technique the liver tissue was crushed around the line of dissection, which makes the fine vasculature of the lobe visible. The individual veins and arteries can then be ligated instead of the whole stump of the lobe. Furthermore, the amount of heparin used during the procedure was decreased to a minimal level. This technique was performed successfully and no complications were seen in the third dog.

The preparation of the cells for transplantation was successful as well. Table 5 presents the amount of cells that were obtained after harvesting and their viability.

	Transplantation day	**Amount of cells**	**Viability (%)**
Paul	Day 1	$51 * 10^6$	92
	Day 2	$2 * 10^8$	94
	Day 3	$2 * 10^8$	95
Aagje	Day 1	$1{,}64 * 10^8$	99,1
	Day 2	$3{,}12 * 10^8$	95
	Day 3	$2{,}52 * 10^8$	96,99

Table 5. Amount of cells at each transplantation day. Overview of the amount of cells transplanted each day. Paul received a total of $4{,}5*10^8$ cells, and Aagje received $7{,}28*10^8$ cells in total.

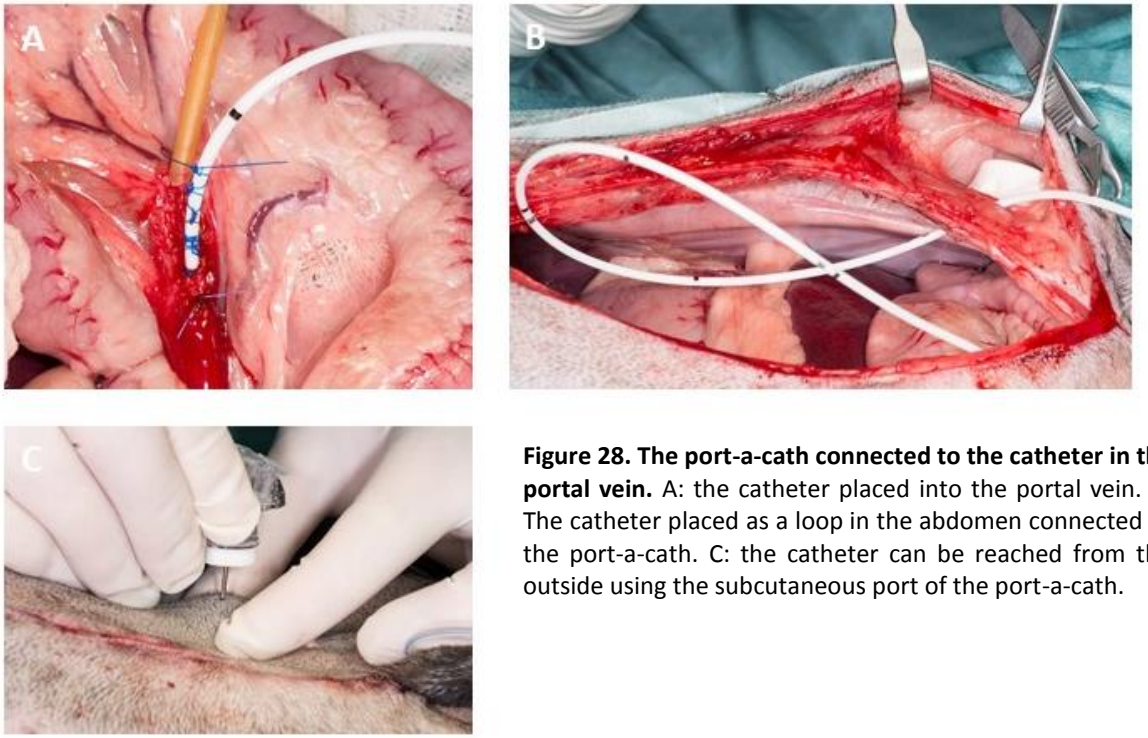

Figure 28. The port-a-cath connected to the catheter in the portal vein. A: the catheter placed into the portal vein. B: The catheter placed as a loop in the abdomen connected to the port-a-cath. C: the catheter can be reached from the outside using the subcutaneous port of the port-a-cath.

Figure 29. A total of 324 wells (24 wells plate) with liver organoids were injected through the port-a-cath. left: 324 wells filled with organoids were transplanted over 3 successive days. Right: the injection of the cells into the port-a-cath leading to the portal vein.

K19 staining

K19 is a ductal marker and stains biliary epithelium and hepatic progenitor cells[4] (Figure 30, B). Since organoids are also derived from ductal structures, they also stained positive (Figure 30, E). In liver biopsies from Paul before transplantation the duct cells were immunoreactive, but the rest of the tissue stained negative (see Figure 30, A and B). Also liver biopsies of Paul 3 days post transplantation the duct cells were immunoreactive for K19. In this biopsy immunoreactivity was also seen in a structure that appears as a bile duct, but inside the duct a immunoreactive structure of debris is seen (Figure 30, C). Also a islet of cells showed immunostaining of the cytoplasm in a liver biopsy after transplantation (Figure 30, D).

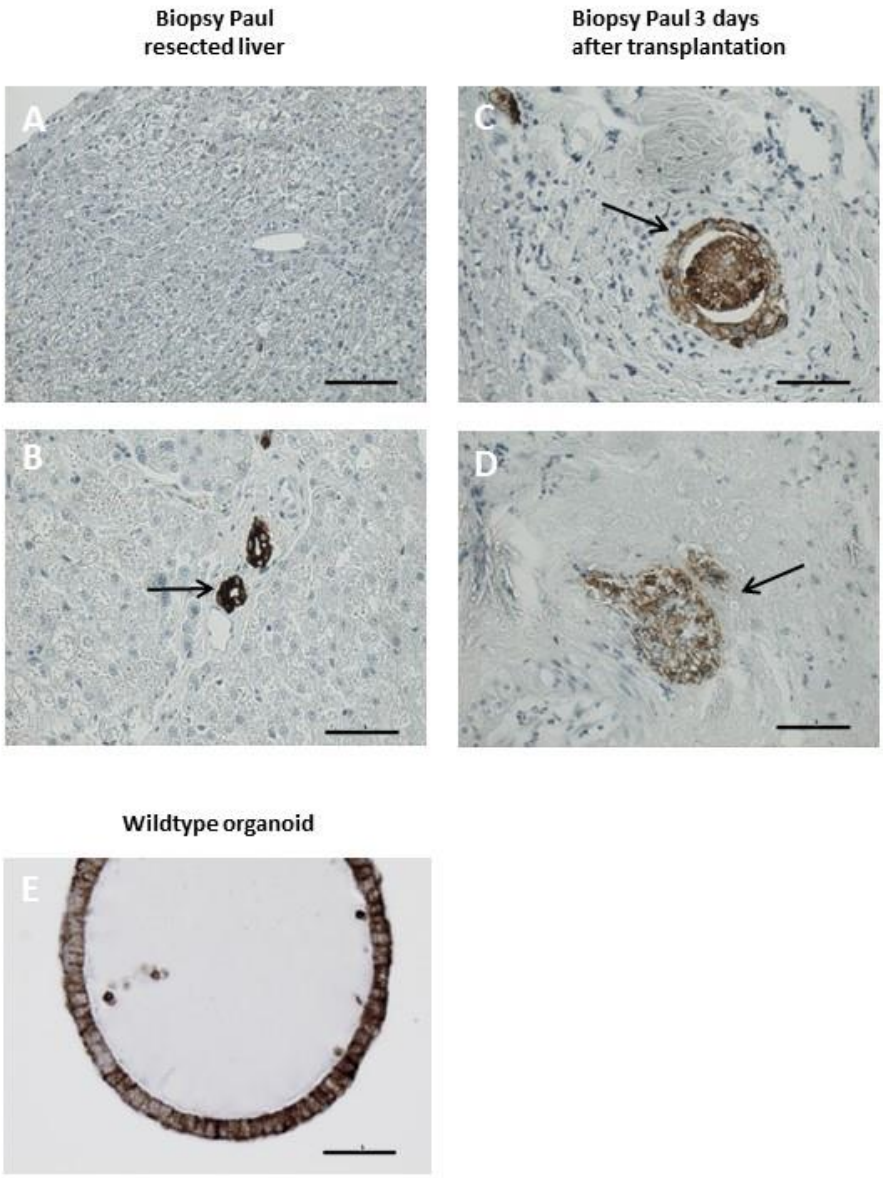

Figure 30. K19 staining. A: negative control (no primary antibody), scale bar represents 100 µm. B: liver biopsy of Paul before transplantation. Arrow shows positive duct cells, scale bar represents 50 µm. C and D: liver biopsies of Paul after transplantation shows next to positive duct cells several positive structures (arrows), scale bars represent 50 µm. E: undifferentiated wild type liver organoid staines positive for K19, scale bar represents 50 µm.

COMMD1 immunohistochemistry and immunocytochemistry

For the COMMD1 staining, three different antibodies were used. We started with antibodies CF504900 and Orb2388. The optimization of the staining with these antibodies was evaluated by a pathologist. Antibody Orb2388 was considered the best for the staining of COMMD1, when using citrate buffer as the antigen retrieval and antibody dilution 1:50. To confirm this protocol, COMMD1 deficient liver tissue was involved in the staining. The stained wild type liver tissue was a lot darker than the COMMD1 deficient liver tissue, which indicates more expression of COMMD1 protein in this tissue. However, the COMMD1 deficient liver tissue also showed weak immunostaining. Several more dilutions of this antibody were tested, but in all of these the COMMD1 liver tissue stained positive. From these results we concluded that the antibody was not specific enough.

Next, antibody NBP2-03755 was tested. Several optimizations were performed that showed that citrate was the most successful antigen retrieval and that the antibody dilution should be between 1:200 and 1:1000. In Figure 31 shows the optimized COMMD1 staining. The tissue used as a negative and isotype control all stained negative. The biopsies of wild type liver showed weak immunostaining in the cytoplasm and strong immunostaining in the sinusoids (Figure 31, A). Liver biopsies of Paul before transplantation were completely negative (Figure 31, B). In the transduced Paul organoids the positivity was seen in the cytosol, but the inner membrane of the organoids were more immunoreactive (Figure 31, D). The liver biopsies of Paul after transplantation showed a lot of similarities with the biopsies before transplantation. However, in the biopsies from 3 and 7 days after transplantation there were dark brown areas (Figure 31, arrow heads). These brown areas didn't involve complete cells, it seems more like an artefact. The biopsy from 1 month after transplantation showed a different kind of positivity. A few islets of cells showed weak immunostaining (Figure 31, arrows).

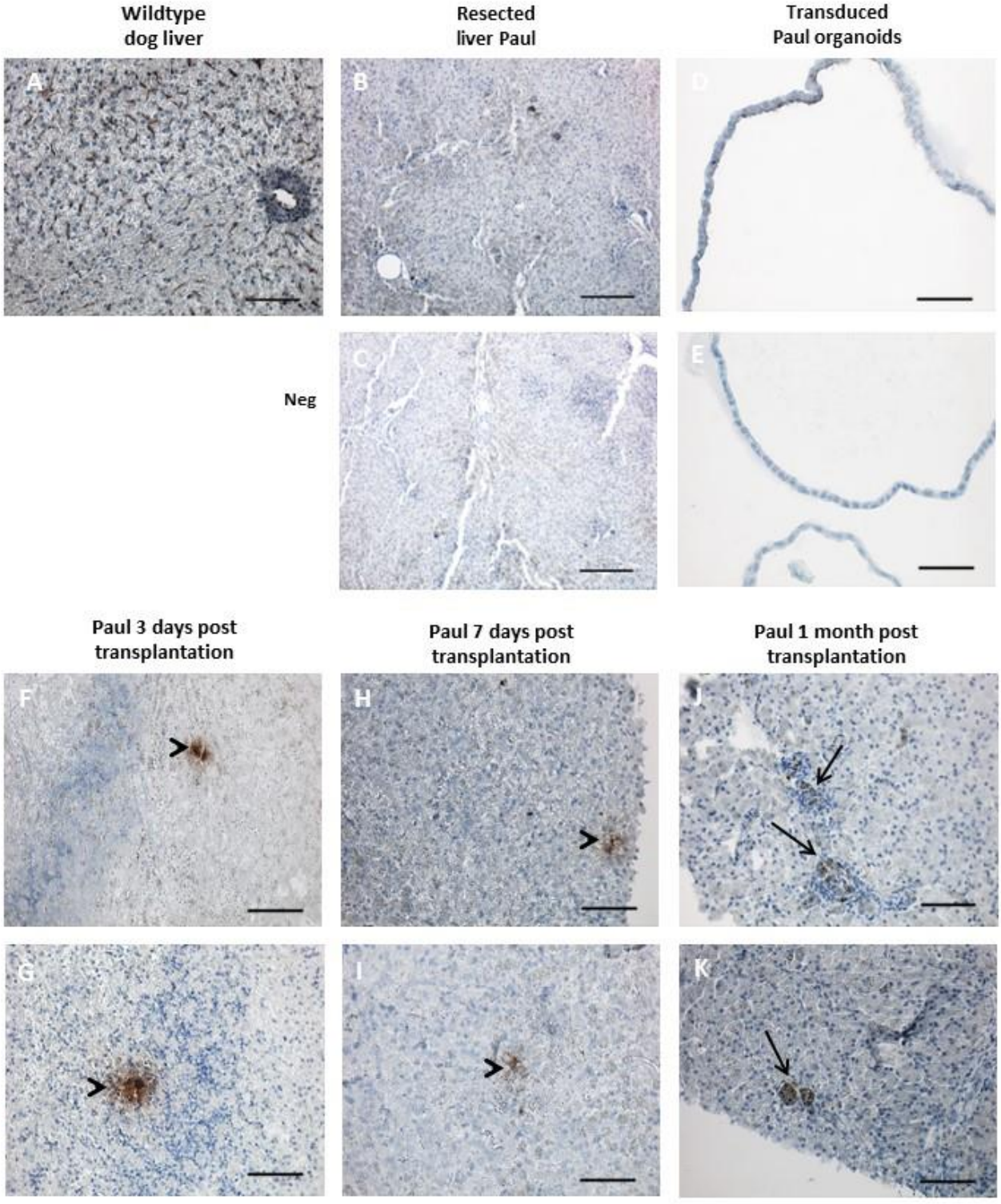

Figure 31. COMMD1 staining with NBP2-03755. A: wild type liver shows positive staining, especially in the sinusoids. Scale bar represents 100 µm. B: liver biopsy of COMMD1 deficient dog shows no positivity when stained for COMMD1. Scale bar represents 200 µm. C: negative control (no primary antibody), scale bar represents 200 µm. D: transduced organoids show some positive staining for COMMD1, scale bar represents 50 µm. E: negative control (no primary antibody), scale bar represents 50 µm.
F t/m K: post transplantation the biopsies are mostly negative (just like biopsies before transplantation) with some brown spots. The arrow heads show spots that are not well defined and look more like an artefact. The arrows show spots where there are cells that look more positive than the surrounding cells. Scale bars represent 100 µm.

dsRed immunohistochemistry and immunocytochemistry

Two different antibodies were tested to detect the presence of dsRed protein in transduced liver organoids, and liver biopsies of Paul after transplantation. Antibody Sc-390909 was tested on Paul transduced organoids, wild type organoids, a liver biopsy of Paul before transplantation and on liver biopsies of Paul 3 days after transplantation. Negative and isotype control showed no staining. Staining with an antibody dilution of 1:50 generated strong immunostaining of the cytoplasm in both biopsies of Paul before and after transplantation (Figure 32). Wild type and transduced Paul organoids also showed strong immunostaining of the cytoplasm, but showed variability between the organoids in the same section (Figure 32). This staining couldn't be specific for dsRed protein. Wild type organoids stained positive while these organoids should not express dsRed protein. In addition, biopsies of Paul before transplantation were just as positive as biopsies of Paul 3 days after transplantation. Before transplantation, the liver tissue didn't have expression of dsRed protein. After transplantation we expected to see islands of cells which are immunoreactive.

Another antibody (Ab62341) for the dsRed protein was tested. The negative controls for this staining showed no immunoreactivity. Staining with an antibody dilution of 1:200 showed immunostaining of the cytoplasm in both biopsies from tissue before and after transplantation (Figure 33, B and E). Increasing the dilution (1:600) still exhibits weak immunoreactivity in both biopsies, restricted to the cytoplasm (Figure 33, C and F). The transduced organoids also showed immunoreactivity of the cytoplasm, but not all cells within an organoid and not all organoids exhibited this reactivity (Figure 33, H and I). The wild type organoids remained negative in both dilutions (Figure 33, J and K). This antibody was not specific for dsRed protein, since there was immunostaining in liver biopsies before transplantation.

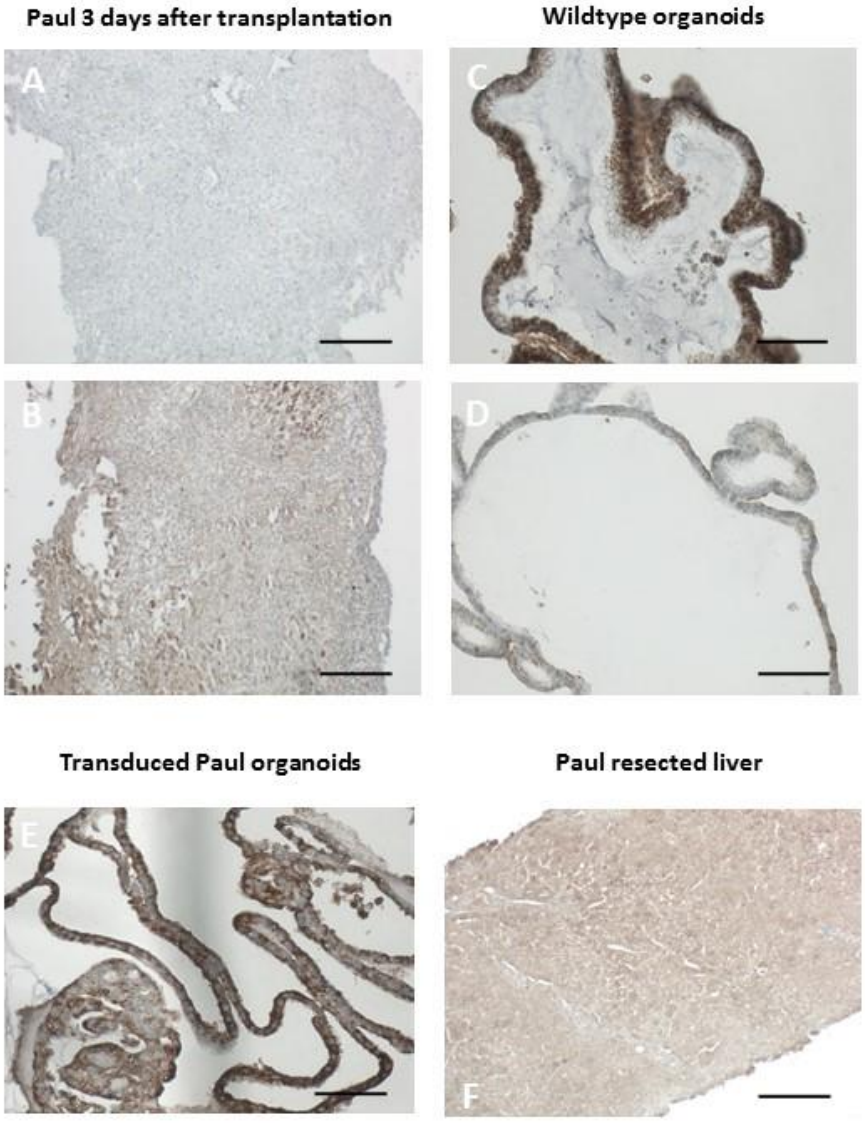

Figure 32. dsRed staining with Sc-390909.
A: negative control (no primary antibody), scale bar represents 200 µm. B: liver biopsy of Paul stained for dsRed shows positivity of the complete tissue (aspecific), scale bar represents 200 µm. C and D: wild type organoids also stain positive, although there is variation through the coupe, scale bar represents 50µm.
E: transduced Paul organoids stain just as positive as the wild type organoids, scale bar represents 50 µm. F: liver biopsy of Paul before transplantation also stains completely positive, scale bar represents 200 µm.

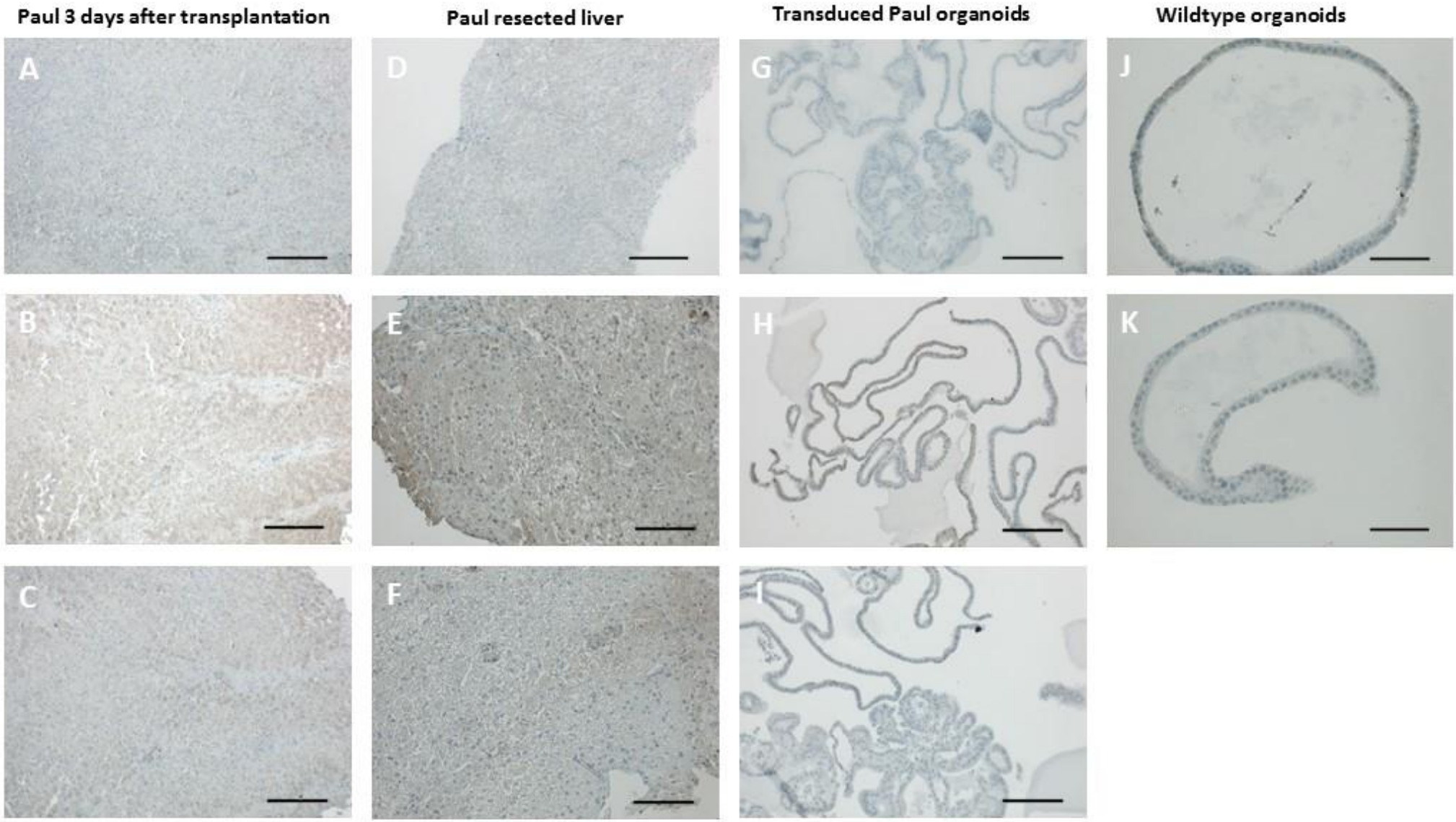

Figure 33. dsRed staining with Ab62341. A and D: negative control (no primary antibody), scale bars represents 200 µm. B and C: liver biopsy of Paul 3 days after transplantation, stained for dsRed with antibody dilution 1:200 (B) and 1:600 (C), scale bar represents 200 µm. E and F: liver biopsy of Paul before transplantation, stained with antibody dilution 1:200 and 1:600 (F), scale bars represent 100 µm. G: negative control (no primary antibody), scale bar represents 100 µm. H and I: transduced Paul organoids stained for dsRed with antibody dilution 1:200 (H) and 1:600 (I), scale bars represent 100 µm. J and K: wild type liver organoids stained for dsRed with antibody dilution 1:200 (J) and 1:600 (K), scale bars represent 50 µm.

General discussion part B

Two COMMD1 deficient dogs were transplanted with genetically corrected liver organoids. One dog was transplanted with vehicle as a negative control. Because of postoperative bleeding from the resection site in dog 1 and 2, the surgical protocol for lobectomy was altered. In dog 3 a finger fracture technique was applied as well as a reduction in heparin administration throughout the procedure. Dog 3 had an uneventful recovery from the surgery.

The patient specific liver organoids were genetically corrected using a viral transduction. After transduction of the organoids it took 5 passages, before 95-100% of the organoids were red fluorescent under the microscope. The red fluorescence indicates that the organoids have incorporated the vector into their genome, which contains the dsRed protein. However, when staining the transduced organoids for COMMD1 (which gene is also in the vector), we saw that not all cells in the organoids were positive and also some organoids were more positive than the others. This means that when 100% of the organoids are red fluorescent under the fluorescence microscope, which indicates the incorporation of the vector into the genome, they don't all express COMMD1.

The dsRed gene in the vector makes the transduction visible. By looking under the fluorescent microscope, the transduction efficacy can be evaluated. Also, when transplanting these organoids into a patient, we could stain biopsies for this protein to track the cells. None of the tested antibodies for the red fluorescent protein in this study were specific.

Staining of the COMMD1 protein is the second option for cell tracking. Also this staining can confirm the functionality of the transplanted organoids. We tested a few antibodies for this protein, of which the first two weren't specific enough. These antibodies resulted in positive staining of COMMD1 deficient liver tissue, while no COMMD1 protein should be present there. The COMMD1 deficient liver tissues showed no immunoreactivity with the last antibody, while the wild type liver tissues were stained positive. COMMD1 is mainly a cytosolic protein, although a small fraction has also been detected in the membranous fraction[11]. The localization of the protein partly overlaps with the vesicular compartment of endosomes and lysosomes[11]. Next to this, an interaction between COMMD1 and ATP7B has also been confirmed[75]. For these reasons, we would expect the COMMD1 staining to be primarily visible in the cytosol of the hepatocytes. However, in the wild type liver tissue the immunostaining is primarily seen in the sinusoids. In the transduced organoids you see immunostaining at the inner membrane, but also some positivity can be seen in the cytosol. This could raise the question if the antibody is specific for COMMD1 and does not cross react with another protein that is present in the sinusoids. The fact that the staining is completely negative in liver tissue from COMMD1 deficient dogs does however imply that the antibody is specific for COMMD1 alone, since this is the only difference between the wild type liver tissue and the COMMD1 deficient liver tissue.

We expect that the transplanted liver organoids will engraft in the liver tissue and will express the COMMD1 protein. Because these cells have a genetic correction, we expect them to have a growth advantage compared to the patient's own hepatocytes/stem cells. After transplantation the organoids will engraft and proliferate and eventually the liver tissue will be (partly) replaced with genetic corrected hepatocyte-like cells. In the liver biopsies from Paul after the transplantation, we would therefore expect to see sporadic islets of cells that are positive. However, as mentioned before, the 100% transduction of the organoids doesn't seem to lead to a 100% expression of the COMMD1 protein (according to the staining). This could be a reason why we miss transplanted cells when we stain them for COMMD1. dsRed could therefore be a better method to find back the transplanted cells, since all of these cells do express the dsRed protein (fully red fluorescent organoids were transplanted). In the liver biopsies after transplantation we do see some positive areas. However, the biopsies from 3 and 7 days post transplantation show areas that are not well restricted to complete cells. It seems that these areas are artefacts. It is unclear how these artefacts have developed. In the liver biopsy from one month post transplantation, we see a different kind of positivity. A few islets of cells with weak immunostaining in the cytoplasm are found in these biopsies. These cells could be derived from the transplanted organoids.

The K19 staining is very specific for bile duct epithelium and hepatic progenitor cells, and shows no a-specific staining. K19 is a marker of canine hepatic progenitor cells[76,77], and therefore also undifferentiated organoids are stained positive. When differentiating the organoids towards hepatocyte-like cells, the expression of the K19 protein will decrease (*Nantasanti et al.* 2015 Unpublished data). This is why this might not be the right protein to track transplanted differentiated organoids in the tissue. Next to this, it is hard to distinguish bile ducts from the organoids.
As also mentioned in part A, the differentiation of organoids lead to a more functional copper excretion pathway. Therefore, but also because we eventually want to repopulate the liver with the organoids, it is important that the differentiation process is optimal. Our protocol leads to more hepatocyte-like cells, but they are not the same as freshly isolated hepatocytes. However, it is also possible that the organoids will differentiate more after they have engrafted in the liver tissue, since this should be the optimal environment for hepatic differentiation. This was also seen in the study of *Huch et al.* where transplanted cells didn't have any ductal expression after transplantation and engraftment[48], contrary to what was seen *in vitro*. Differentiation of the organoids to fully mature hepatocytes might therefore not be necessary. On the other hand, in the same study they also tried to transplant undifferentiated organoids in mice, but this didn't lead to engraftment of the cells[48]. This indicates that the cells need to be differentiated before transplantation can be successful, however *Huch et al*. only found engraftment of the differentiated cells in 5 of the in total 15 mice that were transplanted[48]. The results of our study may be different since the cells were administered through the portal vein of the dog, instead of intrasplenically in a mouse in the study of *Huch et al.,* and also we injected cells on three successive days.
For future research, transplantation of both undifferentiated and differentiated organoids need to be repeated, to see which of these cells are most suitable. Next to this, more dsRed antibodies should be tried and optimized until a specific antibody has been found. It also would be a good suggestion to do a double or triple staining with for example COMMD1 and dsRed.

Conclusion

The procedure we present here to genetically correct patient specific liver organoids and transplant them back into the patient, has been successfully performed on two COMMD1 deficient dogs. We have developed a protocol for staining of COMMD1. The biopsies of these patients after transplantation show COMMD1 positive cells that could be derived from the transplanted organoids. A staining for the dsRed protein still has to be optimized. The cell transplantation will be performed in three more COMMD1 deficient dogs. Liver biopsies of more time point post transplantation and more different transplanted dogs will be stained for COMMD1 and dsRed to further confirm our hypothesis.

Part C: Breeding program

Purpose of breeding program

For the transplantation project (part B) we obtained DEC approval (2014.III.12.112) to transplant 9 COMMD1 deficient dogs. These dogs were not yet available. To this purpose we started a breeding program to create more COMMD1 deficient dogs for the transplantation study. Paul is a COMMD1 deficient dog present at the clinic. We also had two female dogs who were carriers of the genetic disorder. Both female dogs were inseminated and got pregnant. The first female dog gave birth to 5 puppies. Three of these puppies were COMMD1 deficient, the other two were carrier of the disorder (Figure 34). The second female dog gave birth to two puppies, of which one did not survive. The dead puppy appeared to be COMMD1 deficient and the living puppy is a carrier for the disorder.
Paul was the first dog to be transplanted with differentiated transduced liver organoids. After this one of the deficient pup (Antonio) was transplanted at seven months of age with vehicle, as a negative control. In June another deficient pup (Aagje) was transplanted at seven months of age with liver organoids. In order to obtain new COMMD1 deficient dogs, one of the deficient pups will also be used for breeding purposes.

Organization

As the transplantation study was also part of my honours program, I was asked to coordinate the care of the mother and pups before, during and after the birth. I assembled a team of 10 bachelor or master students. These students were scheduled to visit the mother (and later pups) four times a day and they were asked to fill out forms each time about the well-being of the mother and pups. I set up the organization of this team and coordinated the care of mother and pups, until the pups had to be weaned. In Appendix 2 you can find the scenario we created for this project.

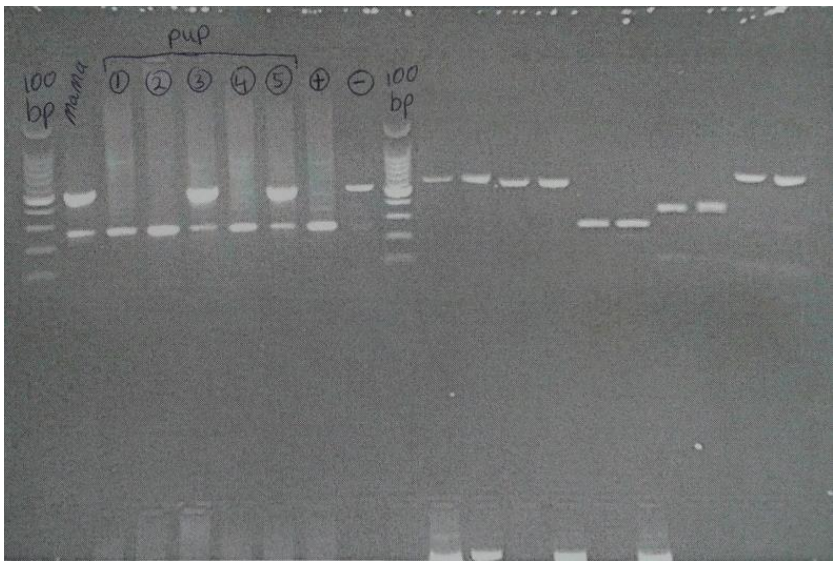

Figure 34. COMMD1 PCR results. This gel shows that three of the five pups (pup 1, 2 and 4) are homozygous for the mutated gene and therefore are COMMD1 deficient.

Figure 35. COMMD1 deficient pups. Picture of two COMMD1 deficient pups: Aagje (left), who has been transplanted in June, and Aron (right), who has not yet been transplanted.

General Discussion

This research explored two main applications for liver organoids: disease modeling and transplantation. A major advantage of using liver organoids for transplantation is that they can be isolated from liver biopsies or fine needle aspiration biopsies (FNAB) from the patient's own liver[52]. When transplanting the cells back, there will be no need for immunosuppression[52]. However, culturing liver organoids is a relatively expensive method in comparison to 2D cell lines. For research purposes, the liver organoids can be very suitable because they can be cultured for over a year and they remain their differentiation potential over time[52]. The current method for culturing organoids is not well standardized. Wells can't be seeded with exactly the same amount of cells/organoids and the compounds of Matrigel are variable. When performing experiments on these organoids, one should consider these variables. An obstacle in using these liver organoids for disease modeling is that the differentiation protocol still needs to be improved. Since cell-cell contact is very important in the differentiation process[39], co-culturing of liver organoids with other cell types could enhance the differentiation. For instance, hepatic stellate cells have shown to have a positive effect on the differentiation of HPC-like cells[78]. Also, macrophages have shown to be involved in the specification of hepatocyte differentiation[79]. Co-culturing liver organoids with these cell types could lead to an enhanced differentiation towards hepatocyte-like cells. The extracellular matrix is also very important regulating the cellular shape and the liver-specific function[39]. Adding extracellular matrix compounds, like collagen, into the Matrigel could therefore also enhance the differentiation. For transplantation purposes, it might be less important for the organoids to be fully differentiated towards hepatocytes because, but this requires further research.

New therapeutic methods developed in studies on rodents, should preferably be tested on a large animal model before application in the human clinic. An accepted animal model requires a known aetiology of the disease and this must be comparable to the aetiology in humans[56]. Canine fibrotic liver diseases have recently been shown to have many similarities with the human counterparts in both pathophysiology and molecular mechanisms[80]. For this reason, the dog could function as a large animal model for liver diseases in humans. COMMD1 deficiency in the Bedlington terrier is a well-studied disease which leads to chronic hepatitis and presents a Wilson's disease like phenotype[7,12]. Due to a dog's life-span, size, and the possibility to easily approach the liver and take multiple liver biopsies, these dogs are suitable as a disease model for Wilson's disease, but also for chronic liver diseases in general. Recently, a method for culturing human liver organoids has been established[81]. A human patient with a metabolic liver disease, would be a good candidate for liver organoid transplantation. This type of liver diseases is associated with a deficiency of a hepatic protein or enzyme[72], which is very comparable to COMMD1 deficiency in dogs. The introduction of 5-15% of the missing protein is often enough to correct the metabolic disease[72]. As performed in this transplantation study on dogs, the patient's own HPCs can be isolated, cultured as three dimensional liver organoids and genetically corrected. After expansion and differentiation *in vitro*, these cells can then be transplanted back into the patient. The port-a-cath, which we used in this transplantation study, has also been successfully used in the human clinic for hepatocyte transplantation[82]. When the transplantation of liver organoids is successfully in the dog model, it could also be a very promising method to treat liver diseases in humans.

General Conclusion

Culturing patient specific liver organoids is a very promising technique. The cells can be isolated from the diseased liver and therefore patients specific aspects of the disease can be studied and drugs can be tested (personalized medicine). In case of copper toxicosis, we have not yet succeeded to develop a repeatable disease model. More research on optimization of the differentiation protocol and the standardization of the culture method should be done. Next to this, a more sensitive copper sensor should be used, which is more suitable for accurately measuring minor shifts in intracellular copper concentrations. Liver organoids are also very promising to be used for transplantation purposes. The organoids can be cultured from the patient's own liver, and a genetic correction can be done before transplanting the cells back. We have found that we can successfully culture patient's specific COMMD1 deficient canine liver organoids and transplant the cells back using a port-a-cath connected to the portal vein, after genetic correction. With an optimized COMMD1 staining used on liver biopsies taken after transplantation, we found an indication that COMMD1 positive cells have engrafted in the liver tissue. Liver biopsies of later time-points after transplantation still have to be stained for COMMD1, and the procedure has to be repeated on more dogs. Also, the dsRed staining remained to be optimized, which can also be used to track the transplanted cells in the liver tissue. However, since there is now a published culture method for human liver organoids[47], these results on transplantation of canine liver organoids are very promising for use in human medicine as well.

Acknowledgments

I would like to thank my supervisor Hedwig Kruitwagen, for guiding me throughout this honours program and for her enthusiasm during this research project. I would also like to thank Baukje Schotanus, who was my supervisor during Hedwig's maternity leave and Monique van Wolferen for sharing her experience in the laboratory. I thank my other supervisor Bart Spee for all his advice on my experiments during this year. Next, I would like to thank Loes Oosterhoff for sharing her experience with the organoid culturing and other organoid-related experiments on the laboratory, and also for her support and sociability throughout this period. I would like to thank all employees and students on the laboratory, for their collaboration and sociability during this honours program.

Courses

During this honours program I participated in two courses: 'Modern methods in Data analysis' and 'Writing a scientific paper'. The 'Modern methods in Data analysis' course was preceded by an short course 'Introduction to statistics and R'. The statistics course was successfully completed with the final grade of 8,8. The course 'writing a scientific paper' was also successfully completed.

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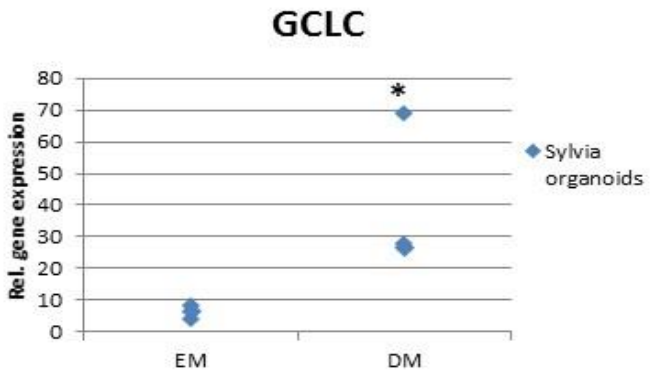
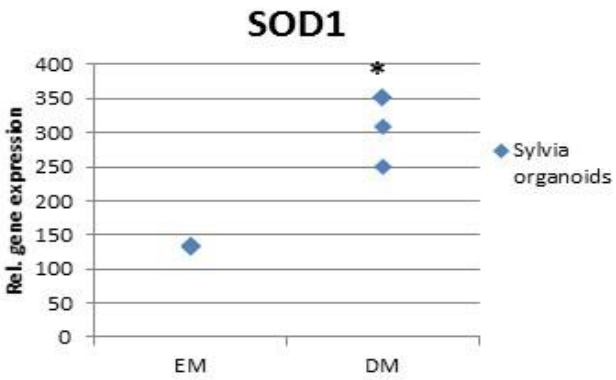
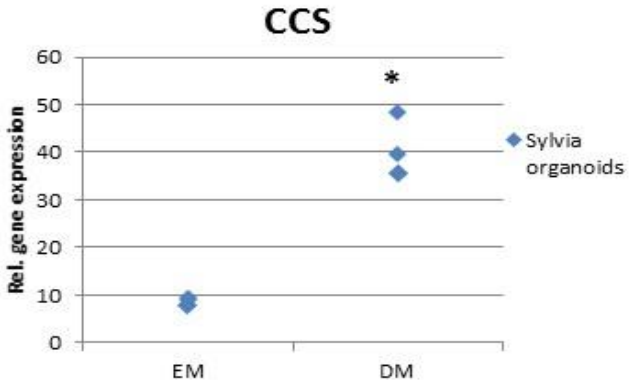
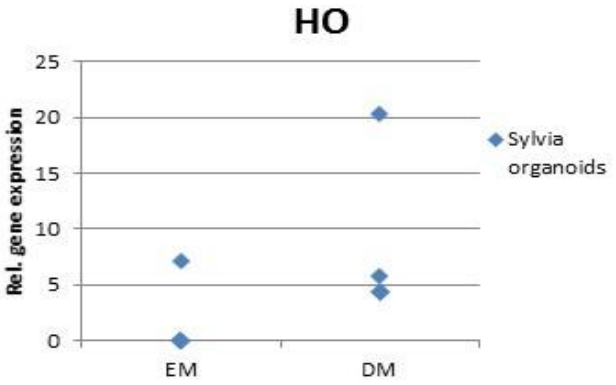
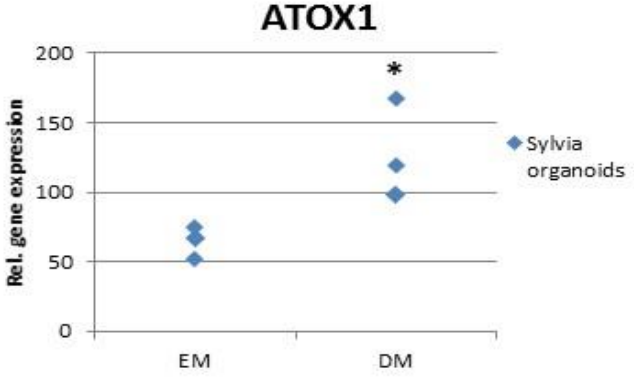
Appendix 1: Gene expression profiles Sylvia organoids

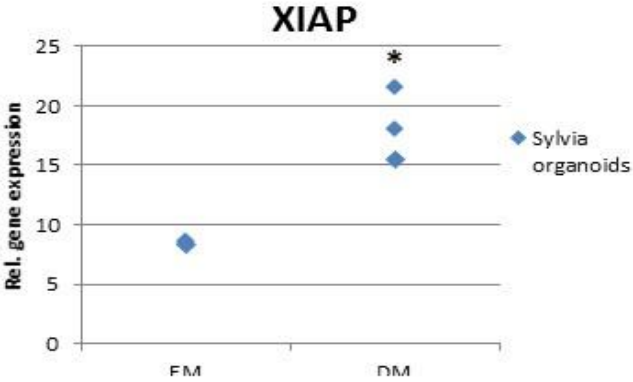


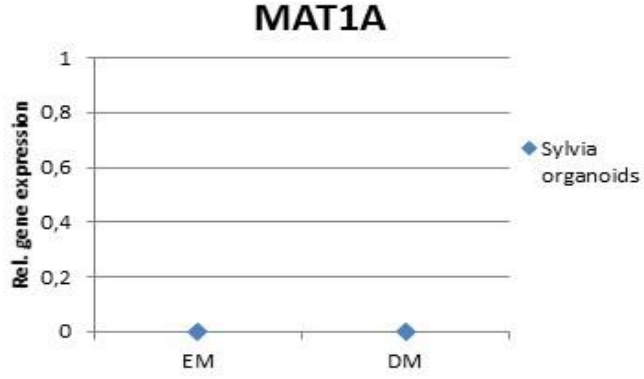


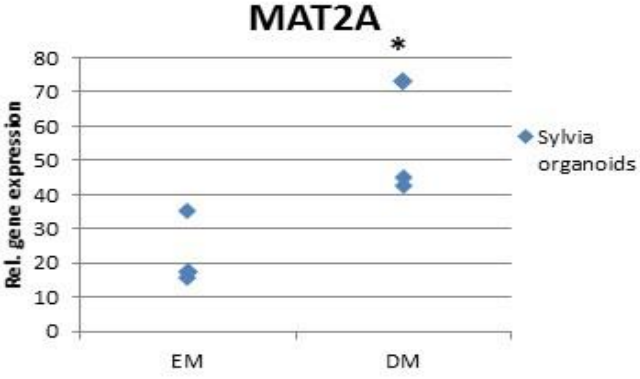


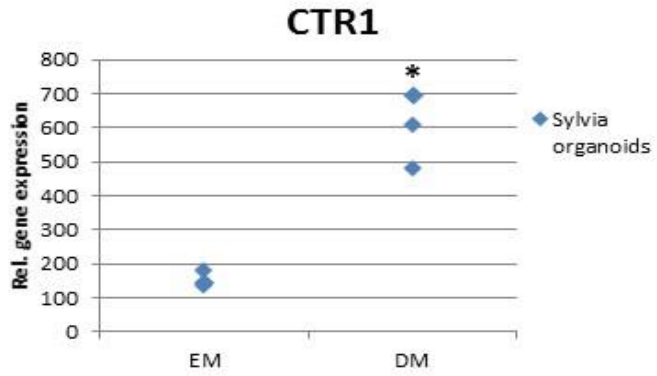











CTR1
Rel. gene expression
800
700
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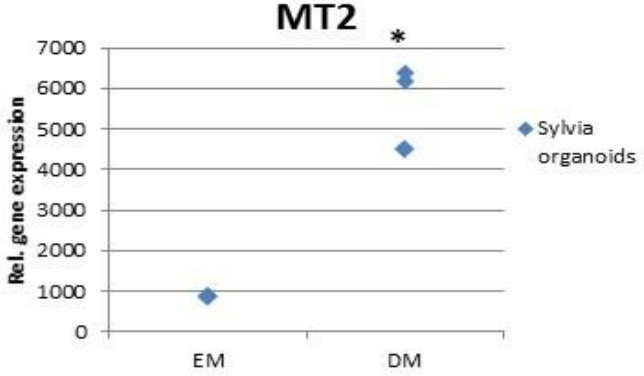
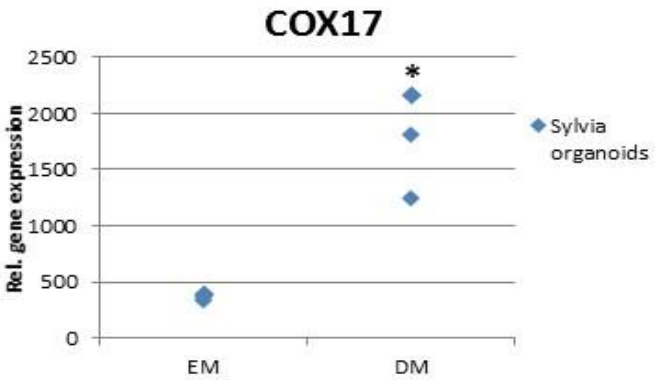
500
400
300
200
100
0
EM
DM
*
Sylvia organoids


MT2
Rel. gene expression
7000
6000
5000
4000
3000
2000
1000
0
EM
DM
*
Sylvia organoids


COX17
Rel. gene expression
2500
2000
1500
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EM
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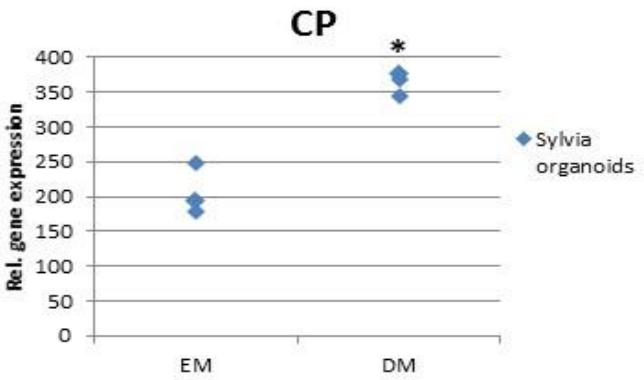
*
Sylvia organoids


CP
Rel. gene expression
400
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300
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150
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EM
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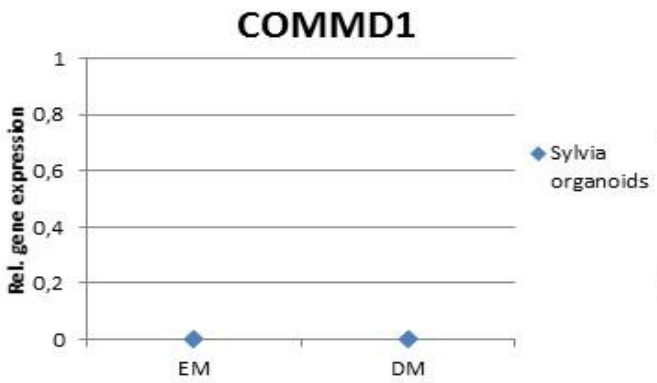
Sylvia organoids


COMMD1
Rel. gene expression
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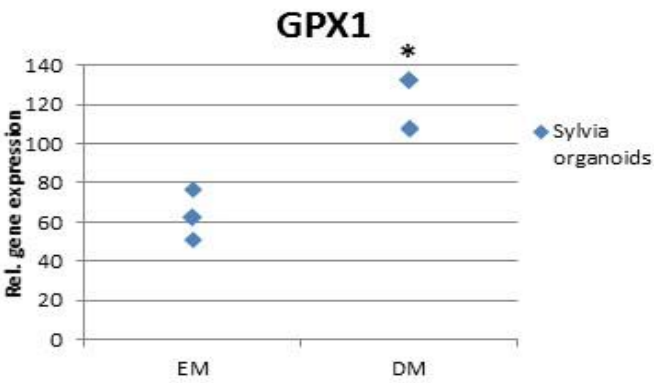
0,6
0,4
0,2
0
EM
DM
Sylvia organoids


GPX1
Rel. gene expression
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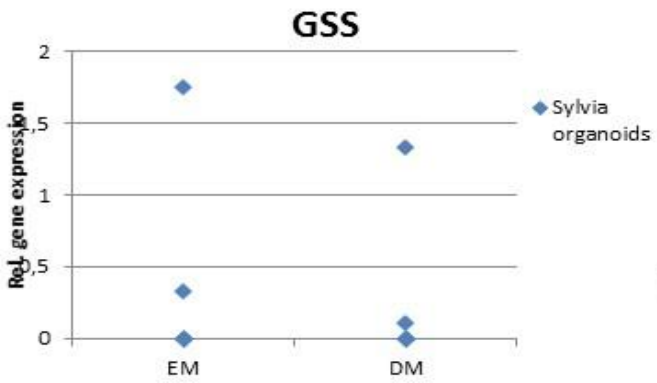
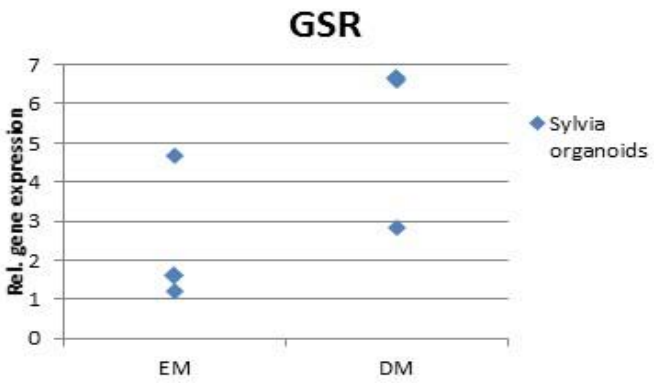
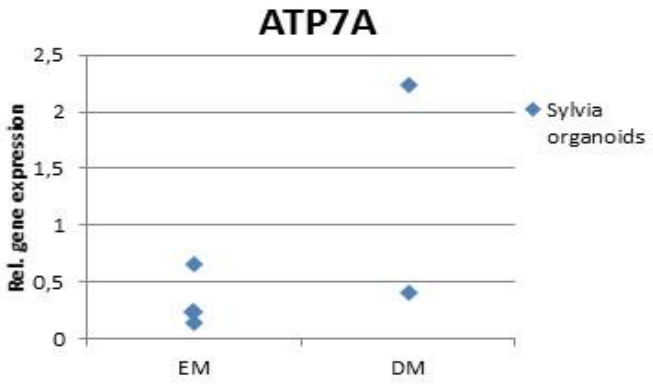
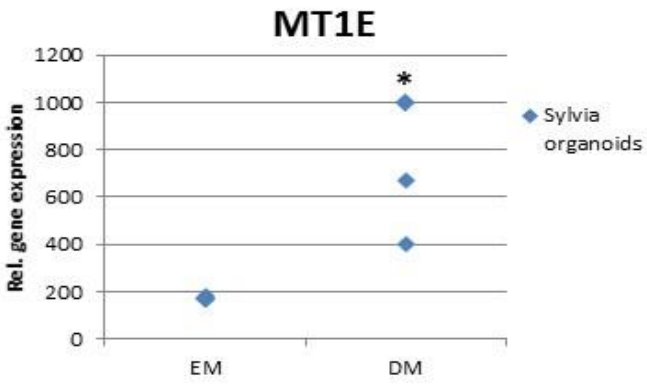
120
100
80
60
40
20
0
EM
DM
*
Sylvia organoids


GSS
Rel. gene expression
2
1,5
1
0,5
0
EM
DM
Sylvia organoids


GSR
Rel. gene expression
7
6
5
4
3
2
1
0
EM
DM
Sylvia organoids


ATP7A
Rel. gene expression
2,5
2
1,5
1
0,5
0
EM
DM
Sylvia organoids


MT1E
Rel. gene expression
1200
1000
800
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*
EM
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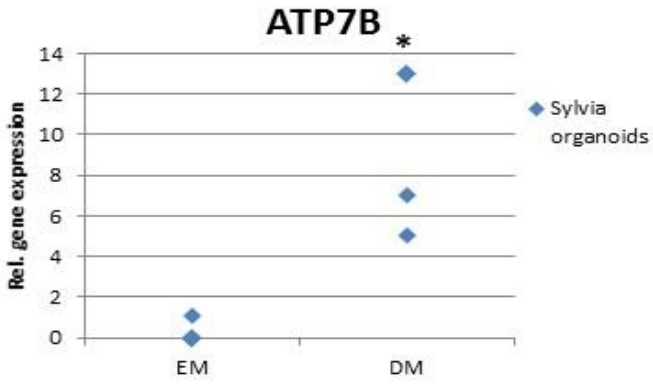
Sylvia organoids


ATP7B
Rel. gene expression
14
12
10
8
6
4
2
0
*
EM
DM
Sylvia organoids

Appendix 2: Scenario organization breeding program

Verzorging van een hond en haar pups vanaf kort voor de geboorte tot 6 weken erna.

Hoofddoel:

Met inzet van studenten (rondom hun reguliere onderwijs) de partus van een hoogdrachtige teef begeleiden en haar met de pups verzorgen tot speenleeftijd. Dit op het niveau waarop een eigenaar dat zou doen binnen een geïnstitutionaliseerde omgeving om het welzijn van de moeder en pups optimaal te waarborgen.

Einddoel: gezonde, goed groeiende en goed gesocialiseerde honden.

Beschrijving functies:

Coördinator kraamzorgteam: HP student betrokken bij onderzoeksproject,

- Draaiboek verder uitwerken (wat moet er wanneer gebeuren)
- Studenten (vrijwilligers) benaderen en rooster maken
- Maakt taakverdeling voor de geroosterde diensten (wat moet je doen in de dienst waarvoor je ingeroosterd bent?)
- Kennismaking met dierverzorgers / met de kennel / met de teef
- Afstemming met dierverzorgers over de proefdierverplichtingen / registraties
- beheerder van Whatsapp groep
- beheert voorraden van verbruiksartikelen; van voer (moeder en pups, kunstmelk) tot schoonmaakmiddelen in de partusruimte)

Vrijwilliger kraamzorgteam: student DGK, eind bachelor (na blok 18) / master GD

- Werkzaamheden in van tevoren afgesproken shifts, commitment aan deze taak is noodzakelijk
- Verzorgen moederdier vanaf kort voor de partus tot 6 weken erna (voeden, uitlaten, temperaturen rondom partus etc.)
- Begeleiding partus (live in kleinere subteams, max 2 studenten / shift, allen per webcam)
- Monitoring en verzorgen pups (bijv. wegen, bijvoeren als nodig). Later evt. ook een rol bij socialisatie van de pups (socialiseren/uitlaten/trainen)
- Participeren in schoonmaakrooster partusruimte en voorruimte

Huismeester: Hans van Baar (1699)

- Verstrekt XS passen voor toegang in afgesloten gedeelten en toegang tot gebouw buiten kantooruren
- Zorgt voor functioneren webcam
- Is te benaderen bij technische problemen in de partusruimte.

Dierenartsen discipline voortplanting: Karin Albers-Wolthers (4126 / 06-19020913) en Jeffrey de Gier (1477 / 06-53784462)

- Achterwacht mbt partus (altijd contact zoeken bij aanvang en tijdens partus svp) en neonatale zorg
- Antiparasitaire behandelingen (eerste 6 levensweken pups) en vaccinaties (op 6, 9 en 12 weken leeftijd)

Dierverzorgers/-verplegers : avond en nacht Proefdierafdeling (overdag: 1006) en IZA/ZPA/SGMN

- Aanspreekpunt bij vragen over de verzorging van de moeder en pups: overdag: locatie spullen, voer etc. Contact: Inge van Duiven, tel 1006.
- **Alleen in geval van noodzaak tot bijvoeden**: dierverplegers van de nachtdienst (IZA/ZPA/SGMN) nemen de nachtvoeding voor hun rekening
- **Alleen indien noodzakelijk**: toedienen oxytocine bij niet vorderende partus, dit alleen op indicatie vanuit de begeleidende achterwacht voortplanting (mag namelijk niet door studenten worden toegediend)

Proefdierdeskundigen

- Raadplegen bij vragen mbt proefdiergebruik: altijd via Hedwig Kruitwagen

Manager OBP, Jesje Zijlstra Leidinggevende van de dierverzorgers/-verplegers

- Vragen over inzet dierverplegers, bijvoorbeeld voor oxytocine injecties, sondevoeding ‘s nachts / roosters van dierverplegers etc.

Wie is wie?

Coördinator kraamzorgteam

Coördinator van het kraamzorgteam is Christel Vinke (student olv dierenarts-onderzoeker Hedwig Kruitwagen). Zij is ook eerste aanspreekpunt en participeert in het 24/7 rooster.

Mailadressen: C.R.Vinke@uu.nl
H.S.Kruitwagen@uu.nl

Mobiel nummer: 06 36170755 (Christel); 06 45612598 (Hedwig)

Vrijwilligers voor het kraamzorgteam (bereikbaarheidsgegevens in bijlage):

Er wordt een 'smoelenboek' gemaakt, met namen en foto's van de studenten (incl Christel), dat naast de ingang van de partusruimte komt te hangen. Zo is duidelijk wie er lid van het kraamzorgteam is en dus de partusruimte mag betreden. Het team bestaat uit 8-10 studenten.

Voorbereiding Informatiebijeenkomst:

- Doel vd fok, doel van de zorg
- Wat verwachten wij van de studenten (commitment aan deze taak)
- Proefdieren; artikel 12 functionaris en de registratieverplichtingen
- 24/7 rooster
- Sondevoeding van pups
- Rondleiding kliniek: wat/wie is waar?

Actiepunten:

- Hedwig:
  - afstemming met artikel 14 mannen over kraamzorgteam, inzet vrijwillige studenten (zie aanmeldingsformulier stagaires/studenten): na infobijeenkomst sturen naar art. 14, Inge, Ronald Jan, Ludo,
  - organiseren bijeenkomst met vrijwilligers en start werkzaamheden, o.a. zorgen dat iedereen toegang heeft (xs-passen via Hans van Baar: na infobijeenkomst ma 6 oktober)
  - zorgen dat webcam weer in de lucht is en iedereen de URL sturen : na infobijeenkomst.
- Jesje: afstemming met dierverplegers IZa/ZPa/SGMN over eventuele sondevoedingen/injecties oxytocine, informeren dat zorg voor teef vóór de partus door DVers wordt gedaan (voeren, schoonmaken)
- Christel: voorbereidingen: voorraden, temperatuurlijst, communicatiestructuur (vastlegging dagelijkse bevindingen, overdracht tussen diensten etc → dagboekvorm). Inlezen, inhoudelijk voorbereiden (partus, neonatale zorg etc.), roosters maken.

Verloop begeleiding

1. De hoogdrachtige teef wordt gedurende de laatste 10-14 dagen voor de verwachte partusdatum gehuisvest in de speciaal ingerichte partusruimte. De verzorging valt dan nog (tot de start van de partus) onder de reguliere verzorging van de proefdieren door de dierverzorgers (v.w.b. schoonmaken ruimte en voeden van het dier).
   Actiepunten:
   - Hans van Baar: webcam aansluiten / werkend maken
   - Dv/art12: in welzijnslogboek aantekenen wat de reden is van de solitaire en dus niet groepshuisvesting (gewenning werpruimte hoogdrachtige teef icm verruimd voedingsregime en monitoring partus)
2. Monitoring van de teef rondom de geboorte geschiedt (met behulp van onder andere een webcam) door meerdere betrokkenen: clinici discipline VPL, studenten in de VPL dienst, dierverpleging IZa en het kraamzorgteam. In principe zijn alleen 2 studenten vh kraamzorgteam fysiek aanwezig bij de partus.
   Actiepunten:
   - Studenten monitoren (volgens een te maken rooster, Christel) het temperatuurverloop van de teef vanaf zaterdag 11 oktober
     - 3 dd temperatuur opnemen en aantekenen op temperatuurverloopkaart.

Wij stellen ons een optimale verzorging van de moeder en pups als volgt voor, dit verdient nadere uitwerking op details. Hier is het doel te beschrijven en aan te geven hoeveel tijd er nodig is om een nest pups naar behoren op te vangen:

Om te beginnen is de betrokkenheid van 1 persoon die naast de verzorging van het nest geen overige verantwoordelijkheden heeft randvoorwaardelijk. Deze persoon fungeert als een “spin-in-het-web”. Deze persoon kan het merendeel van het werk uitvoeren, maar ook door anderen uit te voeren werkzaamheden coördineren en controleren. Omdat het gezien kan worden als een relatief kortlopend “project” is het aantrekken van een student (idealiter DGK, maar paraveterinair kan uiteraard ook) waarschijnlijk praktisch. Voor teef 085 en teef 777 is hiervoor Christel Vinke, HP-student olv onderzoeker Hedwig Kruitwagen, aangetrokken. Deze persoon voelt zich verantwoordelijk voor het nest, dit zou ervoor moeten zorgen dat er geen werkzaamheden “tussen wal en schip vallen”, vooral van belang tijdens azz. Om de betrokkenheid te vergroten is het ideaal als deze persoon ook aanwezig is bij de geboorte van de pups en bereid is ook te participeren in de zorg tijdens azz-uren. Voor teef 085 en 777 is afgesproken dat Christel voornamelijk coördinerende werkzaamheden uitvoert en door de weeks dagelijks teef en pups bezoekt om te controleren of de voorraad intact is en de administratie correct verloopt. Participatie in de diensten vnl. op reserve-basis of als er gaten in het rooster ontstaan.

Week -2 en -1: 10-14 dagen voor de partus wordt de hond gehuisvest in de speciale partusruimte, alwaar de verzorging (voeren, schoonmaken, uitlaten) bij de dierverzorgers ligt. Vanaf dat moment is monitoring door bezoeken (CAVE aantal aanwezigen/keer) en middels webcam mogelijk. De teef wordt vanaf dan 3-4 keer per dag bezocht door de kraamzorgteam studenten, die beoordelen hoe het met de hond gaat. Ze letten onder andere op gedragsveranderingen, vaginale uitvloeiing en anorexie. Dit wordt genoteerd op een checklist bij het hok en geparafeerd. Vanaf ongeveer D55 (zaterdag 11 oktober) wordt 3 maal daags de rectale temperatuur van de teef gemeten om het verloop te kunnen volgen ivm de kortdurende temperatuurdaling die voor de partus vaak wordt gezien. Dit is bij te houden op een temperatuurlijst.

De studenten dienen zich in deze periode ook goed in te lezen over het verloop van de partus bij de hond en de neonatale zorg.

Dag 0: tijdens de partus zijn er continue studentvrijwilligers aanwezig op basis van een rooster. Niet meer dan 2 studenten tegelijk. Zij begeleiden de partus "als eigenaar" en zoeken contact met de achterwacht (dierenartsen discipline voortplanting) bij problemen / vragen. Bij start van de partus wordt meteen contact op genomen met Christel, die vervolgens Jeffrey de Gier of Karin Albers-Wolthers op de hoogte zal houden. De start van de partus wordt gedefinieerd als de uitdrijvingsfase maar bij twijfel wordt eerder contact gezocht.

Weken 1 -3: kort na de geboorte en de periode tot openen ogen en oren zijn de pups erg kwetsbaar. Vooral onvoldoende opname van moedermelk kan snel ernstige negatieve gevolgen hebben. Daarom moet in deze periode vaak iemand het nest bezoeken voor verzorging en monitoring (8 uur / dag; 7 dgn / week). Niet in 1 aaneengesloten "dienst" van 8 uur, maar opgesplitst in meerdere kortere shifts van steeds 1-2 uur (afhankelijk van de hoeveelheid benodigde zorg)

- Werkzaamheden:

    - schoonmaken partusruimte (iedere ochtend door de student die geroosterd is om 8:00)

    - voeding/verzorging moederdier (middels checklist en paraaf):Er wordt onder andere gelet op vaginale uitvloeiing van teef, gedrag, rectale temperatuur en de melkklierpakketten.

    - verzorging pups: monitoring (wegen 1x per dag) en extra aandacht geven (aanleggen tijdens zogen, als nodig) aan pups die suboptimaal groeien. Sondevoeding bij pups die onvoldoende groeien (8 maal daags).

    - voorraadbeheer spullen partusruimte, denk aan materiaal, voer, "hygienesluis", weegschaal, medische materialen etc. (*denk vooruit, in het weekeinde zijn veel spullen bijvoorbeeld niet/moeilijk te verkrijgen → Christel controleert vrijdags de voorraad voor weekend)*

Weken 4 t/m 6 als de pups goed drinken en groeien is dit een relatief rustige periode. In de vierde week worden de pups gewend aan het eten van brokjes. In deze periode is er cumulatief ongeveer 4-6 uur werk per dag, maar ook weer verdeeld over de dag: 4 x daags 1-1,5 uur. Vooral schoonmaken en voeden van moeder en pups, vanaf de vierde week moeten de pups 4 keer per dag gevoerd worden. Daarnaast de coördinerende taken.

De pups worden gespeend op 7 weken leeftijd. Vanaf dan wordt de dagelijkse verzorging (voeren, schoonmaken, uitlaten) weer overgenomen door de dierverzorgers. De taken van het kraamzorgteam stoppen op dat moment. De periode na het spenen is wél belangrijk voor socialisatie, maar daarover worden nog nadere afspraken gemaakt mbt wie wat doet. Mogelijk dat er vanuit de studenten van het kraamzorgteam belangstelling is voor participatie hierin.

Weken 7 en 8: start socialisatie, erg van belang. Daarvoor is 1 uur per dag per pup nodig, naast de algemene voeding en verzorging. Bij 4 pups is dat cumulatief 6 uur per dag.

Weken 9 t/m 16: dagelijks socialiseren, voeding en verzorging gaan mee met de overige honden in de kennel. In deze periode vooral aandacht voor socialisatie. 0,5 – 1 uur per pup per dag. In een afbouwend schema . Aan het einde van deze periode invoegen in het standaard agility programma. Einde inzet extra personeel. Bij vier pups dus van 4 uur naar 2 uur per dag. Geen weekenddiensten meer.

Bij onvoorziene omstandigheden, denk vooral aan het bijvoeden per voedingssonde van de pups bij te weinig groei zal *ad hoc* een oplossing gevonden moeten worden en zal meer aanspraak gedaan worden op anderen, vooral voor de late avond en nachtvoedingen. Dit is in bovenstaande schema niet meegenomen.

Overzicht taken en regels Kraamzorgteam 2015

Algemene regels:

- Denk aan je hygiëne voordat je gaat werken met de teef en pups.
- Vul elke keer de checklist in en zet een paraaf als je alles hebt ingevuld.
- Als je iets extra's kwijt moet over de gezondheid van de teef en pups, schrijf dit dan op in het logboek. Maak er geen lang verhaal van, houd het beknopt!
- Er is een ante- en een post-partum rooster en checklist. Het spreekt voor zich dat het ante-partum rooster overgaat in het post-partum rooster zodra de partus geweest is.
- De whatsapp-groep is alleen bedoeld voor serieuze vragen/opmerkingen over het kraamzorgteam.

Taken ante-partum:

- Tot 9 maart bestaan de dagelijkse bezoeken uit het monitoren van de gezondheid en prodromi van de teef. Let op eventueel veranderd gedrag (hijgen, onrust, graven etc.), vaginale uitvloeiing en anorexie (zie checklist).
- Vanaf 9 maart wordt de teef 3x per dag getemperatuurd (om 8, 12 en 17u). Het temperatuurverloop wordt bijgehouden op temperatuurlijst. Verder staan de werkzaamheden hier ook uit het monitoren van de gezondheid en prodromi van de teef. **Bij afwijkende gezondheid van de teef MOET contact worden opgenomen met Christel.**
- Uitlaten is in principe toegestaan op het terrein van de Munsterlaan (tot aan hoek Yalelaan en rechtsaf tot aan oude drafbaan). Heb je dus tijd over en vind je het leuk om haar uit te laten, dan kan dat. Kijk wel even in het logboek of dit niet al 3x gedaan is die dag.
- Schoonmaken en voeren wordt voor de partus gedaan door de dierverzorgers. Na de partus is het kraamzorgteam hier ook verantwoordelijk voor.

Taken post-partum:

- Teef: (aan de hand van checklist)
    - Melkklierpakketten voelen (warmte, roodheid, zwelling → mastitis?)
    - Algemene gezondheid teef beoordelen. (algemene indruk, eet ze goed? Etc.)
    - 1x per dag temperaturen. Bijgehouden op temperatuurlijst. (tot 1 week na partus tijdens 8u shift)
    - Letten op vaginale uitvloeiing (aspect belangrijk!)

  Bij afwijkende gezondheid van de teef MOET contact worden opgenomen met Christel.
- Pups:
    - Wegen:
        - Tijdens de partus (pup voor pup) als ze zijn droog gelikt
        - Na de partus het hele nest
        - 12 uur na partus
        - Herhaling 1x per dag (8u shift)
    - Algemene indruk beoordelen
    - Knuffelen (wennen aan contact)
    - Aanleggen bij tepel indien nodig
    - Sondevoeding indien nodig
    - Als ze ouder worden: spenen
    - Knuffelen en spelen!

  Bij afwijkende gezondheid van de pups MOET contact worden opgenomen met Christel.
- Voeren teef (en pups indien nodig):
    - Post partum mag de voerbak van de teef nooit leeg zijn! Vink op de checklist af als je hem bijvult (dan is duidelijk of de teef blijft eten).
- Schoonmaken:
    - Als er viezigheid is bij jouw shift, dan ruim je dit op.
    - De shift van 8u zal iedere dag een grote schoonmaaktaak hebben: schoonspuiten, dweilen etc. Als de pups uit de werpkist gaan, wordt het steeds viezer in de ruimtes. Ik zal dus later in het rooster op de shift van 8u 2 mensen inroosteren. De eerste persoon gaat sowieso en de tweede persoon mag gaan indien dat jullie handig lijkt (onderling afspreken).

Partusregels

Voor de partus geldt het volgende:

- Op het moment dat het begin van de partus wordt vastgesteld, mag degene die bij dat tijdslot hoort samen met de persoon die de volgende dag hetzelfde tijdslot heeft bij de teef blijven. (onafhankelijk van wie het begin van de partus heeft opgemerkt! (bv via de webcam)). Op het moment dat het volgende tijdslot begint, worden deze twee personen afgelost door degene die het volgende tijdslot heeft + degene die de volgende dag dat betreffende tijdslot heeft etc. etc.
- Maak het begin van de partus meteen kenbaar bij de rest van groep en bij Christel (bellen, whatsapp-groep etc.)
- Het tijdslot dat begint om 22:00 duurt tot de volgende ochtend 8:00.
- Wanneer iemand niet kan komen wanneer hij gebeld wordt, dan wordt contact opgenomen met Christel (0636170755), die vervolgens bepaalt wie er dan gebeld wordt.
- Het is niet mogelijk om zelf aanpassingen in dit systeem te maken. Er mogen maar maximaal 2 personen tegelijk bij de teef aanwezig zijn tijdens de partus, dus niet iedereen kan de partus zien! (wel via de webcam natuurlijk).
- **Wanneer je denkt dat er iets misgaat, bel dan Christel die contact opneemt met de dierenarts van de discipline voortplanting**

Voorbeeld:

17-10-2014		18-10-2014	
8:00	persoon a	8:00	persoon e
12:00	persoon b	12:00	persoon f
17:00	persoon c	17:00	persoon g
22:00	persoon d	22:00	persoon h

19-10-2014		20-10-2014	
8:00	persoon i	8:00	persoon c
12:00	persoon j	12:00	persoon d
17:00	persoon a	17:00	persoon e
22:00	persoon b	22:00	persoon f

Wanneer de partus wordt geconstateerd ’s ochtends om 8:00 door persoon a, dan laat zij dit aan iedereen weten via de whatsapp-groep en ze belt Christel. Persoon e mag bij persoon aansluiten om de teef te begeleiden. Persoon a en e blijven bij de teef tot 12:00. Op dat moment worden ze afgelost door persoon b en f. Om 17:00 worden deze twee weer afgelost door persoon c en g, en vervolgens om 22:00 door persoon d en h. Persoon d en h blijven de hele nacht op de faculteit en worden ’s ochtends afgelost door persoon i en c. etc. etc.

Taken tijdens de partus

- Doe niet te veel! In principe kan moeder alles zelf, dus grijp niet te snel in.
- Houd tekenen van dystocia in de gaten, wanneer je denkt dat er sprake is van dystocia → bel Christel!
- Zodra een pup geboren wordt, moet deze gewogen worden. Als de hele partus voorbij is, worden alle pups nogmaals gewogen.
- Houd in de gaten hoeveel placenta’s naar buiten komen.
- Zorg ervoor dat de pups bij een tepel komen. Is de teef erg onrustig, leg haar dan eventueel rustig neer zodat de pups een tepel kunnen pakken en kunnen drinken.