UNIVERSIDADE D
COIMBRA

André Filipe Tavares Ribau

PATIENT-DERIVED XENOTRANSPLANTATION (PDX) OF HUMAN TUMOR TISSUES

VOLUME 1

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Doutor Hugo João Marques Prazeres do Instituto de Oncologia de Coimbra Francisco Gentil como co-orientador institucional o Professor Doutor Rui de Albuquerque Carvalho do Departamento Ciências da Vida da Faculdade de Ciências e Tecnologia. Dissertação apresentada no departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia.

Novembro de 2020

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List of abbreviations

CAM: Chorioallantoic membrane assay

PDX: Patient derived xenograph

DMEM: Dulbecco's Modified Eagle Medium

mm^3: Millimeters Cubed

µl: microliter

PenStrp: Penicillin-Streptomycin

H&E: Hematoxilin and eosin

pH: Hidrogenionic potential

µm: micrometers

mm: milimeters

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

mRNA: Messenger RNA

tRNA: transporter RNA

rRNA: ribossomic RNA

MMP´s: matrix metalloproteases

ECM: extracellular matrix

VEGF: Vascular endothelial growth factor

TSP-1: Thrombospondin-1 protein

TGF-β: growth factor beta

TP53: Gene TP53

(MAP)-kinase: mitogen-activated protein kinase

RB: retinoblastoma protein

PI3-kinase: phosphatidylinositol 3-kinase

TAMs: Tumor-associated macrophages

CAF: cancer-associated fibroblasts

TAN: tumor-associated neutrophils

PDGFs: Platelet-derived growth factor

CD8+ T: CD8+ (cytotoxic) T cells

$CD4^+$ T: $CD4^+$ Helper T cells

FGF: fibroblast growth factors

PET: Positron emission tomography

MRI: Magnetic resonance microscopy

ºC: degree Celsius

PCR: Polymerase chain reaction

EGF: epidermal growth factors

IGF: insulin-like growth factors

RCC: Renal cell carcinoma

ccRCC: clear cell renal cell carcinoma

HIF: hypoxia-inducible factors

mTOR: mammalian target of rapamycin protein

p: probability

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Abstract

In this study, we sought to develop a method to simulate the tumor environment "*in vivo*" using a living organism. The organism used was the chicken egg and we used tumor fractions coming directly from the patients followed at the Portuguese Oncology Institute of Coimbra Francisco Gentil. We studied different types of benign and malignant tumors (tumor of the kidney, ovary and breast). This method is called “patient derived xenograph”, because a tumor piece of the patient was directly inoculated into the egg.

The eggs were incubated and on the ninth day of incubation were inoculated with a tumor fragment and then sealed with adhesive tape. Prior to inoculation, the tumor fragments were submerged in DMEM for one hour and then inoculated into the chorioallantoic membrane of the embryo. In this study, we optimized the CAM assay by the “*in ovo”* method to obtain a greater number of viable transplants and developed protocols for collecting and pre-treating the tumor tissue. The collection protocol is essential for obtaining viable tumor fragments that include healthy tumor and non-tumor tissue. Through the obtained results, we verified greater viability in the fragments belonging to the kidney tumor (Renal Cell Carcinoma) in relation to fragments belonging to the ovary and breast tumor. Some of the contaminations may be due to the incubator environment not being entirely sterile or due to the space where the pathologists cut the tumor into fragments also so as not to be totally sterile. Through the results of the three types of tumor, the tumor fragments corresponding to the malignant tumors presented greater viability compared to the fragments corresponding to the benign tumors. The developed method may allow making comparisons in later studies to evaluate *ex vivo* the action of some drugs.

Keywords: Tumor; chorioallantoic membrane; patient-derived xenograph; “*in ovo”;* tumor fragments

Resumo

Neste estudo, procuramos desenvolver um método para simular o ambiente tumoral "*in vivo*" usando um organismo vivo. O organismo utilizado foi o ovo de galinha e foram utilizadas frações tumorais provenientes diretamente dos pacientes inseridos no Instituto Português de Oncologia de Coimbra Francisco Gentil. Estudamos diferentes tipos de tumores benignos e malignos (rim, ovário e mama). Este método chama-se xenotransplantação derivada do paciente, pois era feita a inoculação de uma peça tumoral do paciente diretamente no ovo.

Os ovos foram incubados e no nono dia de incubação foram inoculados com um fragmento de tumor e selados com fita adesiva. Antes da inoculação, os fragmentos do tumor foram submersos em DMEM por uma hora e depois inoculados na membrana corioalantóica do embrião do ovo. Neste estudo, otimizamos o ensaio CAM pelo método "*in ovo"* para obter um número maior de transplantes viáveis e desenvolvemos protocolos para coleta e pré-tratamento do tecido tumoral. O protocolo de coleta é essencial para a obtenção de fragmentos tumorais viáveis que incluam tecido tumoral e não tumoral saudável. Através dos resultados obtidos, verificamos maior viabilidade nos fragmentos pertencentes ao tumor renal (Carcinoma de Células Renais) em relação aos fragmentos pertencentes ao tumor de ovário e mama. Algumas das contaminações podem dever-se ao fato de o ambiente da incubadora não ser totalmente estéril ou devido ao espaço em que os patologistas procedem à fragmentação do tumor em fragmentos também não serem totalmente estéreis. Pelos resultados dos três tipos de tumor, os fragmentos tumorais correspondentes aos tumores malignos apresentaram maior viabilidade aquando comparados aos fragmentos correspondentes aos tumores benignos. O método desenvolvido pode permitir comparações em estudos posteriores para avaliar *"ex vivo*" a eficácia de alguns fármacos.

Palavras-chave: Tumor; Membrana corioalantóica; xenotransplantação derivada do paciente; "*in ovo",* fragmentos tumorais

Chapter I

1. Cancer hallmarks

Cancer is a disease characterized by uncontrollable cell growth. This disease has rules that are transversal to any type of tumor (Hanahan & Weinberg, 2011). Some of these rules are the possibility of inducing the growth of new blood vacuums called angiogenesis, the possibility of preventing cell death called apoptosis, the ability to move to other tissues called metastasis, promoting a support in proliferative signaling. All of these capabilities are combined with several mutations that occur in the genome, with inflammation reactions, changes in metabolic reactions and the ability to resist the immune system (Hanahan & Weinberg, 2011).

1.1 Sustaining proliferative signaling

Normal cells control their growth through control points that exist in mitosis. Cancers cells have the ability to produce their own proliferative signals by interfering directly with the tyrosine kinase domains (Hanahan & Weinberg, 2011). To control their own sustaining system, cancer cells use two main mechanisms. They can produce their own proliferative signals or send signals to stimulate other cells, which reciprocate by sending growth factors to cancer cells (Cheng et al., 2008). Two of the most activated metabolic pathways in cancer cells are the mitogen-activated protein (MAP)-kinase pathway and the phosphoinositide 3-kinase (PI3-kinase) (Davies & Samuels, 2010; Jiang & Liu, 2008; Yuan & Cantley, 2008)**.** Cancer cells also have the ability to influence neighboring normal cells to produce growth factors that will be used in the growth of cancer cells (Cheng et al., 2008). Another way of regulating growth is at the level of cell membrane receptors. Cancer cells have the ability to increase the number of receptors on their cell membrane, making the cell hyperresponsive even though the amounts of binding factors are low.

1.2 Evading tumor suppressors

Cancer cells have another barrier that they have to overcome which is to avoid the actions of several tumor suppressors. At the genomic level, cancer cells have the ability to activate oncogenes (these genes enhance tumor malignancy), inactivate tumor suppressor genes (these genes inhibit tumor action) and the activation of genes that are involved in DNA repair. Two of the many tumor suppressors spoken of in tumor cells are RB (retinoblastoma-associated) and TP53. These two proteins act in central pathways

that activate senescence and apoptotic programs (Burkhart & Sage, 2008; Deshpande et al., 2005; Sherr & McCormick, 2002)**.** TP53 receives signs of stress and other abnormalities that may be occurring in the cell (from levels of oxygen, glucose, degree of damage to the genome, among other reasons) and is activated when these conditions are not normalized triggering apoptosis. The RB protein has the ability to block the division of DNA preventing the passage from the G1 phase to the S phase of the mitotic cycle. The dephosphorylation of the Rb protein inhibits the dimerization of the E2 promoter binding protein (E2F-DP), as this protein promotes the passage to the S phase. This binding of the Rb protein to the E2F attracts the histone deacetylase (HDAC) protein for chromatin preventing further division of DNA (Sherr & McCormick, 2002) (Burkhart & Sage, 2008) (Deshpande et al., 2005).

1.3 Resisting cell death

In relation of the cell death, cancers cells have mechanisms to resist (Adams & Cory, 2007; Evan & Littlewood, 1998; Lowe et al., 2004). One of the mechanisms to induce apoptosis is the action of TP53 that is a tumor suppressor. One of the mechanisms that cancer cells use to circumvent this situation is the loss of this protein (Junttila & Evan, 2009; Lowe et al., 2004).

During the mitosis process, we check for control points regarding cell growth. Two of these points are called senescence (corresponds to a process of non-proliferation of the cell) and the crisis (corresponds to cell death). Cancer cells are able to overcome these barriers and produce unlimited growth capacity, while normal cells have controlled growth cycles and obey these two mitotic control points. Normally, when cells enter in the senescence phase, some of them try to circumvent this barrier and enter in the crisis phase (in this phase most of them die). In some occasions some cells can circumvent this barrier and demonstrate unlimited divisions. This situation is defined as immortalization and one of the keys to do this is the action of an enzyme called telomerase (Blasco, 2005; Shay & Wright, 2000).

1.4 Enabling replicative immortality

Cells have a short life span, while cancer cells have a very long-life cycle compared to previous ones. During this proliferation process, there are two barriers to stop cell growth, senescence (non-proliferative but viable state) and crisis (cell death). For example, in a cell culture, cells after some time of growth enter the senescence phase and if there are cells that manage to circumvent this barrier, they enter the crisis

phase. On rare occasions, cells can be found that can overcome the crisis phase and exhibit unlimited replicative growth (Blasco, 2005).

Along the mitotic divisions in the cell, telomeres are losing part of their DNA. As long as there is this erosion of the telomeres, there is no problem, as these are a set of hexanucleotide repeats (Shay & Wright, 2000). When mitotic divisions begin to affect chromosomal DNA, it induces the loss of some functions in the cell. Thus, the length of the telomeres DNA can determine the cell's lifetime (Blasco, 2005).

Telomerase is an enzyme that add repetitive segments of DNA to the end of the telomeric DNA. This enzyme is practically absent in normal cells and practically present in most cancer cells. This enzyme therefore promotes a certain immortality to the cells, because by adding segments of DNA to the telomeres it makes the chromosomal DNA never affected.

1.5 Promote angiogenesis

All tissues need to receive nutrients and excrete metabolic waste, however in cancer cells at some point they need to activate angiogenesis to support their growth. Angiogenesis corresponds to the growth of blood vessels from existing vessels (Hanahan & Folkman, 1996). Some of these factors involved in this process are the VEGF protein (vascular endothelial growth factor) that potentiates angiogenesis and the TSP-1 protein (thrombospondin-1) that inhibits angiogenesis. The metabolic signaling of VEGF protein is through various types of receptor tyrosine kinases is regulated by hypoxia and by oncogene signaling like the RAS and MYC oncogenes (Carmeliet, 2005; Ferrara, 2010; Gabhann & Popel, 2008). Other factors involved in the regulation of angiogenesis are fibroblasts (FGF) and matrix metalloproteinases-9 (MMP-9) (Kessenbrock et al., 2010)(Baeriswyl & Christofori, 2009). TSP-1 proteins act as angiogenesis blockers by binding to transmembrane endothelial receptors producing suppressive signals (Kazerounian et al., 2008). Angiogenesis occurs at very early stages of the tumor, producing a large dense neovascularization of blood vessels and an irregular blood flow (Hanahan & Folkman, 1996).

2. Tumor heterotypic interactions

2.1- Tumor heterogeneity

Nowadays all the tumors are different which is more difficult to choose the best therapy to apply. Tumor heterogeneity is one of the themes that has gained importance over recent times. This concept is characterized by differences in morphological, cellular, genomic between the tumors of each patient (Hamburger & Salmon, 1977). This process of heterogeneity was due to the instability of mutations that each tumor can acquire, besides that it is not only affected genomic, but also the proteomic and metabonomic that can originate different combinations generating different tumor responses (Hamburger & Salmon, 1977). The advancing of diagnostic techniques can increase the efficacy of the treatments applied for which type of tumor. Recent techniques, such next-generating DNA sequencing, give us great reliability in the results to understand the differences between many types of tumor. Some of therapies applied with this diagnostic technique reveals more efficacy in some subgroups (Daniel et al., 2009; DeVita & Chu, 2008; Dulbecco, 1952). Next-generating sequencing is a set of methods (like Illumina for example), which allows sequencing of thousands of DNA molecules at the same time. Initially, the DNA molecule is divided into several DNA fragments. Afterwards, these fragments are amplified using the PCR (polymerase chain reaction) method, then these fragments will be sequenced and analyzed by a computer program (Yohe & Thyagarajan, 2017).

The study of the metabolic and genomic of which tumor is very important to increase the efficacy of the treatments because, unfortunately, many treatments remain ineffective in some tumors. More knowledge is necessary about the metabolic pathways, genomic mutations, cancer factors, activation of some proteins and the inhibitory agents to have a better understanding around this disease. Behind this, a very promising technique called PDX (patient derived xenografts cancer model) show to be a feasible technique to study the nature of the tumors and to test the reaction of certain pharmacologic agents in some groups of tumors(Julien et al., 2012; Reyal et al., 2012; Stephens et al., 2012; Tentler et al., 2012).

Another concept to take into account is the cancer stem cell paradigm, which is the presence of different subpopulations inside the tumor and one of that subpopulations correspond to cancer stem cells (these cells have the potential to generate the tumor). Through this paradigm, the concept of tumor heterogeneity is very important because it determines the identity of each tumor and before introducing a good therapy, it´s

important to know better the identity of the tumor (Clarke et al., 2006; O'Brien et al., 2010; Valent et al., 2012).

Tumors can be divided into groups and within the same group there are differences between the tumors. Some conventional concepts and therapies have helped to reduce the impact of this disease on society; however, it is necessary to develop other personalized methods to increase the effectiveness of the treatments. Personalized medicine is one of the great promises and its development will play a fundamental role in this process.

In a broader perspective, the development of the technique patient-derived xenografts, the cancer stem cells paradigm and the next generating sequencing will provide tools to understand the biology and the interactions involved in this disease.

2.2 - Tumor microenvironment

The tumor microenvironment includes fibroblast, immune cells, lymphatic and blood vessels, normal cells, extracellular matrix (ECM) and a complex mixture of signaling molecules (Chen et al., 2015)**.** Some alterations of the cellular microenvironment can contribute to tumor initiation, metastasis and angiogenesis (Chen et al., 2015).

We know that some characteristics and processes are common of most tumors like hypoxia, Warburg effect (Danhier et al., 2010), great permeability (Blagosklonny, 2004) and angiogenesis (Jubb et al., 2006). The extracellular matrix (ECM) is composed by macromolecules such as matrix metalloproteases (MMP´s), collagen, enzymes, laminin, fibronectin and glycoproteins, forming an environment that provides structure properties to the surrounding cells (Theocharis et al., 2016). The ECM have a role in the cell-cell communications, cell adhesion and cell proliferation (Frantz et al., 2010). For example, the tumor cells secreted MMP´S to degrade ECM proteins and change the tumor microenvironment by releasing chemokines and angiogenic factors. Normally, tumors are stiffer than the surrounding normal tissues due to the presence of cancer associated fibroblasts (CAF) (Weigelt & Bissell, 2008). Collagen, elastin fibers, lysyl oxidase and transglutaminase can promote more rigid fibrils (Levental et al., 2009).

Other cells present in this microenvironment are the immune cells, such as: natural killers, B lymphocytes, T lymphocytes, macrophages, neutrophils and dendritic cells. There are different subtypes of T lymphocytes, such as: CD8+ T cells which are supported by CD4+ T helper 1 cells and the binding of these two can release cytokines,

interferon gamma and other molecules (Fridman et al., 2012). B lymphocytes can be found at the invasive margin of some tumors, but more frequently as secondary or tertiary structures. In some types of tumors (like breast cancer), the infiltration of B lymphocytes has been associated with a good prognosis (Coronella et al., 2001). Natural killers' cells belong to the innate immune system and are usually found outside the tumor area. Many studies reported an hyperresponsiveness on natural killers cells induced by malignant cells through the growth factor beta (TGF-β) (Fridman et al., 2012).

Two of the most abundant myeloid cells are the macrophages associated with the tumor (TAM) and neutrophils associated with the tumor (TAN). TAM can produce angiogenic factors and accumulate in hypoxic or necrotic areas and the TAN can produce pro- or anti- tumor activity (Qian & Pollard, 2010; Shojaei et al., 2008).

Other molecules present in tumor microenvironment are soluble factors such VEGF, FGF, platelet-derived growth factors (PDGFs) and chemokines. These stimulate endothelial cells and other factors to promote the neovascularization for cancer growth (Carmeliet & Jain, 2011).

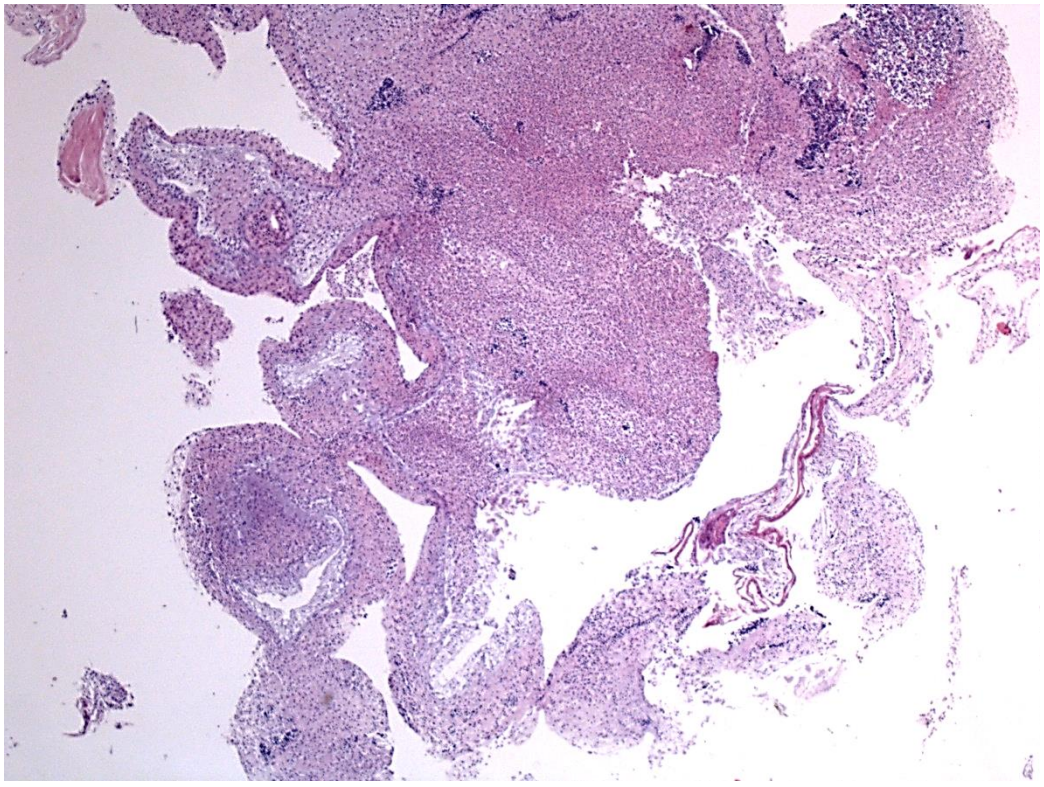

Figure 1: Ovarian tumor [own authorship]

3- Patient-derived xenograft (PDX) models

PDX is a model that consists in the implantation of tumor tissue/cells from a patient into an immunodeficient organism. There are diverse organisms that can be used, such as: mouse, zebrafish, and chicken embryo.

One of the advantages of PDX is the preservation of the tumor heterogeneity and genomic integrity likely to increase the success of treatments (Lai et al., 2017). Nowadays, the PDX prove to be one of the greatest methods to study the pathobiology of cancer and therapies involved. There are two types of implantation, the orthotopic and the heterotopic implantation. In the orthotopic implantation, the tissue from tumor patient is implanted in a similar anatomic site where the tumor was in the patient (Cutz et al., 2006). In the heterotopic implantation the tumor tissue is implanted to a place unrelated with the original tumor site (Reddavid et al., 2020).

PDX tumor models can be used with tumor fragments or with single cell-suspensions. The first has the advantage to maintain most of the interactions between cells, preserving the architecture of the tumor, the second allows the study of distinct tumor subpopulations in an isolated way (Zvibel et al., 2002).

The PDX also have their shortcomings, such the engraftment frequency and variable growth rates depending on the types and subtypes of tumors. Some tumors are difficult to engraft, and the reasons can be the conditions of the transport (mechanical stress), the absence of cancer stem cells, the incompatibility with human cells or other barriers related to the xenotransplantation. One of the types of tumors that appears to be difficult to establish is the breast cancer comparing with the ovarian, lung and colorectal cancer (Eppert et al., 2011; Quintana et al., 2008; Tentler et al., 2012).

The current work is centered in the chicken chorioallantoic membrane as a model for PDX, which will be the focus of the remaining chapters of the introduction.

4 - Chicken chorioallantoic membrane (CAM) model

4.1 - Structure and development of the CAM

The chicken chorioallantoic membrane is one of the PDX models that can be used to study the pathobiology of cancer (DeBord et al., 2018) . The chorioallantoc membrane is a vascular membrane, especially a lymphatic system, found in eggs and it is formed by the fusion of the allantois and the chorion. This membrane is responsible

for the respiratory system of the embryo and have an important role to bone development, transporting calcium from the eggshell (Gilbert, 2000). The CAM is formed with three different layers: the chorionic epithelium (originated from the ectoderm layer), the mesenchyme and the allantoic epithelium (originated from the endoderm layer). The epithelium layer limits the stroma and is within the stroma that the blood vasculature and lymphatics reside (Fáncsi & Fehér, 1979).

The development of the CAM starts on day 3 of the embryonic development and is completed at day 10 and on day 13 is fully differentiated. At day 5, the CAM is closed to surface of the eggshell and starts to remove calcium and oxygen across the porous shell.

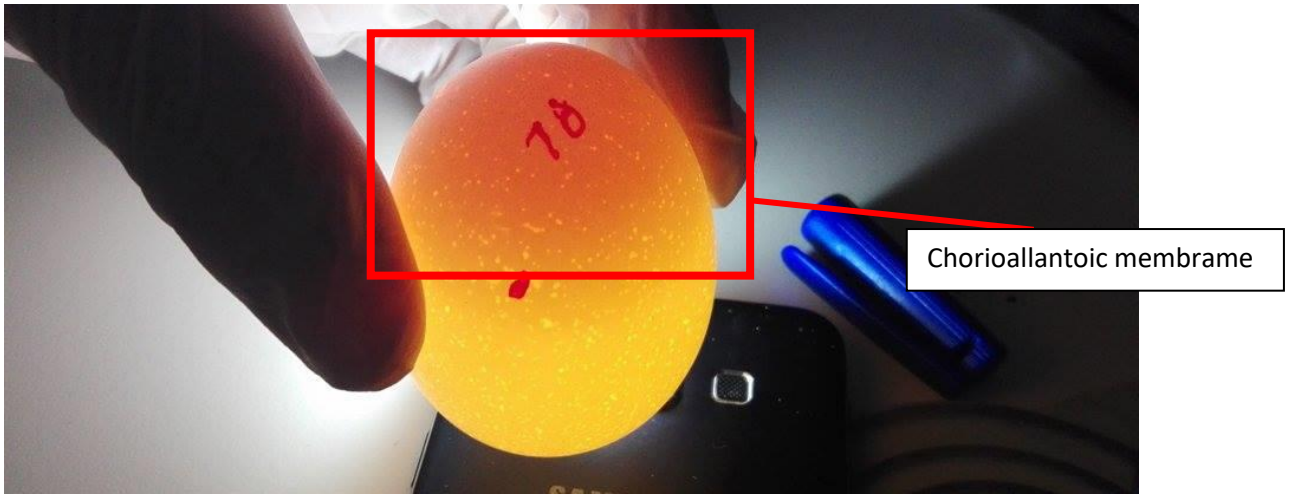

Figure 2: The beginning of the formation of the chorioallantoic membrane. [own authorship]

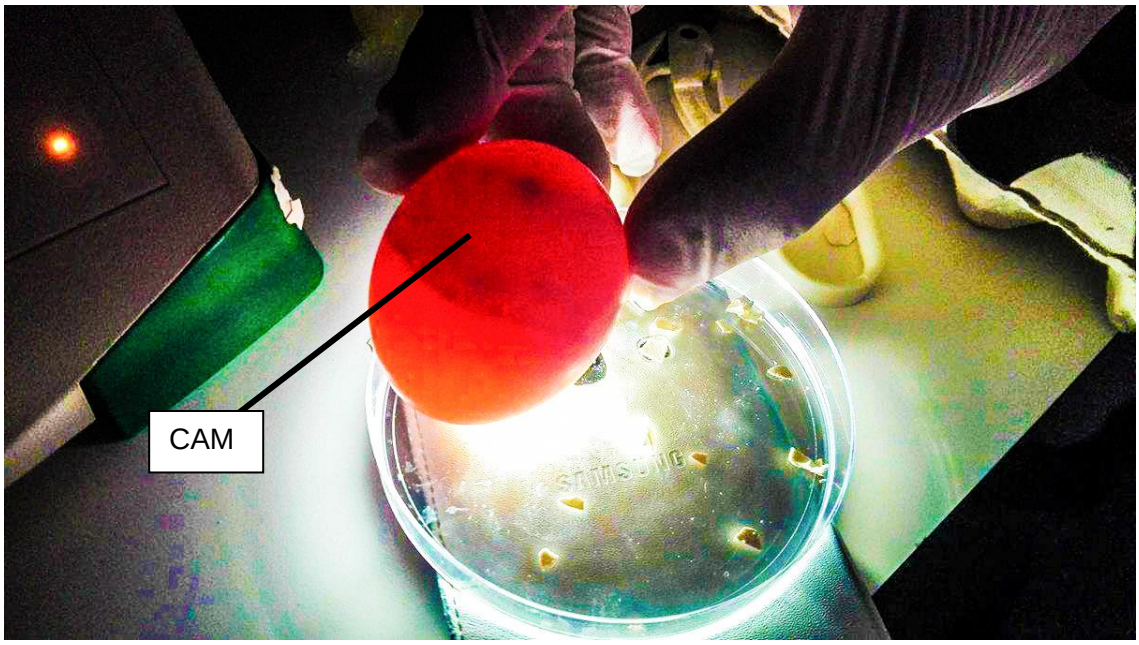

Figure 3: The advanced egg development on the sixteenth day. [own authorship]

It is important to open a fertilized egg for cultivation "*ex ovo*" by day 3, because after that, the rupture of the shell can cause the rupture of the CAM. In experiments "*in ovo*" the opening of a small window should be at day 4 and 5, though it is necessary to be careful to avoid damage of the CAM vessel (Fáncsi & Fehér, 1979).

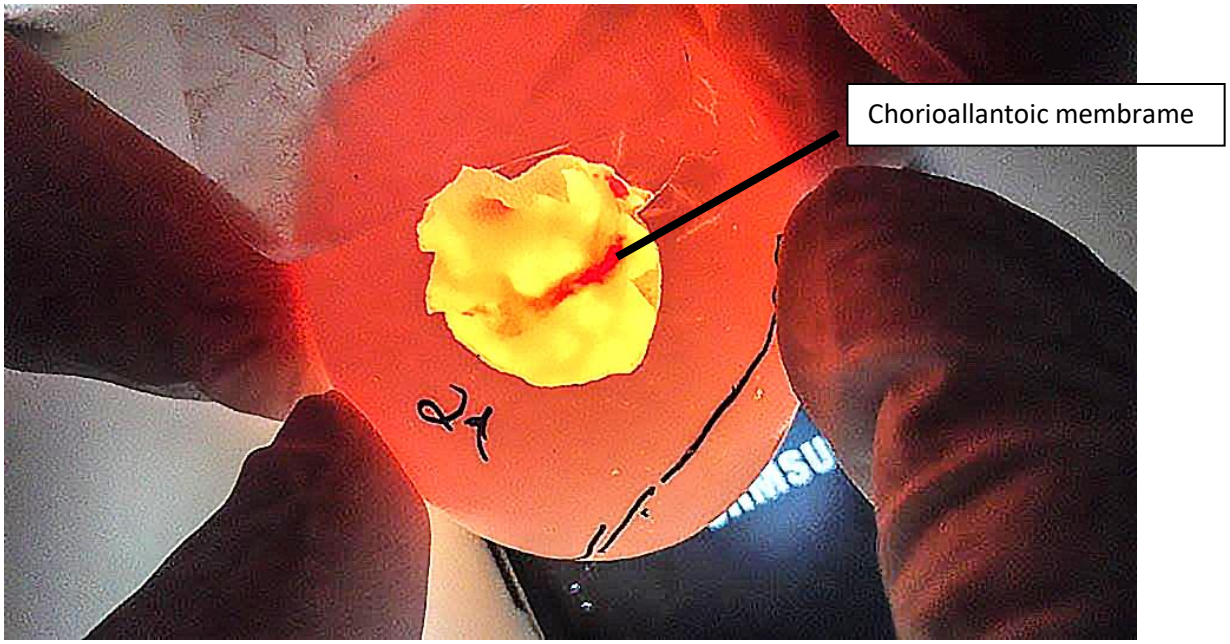

Figure 4: A window in the egg on the fifth day of embryonic development. We can see the formation of the chorioallantoic membrane in red. [own authorship]

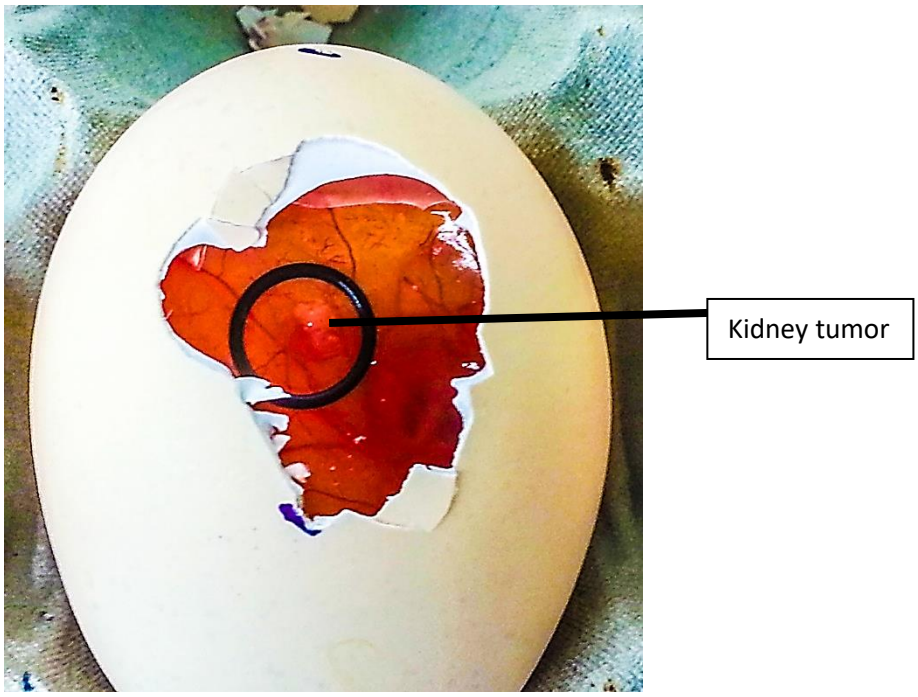

Figure 5: egg with a tumor on the 6th day of incubation [own authorship]

4.2 - Advantages and limitations of the CAM model

This model has many advantages that make you very interested in comparison with other models "in vivo" (Nowak-Sliwinska et al., 2014). One of these advantages is the ease of handling it, so it does not require a lot of laboratory equipment to be able to handle it, so neither does it require great costs associated with these same laboratory experiments. This also applies to diagnostic techniques where often with electromagnetic radiation in the visible area it is possible to examine a large number of reactions in the embryo (this is due to the small thickness of many layers of the embryo which facilitates fluorescence testing). However, this does not invalidate the need to have a great deal of care to handle it, from temperature, humidity, sterility, sensitivity to handling so as not to damage it. Embryonic development time is also an advantage in providing quick results.

Two strengths of this model are its applicability for studying angiogenesis and its natural immunodeficiency. Throughout the embryonic development, it develops a

complex network of blood vessels which allows to see tumor angiogenesis processes clearly and thus study with greater clarity. Another beneficial factor of this model is the temporary absence of the immune system. This makes it possible to reduce unwanted side reactions, making it possible to analyze and study the experience in question with greater confidence.

Another advantage is that it is an "in vivo" experience. In fact, many tumor studies are ineffective when used for the implementation of a drug, because it is not often possible to simulate the patient's environment with that of the model under study. The egg shows an advantage, so we simulate an environment of a living being and even introduce a tumor piece as a whole, in order to have the least possible disturbances and the maximum possible similarity to the environment.

In bureaucratic terms there is also an advantage so the egg does not require authorizations or protocols by other institutions for its use, although some precautions are necessary for its use (Nowak-Sliwinska et al., 2014).

This model can also be useful to study the reaction and development of external tissues inside him and the relations with drugs, hormone or other agents (DeBord et al. 2018). Therefore, this model allows the study of interactions between many biological pathways in a macroscopic view. This model has also a great applicability of distinct imaging modalities that range from microscopic- to magnetic resonance- to positron emission tomography imaging (MRI and PET) (DeBord et al., 2018; Ribatti, 2010)

For applications related with transplantation, the immune system of the embryo is to slow developing and starts to develop on day 15, which allow the researchers making experiments until that day without worrying about side reactions with immune cells.

So far, we have seen that this model has many advantages, but also its limitations. One of the advantages we saw earlier is the extensive network of blood vessels, however this can be a limitation as it can prevent distinguishing which are the new vessels to be originated from those that already exist (Nowak-Sliwinska et al., 2014; Ribatti, 2012).

Another disadvantage is contamination, often due to impurities in the skin that must be cleaned very well. Many experiments have had a very low success rate on embryo viability and the main reason for these results is fungal contamination (Nowak-Sliwinska et al., 2014)

Another unfavorable point is its fragility. The egg, although naturally adapted to nature, is also very sensitive to external factors. Whatever tissue is to be inserted into the egg for further study, it must be small in size to fit inside the egg. The shell itself is fragile so any external damage to its structure can easily break it making the egg

unfeasible for future experiments. Even the internal content itself, if it undergoes strong oscillations, quickly damages developing tissues leading to its unfeasibility.

For therapeutic purposes the egg shows a limitation that has a great impact on the desired end result. The metabolism of the egg is not the same as that of a mammal in which the human is inserted. For further testing of the efficacy of some drugs in the action of some tumors, the egg may not be the most similar environment compared to humans. The egg compared to a mammal differs in the presence of different proteins, different molecular processes up to metabolic interactions, constituting an unfavorable scenario in the confidence of the final results.

Finally, we also have to take into account that there is a limit on the compatibility of some agents, such as proteins, hormones, primers, antibodies to be used in these experiments (Lokman et al., 2012; Nowak-Sliwinska et al., 2014).

Chapter II

5 - Aims

The aim, of this work was to optimize a method for the xenotransplantation of patient-derived tumor fragments into the chorioallantoic membrane of the chicken egg. We intended to apply this process to maintain the viability of tumor tissue from various types of cancers. By optimizing this method, we aimed to develop a model that is more reliable in recapitulating tumor biology, since it is based in patient-derived material and the conditions regarding the tumor micro environment are kept.

By increasing the yield of this method, we intend to potentiate further studies on the *ex vivo* evaluation of the action of drugs against specific tumors, contributing to the advancement of new therapies in the fight against this disease.

Chapter III

6 - Materials and Methods

6.1 - Materials

Biological materials:

Eggs: Fertilized chicken eggs
Tumor tissues from ovarian, kidney and breast

Other materials and consumables:

20 G needles, 30 G needles, ribbon, paper, distilled water, DMEM (Dulbecco's Modified Eagle Medium), silicon rings, scissors, tweezers, petri dishes, incubator (37 °C, 60% humidity), tugon tube, egg candler, microsurgical kits, sterile forceps, push pin, dissection scissors, needle nose forceps, 2-20 µl pipette, 20-200 µl pipette, laminar flow chamber, suction bomb, thermometer, pencil, eppendorf, optical microscope, biofreeze solution.

6.2 - Methods

The described methods correspond to an optimization process at the level of the implemented protocols, since it was essential to overcome certain barriers in order to have the results of this study. In relation to the state of the art, the main optimized steps were introduced in the pre-incubation process of the tissues with a specific solution (culture medium + antibiotics). The importance of this optimization served to increase the percentage of tissue survival in the first stages of transplantation, as it might not have blood vessels to support tissue nutrition during some tissue and to protect the tissue from factors coming from the external environment. Antibiotics play an essential role, as one of the greatest difficulties is preventing contamination. This was due to the fact that throughout our studies there are stages in which we are unable to guarantee sterility in its entirety.

6.2.1 - Preparing the eggs for xenografting tumor cells

Before starting the incubation, the dirt, feathers and droppings are carefully removed from the eggshells mechanically by paper drying, which has a rough structure rather than a soft surface. Never clean the eggs with ethanol because it significantly reduces the survival rate of the embryos. Incubation of the eggs from a local commercial hatchery ensuring 95% fertilization.

After fertilizing the eggs, they are applied inside an incubator (with horizontal rotation of the eggs) at 37ºC and at a humidity of 60% for 10 days. After these 10 days of incubation, the eggs are removed from the incubator and placed on a supported table (Figure 2.3).

Use a tube lamp or other suitable light source to candle the eggs by shining the light at the blunt end of the egg where the air sack is located. The embryo must be located near the bottom of the egg and the air sack on its right. Localize and mark using a pencil the allantoic vein that is located at the top of the eggshell, right where several blood vessels cross (Figure 2.4). Clean the area, including and around the mark, using paper dipped in distilled water (Figure 2.5). Drill a small hole through the eggshell into the air sack using a 30-gauge syringe needle (Figures 2.6 and 3A). Make another hole near the allantoic vein, which penetrates the shell membrane but not the CAM, using a 30-gauge syringe needle (Figures 2.7 and 3B). The CAM is attached to the inner surface of the shell, so care should be exercised at this point. Use a 20-gauge syringe needle with a small hook on the end to make a third very small hole in the eggshell membrane (Figure 3C).

To suction, create with an automatic pipette aid fitted with a piece of tygon tubing laced against the hole in the airsac. To separate the CAM from the shell and let it drop, apply a mild vacuum to the hole over the air sack so the blood vessel drops down, away from the eggshell and attaches to the embryo (Figures 2.8 and 3D).

After the CAM fell, we were able to open a window on the egg. For this we use a needle and open a $1cm^2$ window. To open a window in the $1cm^2$ egg, we use a needle (Figures 2.9 and 3E). Then we seal this window with tape and place the eggs in the incubator without a rotating option at 37 °C and 60% humidity.

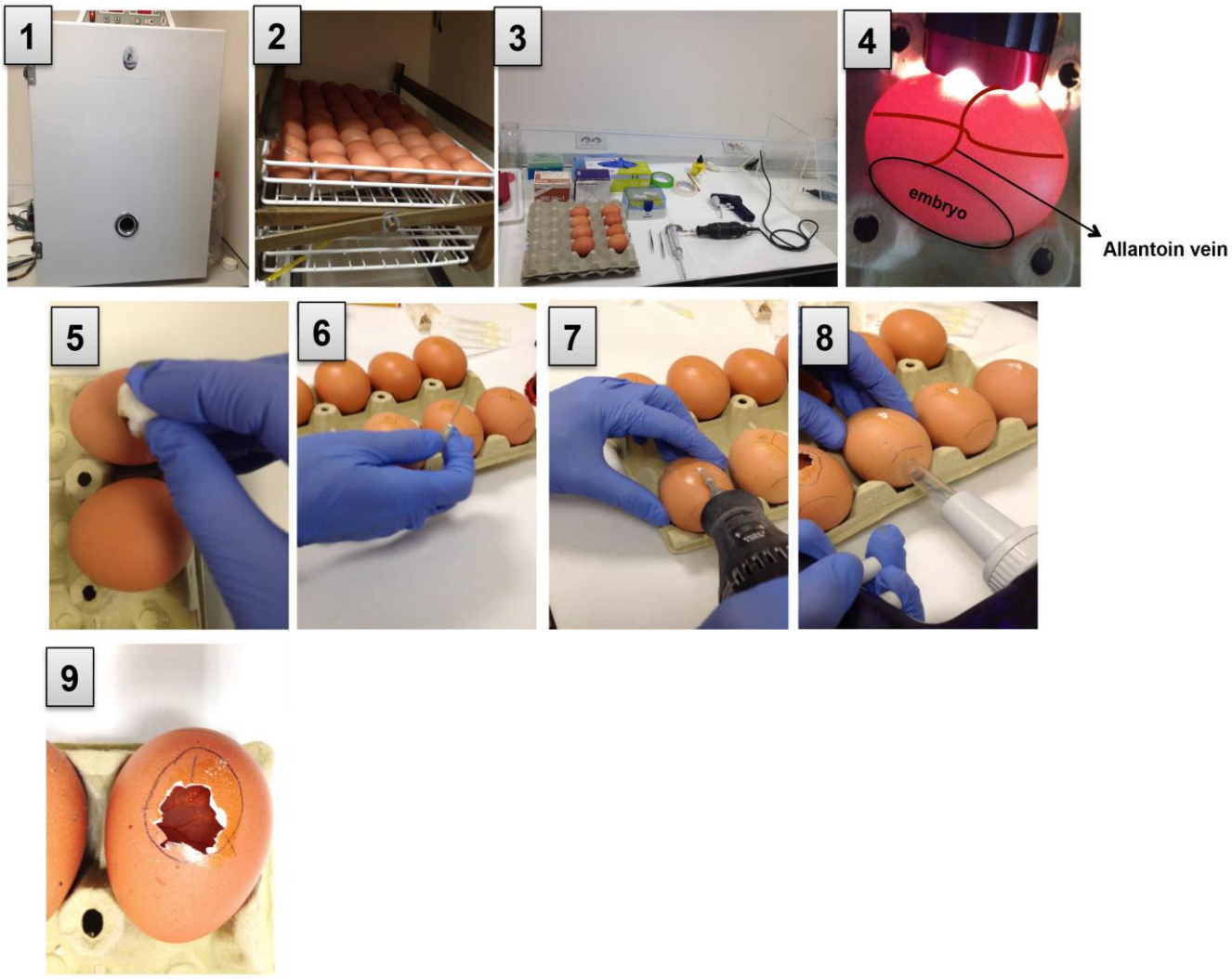

Figure 6: A scheme of images that represent the incubation process of the egg until its preparation to insert the tumor tissue. 1 and 2, corresponds to the incubation of eggs. 3 Preparation of all the materials needed to work on the egg after the minimum incubation time. 4. Verification and marking of the air sac and allantoic vein. 5. Clean the egg with a paper or cotton wool soaked in distilled water. 6. Preparation of a needle and execution of the first drilling (air bag). 7. Execution of the second and third drilling in the egg. 8. Insert the air pump into the hole in the air bag and suction to force the air bag to move to the shell. 9. Opening a window in the egg shell in the area marked with the CAM (adapted (Crespo & Casar, 2016))

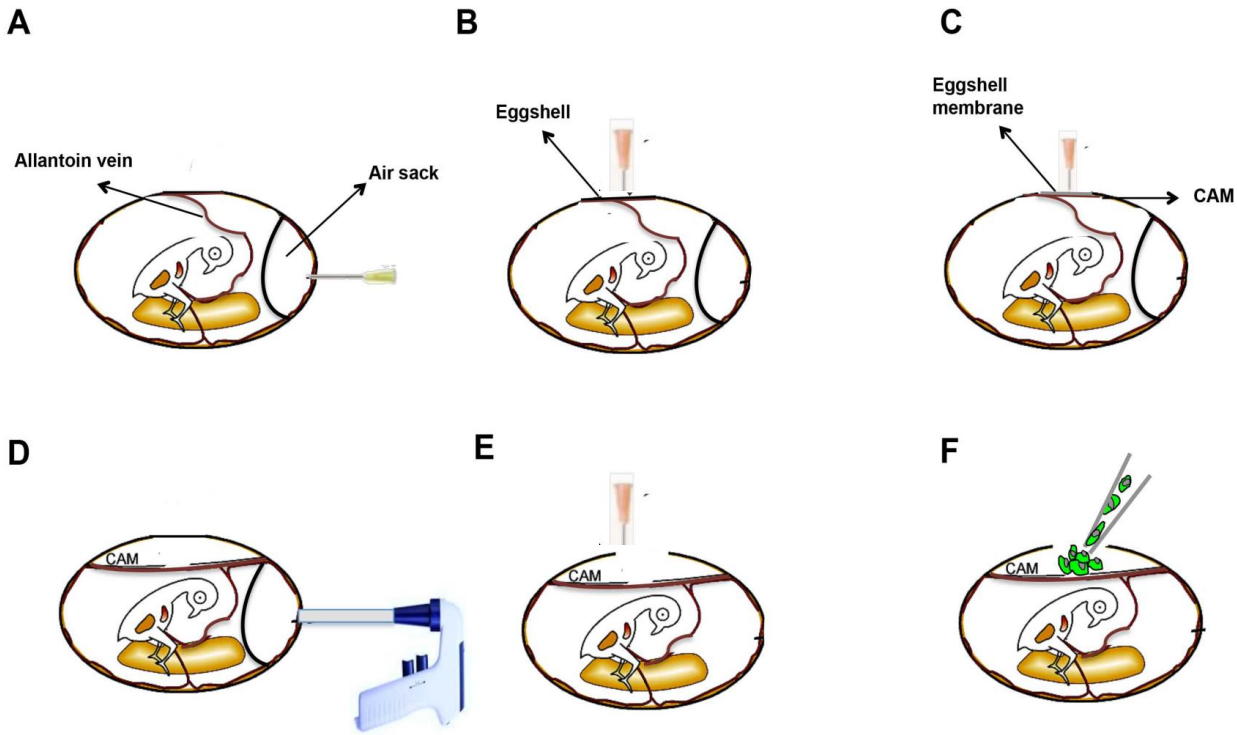

Figure 7: A diagram that includes 6 processes from creating the hole in the eggshell to introducing the tumor into the CAM. A. Verification of an allantoic vein against the eggshell. This usually occurs on the tenth day of embryonic development. After seeing the position of the vein and the air sac, the skin is punctured at the air sac site with a needle. B. Drilling another hole in the area of the bark where the allantoic vein occurs. C. Make a third hole in the egg shell in the same area as the second hole a thinner needle compared to the needles used in the first two holes. D. Inserting a pump in the air bag hole, in order to produce a vacuum to force the release of the CAM from the shell. E. Cut a small section in the egg shell, so that it is possible to see the exposed CAM. F. Placing tumor tissue on the CAM (adapted (Crespo & Casar, 2016)).

6.2.2 - Preparing tumor fragments for grafting

The process starts with patient surgery, where surgeons extract a tumor portion. This portion is then sent to pathologists by cutting a small fragment that will be used in this method. This fragment will be placed in an eppendorf with a freezing liquid (biofreeze) to be stored.

3

6.2.3 - Tumor fragment inoculation in the CAM

Place the eppendorf (containing the tumor fragment and the freezing liquid) in the incubator for one and a half hours to thaw. After this defrosting time, remove the freezing liquid from the eppendorf and place 200 µl of DMEM and PenStrp (this step is done inside the laminar flow chamber). The eppendorf is then placed in the incubator again for an hour and a half.

After that time, we removed the glue tape and put a silicone ring (this is to locate the tumor). We then removed the eppendorf tumor fragment into a petri dish along with the DMEM liquid to be cut with the scalpel to a volume of 3 mm^3. After we had this tumor volume, we placed the fragment inside the egg (on the CAM), sealed the egg with the tape glue and put the egg in the incubator for 4 days. After that, remove the fragment from the egg and insert it into some cassettes to start the processing.

After incubation in the egg, tissues were removed and subjected to histology processing and microscopic examination, as described below:

Figure 8: Putting the eppendorf with the tumor in the incubator to melt the biofreeze [own authorship]

6.2.4 - Histology

Histology nowadays represents a very important process in the treatment of any disease, it is characterized by the verification of tissues through a microscope. This research process allowed us to study cell structures more clearly, reactions between antibiotic factors with certain cells for therapeutic purposes, cell behavior under certain conditions and many other purposes (Kanitakis, 2002). Medicine today uses this method massively in research to optimize the most appropriate therapies. Histology in practice corresponds to the preparation and staining of tissues and / or cells for later verification through a microscope. Histology in the area of cancer played an essential role both in the study of the evolution of each tumor in each patient and in the selection of the best drug to combat each tumor specifically (Kanitakis, 2002). There are 5 stages: fixation, processing, incorporation, cutting and coloring (Titford, 2009). Each of these steps has improved over time, through advances in molecular and cellular biology (in order to develop immunological techniques), molecular chemistry (developing chemical tests from staining to cell preservation) and in medicine (in order to understand the physiology of tissues and organs) (Yamamoto, 2019).

6.2.4.1 - Fixation

Fixation is the first histological process and consists of structural preservation of the tissue. From this stage, processes such as autolysis (carried out by proteolytic enzymes) and putrefaction (carried out by bacteria or fungi) are considerably delayed (Titford, 2009; Young et al., 2011).

In fixation we have several factors that affect the effectiveness of this process such as pH, temperature, osmolarity, fixative (there are several fixatives, but one of the most used fixatives is formaldehyde), sample size, amount of fixative, among others (Kanitakis, 2002).

Regarding the pH for most fixations, it is 4.9.

The increase in temperature helps to increase the speed of this reaction, however in excess it can burn the sample in question. The sample size also has an impact because it will vary the amount of fixer to impregnate.

In relation to osmolarity, hypertonic solutions induce a reduction in the sample size, while hypotonic solutions induce greater volume in the sample. In general, hypertonic solutions have worked well for most fixation processes.

The fixative quantity in relation to the sample size is usually the fixative is fifteen times the sample quantity (Buchwalow & Böcker, 2010)

Fixation, as already mentioned, consists of preserving the sample, avoiding its degradation processes, however it also causes protein denaturation, making them dysfunctional (Young et al., 2011). At the genetic level, some fixators showed denatures at the level of DNA, mRNA, rRNA, tRNA (Anderson, 2011; Kanitakis, 2002)

6.2.4.2 - Dehydration

The dehydration process has a fundamental role to make the subsequent histological evaluation of the tissue. This process consists of the removal of water inserted in the tissue in a gradual way (Crespo & Casar, 2016; Young et al., 2011). This process will be decisive in the effectiveness of later stages of histology such as fixation. The removal of water will further solidify the fabric in question, which will later facilitate its cut to a certain thickness. The agent that was used to perform this dehydration was ethanol (Crespo & Casar, 2016; Young et al., 2011). Successive baths with ethanol are carried out to dehydrate the tissue and in each bath the percentage of ethanol is increased, so that the water removal is progressive, causing minimal impact on the tissue (avoiding changes in tissue morphology) (Titford, 2009; Young et al., 2011).

6.2.4.3 - Clearing

Starting from here the tissue already contains little or no water, however it is an intermediate step before the fabric is impregnated by an agent that releases the solidity. This step is crucial for the incorporation process, since paraffin and ethanol are practically immiscible (Young et al., 2011). It is necessary to use another solvent that makes this transition. Many researchers call the agent at this stage the "cleaning agent". In this work, xylene was used. Thus, baths are made successively with this cleaning solvent, in order to remove the ethanol and thus leave the fabric prepared to receive the oil from the next historic step (Young et al., 2011).

6.2.4.4 - Embedding

The incorporation process uses a histological technique that provides structure and solidification to the sample to be subsequently sectioned so that it is possible to check it under the microscope (Musumeci, 2014). There are several histological waxes that can be used, with paraffin being the most used histological wax. (Titford, 2009).

Paraffin is a histological wax with very specific properties that can cut a tissue 2 µm thick. In more complex cellular tissues, other histological waxes such as plastic resin can be used to produce a good morphology to facilitate microscopic visualization (Musumeci, 2014).

In the first step, the paraffin is heated until it becomes liquid and then it is collected in the container where a sample is deposited. Then, a cassette that identifies the sample is placed and more liquid paraffin is placed. The paraffin block after it is cooled is important to have an identification cassette inserted to distinguish as several variables that may have experience. Normally, a paraffin is liquid at 60ºC and then solid at 20ºC, where when solidifying it gives consistency so that the sample sections are cut with consistency.

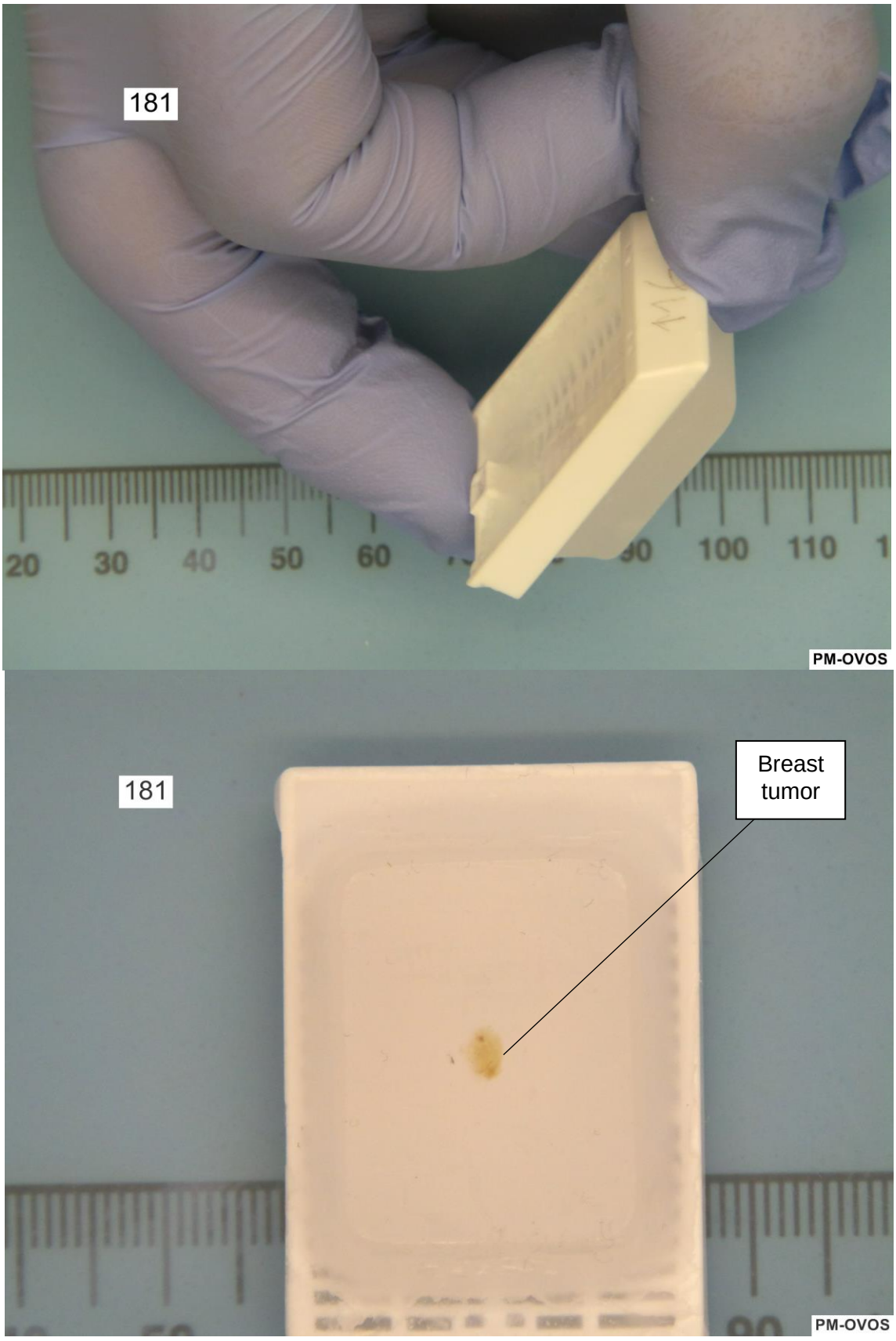

Figure 9: Breast tumor fixed in paraffin. [own authorship]

6.2.4.5 - Microtomy

After the paraffin is impregnated with the tissue, it is necessary to cut or block the tissue into very thin pieces. This is essential for the light from the microscope to pass through the tissue for later observation. For this purpose, a microtomy machine is used that cuts in sections from micrometers (μm) to millimeters (mm). After this cut, the pieces can now be placed later on glass slides with an identification on the tip (Musumeci, 2014).

6.2.4.6 - Coloration

This step is very important to discriminate any structure present in the sample in question. Coloration is a process that must be well applied to obtain the maximum staining of the desired sample, in order to clearly differentiate the various cell compartments and to distinguish tumor cells from other cells well. The better coloration we get, the better the check of the microscope will be. In addition, even if the staining is performed well, it is worthless if each of the other 4 stages of histology is not done well. Two dyes widely used in medicine are hematoxylin and eosin (H&E) for staining some cellular constituents namely nucleus, cytoplasm and connective tissue. Hematoxylin is a basic pH dye and stains the nuclei, providing a bluish color while eosin provides a pink color to the cell nucleus (Kanitakis, 2002). These dyes play a fundamental role in the study of cancer, as they allow the tumor cells to be distinguished from other cells quite clearly at the end of the histological process (Musumeci, 2014). There are several staining techniques besides this one, like differential staining, double staining and multiple staining (Eppert et al., 2011).

6.2.4.7 - Gross and Microscopic Examination

This final step will depend a lot on the effectiveness of the previous histological steps. The researcher will take his interpretations according to what he will observe under 7the microscope. These interpretations will depend on the type of microscope to be

used, which may be optical or electronic, among others (Musumeci, 2014). For pathologists, observation under the microscope is a daily and absolutely essential task for monitoring patients.

Figure 10: Several tumors prepared in slides to perform microscopic visualization [own authorship].

Chapter IV

Results

We studied the feasibility of three types of tumor tissues (kidney: benign lesions and renal cell carcinoma, ovarian: benign conditions and serous adenocarcinoma and breast: benign lesions and ductal carcinoma) in the form of fragments, incorporated into a living organism (egg) environment. The tumor fragments come from several patients aged 40 to 71 years. We divided the tumor fragments of patients by tissue origin (kidney, ovary and breast) and malignancy (malignant or benign). We used tumor fragments with an approximate volume of 3 mm^3 and performed the *in ovo* method, that is, all the corresponding organic material of the egg was incubated inside the egg.

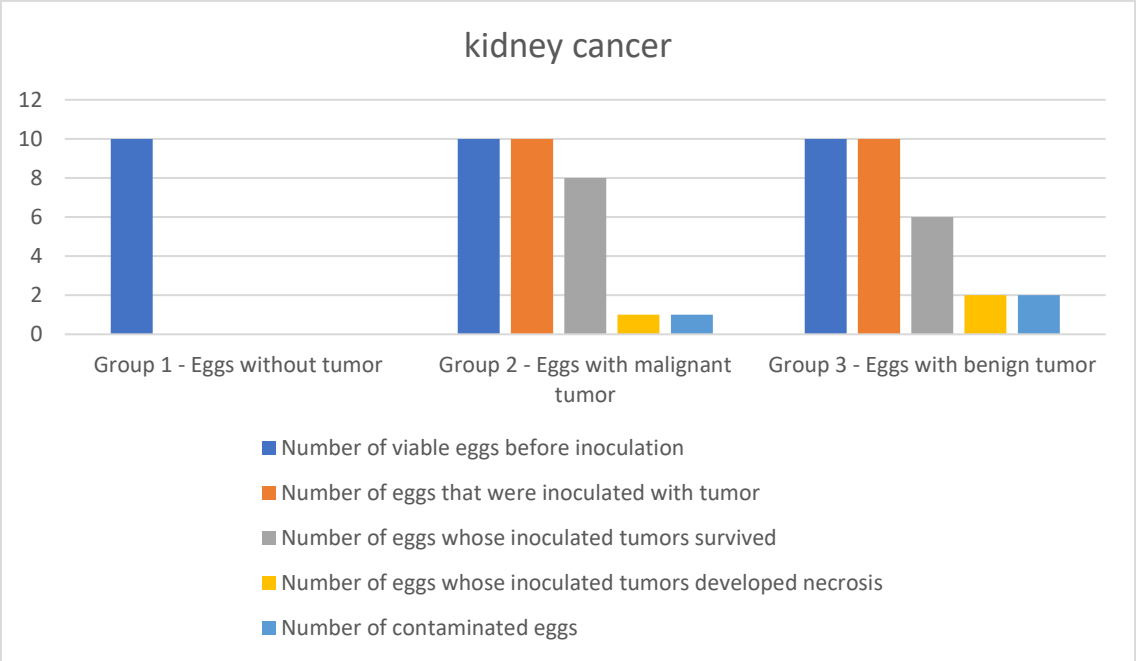

Figure 11: Graph of the viability of kidney tumor fragments. This graph corresponds to the viability of tissue fragments, on the chorioallantoic membrane of the embryo, corresponding to kidney tissues. Group one corresponds to the control group which are the eggs without inoculations of tumor fragments. Group two corresponds to eggs with tumor fragments corresponding to malignant kidney tumors (Renal Cell

Carcinomas). Group three corresponds to eggs with tumor fragments of benign kidney tumors.

According to the chart, all eggs that were incubated in group one was viable. In group two, the ten incubated eggs were viable for inoculation of the tumor fragments. Eight of the ten viable eggs contained viable tumor fragments, one egg contained a tumor fragment with necrosis and one egg developed a contamination with a fungus.

In group three, the ten eggs incubated were viable for inoculation of the tumor fragments, two eggs contained tumor fragments with necrosis and two eggs developed contamination. Thus, the overall viability rate for the fragments belonging to benign tumor was 60%, whereas in the malignant tumor fragments it was 80%.

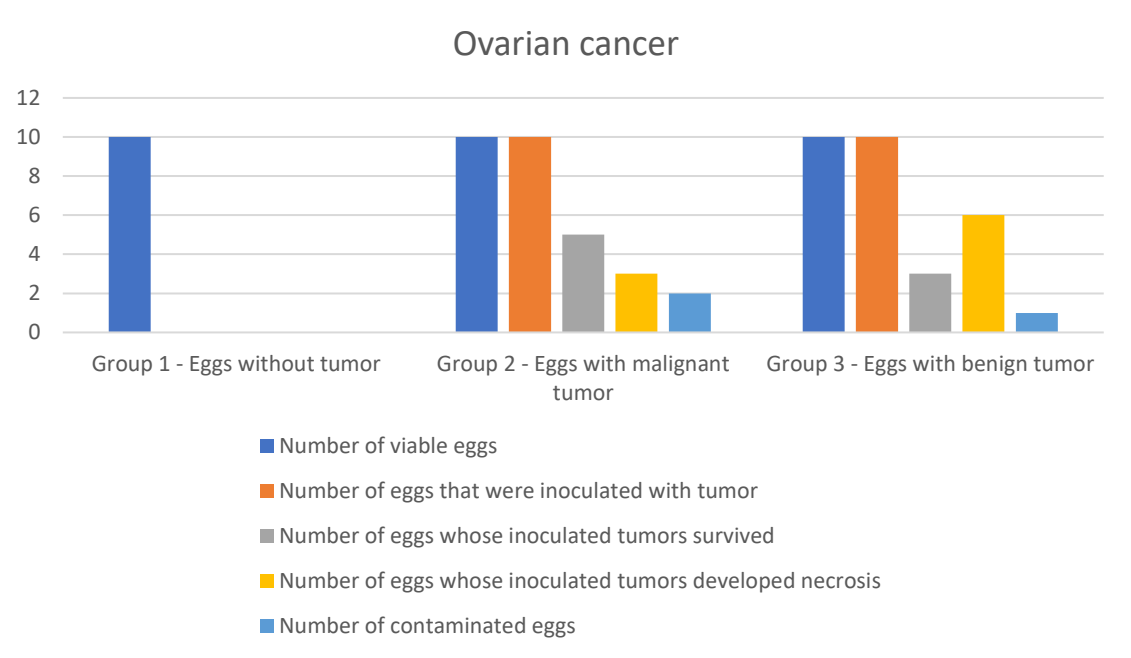

Figure 12: Graph of the viability of ovarian tumor fragments. This graph presents the viability of ovarian tissue fragments, on the embryo's chorioallantoic membrane, corresponding to ovarian tissues. Group one corresponds to eggs without inoculations of tumor fragments. Group two corresponds to eggs with tumor fragments corresponding to malignant ovarian tumors (Serous Adenocarcinomas). Group three corresponds to eggs with fragments of benign lesions.

According to the chart, all eggs that were incubated in group one was viable. In group two, the ten incubated eggs were viable for inoculation of the tissue fragments. Six of the ten viable eggs contained viable tumor fragments, three eggs contained tumor fragments with necrosis, and two eggs developed contamination of a fungus. In group three, the ten eggs incubated were viable for inoculation of the tissue fragments, three of the ten viable eggs contained viable tissue fragments. Six eggs contained tissue fragments with necrosis and one egg developed contamination of a fungus. Thus, the viability rate for the fragments belonging to benign lesions was 30%, whereas in the malignant tumor fragments it was 50%.

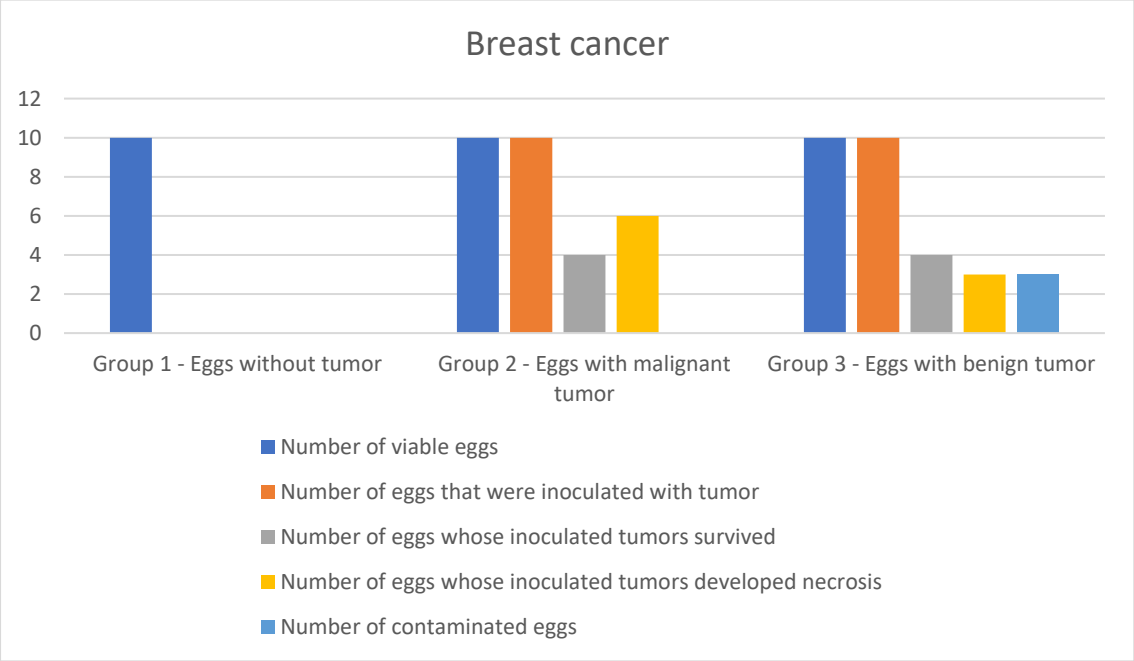

Figure 13: Graph of the viability of breast tumor fragments. This plot corresponds to the viability of tissue fragments, on the chorioallantic membrane of the embryo, corresponding to breast tissues. Group one corresponds to eggs without inoculations of tumor fragments. Group two corresponds to eggs with tumor fragments corresponding to breast malignant tumors (ductal carcinomas). Group three corresponds to eggs with tumor fragments of breast benign tumors.

According to the chart, all eggs that were incubated in group one was viable. In group two, the ten incubated eggs were viable for inoculation of the tumor fragments. Four of the ten viable eggs contained viable tumor fragments, six eggs contained tumor fragments with necrosis and no egg developed contamination of a fungus. In group three, the ten eggs incubated were viable for inoculation of the tissue fragments, four of the ten viable eggs containing viable tissue fragments. Three eggs contained tissue fragments with necrosis and three eggs developed contamination of a fungus. Thus, the viability rate for the fragments belonging to benign lesions was 40%, whereas in the fragments of malignant tumors it was 40%.

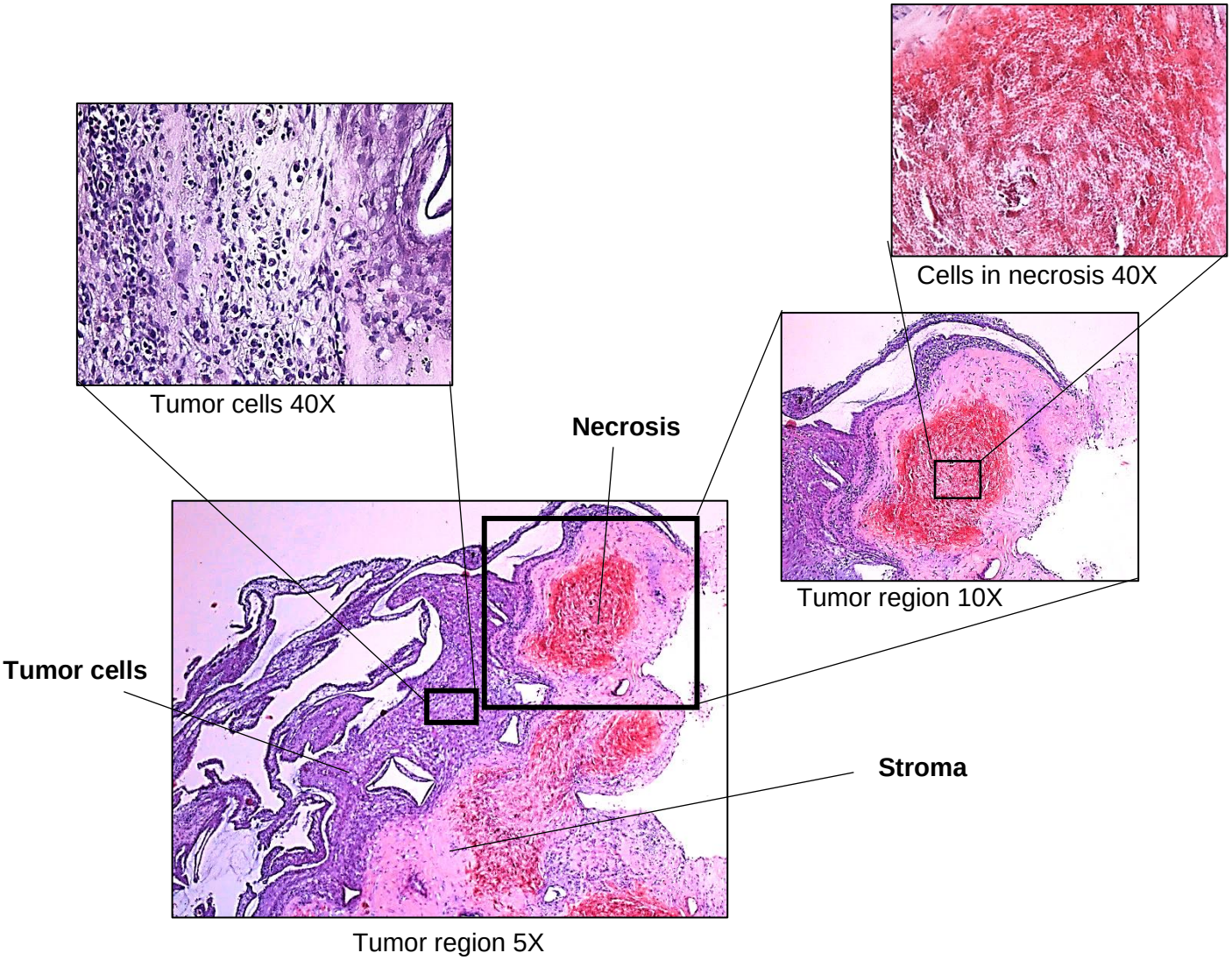

Figure 14: Microscopic visualization of a benign kidney lesion under various enlargements**.** In the image of the upper left corner, it is possible to observe, in a clear

and well-stained manner, viable tumor cells with a high proliferation rate, under a magnification of 40X. In the image of the upper right corner one can observe, in a neat and well-colored way, cells in a state of necrosis, under a magnification of 40X. This fragment was treated with DMEM for one hour and incubated for 5 days. **[own authorship]**

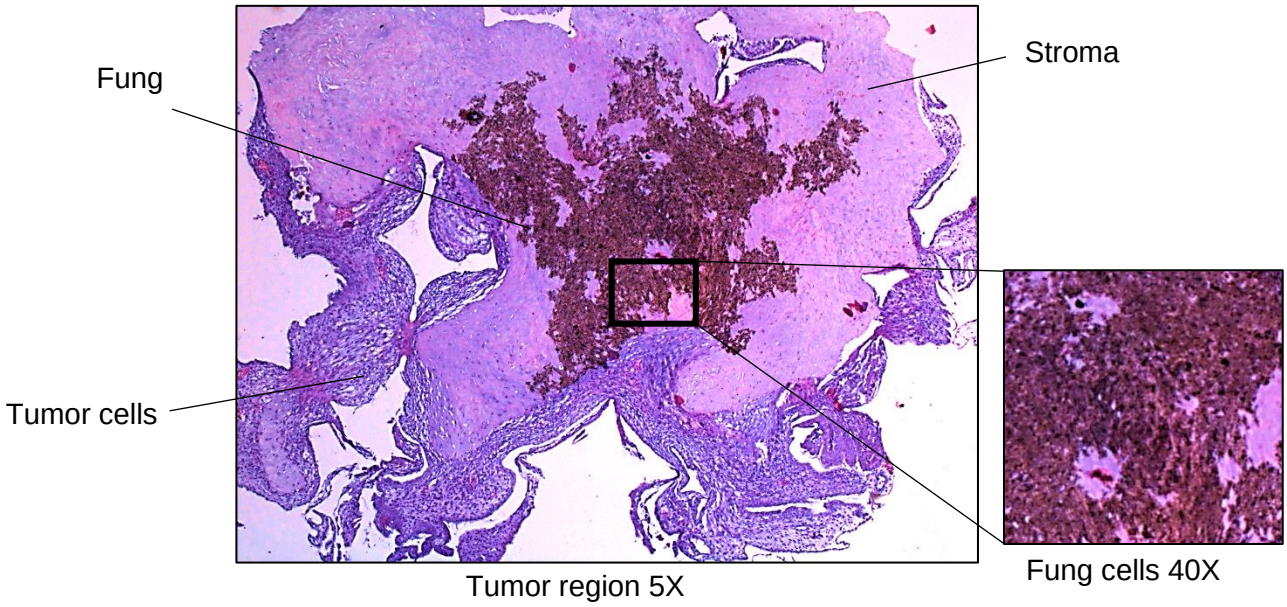

Figure 15: Microscopic view of a malignant ovarian tumor showing contamination. In the image of the right corner we observed the fungus under a magnification of 40X. This fragment was treated with DMEM for one hour and incubated for 5 days **[own authorship].**

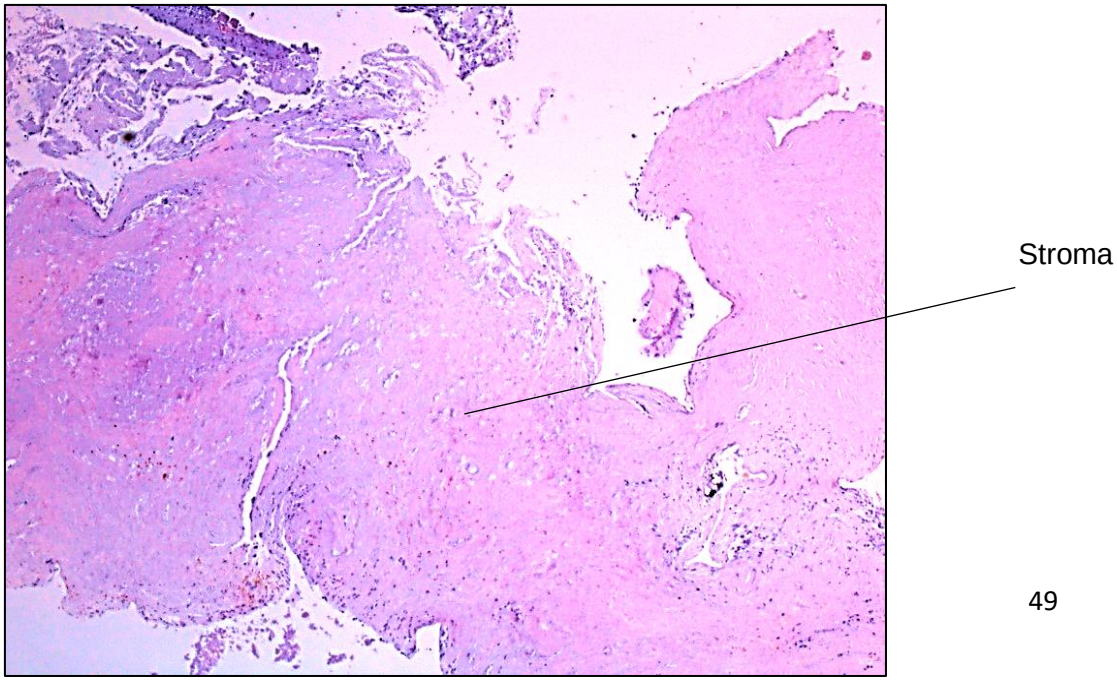

Figure 16: Microscopic view of stroma at a magnification of 10X, corresponding hypothetically to a malignant breast tumor. This fragment was treated with DMEM for one hour and incubated for 5 days **[own authorship].**

Figure 17: Inoculation of kidney tumor tissue into the chorioallantoic membrane [own authorship].

Figure 18: Inoculation of breast tumor tissue into the chorioallantoic membrane [own authorship].

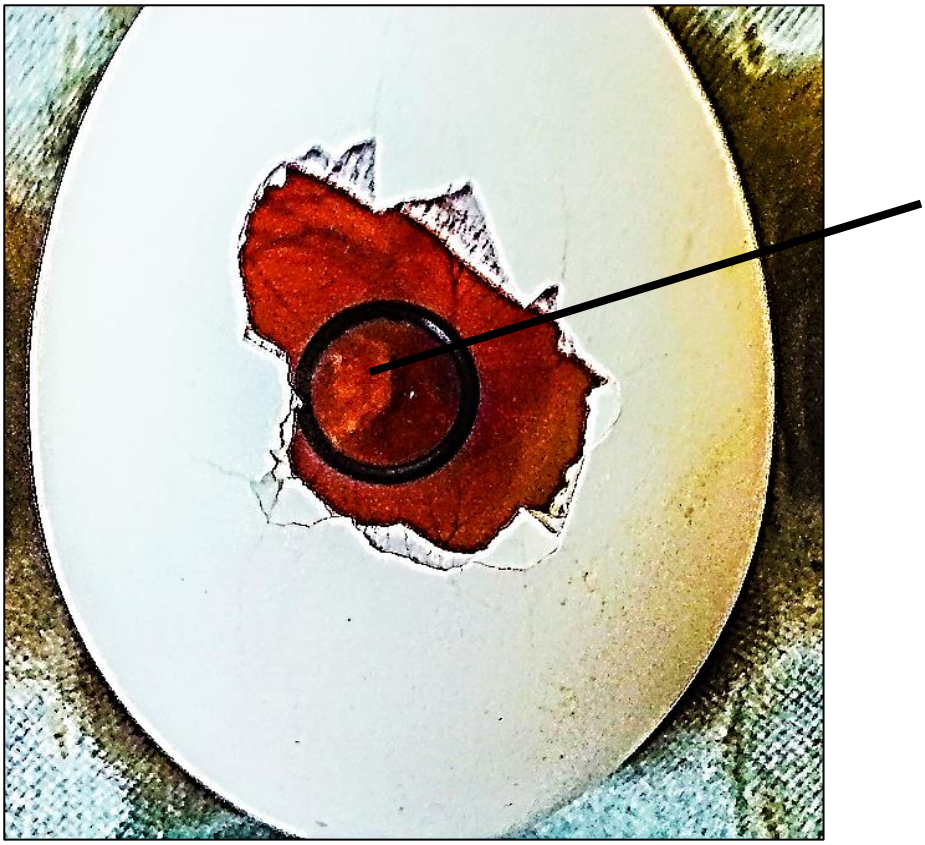

Figure 19: Inoculation of ovarian tumor tissue into the chorioallantoic membrane [own authorship].

Through the Fisher test we analyzed the influence of the tumor organ-of-origin on the viability of the tumor, irrespective of the malignant/benign nature of the lesion. In table 1, in which data for comparison of viability of kidney and ovary tumors is summarized, we obtained significant difference ($p = 0.030146$), the same holding true for comparison of viability of kidney and breast tumors (table 2) where $p = 0.030146$. There were no significant differences between viability of ovary and breast-derived tumors (table 3; $p = 0.268143$). This allows us to say with 95% confidence that the kidney tumors show significantly improved viability and ability to survive CAM inoculation and culture. From the statistical data, we found that the viability of ovarian and breast tumors is just as likely to be successful.

Table 1: Distribution of viability of kidney and ovary tumors after being inoculated into the egg

	Kidney	**Ovary**	Total
tumor survival	14	8	22
tumor death	3	9	12
Total	17	17	

Table 2: Distribution of viability of kidney and breast tumors after being inoculated into the egg

	Kidney	**Breast**	Total
tumor survival	14	8	22
tumor death	3	9	12
Total	17	17	

Table 3: Distribution of viability of ovary and breast tumors after being inoculated into the egg

	Ovary	**Breast**	Total
tumor survival	8	8	16
tumor death	9	9	18
Total	17	17	

We also performed an analysis of the significance of differences between malignant tumors and benign lesions, since the former are potentially more likely to survive within the egg compared to benign lesions of the same tissue type. Using the Fisher's test we compared viability data from malignant *versus* benign lesions in the setting of kidney, ovary and breast tumors (table 4; p = 0.370588, table 5; p = 0.193501 and table 6; p = 0.302345, respectively. Through these analyses we see no statistical significance to claim that malignant tumors are more likely to survive inoculation and culture than benign ones.

Table 4: Distribution of viability of malignant and benign kidney tumors

	tumor survival	**tumor death**	Total
Group 2 - Eggs with malignant tumor	8	1	9
Group 3 - Eggs with benign tumor	6	2	8
Total	14	3	

Table 5: Distribution of viability of malignant and benign ovary tumors

	tumor survival	**tumor death**	Total
Group 2 - Eggs with malignant tumor	5	3	8
Group 3 - Eggs with benign tumor	3	6	9
Total	8	9	

Table 6: Distribution of viability of malignant and benign breast tumors

	tumor survival	**tumor death**	Total
Group 2 - Eggs with malignant tumor	4	6	10
Group 3 - Eggs with benign tumor	4	3	7
Total	8	9	

Everything that was done in this experiment was approved by the ethics committee of the IPO of Coimbra and compliance with the data protection law rule was respected.

Chapter V

8 - Discussion and Conclusion

In this study, we sought to develop a method to culture and maintain tumor tissues and their respective microenvironments "*ex vivo*" using a xenotransplant into living organism. The organism used was the chicken egg and we used tumor fragments from the patients directly. Sometimes we were able to inoculate the fresh tumor fragment directly into the chorioallantic membrane of the egg, that is, the tumor fragment did not undergo any intermediate treatment. If it were not possible to inoculate the fresh tumor into the egg, we would have to use tumors preserved in a biofreeze solution. The inoculation of either fresh or frozen tumor fragments is an important factor because by freezing the fragment, we are subjecting the tissue to another stress that can influence the final result. The ideal is to inoculate the fragment in fresh, that is, as soon as the surgical specimen comes from the operation room, and proceed to cut the tumor tissue into fragments in an appropriate culture medium and then immediately inoculate the egg. This is to reduce possible tumor disturbances and thus retain most of the viability of the tissue. However, this setting requires a synchronization of the pre-incubation of receiver eggs and the patient's surgical intervention, which is very difficult to operate.

Our focus was to be able to inoculate fresh tumors in the eggs avoiding the maximum biological stress in the tumor piece, in order to make the results as reliable as possible. One of the most difficult steps in this process was to be able to inoculate fresh tumors into the eggs, as it required a certain "timing" between arriving a fresh tumor from the surgeries and having eggs at the stage when he was ready to receive the tumor piece. The great advantage of this method is the possibility of using human tissue coming directly from the patient in the form of fragments and not in the form of cells in suspension. By using tumor fragments, we were able to preserve most of the surrounding cellular interactions of the tumor and thus retain the tumor's heterogeneity and surrounding stroma. The cells in suspension for not preserving the tumor structure and heterogeneity may assume different behaviors, indicating an assumption that would not happen with the tumor fragments (Lai et al., 2017).

The PDX-CAM model requires a lot of optimization to obtain minimum results in the order of 40% viability (Ishihara et al., 2020). In addition to the necessary equipment and after several optimizations in the egg viability rate was around 60%, because this

model is very sensitive and can easily fail in the viability of the egg, or not be guaranteed the conditions of the environment or the incorrect manipulation of the egg (Ishihara et al., 2020). In the literature, during the experiences of many researchers, the viability of this method is one of the major barriers to not obtaining a greater number of results, either because of contamination or viability or the egg or tumor. One of the barriers that conditions the viability of this experiment is the contamination that impairs both the viability of the egg and the tumor. Adding the eggs with malignant and benign tumors of each of the three cancers (kidney, breast, ovary) we verify that there are the same number of contaminations in each one. Through these results we can assume that the contamination does not originate in the biology of the tumors, but probably in the applied methodology. The observed contaminations may be due to the incubator's environment not being entirely sterile or due to the space where the pathologists cut the tumor piece into fragments also not to be totally sterile. In any case, we did regular washes of the incubator with a decontamination solution (Korsolex) and 100% alcohol, at least once a week and then we reinforced this cleaning twice a week. To dry, we placed the open incubator inside the laminar flow chamber. All of these actions were performed with great care to increasingly decrease the likelihood of any type of contamination appearing, whether fungal or bacterial in nature. All tumor fragments were pre-incubated in DMEM with Penicillin and Streptomycin for 90 minutes before tissue being inoculated into the egg. The DMEM solution was essential for the viability of the tumor, as it contains a small percentage of antibiotics, which allows to control the growth of fungi, and has a large amount of nutrients (from amino acids to vitamins) that are essential for the supplementation of tissues in study. In fact, when the tumor piece ends up suffering a set of biological stresses when it is cut and extracted from the tissue where it had formed, it is therefore essential to receive a nutritive solution to mitigate those same biological stresses. Because this method has a low success rate it is convenient to have a number of eggs in reserve for these experiments. In relation to this, we found that sometimes some eggs brought some impurities and this could also be a source of contamination. According to the literature, cleaning the egg with disinfectant solutions can decrease the viability of the egg itself (Sys et al., 2013). This action of disinfectant solutions on the egg was due to the fact that the egg is permeable. Thus, we chose to clean the egg with a paper soaked in distilled water to avoid any kind of reaction with the egg. This situation is one of the barriers by which it is not difficult to get around to guarantee 100% cleanliness in relation to the residues that the eggshell may contain. Another important technical factor in this study is the sensitivity or fragility of the egg from the shell to its internal organs, so it is important to be extremely careful when handling the egg. One of the most critical steps in handling the egg is when it introduces the fragment into the

chorioallantic membrane, as there is a strong risk of accidents in that membrane (Ishihara et al., 2020). Incorrect placement of the tumor piece within the egg can cause bleeding from the chorioallantic membrane veins, for example, or the tumor piece to sink into the egg. In our study, we optimize the method to obtain a larger number of viable results and developers' protocols for collecting and pre-treatment of the tumor fragment from the removed surgical tissue of the patient. The pre-treatment protocol is essential to maintain the viability of tumor fragments using healthy tumor and non-tissue tumor. Using this method, one can make comparisons in later studies to evaluate the action of some drugs on the patient's tumor, in an *ex vivo* setting.

This experiment did not take into account the species of eggs concerned, which could also affect the growth rate and the viability of the experiments. There are species with a faster development than others with a slower development and may affect the viability of some types of tumor. Another factor that can influence is the size of the embryo between the different species, since a larger embryo allows to have a larger chorioallantic membrane, which means that there is more space to implement the tumor piece and have more probabilities of vascularizing it with the respective membrane.

Throughout this experience, we verified that there was a fundamental point to approach and study that corresponds to tumor viability. Over the years, researchers have carried out several experiments in order to enhance this point of the method. One of the factors studied was the culture medium in which the tumor tissue is placed before being inoculated into the egg (KAUFMAN et al., 1956). One of the culture medium more used in this experiments is with DMEM. The time of incubation of the tumor fragments in the DMEM solution can be an important factor as said before, since the absorption of certain nutrients present in the solution can vary between the different types of tumors. In addition, passing the tumor piece through an intermediate nutrient solution can be beneficial, as the lesions that the tumor suffered when being transplanted from the patient can be repaired by certain essential nutrients.

Another factor studied was the origin of the transplanted tumor tissue. Some studies show that tissues of conjunctive origin are very likely to be successfully transplanted for CAM (KAUFMAN et al., 1956)(Sommers et al., 1952). Thus, it seems to me that the success of the transplant depends also on the characteristics of the tumor biology and not only on the specifications of the experimental techniques used. The increase in the efficiency of tumor viability after its transplantation has evolved so much that there are types of tumors that have reached almost 100% success rate in this model of PDX-CAM. One of these types of tumor was renal cell carcinoma (Fergelot et al.,

2013) (Ferician et al., 2015). From the results of our experience, we verified that in fact the tumor parts with renal origin were those in which we obtained a greater number of live tumors after inoculation in the egg compared to the ovarian and breast tumor. So, this experimental method can become very important as a test to check the action of drugs because we know that some tumor types are more likely to survive in the egg, considering reaching 100% according to the literature. The greater viability in the fragments belonging to kidney cancer in relation to fragments belonging to ovarian and breast cancer may be due to the fact that kidney tumor is usually highly vascularized and thus may have the potential to induce bold vessel growth in the CAM faster than other types of tumors.

We didn't observe significant differences in viability amongst xenografts from malignant versus benign lesions, despite it is conceivable that the inherent features of a malignant tumor, with fast growth, loss of a polarized organization, invasion of adjacent tissues and ability to give rise to metastases might make them more capable of surviving the transplant and culture conditions. Benign tumors, on the other hand, are formed by adult cells that grow in an orderly and slow fashion and that do not invade adjacent tissues, making them theoretically less prone to survive transplant and culture (Heidari & Gobato, 2019). In this experiment, there was no significant difference in the survival of tumors in the egg because it was malignant or benign. We found a greater survival of malignant tumors in the tumor of the kidney and ovary, while in the breast it remained the same. This prediction that malignant tumors are more likely to survive inside the egg than benign tumors could be seen more clearly if the sample number were larger, allowing to assert with more certainty that there is indeed a different impact depending on the malignancy of the tumor.

In terms of the necroses presented in the results of this experiment, we have some hypotheses that justify this situation. The first hypothesis relates to the intermediate steps from the extraction of a part of the patient's tumor to the inoculation of the tumor piece in the egg. The tumor piece suffers several injuries from the patient to the inoculation in the egg, as it is necessary to make several cuts in the tumor piece until reaching the size of $3mm^3$. These cuts create stress at the tissue level, as they can affect blood vessels and thus impair the metabolism of some tumor cells. Blood vessels are essential for bringing essential nutrients to cells and for releasing metabolic waste from them. When we cut the blood vessels, we induce a strong probability that some cells will enter the necrosis phase. In addition, another important factor is what part of the tumor we are inoculating in the egg, since the tumor cells are not all the same which can influence their viability. Another hypothesis for the appearance of cells with necrosis in

this experiment is the exposure of the tumor piece to the surrounding oxygen. When the tumor is removed from the patient, it is exposed to oxygen from the environment, which can impair its viability. One of the common factors in the various types of tumors is their growth in an anaerobic environment. One of the metabolic pathways most used by tumor cells is glycolysis and not oxidative phosphorylation, because glycolysis can be triggered in a medium without oxygen and oxidative phosphorylation in a medium with oxygen. The presence of oxygen negatively affects the metabolism of tumor cells, which can also induce necrosis. At the level of necrosis in malignant and benign tumors, from our results, we found that there is a greater probability that malignant tumors will develop fewer cells in necrosis than benign tumors. We verified this rule in the ovarian and kidney tumor, although in the breast tumor the opposite effect was verified.

The region where the tumor is placed in the embryo can also be important. Once the tumor is transplanted, it remains in an avascularization process for approximately 72 hours. After the embryo's vessels penetrate the tumor, a rapid tumor growth begins (Knighton et al., 1977). In theory, a greater viability of the tumor fragment is obtained if it is placed in a region of the embryo where it presents a greater number of veins, because it increases the probability of the fragment to establish angiogenesis with the vessels of the embryo and thus to obtain metabolic support for its development (Knighton et al., 1977). Some of the movements of the embryo can move the fragment to other regions and thus, hamper its development within the embryo. This may justify the presence of necrosis in some of the fragments.

The developed model for patient-derived tumor transplantation into the chick embryo is inexpensive and rapid. The established protocol can be used to monitor and study cancer biology (growth, invasion, metastasis, angiogenesis and other aspects of the malignant phenotype) in various types of tumor. It is a model that can serve as an alternative to assays in mice. In addition, it is a model with the potential to study *ex vivo* the effect of drugs against the patient's tumors, in several types of malignancies and thus contribute to the development of new therapies. At the ethical level the National Institutes of Health, EUA and the New England Medical Center and Tufts (IACUC, 2001) decided the chicken egg is devoid of any ethical restrictions, as they consider that the embryo does not feel pain during embryonic development until the fourteenth day (Ribatti, 2016).

Chapter VI

9 - Future perspectives

In the future, the method we have developed can be used for a variety of purposes, including studying the response to anticancer drugs in a given patient (Figure 20). Another of the applications is the use of the egg for the regeneration of small tissues, as there are already protocols from other researchers that have been highly optimized for this purpose.

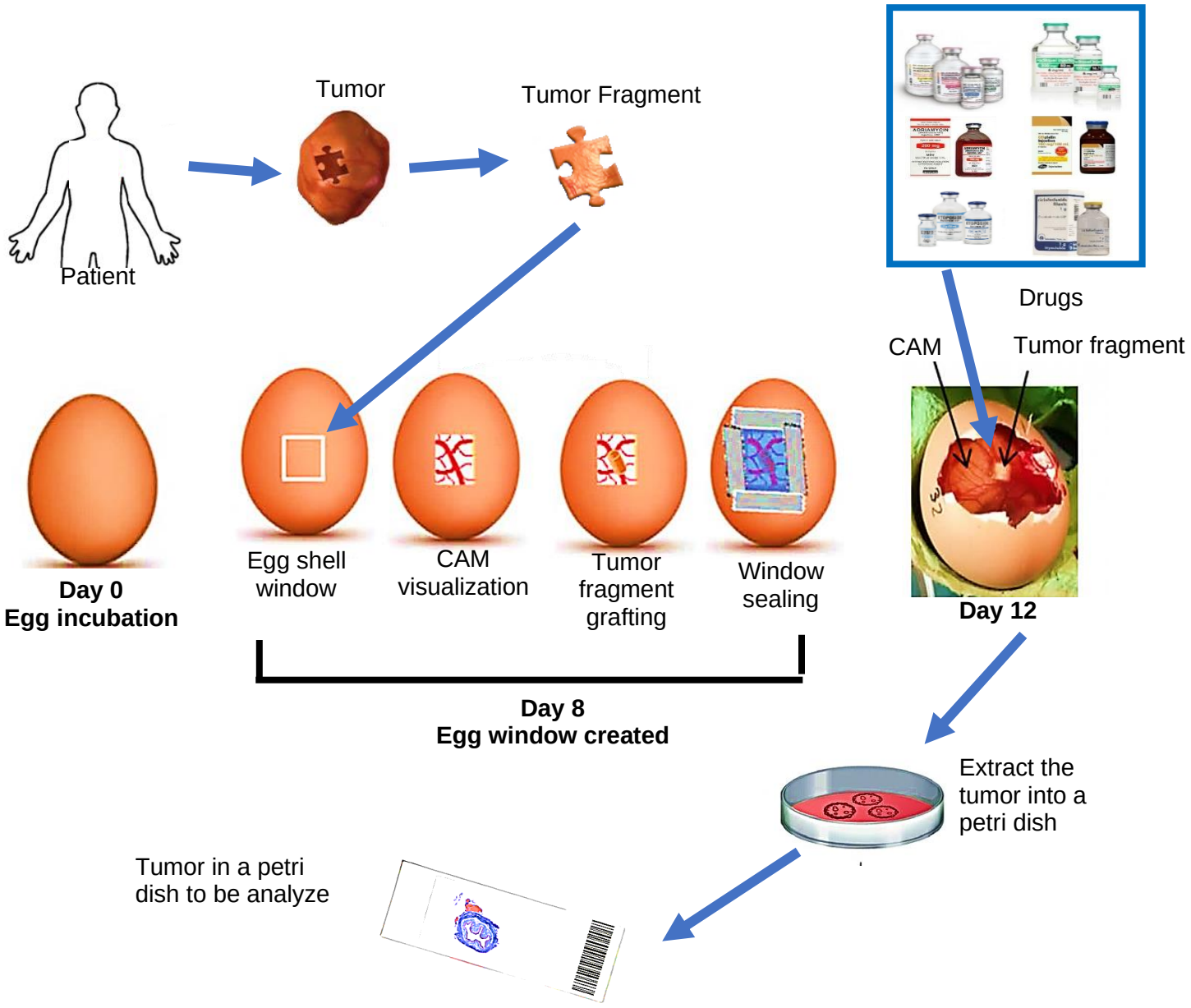

Figure 20: The application of the method developed in this work for testing various types of drugs in a patient's tumor [adapted(Moreno-Jiménez et al., 2016)]

In terms of future work, our intention is to optimize this method so that we can use it in the study of the effect of several drugs against a given tumor. The action of each drug can be tested *ex-vivo* in a tumor fragment treated with the same drugs and regimens for which the patient is eligible. The "*ex vivo"* effect could have a correlation with clinical efficacy of the treatment and inform the oncologist about the most effective therapeutic option. This model could also be used in pre-clinical studies of therapies under development. Since the treated material is actual patient tumor, the model could potentially be very faithful in recapitulating clinical efficacy of the compounds. The method also allows the study of a drug alone or addressing the combined action of different drugs for the same type of tumor, allowing assessment of combinatory therapies. Thus, with future studies, the model herein developed may be used in multiple applications related with cancer treatment in keeping with a framework of personalized treatment, as it can assess therapies in each particular patient's tumor.

This future work may enhance the development of new therapies or contribute to the evolution of existing therapies.

We have already managed to develop a support through which the drug will be inserted into the egg. The goal will be to place the drug inside this support and it will be released by gravity until it reaches the tumor tissue (figure 21 illustrates what is being described). From now on, it will be necessary to test from optimizations of this support to distribute the drug in the tumor in a dose, through its permeability. Its permeability will depend on the density of material the support is made of.

Figure 21: Egg prepared with drug holder support

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