Marta Carolina Jesus Teixeira

Bachelor Degree in Biochemistry

Patient-derived explant cultures for cancer modelling

Dissertation to obtain Master Degree in

Biochemistry for Health

Supervisor: Vítor Espírito Santo, PhD, iBET, ITQB-NOVA

Co-supervisor: Catarina Brito, PhD, iBET, ITQB-NOVA

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“Transforma as pedras onde tropeças nas pedras da tua escada.”

Sócrates

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Abstract

Cancer remains one of the deadliest diseases in the world. The process of drug discovery to find new drugs for oncology treatment takes more than a decade of research and it is associated with high attrition rates during clinical trials. One of the most common reasons claimed for the poor efficiency of new developed drugs is the lack of physiological relevance of the *in vitro* models used to select the novel compounds and to evaluate their potency. The role of tumor microenvironment on tumor progression and drug sensitivity is being increasingly studied and it is expected to provide significant clues for the development of novel therapies. Therefore, the incorporation of microenvironment features on cancer cell models during pre-clinical stages of research has the potential to improve significantly their predictive value.

The developments in culture technologies and analysis techniques have increased the number of cell models available to evaluate drug efficacy. Patient-Derived Explants (PDE) have been proposed as potential models. These models consist of fragments of the original tumor tissue after mechanical and/or enzymatic digestion and offer the advantage to preserve the spatial organization, architecture and heterogeneity of tumor microenvironment. However, explant cultures reported in the literature are typically short-term cultures and are only amenable to short-term drug treatments. The goal of this thesis was to use this *ex vivo* model, optimize its culture parameters and evaluate its potential to be used in cyclic chemotherapy treatments.

This work was performed within the scope of iNOVA4Health, a translational medicine program coordinated by iBET in collaboration with *Instituto Português de Oncologia de Lisboa, Francisco Gentil* (IPOLFG). Treatment of ovarian cancer remains an unmet clinical need, particularly due to recurrence of tumors and acquisition of drug resistance to chemotherapy treatments. For this reason, during this thesis we focused on implementing and optimizing PDE cultures of ovarian carcinoma derived from patients treated at IPOLFG.

In this work, 11 cases of ovarian cancer were successfully cultured as PDE. Received samples included all main malignant ovarian carcinoma types (endometrioid, clear cell, mucinous, undifferentiated and the most prevalent, serous). Fresh ovarian cancer samples were mechanically dissociated into PDE and cultured in dynamic conditions. The preservation of cell populations, cell viability and proliferation were assessed by a panel of readouts. PDE cultured in dynamic conditions retained the original tumor architecture and main cellular components - epithelial cells, fibroblasts and immune cells - for at least 28 days. To validate the *ex vivo* model, PDE cultures were exposed to cyclic chemotherapy treatment (paclitaxel, carboplatin and the combination of both). After two cycles (1 cycle of 24 hours *per* week), PDE showed low cellularity and cell viability, compared to untreated controls, reflecting the action of the compounds.

Altogether, we established PDE dynamic cultures in which tumor architecture and heterogeneity is preserved, replicating the original tumor features. Moreover, we demonstrated the feasibility of performing *ex vivo* drug efficacy studies employing cyclic drug exposure regimens.

Keywords: Ovarian cancer; Patient-derived explants; Tumor microenvironment; 3D cancer model.

Resumo

O cancro continua a ser uma das doenças com maiores índices de mortalidade do mundo. A descoberta de novos fármacos para o tratamento oncológico é um processo que ultrapassa uma década de investigação e que está associada com elevadas taxas de fracasso durante os ensaios clínicos. Uma das principais razões para a baixa eficácia destes fármacos está associada ao pobre nível de relevância fisiológica dos modelos celulares utilizados para selecionar estes compostos e para avaliar a sua eficácia. O papel do microambiente tumoral na progressão tumoral e na sensibilidade aos fármacos é um tópico que despertou elevado interesse à comunidade cientifica e é expectável que traga pistas significativas para o desenvolvimento de novas terapias. Portanto, a incorporação de determinados elementos do microambiente em modelos celulares de cancro em fases de investigação pré-clínica apresenta um elevado potencial para melhorar significativamente o valor preditivo destes modelos.

O desenvolvimento em novos métodos de cultura celular e técnicas de caracterização permitiu um aumento do número de modelos celulares disponíveis para avaliação da eficácia de fármacos. Explantes derivados de pacientes (PDE) foram propostos como potenciais modelos. Estes modelos consistem, essencialmente, em fragmentos do tumor original obtidos após processamento mecânico e/ou enzimático da amostra recolhida. Os PDE apresentam a vantagem de preservar a organização espacial, arquitetura e heterogeneidade do microambiente tumoral. No entanto, os PDE descritos na literatura são normalmente utilizados em culturas curtas, apenas indicados para avaliações de eficácia de tratamentos de curta-duração. O objetivo desta tese foi a utilização e otimização deste modelo *ex vivo*, e avaliação do seu potencial para utilização em tratamentos de quimioterapia cíclicos *in vitro*.

Este trabalho foi desenvolvido no âmbito do projeto iNOVA4Health, um programa de medicina translacional coordenado pelo iBET em colaboração com o *Instituto Português de Oncologia de Lisboa, Francisco Gentil* (IPOLFG) e outros institutos. O tratamento eficaz do carcinoma de ovário ainda se apresenta como uma necessidade clínica, particularmente devido à recidiva dos tumores e à aquisição de resistência aos tratamentos com quimioterapia. Por estes motivos, o projeto desta tese focou-se na implementação e otimização de culturas PDE de carcinoma de ovário, a partir de amostras de pacientes operados e tratados no IPOLFG.

Durante o decorrer deste trabalho, foram cultivados com sucesso 11 casos de cancro de ovário. As amostras incluíram todos os principais tipos de carcinoma de ovário maligno (endometrióide, célula clara, mucinoso, indiferenciado e o mais prevalente, seroso). As amostras de carcinoma de ovário recolhidas durante a cirurgia de remoção no IPOLFG. Foram transportadas para o iBET, onde foram dissociadas mecanicamente em PDE e cultivadas em condições de cultura dinâmicas. A preservação das populações celulares, viabilidade e proliferação celulares foram avaliadas por um painel de métodos de caracterização. PDE cultivados em agitação retiveram a arquitetura original do tumor e as principais componentes

celulares - células epiteliais, fibroblastos e células imunitárias - durante pelo menos 28 dias. Para validar o modelo *ex vivo*, as culturas de PDE foram expostas a tratamentos cíclicos de quimioterapia (com paclitaxel, carboplatina e a combinação de ambos). Após dois ciclos (24 horas de tratamento, uma vez por semana), as culturas de PDE mostraram baixa celularidade e viabilidade celular, em comparação com controlos não tratados, refletindo a ação dos compostos.

Em resumo, neste trabalho foram estabelecidas culturas dinâmicas de PDE que preservam a arquitetura do tumor e a heterogeneidade tumoral, replicando características originais do tumor. Além disso, foi demonstrado que estas culturas são compatíveis com estudos de eficácia de fármacos *ex vivo* aplicando regimes de exposição cíclica ao fármaco.

Palavras-chave: Cancro do ovário; Explantes derivados de paciente; microambiente tumoral; Modelos de cancro 3D.

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Abbreviations and Symbols

2D – Two-dimensional

3D – Three-dimensional

CAFs – Cancer-associated Fibroblasts

CD – Cluster of Differentiation

CO_2 – Carbon Dioxide

Ctrl – Control

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DPBS – Dulbecco's Phosphate Buffer Saline

ECM – Extracellular Matrix

FBS – Fetal Bovine Serum

FDA – Fluorescein-diacetate

FDA – Food and Drug Administration

H&E – Hematoxylin and Eosin stain

HT – HypoThermosol

IHC – Immunohistochemistry

IPOLFG – Instituto Português de Oncologia de Lisboa, Francisco Gentil

LDH – Lactate Dehydrogenase

LDHcum – Lactate Dehydrogenase cumulative

n.a. – Not Applicable

NAD^+ - Nicotinamide Adenine Dinucleotide (oxidized)

NADH – Nicotinamide Adenine Dinucleotide (reduced)

NK – Natural Killer

OVC – Ovarian Carcinoma

P/S – Pen Strep (penicillium and streptomycin)

PDE – Patient-derived Explants

PFA - Paraformaldehyde

PI – Propidium Iodide

rpm – Rotations *per* minute

RT – Room Temperature

SD – Standard Deviation

SOC – Standard of Care

WHO – World Health Organization

% - Percentage

µg – Microgram

µL – Microliter

µM – Micrometer

µmol – micromole

g – gram

g – g force, g from gravitational

mL – milliliter

mm^2 – square millimeter (area)

mm^3 – cubic millimeter (volume)

nm – nanometer

ºC – Celsius (temperature)

U/mL – Units *per* volume

I. INTRODUCTION

I.1. Cancer and drug discovery

Cancer remains one of the deadliest diseases in the world, being responsible for 8.8 million cancer-related deaths in 2015, according to the World Health Organization (WHO). Cancer development is characterized by a series of abnormal molecular events, such as the acquisition of genomic instability, the production of atypical levels of epigenetic proteins, reprogramming of the energy metabolism and escape of the immune destruction, which can lead to uncontrolled growth (Xu, Farach-carson, & Jia, 2014). Cancer cells tend to overcome the spatial constraints typically found in normal tissues through mutations in oncogenes and tumor suppressors, leading to the acquisition of key hallmarks of disease, such as enhanced proliferative signaling, resistance to cell death, induction of angiogenesis and activation of invasion and metastasis (Xu et al., 2014).

Disease progression and drug response are now recognized to be highly dependent on the tumor microenvironment (Santo et al., 2017). The cellular components of the tumor microenvironment are heterogeneous and consists of tumor cells, stromal fibroblasts, endothelial cells of the blood and lymphatic circulation and innate and adaptive infiltrating immune cells (Santo et al., 2017; Xu et al., 2014). The tumor cells can induce a phenotypic modification in healthy fibroblasts to become cancer-associated fibroblast (CAFs) (Wang et al., 2017), with cancer-promoting features such as secretion of matrix components, growth and inflammation factors (Estrada et al., 2016). In the tumor microenvironment, cells are distributed and embedded in a non-cellular fraction, mainly composed of extracellular matrix (ECM) molecules, growth factors, cytokines, chemokines and exosomes. These components interact with tumor cells and influence their behavior, namely proliferation, migration and cellular response to therapy, including resistance (reviewed by Santo et al., 2017). Those interactions between the cellular component of the tumor and the non-cellular fraction are critical for tumor initiation and progression stimulating genetic and epigenetic changes within the cells (Spill et al., 2016; Yiu et al., 2014).

The ECM is an important component of tumor microenvironment composed by glycoproteins, proteoglycans and collagens. It acts as a scaffold, providing the architecture that offers mechanical support for cell attachment. Additionally, it confers biochemical and biomechanical signals influencing tumor cell proliferation and differentiation, cell migration and modulation of immune and vascular activities. Altogether, it plays a determinant role in cancer progression (Mitra, Mishra, & Li, 2013; Santo et al., 2017; Xu et al., 2014).

The influence of tumor microenvironment on disease progression and drug response has been demonstrated in different studies. The cross-talk between the tumor and stromal cells leads to the alteration of sensitivity to certain drugs, potentially leading to drug resistance (Pompili et al., 2016).

The discovery and development of new drugs is a long and expensive process that can take up several years of research (Santo et al., 2017). Candidates to new anticancer drugs that enter clinical trials have only a 5% probability of receiving approval from the Food and Drug Administration (FDA), despite robust indications of efficacy in existing *in vitro* preclinical models (Hickman et al., 2014). These high attrition rates at such advanced stage of drug discovery programs have raised the debate over the efficiency of the current industry methodologies in research and development platforms prior to clinical studies (Santo et al., 2017). Preclinical research is one the key stages of drug discovery programs and its goal is to validate the efficacy, specificity and safety of hit compounds. It is usually performed in *in vitro* cell cultures and *in vivo* models, before moving into clinical trials. Numerous efforts have been made in the last decade towards the development of *in vitro* preclinical models that are more representative of their original counterparts (Hickman et al., 2014; Horvath et al., 2016).

Conventional cell models commonly used in cancer and drug discovery are reductionist and do not incorporate the complexity and heterogeneity of human cancer *in vivo* (Hickman et al., 2014). These cell models should be more reflective of the disease and help eliminate drug candidates that lack efficacy or safety at preclinical stages (Santo et al., 2016). Nevertheless, the complexity of tumor microenvironments poses a difficult challenge to develop biomimetic *in vitro* models that are feasible for application in medium- and high-throughput drug discovery approaches.

I.2. Models available for drug screening

Experimental cellular models are used to better explain tumor behavior, to evaluate the effect of chemopreventive agents and to study the efficacy of antitumor drugs (Arantes-Rodrigues et al., 2013).

In preclinical studies, the efficacy of a new antitumor drug is firstly evaluated in *in vitro* tests using cell lines, and afterwards in *in vivo* tests using animal models, which are crucial to determine the safety and potential usefulness of the drug (Arantes-Rodrigues et al., 2013; Breslin & Driscoll, 2013). In the last stage, clinical trials evaluate the most effective compound through several phases until the antitumor agent is proven to be safe and efficacious enough to be commercialized (Breslin & Driscoll, 2013).

Typically, conventional *in vitro* models consist of isolated cancer cells lines used to study specific cellular and molecular pathways involved in cancer development and to evaluate drug efficacy (Arantes-rodrigues et al., 2013). However, a consequence of using cell lines cultured over years is that they are no longer representative of the original tumor (Majumder et al., 2015). *In vivo* models are also a “gold standard” in cancer drug discovery. Xenografts are developed by injecting subcutaneously or orthotopically human tumor cells in immunosuppressed mice, which can mimic the original tumor at the molecular complexity and tumor heterogeneity levels, and then passed *in vivo* directly from mouse to mouse (Pompili et al., 2016). The efficacy of a drug in a patient can be compared with the drug effects in the

xenograft model generated from cell lines or patient-derived material. Although this model is a good predictor of clinical activity for drugs and it also provides a readily accessible source of target human tumor cells, one of the major limitations is that the tumors generated fail to retain important features of the disease, particularly the human stromal component of tumors. These xenografts should be characterized at the molecular and phenotypic level for the target of the compound to be tested before they can be use as models for chemosensitivity or targeted-therapy studies (Kumar, 2016).

I.3. *In vitro* and *ex vivo* cell models

I.3.1. From 2D to 3D

Traditional 2D cultures (cells cultured in monolayers in adhesive plasticware) are the most used culture strategy in drug development because cells can be easily manipulated, and grow quickly under reproducible protocols, with the ability to implement automated systems which enable high throughput systems (Kim, 2005; Pampaloni, Reynaud, & Stelzer, 2007). Despite these advantages, 2D cultures fall short on the reflection of *in vivo* physiology, as they lack a three-dimensional environment, and they do not recapitulate the *in vivo* cell-cell and cell-ECM interactions found in the tissue microenvironment. Moreover, monolayers have an artificial exposed surface area to *in vitro* drug treatments, which is not comparable to the gradients of drug diffusion naturally occurring within tissues and to the heterogeneous drug concentration sensed by the cells distributed within the tissue (Nath & Devi, 2016).

Developments in culture technologies have enabled the increased use of 3D cell cultures in cancer drug discovery, which has contributed to fill the gap between the models developed *in vitro* and the *in vivo* microenvironment. In 3D cultures, each cell is surrounded by other cells and potentially by ECM, having access to structural support and nutrients from all directions. This contrasts with the 2D environment, in which the cell only sticks on one side and is exposed to nutrients of culture medium from another. The cell-cell and cell-ECM interactions found in 3D cultures stimulate the production and distribution of soluble factors that recognize and activate cell signaling pathways, so that differentiation and proliferation occurs. Moreover, they are also relevant in the diffusion and action of drugs (Santo et al., 2016). Therefore, the ideal *in vitro* cell model must mimic key environmental and architectural cues of the target tissue, in order to be used as a predictive tool (Davies et al., 2015).

There are several methods to generate 3D cell cultures (figure I.1). Multicellular tumor aggregates or spheroids are the best characterized models of cancer and they are used to evaluate some aspects of tumor biology, such as diffusion and effects of nutrients and oxygen on tumor development. They can be generated either by static cultures (forced-floating and hanging drop methods), or using agitation-based conditions (rotatory shakers and stirred-tank culture systems) (Santo et al., 2016).

The forced-floating method (figure I.1a) consists in preventing cell attachment to the vessel surface, (by chemical modifications with polymers), resulting in forced-floating of cells

and a subsequent aggregation step normally induced by centrifugation. This method presents several advantages, as it is relatively simple, inexpensive and reproducible. Moreover, the size of spheroids is adjustable, and the spheroids produced are easily accessible and suitable for high-throughput drug testing. On the contrary, the time and work necessary to coat the plates before cell seeding is relatively labor intensive and there is variability in cell size and shape if not as fixed cell number *per* well (Breslin & Driscoll, 2013).

The hanging-drop method (figure I.1b) uses a small aliquot of a single cell suspension hanging on a glass cover that is inverted on a culture well that is kept due to surface tension caused by the gravity force. The cells accumulate at the tip of the drop and are allowed to proliferate (Breslin & Driscoll, 2013). The size of the spheroids can be regulated in a fast and simple way, depending on the cell density of the seeding suspension. With this technique it is also possible to produce homogenous spheroids in a limited space, thus being suitable for high-throughput drug testing (Breslin & Driscoll, 2013; Nath & Devi, 2016). It is also known to promote the formation of tissue-like structures with strong ECM deposition (Lamichhane et al., 2016). However, the surface tension does not hold higher volumes of the liquid drop, which increases the cost and the labor-intensity of the method. Moreover, it is not suitable for scale-up and it is difficult to make medium exchange without damaging the spheroids.

The rotatory shakers (figure I.1c) involves placing a cell suspension in an Erlenmeyer flask with specific amount of medium, which is then rotated in a gyratory rotation incubator until spheroids are produced (Kim, 2005). Alternatively, dynamic cultures also comprise the use of stirred suspension culture systems (such as spinner flasks). In these, cells cultured in suspension aggregate due to the mechanical stirring and consequent cell-cell shocks. Hydrodynamic conditions inside the vessel can be simply adjusted by changes in the stirring conditions of the culture or cell inoculum. The stirred conditions contribute for an improved mass transport of nutrients and oxygen to the spheroids (Kim, 2005). If defined within an ideal range, stirring rates do not affect cellular physiology and viability. However, these cultures require considerably higher volumes of culture medium (Breslin & Driscoll, 2013).

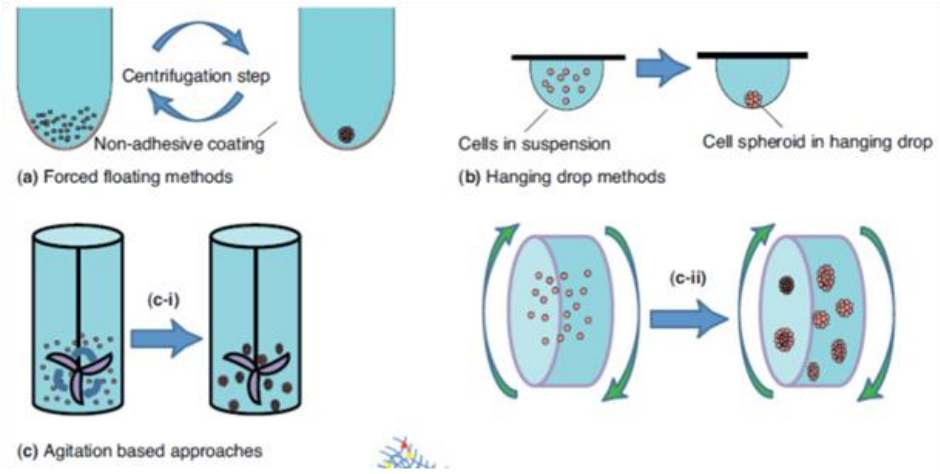

Figure I.1. Cell aggregation approaches. (A) Forced-floating methods or **(B)** Hanging-drop methods or **(C)** Agitation-based approaches (adapted from Breslin & O'Driscoll, 2013).

3D cultures can also be set-up by embedding of tumor cells or spheroids in artificial matrices, such as Matrigel, collagen and other polymers. For instance, Stock et al (Stock et al., 2016) reported the embedding of tumor mono- and co-cultures in either Matrigel, a basal membrane that induces polarization of normal epithelial cells, providing a localized tumor environment, or collagen I, an interstitial stroma matrix compartment, providing an invasive growth environment (Stock et al., 2016). In our group, we have previously implemented a co-culture tumor cell model based on microencapsulated tumor spheroids in the presence or absence of stromal cells into inert hydrogels, such as alginate capsules (Estrada et al., 2016). The inert alginate microencapsulated mimics the glycosaminoglycan structure present in the stromal compartment and keeps the tumor spheroids and stromal cells close to each other.

I.3.2. Recapitulating elements of microenvironment to increase physiological relevance

When implementing preclinical models recapitulating microenvironment there are elements that need to be considered: cell-cell and cell-ECM interactions; tridimensionality and generation of diffusion and gradients of molecules; preservation of stromal components (Spill et al., 2016).

The establishment of physiologically relevant 3D tumor models can improve the predictive value of preclinical research and contribute to an early and successful decision during the development of targeted therapies for cancer. It is important to incorporate different elements of the tumor niche into the model to increase the predictive value of tumor models *in vitro*, since the tumor microenvironment plays an important role in tumor cell response to therapy (Santo et al., 2017).

In order to mimic the events of tumor progression *in vitro*, we can establish co-cultures between relevant cell types: tumor and stromal cells. The implementation of standardized co-cultures that include different cell types remains challenging, as different cell types require distinct culture conditions. By following a deconstructive strategy and reducing the tumor ecosystem to the few essential components that are involved in tumor biology, one may be successful enough to establish models with predictive power superior to conventional 2D cultures (Santo et al., 2017).

The use of artificial matrices to support cell cultures can also contribute to recapitulate cell-ECM interactions found in native tumors, as described in the previous section. By embedding cells in these materials, they will sense physical, chemical and mechanical signals essential for their fate (Alemany-Ribes & Semino, 2014).

I.3.3. The need for patient-derived material

Unfortunately, the traditional *in vitro* preclinical approaches that use cell lines and spheroids generated from established cell lines are limited since they represent a limited level of heterogeneity of original tumors and only selected sub clones that were demonstrated to thrive in *in vitro* cultures. (Davies et al., 2015; Majumder et al., 2015).

Cultures of patient-derived cancer cells are being recognized as important *in vitro* models for preclinical drug discovery as they incorporate the characteristics of human carcinomas that should be integrated into early steps of drug discovery, such as acquired resistance and intrinsic heterogeneity, tumor sites and stages of disease (Santo et al., 2017). However, the availability of patient-derived material is limited for most research and drug screening facilities, which compromises its wide spread use (Santo et al., 2017).

Many cell models using patient-derived tumor material have been recently described in the literature, such as organoids, explants and tissue slices (Santo et al., 2017).

Organoids established from primary tumor are organ-like structures that mimic *in vitro* the tissue of origin and are obtained by the differentiation of pluripotent stem cells (Walsh et al., 2016). Explant culture model enables evaluation of tumor response to therapy while maintaining the tumor microenvironment. The fresh human tumors can reflect the tumor growth *in vitro* and both tumor and stroma have the potential to be retained intact and viable (Karekla et al., 2017).

Tumor slices capture the aspects of tumor architecture and heterogeneity, and maintain the native cellular elements such as immune, vascular and mesenchymal cells (Hickman et al., 2014). However, there is no standardized approach to cultivating this and there is a dearth of information about the impact of slicing on tissue integrity and function (Davies et al., 2015).

Hence, given the important role of the tumor microenvironment on drug sensitivity and other aspects of tumor biology, fresh tumor samples would appear more suitable to study individual patient tumors than more simplistic models, such as established immortalized cell lines (Hickman et al., 2014; Mitra et al., 2013). These methods can be used to inform mechanisms of drug action by evaluating biomarkers of drug response and then can effectively predict patient response to therapy and can be used for monitoring crucial biomarkers (Karekla et al., 2017).

I.3.4. Cultures of tumor explants

The tumor explant models can be used to reliably and simply reflect tumor growth *ex vivo*, preserving the tumor and stroma intact and viable. Explants offer the possibility to preserve the original microenvironment and are an alternative tool to incorporate stromal elements in cell models used for preclinical drug discovery. Thus, explants can be used to study mechanisms of drug action, predicting patient response to therapy and monitoring clinically relevant biomarkers (Karekla et al., 2017; Santo et al., 2017).

Since anticancer drugs exert their effects by changing both cancer cells and tumor microenvironment, it is important to explore cell models that conserve cellular components, such as immune cells, fibroblasts and vascular components of their original architecture (Majumder et al., 2015).

However, the major disadvantage of the explant cell model is the lack of reproducibility due to natural heterogeneity of donor tissues. Also, it is difficult to maintain the culture for more than 3 weeks without loss of cell components (Nath & Devi, 2016).

Table I.1. Advantages and limitations of 2D and 3D cell cultures, and the culture of patient-derived explants for the screening of drugs. Adapted from (Das et al., 2015).

Method	Advantages	Limitations
Cell lines cultured in 2D	Easy to culture Good viability Economical Easy to image and suitable for endpoint assays	Few cell-cell and cell-ECM interactions No histological morphology of original tumor Do not replicate heterogeneity
Cell lines cultured in 3D	Oxygen and nutrient gradients Many cell-cell and cell-ECM interactions Distinct histological morphology of original tumor	Assays are laborious Lack tumor vasculature Relatively expensive
Patient-derived Tumor Explants	Recapitulate tumor heterogeneity and architecture Recapitulate tumor microenvironment	Reduced access to biological samples Long-term culture with poor cell viability No standardized methods available Reduced reproducibility

I.3.5. Cryopreservation and cold storage of PDE

Although patient-derived models are considered to be of high value, the availability of the relevant tissues is a limiting factor (Horvath et al., 2016). Recently, more attention has been given to effective methodologies and protocols to preserve and transport this fresh material between hospitals and laboratories, but also for the creation of biobanks with potential preservation of samples for later trials, being a requirement in biotechnology companies, cellular therapy and not only in cellular models of cancer.

Tissue is often conserved for cancer research either as formalin-fixed paraffin embedded samples or flash frozen in liquid nitrogen. Walsh et al., (2016), preserved the tissue by two methods of freezing and evaluated the viability of both xenografts and organoids. Therefore, flash freezing in liquid nitrogen is the current method for tissue but slow freezing in dimethyl sulfoxide (DMSO) preserve tissue viability. Although, Alkema et al., (2015) shows that

cryopreserved tumor tissue achieved 94% of viability with serum and DMSO, compared to 91% using fresh tumor tissue.

While the freezing process affects the metabolic rate of cells (Walsh et al., 2016), the hypothermic preservation can be an efficient alternative to cryopreservation, for example during shipment of the cells. During this short-term storage, cells are maintained at low temperatures between 4º and 10ºC, which can affect cell physiology by reducing their metabolism, energy storage degradation and oxygen demand (Correia et al., 2016).

It is important to have a preservation solution that was carefully formulated to provide the optimal concentration of ions and impermeable molecules to maintain ionic and osmotic balance, preventing the formation of free radicals, inhibit acidosis and prevent cell swelling at low temperatures, therefore facilitating the preservation of cell homeostasis.

Commercially available solutions include HypoThermosol (HT), that is an optimized hypothermic preservation solution enabling improved and extended preservation of cells, tissues and organs. HT consists of key ions at concentrations that balance the intracellular state at hypothermic temperatures and also additional components, such as pH buffers, energy substrates, free radical scavengers and osmotic/oncotic stabilizers.

Correia et al., (2016) used HypoThermosol solution for hypothermic storage at 4ºC of cardiomyocytes and reported high levels (> 70%) of cell viability 3 days after thawing.

I.4. Ovarian Cancer

Ovarian cancer is an aggressive tumor in the female genital tract and is the leading cause of death of gynecological cancer in developed countries; it is usually one of the most frequent causes of fatal malignancy in female patients, despite advances in treatment. It was estimated that nearly 240,000 women in Europe were diagnosed with ovarian cancer in 2012 (Ferlay et al., 2013).

Ovarian cancer presumably originates from the ovarian surface epithelium and/or fallopian tube. Most of the patients do not show specific symptoms, making it difficult to detect early. For this reason, most patients are diagnosed at an advanced stage and with tumor masses in the abdomen beyond the pelvis, contributing to the poor prognosis of the disease (Koshiyama, 2017; Worzfeld et al., 2017).

Ovarian cancer is also characterized by a frequent acquisition of chemoresistance. This behavior strongly contributes for tumor relapse and poor prognosis (Thibault et al., 2014).

I.4.1. Types of cancer ovarian

Recently, the characteristics of the various subtypes of ovarian cancer have been uncovered through histopathological, molecular and genetic studies. Shih and Kurman (Koshiyama, 2017) proposed to classify epithelial ovarian cancers according to their genetic

alterations and tumorigenesis pathways. Based on this, there are two types of ovarian cancer: Type I carcinomas, which includes endometrioid, clear cell and mucinous are often early diagnosed, slow-growing, indolent neoplasms that may arise from a precursor lesion and resistant to conventional chemotherapies but may respond to hormonal treatment. Type II carcinomas are frequently diagnosed at a late stage. They are aggressive and very unstable neoplasms that may develop *de novo* from serous intraepithelial carcinomas and/or ovarian surface epithelium and respond to conventional chemotherapies (Koshiyama, 2017; Thibault et al., 2014).

High-grade serous tumors are the predominant subtype and present the worst prognosis. They are characterized by TP53 gene mutations and a high Ki67 proliferation index. Other subtypes, like endometrioid and clear cell tumors, arise from endometriosis, which they are influenced by the microenvironment in the precursors. Type I mucinous tumors are metastases coming from gastrointestinal cancer (Alkema et al., 2015; Koshiyama, 2017; Thibault et al., 2014).

In terms of distinct morphological features (figure I.2), which reflect differences in gene expression and clinical characteristics, serous carcinoma would include finger-like structures that consist of central stroma cores giving rise to smaller branches lined by a malignant epithelium with minimal cytoplasm and very large, high-grade, nuclei (Ince et al., 2015). On the other hand, due to its similarity to the endometrium, endometrioid carcinomas have glands that are organized around central lumina surrounded by elongated malignant epithelial cells with abundant cytoplasm (Ince et al., 2015). The formation of microcysts, glands and/or papillae that are lined with cells with abundant cytoplasm leads to a distinct subtype, clear cell carcinoma, which appears clear in hematoxylin and eosin stains due to excess of cytoplasmic glycogen. Instead of glycogens, mucinous carcinoma has high levels of mucin in their cytoplasm, which is a glycosylated protein (Ince et al., 2015).

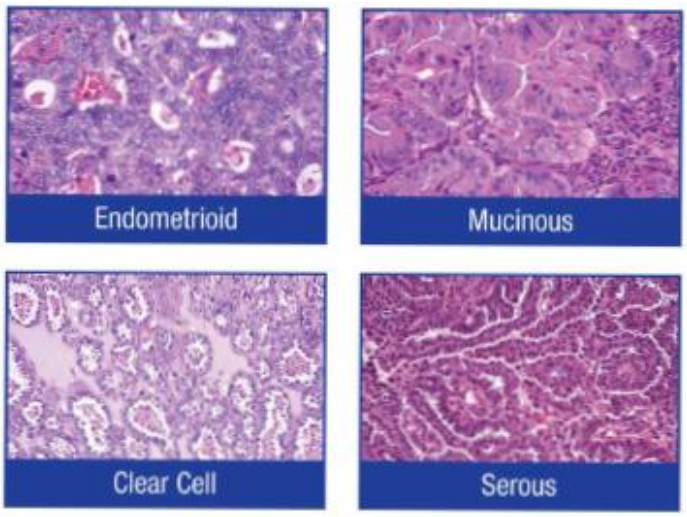

Figure I.2. Representative examples of the main types of ovarian carcinoma stained with hematoxylin and eosin (H&E). Endometrioid, mucinous, clear cell and serous carcinoma. Scheme adapted from Jones & Drapkin (Drapkin, Jones, & Drapkin, 2014)

I.4.2. Features of ovarian tumor microenvironment

Ovarian tumors are composed by a mixture of epithelial, stromal, immune and endothelial cells and are characterized by a unique tumor microenvironment. Its particular properties enable specific and efficient metastatic routes, impairing immune surveillance and mediating therapy resistance (E Lengyel, 2015; Thibault et al., 2014; Worzfeld et al., 2017).

Indeed, tumor cells separate from the ovary and/or fallopian tube and are transported through the peritoneal fluid to the surfaces of the organs in the abdominal cavity, where metastatic colonies grow (Balkwill et al., 2003).Subsequently, epithelial tumors, like ovarian carcinoma, are heterogeneous in cell population and macrophages and lymphocytes B and T are the most common immune cell types, but other cells are also present, such as natural killer (NK), fibroblasts, adipocytes and mesothelial cells (Lamichhane et al., 2016; Worzfeld et al., 2017). Cytokines and growth factors are also released by these cells to the tumor niche, which plays an important role in tumor growth and progression, cancer dissemination and immune escape (Worzfeld et al., 2017).

The stromal components present in the tumor microenvironment do not develop mutations or genetic aberrations as do tumor cells, leading to a possible and better target for therapy (Hansen, Coleman, & Sood, 2016).

I.4.3. Current therapies and alternatives

Treatment options against ovarian cancer are resection surgeries, chemotherapy, and in some cases immunotherapy. An early detection, accurate diagnosis and effective treatment are crucial for increasing ovarian cancer survival.

The use of platinum drugs, namely carboplatin and a taxane, usually paclitaxel, either in combination or single therapies is the gold standard for optimal chemotherapeutic response (Lopes-Coelho et al., 2016). The cisplatin was modified with a cyclobutane bicarboxylic acid replacing two chlorine atoms as the leaving groups, originating carboplatin, leading to a decreased toxicity. The ability of cancer cells to respond to a chemotherapeutic agent is believed to be due to its apoptotic capacity. Despite modern chemotherapy, most women with advanced ovarian cancer will relapse (Balkwill et al., 2003).

In general, ovarian cancer cells are sensitive to chemotherapy once the treatment above described has initiated and then they become chemoresistant either during the treatment or at various times following therapy, due to acquisition of several mutational events in epithelial cells for the formation of chemoresistant tumors, which could be responsible for any recurrences following treatment (Thibault, Castells, Delord, & Couderc, 2014).

Therefore, intrinsic and acquired resistance to platinum-based therapy is a major obstacle in the treatment of patients with epithelial ovarian cancer (Alkema et al., 2015). High-grade serous carcinoma acquires resistance due to cyclic exposure to chemotherapy and clear cell is intrinsically resistant, leading to an incurable disease (Lopes-Coelho et al., 2016).

Whereas ovarian cancer is a very aggressive tumor of the female genital tract, one reason any chemotherapy drugs that have not been effective in patients with this disease might be the unusual metastatic pattern, which rarely metastasizes hematogenously (Kenny et al., 2015).

Therefore, new treatments are currently under evaluation. Immunotherapy is a field of targeted therapy and there is a need to identify specific targets so that the host response can be optimized by an immunotherapeutic manipulation. Women with ovarian cancer have inefficient immune responses. So, it is important to keep immune cells in explant cultures and maintain the interactions with tumor and other stroma cells. The impact of the presence of immune cells in the tumor microenvironment on drug response also needs to be fully assessed (Balkwill, Jr, Berek, Gore, & Hamilton, 2003)

I.5. Thesis Aim

The main goal of this thesis was to develop a more physiological relevant ovarian cancer model which preserves tumor architecture and microenvironment, using patient-derived tumor explant cultures, for early and fast assessment of chemotherapy efficacy, after resection surgeries performed at *Instituto Português de Oncologia de Lisboa, Francisco Gentil* (IPOLFG).

To attain this, we established four specific objectives:

1. Establishment of ovarian carcinoma patient-derived explant (PDE) cultures by implementing a tissue processing and an explant culture methodology using dynamic cultures.
2. Characterization of PDE cultures and preservation of the histological, morphological and molecular features of original tumors.
3. Evaluation of the potential of PDE of ovarian carcinoma as feasible models for assessment of drug efficacy. This objective involved optimization of different read-outs of drug efficacy.
4. Implementation of storage conditions of the PDE cultures of ovarian carcinoma, exploring cryopreservation and cold storage protocols.

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II. MATERIALS AND METHODS

II.1. Sample collection and processing of tumor tissue

Fresh ovarian cancer specimens were collected from patients with signed informed consent that underwent surgery at Instituto Português de Oncologia de Lisboa, Francisco Gentil (IPOLFG).

A total of 11 specimens were collected and labelled OVC1 through OVC11, with the abbreviation from ovarian carcinoma (OVC) followed by the isolation number.

Tumor specimens were transported in culture medium from the surgery room at IPOLFG to iBET laboratories. Tumor samples were processed up to 5 hours after surgery, and then washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco) and weighed (AX224, Sartorius). Next, the tumor sample was immersed in Dulbecco's Modified Eagle Medium (DMEM) (41965-039, Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (10270-106, Gibco) and 1% PenStrep (P/S, penicillin and streptomycin) (15140-122, Gibco), and cut into fragments of approximately 1.0±0.5 mm^2 using two disposable sterile scalpels (0503, Swann-Morton) on a Petri dish (353003, Corning).

II.2. Establishment of patient-derived explant cultures

Patient-derived explant (PDE) cultures were placed in culture medium (DMEM) supplemented with 10% FBS and 1% P/S at a 5 PDE/mL concentration in Erlenmeyer cultures (Corning) with a total volume of 20 mL. PDE were cultured in an orbital shaker (IKA KS 260 basic) at 100 rpm placed in an incubator (Nuaire US Autoflow) at 37ºC with humified atmosphere containing 5% CO_2 for approximately 30 days. Culture medium was changed once a week (50% of the total volume). During change of culture medium, agitation was briefly stopped so that PDE could sediment and avoid the disposal of tumor fragments. PDE were collected at the day of isolation, and at days 10, 20 and 30 for culture characterization.

II.3. Characterization of cell viability

II.3.1. Live/Dead assay

The cell plasma membrane integrity during culture was assessed using fluorescein diacetate (FDA, 1:1000 dilution) (Molecular Probes) to label live cells and propidium iodide (PI, 1:1000 dilution) (Molecular Probes) for dead cells. FDA is a non-polar and a non-fluorescent ester that is converted to highly fluorescent fluorescein by intracellular esterases. Fluorescein, being highly polar, becomes trapped within cells with intact membrane, measuring both their enzymatic activity and membrane integrity, and confers to them green fluorescence when excited by blue light. PI is a polar and a fluorescent molecule that can only enter cells with

damaged membrane, therefore nucleus of dying/dead cells stain red upon intercalation between DNA bases.

A small volume of PDE cultures was collected and incubated for 5 minutes at room temperature (RT) with a solution composed of FDA/PI probes in DPBS and then visualized using a fluorescence microscope (Leica DMI6000). Hence, with FDA/PI staining viable cells present green fluorescence, and non-viable cells present red fluorescence. Image analysis was performed with open access Image J Software.

II.3.2. Resazurin reduction capacity

Resazurin reduction capacity of cells present in the PDE was assessed using the indicator PrestoBlue Cell Viability Reagent (A13262, Invitrogen). The active ingredient of PrestoBlue reagent (resazurin) is a blue, non-toxic and non-fluorescent dye, that when in contact with a viable cell is reduced, becoming bright red fluorescent resorufin. Fluorescence can be measured with excitation wavelength 570 nm and emission wavelength 590 nm. The amount of fluorescence produced is proportional to the number of live and metabolically active cells.

A volume of 1 mL of culture was collected and the medium was dispensed, leaving only the PDE. 400 µL of fresh medium were added and incubated for 1 hour with 40 µL of PrestoBlue reagent, at 37ºC. After this, 150 µL were collected to a 96-well black fluorescence reading plate (Corning) and fluorescence reading was performed in an Infinite 200 PRO NanoQuant plate reader (TECAN) at 560/590 nm. The blank control consisted of fresh culture medium, incubated with the PrestoBlue Cell Viability reagent.

Resazurin reduction capacity of cells in PDE along culture was calculated and presented in % relative to the same culture condition at day 0, considering the dilution factor of the culture. Data is presented as mean ± SD from at least 3 replicates.

II.4. Characterization of cell death

II.4.1. LDH activity in culture supernatants

Lactate dehydrogenase (LDH) is an intracellular enzyme that is only accessible in the culture supernatant when the cell membrane is damaged; this assay is then useful to monitor cell death throughout culture. The LDH assay is based on the physiological role of the LDH enzyme. It is responsible for the pyruvate conversion into lactate, by NADH oxidation to NAD+, as indicated in the equation 1:

$$\text{Piruvate} + \text{NADH} + H^+ \xleftrightarrow{\text{LDH}} \text{L-Lactate} + NAD^+ \qquad \text{(Equation)}$$

The decrease in NADH concentration was determined spectrophotometrically at 340 nm, following the rate of oxidation of NADH to NAD+ coupled with the reduction of pyruvate to

lactate. U represents the µmol of NADH consumed in reaction *per* minute, and when normalized by the volume of culture, gives rise to LDH activity (U/mL).

Preliminary data showed that the LDH is a stable enzyme in the cell culture supernatant at 37ºC for 7 days. Thus, the cumulative value of LDH (LDHcum) was estimated for each timepoint and time interval, respectively.

A volume of 1 mL of culture was collected and centrifuged (Eppendorf) at 1000g for 5 minutes. 20 µL of the culture supernatant were dispensed into each well of the plate, together with 234 µL of NADH (Nicotinamide adenine dinucleotide reduced) (Sigma-Aldrich). Absorbance reading was performed in an Infinite 200 PRO NanoQuant plate reader (TECAN) at 340 nm and 37ºC. Then, 46 µL of sodium pyruvate (Sigma-Aldrich) were added and absorbance was measured again. The blank control consisted of fresh culture medium.

II.5. Morphological characterization

II.5.1. PDE surface area

Analysis of PDE surface area was performed along the culture period, at days 0, 10, 20 and 30. Briefly, the PDE collected for assessment of resazurin reduction capacity were observed by phase contrast microscopy. The dimensions were measured using ImageJ software by manually defining the fragment boundaries for automatic calculation of area. Data is presented as mean ± SD from at least 15 different PDE, one measurement per each PDE.

II.5.2. Measurements of PDE concentration

PDE concentration was calculated by sampling the culture at days 0, 10, 20 and 30. PDE concentration was counted by phase contrast microscopy and concentration measured using ImageJ software by manually defining the fragments and counting them, considering the dilution factor of the culture. Data is presented as mean ± SD from at least 3 technical replicates.

II.6. Immunohistochemistry analysis

A 1 mL volume of culture was collected, and medium was removed. This sample was fixed in 4% paraformaldehyde (PFA) (Sigma) and 4% sucrose in PBS for 1 hour at RT. Fixed samples were then sent to IPOLFG to be processed and to perform immunohistochemistry (IHC) analysis of proliferation and apoptosis of PDE cultures along culture time. Histopathological scoring was then performed by specialist clinicians, depending on the staining levels for Ki67 (proliferation) and cleaved caspase-3 (apoptosis). Classification not applicable (n.a.) indicates that a score could not be given due to insufficient amount of histological material

for quantitative analysis (PDE count<5), or that the collected PDE for analysis were depleted of epithelial cells and were mostly composed of stromal cells.

Other immunohistochemistry characterization was performed, namely for the identification of immune cells in PDE cultures along time.

Processing, characterization and quantification of the samples was performed at IPOLFG and will be included in the thesis as relevant results for the completion of the proposed workplan. Full detailed protocols will not be listed as they were done in collaboration with partners from IPOLFG.

II.7. Drug treatment of PDE with chemotherapeutic agents

Two high serous ovarian carcinomas were chosen to perform and optimize *ex vivo* chemotherapy treatment (OVC6 and OVC7). PDE cultures with an initial concentration of 5 PDE/mL in 20 mL of culture medium were maintained in culture for 7 days prior to chemotherapy treatment, since during the first week PDE cultures go through a period of culture adaptation. During this period, after tissue processing PDE become more homogenous in size while simultaneously remaining representative of the original tumor. PDE cultures were then incubated with standard of care chemotherapeutic agents, carboplatin (25 µg/mL), paclitaxel (10 µg/mL) and a combination of both, aiming to optimize read-outs of drug efficacy.

The cyclic chemotherapy treatments were performed by exposing PDE cultures to 24-hour cycles of carboplatin, paclitaxel and a combination of both drugs, once a week, with complete exchange of culture medium after the 24-hour cycle, mimicking the chemotherapy cycles of the patient treatment regimen. PDE were submitted to at least 2 cycles of chemotherapy exposure.

Evaluation of *ex vivo* chemotherapy efficacy was assessed along the culture with timepoints taken before and after treatment for evaluation of resazurin reduction capacity, LDH activity in the supernatant, live/dead staining, PDE area, concentration and morphology, proliferation and apoptosis status by immunohistochemistry. PDE were exposed to standard of care chemotherapy drug once a week, for 24 hours (exposure between day 0 and 1 and day 7 and 8 of treatment). Cultures were sampled at days 0, 1, 7 and 8 of treatment and incubated with FDA (green, live) and PI (red, dead) (see figure III.1).

II.8. Cryopreservation and cold storage methods for preservation of PDE

II.8.1. Cryopreservation methodologies

At the day of tumor processing, PDE were collected and distributed into several cryovials for cryopreservation assays, using either 1 mL of CryoStor10 solution (Sigma) or standard cell cryosolution of FBS with 10% DMSO (Dimethyl Sulfoxide) (Sigma). Samples were

frozen to -80ºC in an isopropanol-based freezing system, ("Mr. Frosty", Nalgene) at a rate of cooling very close to 1ºC *per* minute, and stored in -80ºC until thawing.

After 1 to 4 weeks, PDE of OVC6 and OVC10 were thawed and cultured following the same experimental set-up as the control culture for approximately 30 days.

The characterization of the cultures and the timepoints were the same of the control culture, i.e. the non-thawed PDE.

II.8.2. Cold storage methodologies

HypoThermosol (BioLife Solutions) is a hypothermic preservation medium that allows improved and extended preservation of cells, tissue or organs. This reagent is formulated to address the molecular-biological response of cells during this preservation process. It is composed of essential ions at concentrations that balance the intracellular state at low temperatures.

At the day of tumor processing, to mimic the normal features of culture, around 100 PDE were collected and distributed into eppendorfs for cold storage assays, using either 1 mL of HypoThermosol solution or regular culture medium (DMEM) and placed at 4ºC for 7 days. After this, PDE were cultured up to 30 days, under agitation-based conditions at 37ºC, in Erlenmeyers with 20 mL of DMEM culture medium, following the regular experimental set-up.

Cultures were then characterized for cell viability, proliferation and apoptosis, and compared with the control PDE samples.

Immunohistochemistry and scoring of proliferation and apoptosis were performed by clinicians specialized in gynecological malignancies, as previously described in section II.6.

II.9. Statistical analysis

The one-way ANOVA statistical test or two-yay ANOVA statistical test, followed by the Tukey's multiple comparison test, were used for comparisons between more than two groups. Data are shown as mean ± standard deviation of the mean. Statistical analysis was carried out using GraphPad Prism 6 software.

III. RESULTS AND DISCUSSION

The aim of this work was to generate an "*ex vivo*" ovarian cancer cell model that could better mimic the *in vivo* tumor microenvironment and heterogeneity, by using a patient-derived explant (PDE) culture strategy. The main goals of this thesis were addressed by pursuing three main tasks:

(1) Culture and characterization of PDE of ovarian carcinoma in agitation-based cultures and characterization of cell viability and proliferation, and preservation of tumor microenvironment features:
   (i) optimization of tumor processing methodology;
   (ii) culture and characterization of PDE of ovarian carcinoma;

(2) Performance of repeated *ex vivo* drug treatment and evaluation of drug efficacy;

(3) Optimization of protocols for cryopreservation and cold-storage of PDE cultures of ovarian carcinoma.

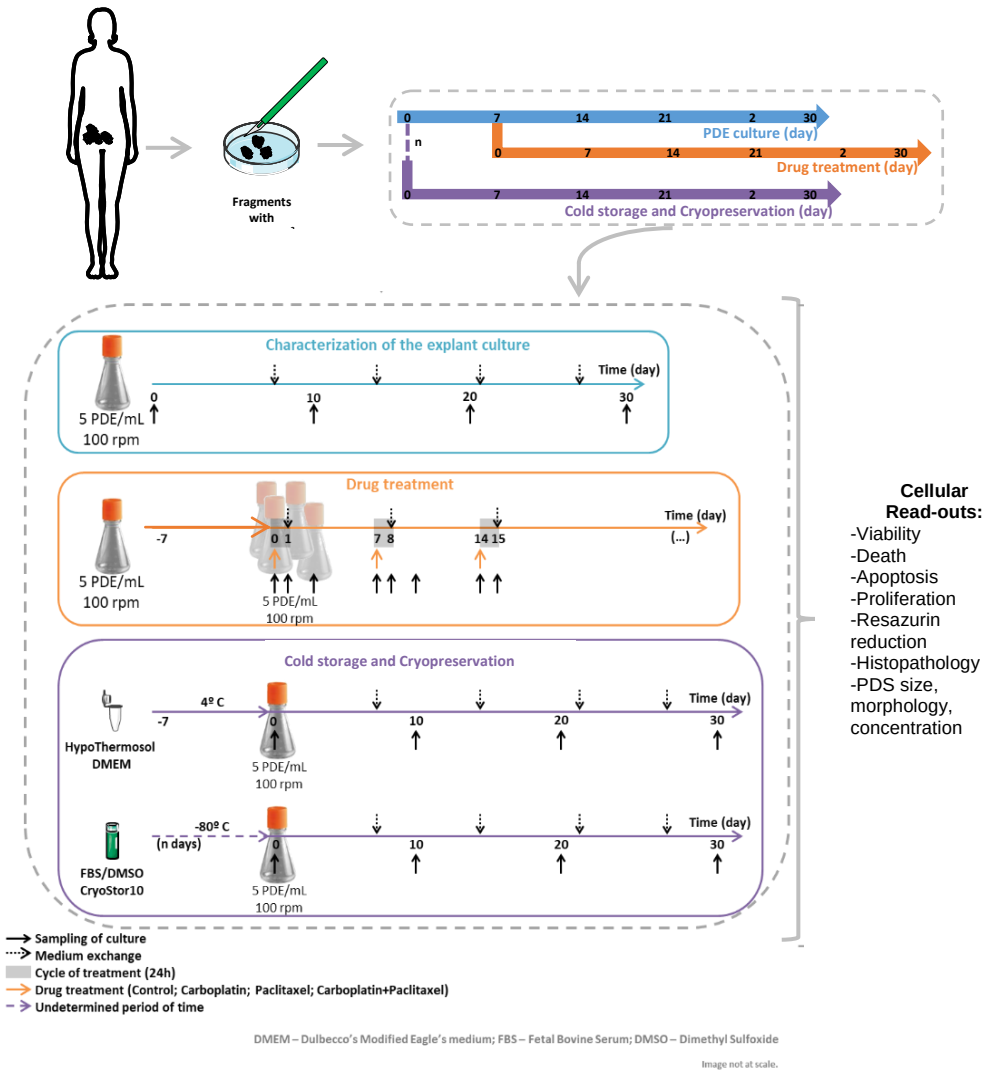


Figure III.1. Schematic representation of the strategy pursued to establish long-term PDE cultures from ovarian carcinoma. Tumor samples collected from IPOLFG were cut into fragments with approximately 1±0.5 mm^3 of volume, denominated Patient-derived Explants (PDE). PDE were cultured for

up to 30 days in agitation-based conditions, with culture media exchange once a week and frequent sampling for culture monitoring. Cultures were also exposed to a cyclic chemotherapy treatment (carboplatin, paclitaxel and combination of both) for drug efficacy and optimization of read-outs. Validation of protocols of cryopreservation and cold storage were also performed using different commercially-available solutions.

III.1. Culture and characterization of PDE of ovarian carcinoma

III.1.1. Optimization of tumor processing methodology

The main goal of the thesis comprised the culture of patient-derived ovarian cancer samples, aiming to preserve tissue architecture, microenvironment composition, and phenotype. To attain this, we used agitation-based culture systems to culture PDE (obtained after mechanical dissociation of tumor fragments collected from surgical specimens of the patient). We hypothesized that by culturing tumor explants under dynamic conditions, we could avoid diffusional limitations and contribute towards the preservation of cell viability and tumor phenotype. The samples of ovarian cancer were collected and fragmented as described in the section of “Materials and Methods” of this thesis. Culture of PDE (typically at a starting fragment initial concentration of 5 PDE/mL) was followed for 30 days, with continuous monitoring of culture progression at days 10, 20 and 30.

During the timeframe of this thesis, 11 samples were obtained from surgical resections of ovarian cancer patients treated at IPOLFG. Sample name was sequentially attributed by isolation date, which the abbreviation from ovarian carcinoma (OVC) followed by the isolation number. Different subtypes of carcinoma were processed: 5 were high-grade and 1 low-grade serous carcinomas, 1 mucinous borderline tumor, 1 endometrioid, 1 clear-cell carcinoma, 1 undifferentiated and 1 carcinosarcoma, with sampled epithelial component of high-grade serous carcinoma architecture, as indicated in table III.1. Two of the samples, OVC1 and OVC8 were isolated from patients who were submitted to chemotherapy treatment prior to surgical resection.

Table III.1 depicts a detailed characterization of each sample received from IPOLFG.

Table III.1. Clinical annotation and tissue processing characteristics of ovarian cancer samples received from IPOLFG.

Case reference	Histological sub-type	Chemotherapy prior to surgery (Y/N)	Weight (g)	Fragment number at isolation	Maximum time of culture (Day)	PDE Drug treatment (Y/N; number of cycles)	Cryopreservation of PDE at day of isolation (Y/N; cryosolution)	Cold storage of PDE at day of isolation (Y/N; cold storage solution)
OVC1	High grade Serous carcinoma	Y	0.10	60-70	28	N	N	N
OVC2	Mucinous borderline tumor	N	1.8	~150	40	N	N	N
OVC3	High grade Serous carcinoma	N	0.66	~120	30	N	N	N
OVC4	Clear cell carcinoma	N	1.34	~200	30	N	N	N
OVC5	High grade Serous carcinoma	N	0.90	~400	28	N	N	N
OVC6	Carcinosarcoma Epithelial component sampled - high grade serous carcinoma	N	3.55	~700 (+600)	30	Y; 2 cycles	Y; FBS/DMSO (600 PDE)	N
OVC7	High grade Serous carcinoma	N	0.55	~430	21	Y; 3 cycles	N	N
OVC8	High grade Serous carcinoma	Y	1.37	~800	30	N	Y; FBS/DMSO (120 PDE) and Cryostor10 (120 PDE)	Y; HT (100 PDE)
OVC9	Endometrioid carcinoma	N	1.06	~710	30	N	Y; FBS/DMSO (200 PDE) and Cryostor10 (280 PDE)	Y; HT (100 PDE)
OVC10	Undifferentiated	N	0.85	~875	30	N	Y; FBS/DMSO (160 PDE) and Cryostor10 (360 PDE)	Y; HT (100 PDE) and DMEM (100 PDE)
OVC11	Low grade Serous carcinoma	N	0.66	~375	30	N	N	N

Tumor samples were processed up to 5 hours after surgery. During this period the tumor specimens were kept in culture medium. Then, tumor specimens were mechanically dissociated into fragments of approximately 1 mm^3 of volume.

We managed to isolate and establish PDE cultures from all the collected ovarian carcinoma samples. The duration of the culture was dependent on the amount of initial material, drug testing and characterization assays that were performed for each case. For the first cases, the entire sample was dedicated to the characterization of culture progression and to understand whether PDE cultures could preserve cell viability for up to one month of culture, while retaining the tumor heterogeneous architecture and microenvironment. Explant sample collection and read-outs were optimized, and an increasing amount of material per isolation was observed. OVC7 was the exception for not having enough material to analyze during 30 days of culture.

Patient-derived material was obtained from IPOLFG, following the ethical guidelines and preservation of patient data and confidentiality previously approved by the ethical committee at the hospital. All the samples used for the research activities of this project were obtained after signed consents from the patients.

Figure III.2 depicts experimental details regarding tissue processing and the obtained tumor fragments.

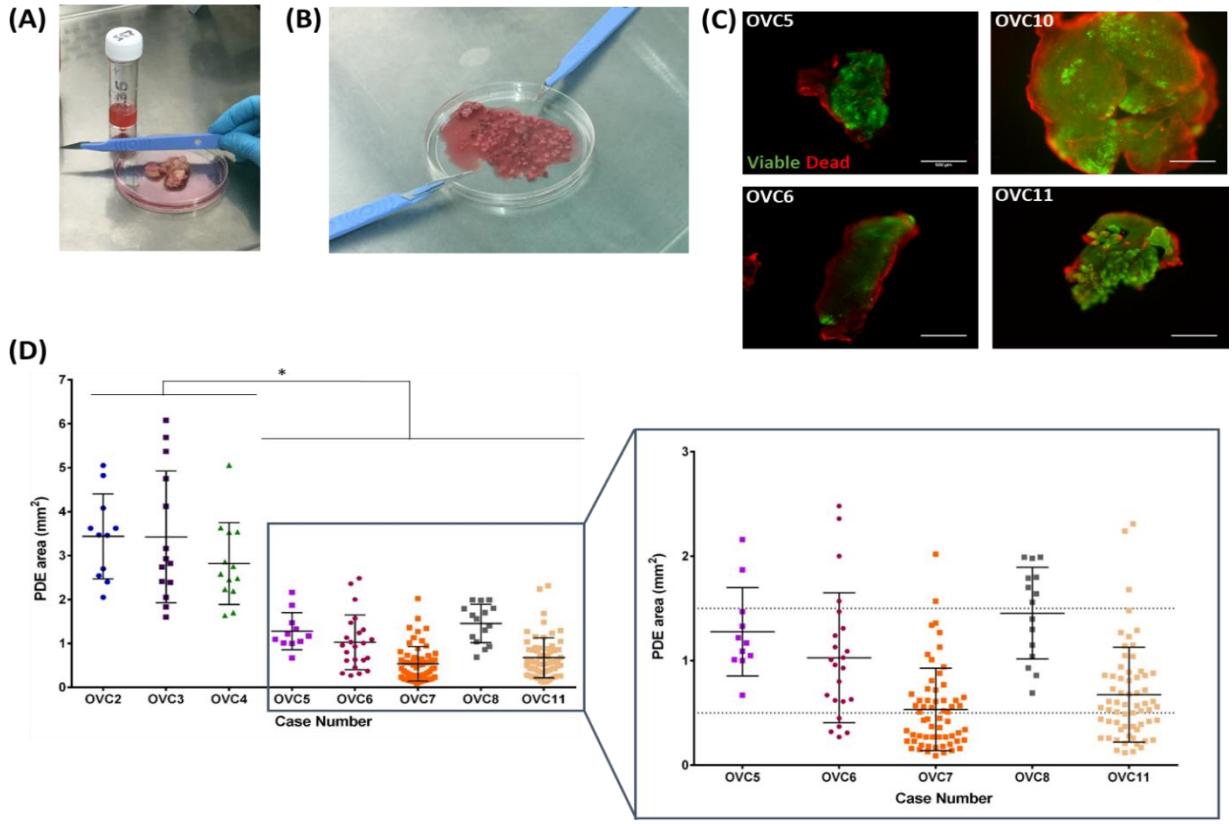


Figure III.2. Processing of ovarian carcinoma samples for the generation of PDE cultures. (A) Macroscopic image of a tumor sample obtained after surgical resection of an ovarian carcinoma (OVC6) before mechanical

dissociation; **(B)** Macroscopic image of the tumor sample after fragmentation; **(C)** Representative images of live/dead fluorescent assay of PDE from ovarian carcinoma, after tissue processing (day 0 of culture): PDE were incubated with fluorescein diacetate (FDA, green) for live cells and propidium iodide (PI, red) for the dead cells. Scale bars represent 500 µm; **(D)** Measurement of PDE surface area at day 0. The figure insert is the result of the cases processed after implementation of the optimized protocol. Data is presented as mean ± SD from at least 10 PDE. One-way ANOVA statistics test was applied to compare the average surface area of the tumor explant for each case of ovarian carcinoma. * ($p<0.001$) represents a statistically significant difference between PDE areas of earlier cases and the five most recent serous cancer cases. Statistical analysis was carried out using GraphPad Prism 6 software.

Figure III.2A is a representative image of the ovarian tumor sample supplied after surgery in the IPOLFG, prior to tissue processing.

Figure III.2B shows the patient-derived tissue after sectioning. Samples from serous subtypes have always been easier to cut, leading to PDE microscopically more compact. On the other hand, the samples diagnosed with the mucinous subtype were difficult to cut, forming a thread-like structure between the fragments, probably due to the excess of mucus, creating more irregular and sticky fragments.

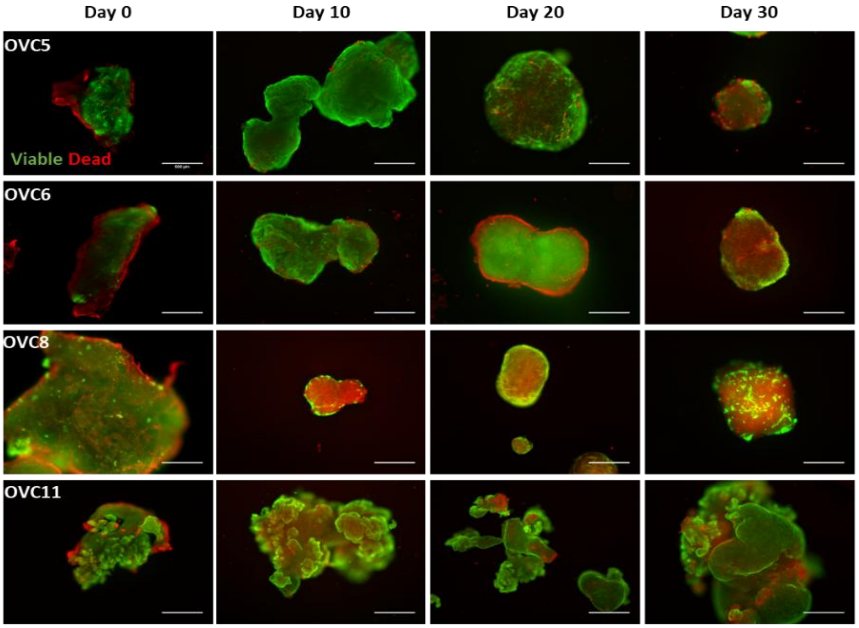
Live/dead assays were performed in the PDE cultures after tumor fragmentation, and representative results on four different cases are presented in figure III.2C. FDA/PI dual staining was used to evaluate cell viability during culture time using fluorescence microscopy, with a protocol described in Materials and Methods (section II.3.1.). At the day of isolation, most of the tissue remained viable after manipulation but a layer of dead cells was visible on the surface of the PDE, as a consequence of PDE mechanical processing.

Measurements of PDE surface area at day 0 of culture revealed an optimization of the cutting procedure in the last cases, especially in the serous ones, as indicated in figure III.2D. All the samples presented a heterogeneous distribution of fragment areas at day 0, however after the optimization period, PDE were consistently smaller and presented average PDE areas ranging from $1\pm0.5 mm^2$ (insert of figure III.2D).

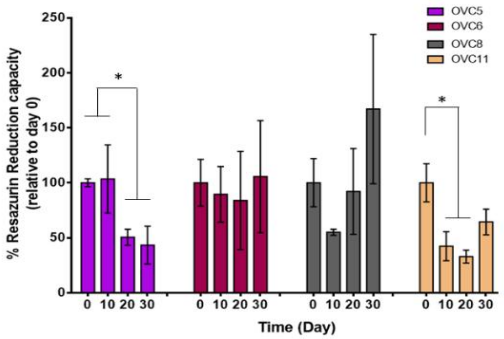
III.1.2. Culture and characterization of PDE of ovarian carcinoma

After isolation, tumor fragments were placed in cultured, in agitation-based culture conditions, for up to 30 days, as indicated in the Materials and Methods (section II.2.). Analysis of the culture was performed to evaluate cell viability, proliferation and apoptosis. Results are shown in figure III.3.

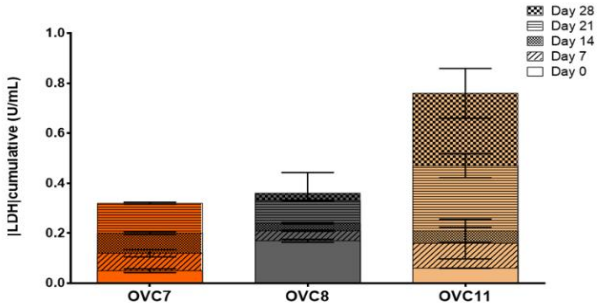
(A)



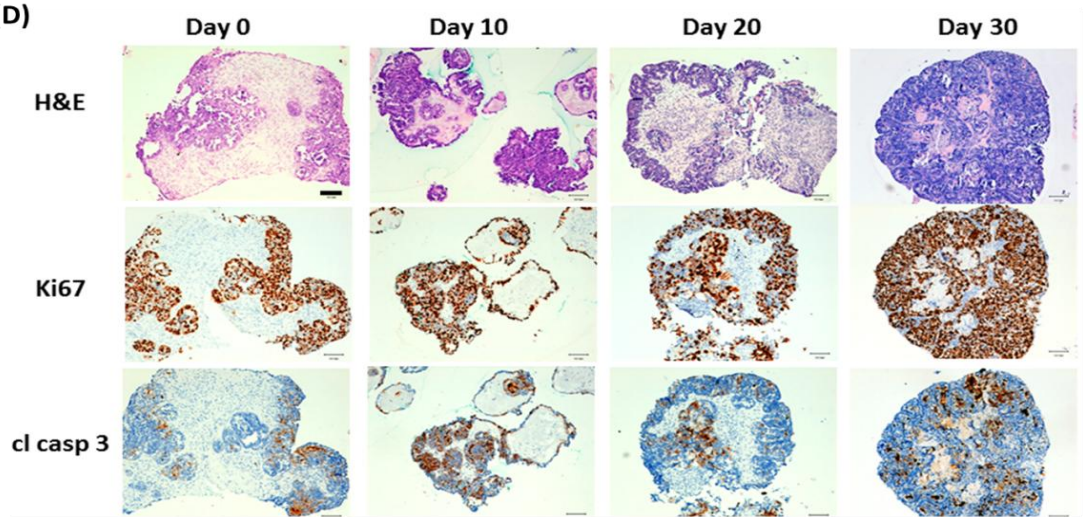
(B)



(C)



(D)



(E)

		Culture Progression			
		d0	d10	d20	d30
Ki67 (%)	OVC5	25-50	>75	50-75	na
	OVC6	>75	75-100	50-75	75
	OVC8	50-75	50-75	na	na
	OVC11	5	1	1	na
Cl Casp 3 (%)	OVC5	0	5-25	<5	na
	OVC6	10	25-50	<5	<5
	OVC8	0	5-25	na	na
	OVC11	<5	5	<5	na

Figure III.3. PDE cultures from ovarian carcinoma can be preserved under agitation conditions for up to 30 days of culture with preservation of cell viability and proliferation, and low levels of cell death. (A) Representative images of live/dead fluorescent assay of PDE cultures of serous ovarian carcinoma. Samples were collected along the culture period and incubated with FDA (green, live) and PI (red, dead). Scale bars represent 500 µm. **(B)** Measurements of resazurin reduction capacity (in %) of the PDE along culture time relative to day 0. Data is presented as mean ± SD from at least 3 replicates. Two-way ANOVA statistical test (Tukey's multiple comparison test) was applied to compare the mean values of resazurin reduction capacity along culture period. * ($p<0.05$) represents a statistically significant difference; **(C)** Quantification of cumulative LDH activity in the culture supernatants of PDE cultures. Data is presented as cumulative mean ± SD from at least 2 technical replicates. **(D)** Haematoxylin & Eosin (H&E) staining and immunohistochemistry analysis for proliferation (Ki67+ cells) and apoptosis (cleaved caspase 3+ cells) of PDE cross-sections from OVC6, maintained in agitation-based conditions and collected at days 0, 10, 20 and 30. Scale bars represent 100 µm. **(E)** Pathological score-based analysis of cell proliferation and apoptosis in PDE cultures from serous ovarian carcinomas. Statistical analysis was carried out using GraphPad Prism 6 software.

During the 30 days of culture, PDE were composed mainly by viable cells (green). Some cell death (red) was also identified, to a lower extent throughout the culture (figure III.3A). Cell death typically observed at day 0 in the superficial layer of the PDE disappeared with culture time. Ratios of live/dead cells also seemed to be reduced for most cases at day 30 in comparison with previous culture time points, particularly for high-grade serous carcinoma samples (OVC5, OVC6, OVC8). These same cases also showed a reduction of fragment size and an acquisition of a rounder shape. OVC11, a low grade serous carcinoma, showed a distinct behavior, with higher cell viability throughout cultures and a more irregular shape that was not altered during culture.

Resazurin reduction capacity was also determined along the culture time as an indication of cell metabolic activity. The values of resazurin reduction capacity relative to day 0 (figure III.3B) were coherent with live/dead assays. After 30 days of culture, PDE cultures presented a similar capacity to reduce resazurin in comparison with the PDE cultures immediately after tumor processing (day 0 of culture). The only exception was OVC5.

The quantification of LDH activity in the supernatant of the culture medium was also evaluated as a read-out of cell death for some isolated cases (OVC7, OVC8 and OVC11), as shown in figure III.3C. OVC7 culture was terminated by day 21, as mentioned before, due to total explant usage for other characterization read-outs, while OVC8 and OVC11 were cultured until day 28. The 3 cases showed an increasingly cumulative LDH activity in the culture medium over time. OVC8 showed apparent higher LDH values at day 0, which could be related with the chemotherapy treatment applied to the patient prior to surgical resection of the tumor. Overall, OVC11 presented the highest level of cumulative LDH activity in culture supernatants, which could be associated to its low-grade serous carcinoma sub-type. These observations are still limited by the low number of cases assessed. These possible correlations of LDH activity with tumor subtype and chemotherapy treatment prior to surgical resection must be confirmed in the future with a higher subset of cases.

Figure III.3D shows proliferation and apoptosis status by immunohistochemistry for Ki67 and cleaved caspase 3 and figure III.3E lists the quantification obtained by pathological analysis. For the OVC5, OVC6 and OVC8 (all high grade serous carcinoma), proliferation levels of PDE cultures remained high in culture for several weeks (>50%). The percentage of Ki67+ cells along time correlated well with the levels detected in the PDE at day 0. It was also found that up to day 10 of culture, there is an increase

in cell apoptosis levels (detected within a range of 5 to 50%) and a stagnation after that, suggesting that there is an adaptation period of the culture. There seems to be a balance between proliferation and apoptosis, which was demonstrated by the levels of cell viability detected in figure III.3A and resazurin reduction capacity in figure III.3B. Even so, overall cell viability of the tumor explants was not significantly altered, probably due to the high proliferation levels. On the other hand, OVC11 was representative of low grade serous carcinoma and presented different proliferation and apoptosis characteristics. As predictable for this sub-type, the levels of Ki67+ and cleaved caspase 3+ cells were low and never exceeded 5% of the epithelial cells during the culture period, preserving the values measured for the original tumor.

The size of the PDE cultures was also monitored along time (figure III.4).

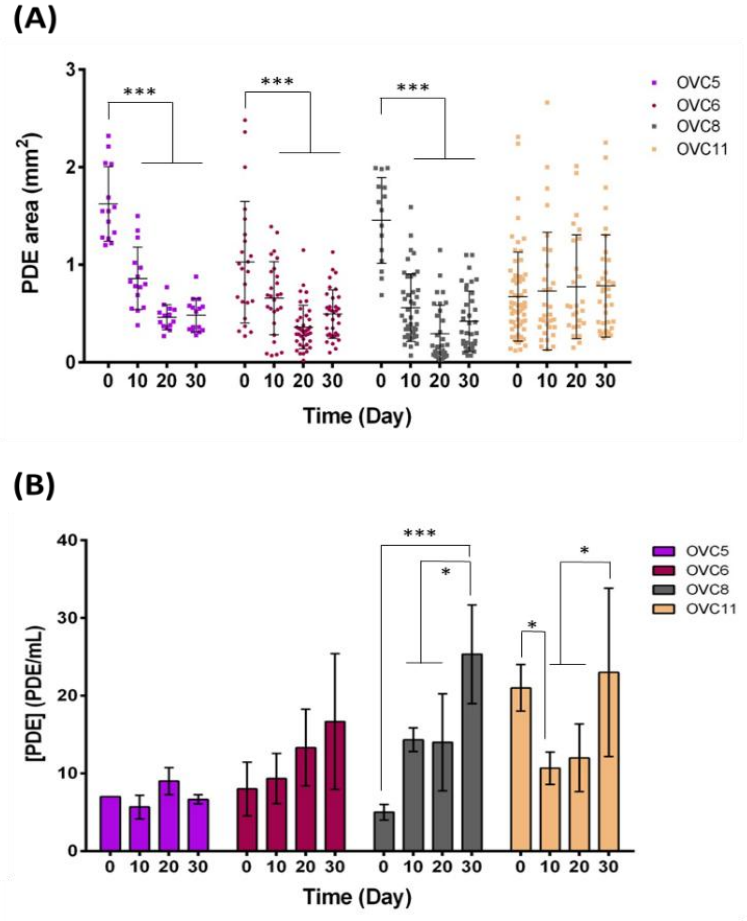


Figure III.4. Morphological characterization and fragment concentration dynamics of PDE cultures of serous ovarian carcinoma. (A) Measurement of PDE surface area of cases OVC5, OVC6, OVC8 and OVC11 from the day of tumor processing up to 30 days of culture. PDE were imaged with the bright field mode in an inverted fluorescence microscope and PDE area was measured by adjusting the threshold until the border of each PDE, which was then quantified using the area measurement algorithm from ImageJ open source software. Data is presented as mean ± SD from at least 15 different PDE. Two-way ANOVA statistical test (Tukey's multiple comparison test) was applied to compare the mean values of PDE size along culture period. *** ($p<0.001$) represents a statistically significant difference; **(B)** Measurement of PDE concentration of cases OVC5, OVC6, OVC8 and OVC11 from the day of tumor processing up to 30 days of culture. Data is presented as mean ± SD from at least 3 technical replicates. Two-way ANOVA statistical test (Tukey's multiple comparison test) was applied to compare the mean values of PDE concentration along culture period. * ($p<0.05$) and *** ($p<0.001$) represents a statistically significant difference. Statistical analysis was carried out using GraphPad Prism 6 software

The determination of the surface area of the explants for up to 30 days provides an indication of PDE size and is important to understand the dynamics and behavior of the culture. Potentially, PDE size can provide clues on cell proliferation/death and efficacy of drug treatment. Size of PDE can also have an impact on drug diffusion. At day 0, PDE were typically more heterogeneous, with higher dispersion of surface area measurements. The average surface area was also higher than 1 mm^2 for the high serous carcinoma cases (OVC5, OVC6, OVC8). Cultures from this subtype presented significantly decreased areas at day 10 and a more homogeneous size dispersion. This culture feature was preserved with no significant changes until day 30. OVC11 was a low grade serous carcinoma and showed a distinct behavior. Surface areas remained constant over time and cultures did not become more homogeneous.

Figure III.4B depicts culture dynamics, focusing on the evolution of PDE concentration with time. Despite some visible oscillations in most of the cultures, only OVC8 showed a statistically significant increase in PDE concentration from the starting to the end-point of culture (absolute value 23 ± 10 PDE/mL).

Altogether, by combining area measurements with PDE concentration and live/dead staining, the tendency of decreasing PDE surface area during the first day of culture (day 0 to day 10) is justified by the shedding of the layer of dead cells resultant from tissue processing. Once PDE stabilize as an adaptation their new environment, cultures do not undergo significant changes.

PDE cultures of ovarian carcinoma were evaluated for their ability to maintain the histopathological features of the original tumors from which they were derived at the anatomical pathology unit at IPOLFG. They performed an analysis of sequential series of cross-sections of individual PDE, showing that the culture of PDE in agitation-based culture systems does not lead to the formation of necrotic cores or specific gradient of cells (figure III.5).

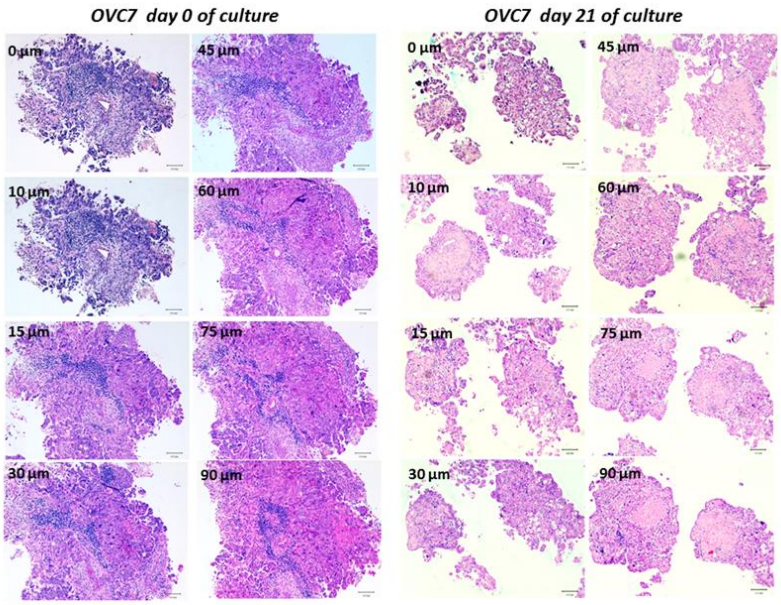


Figure III.5. Histological characterization of PDE cultures for identification of PDE morphology and intra-PDE heterogeneity. H&E staining of cross-sections at different depths of representative PDE cultures of high grade serous carcinoma (OVC7) collected at day 0 and day 21 of culture. Scale bars represent 100 µm.

The phenotype and morphology of each individual PDE was similar regardless of the depth of the cross-section and heterogeneity of tumor microenvironment preserved after 21 days of culture. Hence, ovarian cancer tumor architecture and heterogeneity was preserved in ovarian PDE.

Concerning the preservation of tumor microenvironment components in PDE cultures, partners from IPOLFG performed immunohistochemistry analysis indicative of specific immune cell populations, as illustrated in figure III.6.

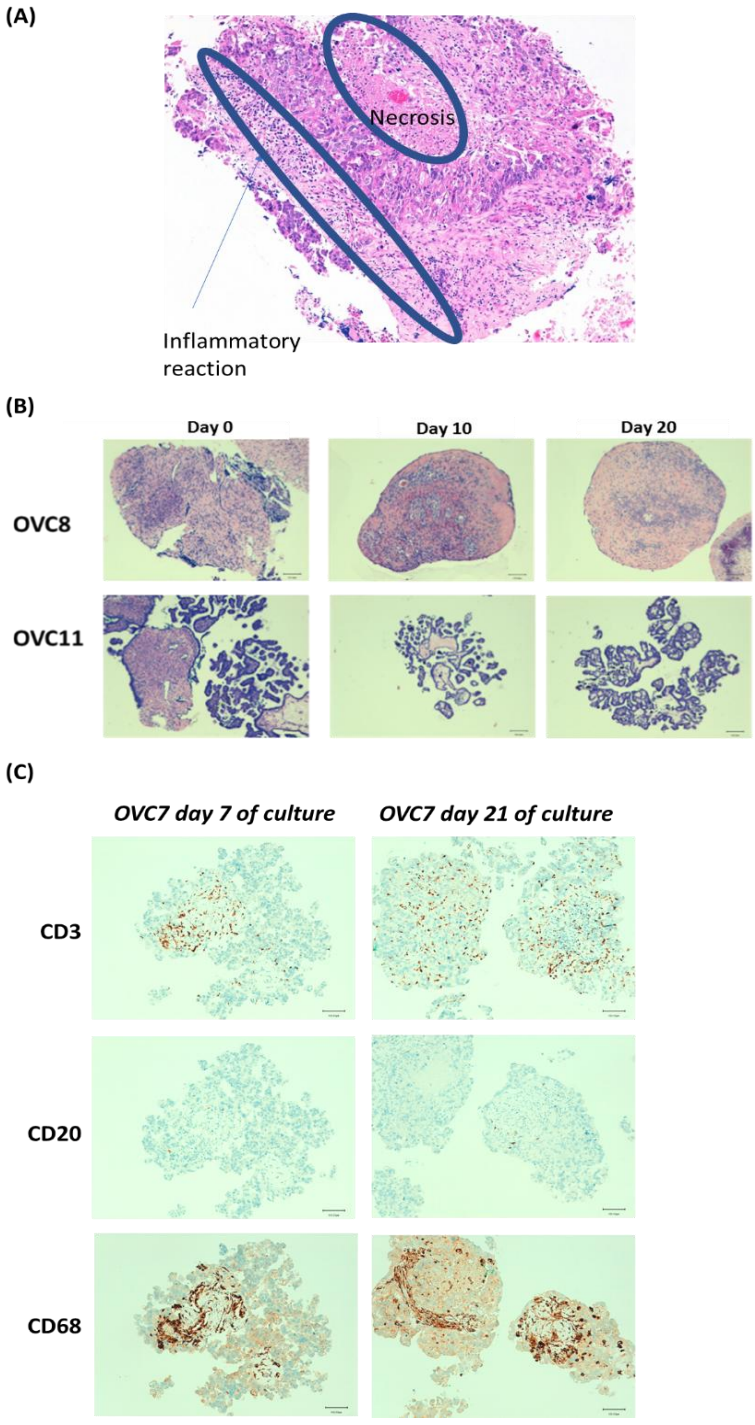


Figure III.6. Histological evaluation of cross-sections from PDE of high grade serous carcinoma (OVC7) cultured up to 21 days in agitation-based culture systems. (A) H&E staining of PDE at day 0 of culture (40x magnification). Necrosis and inflammatory reaction regions are highlighted; **(B)** H&E staining of representative PDE

cultures of high-grade serous ovarian carcinoma (OVC8) and low-grade serous ovarian carcinoma (OVC11) collected at days 0, 10 and 20. Scale bars represents 100 µm. **(C)** Immunohistochemistry analysis of cross-section of PDE cultures from OVC7 at day 7 and day 21. Immune markers (CD3 and CD20 for lymphocytes T and B, respectively and CD68 for macrophages). Scale bars represent 100 µm.

This particular tumor sample (OVC7) presented a quite heterogeneous microenvironment, with necrotic regions and a strong immune infiltrate. Figure III.6B represents the difference between high-grade serous (OVC8) and low-grade serous carcinoma (OVC11), which shows heterogeneity in the morphology and histological differences between two subtypes of ovarian cancer, along culture, but with preservation of epithelial and stromal components in both subtypes. In figure III.6C, after tissue processing and culturing of PDE up to 21 days, we could still detect high numbers of macrophages and lymphocytes T and B, indicating the maintenance of immune components. Histopathological evaluation of several cases has also demonstrated that the fibroblasts remained viable and blood vessels were preserved during 30 days of culture.

III.2. Performance of repeated *in vitro* chemotherapy cycles and evaluation of drug efficacy

We have performed chemotherapy tests using some of the established PDE cultures from high-grade serous carcinoma. Our aim was to evaluate the feasibility to use these PDE cultures as an *ex vivo* representative model for evaluation of drug efficacy in the most prevalent subtype of ovarian carcinoma. We performed cyclic chemotherapy treatment with carboplatin and paclitaxel and the combination of both in two high-grade serous carcinoma cases (OVC6 and OVC7) to validate quantification methods of *ex vivo* drug efficacy.

Figure III.7 shows the results obtained for drug treatment of ovarian carcinoma samples. Patients that undergo chemotherapy treatment for ovarian cancer are typically submitted to 3 cycles of combination therapy using carboplatin and paclitaxel. In our work, our goal was to mimic this therapeutic regimen and to implement repeated cycles of chemotherapy using the proposed *ex vivo* model that preserves microenvironment complexity. Optimization of read-out measurements of drug efficacy was also carried out.

We started the treatment at day 7 of culture as PDE already presented a stable and homogeneous size dispersion and levels of cell viability, proliferation and phenotype were comparable with the original tumor.

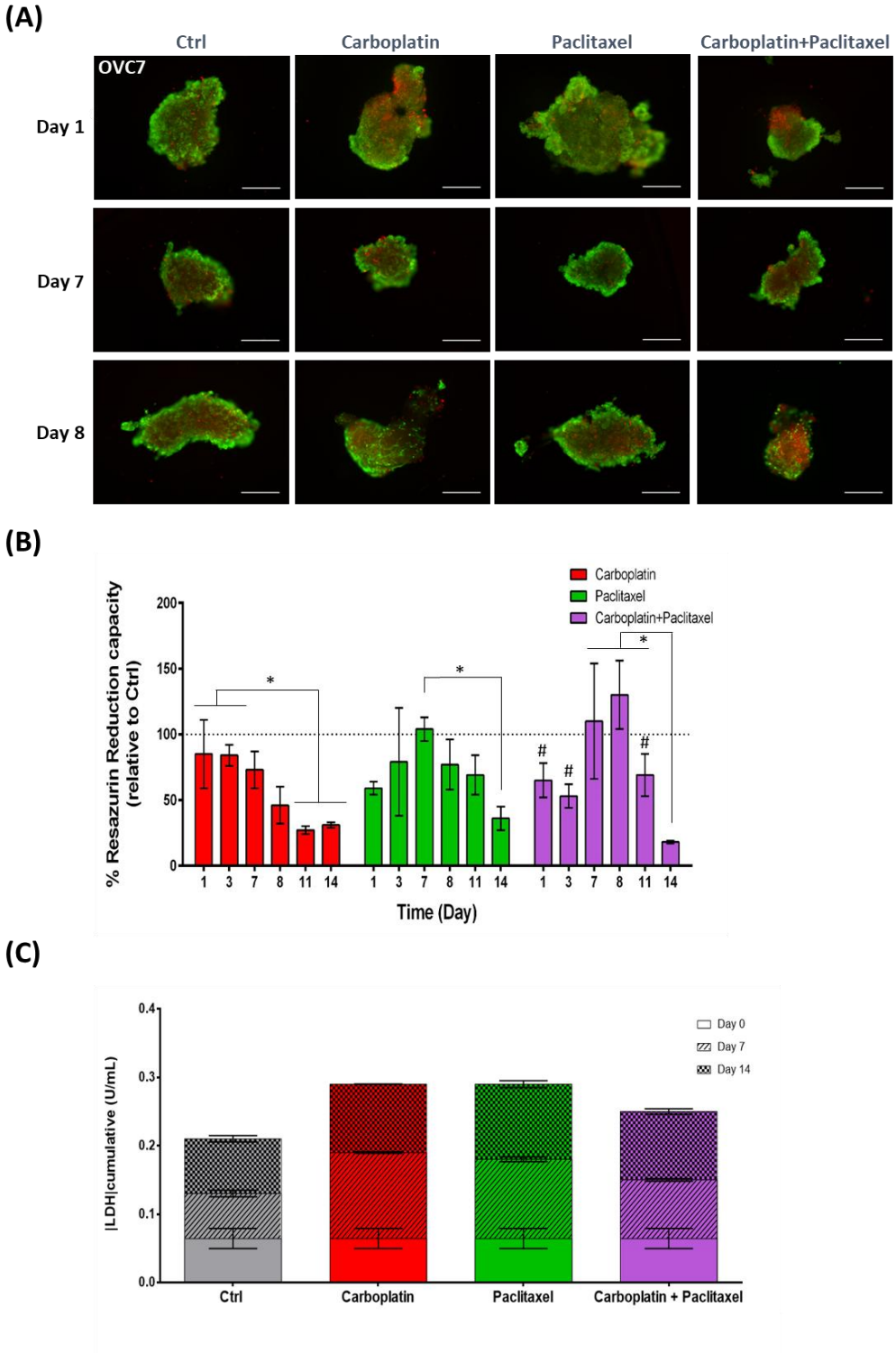


Figure III.7. PDE cultures of serous ovarian carcinoma are amenable for *ex vivo* evaluation of chemotherapy efficacy. (A) Representative images of live/dead fluorescent assay of PDE cultures from OVC7 treated with chemotherapy. PDE cultures were maintained for 7 days in culture after isolation to enable cell adaptation. Scale bars represent 500 µm; **(B)** Measurements of resazurin reduction capacity (in %) of the PDE along treatment time relative to control of treatment, on each timepoint. Data is presented as mean ± SD from at least 3 technical replicates. Two-way ANOVA statistical test (Tukey's multiple comparison test) was applied to compare the mean values of resazurin reduction capacity along treatment time. * ($p<0.05$) represents a statistically significant difference between the non-treated control condition and the chemotherapy-challenged PDE cultures; **(C)** Quantification of cumulative LDH activity in the culture supernatants of drug-treated OVC7. Data is presented as cumulative mean ± SD from at least 2 technical replicates.

Live/dead fluorescence staining (figure III.7A) of treated PDE cultures revealed a slight increase in death staining (red) after two cycles of treatment, in comparison with the control conditions, thus suggesting the action of the drugs on the culture of the tumor explants.

Figure III.7B shows the dynamic of resazurin reduction capacity of PDE cells exposed to chemotherapy agents, herein presented relative to the starting day of treatment for each condition. The effect of the compounds after the 1st cycle of chemotherapy exposure was only detected for paclitaxel-exposed PDE cultures. The capacity to reduce resazurin was significantly decreased for all the conditions during the 1st week of treatment, including the control PDE cultures. At day 14 of treatment, all the chemotherapy-exposed conditions presented resazurin reduction capacity below 14% relative to the beginning of the cyclic drug assay, indicating low metabolic activity at this stage. All the treated conditions presented a statistically significant lower capacity to reduce resazurin relatively to non-treated PDE cultures at the end of the 2nd week of cyclic treatment, indicating the effect of the treatment on cell viability after repeated exposure of the drugs. The stronger effect of the compounds on cell viability after the 2nd cycle of *ex vivo* chemotherapy confirms the results obtained with live/dead assays, which indicated an enhanced PI staining of cultures after exposure to the 2nd 24 hours period of chemotherapy (between day 7 and day 8).

The LDH quantification in the culture supernatant was also performed. At day 7 of treatment (prior to exposure to the 2nd cycle of chemotherapy), carboplatin-treated PDE cultures presented the highest levels of LDH activity and all the treated conditions showed a trend of higher LDH activity values than the control cultures. At day 14, this trend was more pronounced. These data corroborated the results from the resazurin reduction capacity assay.

Immunohistochemistry analysis of treated samples also confirmed the effect of the drugs. Morphological evaluation by H&E staining showed that after 14 days of treatment, few or no epithelial cells were left in the treated conditions (figure III.8).

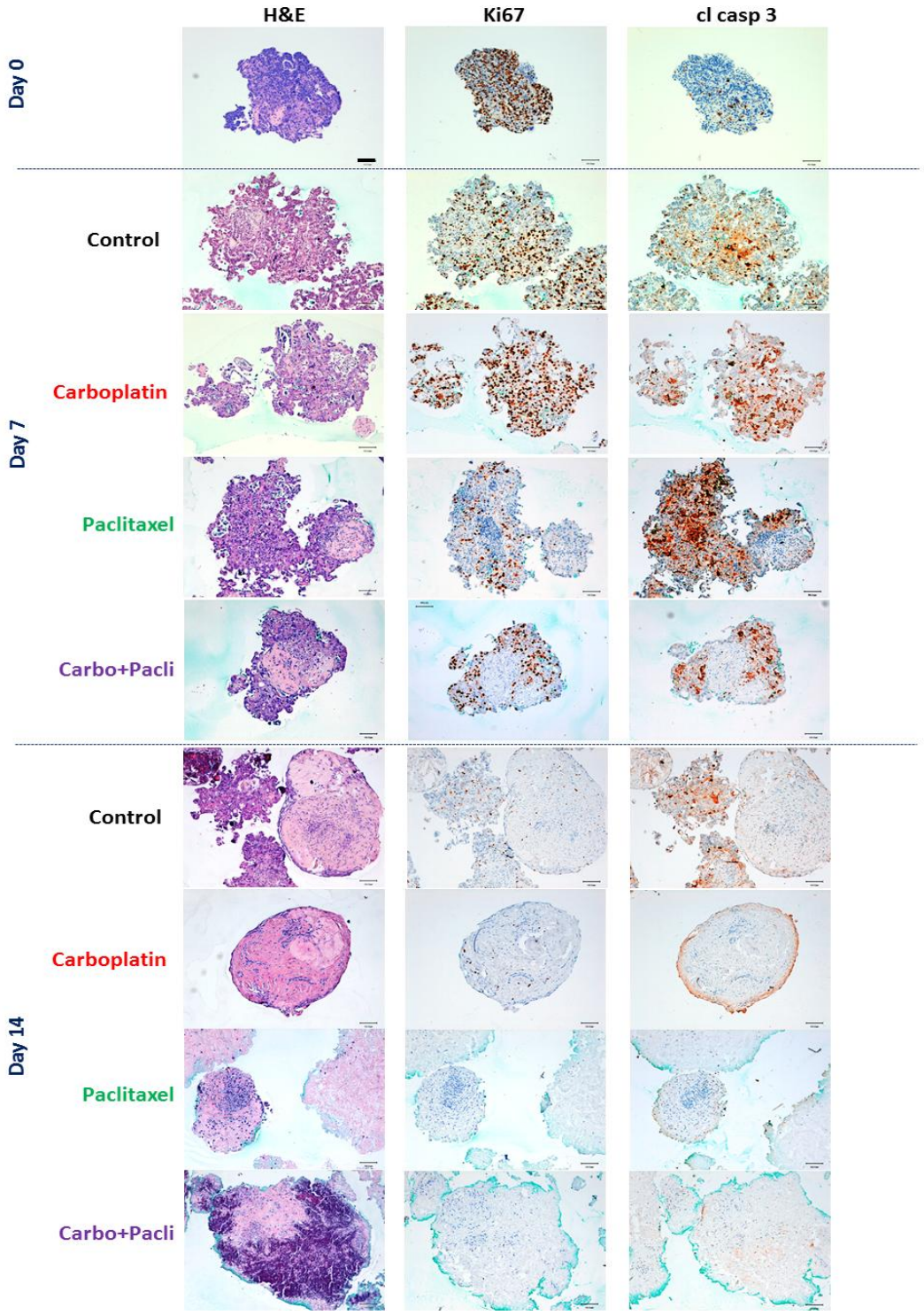


Figure III.8. H&E staining and immunohistochemistry analysis for proliferation and apoptosis of representative cross-sections of PDE (case OVC7) treated with chemotherapy agents. Scale bars represent 100 µm.

Immunohistochemistry analysis performed at IPOLFG by our partners revealed that at day 7, treated cultures showed upregulated levels of apoptosis in comparison with the control, particularly paclitaxel-treated cultures. Ki67+ cells were also identified demonstrating that the 1st cycle of the treatment only had partial success. After the application of the 2nd cycle, the effect of the compounds was clearly enhanced by a significant loss of cellular content in PDEs (none to few epithelial cells could be

identified with most of them being Ki67- and cleaved caspase 3-). This data corroborates the results obtained by measurement of resazurin reduction capacity and cumulative LDH activity in culture supernatants.

The size of the PDE was also evaluated along time, as a measurement of *ex vivo* drug efficacy (figure III.9).

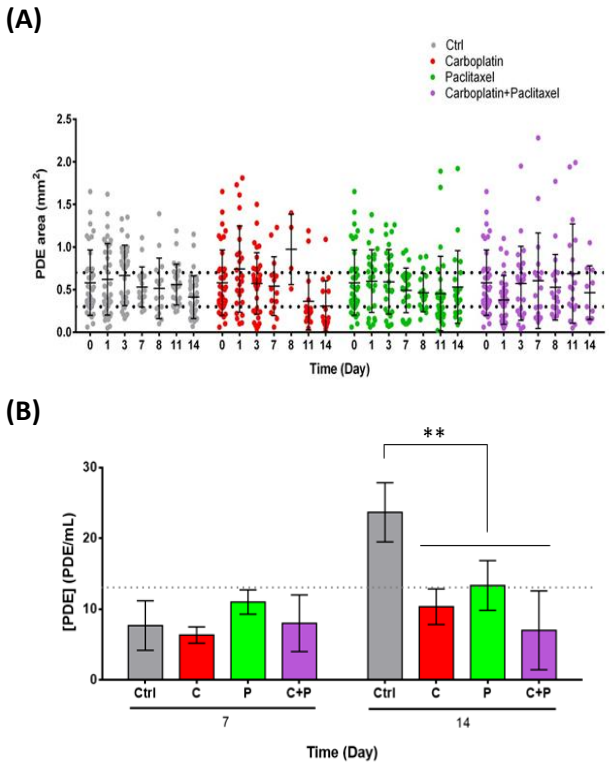


Figure III.9. PDE area and concentration as possible read-outs for evaluation of *ex vivo* chemotherapy efficacy in PDE cultures of serous ovarian carcinoma (OVC7). **(A)** Measurement of PDE surface area during the 14 days of drug exposure. PDE were imaged with the bright field mode in an inverted fluorescence microscope and PDE area was measured by adjusting the threshold until the border of each PDE, which was then quantified using the area measurement algorithm from ImageJ open source software. Data is presented as mean ± SD from at least 10 different PDE cultures of OVC7; **(B)** Measurement of PDE concentration of OVC7 cultures treated with chemotherapy. Initial PDE concentration at the beginning of the treatment period was 13 ± 2.6 PDE/mL, as indicated by the grey dashed line. Data is presented as mean ± SD from at least 3 technical replicates. Two-way ANOVA statistical test (Tukey's multiple comparison test) was applied to compare the mean values of PDE concentration in culture during chemotherapy treatment. ** ($p<0.01$) represents a statistically significant difference between non-treated and all treated PDE cultures at day 14. Statistical analysis was carried out using GraphPad Prism 6 software.

For OVC7, mean PDE surface area was not a feasible method for assessment of drug efficacy as no changes were observed during treatment and challenged PDE presented similar sizes to non-treated PDE.

PDE concentration revealed significant differences between treated and non-treated cultures at day 14 (after exposure to 2 cycles of chemotherapy). Non-treated PDE dissociated during culture, achieving a PDE concentration of 23 ± 3 PDE/mL, while treated PDEs maintained similar PDE concentration throughout the treatment.

Histological analysis from PDE cultures of high grade serous carcinoma showed that tumor fragmentation of original explants to smaller PDE during culture could happen due to the dissociation of epithelial cell-enriched papillae from the main fragment. By treating these tumor explants with chemotherapy, these epithelial papillae are targeted, thus inhibiting the dissociation step. Further analysis will have to be performed in future cases to confirm this phenomenon and to validate PDE concentration as a valid read-out measurement for assessment of drug efficacy.

From all read-outs used in this work to evaluate drug efficacy, no differences were observed between mono-therapy and combination therapy.

Overall, this study showed that it is possible to evaluate drug efficacy in PDE cultures and *ex vivo* models are promising for assessment of efficacy in repeated drug treatments.

III.3. Cold-storage and cryopreservation protocols of PDE cultures of ovarian carcinoma

We also investigated the possibility to preserve the tumor explants at the day of isolation using either cryopreservation or cold storage methodologies. Our aim was to validate procedures for the maintenance of tumor explants for future studies and to enable, for instance, transportation of tissue between laboratories without major impact in cell viability.

Samples from cases (OVC6, OVC8, OVC9 and OVC10) were cryostored and other 4 cases were (OVC8 to OVC11) cold-stored at 4ºC for up to 7 days using different commercial solutions. Figure III.10 shows a panel of live/dead assays and histological analysis of cold stored samples for case OVC10.

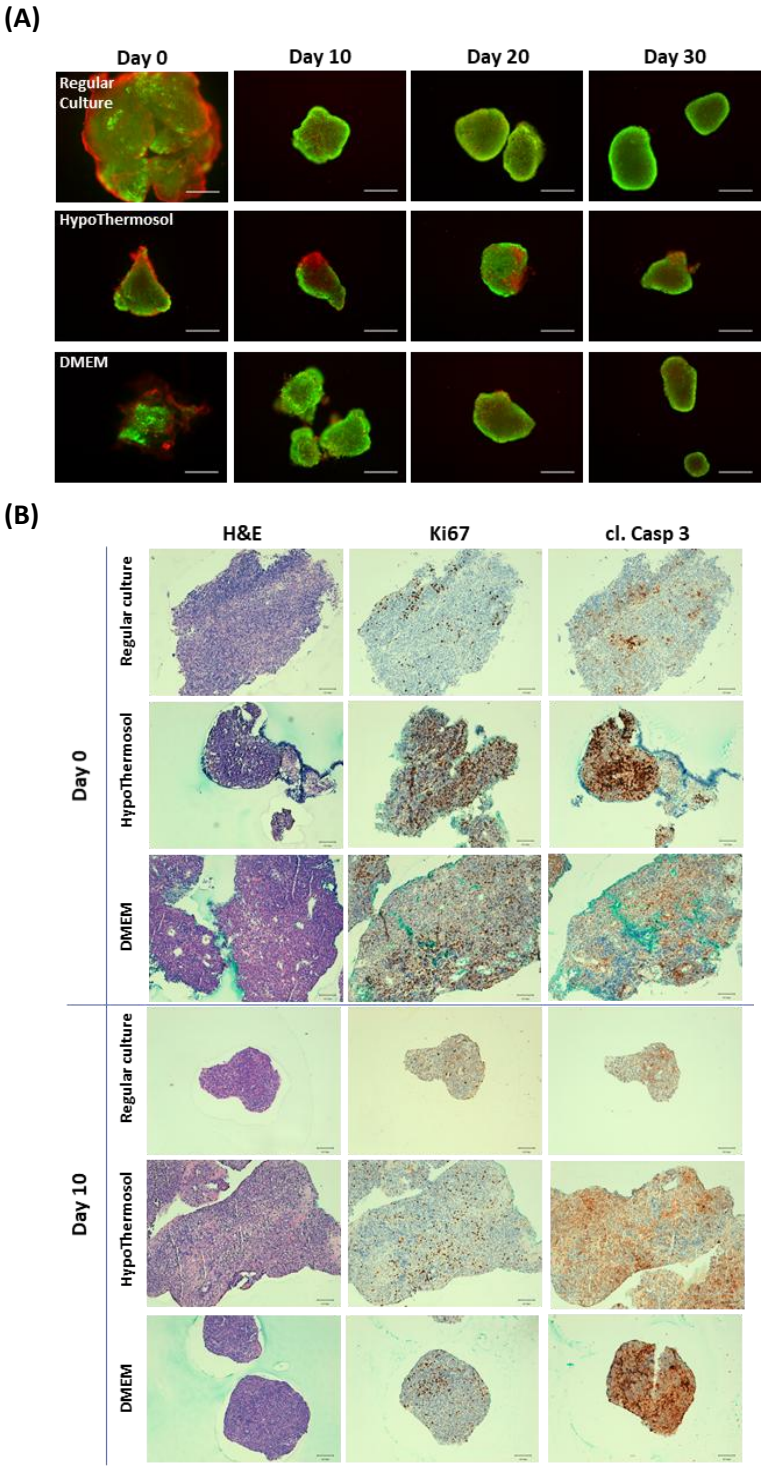


Figure III.10. Cold storage assays of PDE cultures of ovarian carcinoma (case OVC10). (A) Representative images of live/dead fluorescent assay of PDE cultures from OVC10. PDE cultures were maintained for 7 days in HypoThermosol solution and DMEM at 4ºC after isolation. Then were cultured up to 30 days, following the regular experimental set-up. Cultures were sampled at day 0, 10, 20 and 30 and incubated with FDA (green, live) and PI (red, dead). Scale bars represent 500 µm; **(B)** Cold-stored PDE were cultured in the same conditions as the regular control PDE cultures. These were sampled along the culture for histology (H&E) and immunohistochemistry analysis of Ki67 and cleaved caspase 3 markers in representative cross-sections of the cultures. Scale bars represent 100 µm.

Case OVC10 was identified as an undifferentiated tumor in the ovaries and it was cultured as described in Materials and Methods. Briefly, after tumor processing, PDE were stored in 2 different solutions: (i) HypoThermosol, commercially available solution; (ii) regular culture medium DMEM, supplemented with 10% FBS and 1% P/S. PDEs were maintained at 4ºC at a concentration of 25 PDE/mL for 7 days. After this period, PDE were collected and washed from their cold-storage solutions. These were then cultured for up to 30 days, under agitation-based conditions, following the same experimental set-up of the control PDE cultures. Samples were then characterized by performing live/dead assays and by IHC for evaluation of morphology, proliferation and apoptosis. Live/dead assays identified higher numbers of PI-positive cells for the samples stored in HypoThermosol. Histological analysis performed by our partners at IPOLFG (figure III.10B) suggests that PDE morphology is partially lost and that these cultures do not fully represent the histopathology of the original tumor. On the contrary, samples stored in DMEM on the same conditions presented high cell viability, proper morphology and preservation of tumor microenvironment. However, for both HypoThermosol- and DMEM-stored samples, levels of cleaved caspase 3+ cells were strongly enhanced in comparison with the control cultures.

For the cryopreservation studies, 2 solutions were assessed: (i) CryoStor10, commercially available solution; (ii) typical cell freezing solution FBS/DMSO (figure III.11).

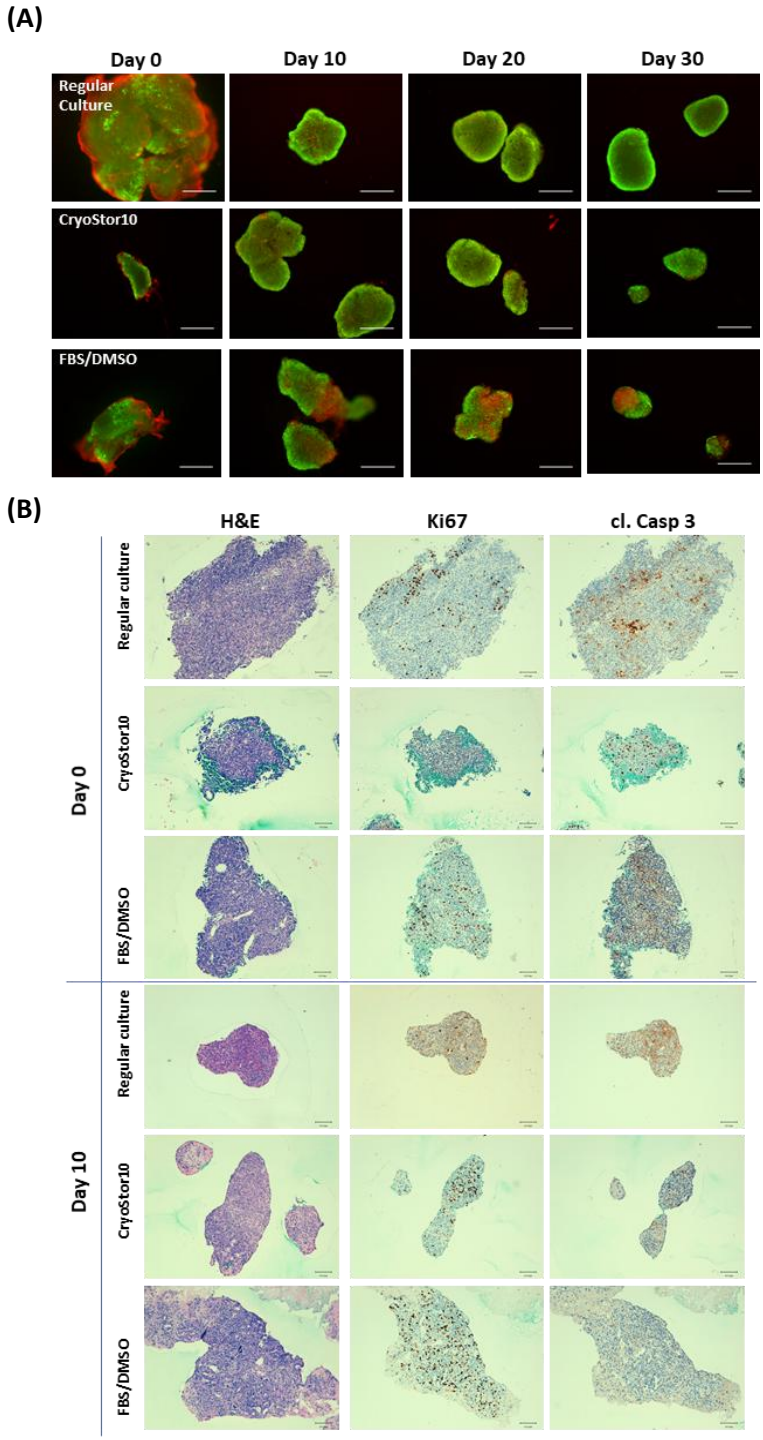


Figure III.11. Cryopreservation assays of PDE cultures of ovarian carcinoma (case OVC10). (A) Representative images of live/dead fluorescent assay of PDE cultures from OVC10. PDE cultures were maintained at -80ºC after isolation. After thawed, they were cultured for up to 30 days, following the regular experimental set-up. Cultures were sampled at day 0, 10, 20 and 30 and incubated with FDA (green, live) and Pi (red, dead). Scale bars represent 500 µm; **(B)** Cryopreserved and thawed PDE were cultured in the same conditions as the regular control PDE cultures. These were sampled along the culture for histology (H&E) and immunohistochemistry analysis of Ki67 and cleaved caspase 3 markers in representative cross-sections of the cultures. Scale bars represent 100 µm.

After 1 to 4 weeks of cryopreservation, the PDE were thawed and cultured following the same experimental set-up as control cultures. Live/dead assays showed that cryopreserving PDE in Cryostor10 led to better results than FBS/DMSO, with higher percentage of live cells. From immunohistochemistry analysis (figure III.11B) performed at IPOLFG, it was detected that cultures previously cryopreserved with Cryostor10 demonstrated similar levels of proliferation and apoptosis in comparison with the control cultures.

PDE cultures previously cryopreserved or cold stored at 4ºC were thoroughly analyzed by live/dead assays and histological evaluation of morphology (H&E staining), proliferation (Ki67+ immunostaining) and apoptosis (cleaved caspase 3+ immunostaining) of epithelial cells. Live/dead analysis of cryopreserved or cold stored PDE cultures was performed at iBET using a quantitative scale that ranged from [-] (indicative of strong PI and low FDA staining) to [+++], which represented strong FDA staining with absence of PI staining. Intermediate levels of live and dead stained cells were classified either as [+] or [++].

Quantification of proliferative and apoptotic epithelial cells was presented in % of Ki67+ and cleaved caspase 3+ epithelial cells relative to total epithelial cells in the sample. Samples were scored at the Anatomical Pathology Unit at IPOLFG by expert clinicians in gynecologic malignancies. A minimum number of 5 PDE was considered to perform the analysis. Samples were not considered for this analysis when the amount of material left for histological analysis was lower than 5 PDE or when the PDE were depleted of epithelial cells and were mostly composed by stromal cells. These results are summarized in table III.2.

Table III.2. Quantification of viability, proliferation and apoptosis of cryopreserved and cold stored PDE cultures from OVC10.

OVC10 (Undifferentiated sub-type)		Culture Progression			
		Day 0	Day 10	Day 20	Day 30
FDA/PI	Ctrl	+	+++	+++	+++
	CryoStor10	+	++	+++	+++
	FBS/DMSO	+	++	++	+
	HypoThermosol	+	+	++	++
	DMEM	+	++	++	++
Ki67 (%)	Ctrl	25-50	<5	<5	<5
	CryoStor10	25-50	10-25	na	na
	FBS/DMSO	10	10-25	<5	<5
	HypoThermosol	25-50	<5	na	na
	DMEM	10-25	10	<5	<10
Cl Casp3 (%)	Ctrl	10	25	0	<5
	CryoStor10	10	<5	na	na
	FBS/DMSO	<10	<5	<5	10-25
	HypoThermosol	>75	>75	na	na
	DMEM	50-75	>75	<25	<10

There is an indication that the cryopreservation protocols were more successful in preserving the original features of the tumors, particularly when using Cryostor10 as a cryopreservation agent. Cold stored samples were less successful, as apoptosis was significantly upregulated in comparison with the control conditions.

These results demonstrate that the used cryopreservation methods enable the preservation of most of the features of the original tumor sample. This is quite useful for the build-up tumor explants biobanks for future drug assays and cancer biology characterization.

IV. CONCLUSIONS

In this work, the feasibility of using patient-derived explant technology in agitation-based culture systems was demonstrated.

We were successful in establishing and characterizing cultures of PDE from ovarian carcinoma for up to 30 days. These cultures remained viable, with long-term maintenance of microenvironment components, such as fibroblasts and immune cells, and preserved the heterogeneity and architecture of the original tumor. Particularly, PDE cultures from the high-grade serous carcinoma, as this was the most prevalent sub-type, were assessed for evaluation of chemotherapy efficacy. The performance of cyclic challenge of PDE cultures with chemotherapy agents was possible and we have implemented cell viability, cell death, morphological and molecular read-outs of drug efficacy.

Moreover, we tested protocols for cold storage and cryopreservation of tumor fragments and cryopreservation with the commercially available solution CryoStor10 was quite successful in the preservation of tumor original features along culture post-thawing of PDE.

In conclusion, the results suggest that the implemented methodology can be employed in drug assays with standard of care and has the potential to assess novel compounds currently in the drug discovery stage.

V. FUTURE PERSPECTIVES

The results described in this thesis are planned to be part of a future publication about preservation of histopathological features by PDE cultures of ovarian carcinoma and their use in cyclic *ex vivo* chemotherapy treatments.

The strategies presented herein contributed to the progress of the state-of-the-art on patient-derived explant models, although further improvements can be pursued aiming at increased predictability in preclinical studies.

For validation of the developed PDE explant cultures for the robustness and sensitivity, assays with increase number of replicates will be performed.

Further cyclic chemotherapy treatments could also be performed to validate the quantification methods of *ex vivo* drug efficacy, thus demonstrating the feasibility to use these models as promising tools for preclinical research. Specifically, the application of these models to evaluate chemoresistance to carboplatin treatment after cyclic exposure for high grade serous carcinomas, as these cancers are known to develop chemoresistance for this type of treatment and represent a current unmet clinical need.

Moreover, the PDE models herein proposed could be further used to explore the role of tumor microenvironment on drug response and acquisition of resistance mechanisms, particularly for immune cells. Evaluation of therapeutic efficacy of novel immunotherapies for ovarian cancer would also be an interesting application for the PDE models.

VI. REFERENCES

Alemany-Ribes, M., & Semino, C. E. (2014). Bioengineering 3D environments for cancer models. *Advanced Drug Delivery Reviews*, *79*, 40–49. https://doi.org/10.1016/j.addr.2014.06.004

Alkema, N. G., Tomar, T., Duiker, E. W., Meersma, G. J., Klip, H., Zee, A. G. J. Van Der, … Jong, S. De. (2015). Biobanking of patient and patient- derived xenograft ovarian tumour tissue : efficient preservation with low and high fetal calf serum based methods. *Nature Publishing Group*, (June), 1–12. https://doi.org/10.1038/srep14495

Arantes-rodrigues, R., Colaço, A., Pinto-leite, R., & Oliveira, P. A. (2013). In Vitro and In Vivo Experimental Models as Tools to Investigate the Efficacy of Antineoplastic Drugs on Urinary Bladder Cancer, *1296*, 1273–1296.

Balkwill, F., Jr, R. C. B., Berek, J., Gore, M., & Hamilton, T. (2003). Current research and treatment for epithelial ovarian cancer, *39*, 1818–1827. https://doi.org/10.1016/S0959-8049(03)00511-2

Breslin, S., & Driscoll, L. O. (2013). Three-dimensional cell culture : the missing link in drug discovery. *Drug Discovery Today*, *18*(5–6), 240–249. https://doi.org/10.1016/j.drudis.2012.10.003

Cláudia Correia, Alexey Koshkin, Madalena Carido, Nuno Espinha, Tomo Saric, Pedro Lima, Margarida Serra, P. A. (2016). Effective Hypothermic Storage of Human Pluripotent Stem Cell-Derived Cardiomyocytes Compatible With Global Distribution of Cells for Clinical Applications and Toxicology Testing. *Stem Cells Translational Medicine*, 658–669.

Das, V., Bruzzese, F., Konec, P., Iannnell, F., Budillon, A., & Hajdu, M. (2015). Pathophysiologically relevant in vitro tumor models for drug screening, *0*(0). https://doi.org/10.1016/j.drudis.2015.04.004

Davies, E. J., Dong, M., Gutekunst, M., Närhi, K., Wedge, S. R., Hällström, T. M., … Weerden, W. M. Van. (2015). Capturing complex tumour biology in vitro: histological and molecular characterisation of precision cut slices. *Nature Publishing Group*, (July), 1–17. https://doi.org/10.1038/srep17187

Drapkin, R., Jones, P. M., & Drapkin, R. (2014). Modeling High-Grade Serous Carcinoma : How Converging Insights into Pathogenesis and Genetics are Driving Better ... Modeling high-grade serous carcinoma : how converging insights into pathogenesis and genetics are driving better experimental platforms, (August 2013). https://doi.org/10.3389/fonc.2013.00217

E Lengyel, et al. (2015). Epithelial Ovarian Cancer Experimental Models, *33*(28), 3619–3633. https://doi.org/10.1038/onc.2013.321.Epithelial

Estrada, M. F., Rebelo, P., Davies, E. J., Pinto, M. T., Pereira, H., Santo, V. E., … Brito, C. (2016). Modelling the tumour microenvironment in long-term microencapsulated 3D co-cultures recapitulates phenotypic features of disease progression, *78*, 50–61. https://doi.org/10.1016/j.biomaterials.2015.11.030

Ferlay, J., Steliarova-foucher, E., Lortet-tieulent, J., & Rosso, S. (2013). Cancer incidence and mortality patterns in Europe : Estimates for 40 countries in 2012. *European Journal of Cancer*, *49*(6), 1374–1403. https://doi.org/10.1016/j.ejca.2012.12.027

Hansen, J. M., Coleman, R. L., & Sood, A. K. (2016). Targeting the tumour microenvironment in ovarian cancer. *European Journal of Cancer*, *56*, 131–143. https://doi.org/10.1016/j.ejca.2015.12.016

Hickman, J. A., Graeser, R., Hoogt, R. De, Vidic, S., Brito, C., Gutekunst, M., & Kuip, H. Van Der. (2014). Three-dimensional models of cancer for pharmacology and cancer cell biology : Capturing tumor complexity in vitro / ex vivo, 1115–1128. https://doi.org/10.1002/biot.201300492

Horvath, P., Aulner, N., Bickle, M., Davies, A. M., Nery, E. Del, Ebner, D., … Carragher, N. O. (2016). Screening out irrelevant cell-based models of disease. *Nature Publishing Group*. https://doi.org/10.1038/nrd.2016.175

Ince, T. A., Sousa, A. D., Jones, M. A., Harrell, J. C., Agoston, E. S., Krohn, M., … Mills, G. B. (2015). Characterization of twenty-five ovarian tumour cell lines that phenocopy primary tumours. *Nature Communications*, *6*(May), 1–14. https://doi.org/10.1038/ncomms8419

Karekla, E., Liao, W.-J., Sharp, B., Pugh, J., Reid, H., Le Quesne, J. P., … Pringle, J. H. (2017). Ex vivo explant cultures of non-small cell lung carcinoma enable evaluation of primary tumor responses to anticancer therapy. *Cancer Research*, canres.1121.2016. https://doi.org/10.1158/0008-5472.CAN-16-1121

Kenny, H. A., Lal-nag, M., White, E. A., Shen, M., Chiang, C., Mitra, A. K., … Lengyel, E. (2015). Quantitative high throughput screening using a primary human three-dimensional organotypic culture predicts in vivo efficacy. *Nature Communications*, *6*, 1–10. https://doi.org/10.1038/ncomms7220

Kim, J. Bin. (2005). Three-dimensional tissue culture models in cancer biology, *15*, 365–377. https://doi.org/10.1016/j.semcancer.2005.05.002

Koshiyama, M. (2017). Subtypes of Ovarian Cancer and Ovarian Cancer Screening, 1–10. https://doi.org/10.3390/diagnostics7010012

Kumar, S., Bajaj, S., & Bodla, R. B. (2016). Preclinical screening methods in cancer. *Indian Journal of Pharmacology*, *48*(5), 481–486. http://doi.org/10.4103/0253-7613.190716

Lamichhane, S. P., Arya, N., Kohler, E., Xiang, S., Christensen, J., & Shastri, V. P. (2016). Recapitulating epithelial tumor microenvironment in vitro using three dimensional tri-culture of human epithelial , endothelial , and mesenchymal cells. *BMC Cancer*, 1–12. https://doi.org/10.1186/s12885-016-2634-1

Lopes-coelho, F., Gouveia-fernandes, S., Gonçalves, L. G., Nunes, C., Faustino, I., Silva, F., … Serpa, J. (2016). HNF1 β drives glutathione (GSH) synthesis underlying intrinsic carboplatin resistance of ovarian clear cell carcinoma (OCCC). *Tumor Biology*, 4813–4829. https://doi.org/10.1007/s13277-015-4290-5

Majumder, B., Baraneedharan, U., Thiyagarajan, S., Radhakrishnan, P., Narasimhan, H., Dhandapani, M., … Majumder, P. K. (2015). Predicting clinical response to anticancer drugs using an ex vivo platform that captures tumour heterogeneity. *Nature Communications*, *6*, 6169. https://doi.org/10.1038/ncomms7169

Mitra, A., Mishra, L., & Li, S. (2013). Technologies for deriving primary tumor cells for use in personalized cancer therapy. *Trends in Biotechnology*, *31*(6), 347–354. https://doi.org/10.1016/j.tibtech.2013.03.006

Nath, S., & Devi, G. R. (2016). Three-dimensional culture systems in cancer research : Focus on tumor spheroid model. *Pharmacology and Therapeutics*, *163*, 94–108. https://doi.org/10.1016/j.pharmthera.2016.03.013

Pampaloni, F., Reynaud, E. G., & Stelzer, E. H. K. (2007). The third dimension bridges the gap between cell culture and live tissue. *Nature Reviews Molecular Cell Biology*, *8*(10), 839–845. https://doi.org/10.1038/nrm2236

Pompili, L., Porru, M., Caruso, C., Biroccio, A., & Leonetti, C. (2016). Patient-derived xenografts: a relevant preclinical model for drug development. *Journal of Experimental & Clinical Cancer Research*, 1–8. https://doi.org/10.1186/s13046-016-0462-4

Santo, V. E., Estrada, M. F., Rebelo, S. P., Abreu, S., Silva, I., Pinto, C., … Brito, C. (2016). Adaptable stirred-tank culture strategies for large scale production of multicellular spheroid-based tumor cell models, *221*, 1–12.

Santo, V. E., Rebelo, S. P., Estrada, M. F., Alves, P. M., Boghaert, E., & Brito, C. (2017). Drug screening in 3D in vitro tumor models: overcoming current pitfalls of efficacy read-outs. *Biotechnology Journal*, *12*(1). https://doi.org/10.1002/biot.201600505

Spill, F., Reynolds, D. S., Kamm, R. D., & Zaman, M. H. (2016). Impact of the physical microenvironment on tumor progression and metastasis. *Current Opinion in Biotechnology*, *40*, 41–48. https://doi.org/10.1016/j.copbio.2016.02.007

Stock, K., Estrada, M. F., Vidic, S., Gjerde, K., Rudisch, A., Santo, V. E., … Graeser, R. (2016). Capturing tumor complexity in vitro: Comparative analysis of 2D and 3D tumor models for drug discovery. *Scientific Reports*, *6*(1), 28951. https://doi.org/10.1038/srep28951

Thibault, B., Castells, M., Delord, J., & Couderc, B. (2014). Ovarian cancer microenvironment : implications for cancer dissemination and chemoresistance acquisition, 17–39. https://doi.org/10.1007/s10555-013-9456-2

Walsh, A. J., Cook, R. S., Sanders, M. E., Arteaga, C. L., & Skala, M. C. (2016). Drug response in organoids generated from frozen primary tumor tissues. *Nature Publishing Group*, (January), 1–11. https://doi.org/10.1038/srep18889

Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., & Gong, Z. (2017). Role of tumor microenvironment in tumorigenesis, *8*. https://doi.org/10.7150/jca.17648

Worzfeld, T., Pogge von Strandmann, E., Huber, M., Adhikary, T., Wagner, U., Reinartz, S., & Müller, R. (2017). The Unique Molecular and Cellular Microenvironment of Ovarian Cancer. *Frontiers in Oncology*, *7*(February). https://doi.org/10.3389/fonc.2017.00024

Xu, X., Farach-carson, M. C., & Jia, X. (2014). Three-dimensional in vitro tumor models for cancer research and drug evaluation. *Biotechnology Advances*, 1–13. https://doi.org/10.1016/j.biotechadv.2014.07.009

Yiu, S., Choi, C., Lin, D., Gout, P. W., Collins, C. C., Xu, Y., & Wang, Y. (2014). Lessons from patient-derived xenografts for better in vitro modeling of human cancer. *Advanced Drug Delivery Reviews*, *79–80*, 222–237. https://doi.org/10.1016/j.addr.2014.09.009