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TITLE OF THE DOCTORAL THESIS

The Chick Embryo Chorioallantoic Membrane (CAM) model for Circulating Tumor Cell Culturing for Precision Oncology in Patients with Pancreatic Cancer. A proof of concept.

S.S.D. _____

(Please complete this space with the S.S.D. of your thesis – mandatory information)*

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The Chick Embryo Chorioallantoic Membrane (CAM) model for Circulating Tumor Cell Culturing for Precision Oncology in Patients with Pancreatic Cancer. A proof of concept.

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Premises

The current proposal is aimed at developing a novel technique for culturing of Circulating Tumor Cells (CTCs) in pancreatic ductal adenocarcinoma (PDAC) that will allow for expansion of the population of isolated CTCs resulting in the ability to further study them.

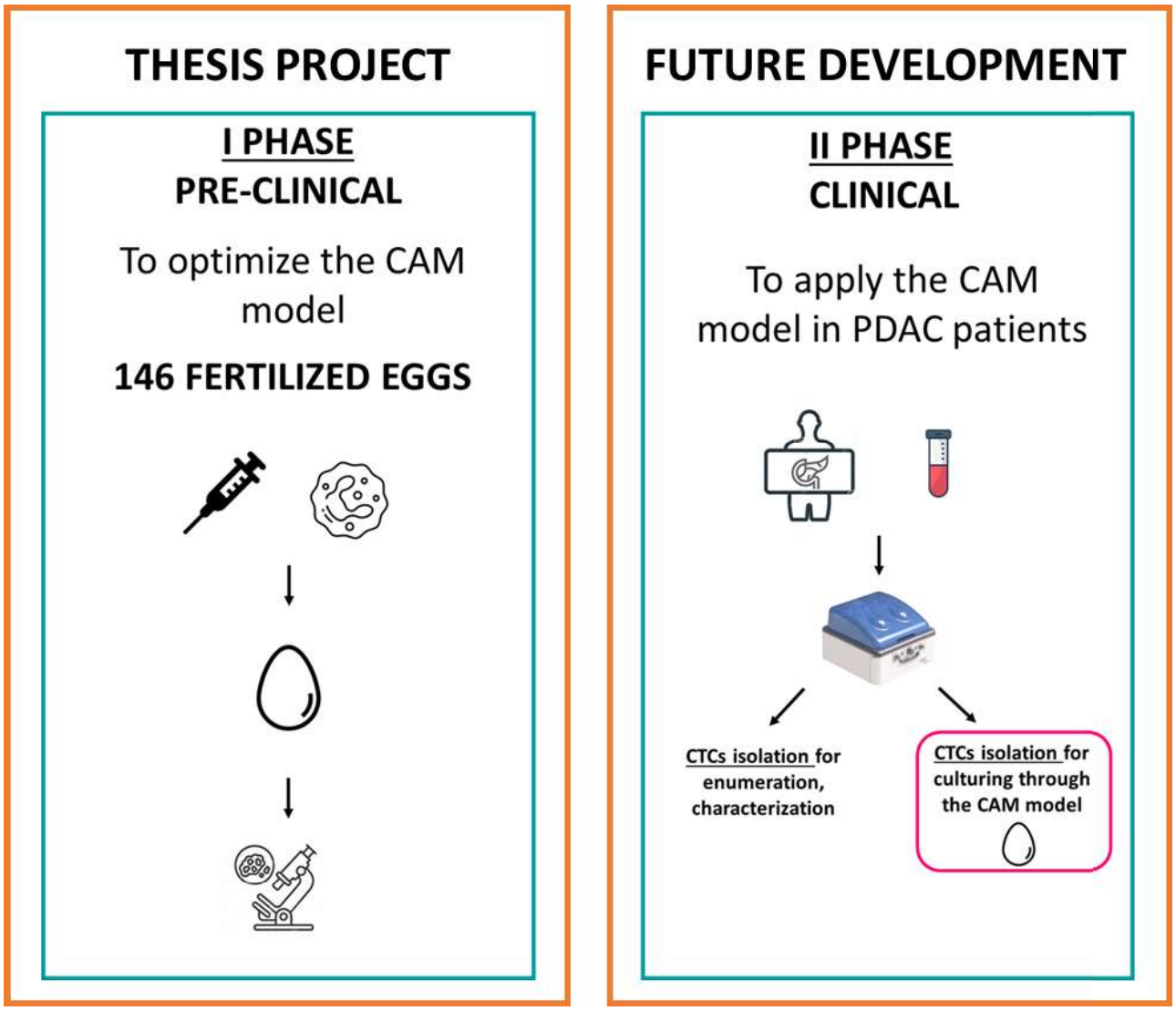
This study describes and analyzes preliminary (pre-clinical) experiments aimed at developing and optimizing the CAM assay through implantation of human pancreatic cancer cell lines (MIA PaCa-2) onto the CAM at progressively decreasing concentrations. The optimal technique established during this pre-clinical phase will then be applied in the subsequent clinical phase to culture and expand circulating tumor cells (CTCs) isolated from patients diagnosed with locally advanced or metastatic pancreatic ductal adenocarcinoma (PDAC).

The possibility of CTCs expansion and characterization, will allow to use them as a prognostic biomarker. The novelty of this project is the combination of two powerful techniques, represented by the ISET® technology for viable CTCs isolation and the CAM model for the subsequent culturing step.

The experimental design and planning were carried out under the supervision of Dr. Christopher Wolfgang, director of the new Division of Hepatobiliary and Pancreatic Surgery in the Department of Surgery at NYU Langone Health, and Dr. Ammar Asrar Javed, Research Assistant Professor of Surgery, NYU Langone Health. The author personally performed all the experiments for the optimization of the CAM assay. Data analysis, interpretation, and preparation of the manuscript were conducted by the author with contributions and critical revisions from the co-authors. All the results

presented in this thesis, unless otherwise specified, derive from the author's direct experimental activity.

Flow Chart


THESIS PROJECT
I PHASE
PRE-CLINICAL
To optimize the CAM model
146 FERTILIZED EGGS
FUTURE DEVELOPMENT
II PHASE
CLINICAL
To apply the CAM model in PDAC patients
CTCs isolation for enumeration, characterization
CTCs isolation for culturing through the CAM model

Context and Hypothesis

Rationale

Cancer is a leading cause of deaths globally (~10 million deaths in 2020), with 90% of deaths resulting from distant spread of disease forming metastasis. (1) While much work has been done, our understanding of the metastatic cascade remains limited especially in terms of circulating tumor cell biology. Cancer progression is based on the stepwise accumulation of somatic mutations followed by clonal selection. Furthermore, as a result of interaction between the tumor cells and host cells, tumor cells undergo multiple changes that allow them to escape into circulation, evade the host immune system, and seed distant organs. (2) The complexity of these processes and its variability among individuals and cancer types makes it challenging to define a single triggering cellular event or events that drive these processes. Nowadays, a majority of studies focusing on understanding tumor biology are based on the analysis of primary tumor or metastatic sites, omitting an important aspect of the metastatic cascade which is the circulating disease i.e. circulating tumor cells. Knowledge gained from these studies is translated to clinical decision making and while it has shown promise for tumors that have targetable mutations, for those that don't this approach has failed to significantly improve treatment strategies and survival. Additionally, one of the limitations of using this approach is that it provides a snapshot of tumor biology and tools for non-invasive serial assessment of changes in tumor biology are inadequate.

In particular, Pancreatic Ductal Adenocarcinoma (PDAC) is one of the leading causes of cancer-related deaths in the United States, with a 5-year overall survival of only 12% across all stages of disease. (3) While the incidence of other major malignancies is on the decline, that of PDAC is on the rise, ranking second among cancer-specific mortality by 2030. (3) The underlying biological features of PDAC that drive poor outcomes are the propensity for early systemic spread and only a modest impact of

current systemic treatment paradigms. (4) The only potentially curative therapy is local control through oncologic resection and systemic control through chemotherapy. Despite improvements in systemic therapies their impact on survival has been modest at (best. (5) The majority of patients who undergo successful surgical resection in addition to systemic therapy experience treatment failure though systemic progression of the so called “minimal residual disease”. (6) Early recurrence (<12 months) is reported in 15 to 25% of the patients who undergo surgery, suggesting that the patients had aggressive micrometastatic disease. (6) Effective control of systemic disease remains the greatest challenge to improve long-term survival in these patients. Given the systemic nature of clinically localized PDAC, a surgery-first approach is becoming less common in favor of a neoadjuvant approach. (7; 8; 9; 10) Chemotherapy is added to surgery with the intent of eradicating micrometastatic disease, identified in pancreatic cancer patients liver and blood, thus improving median survival.

In the absence of reliable subclinical measurement of treatment response, failure can only be assessed clinically as either clinical progression of disease (seen on standard cross-sectional imaging) or poor treatment response (seen on histopathological evaluation of the surgical specimen). This becomes a particular problem in the treatment of potentially curable patients with neoadjuvant - in which case failure of systemic therapy may only be determined upon progression to incurable disease.

The current clinical diagnostic modalities, such as tissue biopsies, imaging and tumor marker, have a limited function in predicting tumor progression. Moreover, since the current therapeutic strategies are mainly based on primary tumor biology and not on the metastatic process, surgery or systemic therapy for the primary tumor will not necessarily remove micrometastatic circulating disease. (11)

The main limitation of focusing on the primary tumor is that it provides a static view of tumor biology, which in fact is dynamic in nature.

(12) In this context, the possibility to capture and monitor circulating tumor heterogeneity over time and to modulate therapies could lead to personalized approaches in the management of these patients resulting in an improvement in survival. The term liquid biopsy represents the analysis of various biological fluids components (13), which have the potential to provide information about features of the primary tumor and circulating disease. The generated data concern gene mutations (13), transcriptome (14), epigenome (15), proteome (16) and metabolome. (17) Their integration, as through the application of the artificial intelligence, (18) could provide a wider range of applications as compared to the traditional assessment made using tumor tissue obtained and analyzed by pathology. Of all the organic fluids, blood is the most frequently used, offering several sources of cancer-derived material, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes. (19) These components represent the status of the primary tumor and its progression through metastasis formation, providing a dynamic assessment of tumor evolution. In particular, CTCs are cells shed from primary tumors and/or metastatic deposits into the blood stream and they are considered to be the main drivers of metastasis. (20) CTCs were identified and reported for the first time by Ashwort (21) in 1869 and their metastatic potential was described over twenty years later by Stephan Paget through the "seed and soil" hypothesis (22), according to which the metastatic cascade begins when some tumor cells from the primary tumor are able to enter in the circulation as seeds and grow in selected organs with a microenvironment conducive to their growth. It was over a century later that CTCs were definitively recognized for their relevant role in driving the metastases. (23)

CTCs enumeration and analysis in epithelial tumors can contribute to the prognostic evaluation, patient stratification, and real-time monitoring of treatment efficacy, as well

as to the identification of therapeutic targets and tumor resistance mechanisms. CTCs, in fact, could function as an efficient and reliable drug screening system for guiding clinical decisions, especially for oncological patients that have limited windows of opportunity. Moreover, CTCs can help understand disease biology and the metastatic cascade. However, studying CTCs has some important limitations. Despite a huge progress in the development of techniques for their detection and molecular characterization, the rarity of CTCs in circulation remains a limiting factor to investigate on their functional properties. (24; 25) Therefore, research on CTCs expansion, has to be promoted to increase their applicability in the clinical setting. Over the last decade, various groups have provided the basis to culture CTCs in a clinical actionable manner demonstrating its feasibility. However, such techniques have not yet been standardized and integrated into clinical practice.

There are two main steps to overcome to be able to culture CTCs successfully. The cells have to be enriched, which means concentrated to facilitate their isolation from other blood cell components, (26; 27) and they have to remain viable. For these reasons, it is crucial to rapidly process (< 24 h) the blood without any cell fixation or permeabilization. (26)

As already described, there are different techniques to isolate and to enrich viable CTCs. At this point, the greatest obstacle to study them in further detail is the possibility to culture them. In fact, due to CTCs rarity, expansion remains crucial for their functional study. The difficulties to culture CTCs in a way that they could be routinely used in clinical practice as dynamic and real time biomarkers, reflect the lack of knowledge of their heterogeneity and biology.

The following are the clinically oriented studies on different techniques to culture CTCs and are stratified by the approach to culturing.

In vitro CTCs culturing methods

In vitro culturing could be a reliable method to assess the molecular profile of CTCs and to test response to therapy. In this paragraph we will describe how in the majority of the studies on different cancer types, the authors were able to successfully culture CTCs for a short period of time however only few of them were able to established long-term culture.

Breast cancer

In 2013, for the first time CTCs from metastatic breast cancer patients were cultured short-term, describing how the metastatic cascade can be sustained by CTCs negative for EpCAM markers. (28) The authors were able to isolate EpCAM CTCs in 8 of 32 patients through multiparametric FACS analysis, of which 3 were viable. These were firstly cultured in stem cell culture medium till day 7, and then transferred in medium for epithelial cells. Here, both in vitro and in vivo models were used to identify the CTC subpopulation with metastatic capacity, to be targeted for therapeutic intervention. In another study, the authors were able to culture CTCs as long-term tumor spheres from 6 metastatic estrogen receptor positive (ER)- breast cancer patients from a total of 36 patients. (29). All of the cultures showed different drug sensitivity, mimicking the high heterogeneity of ER+ breast cancer, which usually is diagnosed in advanced stages. In 2015, 226 blood samples from 96 breast cancer patients with different stages were cultured in a laser-ablated, microwell- assay from whole blood, after red blood cell lysis and in hypoxic conditions. (30) At day 14, CTCs showed cluster formation, whose growth was inversely related with systemic therapy duration, working as a predictor of treatment response. A total of 43.3% of the early stage (IA- IIIC) patients formed clusters, which were more commonly in patients with involvement of lymph nodes and before the start of adjuvant therapy. Moreover, cluster formation from post-treatment

samples was associated with early radiological progression of disease. The same group integrated the laser-ablated, microwell-assay with microfluidics to improve formation of CTC clusters without pre-enrichment and subsequent drug screening in situ. Using the blood of a metastatic ER+ patients, a permanent CTCs cell line was established, with copy number alteration (CNA) profiling indicating high concordance between the original CTCs and the established CTC. Here, functionally relevant mutations were detected in the CTC line, primary tumor, and metastasis, showing how that the CTC line can provide novel insights into the biology and therapeutic response. (31) Recently, doxorubicin resistance was proved to be increased in CTCs from advanced breast cancer patients under high culture condition through microfluidic technology. (32)

Lung cancer

In 2014, through an immunoaffinity-based microfluidic device, CTCs from early stage lung cancer were captured and co-cultured in a 3-D model that simulates the tumor microenvironment. (33) The authors successfully expanded CTCs from 14 of the 19 patients for a short period of time, and their genomic profiles matched to those of the primary tumors. Being able to isolate and culture CTCs from early stage cancer, could permit to better understand the metastatic cascade. Similarly, in 2019, one permanent cell line was established from an early stage lung cancer patient. (34) In vitro characterization showed that the cell line was in the intermediate stage of EMT with stem cell characteristics, and it was drug resistant. In vivo studies demonstrated the capacity of the cells to induce tumorigenesis and metastasis, potentially due to the low expression level of CX3CL1 and high expression level of CXCL5, representing an important mechanism for metastasis formation and a potential target for therapy.

In 2016, a CTC line was established from a small cell lung cancer (SCLC) patient, for a short time and used for drug sensitivity testing as a real-time prediction of the response to treatment. This line expressed EMT markers in different proportions, without stem cell markers. (35) In 2020, CTCs were isolated from 22 patients with all stages of SCLC, through Ficoll-Paque® density gradient and CD45 RosetteSep® for negative selection and then cultured in a custom-made culture plates coated with self-assembling binary colloidal crystals. (36) CTC spheroids were observed after 14 days and continued to enlarge till day 40, with a success rate of 82%. Some of the expanded CTC were tested for drugs sensitivity, showing concordance with the patients' clinical response. Very recently, a new Chip technology to capture and culture CTCs was set up through a lung cancer cell line spiked in porcine whole blood. The acoustic bubble for trapping, rotation and culture CTC spheroid on a tumor-on-a-chip platform (ABSTRACT) creates microcavity structures in which air bubbles can be captured in the microchannel structure. In this way, CTCs are trapped and separated from blood flow due to the difference in size and density. Moreover, under rotational motion, CTCs can aggregate and form spheroids. (37) When the spheroid has reached the desired size, by turning off the rotating motion, it is released in the culture chamber in the same chip. During all the process, the medium reaches the spheroid in a controlled way. Thus, using the same platform to isolate and culture CTCs, the ABSTRACT technology could also be used to personalize drug testing while monitoring the metastatic progression and simplifying the complexity of the CTCs processing.

Prostate cancer

In 2014, CTCs from one patient with metastatic prostate cancer were isolated and enriched by negative selection and red and white blood cell depletion by density

gradient, to be then cultured as organoids as a long-term culture. (38) The 3D culture system confirmed a concordance in gene expression with the primary tumor, the heterogeneity of the prostate cancer and it showed the ability to cause growth tumor in mice. In 2021, diagnostic leukapheresis (DLA) was used in 40 metastatic prostate cancer patients with > 5 CTCs/7.5 mL blood. (39) The author's idea was to prove that starting from a higher number of CTCs would improve the ability to grow them. Through DLA, the authors were able to isolate a median yield of 5312 CTCs, but the culturing results showed organoids formation in only 35% of the collected samples and only one long term culture was established. Even though the cultured CTCs showed the same drug sensitivity as the patient's clinical course, this technique is limited to by the excessive blood volume needed.

Colon-Rectal cancer

In 2015, a permanent cell line was established from a patient with metastatic colon cancer, allowing, for the first time, functional studies on CTCs biology and their role in drugs screening. (40) The cell line, in fact, formed endothelial tubular structures in vitro, and tumors in vivo after xenografting in immunodeficient mice. The cell line expressed an intermediate epithelial/mesenchymal phenotype and it shared the main genomic characteristics of the primary tumor and lymph node metastases. The same group was then able to culture several cell lines from the same patient at different time points with the aim to define colon cancer evolution through CTCs as real time biomarkers. (41) Expression changes were identified during tumor progression and treatment pressure by the comparison of the CTCs cell line evolution during the time and treatment. Driven by the same aim to use CTCs as prognostic biomarker for response to therapy, in 2017, three long term cell lines were established after 4 attempts

from metastatic chemo-naïve patients. (42) Here, the CTCs expressed drug metabolizing pathways as the most upregulated feature, compared to primary CRC cells grown under similar conditions, showing high resistance to conventional cytotoxic compounds. Moreover, CTCs had cancer stem cell markers and were tumorigenic in subcutaneous xenografts. In 2022, to determine the predictive value of CTCs-based precision oncology, CTCs were isolated through density gradient medium and cultured short term as cell line from 36 patients with refractory unresectable rectal cancer or refractory unresectable colorectal cancer liver metastases. (44) Patient were then treated combining the CTCs cell lines drug testing results and gene expression analysis, which showed an overall calculated predictive accuracy of 47.22% (95% CI 0.30–0.64).

Pancreatic cancer

In 2014, for the first time, CTCs from surgically resectable pancreatic ductal adenocarcinoma (PDAC) patients, after isolation through MetaCell, were maintained in a viable state for 14 days on the membrane. (44) Moreover, some of the CTCs invaded through the membrane and grew on the bottom of the 6-well plate, demonstrating their plasticity. In the same year, (45) CTCs were isolated from 18 patients with metastatic PDAC through a geometrically enhanced mixing chip. Even though the authors were able to keep the cells alive on the culture dish for 4 months, they did not proliferate. In 2022, CTCs were isolated from 41 blood samples from 31 metastatic PDAC patients and cultured through a laboratory-developed biomimetic cell culture system without using size elimination or epithelial marker capture methods to preserve CTC heterogeneity. (46) After three weeks, cultured organoids reached the expansion phase, with an efficiency of 87.8% and were tested for sensitivity to a PDAC panel of nine drugs, showing a significant correlation with the patients' clinical response.

Other malignancies

In 2021, CTCs from 60 patients with all stages of hepatocellular carcinoma (HCC) were isolated through density gradient centrifugation and 91% of the enriched CTCs formed 3D spheroids at day 7. (48) In this study, spheroids formation predicted the prognosis more accurately than CTCs enumeration in terms of early recurrence and helped to demonstrate how HCC cells and CTCs expressing EpCAM and β-catenin were associated with spheroid formation in 3D culture and were more tumorigenic and likely to metastasize to the lung in vivo. In 2021, CTCs were isolated and cultured as 3D organoids from advanced-stage head and neck cancer (HNC) patients, who, usually, had a poor response to platinum- based agents. (48) Organoids were then tested with a panel of anti-cancer treatments and the resulting drug sensitivity profiles derived correlated with clinical treatment response to cisplatin in patients.

In vivo CTCs culturing methods

Despite the advanced technology now available to isolate, enrich and enumerate CTCs, their expansion still remains a challenge. In parallel to in vitro experiments, due to the difficulties in establishing in vitro CTCs 2D and 3D cultures, in vivo methods have been developed to culture CTCs faster, while maintaining a higher molecular complexity and heterogeneity, (49) to determine a personalized dynamic model for each single patient. In this section we focus on CTC-derived xenograft (CDX) models and the chicken chorioallantoic membrane (CAM) assay as an alternative to the murine model.

Xenografts

In this section, we will discuss the most relevant studies, where enriched CTCs from cancer patients (or CTCs cell line) were directly injected into immunodeficient mice for expansion of the CTC population.

Breast cancer

The first CDX experiment was described in 2013 from patients with metastatic breast cancer. (50) First the CTCs were enumerated through the SellSearch® system in 350 patients with progression of metastatic breast cancer, following which, the authors, directly transplanted the blood of 110 patients, after depletion of hematopoietic cells, into the femoral medullar cavity of immunodeficient mice, forming bone, lung and liver metastases within 6–12 months, only when the CTCs transplanted were higher than 1000, reflecting a higher CTCs blood concentration. (50) Moreover, a metastasis-initiating cells (MICs) phenotype was described in CTCs expressing CD44, CD47 and MET, which was strongly correlated with decreased progression-free survival. In 2018, in a study on 32 patients, CTCs from one selected patient with advanced metastatic triple-negative breast cancer (TNBC) and high CTCs count, formed detectable tumors in 5 months when injected subcutaneously into mice. (51) Through the characterization of the primary tumor, the metastasis and the CDX the authors confirmed the WNT pathway played a major role in tumor progression and metastasis formation and they were able to identify markers of aggressiveness which could be monitored in CTCs. To investigate the mechanisms of metastatic cascade in TNBC, in 2019, a sequential generation of CDX was established. (52) Through this model the CTCs biology was recapitulated for four sequential generations of mice, allowing the authors to describe a

distinct transcriptomic signature of CTCs consistent throughout the sequential CDX modeling.

Lung cancer

The tumorigenicity of CTCs derived from a patient with a diagnosis of non-small cell lung cancer (NSCLC) was described for the first time in 2016 through the establishment of a CDX model. (53) Blood samples were collected from a male patient with a diagnosis of advanced metastatic NSCLC at multiple time points. A part of the sample was used for CTCs analysis, while the remaining underwent depletion of blood cells and the enriched CTCs fraction was injected in an immunocompromised mouse. Only the post-radiotherapy sample formed a tumor with mesenchymal phenotype. The CDX analysis matched the blood sample used for CTCs analysis, where CTCs were isolated through size-based method (ISET) and not through the CellSearch (EpCAM+), showing the importance of biology of CTCs undergoing EMT, as the absence of CTCs EpCAM+ does not preclude formation of metastasis. In 2014, CTCs were isolated from 6 chemo-naïve SCLC patients. (54) Only samples with a high count of CTCs (> 400) were able to demonstrate tumor growth in 4 months and the established CDXs showed drug sensitivity that was concordant with the same patient's response to treatment. (54) Another group described the possible transitional role of CDX from SCLC. (55) Through an automated microfluidic chip, the authors were able to grow CDX with an efficiency of 38% versus 89% for patient-derived xenograft. Here, the MYC signature was strongly associated with drug resistance. Moreover, serial CDXs generated from an individual patient at multiple time points accurately recapitulated the evolving drug sensitivities of the patient's disease. In the same year, for the first time, chemo-sensitivity was tested in two phenotypically different CDXs from advanced stage SCLC

patients, one chemo-sensitive and one chemo-resistant. (56) CDXs showed a heterogeneous response to olaparib/AZD1775 (PARP and WEE1 inhibitor, respectively) that diminished when tested at the time of disease progression, suggesting its use early after diagnosis and before relapse of disease. The sensitivity to olaparib/AZD1775 was identified in homologous recombination repair genes and oncogenes that induce replication stress (MYC family members).

Prostate cancer

To investigate the role of EpCAM+ CTCs in carcinogenesis, in 2013, CTCs were isolated from 6 patients with metastatic prostate and 2 patients with breast cancer and were injected in mice with increasing amounts of CTCs. (57) After 10 months, the authors found CTCs in the blood, spleen and bone marrow of all the CDXs, even in mice injected with a very small number of CTCs, but no macroscopic tumor were observed. The identification of human CTCs in the peripheral blood, bone marrow and spleen demonstrated how EpCAM+ CTCs maintain the migratory capacity.

In 2020, a CDX model and a CTCs cell line were established by isolating CTCs with diagnostic leukapheresis from a castration-resistant prostate cancer (CRPC) resistant to enzalutamide, with the main aim to describe the transformation of CRPC into an aggressive neuroendocrine disease (CRPC-NE). (58) The CDX and the CDX-derived cell lines conserved 16% and 56% of the mutations detected in the primary tumor and collected CTCs, respectively. Interesting, evolutionary analysis identified a primary tumor subclone harboring TP53 loss as the driver of the metastatic event leading to the CDX. Being able to describe the acquisition of key drivers of neuroendocrine differentiation, could provide a chance for effective drug screening in CRPC-NE management.

Other malignancies

In 2016, to optimize a tailored approach for patients with advanced melanoma difficult to biopsy, CTCs from 47 blood samples from 40 patients were isolated through RosetteSep Human Circulating Epithelial Tumor Cell Cocktail (Stem Cell Technology) and Ficoll-Paque and then injected in mice. Tumors were detected after 1 month from the injection and the overall success rate for CDX was 13%. (59) In this model, the CTCs tumor tropism was similar to the primary tumor. The results were then integrated with whole exome sequencing (WES) and sequencing of circulating tumor DNA (ctDNA) to monitor responses to therapy and to identify and then follow mechanisms of resistance for *BRAF* wild-type and inhibitor-resistant *BRAF* -mutant tumors

The Chorioallantoic Membrane Assay

The chorioallantoic membrane assay (CAM) uses the chorioallantoic membrane, which surrounds the chick embryo at day 10 of embryo development, as an alternative model to grow tumor in vivo. (60) The incomplete development of the immune system at this stage of chicken development, the rich angiogenic features and the hypoxic environment all allow for rapid tumor formation. The CAM assay, moreover, is less expensive than immune-compromised mice and has less ethical issues, since eggs are not considered "animal" yet. (61; 62; 63)

Nowadays, *in ovo* xenografts from primary or metastatic tumor tissue are a well-established model from cell lines to patient and tumor-derived xenografts. Culture conditions for CTCs derived *in ovo* xenograft, instead, have still to be defined, but they represent a promising tool, in particular for *in vivo* real time drug testing suitable for use in the clinical setting. (64)

Recently, CTCs isolated from patients with all stage breast cancer were initially cultured as tumor spheroids with a success rate of 77.35%. (65) Interesting, the number of tumorspheres increased significantly with tumor progression and aggressiveness of primary tumor. Following this, tumor spheroids from 10 breast cancer patients were engrafted to establish a chicken-egg breast cancer model, with an overall success rate of 50%. Also, the grafting of tumorspheres was directly correlated with aggressiveness and proliferation capacity of the primary tumor, showing how the CAM assay permits to grow in time a personalized preclinical tumor model for drug screening and biomarker discovery. In the same year, to validate the CAM xenograft as a reliable tumor model, CTCs were isolated and enriched from 35 patients with metastatic cancer (breast, prostate and lung) through a microfluidic technology. (66) After 48–72 h of in vitro culture in an ultra-low adherence cell dish with Dulbecco's Modified Eagle's medium, CTCs were engrafted into the CAM of embryonated chicken eggs at day 9 of embryonic development and then resected 9 days after engraftment, with a success rate of 61%. This study confirmed the possibility to realize a personalized tumor model in line with the patient's treatment time.

However, CAM models still have limitations. Obtaining experimental results is hampered by the growth and development of chicken embryos, the short time window for observation and treatment, and species-specific differences. For instance, oxygen diffuses into the blood vessels and surrounding tissues on the CAM surface, whereas in vivo mammalian tumor models and clinical cases frequently experience severe hypoxia, which can promote metastatic progression. In in vivo mammalian models, tumors generally arise within the tissue or organ of the host, providing an environment that more closely mimics the native tumor microenvironment found in humans. Nevertheless, CAM models are the most accessible and reliable preclinical platforms for studying oncogenesis related to various oncological conditions.

The expansion of circulating tumor cells remains a critical challenge in cancer research, yet both CTC-derived xenograft models and the chorioallantoic membrane assay offer valuable, complementary platforms to address this gap. While CDX models provide in-depth, longitudinal insights into tumor progression, heterogeneity, and therapeutic response, their extended timeframes and costs limit rapid clinical application. Conversely, the CAM assay enables faster tumor growth and personalized drug testing in a cost-effective and ethically favorable setting, though it requires further optimization for long-term studies. Together, these models enhance our understanding of CTC biology and hold promise for advancing precision oncology through tailored therapeutic strategies.

The ability to efficiently isolate and culture CTCs serially during the clinical course of a patient, will permit to identify aggressive clones that drive metastatic progression and to perform drug sensitivity analyses to identify the most suitable therapy for each patient and allow us to monitor the course of the disease on a more sophisticated level. In the first section we discuss isolation of viable CTCs, following which we detail the most recognized techniques to culture CTCs, both in the in vitro and in vivo settings. We aim to highlight the importance of CTC culturing to define them as dynamic real-time biomarkers of tumor biology and to predict drug sensitivity for precision approaches in the management of cancer.

Aim of the project

The current proposal is aimed at optimize the CAM model technique for culturing of CTCs from patients with a diagnosis of PDAC. This will allow to expand and to characterize CTCs as a prognostic biomarker. The novelty of this project is the combination of two powerful techniques, represented by the ISET® technology for viable CTCs isolation and the CAM model for the subsequent culturing step.

Originality and Innovative Aspects

Currently, no clinically available test can allow for serial assessment of circulating tumor cell biology and culturing. If we were able to develop this technology, real-time drug sensitivity profiling could allow serial assessment of circulating disease and tailor therapy to eradicate it, resulting in long-term survival.

In this context, we think that the CAM model could represent a promising, fast, low-cost and easy-to-use tool, in particular for *in vivo* real time drug sensitivity profiling that could be used in the clinical practice.

Objectives

Main objective

Pre-clinical phase and preliminary experiments: optimization of the CAM model through the implantation of human pancreatic cell lines on the CAM of 100 fertilized eggs.

Secondary objectives

1. To describe the correlation between the number of the cells implanted on the CAM and their growth patterns.

2. To improve the growth time of the cells implanted on the CAM.

3. To establish the minimal viable cell number required for implantation on the CAM

to achieve sustained growth within the relevant timeframe.

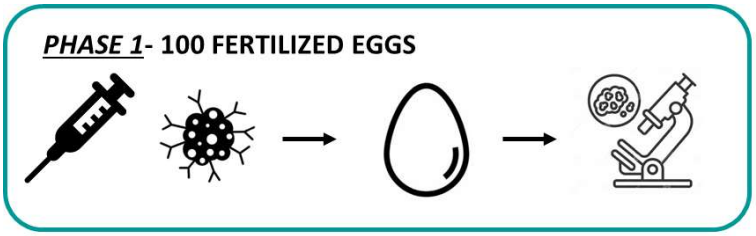
4. To develop complementary strategies to promote and optimize cellular proliferation on the CAM.

The future objective is to validate the CAM assay on 70 patients with a diagnosis of locally advanced or metastatic PDAC (clinical phase). Once the clinical phase will be accomplished, this technique will be utilized to develop a pipeline for pharmacotyping of CTC cultures to identify association between predicted response and clinical course of PDAC patients.

Research design and Methodology

Prior to testing on human sample, we aimed to improve our current technique for the CAM model using the human pancreatic cancer cell line Mia-Pa-Ca 2, till the development of a new protocol.

Pre-clinical phase



To optimize the CAM assay, we implanted a decreasing concentration of human pancreatic cell lines (MIA PaCa-2) into the CAM of 100 eggs, starting with 10^6 cells/egg, and gradually reducing cell concentration till 10 cells implanted, obtaining reproducible results. Similarly to other studies, we implanted the cells at incubation day (ID) 10. Normally the CAM stays attached to the eggshell. To make it to collapse and being usable to cell culture, we used a Dremel point to create a pin hole on the void chamber and another 3 mm hole in a less vascularized area of the eggshell between two big caliber vessels. Once the membrane has collapsed, using a Dremel rotating disc, we

enlarged the 3 mm hole in a $1cm^2$ square as operating window to implant the cells. To avoid debris on the CAM, we were able to remove the hard part of the eggshell first and separately from the inner and softer part. The area for cells implant was carefully selected. We chose a CAM vessel bifurcation as implant site to augment the blood supply.

To set the optimal technique, we analyzed the results coming from different methods to implant the cells. We always mixed the cells with a different proportion of Matrigel (based on the cells concentration and thus on the total amount of cells medium).

The different techniques that we tested are:

a. Direct implant of cells and Matrigel on the CAM through a micropipette.
b. Implant of the cells and Matrigel on the CAM after 15 min of incubation.
c. Injection of the cells solution through a needle on an insulin syringe, after a 3min incubation.
d. Direct implant of cells and Matrigel on the CAM through a micropipette after a 3 min incubation and a gently laceration of the CAM with a sterile cotton swap to improve the cells implant.

As final technique, we implanted the mix of cells and Matrigel through a pipette after a short period of incubation (3 minutes) on a well-defined CAM (vessel bifurcation) area previously lacerated using a sterile cotton swap.

The implanted eggs rested in the incubator in a defined temperature and humidity till ID 18. At day-18 of embryo development, we harvested the CAM and the liver of each embryo. (Figure 1)

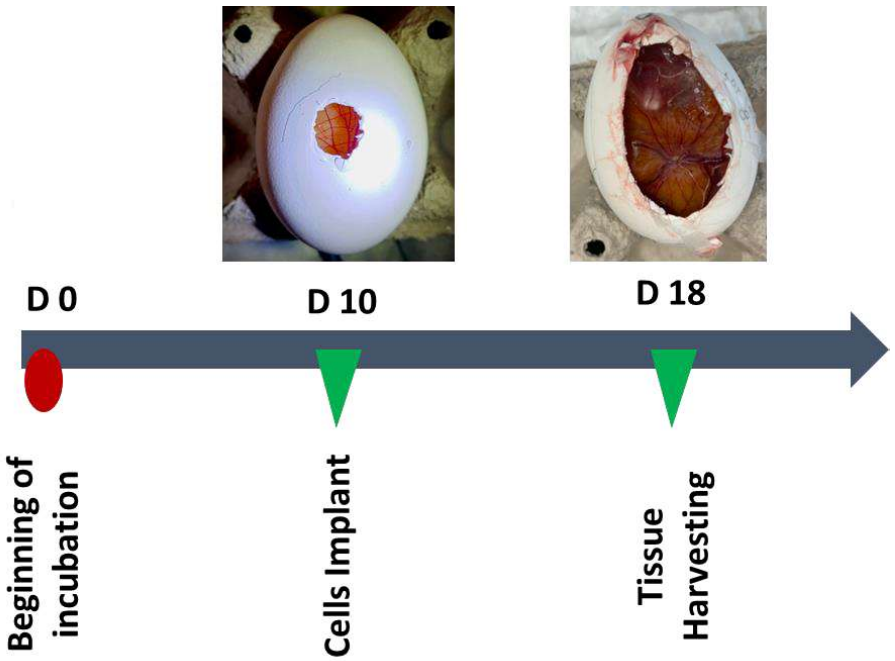


FIGURE 1: TIMELINE FOR CELLS IMPLANT AND TISSUE HARVESTING

The final Protocol for *in ovo* MIA PaCa-2 xenograft

Reagents and Equipements:

1. 30 G needles
2. Parafilm
3. Laboratory tape 1/2" x 500" (VWR, catalog number: 470144-262)
4. Fertilized chicken eggs
5. MIA PaCa-2 cell lines
6. Trypsin 0.05% and 0.25% with EDTA (1 mM), liquid
7. Phosphate-buffered saline (PBS) (1x, pH 7.4), liquid
8. Penicillin-streptomycin (10,000 U/ml)
9. Dulbecco's modified Eagle medium (DMEM)

10. Fetal bovine serum

11. Matrigel

12. Incubator 37 °C, 60% humidity

13. Egg incubator + rotating eggs trays (Hatching Time, CT60)

4. Egg candler

5. Microsurgical kits, sterile forceps, push pin, dissection scissors, needle nose forceps

6. Dremel 100 rotary tool

7. Dremel cut off wheels number 36

9. P2-P20 pipette

10. P20-P200 pipette

Procedure:

Preparing the eggs for xenografting tumor cells

1. Incubate freshly fertilized chicken eggs on their side in a rotating incubator at 37 °C and 60% humidity for 10 days.
2. The eggs are rotated three times per hour.
3. On day 10, place the eggs on their side on an egg rack.
4. Use a tube lamp or other suitable light source to candle the eggs by shining the light at the blunt end of the egg where the air sack is located. The embryo must be located near the bottom of the egg and the air sack on its right. Localize and mark using a pencil the allantoic vein that is located at the top of the eggshell, right where several blood vessels cross. (Figure 2)

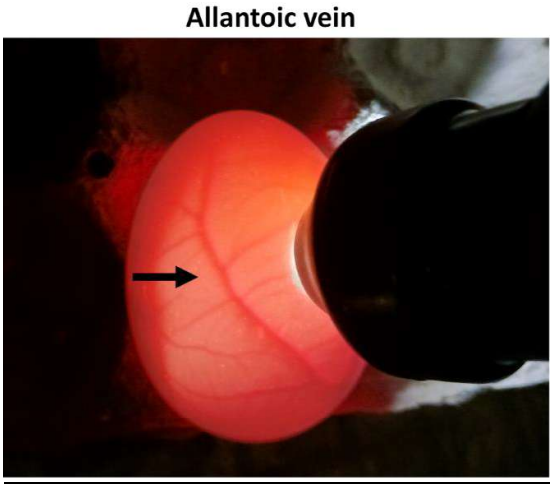


FIGURE 2: IDENTIFICATION OF THE ALLANTOIC VEIN

5. Clean the area, including and around the mark, using a cotton swab soaked with iodine.

6. Identify the attachment of the developing embryo to the chorioallantoic membrane as a dark moving vessel attached to the egg membrane and use a pencil to mark the attachment to avoid interventions in this region.

7. Mark the center of the air sac region. (Figure 3)

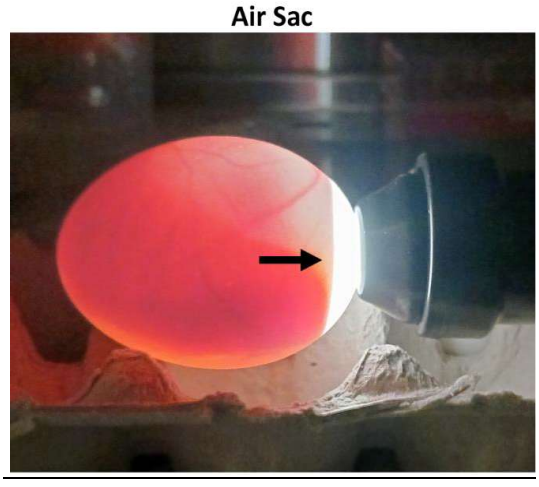


FIGURE 3: IDENTIFICATION OF THE AIR SAC

8. Mark a square area in a less vascularized area, between two big vessel.

9. Use a rotary tool to carefully drill the eggshell within the marked square. Use blunt forceps to remove the eggshell without removing the white outer eggshell membrane right under the shell.

10. Add 30 microliters of HBSS to the square opening over the intact inner and softer eggshell membrane.

11. Use the drill to carefully make a pinpoint drill perforation in the marked cross in the air sac area to allow airflow into the egg without breaking or damaging the shell.

12. Using a 30 gauge needle, make a pinpoint perforation in the outer membrane within the square area.

13. Hold the egg up to the light source to visualize the air sac and apply pressure to an eyedropper rubber bulb.

14. Place the eyedropper bulb over the small perforation within the air sac and release the pressure on the bulb until a separation of the outer membrane and chorioallantoic membranes within the operating window area is observed repeating this step until a complete separation of the membranes is achieved. (Figure 4)

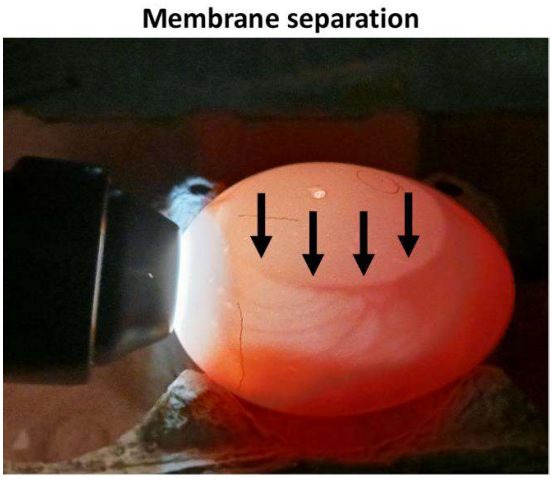


FIGURE 4: MEMBRANE SEPARATION

15. Drow a square area (operating window) about 1.5 cm, including in the area the first square opening. (Figure 5)

16. When all of the eggs have been modified in the same manner, drill the operating window in one egg taking care not to rupture the outer eggshell membrane and use the sticky side of a piece of adhesive tape to remove any loose particles from the shell. Using blunt forceps, remove the eggshell from the drilled area followed by the outer eggshell membrane taking care not to introduce small shell particles inside the egg to minimize contamination.

17. When all of the eggs have been grafted, return them to the humidifying egg incubator for two days without rotation. At the end of the incubation, transfer the eggs to the laminar flow cabinet for seven days without rotation. At the end of the incubation, place the eggs onto the laboratory bench top. Use a syringe to perforate the film dressing of each egg.

FIGURE 5: MAIN STEPS FOR THE OPERATING WINDOW OPENING

Harvesting tumors and chick embryo tissues

1. Perform dissections in an area free of contaminating DNA. To detect metastatic invasion this assay quantifies the presence of ectopic DNA in chicken organs using a highly sensitive PCR approach. Therefore, it is very important to prevent contamination with exogenous DNA.

2. In order not to have cross-contamination of your samples, use three separated sets of surgery tools. One set for cutting the egg, one set for harvesting the primary tumor and one set for removing the internal tissues.

3. Prepare three wash containers for sequential rinsing of the tools between each animal and change their liquid between different experimental groups.

Sequentially these washes are: distilled water, 70% ethanol and 1x PBS.

4. Get the eggs from the stationary incubator.

5. Enlarge the window with a pair of surgical scissors. Make sure not to cut too low, otherwise the CAM will be damaged, resulting in bleeding of the injured vessels.

6. Add 300 - 500 µl of PBSD with a pipette onto the CAM section containing the inoculated material. If no fluorescent probes are used, buffered formaldehyde may be used as this makes the membrane stiffer, facilitating the cutting of the membrane.

7. Make a photograph of the CAM in the egg through a stereo fluorescence microscope, equipped with a digital color camera, using both the GFP or the DSR filter, and no filter. For the tumor grafts, all photographs are made without filters. Illumination from above by LED lights is strongly recommended during photographing.

8. Record tumor cell migration for the labeled cells. The borders of the tumors may be irregular and frayed. Scattered cells may be present near the tumor borders. In some specimens, rows of tumor cells may be observed

9. Cut the membrane with a pair of sterile scissors at the border lying against the shell.

10. Place the harvested CAM in a sterile Petri dish filled with PBSD or buffered formaldehyde.

11. Make a photograph of both the upper side and the lower side of the CAM, both with and without filter.

12. Harvest also the liver of the chicken embryo.

13. Put the membrane and the liver in a labeled container with buffered formaldehyde 4% for 24 h

14. Put the samples in ethanol 70% and leave them in the cold room

15. Embed the CAMs in paraffin and prepare them for histological examination (IHC, H&E).

    Take care to cut the tumor grafts at the center of the nodule, making sure the cut surface is parallel to the bottom of the cassette. If not, the interaction between the CAM and the tumor graft will not be visible.

16. Eventually, part of the tumor could be divided into:

    - Liquid nitrogen
    - Freezing media to be engrafted in the next batch of eggs.

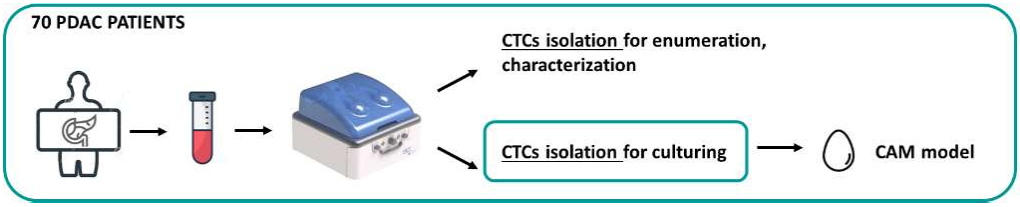
17. Remove the chick embryo from the eggshell by cutting the shell radially into equal halves.

18. Transfer the embryo to a clean weight boat. The animal must be dissected using a clean set of tools.

19. Open the chick embryo cutting through the sternum. Once the embryo is open, collect a piece of the liver. To harvest the lung, cut the rib cage and separate from the breast.

20. Harvested samples can be processed immediately for DNA isolation or stored at -80 °C for later extraction of DNA, if necessary.

Clinical phase



Once the pre-clinical phase will be accomplished, CTCs from 70 patients with a diagnosis of metastatic or locally advanced PDAC will be isolated, characterized and cultured through the CAM model.

From each patient we will collect 15 ml of peripheral blood at the diagnosis, before the start of the systemic therapy.

For each patient:

5 ml blood will be used to fix the isolated CTCs on the membrane of the ISET® TECHNOLOGY (Rarecells) to be counted and phenotypically characterized. CTCs phenotype characterization will be performed through immunofluorescent staining technique with a combination of pan-cytokeratin and vimentin antibodies to assess epithelial and mesenchymal cell traits, respectively. In addition, in order to ensure a high degree of specificity in CTCs assessment, cells will be stained with an antibody ‘‘cocktail’’ (anti-CD45, CD11b, CD14, CD34) to distinguish different white blood cells (WBCs) populations from CTCs. CTCs will be stratified as epithelial-type (pan-cytokeratinþ, vimentin-, CD-), mesenchymal type (vimentin, pan- cytokeratin-, CD-), and epithelial/mesenchymal- type (pan-cytokeratin, vimentin, CD-). Detected cells will be additionally assessed for morphology characteristics (nuclei and cytoplasmic structures). CTCs identification and counting will be performed through the Nikon Ti-E inverted microscope system (Nikon, Japan) based on CD-marker negativity and pan-cytokeratin and/or vimentin positivity.

10 ml blood will be used for the isolation of viable CTCs using the ISET® Technology (Rarecells) and to subsequently culture them through the CAM model. Normally the CAM stays attached to the eggshell. To make it collapse and become usable for cell culture, we will use a Dremel drill to create a pin hole on the void chamber and another 3 mm hole in a less vascularized area of the eggshell between two big caliber vessels. Once we are sure that the membrane has collapsed, we will enlarge the 3mm hole to a $1cm^2$ which will serve as an operating window to inject the cells. The region for cell implantation will be carefully selected. We will choose a CAM vessel bifurcation to augment the blood supply. (Figure 4) The implanted eggs will rest in the incubator in a defined temperature and humidity till day 18 of embryo development. At day-18 of fertilization, prior to hatching, we will harvest the CAM of each embryo. The tissues will be fixed in paraffin and sliced to confirm the presence of the CTCs and enumeration through H&E and immunohistochemistry. Patients and tumor characteristics associated with successful culturing will be identified to optimize our technique even further and accelerate and improve CTC biomass for further analysis.

Results

Considering all the 146 eggs implanted in the pre-clinical phase, we were able to see macroscopic tumor growth in 82% of the eggs, regardless of the technique used for the cells implantation. (Figure 6)

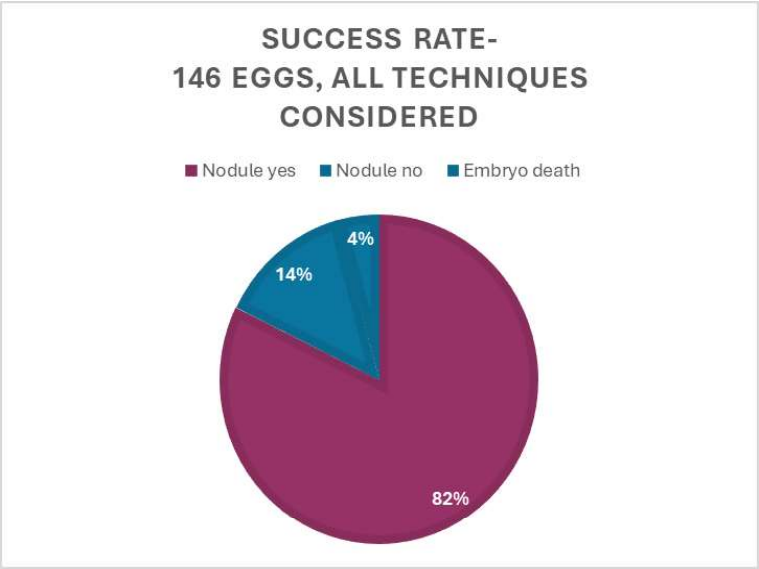


FIGURE 6: SUCCESS RATE CONSIDERING ALL THE 146 EGGS IMPLANTED

Among the 21 CAMs without any cellular growth, in 5 of them (4%) the absence of the nodule was due to the embryo death.

Taking into account only the definitive implantation technique used in 60 embryos, the success rate increased to 90%. Here, no embryo death was reported. (Figure 7)

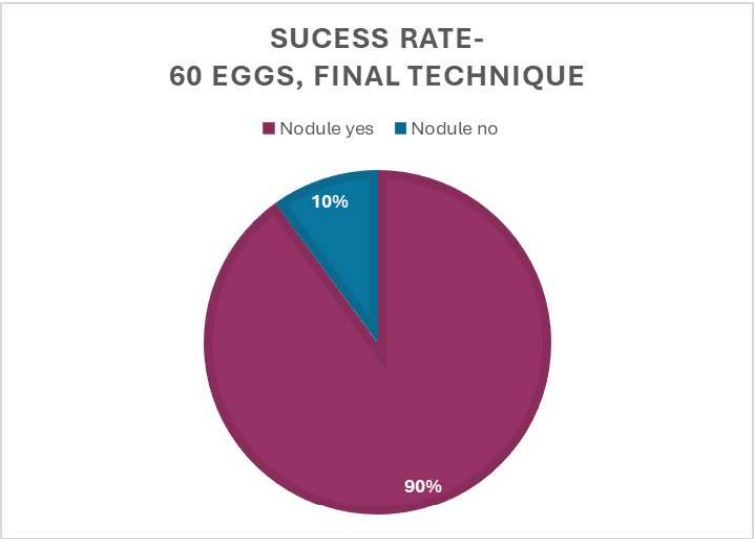


FIGURE 7: SUCCESS RATE CONSIDERING ONLY THE FINAL TECHNIQUE

Looking in detailes: (Figure 8)

- In the embryos with 1000 cells implanted: in a total of 18 embryos, in 14 of them we observed a macroscopic nodule;
- In the embryos with 500 cells implanted: we observed a macroscopic nodule in all of them (9 in total);
- In the embryos with 100 cells implanted: we observed a macroscopic nodule in all of them (17 in total);
- In the embryos with 10 cells implanted: in a total of 16 embryos, in 14 of them we observed a macroscopic nodule.

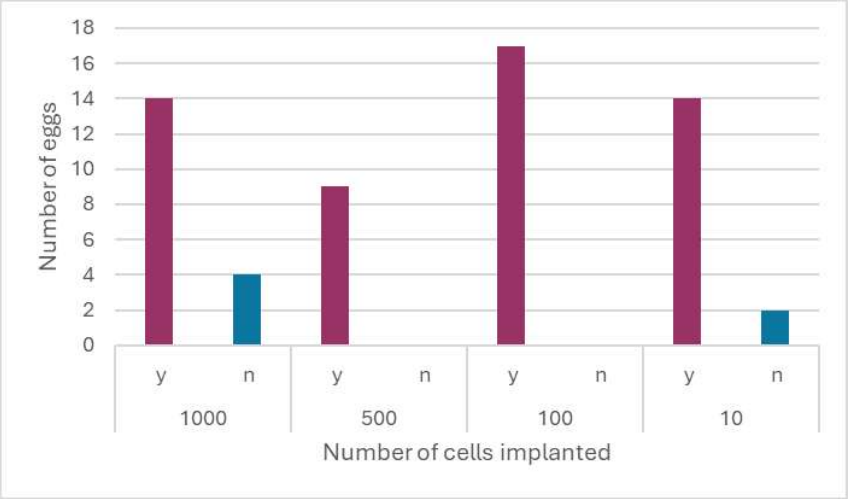


FIGURE 8: FINAL TECHNIQUE: NODULE GROWTH CONSIDERING THE DECREASING NUMBER OF CELLS IMPLANTED
(y: growth on the CAM; n: no growth on the CAM)

The size of the observed nodules shows variability and does not appear to be directly correlated with the number of implanted cells. The factors that could be related to the size of the nodules include: the presence of Matrigel, which is essential for cellular connections; the possible presence of debris mainly derived from the eggshell; and the recruitment of cells such as macrophages. For this reason, refining the technique, even in its smallest details, is a fundamental part of the project. (Figure 9)

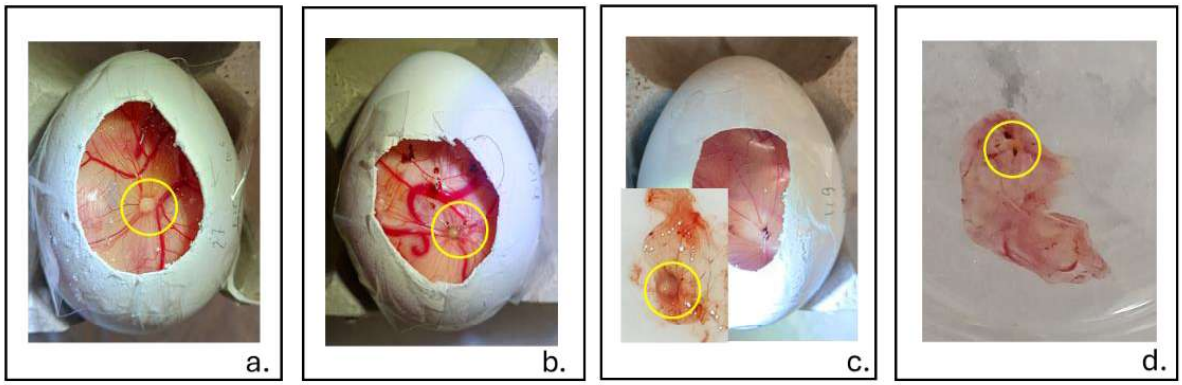


FIGURE 9: SOLID NODULE ON THE CAM AFTER IMPLANTATION OF 10^6 (A.), 10^3 (B.), 500 (C.), AND 10 MIA PA-CA-2 CELLS

In particular:

1. Matrigel: This is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and it mimics the natural extracellular matrix. Its inclusion is vital because it provides structural support for the cells, facilitating their attachment and growth. Without Matrigel, cells may not form stable connections, leading to irregular nodule sizes. Ensuring the right concentration of Matrigel can significantly impact the outcomes, as too little may fail to support cell growth, while too much could inhibit proper interaction.

2. Debris from Eggshell: The potential presence of eggshell debris can also influence the experimental results. If fragments are not adequately removed, they can interfere with the cell environment, impacting cell behavior and nodule development. Cleaning and preparing the workspace and materials meticulously can help mitigate this issue. Using filtration techniques or a thorough rinsing process can ensure that any unwanted particles are eliminated.

3. Recruitment of Macrophages: Macrophages play a crucial role in the immune response and can be recruited to the site of the nodules. Their presence can influence the growth and size of the nodules by either promoting or inhibiting cellular proliferation. Understanding the signals that attract these immune cells can help in designing the experiment. For instance, incorporating specific cytokines or growth factors could enhance their recruitment, thus potentially altering the nodule development.

In light of these factors, the refinement of technique is critical. Each small detail—from the preparation of the Matrigel and the implantation technique to the elimination of debris and the modulation of immune cell recruitment—can dramatically affect the experiment's success. Systematic adjustments and careful observations are essential for achieving consistent and reliable results.

By focusing on these areas, the project can achieve a deeper understanding of the cellular interactions at play, ultimately leading to more effective outcomes in research or therapeutic applications.

To prove the presence of the Mia-PA-Ca 2 cancer cells, we did H&E and IHC analysis, observing growth of tumor cells as shown in the figure. In the H&E analysis, we were able to observe the presence of tumor cells, particularly due to the images of mitoses, which were later confirmed as such by IHC. Specifically, the Mia Pa Ca 2 cells are positive for CK8 and, as demonstrated in the figure, CK8 positive cells were observed on the membrane. (Figure 10)

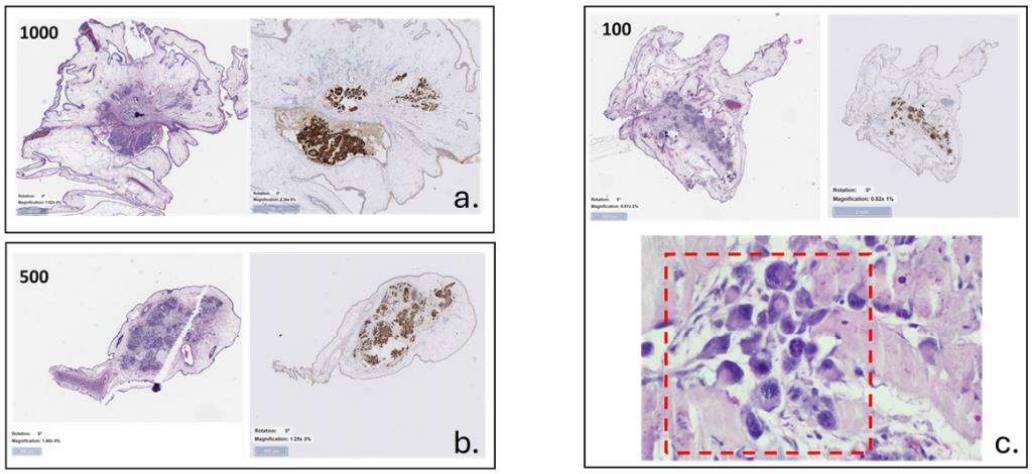


FIGURE 10: H&E AND IHC CONFIRMATION OF THE MIA PACA-2-CELLS GROWTH AFTER IMPLANTATION OF 1000 (A.), 500 (B.), 100 (C.) MIA PACA-2- CELLS.

Discussion

CTCs are recognized as the main driver of metastatic spread (24) through a complex and multi-step cascade, which is not yet well understood. It starts with cell invasion and subsequent entry in the blood stream through active intravasation or passive cell shedding. (67) Upon entry into the circulation, the CTCs, or their clusters, have to survive the shear stress and hostile conditions and have to evade the immune system. This microenvironment potentially induces changes in CTCs, and through natural selection the ones that survive exit the bloodstream either actively or passively. For these reasons, understanding their functional properties could provide a deeper and dynamic knowledge on tumor evolution and, in particular, its dissemination. CTC enumeration and subtyping as prognostic biomarkers have already shown promise, but we are still far from understanding their complete biology. The possibility to culture CTCs, first, will increase the number of cells that are available to be analyze. Comparing them with the primary tumor and the metastatic sites will help to understand the transitional process of the evolving tumors cells and, as a consequence, will permit to define new targets for a tailored therapy. Moreover, if CTCs are expanded in a short

time, they could also function as real-time predictive biomarkers for assessment of disease.

Different techniques to isolate viable CTCs and to culture them have been described, but no single technique is recognized as to be much superior. Viable CTCs can be isolated through antibody-based enrichment methods, where EpCAM is the most common used protein, due to a lack of cancer- specific proteins. Different surface proteins based- microfluidics technologies have been developed, which have the capability not only to isolate, but also to culture the CTCs in the same chip, but their complexity and the need of validation hamper large-scale production and clinical integration. (68; 69; 70) Technologies based on a functional approach seem very promising, because of their capability to isolate and characterize the CTCs and maintaining CTC heterogeneity, however, the culture efficacy has still to be validated. (71) The most commonly used techniques to isolate viable CTCs take advantage of their larger size and lower deformability compare to the other blood cells. The physical properties-based methods are in turn divided on the complexity of the technology applied. Density gradient media have been widely used to isolate blood cells components. They can be adapted to CTCs and combined with RosetteSep® antibody cocktail to improve the recovery rate. (26; 72) Even if the centrifugation might result in a partial loss of CTCs, they are easily applicable and cost effective. Density gradient media are frequently used when the isolation technique needs to fix CTCs for counting and characterization. (30) More sophisticated technologies and available for large scale use, based on physical properties, have already started to gain interest. The ISET device is a highly accurate, non-invasive liquid biopsy technology, which uses a powerful size-based isolation system to detect CTCs from whole blood, keeping them intact and ready for further analysis or culturing.

Once viable CTCs have been isolated and enriched, their expansion is fundamental for further functional analysis. The techniques to culture CTCs can be divided into two main categories with the *in Virto* methods being the most well described. These techniques can be used to assess the molecular profile of CTCs, to test drugs sensitivity and response and to improve our understanding of the metastatic cascade. Even when the low yield of CTCs in the peripheral blood represents a large obstacle, multiple groups were able to describe culturing conditions for CTCs; at least for short term cultures. Considering breast cancer, in 2013, for the first time, short term CTCs cell lines were established from 36 metastatic patients (33). Here the authors were able to identify a specific signature in CTCs Ep-CAM- responsible for brain metastasis. In 2020, it was reported that a long-term cell line was established from a patient with metastatic breast cancer and maintained the same hormone and copy number alterations profile over the culture time. CTCs isolated from all stages breast cancer patients were also able to form clusters in short- and long-term (30) cultures. In 2014, a long-term culture of organoids from CTCs isolated from metastatic ER+ breast cancer patient was described (73) and all the six cultures reflected the typical heterogeneity of ER+ breast cancer. In the same year, multiple organoids lines were established from CTCs isolated from early stage lung cancer patients, which carried mutations of the *TP53* gene identical to those observed in the matched primary tumors (74). Following this in 2019, a permanent cell line with metastatic potential was established from a patient with early stage lung cancer (34). In 2016 a permanent cell with high levels of E-cadherin consistent with EMT but no stem cell markers were developed and in 2020 tumorsphere structures formed in a clinically relevant time frame were tested for drugs sensitivity, showing a strong correlation with the patients' clinical response (36). A permanent cell line was also established from CTCs isolated from a metastatic unresectable colorectal cancer patient, which not only showed a stable phenotype, but also was able to result in

tumor growth when transplanted in mice. (40, 41) Additionally, organoids established from a high CTCs yield prostate cancer patient were able to reflect the classical heterogeneity of prostate cancer. (38) In 2022, CTCs were expanded to form organoids from advanced pancreatic cancer patients with a high success rate, and their drugs sensitivity profiles showed correlation with the patients' clinical response. (46)

Even though cell culture technologies have demonstrated success in *in vitro* cultures of CTCs, they remain as isolated examples. The extremely variable and unpredictable number of CTCs among different cancer types and the different technologies used to isolate and culture the cells, makes it difficult to interpret, compare and reproduce. Moreover, the most promising results were obtained applying the microchip technology, which is not yet usable on a large scale. Considering the difficulties in establishing *in vitro* CTCs cultures, (31; 33), the *in vivo* methods, as the CDX and CAM models, are gaining more importance, mainly because of their ability to better recreate the cancer molecular complexity and heterogeneity, and the possibility to directly test drugs. In breast cancer, three main examples (50; 51; 52) demonstrate how direct subcutaneous mice injection of CTCs enriched fraction after whole blood depletion from the blood cells, are able to metastasize to distant organs. CTCs isolated from breast cancer patients, after being previously cultured as tumor spheroids, were also successfully implanted in the CAM of chicken embryos, where the tumor growth was directly correlated with the aggressiveness of primary tumor. (65) Similarly, CTCs isolated through a microfluidic device and pre-cultured *in vitro* in an ultra- low attachment plate were after implanted in the CAM model, demonstrating genomic concordance with the primary tumor and metastatic potential. (66) Considering lung cancer and SCLC, multiple studies have been able to demonstrate how CTCs, isolated through density gradient medium, where able to grow tumor after subcutaneous mice injection for drug testing (54; 55; 56) and metastatic progression analysis. (53)

Despite the fewer number of studies present in the literature, the *in vivo* models appear to be more promising not only in terms of the rate of success, but also as patient-specific dynamic model for drug testing and assessment of progression of disease. In particular, considering the fundamental importance to have a real time model that is able to permit rapid cells growth, while maintaining their original genome profile, the CAM appear to be the most promising model, in particular if combined with a previous *in vitro* short time culture.

This project describes the possibility of culturing and expanding CTCs after their isolation as live cells, using a technique that is already established and well-documented in the literature, but not yet applied to individual circulating cells. The CAM model, in fact, allows for accelerated growth of implanted cells due to its rich vascularization, which facilitates the transport of oxygen and nutrients, along with the incomplete development of the embryo's immune system. To be effective, the membrane must remain intact and clean, and the embryo should be grown in an environment with controlled temperature and humidity. The complex part of the project is represented by the description of a new implantation technique, building on those already present in the literature, but making modifications to promote the growth not only of a very low number of cells (as few as 10), but also of cells that normally circulate within our body's vascular system and are subjected to continuous stress.

Conclusions

In conclusion, despite rapid advancements in the field, functional analysis of CTCs remains challenging. While the *in vitro* cell cultures are more frequently described in literature the *in vivo* models seem to have more promise. In particular, the CAM assay, is emerging as a technique which facilitates rapid growth of CTCs while maintaining tumor biology. CTCs are promising biomarkers of serial assessment of evolving tumor

biology which are currently limited by the low yield. With the development of isolation and culturing techniques that can allow rapid expansion of CTCs, their potential applications in the clinical setting will expand allowing for near real-time assessment of disease, precision approaches to patient management and improved survival.

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