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Modelling CRS/HIPEC Treatment Response Using Patient-Derived Organoid-
CAF Co-Cultures from Colorectal Peritoneal Metastases

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I.ABSTRACT

Peritoneal metastases from colorectal cancer (CRCPM) represent the second most common site of metastasis in colorectal cancer (CRC) and are associated with a highly unfavorable prognosis. The introduction of cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) has led to improved outcomes in the treatment of this disease; however, approximately 60% of patients experience recurrence following treatment. For this reason, the implementation of personalized therapeutic strategies is of great importance.

Patient-derived organoids (PDO) have emerged as valuable models, as they can faithfully reproduce the morphological and genetic features of the original tumour. These models have been successfully used for drug screening studies, enabling the identification of potential new therapeutic options. However, PDO are derived primarily from the epithelial component of the tumour and do not fully recapitulate tumour complexity, as they lack elements of the surrounding microenvironment. More advanced models incorporating additional cellular components could improve the identification of effective treatments.

Cancer-associated fibroblasts (CAF), a major stromal component of the tumour microenvironment, are known to play a key role in modulating tumour behavior and response to therapy.

To better reproduce the tumour microenvironment of CRCPM, and to evaluate the impact of CAF on response to treatment, I developed three PDO models and two CAF lines from CRCPM patients' tissue. These models were used to establish co-culture systems combining PDO and CAF. Comparative analysis of the response of PDO, CAF, and the co-cultures to the drugs schemes commonly used for HIPEC treatment showed that presence of CAF significantly influences the therapeutic response. In particular, PDO co-cultured with CAF exhibited increased resistance to treatment compared to organoids grown in monoculture conditions.

Overall, this work highlights the importance of incorporating stromal components into in vitro models to more accurately mimic tumour complexity and improve the predictive value of preclinical systems. These findings support the development of more effective, personalized therapeutic approaches for patients with CRCPM.

ABSTRACT

Le metastasi peritoneali da carcinoma del colon-retto (CRCPM) costituiscono la seconda localizzazione più comune di malattia metastatica nel tumore del colon-retto (CRC) e si associano ad una prognosi sfavorevole. L'introduzione della chirurgia cito riduttiva (CRS) combinata con la chemioterapia ipertermica intraperitoneale (HIPEC) ha permesso di migliorare gli esiti di questi pazienti, ma circa il 60% va comunque incontro ad una recidiva dopo il trattamento. Per questo motivo, diventa cruciale poter contare su strategie terapeutiche personalizzate.

In questo contesto, gli organoidi derivati da paziente (PDO) si sono affermati come modelli preziosi, poiché sono in grado di riprodurre fedelmente le caratteristiche morfologiche e genetiche del tumore di origine. Questi modelli sono stati utilizzati con successo in studi di screening farmacologico, consentendo l'identificazione di potenziali nuove opzioni terapeutiche. Tuttavia i PDO derivano dalla componente epiteliale del tumore e non ricapitolano completamente la complessità tumorale, essendo privi degli elementi del microambiente circostante. Modelli più complessi, che incorporano più componenti cellulari, potrebbero migliorare l'identificazione di trattamenti efficaci.

Tra le componenti stromali del microambiente tumorale, i fibroblasti associati al cancro (CAF) occupano un ruolo di primo piano, essendo noti per la loro capacità di modulare sia il comportamento del tumore sia la risposta alle terapie.

Con l'obiettivo di riprodurre meglio il microambiente tumorale delle CRCPM e di comprendere quale peso abbiano i CAF nella risposta ai trattamenti, ho sviluppato tre modelli di PDO e due linee di CAF a partire da tessuto di pazienti con CRCPM. Questi modelli sono stati utilizzati per allestire sistemi di co-cultura PDO-CAF. Confrontando la risposta di PDO, CAF e co-culture agli schemi farmacologici tipicamente impiegati nel trattamento HIPEC, è emerso che la presenza dei CAF condiziona in modo significativo la risposta terapeutica: i PDO coltivati insieme ai CAF si sono infatti dimostrati più resistenti al trattamento rispetto agli organoidi in monocultura.

Nel complesso, questo lavoro evidenzia l'importanza di includere le componenti stromali nei modelli in vitro per rappresentare con maggiore fedeltà la complessità del tumore e migliorare il valore predittivo dei sistemi preclinici. Questi risultati supportano lo sviluppo di approcci terapeutici più mirati ed efficaci per i pazienti con CRCPM.

II. Abbreviations

2D – Two-Dimensional

3D – Three-Dimensional

APC – Adenomatous Polyposis Coli

AUC – Area Under the Concentration–Time Curve

BMP – Bone Morphogenetic Protein

BRAF – B-Raf Proto-Oncogene

CAF – Cancer-Associated Fibroblasts

CBC – Crypt Base Columnar (cells)

CIMP – CpG Island Methylator Phenotype

CIN – Chromosomal Instability

CRC – Colorectal Cancer

CRCPM – Colorectal Cancer–Derived Peritoneal Metastases

CRS – Cytoreductive Surgery

CRS/HIPEC – Cytoreductive Surgery Combined with Hyperthermic Intraperitoneal Chemotherapy

CSCs – Cancer Stem Cells

CXCL12 – C-X-C Motif Chemokine Ligand 12

dMMR – Deficient Mismatch Repair (Mismatch Repair Deficient)

DMEM-F12 – Dulbecco’s Modified Eagle Medium F12

DMSO – Dimethyl Sulfoxide

DOX – Doxorubicin

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EMT – Epithelial–Mesenchymal Transition

FGF4 – Fibroblast Growth Factor 4

FIT – Fecal Immunochemical Test

FOBT – Fecal Occult Blood Test

GSK3 – Glycogen Synthase Kinase 3

HIF-1 – Hypoxia-Inducible Factor 1

HIPEC – Hyperthermic Intraperitoneal Chemotherapy

HSPs – Heat-Shock Proteins
IC50 – Half-Maximal Inhibitory Concentration
IL-6 – Interleukin 6
IL-8 – Interleukin 8
JAK – Janus Kinase
KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog
LGR5 – Leucine-Rich Repeat-Containing G Protein-Coupled Receptor 5
LOX – Lysyl Oxidase
MLH1 – MutL Homolog 1
MMPs – Matrix Metalloproteinases
MMP-2 – Matrix Metalloproteinase 2
MMP-9 – Matrix Metalloproteinase 9
MMP-14 – Matrix Metalloproteinase 14
MMR – Mismatch Repair
MSI – Microsatellite Instability
MSI-H – Microsatellite Instability-High
PD-1 – Programmed Cell Death Protein 1
PDGF-C – Platelet-Derived Growth Factor C
PDO – Patient-Derived Organoids
PDX – Patient-Derived Xenograft
PDTXs – Patient-Derived Tumour Xenografts
PIK3CA – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PM – Peritoneal Metastases
PMI – Peritoneal Body Index
pMMR – Mismatch Repair Proficient
PSCs – Pluripotent Stem Cells
SNAI1 – Snail Family Transcriptional Repressor 1
STAT3 – Signal Transducer and Activator of Transcription 3
TA – Transit-Amplifying (cells)
TGF-β – Transforming Growth Factor Beta
TNM – Tumour, Node, Metastasis (staging system)
TP53 – Tumour Protein p53
uPA – Urokinase-Type Plasminogen Activator
VEGF – Vascular Endothelial Growth Factor

1.Introduction

1.1. Colorectal Cancer

Colorectal cancer (CRC) represents a major global health burden, with over 1.8 million new cases and 881,000 deaths estimated worldwide in 2018 (Bray F. et al., 2018). The disease occurs more frequently in men than in women, with incidence and mortality rates in females being approximately 25% lower than in males. CRC is traditionally considered an age-related disease, as the majority of cases occur in older adults. However, in recent years, a concerning increase in early-onset CRC has been observed in several countries, highlighting a growing and highly relevant issue in current cancer research. Its distribution varies widely by geographic region, with up to a 10-fold difference in incidence and mortality worldwide, and the highest rates observed in more industrialized countries, suggesting an important contribution of socioeconomic development, lifestyle, and access to screening programs. CRC burden is expected to increase by 60% by 2030, reaching over 2.2 million new cases and 1.1 million deaths (Arnold M. et al., 2017). From an etiological perspective, epidemiological evidence indicates male sex and increasing age as major risk factors, and only about 4% are diagnosed before age 50. Hereditary and environmental factors also contribute to CRC development. Most of CRC cases are sporadic, whereas approximately 10–20 % of cases show familial aggregation, likely reflecting a combination of genetic susceptibility and shared environmental exposures; by contrast, high-penetrance inherited syndromes account for only a small proportion of all, (Henrikson et al., 2015). Several lifestyle- and environment-related factors, including smoking (Botteri E. et al., 2008), alcohol abuse (Cai S. et al., 2014), obesity (Kyrgiou M. et al., 2017), and excessive consumption of processed food and red meat (Chan et al., 2011), have been associated with an increased CRC risk. Conversely, preventive strategies based on diet and lifestyle modifications appear to play a key role in risk reduction. Higher intake of fiber, whole grains, calcium, and milk has been associated with a lower risk. Moreover, evidence supports potential protective roles for vitamin D, folate, and fruits and vegetables, highlighting diet as an important modifiable factor in CRC prevention, partly through its effects on inflammation, obesity, and gut microbiota composition (Song M. et al., 2015). In this context, growing evidence highlights the role of the gut microbiota in CRC pathogenesis. Alterations in microbial composition may represent one of the mechanisms through which diet and other environmental exposures influence colorectal carcinogenesis. Moreover, specific bacterial species, such as *Fusobacterium nucleatum* and *Bacteroides fragilis*, have been associated with a

higher risk of CRC development (Nakatsu G. et al., 2015; Wong X. et al., 2018; Kostic et al., 2013).

1.1.1. Diagnosis of CRC

Over the last decade, CRC mortality has decreased by more than 20%, mainly due to advances in diagnostic techniques and the optimization of surgical, neoadjuvant, and palliative therapies (Kekelidze M. et al., 2013). This improvement is partly attributable to earlier detection, as well as to progress in multidisciplinary management. CRC diagnosis is definitively confirmed through colonoscopy with tissue biopsy for histopathological assessment, which remains the reference standard (Barret M. et al., 2013; Kaminski M.F. et al., 2010). Colonoscopy is a structural examination that allows direct visualization of the entire colon and rectum, from the appendiceal orifice to the dentate line, and it represents a key tool not only for diagnosis but also for screening. Importantly, it enables biopsy collection and the removal of precancerous lesions (polypectomy) during the same procedure, contributing not only to early detection but also to cancer prevention. However, colonoscopy is invasive, requires bowel preparation and often sedation, and its diagnostic performance may vary depending on the operator's expertise, while rare complications such as bleeding and perforation can occur (Levin B. et al., 2008). These non-invasive stool-based tests are widely used in screening programs for average-risk individuals, whereas colonoscopy is generally performed at longer intervals and is reserved as a primary screening or follow-up diagnostic procedure, depending on national guidelines and individual risk profiles. CRC is clinically classified according to the TNM staging system, which evaluates the extent of the primary tumour (T), the involvement of regional lymph nodes (N), and the presence of distant metastases (M), providing the basis for stage assignment and treatment planning. In particular, stage III disease may be further stratified into low-risk (T1–3N1) and high-risk categories (T4N1–2 or any T with N2), reflecting differences in prognosis and therapeutic management (Benson A.B. et al., 2018). In addition to conventional histopathological and radiological assessment, molecular characterization has become increasingly relevant in the diagnostic work-up of CRC. Microsatellite instability (MSI), resulting from mismatch repair deficiency (dMMR), can be assessed to identify CRC patients with Lynch syndrome (a hereditary form of CRC caused by inactivating mutations in DNA repair genes) as well as a subgroup of sporadic tumours. This molecular subtype is characterized by high tumour mutational burden and increased neoantigen formation, features that are associated with greater sensitivity to immune checkpoints inhibitors. Consequently, MSI-high/dMMR CRC is

more likely to benefit from immunotherapy approaches such as PD-1 blockade (e.g., pembrolizumab) (Le D.T. et al., 2017).

1.1.2. Pathogenesis of CRC

CRC, like many solid tumours, develops through a multistep process driven by the sequential accumulation of genetic alterations in genes regulating cell growth, differentiation and survival. This progressive model of carcinogenesis is supported by the observation that CRC harbors approximately 80 somatic mutations that are present in tumour cells but absent in normal tissue (Frank D. et al., 2008). Most colorectal cancers arise from a precursor lesion through a gradual multistep process that begins with an aberrant crypt, progresses to a preneoplastic lesion (polyp), and ultimately evolves into invasive carcinoma over an estimated 10–15-year period. The initiation and progression of CRC are thought to involve stem cells and stem-cell-like populations, and cancer stem cells (CSCs)—defined by self-renewal and differentiation capacity—within the intestinal epithelium. These cells are thought to play a key role in tumour maintenance and progression and are closely associated with metastasis, tumour recurrence, and therapy resistance through regulation by intestinal signaling pathways and microenvironmental cues (Nassar D. et al., 2016; Dekker E. et al., 2019).

Colorectal cancer arises predominantly through two distinct molecular pathways (Figure 1) that lead to the formation of precursor lesions: the adenoma–carcinoma pathway and the serrated pathway. These pathways differ not only in the morphology of their precursor lesions, but also in their underlying genetic and epigenetic alterations.

The adenoma–carcinoma pathway is the classical and most frequent route of CRC development, accounting for approximately 70–90% of cases. It is characterized by chromosomal instability (CIN) and originates from conventional adenomatous polyps. This pathway is initiated by inactivation of the *APC* tumour suppressor gene, followed by additional mutations in oncogenes such as *KRAS* and loss of *TP53*, which drive progression from adenoma to carcinoma.

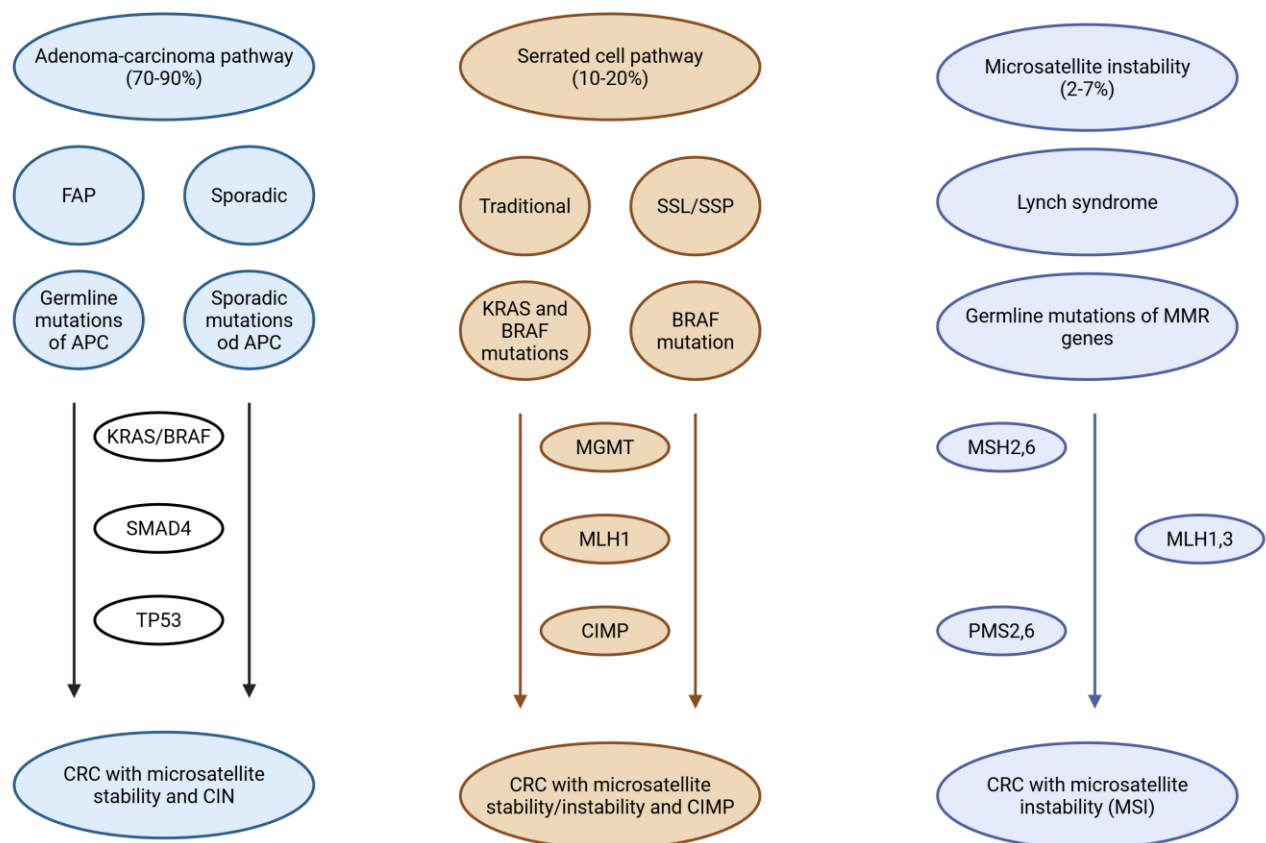


Figure 1: The figure illustrates the pathways leading to the development of colorectal cancer (CRC). (A) The canonical adenoma–carcinoma pathway is characterized by the sequential accumulation of genetic mutations and chromosomal instability (CIN), resulting in tumours that are microsatellite stable (MSS). (B) The serrated pathway is often initiated by mutations in the *BRAF* or *KRAS* genes and subsequently progresses through methylation of tumour suppressor genes. (C) Microsatellite instability (MSI) results from inactivation of genes involved in DNA mismatch repair mechanisms and is a hallmark of Lynch syndrome. Image adapted from “Colorectal cancer” by Evelien Dekker et al., The Lancet, 2019, and created using BioRender.

The serrated pathway, which accounts for approximately 10–20% of CRCs, arises from serrated precursor lesions, such as sessile serrated adenomas/polyps. This pathway is molecularly defined by activating mutations in *BRAF* or *KRAS*, extensive CpG island methylation (CIMP), and frequent microsatellite instability (MSI) due to epigenetic silencing of mismatch repair genes, particularly *MLH1* (Dekker E. et al., 2019).

1.1.3. Comparison between Left-Sided and Right-Sided Colon Cancer

Proximal (right-sided) and distal (left-sided and rectal) CRCs exhibit distinct molecular characteristics (Figure 2). This biological heterogeneity reflects differences in embryologic origin,

tumour microenvironment, and molecular oncogenesis. Right-sided colon cancers appear to follow distinct molecular pathways of oncogenesis and are more frequently characterized by microsatellite instability (MSI/dMMR), CpG island methylation, and a higher tumour mutational burden, together with increased alterations in the EGFR pathway, including higher mutation rates in genes such as *BRAF* and *PIK3CA* (Salem M.E. et al., 2017; Petrelli F. et al., 2017). Many of these features, with the exception of MSI-high status, have been associated with poorer survival, supporting their prognostic relevance in CRC (Tabernero J. et al., 2018). In contrast, left-sided colon and rectal cancers generally show a lower frequency of MSI/dMMR and a higher prevalence of mutations in key tumour suppressor genes, particularly *APC* and *TP53*, supporting the concept of a molecular gradient from the right colon to the rectum (Salem M.E. et al., 2017). Left-sided CRC typically shows lower immune cell infiltration than right-sided tumours and is generally associated with a more favorable prognosis. Moreover, cancers arising from the left colon account for the majority of CRC cases, representing approximately 70% overall (Jess P. et al., 2013).

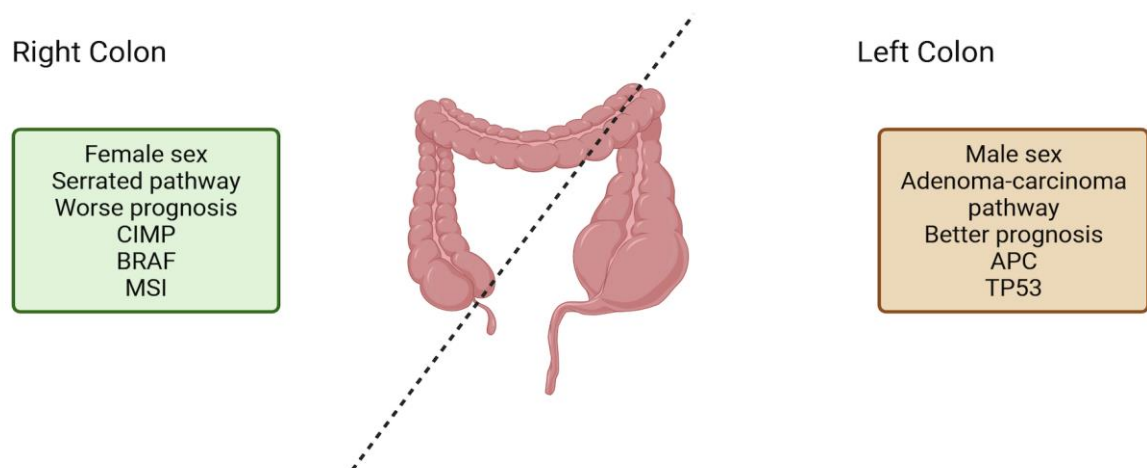


Figure 2: The figure illustrates the main differences between left- and right-sided colorectal cancer. Image created using BioRender.

1.2. Peritoneal Metastases from CRC

1.2.1. Clinical Presentation and Diagnostic Challenges

The peritoneum is the second most frequent site of metastasis in colorectal cancer. Peritoneal metastases (PM) originating from CRC (CRCPM) occur in roughly 25% of cases and are associated with a worse prognosis compared to other common CRC metastatic sites, such as the liver and lungs (Kim YJ et al., 2021; Park SH et al., 2021; Doah KY et al., 2021). This pattern

of dissemination is clinically relevant because peritoneal involvement is often associated with diffuse disease spread and limited therapeutic options.

The peritoneum is the largest serous membrane of the human body, lining the abdominal cavity and covering the intra-abdominal organs, with a surface area comparable to that of the skin. Anatomically, it consists of two continuous layers: the parietal peritoneum, which lines the internal surface of the abdominopelvic cavity, and the visceral peritoneum, which forms the inner layer covering the intra-abdominal organs and the mesentery. Both layers share a similar histological organization composed of a monolayer of mesothelial cells resting on a basement membrane and an underlying submesothelial connective tissue layer known as the submesothelial stroma. This stroma consists of loose connective tissue rich in extracellular matrix components, fibroblasts, blood vessels, and lymphatic channels, providing structural support and contributing to fluid exchange, inflammatory responses, and tissue repair (Van der Wal JB et al., 2007).

The mesothelium itself is composed of a monolayer of mesothelial cells of mesenchymal origin that can display different morphological shapes, ranging from flattened to cuboidal. These cells function as a selective barrier regulating the transport of fluids, solutes, and cells across the serosal surface and play an important role in maintaining abdominal homeostasis, facilitating low-friction organ movement, and participating in inflammatory and repair processes following injury or surgical trauma (Mutsaers and Wilkosz, 2007). These structural and functional properties are also relevant to the pathogenesis of peritoneal metastasis, as they influence tumour cell adhesion, implantation and dissemination within the peritoneal cavity.

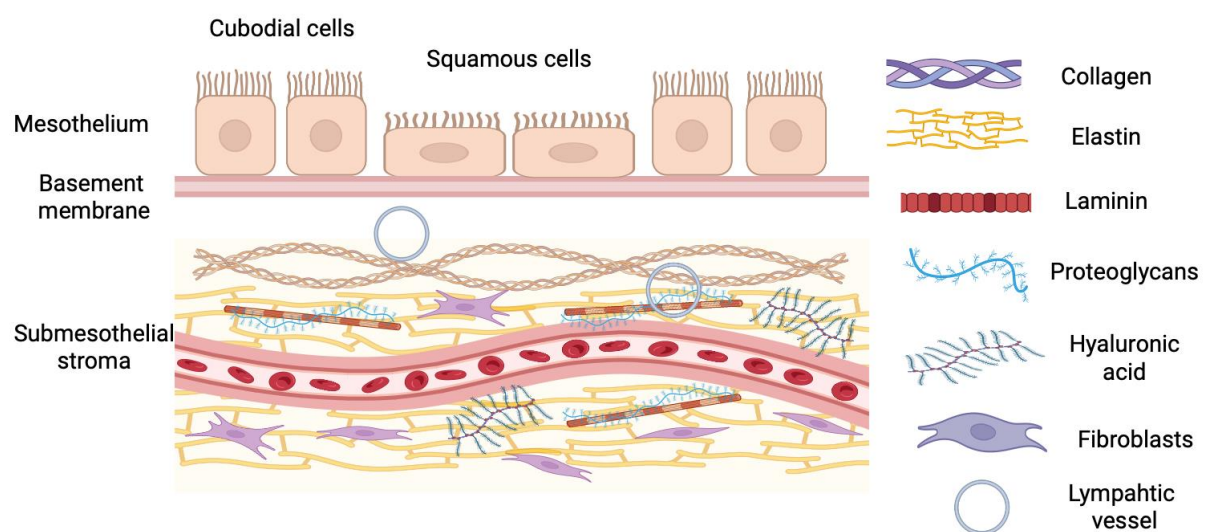


Figure 3: The image shows the structure of the peritoneum. The peritoneum is composed of a monolayer of mesothelial cells, consisting of either flattened squamous cells or cuboidal cells supported by a basement membrane. On the surface of mesothelial cells, microvilli are present, varying in shape, number, and density. The basement membrane is a thin layer composed of type I and IV collagen, proteoglycans, and glycoproteins. Finally, the submesothelial layer is formed by various components of the extracellular matrix (ECM), including collagen, elastin, laminin, proteoglycans, and glycosaminoglycans (GAGs). This layer also contains blood vessels, lymphatic vessels, and fibroblasts. The image was adapted using BioRender from “Pathophysiology of Colorectal Peritoneal Carcinomatosis” by Lemoine et al., World Journal of Gastroenterology, 2016.

1.2.2. The Peritoneal Metastatic Cascade

The development of CRCPM is a complex biological process resulting from interactions between tumour cells and elements of the peritoneal microenvironment. This process is driven by a molecular crosstalk between cancer cells and host components and occurs through a series of sequential and interdependent steps collectively referred to as the peritoneal metastatic cascade (Lemoine et al., 2016).

The metastatic cascade begins with the detachment of tumour cells from the primary colorectal tumour. Individual tumour cells or clusters of malignant cells can separate from the primary tumour mass and gain access to the peritoneal cavity. Several mechanisms may contribute to this detachment, including the downregulation of adhesion molecules such as E-cadherin, epithelial–mesenchymal transition (EMT), increased interstitial fluid pressure within the tumour microenvironment, and mechanical factors associated with surgical manipulation of the tumour (Lemoine et al., 2016).

Once tumour cells are released into the peritoneal cavity, they become subject to the physiological transport mechanisms of peritoneal fluid. The movement of peritoneal fluid is influenced by factors such as respiration, gravity, and intestinal peristalsis, resulting in predictable circulation patterns within the abdominal cavity. These fluid dynamics facilitate the dissemination of free tumour cells and contribute to their distribution to specific anatomical regions that are more susceptible to metastatic implantation (Lemoine et al., 2016).

Following dissemination, tumour cells undergo adhesion to the peritoneal surface, which represents a critical step in the metastatic process. This attachment can occur through two main mechanisms: transmesothelial and translymphatic dissemination. In transmesothelial metastasis, tumour cells adhere directly to mesothelial cells through several adhesion molecules expressed on both tumour and mesothelial cell surfaces. In contrast, in the translymphatic pathway, tumour cells gain access to submesothelial lymphatic vessels through small anatomical openings known as lymphatic stomata, which connect the peritoneal cavity with the underlying lymphatic system. These structures are involved in the absorption of fluids and cells from the peritoneal cavity but may also facilitate tumour dissemination. Tumour implantation is frequently observed in specialized immune structures known as milky spots, which are mainly located in the greater omentum. These structures are considered preferential sites for early metastatic implantation, as they consist of aggregates of immune cells and contain numerous lymphatic vessels, providing a favorable microenvironment that supports the survival and early growth of metastatic tumour cells (Lemoine et al., 2016).

After adhesion to the peritoneal surface, tumour cells invade the submesothelial space. This invasion requires the disruption of the mesothelial monolayer and subsequent interaction with the underlying basement membrane. Tumour cells may induce apoptosis of mesothelial cells or exploit areas where the mesothelial layer is already disrupted. Following penetration of the mesothelium, tumour cells attach to the basement membrane through integrin-mediated interactions and degrade extracellular matrix components using proteolytic enzymes, particularly

matrix metalloproteinases (MMPs). These enzymes facilitate the degradation of the extracellular matrix and the peritoneal-blood barrier, thereby enabling further invasion into the subperitoneal tissue (Lemoine et al., 2016).

The final stage of the metastatic cascade involves tumour proliferation and angiogenesis, which are essential for the establishment and growth of metastatic lesions. As tumour cells proliferate within the submesothelial environment, the formation of new blood vessels becomes necessary to supply oxygen and nutrients. This process is mediated by several molecular factors, including hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), which promote angiogenesis and sustain tumour expansion within the peritoneal cavity (Lemoine et al., 2016).

Importantly, the steps of the peritoneal metastatic cascade do not occur as isolated events but rather represent a continuous and interconnected process in which tumour cells interact dynamically with the peritoneal microenvironment. A deeper understanding of these mechanisms may support the development of more effective preventive, locoregional and targeted therapeutic strategies for peritoneal metastases in colorectal cancer (Lemoine et al., 2016).

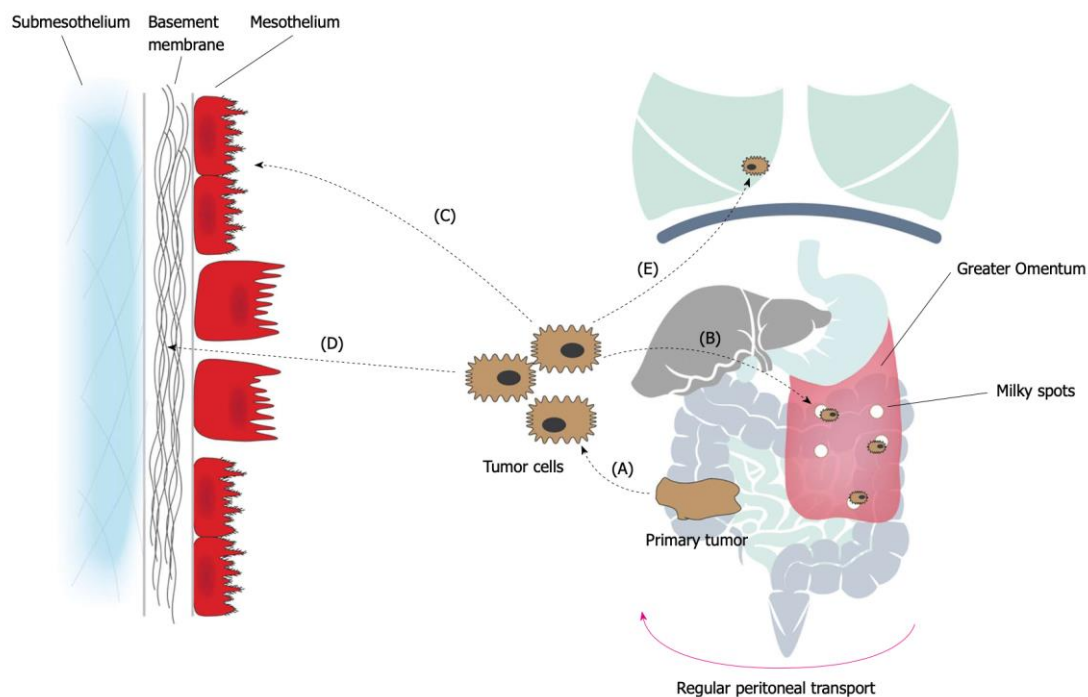


Figure 4: The figure illustrates the events characterizing the peritoneal metastatic cascade: (A) Tumour cells detach from the primary tumour and enter the peritoneal cavity, (B) becoming susceptible to physiological peritoneal transport. (C) The adhesion process begins, with tumour cells attaching along the peritoneal mesothelium through two pathways: the transmesothelial route and the translymphatic route. Tumour cells tend to colonize the milky spots present in the greater omentum. (D) Finally, neoplastic cells invade the submesothelial space through the release of metalloproteinases. Image adapted from “Pathophysiology of Colorectal Peritoneal Carcinomatosis” by Lemine et al., World Journal of Gastroenterology, 2016.

1.3.Cytoreductive surgery and Hyperthermic Intraperitoneal Chemotherapy

1.3.1.Rationale and Therapeutic Objectives

For many years, CRCPM was considered a lethal condition with limited therapeutic options, as systemic chemotherapy provided only modest improvements in survival (McMullen JRW et al., 2017). Historically, no curative surgical strategies were available for this condition. However, the introduction of cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) has changed the therapeutic landscape for carefully selected

patients. This therapeutic strategy, originally developed by Paul Sugarbaker, involves the surgical removal of all visible tumour deposits within the peritoneal cavity, followed by the intraoperative administration of heated cytotoxic chemotherapy directly into the peritoneal cavity to eradicate residual microscopic disease. This sequence is particularly important because the limited tissue penetration of HIPEC makes complete CRS a prerequisite for optimal treatment efficacy. The chemotherapeutic agents used in HIPEC are typically characterized by a relatively large molecular weight, enabling limited penetration into peritoneal tissues, generally reaching a depth of up to approximately 3 mm, which is sufficient to target residual tumour cells while minimizing systemic absorption. The chemotherapeutic solution is delivered under hyperthermic conditions, usually between 41 and 43 °C, which enhances the cytotoxic effects of the drugs and improves their penetration into peritoneal tissues. Clinical studies have shown that this combined approach can substantially improve survival outcomes compared with conventional treatments, with reported median survival ranging from 22 to 63 months and 5-year survival rates of 40–51% in selected patients (McMullen JRW et al., 2017).

The administration of chemotherapeutic agents directly into the peritoneal cavity provides a significant pharmacological advantage compared with systemic chemotherapy. This loco-regional approach allows the delivery of high drug concentrations directly to residual tumour cells within the peritoneal cavity while limiting systemic exposure and associated toxicity. This advantage is largely attributed to the presence of the peritoneal membrane, which acts as a transport barrier and restricts the rapid transfer of drugs into the systemic circulation. As a result, intraperitoneal chemotherapy enables dose intensification at the tumour site. The magnitude of this pharmacokinetic advantage is commonly assessed by comparing the area under the concentration–time curve (AUC) in the peritoneal cavity with that in the systemic circulation (Van der Speeten K et al., 2012).

Hyperthermia exerts its antitumour effects through a variety of cellular and molecular mechanisms. One of the primary mechanisms is the denaturation of proteins, which has been shown to correlate closely with the thermal dose required to induce cell death. Heat exposure leads to the unfolding and aggregation of cytoplasmic and nuclear proteins, impairing their function and disrupting essential cellular processes. In addition, hyperthermia inhibits protein synthesis and alters membrane fluidity, thereby affecting transmembrane transport and cellular homeostasis. At the nuclear level, heat does not directly induce significant DNA damage; however, it interferes with DNA repair mechanisms by inhibiting repair enzymes and altering DNA conformation. Furthermore, hyperthermia induces the expression of heat-shock proteins (HSPs),

which act as molecular chaperones to counteract protein damage, although their protective capacity is limited at higher temperatures. Collectively, these effects contribute to the cytotoxic action of hyperthermia and enhance its therapeutic potential, particularly when combined with other treatment modalities (Hildebrandt et al., 2002).

Moreover, hyperthermia plays a critical role in intraperitoneal chemotherapy, as the use of chemotherapeutic agents under elevated temperatures can increase cytotoxicity at the peritoneal surface while maintaining limited systemic toxicity. In addition, hyperthermia has been shown to enhance the penetration of chemotherapeutic drugs into tumour tissue, thereby improving their therapeutic efficacy (Sugarbaker PH et al., 2005).

The chemotherapeutic agents used for intraperitoneal chemotherapy are selected based on specific pharmacological properties that optimize their effectiveness within the peritoneal cavity. In particular, these agents typically possess a relatively high molecular weight and hydrophilic characteristics, which promote prolonged retention within the peritoneal cavity and limit rapid absorption into the systemic circulation. In addition, many drugs used for this approach demonstrate synergistic effects when combined with hyperthermia, as elevated temperatures can increase cytotoxic activity at the peritoneal surface and improve drug penetration into tumour tissues. Another important consideration is the compatibility of these agents with other chemotherapeutic drugs, which allows the development of combination treatment regimens aimed at enhancing therapeutic efficacy (Sugarbaker et al., 2005).

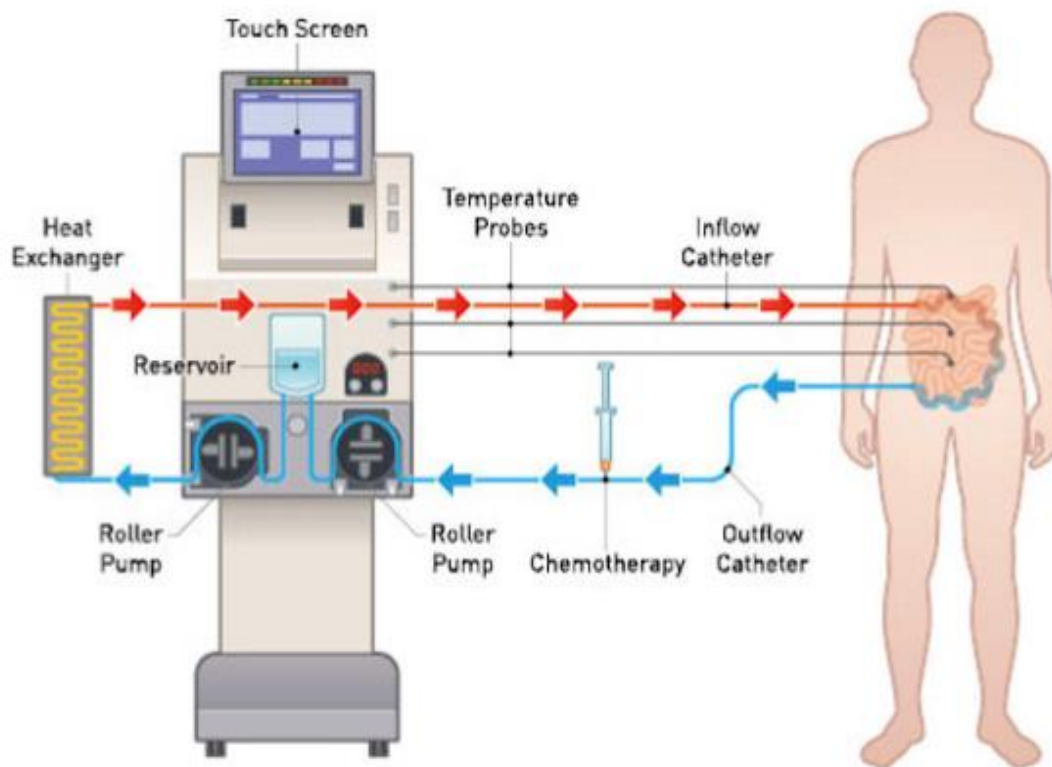


Figure 5: The image illustrates the HIPEC treatment scheme for the management of CRCPM. The peritoneal cavity is perfused with the abdomen either open or closed using a perfusion pump that delivers a solution containing the chemotherapeutic agent(s) (mitomycin C, cisplatin, doxorubicin, and oxaliplatin). The perfusion volume is calculated based on the patient's peritoneal body index (PMI). Image from <https://www.foxchase.org/clinical-care/departments-programs/clinical-departments/surgical-oncology/surgical-techniques/hipec>

At present, no universally accepted standard has been established for intraperitoneal chemotherapy protocols. The administration protocols for intraperitoneal chemotherapy show considerable variability among different institutions, including differences in drug dosage, perfusate volume, temperature, duration, and timing of treatment. This heterogeneity makes it difficult to directly compare clinical outcomes and pharmacokinetic data across studies (Van Ruth et al., 2003).

Clinically, this combined approach has been associated with substantial survival benefit. Indeed, patients with colorectal peritoneal carcinomatosis have a median survival of approximately 4–7 months without treatment, while systemic chemotherapy alone may extend survival to 12–23 months. In contrast, cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (CRS/HIPEC) has been associated with median survival ranging

from 22 to 63 months in selected patients, with reported 5-year survival rates of 40–51% (McMullen JRW et al., 2017).

Drug	Mechanism of action
Mitomycin C (MMC)	It is a cytotoxic antibiotic capable of intercalating into DNA and generating cytotoxic radicals, causing DNA breakage and fragmentation. In addition, MMC can interfere with protein synthesis.
Cisplatin (CDDP)	It belongs to the class of alkylating agents and is therefore able to form bonds with DNA bases, particularly with the nitrogen atom of guanine. This binding inhibits DNA replication and transcription, leading to cellular apoptosis.
Doxorubicin (DOX)	It is an anthracycline antibiotic with antineoplastic activity. It exerts its cytotoxic effect by intercalating into the DNA double helix, forming a drug–DNA complex and inhibiting topoisomerase II.
Oxaliplatin (OHP)	It is an alkylating agent similar to cisplatin and has a similar mechanism of action.

Table 1: The table describes the mechanism of action of different drugs that can be used in HIPEC treatment.

1.4.Organoids

1.4.1.Core Concepts and Biological Characteristics of Organoids

Traditionally, cancer research has relied on a variety of experimental models to study tumour biology and evaluate therapeutic strategies. Among the most commonly used human cancer models are two-dimensional (2D) cancer cell lines and primary patient-derived tumour xenografts (PDXs). Cancer cell lines, derived from primary patient material, have contributed significantly to cancer research; however, their establishment is often inefficient and requires extensive adaptation to in vitro 2D culture conditions. As a result, only a limited number of clones are able to expand, frequently undergoing genetic alterations and failing to accurately reflect the genetic heterogeneity of the original tumour. Furthermore, these models lack important components of the tumour microenvironment, such as stromal compartments. PDXs, generated through the transplantation of freshly derived patient tumour tissue into immunodeficient mice, better preserve the biological characteristics of human tumours and enable pre-clinical testing of novel therapies. Nevertheless, these models also present several limitations, including dependence on animal use, limited engraftment efficiency for certain tumour types, and high costs in terms of time and resources. More broadly, many conventional cancer models fail to faithfully recapitulate the complexity of patient tumours, which contributes to the limited translation of preclinical findings into successful clinical outcomes (Drost J., Clevers H., 2018).

Although Patient-derived xenograft (PDX) models retain many features of the original tumours, they remain subject to important and practical constraints. PDX models are established by transplanting tumour tissue obtained from cancer patients into immunodeficient mice, allowing the tumour to grow and be propagated through multiple in vivo passages. These models are increasingly used in cancer research because they are believed to preserve many of the genomic and biological characteristics of the original human tumours, thereby providing a valuable platform for studying tumour biology and therapeutic response (Ben-David U. et al., 2017). Despite their widespread use, patient-derived xenograft models present important limitations, including genomic instability during passaging, clonal selection driven by the murine microenvironment, and progressive divergence from the original tumour, which may ultimately affect their reliability in modelling tumour biology and therapeutic response. In addition, these models rely on animal systems, show limited engraftment efficiency for certain tumour types, and require considerable time and resources. Moreover, mouse-specific tumour evolution may further affect their biological relevance (Ben-David U. et al., 2017; Byrne, 2017).

In this context, organoid technology has emerged as a valuable alternative for modelling epithelial tumours in vitro. Organoids are in vitro culture systems derived from epithelial cells isolated from tissue-specific niches, such as intestinal crypts, where stem cells are responsible for continuous tissue self-renewal. Under appropriate culture conditions, these cells can expand long term and give rise to complex three-dimensional structures that undergo crypt-like budding and generate villus-like epithelial domains. Organoids produce multiple differentiated epithelial cell types and display key features of the native intestinal epithelium, including its architecture, cellular composition, and self-renewal dynamics. Importantly, this culture system can be applied to a variety of tissues, enabling the growth of both normal and pathological samples, such as adenomas, colorectal cancer, and Barrett's epithelium. Furthermore, organoid-based models provide a platform for the study of inflammatory, infectious, and neoplastic processes, as well as for applications in pharmacological and regenerative research (Sato T. et al., 2011; Fujii M. et al., 2016).

1.4.2. Principal methodologies used for the development of organoids

Organoid research has experienced rapid progress in the past decade, reflecting its increasing relevance in biomedical research (Takahashi et al., 2021). Organoids can be generated from different types of stem cells, mainly through two complementary approaches, highlighting their versatility as in vitro models. They can be derived from pluripotent stem cells (PSCs), including embryonic and induced pluripotent stem cells, as well as from adult stem cells (aSCs), also known as tissue-specific stem cells. While PSC-derived organoids are obtained through direct differentiation, thereby recapitulating key steps of embryonic development, aSC-derived organoids originate from tissue biopsies and retain key characteristics of the tissue of origin, making them particularly suitable for the study of adult tissue homeostasis and patient-specific disease models (Huch M. et al., 2015; Takahashi et al., 2021).

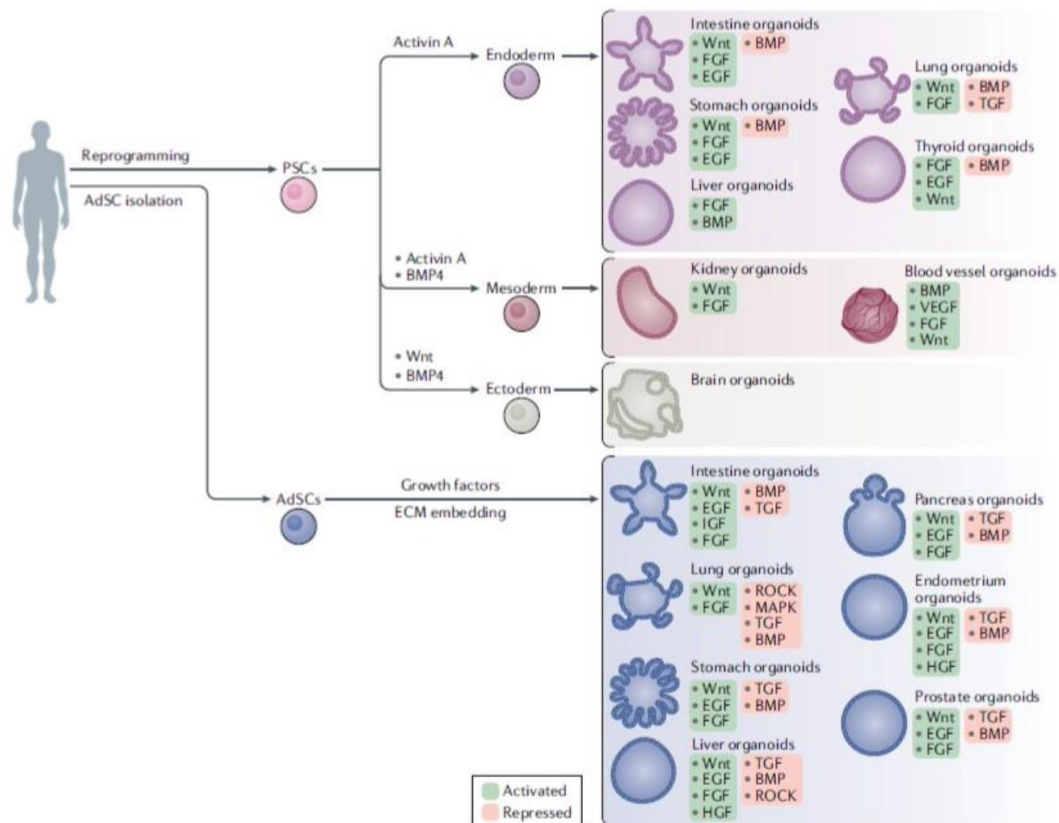


Figure 6: The image shows the steps and pathways involved in the development of human organoids derived from PSCs and aSCs, as well as the main factors used for their growth and maintenance. Image from “Human organoids: model systems for human biology and medicine” by Kim J. et al., Molecular Cell Biology, 2020.

1.4.3.Organoids Derived from PSCs

Organoids can be generated from a wide variety of organs using pluripotent stem cells, including the brain, retina, stomach, lung, liver, pancreas, and intestine (Clevers H. et al., 2016). Among these, intestinal-derived organoids represent one of the best-characterized PSC-derived systems. In pluripotent stem cell (PSC)-derived models, intestinal organoids are generated through a stepwise differentiation process that recapitulates embryonic development. Human PSCs are first induced into definitive endoderm through Activin signaling, after which exposure to Wnt3A and FGF4 promotes specification toward mid- and hindgut lineages. These cells subsequently self-organize under three-dimensional culture conditions to form intestinal organoids that exhibit key structural and cellular features of the native epithelium (Clevers H. et al., 2016).

Following lineage specification, the resulting cell populations undergo morphogenesis when embedded in an extracellular matrix, forming spheroid structures that expand and develop into

organoids. During this process, the cells proliferate and organize into complex epithelial architectures, increasing in size and cellular complexity over time. These organoids can be maintained and expanded in culture for extended periods while preserving key structural and functional characteristics of the intestinal lineage being modeled (McCracken K.W. et al., 2014).

1.4.4.Organoids derived from aSC

As mentioned earlier, organoids can be generated not only from pluripotent stem cells but also from organ-specific adult stem cells (aSCs), which possess the capacity for long-term self-renewal and tissue regeneration (Clevers H, 2016). The establishment of aSC-derived organoids relies on the in vitro recreation of the stem cell niche, achieved through the use of defined growth factor cocktails that mimic the physiological microenvironment. Among the signaling pathways involved, Wnt signaling plays a central role in regulating epithelial adult stem cell activity, representing the key pathway for maintaining stem cell fate and promoting proliferation of crypt base columnar (CBC) stem cells and transit-amplifying (TA) cells (Clevers H, 2016). In particular, these cells express the protein *LGR5*, which is widely recognized as a specific marker of intestinal adult stem cells (Barker N et al., 2010). Activation of this pathway, typically through factors such as Wnt3A and R-spondin or through inhibition of GSK3, is essential for maintaining stemness and supporting organoid growth (Clevers H, 2016). A well-established example is the generation of intestinal organoids from single *Lgr5*-positive stem cells, which are embedded in an extracellular matrix such as Matrigel and cultured in a serum-free medium supplemented with R-spondin-1, epidermal growth factor (EGF), and the BMP inhibitor Noggin (Sato et al., 2009; Clevers H, 2016). Under these conditions, stem cells self-organize into three-dimensional structures that recapitulate the architecture and cellular composition of the native tissue (Grün et al., 2015) and can be expanded long-term. This long-term expandability represents a major experimental advantage, as it enables the development of stable in vitro models for mechanistic studies, disease modelling and drug testing. Indeed, organoids have been shown to be maintained in culture for periods exceeding 1.5 years, with stable genetic and phenotypic characteristics, and can be passaged repeatedly without loss of integrity (Sato T and Clevers H, 2013).

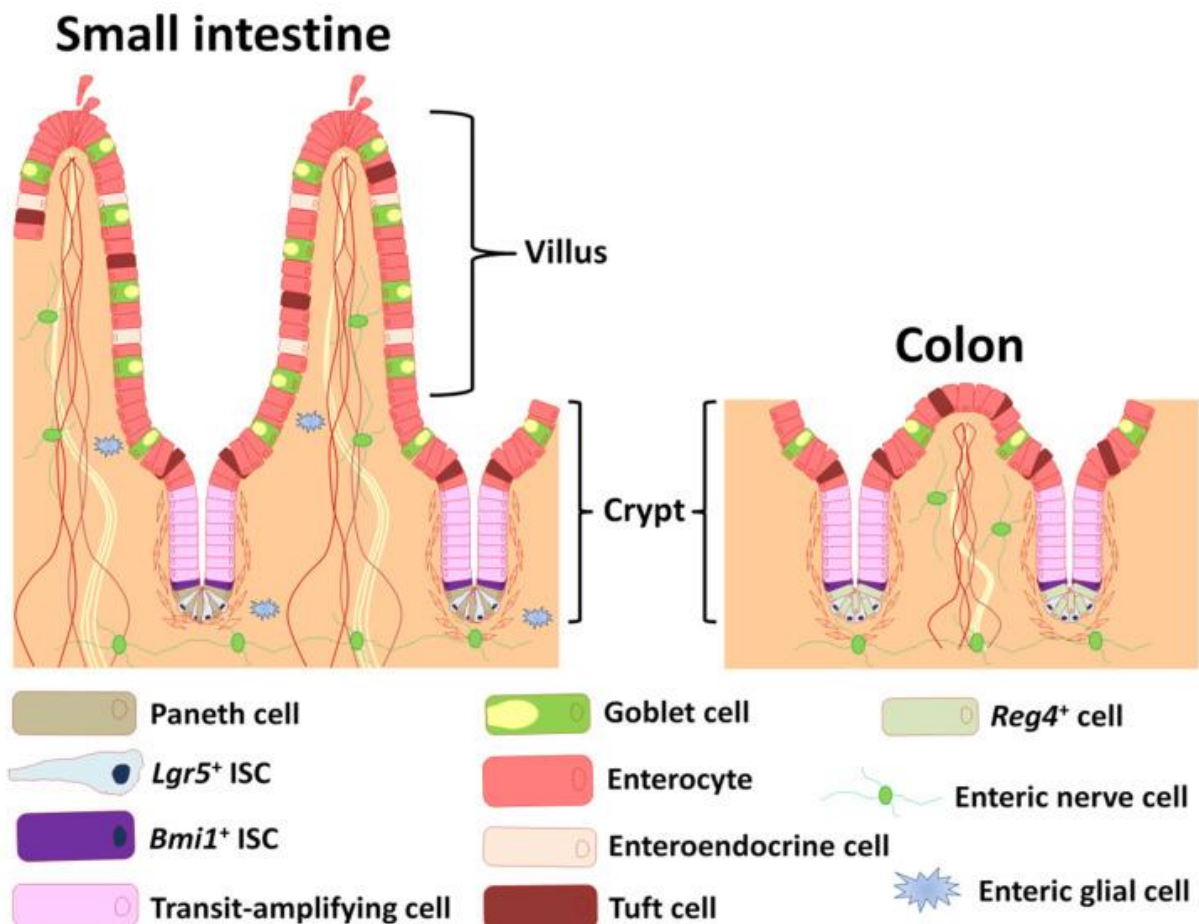


Figure 7: The figure illustrates the anatomy of the intestinal epithelium, characterized by the presence of different cell types. At the base of the crypt, *LGR5*⁺ stem cells are located, interspersed with Paneth cells that provide support to the stem cells. Along the sides of the crypt are the transit-amplifying (TA) proliferative cells, which subsequently differentiate and migrate toward the apical region, giving rise to the various differentiated cell types: enterocytes, goblet (mucus-secreting) cells, enteroendocrine cells, and M cells. In the underlying stroma, cells of the nervous system can be observed, including enteric neurons and glial cells. In addition, this layer contains mesenchymal cells, myofibroblasts, and smooth muscle cells, which promote the release of BMP and its respective inhibitors. Unlike the small intestine, the colon does not present villi. The cell types found in the colon are the same as those in the small intestine, except for Paneth cells. Image adapted from “Modelling Intestinal Stem Cell Function with Organoids” by Toshio Takahashi, International Journal of Molecular Sciences, 2021.

1.4.5. Organoids in cancer research

Organoid technology has significantly advanced biomedical research by providing more physiologically relevant *in vitro* models that better recapitulate human tissue architecture and function. Traditional cancer models, such as cell lines and animal models, often fail to accurately represent the complexity of human tumours, thereby limiting their translational value. In contrast, three-dimensional (3D) organoid culture systems have enabled the development of

improved human disease models, enhancing the translation of basic research findings into clinical applications. Organoids can be derived from both healthy and tumour tissues and maintain key phenotypic and genetic characteristics of their tissue of origin, enabling more accurate disease modelling. Importantly, their derivation from patient samples allows drug screening and supports the development of personalized therapeutic strategies, highlighting their role in translational research and precision medicine (Drost and Clevers, 2018).

Tumour development is characterized by the progressive accumulation of genetic and epigenetic alterations, resulting in a dynamic and evolving population of cancer cells. This process, often described as clonal or Darwinian evolution, leads to the emergence of genetically diverse subclonal populations within the same tumour (Stratton et al., 2009). Large-scale sequencing studies have shown that cancer genomes harbor numerous somatic mutations, including driver mutations that confer a selective growth advantage and passenger mutations that do not contribute directly to tumour progression (Greenman et al., 2007). This genetic diversity underlies intratumour heterogeneity, which has important implications for therapeutic response. In particular, resistant subclones may already be present prior to treatment and can be selectively enriched under therapeutic pressure, ultimately leading to treatment failure and disease progression (Stratton et al., 2009).

Building on this complexity, organoid technology provides a platform to capture and study tumour heterogeneity across multiple patients. Tumour organoid cultures can be expanded long term, cryopreserved, and stored as living organoid biobanks, enabling the establishment of large collections of patient-derived tumour models. These biobanks preserve the genetic and phenotypic characteristics of the original tumours and allow systematic investigation of cancer at a population level. Importantly, they enable the integration of genomic and functional data, making it possible to correlate tumour-specific genetic alterations with drug responses. This represents a major opportunity for the scientific community, as increasing the number of biobanked organoids enhances the statistical power required to identify clinically relevant associations. Furthermore, organoid biobanks support a wide range of applications, including drug screening, biomarker discovery, personalized medicine, and toxicity testing, as well as the development of novel anticancer therapies (Van de Wetering et al., 2015).

A further development of three-dimensional culture systems involves the incorporation of organoids with components of the surrounding microenvironment. While conventional organoid models primarily recapitulate the epithelial compartment, organoid co-culture systems aim to restore key interactions with stroma, immune and other niche-associated cells. Organoid formation and stem cell behavior are regulated by niche-derived signals, including biochemical

cues, cell–cell interactions, and extracellular matrix components, underscoring the critical role of the microenvironment in tissue homeostasis and disease processes (Yin et al., 2016). However, current organoid models do not fully recapitulate the *in vivo* environment and lack interactions with additional cell types, such as immune and mesenchymal cells. To address this limitation, co-culture systems incorporating components of the tumour microenvironment, including stromal and immune cells, are being developed to improve the physiological relevance of these models, although further optimization is still required. The rapid implementation of organoid technology has led to significant advances in cancer research, highlighting the growing importance of these systems for studying tumour biology and enhancing translational applications (Kretschmar, 2021).

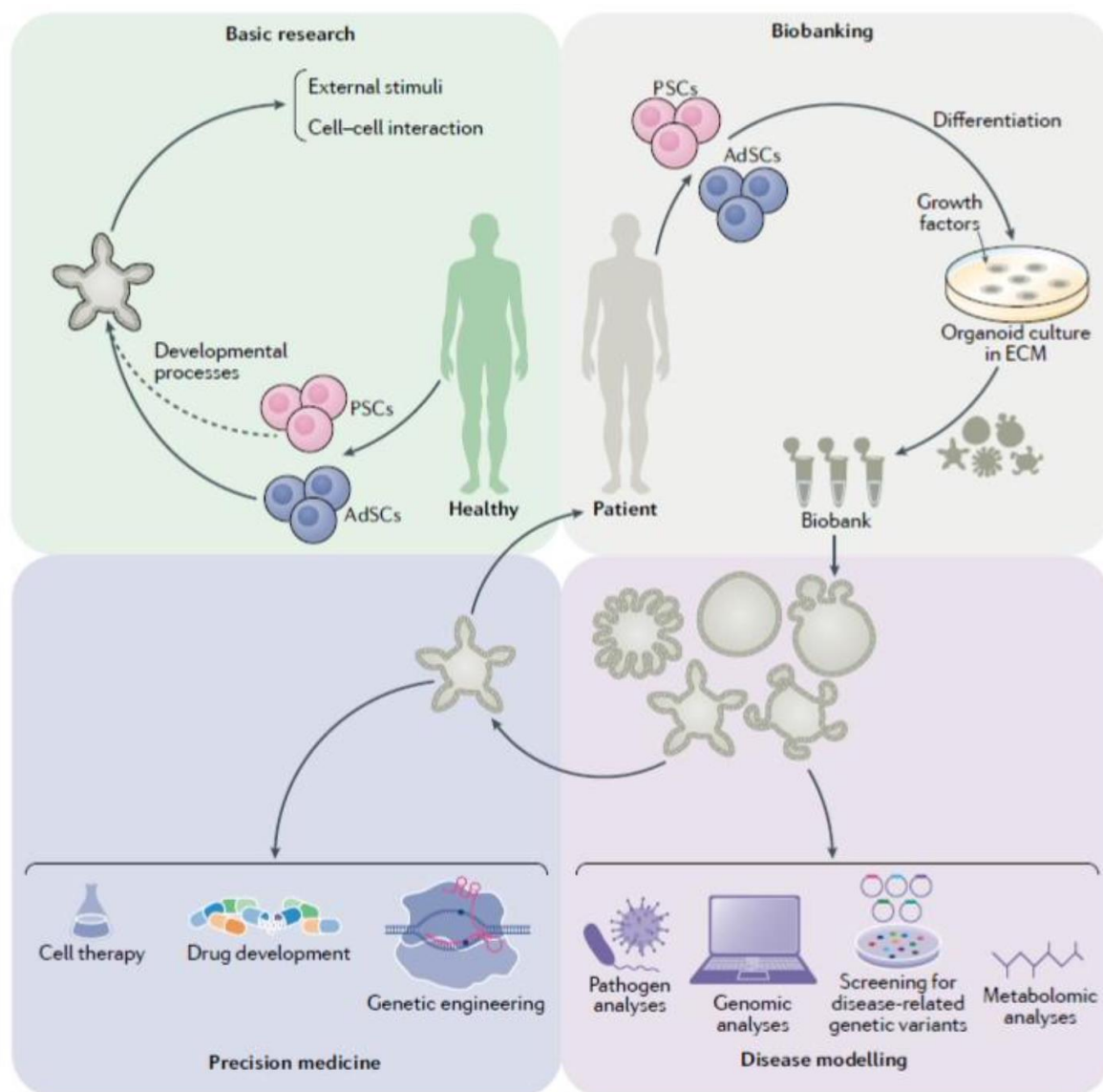


Figure 8: The image represents the potential applications of organoids derived from both PSCs and aSCs. Organoids can be used for: (1) basic research, including biological studies aimed at understanding various cellular processes, responses to external stimuli, cell–cell interactions, and mechanisms of homeostasis; (2) biobank generation, where patient-derived samples can be used to generate organoids

and stored for future research; (3) disease modelling, to investigate the mechanisms of human diseases such as infections, genetic disorders, and cancers, using different approaches such as omics techniques and drug screening analyses; (4) precision medicine, in which patient-derived organoids can be used to predict drug responses or for regenerative medicine applications. Image adapted from “Modeling Intestinal Stem Cell Function with Organoids” by Toshio Takahashi, International Journal of Molecular Sciences, 2021

1.4.6. Limitations and future perspectives

Organoid systems have emerged as powerful *in vitro* models capable of recapitulating key structural and functional features of human tissues; however, they still present several important limitations. Although they capture important aspects of tissue biology, most current organoid models represent only a simplified and partial version of native tissues and lack full control over cell composition, spatial organization, and cell–cell or cell–matrix interactions. Furthermore, significant gaps remain between *in vitro* organoid systems and *in vivo* physiology, particularly due to the absence of critical elements of the tissue microenvironment. For instance, many organoids lack vascularization, which restricts nutrient and oxygen diffusion, thereby limiting their size, maturation, and functional complexity. Similarly, the absence of supporting cell types, such as immune and mesenchymal cells, further reduces their ability to accurately model physiological and pathological processes. In addition, organoid formation relies heavily on intrinsic self-organization, a process that remains difficult to precisely control, resulting in variability and limited reproducibility across systems. Despite these challenges, significant advances in bioengineering and molecular technologies are expected to overcome current limitations. Strategies such as the development of biomimetic scaffolds, microfluidic systems, and controlled delivery of biochemical cues aim to better replicate the *in vivo* microenvironment and improve structural organization. Moreover, emerging approaches including targeted genome editing and synthetic biology offer the possibility to reprogram cellular behavior and enhance control over organoid development and function. Together, these innovations may not only improve biological fidelity, but also increase the standardization, scalability and translational relevance of organoid platforms. Although challenges related to standardization, scalability, and system complexity remain to be addressed (Yin X. et al., 2016).

1.5.Cancer-Associated Fibroblasts (CAF)

1.5.1.Overview of CAF Phenotypes and Origin

Cancer-associated fibroblasts (CAF) represent a key stromal component of the tumour microenvironment, where they actively contribute to tumour development through dynamic interactions with cancer cells and infiltrating immune populations. These cells are primarily of mesenchymal origin, arising from fibroblastic lineages derived from the mesoderm, although their

precise origin remains incompletely understood (Sahai E. et al., 2020). CAF are now recognized as a highly heterogeneous population in terms of both cellular origin and functional state. Multiple sources have been proposed, including bone marrow–derived fibroblasts, adipocyte-derived fibroblasts, and other mesenchymal cell types such as pericytes, highlighting the complexity and diversity of CAF origins.

Functionally, CAF contribute to tumour progression by remodelling the extracellular matrix and engaging in extensive signalling crosstalk with cancer and immune cells, partly mediated by cytokines and growth factors such as IL-6 and members of the TGF- β family (Sahai E. et al., 2020). In addition to supporting tumour growth, CAF modulate immune responses, promote invasion and metastatic dissemination and contribute to the establishment of a tumour-permissive microenvironment. CAF have been implicated in the development of therapy resistance, as anticancer treatments can induce their activation and enhance their tumour-promoting functions (Sahai E. et al., 2020).

1.5.2. Biological Functions of CAF in CRC

Cancer-associated fibroblasts (CAF) are active regulators of the tumour microenvironment and play a pivotal role in shaping the tumour microenvironment through active remodelling of ECM. These cells are among the most effective mediators of ECM deposition and restructuring within the tumour stroma, contributing to profound alterations in tissue architecture and mechanical properties (Sahai et al., 2020). Through their intrinsic fibroblastic activity, CAF remodel the surrounding matrix by depositing ECM components, secreting matrix-crosslinking enzymes, and exerting contractile forces, thereby increasing tissue stiffness and altering cell–matrix interactions (Rimal et al., 2022; Sahai et al., 2020). These biomechanical and structural changes create a dynamic microenvironment that supports tumour progression and facilitates cancer cell invasion (Sahai et al., 2020; Rimal et al., 2022).

CAF also regulate tumour behavior through the secretion of soluble mediators that sustain tumour-stroma crosstalk. Among these, interleukin-6 (IL-6) and members of the transforming growth factor-beta (TGF- β) family, such as activin A, play key roles in promoting a pro-tumorigenic environment (Sahai et al., 2020). CAF secrete chemokines such as CXCL12, which support tumour–stroma interactions and contribute to the establishment of a tumour-promoting niche (Rimal et al., 2022). Through the release of these soluble factors, CAF influence cancer cell phenotype (Sahai et al., 2020), enhancing cancer cell proliferation, migration, and invasion through paracrine signalling and direct interactions with tumour cells (Rimal et al., 2022).

Furthermore, CAF play a crucial role in inducing epithelial–mesenchymal transition (EMT) in tumour cells, a process that enhances cellular plasticity and promotes tumour progression. CAF alter the epithelial phenotype of cancer cells towards a mesenchymal-like state, increasing their migratory and invasive capabilities (Rimal et al., 2022). One of the key mechanisms underlying this process involves IL-6-mediated activation of the JAK/STAT3 signalling pathway. In addition, CAF-derived exosomes contribute to EMT induction through the delivery of regulatory molecules such as *SNAIL* (Rimal et al., 2022).

Finally, CAF are increasingly recognized as important mediators of therapy resistance. Anti-cancer treatments can induce activation and expansion of CAF populations, leading to functional changes that enhance their tumour-promoting properties (Sahai et al., 2020). Through modulation of the tumour microenvironment and direct interactions with cancer cells, CAF support tumour cell survival under therapeutic stress and reduce treatment efficacy. IL-6-mediated signalling and EMT have been implicated in promoting resistance to chemotherapy (Rimal et al., 2022).

1.5.3.CAF-Based Preclinical Models

CAF-based co-culture systems have emerged as advanced preclinical models that better recapitulate the tumour microenvironment compared to conventional organoid monoculture systems. The incorporation of patient-derived CAF into cancer cell models, particularly three-dimensional organoids-based models, enables the reconstruction of a more physiologically relevant tumour stroma, where CAF organize into structured networks that guide tumour cell spatial organization and promote multicellular architectures resembling *in vivo* tumour tissue.

Beyond improving structural complexity, these models provide a valuable platform for personalized therapy testing. as organoids directly derived from patient, can be exposed to clinically relevant drug regimens in the presence of their corresponding CAF populations. CAF-containing systems significantly alter therapeutic outcomes compared to monocultures, highlighting the importance of stromal components in modulating tumour behaviour and improving the predictive value of drug sensitivity assays (Varinelli et al., 2024).

CAF also contribute to drug resistance and metabolic reprogramming within the tumour microenvironment. CAF reduce treatment efficacy through mechanisms such as cytokine-mediated signalling, extracellular matrix remodelling, and metabolic coupling with cancer cells. Through the release of metabolites such as lactate and pyruvate, CAF support tumour growth

and provide metabolic substrates that help sustain cancer cell survival under stress conditions induced by, for example, therapeutic intervention (Rimal et al., 2022).

1.5.4.CAF-Tumour Interactions in Peritoneal Metastasis Microenvironment

In co-culture models of colorectal cancer peritoneal metastases, CAF actively organize the metastatic niche through direct interactions with tumour cells. When co-cultured with PDO, CAF progressively arrange into a stromal network surrounding PDO, invading the matrix and forming stromal tracks along which tumour cells reorganize into aggregates. Immunofluorescence analyses show colocalization of PDO and CAF, indicating the formation of an integrated tumour–stroma architecture (Varinelli et al., 2024).

Beyond their structural role, CAF can secrete a wide range of bioactive molecules, contributing to a tumour-supportive peritoneal microenvironment. They express high levels of vascular endothelial growth factor (VEGF), promoting angiogenesis, as well as matrix remodelling enzymes such as metalloproteinases (MMP-2, MMP-9, MMP-14), lysyl oxidase (LOX), and urokinase-type plasminogen activator (uPA), which facilitate extracellular matrix degradation and tumour dissemination. Additionally, CAF-derived cytokines, including IL-6, IL-8, VEGF, and PDGF-C, enhance tumour cell survival, angiogenesis, and resistance to therapy (Demuytere et al., 2020; Zhang et al., 2025).

These stromal functions also have important therapeutic implications: in co-culture systems, CAF significantly influence the response to HIPEC treatment by increasing the half-maximal inhibitory concentration (IC₅₀) of chemotherapeutic agents and reducing treatment-induced apoptosis, as reflected by decreased CASPASE3 activation (Varinelli et al., 2024). This is consistent with broader evidence showing that CAF promote therapy resistance through cytokine-mediated signalling and the formation of a dense stromal barrier that limits drug penetration. Collectively, these findings highlight the role of CAF in reducing tumour responsiveness to intraperitoneal chemotherapy (Varinelli et al., 2024; Zhang et al., 2025).

2.THESIS AIM

The introduction of the CRS-HIPEC technique has led to a significant improvement in survival among patients affected by CRCPM. However, recurrence rates remain high, mainly due to the resistance of tumour cells to the drugs commonly used for intraperitoneal perfusion. Based on these considerations, the aim of this thesis is to develop and validate more physiologically relevant in vitro models of colorectal cancer peritoneal metastases (CRCPM) to improve the prediction of therapeutic response and support the development of personalized treatment strategies. Specifically, this study aims to:

1. Establish and characterize patient-derived organoids (PDO) and cancer-associated fibroblast (CAF) lines derived from CRCPM patient tissues.
2. Develop co-culture systems combining PDO and CAF to better recapitulate the tumour microenvironment.
3. Evaluate the potential of co-culture models as more predictive preclinical platforms for personalized therapy in CRCPM.

3.MATERIALS AND METHODS

3.1.HUMAN TISSUES

Peritoneal tissues used in the study were collected from patients with peritoneal metastases from colorectal cancer who underwent surgical resection at the Peritoneal Malignancies Unit of Fondazione IRCCS Istituto Nazionale dei Tumori in Milan (INT). The patients were staged according to the World Health Organisation (WHO) classification (I. Ubink et al 2016). All patients provided informed consent for the use of their tissues, with the study protocols adhering to ethical regulations and approved by the institutional review board of INT (INT #134/13; 149/19), in accordance with the Declaration of Helsinki principles, 2009.

The main clinical, pathological, and genetic characteristics of patients enrolled in this study are presented in table 2.

Metastatic lesions were harvested and one part (1 cm in diameter) was placed in ice cold DMEM-F12 (ThermoFisher scientific) containing gentamicin (50 ng/ml, ThermoFisher scientific) for the development of both patient-derived organoids (PDO) and cancer-associated fibroblasts (CAF). A second specimen was frozen in liquid nitrogen for molecular and histopathological analyses. Formalin-fixed, paraffin-embedded (FFPE) blocks were prepared for immunohistochemistry (IHC) analyses.

<i>ID</i>	<i>Diagnosis</i>	<i>Grade</i>	<i>Stage</i>	<i>Mutation</i>	<i>MMR status (pMMR-dMMR)</i>
BRI_42	Sigma Mucinous adenocarcinoma	G2	pT4N2M1	TP53 R175H	pMMR
BRI_47	Intestinal mucinous adenocarcinoma	G2	pT3N0M1	KRAS G12V	pMMR
BRI_58	Sigma Mucinous adenocarcinoma	G3	pT4N2M2	KRAS G12V	pMMR

Table 2: Main pathological characteristics of the patients from which PDO and CAF were obtained. Mutational profile was obtained through NGS analysis, mismatch repair (MMR) status (pMMR/dMMR) was determined by IHC analysis, both done by the INT Department of Pathology. Tumour grade and stage were determined following the WHO classification for colorectal cancer.

3.2. Development of Patient-Derived Organoids (PDO)

Three PDO lines, BRI_42, BRI_47 and BRI_58 were developed in this study (Table 2). To obtain the PDO, fresh CRC-PM-derived samples were processed following the protocol described by Fujii et al. (M. Fujii et al., 2016). Tumour samples were cut into small pieces and transferred into a 15ml Falcon tube, and washed repeatedly (10 times) with 7 ml of ice-cold PBS (your SIAL Media & Serum, Rome, Italy) supplemented with gentamicin at a final concentration of 50 µg/ml.

Subsequently, the samples were resuspended in a solution consisting of DMEM-F12 (Corning, NY, USA) containing type II collagenase at a final concentration of 1 mg/ml. The samples were then incubated for 60 min at 37 °C.

Once digestion was completed, the supernatant was collected and transferred into a new 15-ml Falcon tube, while the residual fragments were further treated with trypsin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and EDTA (Thermo Fisher Scientific, Waltham, MA, USA) at final concentrations of 1 mg/ml and 5 mM, respectively and subjected to an additional digestion step at 37 °C for 20 minutes to enhance the release of epithelium-derived cells. After the incubation, the supernatant was recovered and combined with the previous one, then filtered through a sterile 100-µm strainer (CORNING) and centrifuged for 5 minutes at 1200 rpm at 4 °C.

After centrifugation, the pellet was embedded in Matrigel, growth factors reduced (Corning, NY, USA) and dispensed into a pre-warmed 24-well plate (40 µl of cell–Matrigel suspension per well). The plate was placed at 37 °C for 10 minutes to allow Matrigel polymerization. After Matrigel polymerization, PDO were cultured in either DMEM/F12 or in OncoPro medium (Gibco) and supplemented with different growth factors to mimic different niche factors conditions (Table 3). The composition and concentrations of all supplements are reported in Table 3. This allows reproducing four distinct culture conditions that mimic the microenvironment of the colonic. The plate was then incubated at 37 °C with 20% O₂ and 5% CO₂. The medium was changed every two days, and after 1–2 weeks, the culture condition that supported the best PDO growth was selected.

Factor	Description	Manufacturer	Concentration
Gentamicin	Antibiotic	Thermo Fisher Scientific	50 µg/ml
HEPES	Buffer	Thermo Fisher Scientific	10 mM
L-Glutamine	Cell culture supplement	Thermo Fisher Scientific	2 mM
B27	Cell culture supplement	Thermo Fisher Scientific	1:50
Gastrin-1	Recombinant human protein	Sigma Aldrich	10 nM
N-acetylcysteine	Colonic niche factor	Wako	1 mM
EGF	Recombinant human protein	Thermo Fisher Scientific	50 ng/ml
Noggin	Recombinant human protein	Peprtech	100 ng/ml
R-spondin-1	Recombinant human protein	Peprtech	100 ng/ml
Wnt3A	Recombinant human protein	Peprtech	50 ng/ml
Prostaglandin E2	Colonic niche factor	Tocris	100 nM
A83-01	p38 inhibitor	Tocris	500 nM
SB202190	ROCK inhibitor	Sigma Aldrich	10 µM
Onco Factor	Cell culture supplement	Thermo Fisher Scientific	—
OncoPro BSA	Cell culture supplement	Thermo Fisher Scientific	—

Table 3: List, description, and working concentrations of reagents used to prepare the culture media for the development of patient-derived organoids (PDO).

The PDO were expanded every 1–2 weeks using Cell Recovery solution (Corning), which allows Matrigel to be digested while preserving the PDO, at 4 °C for 1 hour (500 µl/well). The cells were then centrifuged at 1200 rpm for 5 minutes at 4 °C, resuspended in Matrigel and seeded in a 24-well plate (40 µl/well), to which 500 µl of medium was added. The culture medium used for each PDO line and the complete supplement composition are summarized in Table 4.

PDO LINE	MEDIUM	BASAL MEDIUM	SUPPLEMENTS ADDED (per 50 mL medium)
BRI_47	C3	DMEM/F12	B27 (1 mL), Gastrin (5 µL), N-acetylcysteine (100µL), A83-01 (5 µL).
BRI_58	O3	OncoPro	Onco Factor (1 mL), BSA (3 mL), Prostaglandin E2 (5 µL), N-acetylcysteine (100µL), Gastrin (5 µL), B27 (1 mL)
BRI_42	O3	OncoPro	Onco Factor (1 mL), BSA (3 mL), Prostaglandin E2 (5 µL), N-acetylcysteine (100µL), Gastrin (5 µL), B27 (1 mL)

Table 4: Culture media used for each PDO line, including supplement volumes added per 50 mL of complete medium. Concentrations of reagents are shown in table 3.

3.3.Isolation and Expansion of CAF

CAF were isolated from tumour tissue following enzymatic and mechanical dissociation as described in Mun et al. (2023). Briefly, tumour tissues were cut into small pieces (3–4 mm), washed five times with ice-cold 1X PBS supplemented with 50 µg/ml gentamicin, and digested with 1 mg/ml collagenase II for 1 h at 37 °C. The resulting cell suspension was filtered with a 100-µm cell strainer to remove debris, yielding a stromal-enriched fraction containing fibroblasts (Mun et al., 2023). The filtered cells were then centrifuged for 5 min at 1200 rpm at 4 °C, the supernatant was discarded, and the pellet was resuspended in 1 ml of CAF medium.

Following dissociation, cells were seeded in parallel across different culture media conditions using a combinatorial seeding strategy to evaluate optimal growth parameters. Cultures were seeded into Petri dishes, topped up to a final volume of 6 ml, and maintained at 37 °C in a humidified incubator with 5% CO₂, with the culture medium changed every 2–3 days. The condition that yielded the best CAF expansion—a fibroblast-selective culture medium consisting of EGM-MV2 medium supplemented with FGF, IGF, hydrocortisone, and FCS—was selected for subsequent experiments. The list and working concentrations of all reagents used to prepare the CAF culture media are reported in Table 5. Under these optimal conditions, fibroblasts rapidly adhered to the culture surface and began expanding.

FACTOR	DESCRIPTION	MANUFACTURER	WORKING CONCENTRATION
FGF	Recombinant Human Basic Fibroblast Growth Factor (bFGF / FGF-2)	PromoCell / PeproTech	10 ng/mL
EGF	Recombinant Human Epidermal Growth Factor	PromoCell / PeproTech	5 ng/mL
IGF	Recombinant Human Long R ³ IGF-1 (Insulin-like Growth Factor-1)	PromoCell / Sigma-Aldrich	20 ng/mL
Hydrocortisone	Synthetic Glucocorticoid Hormone	PromoCell / Sigma-Aldrich	0.2 µg/mL
FBS	Fetal Bovine Serum (Heat-Inactivated)	Thermo Fisher / Sigma-Aldrich / PromoCell	5% (v/v)

Table 5: List, description, and working concentrations of reagents used to prepare the culture media for the development of cancer-associated fibroblasts (CAF).

The CAF were expanded and split weekly. Briefly, cells were washed with 7 ml of 1X PBS, followed by the addition of 2 ml trypsin and incubation for 5 min to allow cell detachment. Detached cells were collected by pipetting and transferred into a 15 ml Falcon tube. To reduce the toxic effects of trypsin, FBS was added at a volume of 100 µl per 1 ml of cell suspension, and the cells were centrifuged at 1200 rpm for 5 min at 4 °C. The supernatant was discarded, the pellet was resuspended in the selected culture medium, and cells were seeded into an appropriate number of Petri dishes depending on pellet size. Finally, each dish was topped up to

a final volume of 6 ml of medium and incubated at 37 °C. The selected culture medium used for each CAF line is summarized in Table 6.

CAF LINE	MEDIUM	BASAL MEDIUM	SUPPLEMENTS ADDED (per 50 mL medium)
BRI_58	CAF 2	EGM MV2	FBS (1 mL) EGF (50 µL) FGF (50 µL) Hydrocortisone (50 µL)
BRI_65	CAF 2	EGM MV2	FBS (1 mL) EGF (50 µL) FGF (50 µL) Hydrocortisone (50 µL)

Table 6: Culture media used for each CAF line, including supplement volumes added per 50 mL of complete medium. Concentrations of reagents are shown in table 5.

3.4.Co-Culture Development

To develop co-culture models including CRCPM-derived PDO and CAF, PDO were recovered from Matrigel (Corning, Corning, NY, USA) using Cell Recovery Solution (Corning). Briefly, 500 µl of cell-recovery solution was added per well and plates were incubated on ice for 1 h to dissolve Matrigel. The PDO suspension was then collected into a 15 ml Falcon tube, and wells were gently scraped using a pipette tip to detach remaining material. To maximize recovery, an additional 500 µl of cell-recovery solution was used to wash the wells and collect residual PDO, and the wash was pooled into the same tube. Samples were centrifuged at 1200 rpm for 5 min at 4 °C, and the supernatant was discarded. The pellet was washed with 2 ml of 1X PBS to remove dead cells, centrifuged again, and resuspended in 1 ml of the selected culture medium. PDO were then quantified by counting 10 µl of the suspension under a microscope, and the appropriate volume was prepared to seed 30–50 PDO per well, which were subsequently resuspended in the co-culture mix for embedding.

The co-culture mix was composed of 75% neutralized Collagen I (CORNING); final concentration 1.25 mg/mL) and 25% Matrigel (Corning) (Varinelli et al., 2024). To prepare neutralized Collagen I, 100 μ L of 10X PBS (Thermo Fisher Scientific), 120 μ L of Collagen I, 2.76 μ L NaOH (Sigma-Aldrich, Merck, Germany), and 776 μ L sterile H₂O were mixed. The final co-culture matrix was then obtained by combining 750 μ L of the neutralized Collagen I solution with 250 μ L Matrigel, yielding a 75:25 Collagen I: Matrigel mixture. A total of 35 μ L of this matrix was used to coat each well of a 96-well plate before seeding PDO suspended in CC2 co-culture medium at a density of 30–50 PDO per well.

CC2 medium was specifically designed for co-cultures, consisting of a mixture of PDO medium and CAF medium optimized to support the growth of both cellular subpopulations simultaneously. To select CC2 as the optimal formulation for CAF and PDO co-cultures, PDO (lines PDO1, PDO2, PDO3, PDO6, and PDO7) and CAF (three independent primary lines) were cultured separately for 5 days using either standard PDO medium (C1, C2, C3, and C6) or CAF medium alone, as well as combined formulations (CC1, CC2, CC3, and CC6) supplemented with varying levels of key factors required for both cell types. At the end of the culture period, a CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA) was performed to assess which medium formulations supported optimal growth in both CAF alone and PDO alone. Based on these results, CC2 was selected because it sustained high, reproducible viability in PDO while maintaining stable and consistent growth in CAF. The list, description, and working concentrations of reagents used to prepare CC2 medium are reported in Table 7.

MEDIUM	BASAL MEDIUM	SUPPLEMENTS ADDED
C1	DMEM/F12	B27, Glutamax, N-acetylcysteine, Prostaglandin-E2, Gastrin-I
C2	DMEM/F12	B27, Glutamax
C3	DMEM/F12	B27, Glutamax, N-acetylcysteine, Prostaglandin-E2, Gastrin-I, A83-01
C6	DMEM/F12	B27, Glutamax, N-acetylcysteine, Prostaglandin-E2, Gastrin-I, A83-01, Noggin
CC1	DMEM/F12	B27, FGF
CC2	DMEM/F12 or OncoPro	B27, FGF, EGF
CC3	DMEM/F12	B27, FGF, Noggin
CC6	DMEM/F12	B27, FGF, EGF, Noggin

Table 7: Composition of co-culture media formulations (CC1–CC6). Concentrations of reagents are shown in table 3 and 5.

CAF were detached by washing with 1X PBS, followed by incubation with trypsin for 5 min at 37 °C. Detached cells were collected and centrifuged at 1200 rpm for 5 min at 4 °C, after which the supernatant was discarded. The pellet was resuspended in 1 ml of the selected culture medium, and cell number was determined by loading 10 µl of the suspension into a C-Slide and counting using an automated cell counter. CAF were then resuspended in CC2 co-culture medium and seeded at a density of 8,000 cells per well directly onto the co-culture matrix coating containing 30–50 PDO per well, ensuring both cell types shared the same 3D environment.

The embedded co-cultures were maintained between 5-8 days to allow structural maturation. After this period the co-cultures were formed and suitable for HIPEC simulation.

3.5.Co-culture characterization

Co-culture models were characterized using an immunofluorescence (IF) approach. IF analyses were performed as previously reported (Varinelli et al 2024). CAF were seeded in an 8-wells chambered coverslip (Ibidi) as described above. Co-cultures were directly fixed in plate with 4 % paraformaldehyde (Sigma-Aldrich) for 15 min at 4 °C, then the plate was swirled and the cocultures were incubated in 4 % paraformaldehyde for other 15 min at 4 °C. Co-cultures were permeabilized with 0.5 % Triton X-100 (Sigma-Aldrich) for 30 min at RT on a rotor (100 rpm) and blocked for one hour with 5 % BSA and 0.1 % Triton X-100 in PBS 1X at RT on a rotor (100 rpm). Primary antibody mouse anti α -Smooth Muscle Actin (α -SMA) conjugated with Alexaflour 488 dye (1:500, Cell Signalling Technology, clone 1A4, #46489) and rabbit anti-pan cytokeratin (1:100, Abcam, clone C-11, ab7753) were used overnight at 4 °C and detected using secondary goat anti rabbit AlexaFluor 568 (1:500, Thermo Fisher, A11036) for one hour at RT. Nuclei were stained with DAPI (1:25000, Thermo Fisher, D1306). Images were acquired on DM600B microscope (Leica, Germany) equipped with a 100 W mercury lamp and analyzed using Cytovision software (Leica, Germany).

3.6.Drugs Used for HIPEC Treatment Simulation

For the in vitro simulation of hyperthermic intraperitoneal chemotherapy (HIPEC) treatments, five different protocols were tested. These regimens represent the primary therapeutic strategies used for treating colorectal cancer peritoneal metastases (CRCPM), where direct intraperitoneal delivery of heated chemotherapeutic agents after cytoreductive surgery maximizes local cytotoxicity against residual microscopic disease while minimizing systemic toxicity (McMullen et al., 2017). The chemotherapeutic agents employed were: mitomycin-C (MMC) (Kyowa Kirin Co., Ltd., Tokyo, Japan), cisplatin (CDDP) (Accord Healthcare Limited, North Harrow, United Kingdom), doxorubicin (DOX) (Accord Healthcare Limited, North Harrow, United Kingdom), and oxaliplatin (L-OHP) (Accord Healthcare Limited, North Harrow, United Kingdom).

L-OHP was administered following both the Wake Forest protocol (Stewart et al., 2008) and the Elias protocol (Elias et al., 2004) (schemes 4 and 5, respectively). In the Wake Forest

protocol (scheme 4), L-OHP was administered for a longer incubation duration at a lower concentration, whereas in the Elias protocol (scheme 5), the drug was applied for a shorter duration at a higher concentration. Drug concentrations and perfusion times for all evaluated HIPEC regimens are summarized in Table 8.

HIPEC Scheme	Perfusion Concentration	Normalized Concentration (mg/L)	Administration Time (min at 42.5 °C)
#1 (MMC)	35 mg/m ² in 2.5 L/m ² of perfusate	$35 \text{ mg/m}^2 \div 2.5 \text{ L/m}^2 = \mathbf{14 \text{ mg/L}}$	60
#2 (MMC + CDDP)	3.5 mg/m ² /L MMC + 25 mg/m ² /L CDDP in 1 L perfusate	MMC: $3.5 \text{ mg/m}^2 \div 1 \text{ L/m}^2 = \mathbf{3.5 \text{ mg/L}}$ CDDP: $25 \text{ mg/m}^2 \div 1 \text{ L/m}^2 = \mathbf{25 \text{ mg/L}}$	60
#3 (DOX + CDDP)	15.25 mg/L DOX + 43 mg/L CDDP in 5 L perfusate	DOX: $15.25 \text{ mg/L} \div 1 \text{ L} = \mathbf{15.25 \text{ mg/L}}$ CDDP: $43 \text{ mg/L} \div 1 \text{ L} = \mathbf{43 \text{ mg/L}}$	90
#4 (L-OHP)	200 mg/m ² in 2 L of 5% glucose	$200 \text{ mg/m}^2 \div 2 \text{ L/m}^2 = \mathbf{100 \text{ mg/L}}$	120
#5 (H-OHP)	460 mg/m ² in 2 L of 5% glucose	$460 \text{ mg/m}^2 \div 2 \text{ L/m}^2 = \mathbf{230 \text{ mg/L}}$	30

Table 8: Drugs and administration parameters used for simulating HIPEC treatment regimens. Abbreviations: **MMC** = mitomycin-C; **CDDP** = cisplatin; **DOX** = doxorubicin; **OHP** = oxaliplatin; **L-OHP** = oxaliplatin low dose; **H-OHP** = oxaliplatin high dose.

HIPEC was performed in vitro on the two CAF lines BRI_58, BRI_65 and on PDO lines BRI_47, BRI_42, and BRI_58, grown either as patient-derived organoids (PDO) alone on Matrigel or as PDO–CAF co-cultures embedded in the co-culture mix. This dual setup was used to

assess how the presence of CAF and the co-culture microenvironment influence the pharmacological response to the HIPEC regimens. All experiments were carried out in three independent biological replicates, each performed in technical triplicate.

3.7. In Vitro HIPEC Simulation and Assays

3.7.1. In Vitro HIPEC Simulation and IC50 evaluation on PDO

HIPEC simulation was performed as in Varinelli et al. (Varinelli et al. 2024). Briefly, to determine the IC50 value of drugs and relative schemes, 5×10^3 PDO were suspended in culture medium and seeded in 96-well plates (100 μ l/well, Costar 3904; Corning, USA) previously coated with 40 μ l of Matrigel (30 – 50 PDO/well). After two days, PDO were incubated with the different drugs. Briefly, all drugs were diluted at the working concentration in basal cell culture medium (DMEM-F12 supplemented with 15 mM HEPES, 2 mM GlutaMAX and 50 μ g/ml of gentamicin and amphotericin B respectively). The final solvent concentration was < 0.1% for all samples, including controls (DMSO for MMC and physiological solution for the other drugs). PDO were incubated at 42.5 °C or at 37 °C in 100 μ l of basal cell culture medium containing the drugs, in a cell culture incubator for the specific time required for each HIPEC scheme (Table 8). Time and temperature used for the HIPEC simulation were the same as in the patient (Table 8). Afterwards, the drugs were removed by washing the wells three times with 1X PBS and cells were incubated for 48 h with cell growth medium. The drug doses routinely used during HIPEC perfusion, concentrations and perfusion time were calculated from the perfusion data recorded during the HIPEC surgery performed at our institute to treat CRC-PM patients (Table 9 and Table 10). The concentrations used to construct the dose–response curves were selected by scaling up and down the concentrations used for patients. For each HIPEC scheme, the clinically relevant drug concentrations were converted in μ M for the in vitro experiments. Concentrations were obtained by normalizing each concentration using 1 L as the final reference dilution volume; the clinically relevant doses were obtained using 1 L (scheme 1 and 2), 5 L (scheme 3) and 2 L (scheme 4 and 5) as the final reference dilution volumes (Table 9). We used the same specific perfusion time already employed in the clinical practice to treat PDO (Table 9). All the experiments were performed in triplicate. HIPEC simulations were performed on PDO and the co-culture models.

HIPEC Scheme	Protocol	Drug	In vitro Concentration	Perfusion Time (min)
#1	Standard	MMC	41.9 μ M	60
#2	Standard	MMC + CDDP	10.4 μ M + 85 μ M + 4.375 μ g/mL	60
#3	Standard	DOX + CDDP	5.61 μ M + 28.6 μ M	90
#4	Wake Forest	L-OHP	252 μ M	120
#5	Elias	H-OHP	579 μ M	30

Table 9: The five HIPEC schemes mainly used in the clinical practice with the corresponding cytotoxic drugs adopted. Clinically relevant concentrations, dilutions, perfusion time and hyperthermia conditions were obtained from recorded perfusion data from the Peritoneal Malignancy Unit of Fondazione IRCCS Istituto dei Tumori di Milano. The mechanism of action of each cytotoxic drug, the synergistic effect with heat and penetration index are also reported.

HIPEC Scheme	Drug Amount (mol)	Patient Dose (M)
#1 (MMC)	0.014 g / 333.33 Da = 4.19×10^{-5} mol	4.19×10^{-5} mol / 1 L = 41.9 μM
#2 (MMC + CDDP)	MMC: 0.0035 g / 333.33 Da = 1.04×10^{-5} mol CDDP: 0.025 g / 301.11 Da = 8.30×10^{-5} mol	MMC: 1.04×10^{-5} mol / 1 L = 10.4 μM CDDP: 8.30×10^{-5} mol / 1 L = 85 μM
#3 (DOX + CDDP)	DOX: 0.01525 g / 543.52 Da = 2.81×10^{-5} mol CDDP: 0.043 g / 301.11 Da = 1.43×10^{-4} mol	DOX: 2.81×10^{-5} mol / 5 L = 5.61 μM CDDP: 1.43×10^{-4} mol / 5 L = 28.6 μM
#4 (L-OHP)	0.100 g / 397.28 Da = 5.03×10^{-4} mol	5.03×10^{-4} mol / 2 L = 252 μM
#5 (H-OHP)	0.230 g / 397.28 Da = 1.16×10^{-3} mol	1.16×10^{-3} mol / 2 L = 579 μM

Table 10: Determination of the clinically relevant dose for each HIPEC scheme tested. * Concentrations were obtained by normalizing each concentration using 1 L as the final reference dilution volume. ** Clinically relevant doses were obtained using 1 L (scheme 1 and 2), 5 L (scheme 3) and 2 L (scheme 4 and 5) as the final reference dilution volumes.

3.7.2. In Vitro HIPEC Simulation of CAF

CAF lines (CAF BRI_58 and CAF BRI_65) were subjected to in vitro HIPEC simulations followed by viability assessment. CAF monolayers at exponential growth phase were harvested by removing the culture medium and washing the cells with 7 mL of 1X PBS. Cells were detached using 2 mL of trypsin and incubated at 37 °C with 5% CO₂ until complete detachment was achieved. The resulting cell suspension was collected into a 15 mL Falcon tube and centrifuged at 4 °C for 5 min at 1200 rpm. Following centrifugation, the supernatant was discarded, and the cell pellet was resuspended in CAF2 medium. Cells were subsequently seeded into 96-well culture plates (Costar 3904; Corning, USA) at a density of 8×10^3 cells per well in 100 μ L of medium and incubated for 24 hours at 37 °C with 5% CO₂ to allow cell adhesion.

The following day, in vitro HIPEC simulation was performed by replacing the culture medium with basal cell culture medium (DMEM/F12 supplemented with 15 mM HEPES, 2 mM GlutaMAX, and 50 ng/mL gentamicin and amphotericin B). Chemotherapeutic treatments were prepared according to five clinically relevant HIPEC schemes (Table 10). Hyperthermic treatment was performed at 42.5 °C, whereas corresponding normothermic controls were maintained at 37 °C. Untreated control wells received vehicle controls consisting of either DMSO (<0.1%) or physiological saline in basal medium, according to the solvent used for each treatment condition.

Immediately after completion of the designated treatment period, drug-containing medium was removed, and cells were washed with 1X PBS to eliminate residual chemotherapeutic agents. Fresh CAF expansion medium was added, and cells were allowed to recover for 48 hours under standard culture conditions (37 °C, 5% CO₂).

Cell viability following HIPEC treatment was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Luminescence was measured using a Tecan microplate reader. All experiments were performed in three independent biological replicates, with each biological replicate evaluated in technical triplicate.

3.7.3. In Vitro HIPEC Simulation of PDO–CAF Co-Cultures

PDO and CAF were co-cultured using three specific PDO-CAF combinations (PDO BRI_47 + CAF BRI_65, PDO BRI_58 + CAF BRI_58, and PDO BRI_42 + CAF BRI_58). Wells of 96-well culture plates (Costar 3904; Corning, USA) were pre-coated with a gel mixture consisting of 25% Matrigel and Collagen I. Organoids (50 PDO/well) were resuspended in CC2 medium (50 μ L; supplemented as detailed in Table 6), seeded onto the gel dome and incubated for 2 hours at 37 °C with 5% CO₂ to allow initial settlement. Subsequently, CAF (8×10^3) resuspended in CAF2 medium (50 μ L) were added to each well, bringing the total working volume to 100 μ L.

Co-cultures were maintained for 5 days under standard incubation conditions (37 °C, 5% CO₂). On day 5, in vitro HIPEC simulation was performed by replacing the culture medium with basal cell culture medium (DMEM/F12 supplemented with 15 mM HEPES, 2 mM GlutaMAX, and 50 ng/mL gentamicin and amphotericin B) containing chemotherapeutic treatments according to the five clinically relevant HIPEC schemes (Table 8 and Table 9).

Hyperthermic treatments were performed at 42.5 °C, whereas corresponding normothermic controls were maintained at 37 °C. Untreated control wells received vehicle controls consisting of either DMSO (<0.1%) or physiological saline in basal medium, according to the solvent used for each treatment condition. Each scheme was applied for its designated clinical perfusion time (Table 9).

Immediately following treatment, drug-containing medium was removed, and wells were washed with 1X PBS. Fresh CC2 medium was added, and cells were incubated for 48 hours under standard culture conditions (37 °C, 5% CO₂). Cell viability following HIPEC treatment was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Luminescence was measured using a Tecan microplate reader. All experiments were performed in three independent biological replicates, with each biological replicate evaluated in technical triplicate.

3.8. Cell Viability Assays

PDO cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which measures intracellular ATP levels as an indicator of metabolically active cells. CellTiter-Glo reagent was added to each well at a 1:1 ratio with the culture medium, and

plates were shaken for 5 minutes (PDO) or 20 minutes (co-culture) to ensure complete lysis and homogeneous signal. Plates were then incubated for 25 minutes at room temperature to stabilize luminescence. A 200 µl aliquot from each well was transferred to a white 96-well assay plate, and luminescence was measured using a TECAN plate reader. Viability values were normalized to untreated control organoids.

3.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 9.4.1 (676), GraphPad Software, San Diego, CA, USA). Data are expressed as mean and standard deviation (SD). For the analysis of treatment response in PDO and CAF monocultures, untreated and treated groups were compared using two-tailed paired Student's t-tests

Dose–response curves were generated using nonlinear regression, and IC50 values were calculated for the most effective treatment schemes for each PDO line. The best-fit curve was selected based on the coefficient of determination (R^2).

Differences in cell viability among PDO monocultures, CAF monocultures, and PDO–CAF co-cultures exposed to different treatment schemes were evaluated using two-way analysis of variance (ANOVA). Post-hoc comparisons are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. All experiments were conducted in triplicate (independent biological replicates).

4.RESULTS

4.1.Development of Patient-Derived Organoids

To evaluate the potential of patient-derived organoids (PDO) as patient-specific models for identifying the most effective HIPEC regimens, three CRC-PM-derived PDO lines (BRI_42, BRI_47, and BRI_58) were successfully established from fresh surgical tissue (left-over from surgery) using previously described protocols for colorectal cancer organoid generation (Fujii et al., 2016; Van de Wetering et al., 2015) as detailed in the Methods section.

Following embedding in Matrigel and culture in organoid medium, all three PDO lines successfully formed stable three-dimensional structures and could be expanded throughout the experimental period before cryopreservation in liquid nitrogen. Bright-field microscopy showed PDO with cystic or compact morphologies, including structures with a central lumen. Morphological heterogeneity was observed among the different PDO lines, with both cystic and compact structures identified. Despite this morphological heterogeneity, all PDO lines displayed stable growth under the established culture conditions.

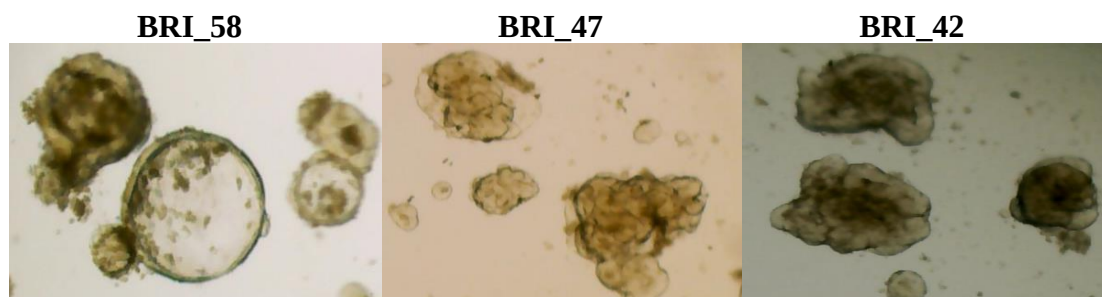


Figure 9: Representative bright-field images of PDO established from CRC-PM from three patients. The models showed heterogeneous cystic and compact three-dimensional morphologies, with a central lumen visible in some PDO. Images were acquired using a light microscope at a total magnification of 40× (magnification – objective 4x, total magnification = 4x (objective) * 10 (ocular)).

4.2. Development of Cancer-Associated Fibroblasts

Primary cancer-associated fibroblasts (CAF) lines, designated BRI_58 and BRI_65, were successfully isolated from CRCPM specimens by combined mechanical and enzymatic dissociation, described in the methods section. Rapid attachment and subsequent outgrowth under selective culture conditions enable the establishment and expansion of primary stromal cultures representative of the CRCPM microenvironment.

Morphological assessment showed that both lines displayed the elongated, spindle-shaped appearance typical of fibroblastic-like cells growing as adherent monolayers. Their morphology remained consistent across the passages examined, and both cultures showed sustained proliferation, supporting their use in subsequent experiments.

CAF BRI_65

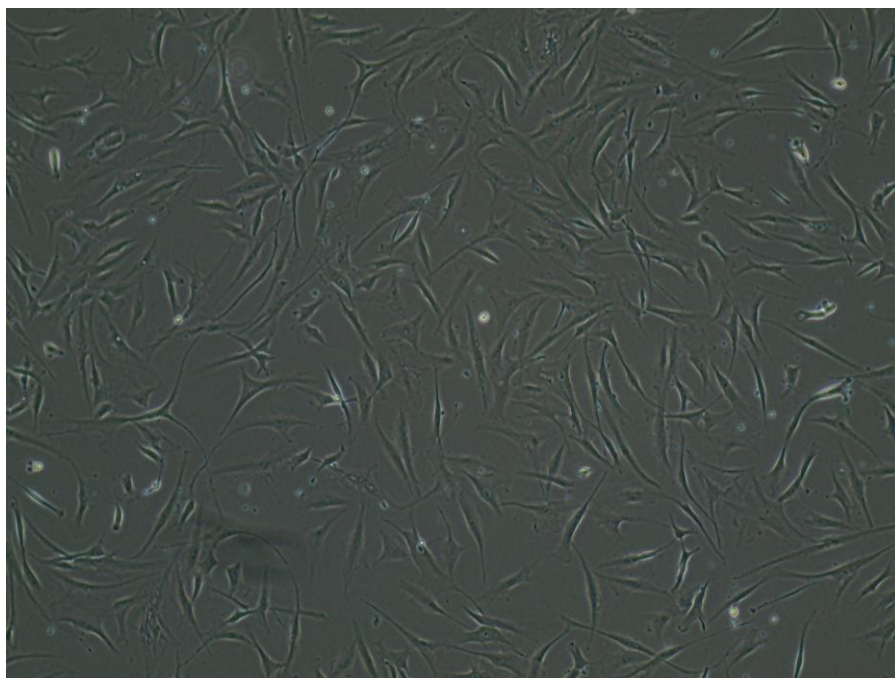


Figure 10. Morphological characterization of primary CAF line BRI_65. Representative phase-contrast micrograph showing primary cancer-associated fibroblasts established from patient sample BRI_65. Cells displayed elongated, spindle-shaped bodies with tapered cytoplasmic extensions and formed dense, whorled parallel bundles. Minimal cellular debris and few rounded cells were observed. Image was acquired using a light microscope at a total magnification of 200× (total= 20x (objective * 10x ocular)).

BRI_65 line exhibited a fibroblast-like phenotype, with elongated, spindle-shaped cells and tapered cytoplasmic extensions adherent to the culture surface (Figure 10). The cells formed

dense, interconnected networks arranged in closely packed, parallel bundles with a prominent whorled organization. Minimal cellular debris and few rounded cells were observed.

BRI_58 cultures also displayed a characteristic fibroblast-like morphology with elongated, spindle-shaped cells (Figure 11).

CAF BRI_58

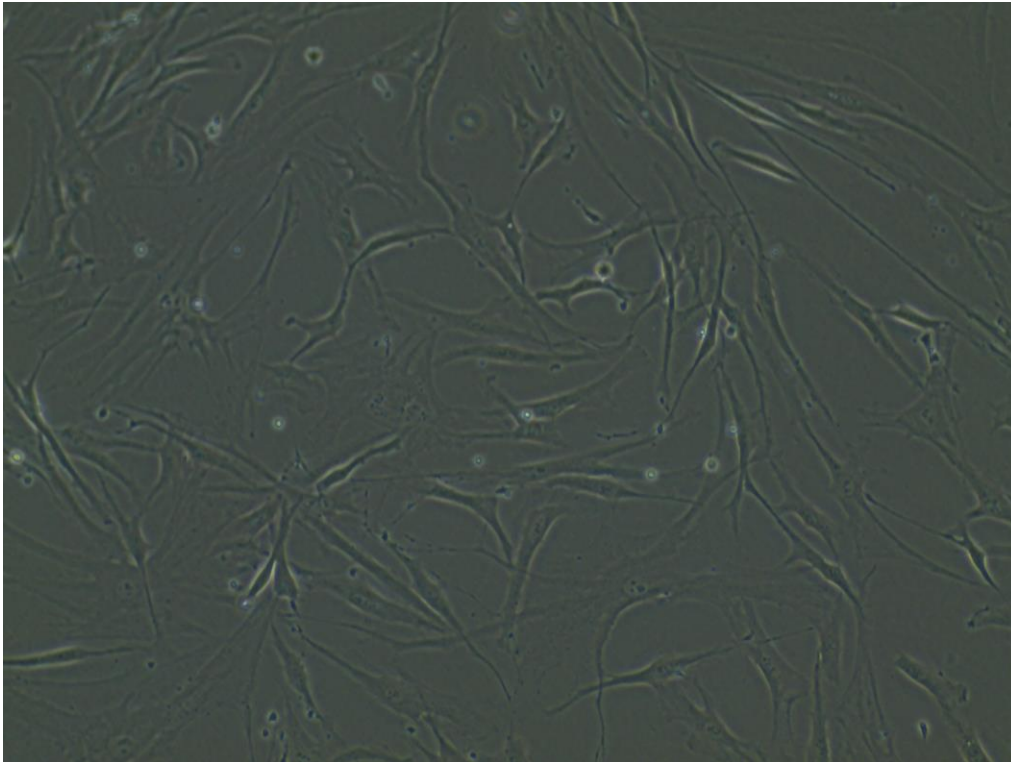


Figure 11. Morphological characterization of primary CAF line BRI_58. Representative phase-contrast micrograph showing CAF established from patient sample BRI_58. Cells displayed elongated, spindle-shaped bodies distributed across the culture surface at relatively low confluence, allowing individual cell outlines to be distinguished. Minimal debris and few rounded cells were observed. Image was acquired at a total magnification of 200 $\times$.

In summary, both BRI_58 and BRI_65 cell lines showed consistent fibroblast-like morphology, however, BRI_65 exhibited a more pronounced whorled spatial organization than BRI_58 under the conditions shown.

4.3. Development of the PDO–CAF Co-culture Model

4.3.1. Selection of the Optimal Co-culture Medium

To identify the culture conditions suitable for establishing a robust PDO–CAF co-culture model, several medium formulations were tested for their ability to support both cellular sub-populations (PDO and CAF). Standard PDO media formulations (C1, C2, C3, and C6) were supplemented with selected growth factors to develop the corresponding co-culture media (CC1, CC2, CC3, and CC6), as described in the methods section. Three CAF lines and five PDO models already available in laboratory (PDO1, PDO2, PDO3, PDO6, and PDO7) were cultured separately in each formulation for 5 days, after which cell viability was evaluated.

Across the tested standard PDO media and combined co-cultures formulations CAF viability remained approximately 50 – 70 % of the control value (Figure 12). CC1 showed the highest mean viability (approximately 70 %) but also showed substantial variability, whereas CC2, CC3 and CC6 yielded mean values of about 50 – 60 %. Overall, the combined formulations did not produce a uniform improvement in CAF viability compared with the standard PDO media.

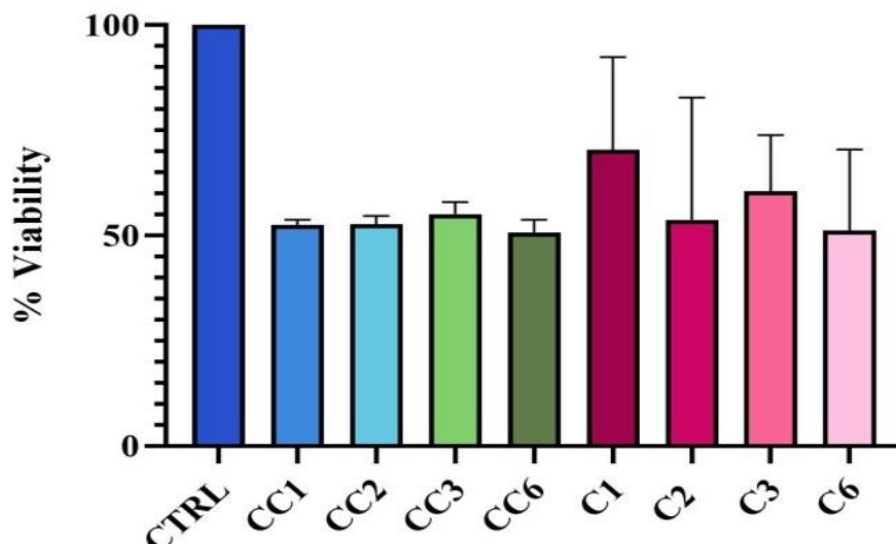


Figure 12: CAF viability across different medium formulations. Cancer-associated fibroblasts (CAF) were cultured for 5 days in standard organoid media (C1, C2, C3, and C6) or combined co-culture media (CC1, CC2, CC3, and CC6). Cell viability was assessed using the CellTiter-Glo assay and normalized to the reference control condition (CTRL, cultured in CAF2 medium). Data analysis was done using Student's t-test. Results are presented as median and SD (n = 3 independent experiments).

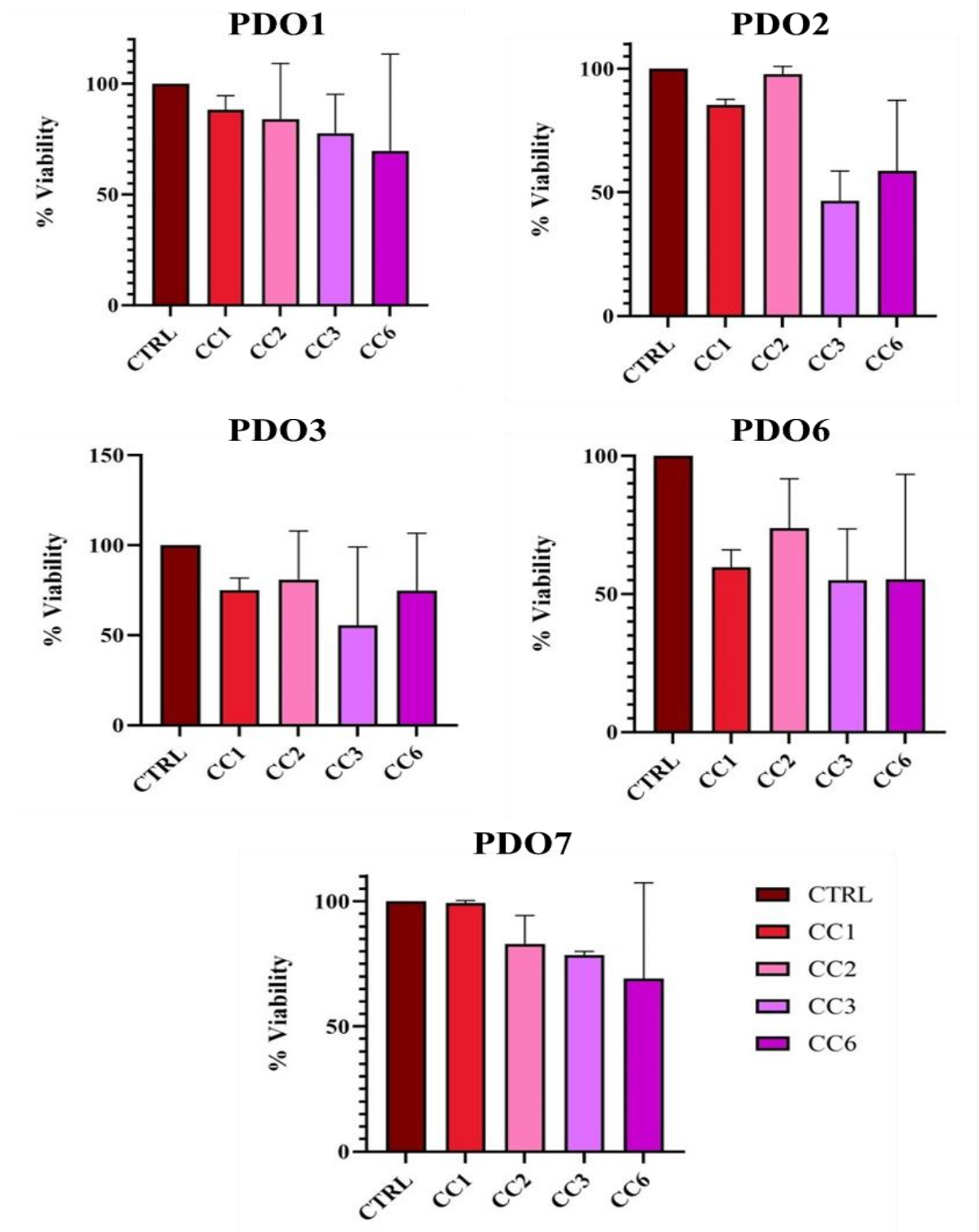


Figure 13:. Effect of combined co-culture media on PDO viability. PDO1, PDO2, PDO3, PDO6, and PDO7 were cultured for 5 days in CC1, CC2, CC3, or CC6. Viability was measured using the CellTiter-Glo assay and normalized to model-specific reference controls (CTRL = 100%). Control media were matched to each organoid model as follows: C1 medium for PDO1, C2 medium for PDO2, and C3 medium for PDO3, PDO6, and PDO7. Data analysis was done using Student's t-test. Results are presented as median and SD (n = 3 independent experiments).

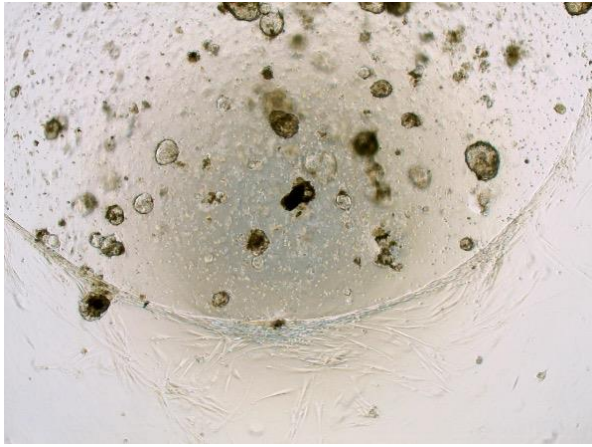
PDO responses were model-specific (Figure 13). CC2 yielded the highest mean viability for PDO2 (approximately 97 %), PDO3 around 82 % and PDO6, about 74 %, whereas CC1 was optimal for PDO1 (approximately 88 %), and PDO7 (approximately 98 %). CC3 and CC6 generally produced lower mean viability and/or greater variability.

Considering the requirements of both cellular compartments, CC2 was selected as the standard medium to use for CAF-PDO co-cultures because it provided the highest mean viability in three out of five PDO models while maintaining acceptable CAF viability.

4.3.2. Selection and Optimization of the Matrix

In addition to culture medium optimization, establishment of a physiologically relevant in vitro 3D co-culture model required a matrix capable for supporting both PDO architecture and CAF behavior and morphology. Standard PDO cultures rely on Matrigel, a basement-membrane extract rich in laminin and type IV collagen that supports PDO development. However, Matrigel alone may restrict fibroblast spreading, migration, and physical interaction. Collagen I, a major fibrillar component of the tumour stroma, was therefore incorporated to provide more permissive mesenchymal environment. and support CAF morphology and motility. To identify a matrix suitable for supporting both cell types simultaneously, cell morphology and spatial organization were evaluated in 100% Matrigel compared to a composite scaffold consisting of 25% Matrigel supplemented with Collagen I.

Matrigel



25% Matrigel+Collagen I

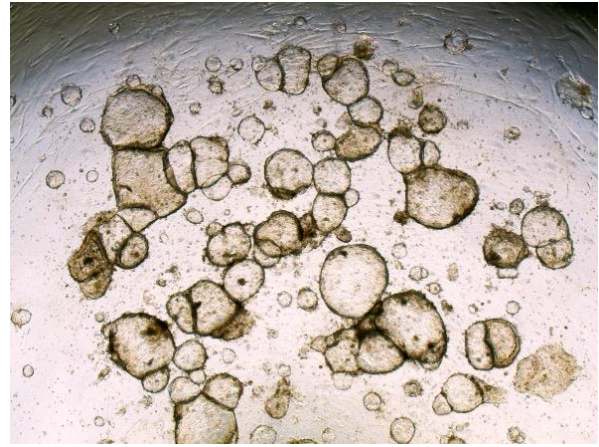


Figure14: Morphological evaluation of PDO and CAF in different scaffolds formulations at day 6. Representative bright-field microscopy images of PDO and CAF cultured in 100% Matrigel (A) or in a composite matrix containing of 25% Matrigel and 75 % Collagen I (B). The composite matrix maintained cystic PDO structures while permitting greater fibroblastic-like elongation and spreading

After 6 days, 100% Matrigel supported well-defined, spherical PDO structures, whereas CAF appeared largely constrained within the matrix and showed limited elongation (Fig 14). In the 25 % Matrigel plus 75 % collagen 1 composite, CAF displayed a more elongated, fibroblast-like morphology and greater spreading, while PDO retained their characteristic three-dimensional architecture.

Because the 25% Matrigel + Collagen I matrix improved CAF attachment and morphological extension without visibly compromising PDO structure it was selected as the standard matrix formulation for all subsequent PDO–CAF co-culture experiments.

4.3.3. Development and morphological Characterization of the Co-cultures

PDO-CAF co-cultures were established in the 25 % Matrigel + 75 % collagen I matrix and maintained in CC2 medium. Bright-field microscopy was used to monitor their organization over time. By day 6, PDO aggregates were closely associated with elongated stromal networks, forming multicellular structures with a mini-tumour-like appearance (Figure 15).

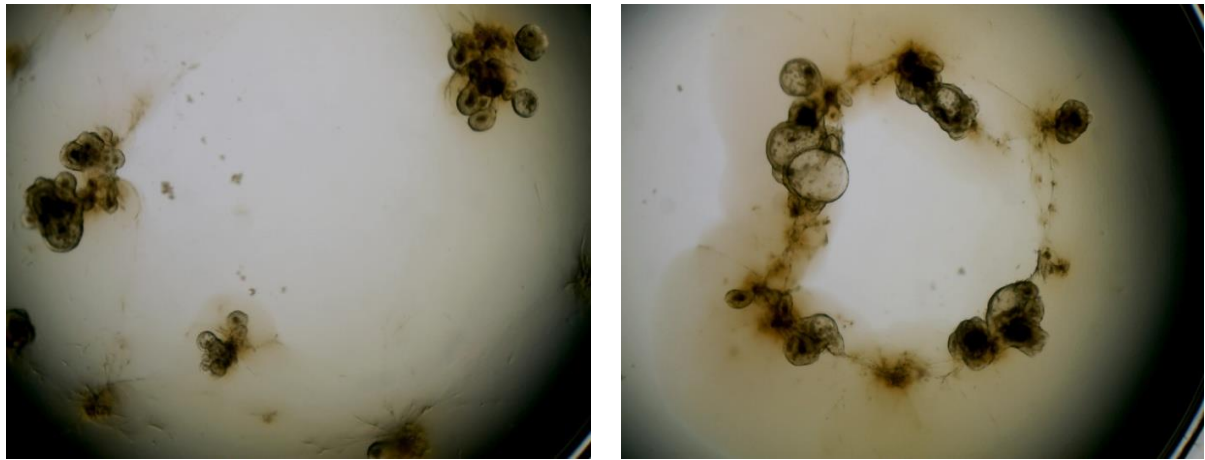


Figure 15. Morphological development and spatial organization of the PDO–CAF co-culture model. Representative bright-field images of PDO co-cultured with CAF in the 25 % Matrigel, 75 % Collagen I matrix after 6 days. Elongated fibroblast-like network was observed around and between PDO aggregates. Images were acquired using a light microscope at a total magnification of $40\times$ and $100\times$ respectively.

To assess the temporal organization of the 3D co-culture model, cultures were monitored from initial seeding to day 7. Progressive changes in the spatial relationship between the stromal and PDO compartments were observed at days 3, 5 and 7. At day 3, elongated CAF-like cells extended from the periphery toward the organoid-containing regions of the matrix, and thin stromal connections were visible between nearby PDO clusters (Figure 16). By day 5, more defined aligned stromal tracks were apparent, with organoids distributed along these structures. By day 7, larger PDO aggregates were surrounded by denser fibroblast-like networks, resulting in more compact multicellular structures.

These longitudinal observations indicate that the composite scaffold supports progressive stromal organization and close spatial association between CAF and patient-derived tumour organoids in vitro.



Day 3



Day 5



Day 7

Figure 16: Longitudinal morphological characterization of BRI_42 PDO and BRI_58 CAF in co-culture. Representative bright-field images of cultures in the Matrigel/collagen I matrix at day 3 (A), day 5 (B), and day 7 (C). Elongated CAF-like cells progressively extended into the matrix, aligned into stromal tracks, and became closely associated with PDO aggregates. Images were acquired at a total magnification of 40X.

Immunofluorescence staining for α -SMA and pan-cytokeratin was used to distinguish the stromal and epithelial compartments, respectively. Pan-cytokeratin-positive PDO clusters were observed in close spatial association with α -SMA-positive CAF networks, supporting the formation of an integrated multicellular structure (Figure 17).

IF analysis - BRI_58 PDO model

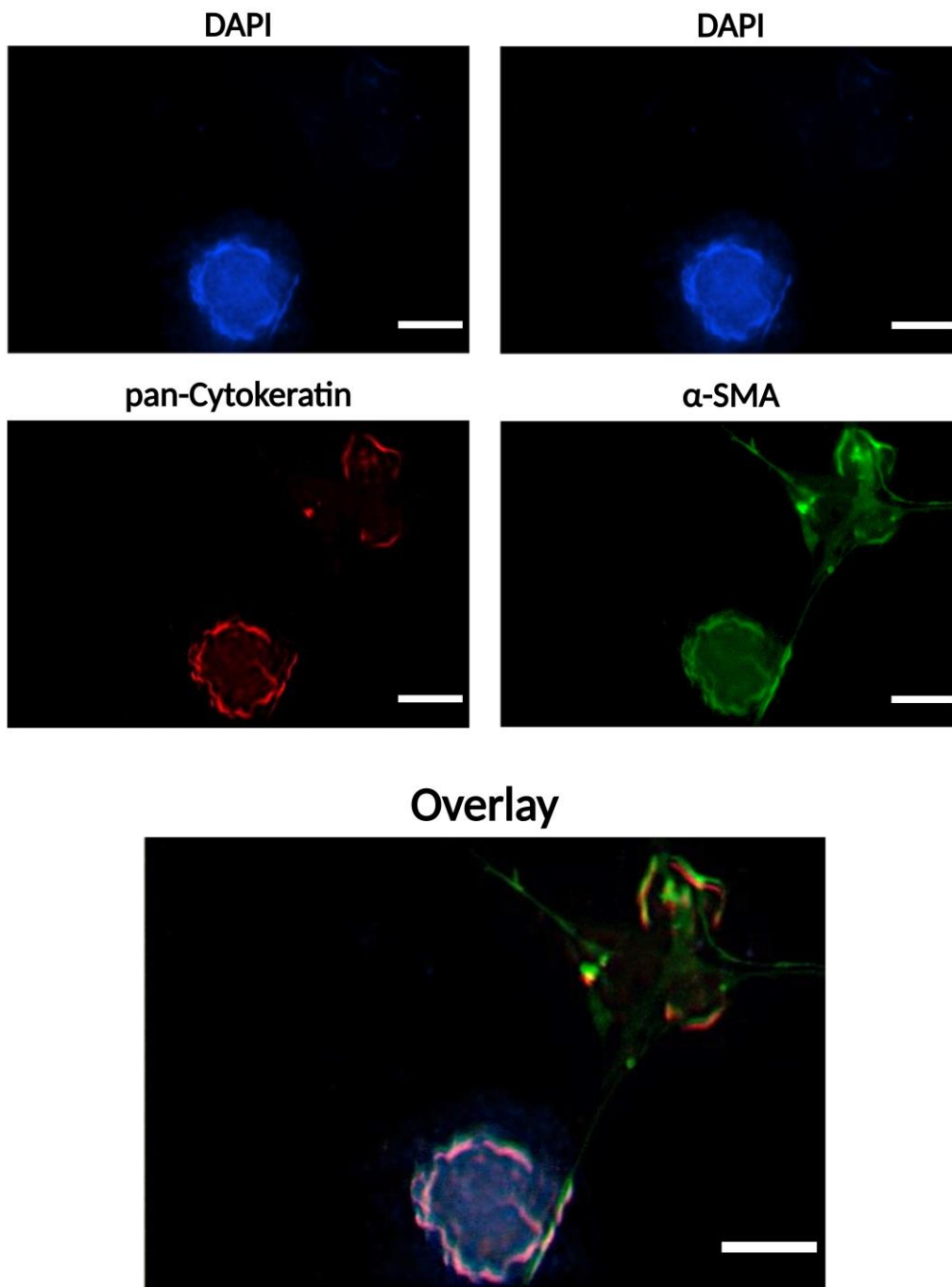


Figure 17: Immunofluorescence characterization of the BRI_58 PDO-CAF co-culture model. Representative immunofluorescence images of patient-derived organoid (PDO) and cancer-associated fibroblast (CAF) co-cultures. Cell nuclei were counterstained with DAPI (blue). Pan-Cytokeratin staining (red) identifies epithelial PDO structures, while α -SMA staining (green) marks activated CAF. Merged panel (Overlay) demonstrates spatial association between pan-Cytokeratin-positive PDO α -and-SMA-positive CAF networks. Images were captured at 10x magnification.

Overall, the Matrigel/Collagen I matrix supported CAF spreading and spatial organization, while maintaining PDO structures, enabling the establishment of a stable 3D PDO-CAFco-culture model. The model recapitulates key structural features of the CRCPM tumour-stroma interface and provides a platform for functional treatment-based studies in vitro.

4.4. PDO Treatment with the Five HIPEC Schemes

To evaluate the sensitivity of CRCPM-derived PDO to clinically relevant HIPEC protocols, BRI_42, BRI_47, and BRI_58 were treated with the five HIPEC schemes described in the Methods section and summarized in the table below. These schemes were selected to reflect clinically used the HIPEC protocols.

<i>HIPEC Scheme</i>	<i>Chemotherapeutic Agent(s)</i>
#1	Mitomycin C (MMC)
#2	Mitomycin C (MMC) + Cisplatin (CDDP)
#3	Doxorubicin (DOX) + Cisplatin (CDDP)
#4	Oxaliplatin (L-OHP — low dose, long-exposure protocol)
#5	Oxaliplatin (H-OHP — high dose, short-exposure protocol)

TABLE 11: Summary of the five HIPEC regimens evaluated.

4.4.1. Differential sensitivity of PDO to HIPEC Schemes

To compare responses to the five HIPEC schemes, viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay and expressed relative to untreated PDO (UNT), which were normalized to 100 %. A vehicle control (CTRL) was included where applicable.

BRI_42 displayed a clear regimen-dependent response. Regimens #1, #2, and #3 produced limited or moderate reductions in viability, with mean values of approximately 70–98 % relative to UNT. In contrast, the oxaliplatin-based regimens #4 and #5 markedly reduced viability to approximately 10 % and 6 %, respectively (Figure 18; $p < 0.001$).

Thus, BRI_42 showed relative resistance to mitomycin C-, cisplatin-, and doxorubicin-containing regimens, but pronounced sensitivity to both oxaliplatin protocols.

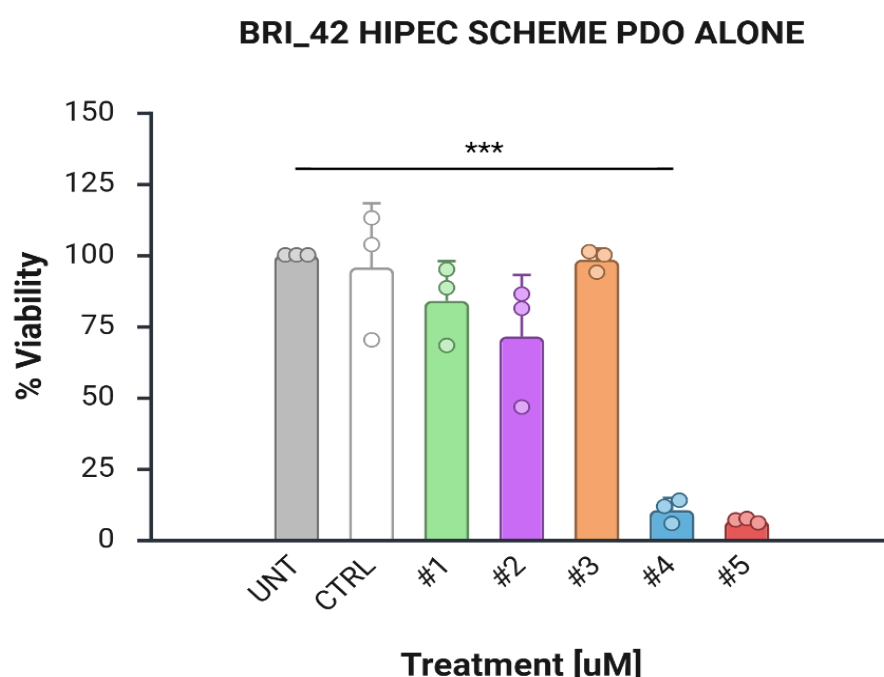


Figure 18: Differential response of BRI_42 PDO to five clinically relevant HIPEC schemes. Viability after regimens #1–#5 was measured using the CellTiter-Glo assay and expressed relative to untreated organoids (UNT = 100%). CTRL indicates the vehicle-treated control (DMSO for MMC, physiological solution for the others). Data analysis was done using Student's t-test. Results are presented as median and SD ($n = 3$ independent experiments).

Bright-field microscopy showed treatment-associated morphological changes consistent with the viability results (figure 19)

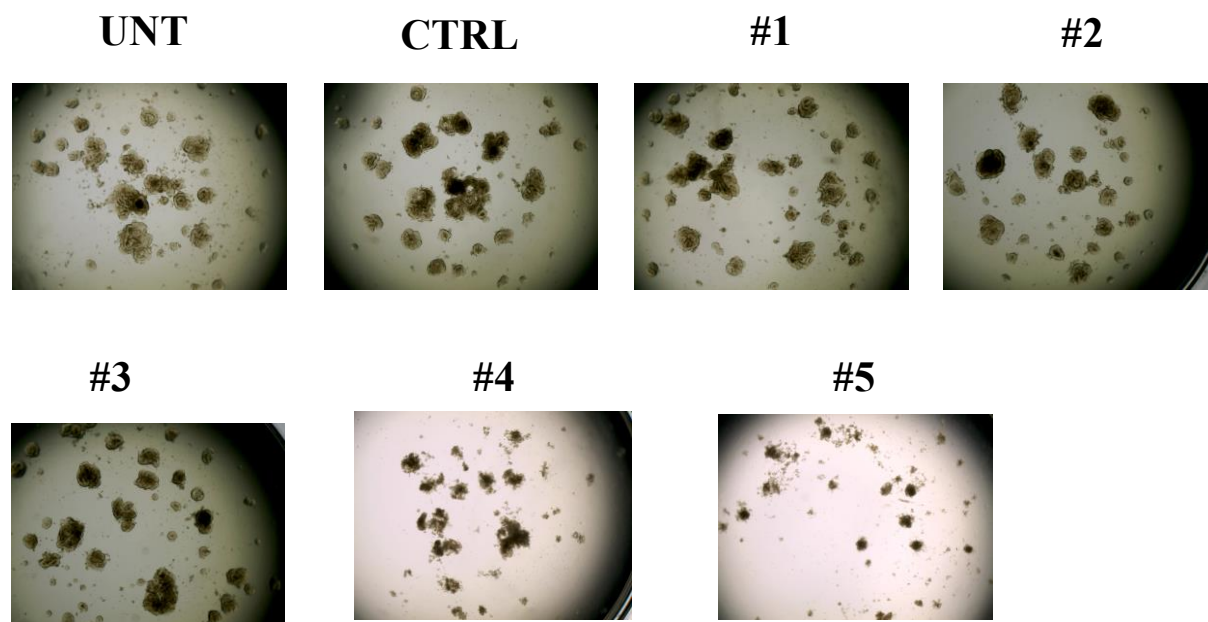


Figure 19: Morphological effects of HIPEC regimens on BRI_42 PDO. UNT and CTRL organoids retained dense, well-defined three-dimensional structures. Organoids exposed to regimens #1–#3 largely preserved their overall architecture, whereas regimens #4 and #5 were associated with marked loss of structural definition, increased compaction, and cellular debris. Images were acquired at a total magnification of 40 $\times$.

BRI_47 showed a similar overall pattern, but was more responsive than BRI_42 to schemes #1-3 (Figure 21). These schemes reduced mean viability to approximately 66 % ($p < 0.05$), 48 % ($p < 0.001$), and 53 % ($p < 0.01$), respectively. The oxaliplatin-based schemes #4 and #5 produced the strongest effects, reducing viability to below 10 % ($p < 0.0001$).

Accordingly, BRI_47 showed partial sensitivity to the mitomycin C-, cisplatin-, and doxorubicin-containing schemes and marked sensitivity to both oxaliplatin protocols.

BRI_47 HIPEC SCHEME PDO ALONE

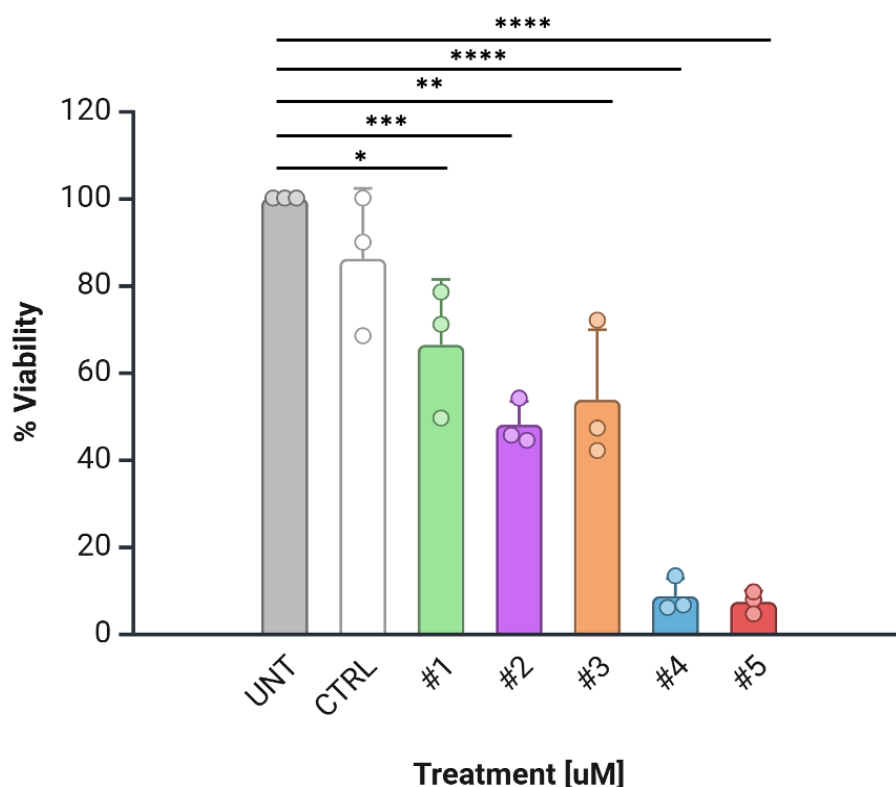


Figure 20: Differential response of BRI_47 PDO to five clinically relevant HIPEC schemes. Viability after schemes #1–#5 was measured using the CellTiter-Glo assay and expressed relative to untreated organoids (UNT = 100%). CTRL indicates the vehicle-treated control (DMSO for MMC, physiological solution for the others.). Data analysis was done using Student's t-test (* $p < 0.05$ ** $p < 0.01$; *** $p < 0.001$). Results are presented as median and SD ($n = 3$ independent experiments).

Bright-field microscopy revealed treatment-associated structural alterations consistent with the viability assay (Figure 21). UNT and CTRL organoids retained dense, spherical structures with well-defined boundaries.

Schemes #1–#3 were associated with progressive compaction and core darkening, although the overall three-dimensional architecture remained recognizable. In contrast, regimens #4 and #5 caused pronounced structural collapse, loss of defined organoid boundaries, and accumulation of cellular debris.

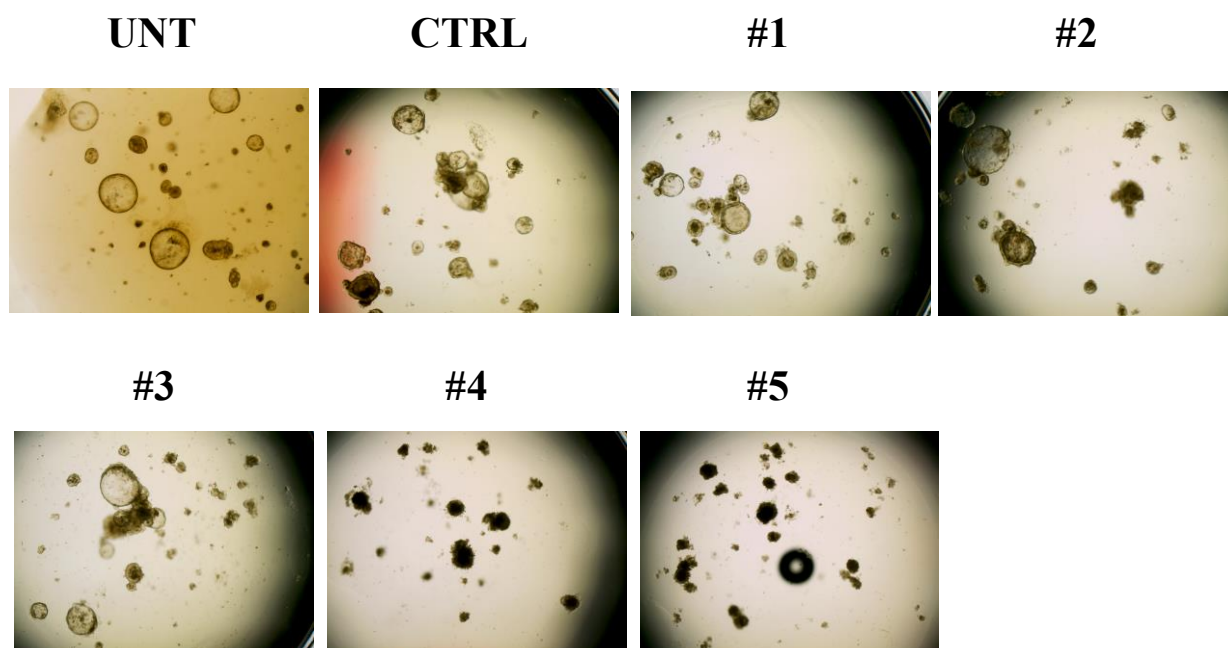


Figure 21: Morphological effects of HIPEC schemes on BRI_47 PDO. UNT and CTRL organoids retained dense, well-defined three-dimensional structures. Schemes #1–#3 caused mild compaction and core darkening while largely preserving organoid architecture. Schemes #4 and #5 were associated with pronounced structural collapse, loss of defined boundaries, and increased cellular debris. Images were acquired at a total magnification of 40 $\times$.

BRI_58 was the least responsive PDO line (Figure 22). Scheme #1 reduced viability to approximately 81 % ($p < 0.05$), whereas regimens #2 and #3 maintained approximately 75 % viability without reaching statistical significance. Schemes #4 and #5 were the most active, reducing viability to approximately 35 % ($p < 0.001$) and 48 % ($p < 0.05$), respectively; variability was greater for scheme #5.

BRI_58 HIPEC SCHEMES PDO ALONE

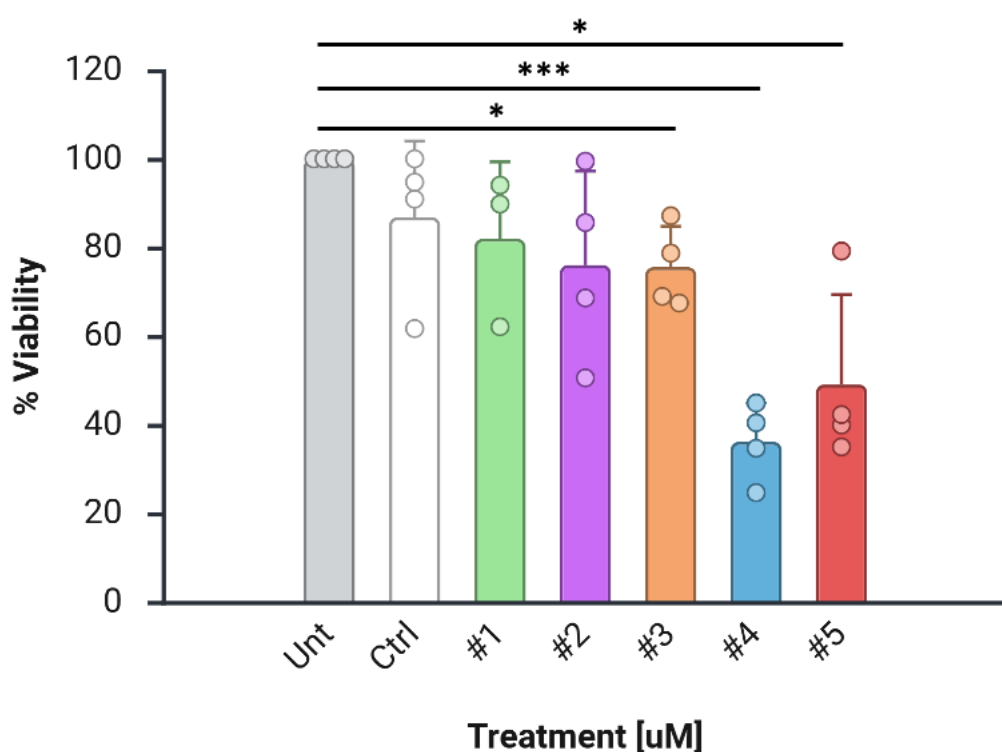


Figure 22: Differential response of BRI_58 PDO to five clinically relevant HIPEC Schemes. Viability after schemes #1–#5 was measured using the CellTiter-Glo assay and expressed relative to untreated organoids (UNT = 100%). CTRL indicates the vehicle-treated control (DMSO for MMC, physiological solution for the others). Data analysis was done using Student's t-test (* $p < 0.05$ ** $p < 0.01$; *** $p < 0.001$). Results are presented as median and SD ($n = 4$ independent experiments).

Bright-field microscopy was consistent with the functional data. BRI_58 organoids displayed a dense, multilobular morphology under baseline conditions. Schemes #1–#3 caused limited visible disruption, whereas treatments #4 and #5 were associated with peripheral disorganization, increased core condensation, and cell shedding (Figure 23). Residual three-dimensional structures remained visible after both oxaliplatin schemes, consistent with partial preservation of viability.

These results indicate that BRI_58 was relatively less sensitive than BRI_42 and BRI_47. Oxaliplatin-based treatments produced the greatest reduction in viability, but their effect remained incomplete compared with the near-total loss of viability observed in the other two PDO lines.

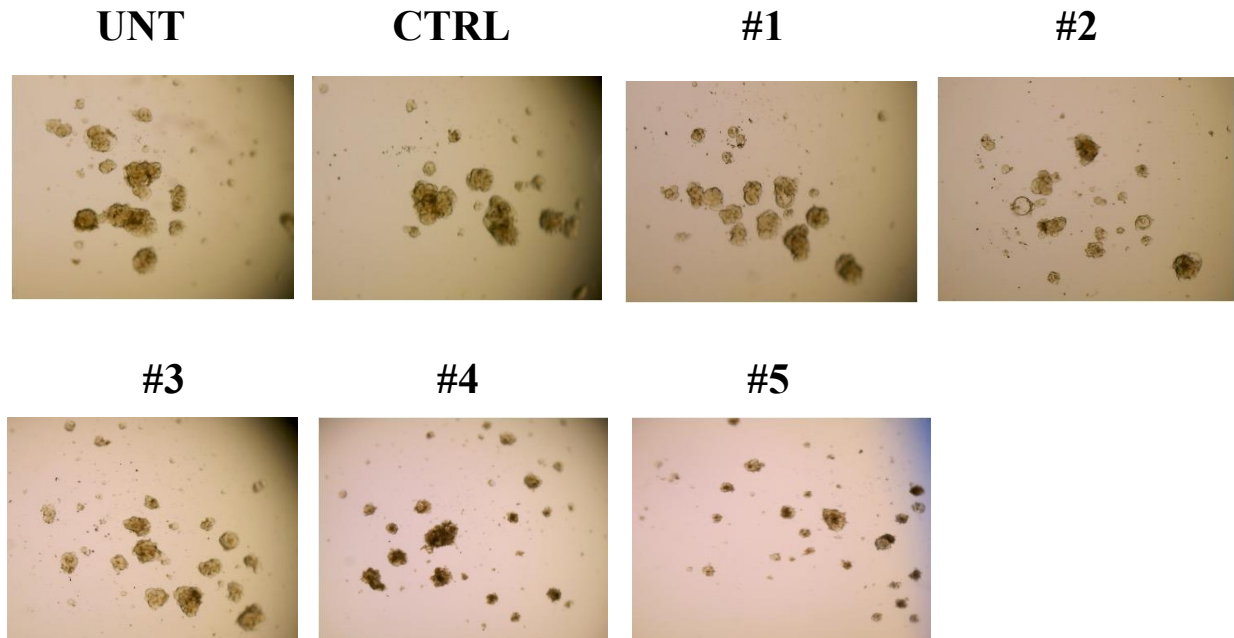


Figure 23: Morphological effects of HIPEC scheme on BRI_58 PDO. UNT and CTRL organoids displayed dense, multilobular aggregates with well-defined boundaries. Schemes #1–#3 largely preserved the three-dimensional architecture, whereas schemes #4 and #5 were associated with core condensation, peripheral disorganization, and increased cell shedding. Residual organoid structures remained visible after treatment. Images were acquired at a total magnification of 40 $\times$.

Overall, the three PDO models exhibited heterogeneous responses to the five HIPEC schemes. BRI_42 and BRI_47 were highly sensitive to schemes #4 and #5, whereas schemes #1–#3 produced smaller and line-dependent effects. BRI_58 was less sensitive overall; scheme #4 produced the strongest reduction in viability, while scheme #5 had a less pronounced and more variable effect.

This variability reflects substantial inter-patient heterogeneity, as no treatment produced an equivalent response across all PDO lines. These findings support the use of PDO as patient-specific preclinical models for comparing candidate HIPEC protocols.

4.4.2.Dose–Response Analysis and IC₅₀ Determination

To further characterize the chemosensitivity of the three PDO lines to oxaliplatin-based regimens, dose–response experiments were performed using the two in vitro HIPEC simulation protocols containing L-OHP: a low-dose/long-exposure schedule (Scheme #4) and a high-dose/short-exposure schedule (Scheme #5). Cell viability was assessed across a range of L-OHP concentrations, and dose–response curves were generated to determine the half-maximal inhibitory concentration (IC₅₀) for each PDO line and treatment schedule.

In both protocols, L-OHP induced a clear, dose-dependent decrease in BRI_42 organoid viability, confirming sensitivity to oxaliplatin in vitro. Under the low-dose/long-exposure condition (Scheme #4), the calculated IC₅₀ was 74.33 μ M ($R^2 = 0.9247$). In contrast, the high-dose/short-exposure condition (Scheme #5) yielded a slightly lower IC₅₀ of 63.48 μ M ($R^2 = 0.9935$). The comparable IC₅₀ values (Scheme #5/Scheme #4 ratio = 0.85) indicate limited schedule dependence in this PDO line. The dose–response curve obtained under Scheme #5 showed a better fit to the nonlinear regression model than that obtained under Scheme #4 ($R^2 = 0.9935$ vs. 0.9247).

Overall, BRI_42 displayed comparable oxaliplatin sensitivity under the two HIPEC simulation schedules, although Scheme #5 yielded a slightly lower IC₅₀ and a higher goodness-of-fit coefficient.

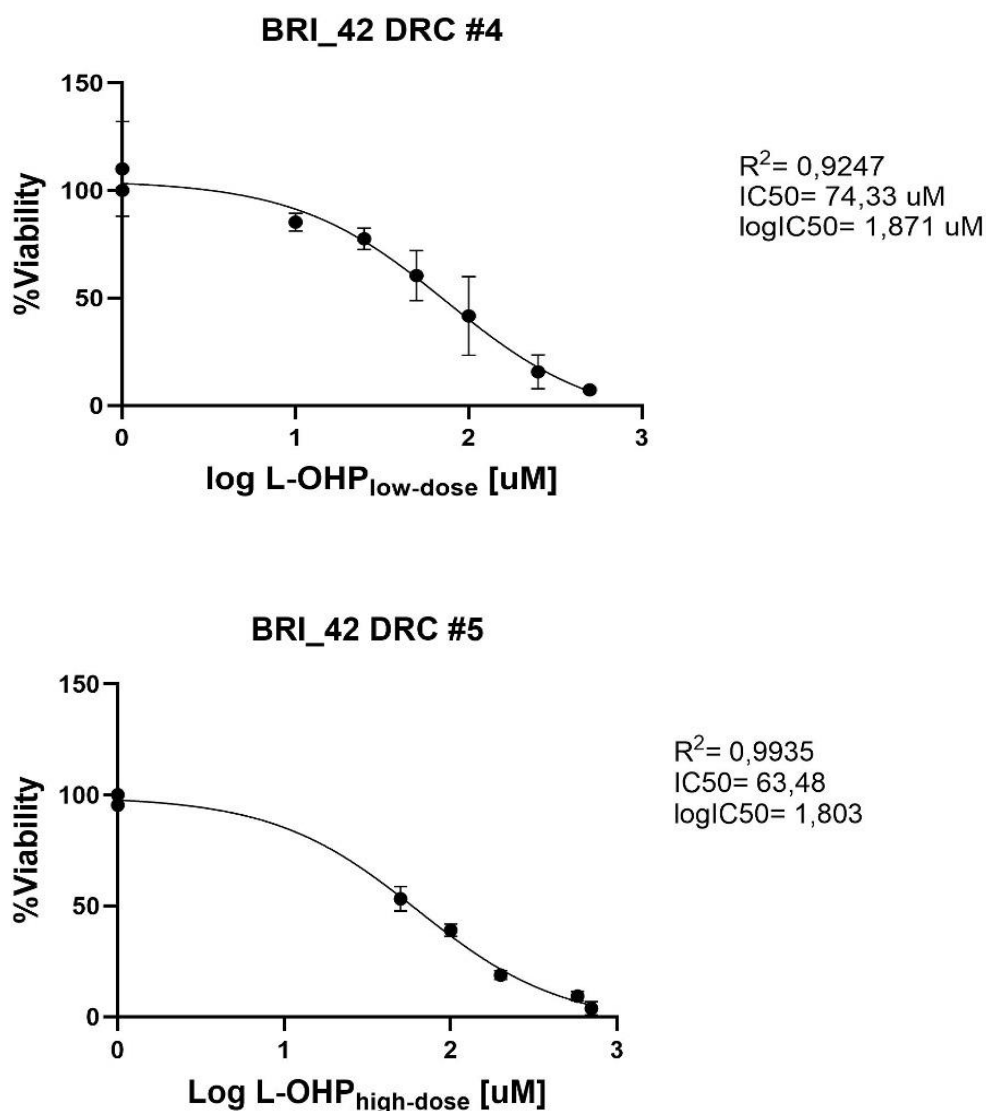


Figure 24: Dose–Response Curves (DRCs) for the BRI_42 PDO line following oxaliplatin treatment using two in vitro HIPEC simulation protocols. PDO were treated with increasing concentrations of L-OHP according to the low-dose/long-exposure schedule (Scheme #4) or the high-dose/short-exposure schedule (Scheme #5). Cell viability was measured, and dose–response curves were fitted using non-linear regression (log concentration vs. normalised response) to determine IC_{50} values. Scheme #4 yielded an IC_{50} of $74.33 \text{ }\mu\text{M}$ ($R^2 = 0.9247$). Scheme #5 yielded an IC_{50} of $63.48 \text{ }\mu\text{M}$ ($R^2 = 0.9935$). Bars represent mean $\pm$ SD ($n = 3$ independent replicates).

Dose–response analysis of the BRI_47 PDO line revealed marked schedule-dependent sensitivity to L-OHP. Under the low-dose/long-exposure condition (Scheme #4), the calculated IC_{50} was $28.80 \text{ }\mu\text{M}$ ($R^2 = 0.9307$). In contrast, the high-dose/short-exposure condition (Scheme #5) yielded an IC_{50} of $104.60 \text{ }\mu\text{M}$ ($R^2 = 0.9550$), corresponding to a 3.63-fold higher IC_{50} than that obtained under Scheme #4. Thus, BRI_47 organoids were more sensitive to prolonged,

low-dose L-OHP exposure than to short-term, high-dose exposure. This schedule-dependent response differed from the limited schedule dependence observed in BRI_42.

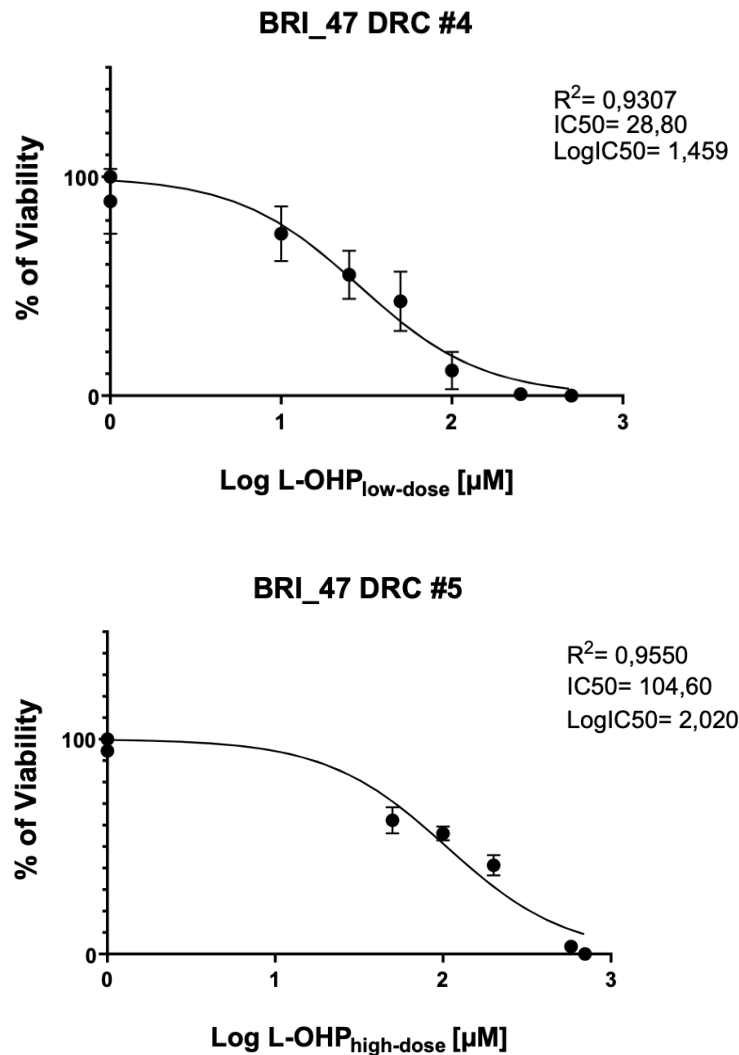
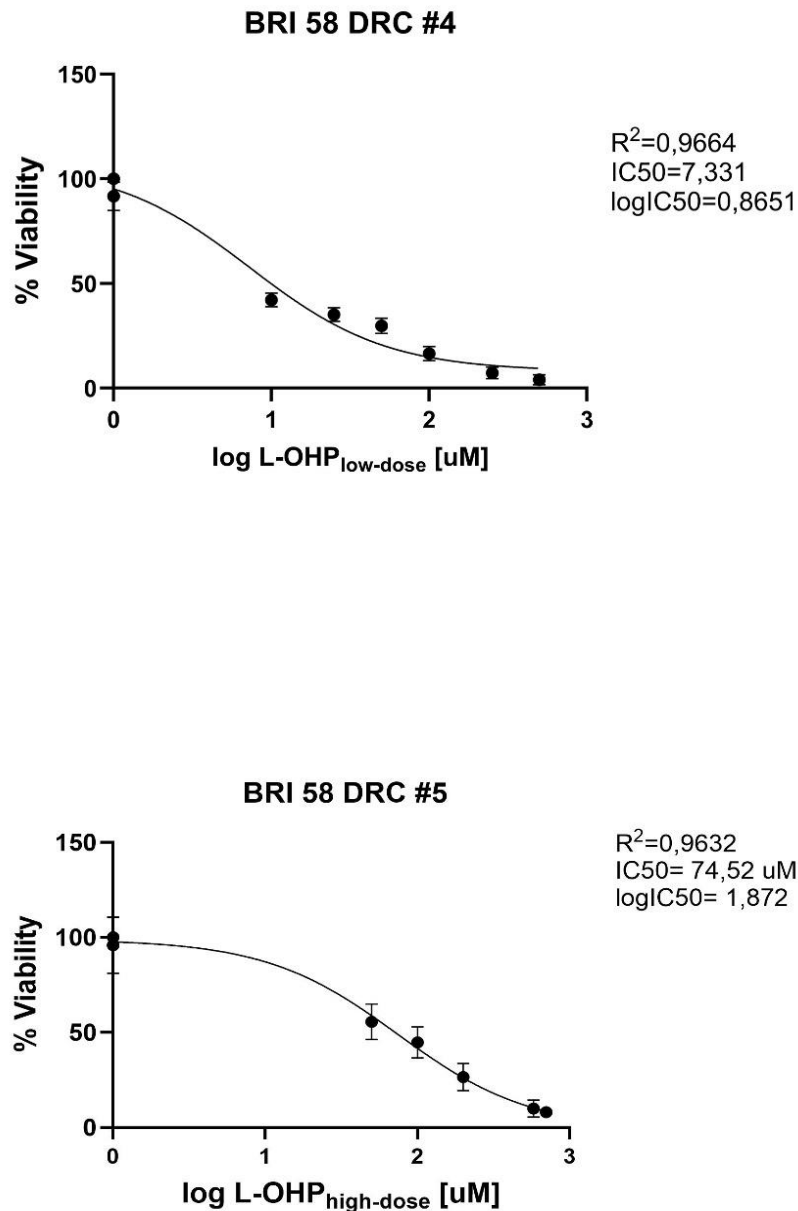


Figure 25: Dose–Response Curves (DRCs) for the BRI_47 PDO line following oxaliplatin treatment using two in vitro HIPEC simulation protocols. PDO were treated with increasing concentrations of L-OHP according to the low-dose/long-exposure schedule (Scheme #4) or the high-dose/short-exposure schedule (Scheme #5). Cell viability was measured, and dose–response curves were fitted using non-linear regression (log concentration vs. normalised response) to determine IC_{50} values. Scheme #4 yielded an IC_{50} of 28.80 μM ($R^2 = 0.9307$), whereas Scheme #5 yielded an IC_{50} of 104.60 μM ($R^2 = 0.9550$). Bars represent mean $\pm$ SD ($n = 3$ independent replicates).

Finally, dose–response analysis was performed on the BRI_58 PDO line following treatment with the two oxaliplatin-based HIPEC simulation protocols (Figure 26). Under Scheme #4, BRI_58 organoids were highly sensitive to L-OHP, with a calculated IC_{50} of 7.33 μM ($R^2 = 0.9664$). In contrast, Scheme #5 yielded a substantially higher IC_{50} of 74.52 μM ($R^2 =$

0.9632), corresponding to a 10.16-fold higher IC_{50} than under Scheme #4. These findings indicate that BRI_58 organoids were considerably more sensitive to prolonged, low-dose L-OHP exposure than to short-term, high-dose exposure. Among the three PDO lines, BRI_58 exhib-



ited the greatest schedule-dependent difference in oxaliplatin sensitivity.

Figure 26: Dose–Response Curves (DRCs) for the BRI_58 PDO line following oxaliplatin treatment using two in vitro HIPEC simulation protocols. PDO were treated with increasing concentrations of L-OHP according to the low-dose/long-exposure schedule (Scheme #4) or the high-dose/short-exposure schedule (Scheme #5). Cell viability was measured, and dose–response curves were fitted using non-linear regression (log concentration vs. normalised response) to determine IC_{50} values. Scheme #4 yielded an IC_{50} of 7.33 μM ($R^2 = 0.9664$). Scheme #5 yielded an IC_{50} of 74.52 μM ($R^2 = 0.9632$). Bars represent mean $\pm$ SD ($n = 3$ independent replicates).

Across the evaluated panel of patient-derived organoids, L-OHP induced a dose-dependent reduction in viability under both treatment schedules; however, the magnitude of the response and its dependence on the exposure schedule varied substantially among PDO lines (Table 12). BRI_42 exhibited comparable chemosensitivity under the two regimens, with a Scheme #5/Scheme #4 IC₅₀ ratio of 0.85. In contrast, BRI_47 and BRI_58 were more sensitive to the low-dose/long-exposure protocol. The IC₅₀ under Scheme #5 was 3.63-fold higher than under Scheme #4 in BRI_47 and 10.16-fold higher in BRI_58. Overall, these findings demonstrate inter-patient heterogeneity in both oxaliplatin sensitivity and schedule dependence across the PDO models.

	Scheme #4		Scheme #5		IC ₅₀ Shift	
PDO	IC ₅₀ (μM)	R ²	IC ₅₀ (μM)	R ²	Scheme #5 / Scheme #4	Primary Sensitivity Driver
BRI_42	74.33	0.9247	63.48	0.9935	0.85×	Equipotent / Schedule-Independent
BRI_47	28.8	0.9307	104.6	0.955	3.63×	Duration-Dependent
BRI_58	7.33	0.9664	74.52	0.9632	10.16×	Strongly Duration-Dependent

Table 12: Comparative IC₅₀ values and sensitivity dynamics of patient-derived organoids (PDO) under HIPEC Schemes #4 and #5. Half-maximal inhibitory concentrations IC₅₀, μM) and goodness-of-fit values (R²) were determined for three PDO models (BRI_42, BRI_47, and BRI_58) exposed to Scheme #4 and Scheme #5. The IC₅₀ shift ratio (Scheme #5 / Scheme #4) highlights the relative potency difference between treatment regimens, categorizing each model's primary sensitivity driver as schedule-independent (equipotent) or duration-dependent.

4.5. Response of CAF to HIPEC Schemes

The responses of the CAF BRI_58 and CAF BRI_65 lines to the five HIPEC regimens, were evaluated using the CellTiter-Glo Luminescent Cell Viability Assay. Viability was expressed relative to untreated cells (Unt), set to 100%, and a vehicle control (Ctrl) was included to account for solvent effects.

Consistent with the overall trends observed in the PDO models, CAF BRI_58 line displayed a marked regimen-dependent response. Scheme #1, #2, and #3 did not induce substantial cytotoxicity, whereas the oxaliplatin-containing schemes (#4 and #5) significantly reduced fibroblast viability, reducing mean survival to approximately 36% and 46 %, respectively (Figure 27; **** $p < 0.0001$ vs Unt). These findings indicate that CAF BRI_58 cells were selectively sensitive to oxaliplatin-based HIPEC regimens while remaining largely resistant to mitomycin C-, cisplatin-, and doxorubicin-containing protocols tested.

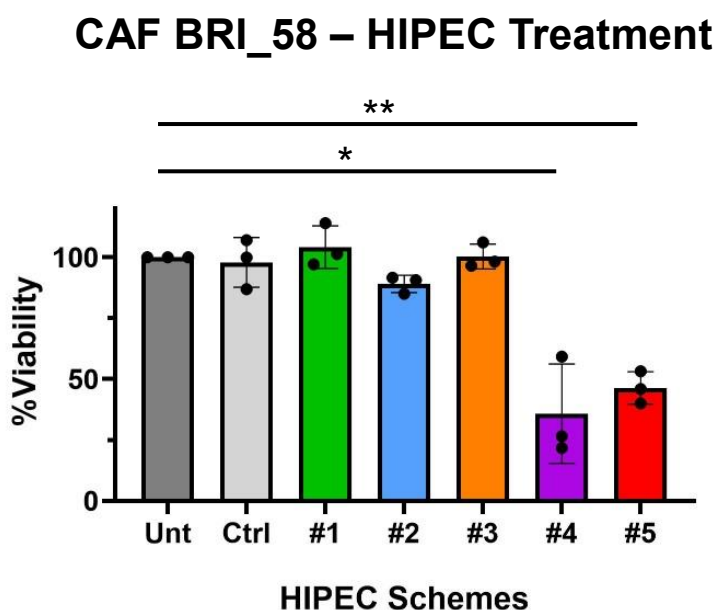


Figure 27: Impact of HIPEC Schemes on CAF BRI_58 Viability. Cell viability of primary CAF BRI_58 cells following treatment with HIPEC Schemes #1-#5. Viability was measured using the CellTiter-Glo assay and expressed as a percentage of untreated cells (Unt, set to 100%). Ctrl denotes vehicle-treated cells (DMSO for MMC, physiological solution for the others). Data analysis was done using Student's t-test (* $p < 0.05$ ** $p < 0.01$; *** $p < 0.001$). Results are presented as median and SD ($n = 4$ independent experiments).

CAF BRI_65 showed a comparable regimen-dependent response, with the oxaliplatin-based treatments producing the greatest cytotoxic effect. Schemes #1, #2, and #3 resulted in moderate or variable viability reductions in viability, with mean values of approximately 92%, 66%, and 78% relative to UNT, respectively. In contrast, schemes #4 and #5 markedly reduced viability to approximately 27 % ($p < 0.01$) and 26 % (* $p < 0.05$), respectively (Figure 28).

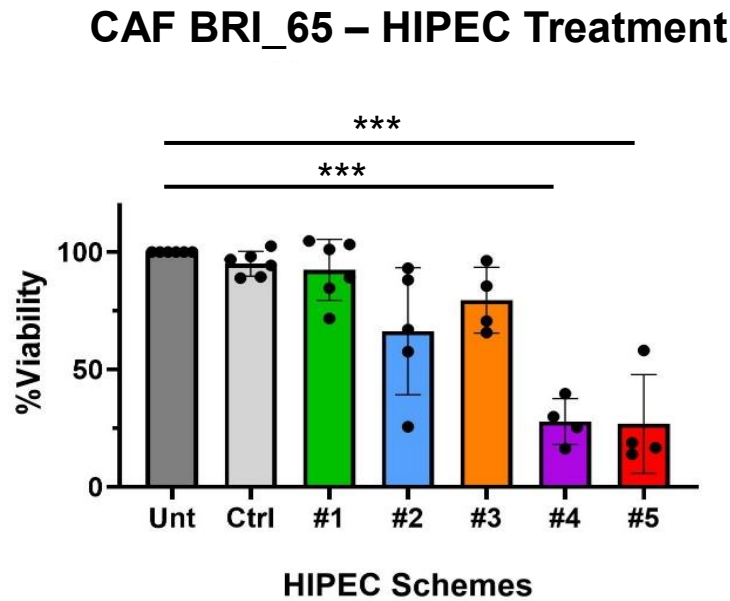


Figure 28: Impact of HIPEC Schemes on CAF BRI_65 Viability. Cell viability of primary CAF BRI_65 cells following treatment with HIPEC Schemes #1-#5. Viability was measured using the CellTiter-Glo assay and expressed as a percentage of untreated cells (Unt, set to 100%). Ctrl denotes vehicle-treated cells (DMSO for MMC, physiological solution for the others). Data analysis was done using Student's t-test (**** $p < 0.0001$). Results are presented as median and SD ($n = 4$ independent experiments).

4.6. Impact of CAF Co-Culture on Organoid Sensitivity to HIPEC Schemes

To investigate the role of the stromal microenvironment in modulating treatment response, the three PDO models were evaluated as PDO monocultures CAF monocultures, and direct PDO/CAF co- Viability following each of the five HIPEC schemes was quantified using the CellTiter-Glo Luminescent Cell Viability Assay and normalized to the corresponding untreated condition, (UNT, 100 %). Vehicle-treated controls were included where applicable.

Across the panel, the effect of CAF co-cultures was PDO-model dependent. Co-culture increased viability after oxaliplatin-based treatment in the BRI_42 and BRI_58 models, whereas

no statistically supported protective effect was detected in BRI_47. Thus, the response of the co-culture could not be predicted solely from the sensitivity of either cell compartment in monoculture.

In the BRI_42-CAF BRI_58 model, PDO monocultures were highly sensitive to schemes #4 and #5, with viability reduced to approximately 5 and 6 %, respectively (Figure 30). The corresponding CAF monoculture retained approximately 30 and 34 % viability, whereas co-culture viability increased to approximately 40 % under Scheme #4 ($***p < 0.001$ vs PDO alone) and 31 % under Scheme #5 ($**p < 0.01$ vs PDO alone; Figure 29). Two-way ANOVA identified a significant interaction between treatment regimen and culture condition ($*p < 0.05$), supporting a CAF-associated protective effect in this model. (Figure 29).

In the BRI_47 model (PDO BRI_47-CAF BRI_65), the interaction between treatment regimen and culture condition was not significant, and the main effect of culture condition was also non-significant (Figure 30). Although mean viability in co-culture was higher than in PDO monoculture under Scheme #5, neither oxaliplatin-based scheme showed a statistically significant CAF-mediated rescue. Both PDO monocultures and PDO-CAF co-cultures therefore remained highly sensitive to Schemes #4 and #5.

BRI_58 displayed the strongest stromal protective effect. Schemes #4 and #5 reduced PDO monoculture viability to approximately 18 and 13 %, respectively. The corresponding CAF monoculture retained approximately 36 and 46 % viability, whereas PDO-CAF co-culture increased viability to approximately 64 and 57 %, respectively ($***p < 0.001$ vs PDO alone for both treatments; Figure 29). A significant interaction between treatment and culture condition was detected ($p < 0.05$). Co-culture also significantly increased viability under Scheme #2, indicating that the stromal protective effect was not restricted to oxaliplatin-containing regimens. Taken together, these findings show that CAF-mediated modulation of HIPEC response is context dependent. CAF markedly attenuated oxaliplatin-induced cytotoxicity in the BRI_42 and BRI_58 co-cultures, whereas no significant protective effect was detected in BRI_47. These results highlight both the contribution of tumour-stroma interactions and the inter-patient heterogeneity of HIPEC responses.

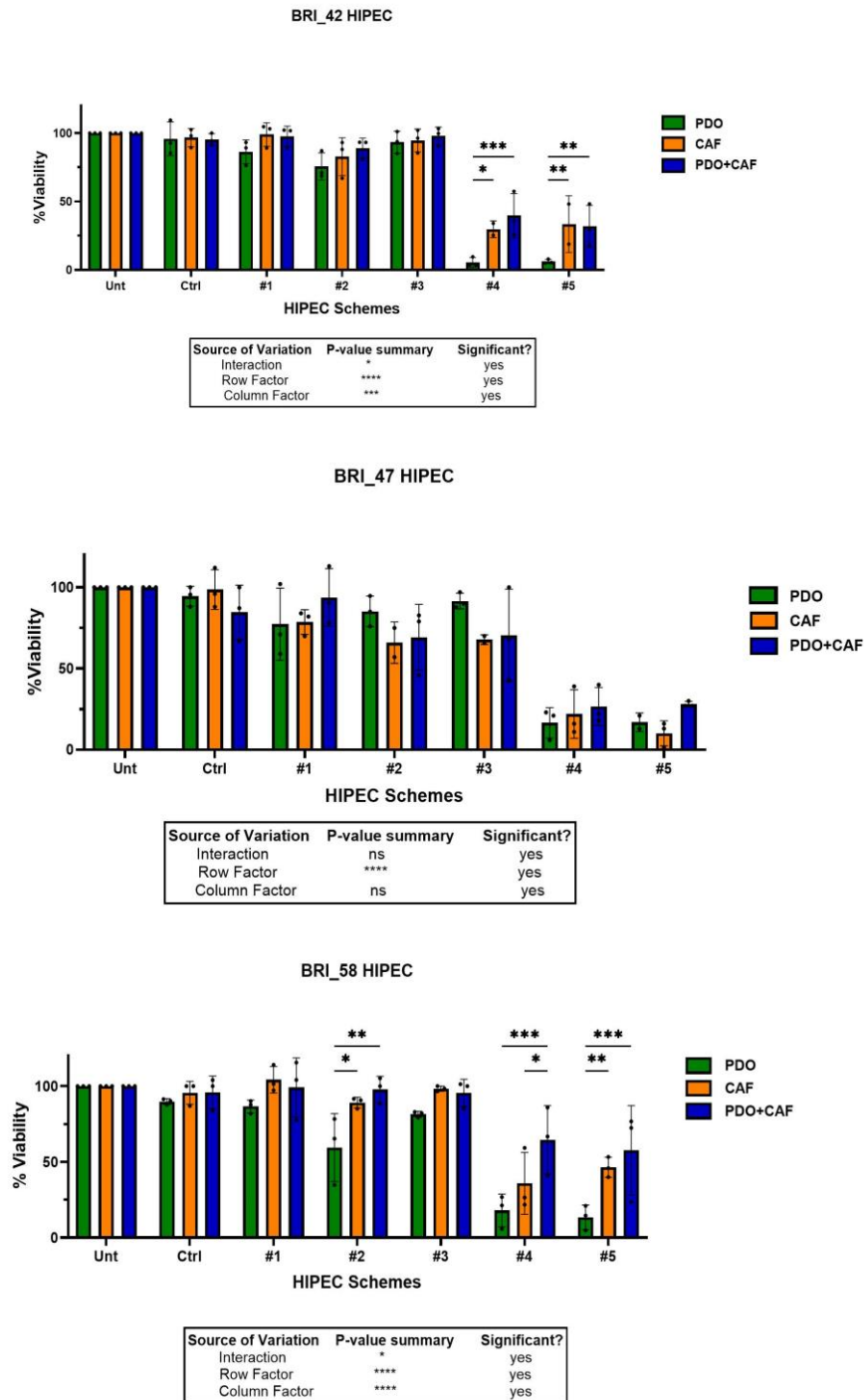


Figure 29: Effect of CAF Co-Culture on the Response of PDO Models to HIPEC Schemes. Relative viability of the BRI_42, BRI_47, and BRI_58 PDO models analysed as PDO monocultures (green), CAF monocultures (orange), or direct PDO-CAF co-cultures (blue) after treatment with HIPEC Schemes #1-#5. Viability was measured using the CellTiter-Glo assay and normalized to the corresponding untreated condition (Unt, 100%); Ctrl denotes vehicle-treated controls (DMSO for MMC, physiological solution for the others.). Bars represent mean $\pm$ SD ($n = 3$ independent experiments). Two-way ANOVA summary tables report the interaction between treatment and culture condition and the main effects of treatment (row factor) and culture condition (column factor). Post-hoc comparisons are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant. Co-culture combinations comprised BRI_42 PDO + BRI_58 CAF, BRI_47 PDO + BRI_65 CAF, and BRI_58 PDO + BRI_58 CAF

5. Discussion

CRCPM remains a clinical challenge, as recurrence rates after CRS/HIPEC is high (Breuer et al., 2021; Varinelli et al., 2024). Preclinical models able to capture the biological heterogeneity of CRCPM and predict individual therapeutic response could help refine treatment selection. We developed PDO and CAF models from CRCPM tissue and established PDO–CAF co-culture systems that recapitulate key features of the tumour–stroma interface, and assessed whether stromal inclusion modifies the response to clinically relevant HIPEC schemes. Our findings support the conclusion that both tumour-intrinsic features and stromal interactions shape HIPEC response in a patient- and regimen-specific manner.

Three PDO models and two CAF lines were successfully derived from CRCPM surgical specimens and maintained stable growth throughout the experiments. PDO showed distinct morphologies, ranging from cystic to compact three-dimensional structures. Such diversity is consistent with the ability of PDO to preserve patient-specific histological and molecular features rather than converging towards a uniform phenotype in culture (van de Wetering et al., 2015; Weeber et al., 2015). This heterogeneity is particularly relevant because the same models showed markedly different treatment sensitivity, supporting their use as individual rather than interchangeable models. Both CAF lines showed the expected spindle-shaped, fibroblast-like morphology, with BRI_65 showing a more organised whorled pattern than BRI_58, suggesting phenotypic differences that may also reflect functional CAF heterogeneity.

We identified culture conditions that support the co-culture of PDO and CAF. While neither the culture medium nor the matrix alone was optimal for both cell types, the combination of CC2 medium and a Matrigel–Collagen I composite matrix represented the best overall compromise, supporting the growth of both PDO and CAF. This is biologically plausible because PDO culture favours epithelial self-renewal within a basement-membrane-like environment, whereas fibroblasts require a more permissive fibrillar matrix for spreading, migration and ECM remodelling. Indeed, CAF elongation was limited in pure Matrigel, while the Matrigel/Collagen I mix preserved PDO architecture while allowing CAF extension. Similar studies have shown that engineered collagen-rich matrices and matched PDO-CAF systems restore stromal programmes that are absent from epithelial-only cultures and can alter drug response (Luo et al., 2021; Marin et al., 2023). The progressive formation of CAF tracks connecting PDO aggregates, and immunofluorescence showed PDO and CAF compartments closely associated. This indicates that the model resembles the real tumours–stroma architecture seen *in vivo* and in previous CRCPM co-culture studies (Varinelli et al., 2024), supporting the interpretation

of the viability results that follow as a real tumor–stroma interaction, rather than two cell populations simply coexisting without influencing each other.

Across all three PDO models, the oxaliplatin-based schemes (#4 and #5) produced the greatest reduction in viability, while mitomycin C-, cisplatin-, and doxorubicin-containing schemes (#1–#3) had more limited and model-dependent effects. The response to oxaliplatin was not uniform across models: BRI_42 and BRI_47 showed almost complete loss of viability, while BRI_58 retained a measurable surviving fraction, indicating partial resistance. This inter-patient variability is consistent with previous CRC-PM organoid studies reporting heterogeneous responses to clinically relevant HIPEC agents (Ubink et al., 2019; Varinelli et al., 2024). Notably, although oxaliplatin-based regimens were the most effective treatments in our three models, our group reported greater efficacy for mitomycin C-based regimens in a larger CRC-PM PDO cohort (Varinelli et al., 2024). These results likely reflect differences in the biological characteristics of the patient cohorts, highlighting that the relative efficacy of HIPEC regimens may vary according to tumors-specific features.

The dose-response and IC₅₀ analyses further showed that oxaliplatin efficacy depends not only on concentration, but also on exposure schedule, and that this dependence varies between models. BRI_42 responded similarly to both oxaliplatin-based schedules, while BRI_47 and BRI_58 were much more sensitive to the low-dose/long-exposure protocol. This observation is clinically relevant because the benefit of high-dose/short-exposure schedule remains debated (Quénet et al., 2021; van de Vlasakker et al., 2021). The stronger effect of prolonged exposure in two models is also consistent with previous CRC-PM-PDO data showing limited efficacy of high-dose/short-exposure oxaliplatin scheme (Varinelli et al., 2024). Mechanistically, prolonged exposure may increase intracellular drug accumulation or allow DNA crosslinks to persist beyond the repair capacity of specific tumor cells; however, drug uptake, platinum–DNA adduct formation, and repair pathways were not directly assessed and this interpretation remains speculative.

Both CAF lines also showed greater sensitivity to the oxaliplatin-based regimens, mirroring the response observed in the PDO. These findings indicate that HIPEC exposure affects not only the epithelial but also the stromal compartment, arguing against the view of CAF as intrinsically resistant bystanders. Nevertheless, residual CAF survival after treatment may remain biologically relevant. Surviving CAF could continue to provide trophic support to tumor cells or acquire a therapy-induced secretory phenotype that promotes disease progression. Recent evidence in colorectal cancer has shown that chemotherapy can reprogram CAF signaling, leading to resistance-associated paracrine responses (Yin et al., 2025).

The co-culture experiments revealed a model-dependent effect: CAF inclusion significantly reduced the efficacy of oxaliplatin-based HIPEC in the BRI_42 and BRI_58 co-cultures, whereas no statistically significant protective effect was observed in the BRI_47 model. Thus, the presence of CAF is not sufficient on its own to confer resistance, and the effect also depends on specific properties of the tumors cells. Protection probably depends on the compatibility of a specific tumour-stroma pairing, including the ability of CAF to produce survival signals and the capacity of the corresponding PDO to respond to them. This interpretation is consistent with studies on PDO-stroma biobanks showing that CAF-mediated resistance is patient-, sub-type- and treatment-dependent (Farin et al., 2023; Wang et al., 2025). Interestingly, the strongest protective effect was observed in BRI_58, which was already the least sensitive PDO model in monoculture and showed the greatest schedule dependence variability. One possible interpretation is that tumour-intrinsic resistance and stromal protection act cooperatively, allowing cells that survive the initial drug exposure to benefit more effectively from CAF-derived rescue signals. This hypothesis would need to be confirmed in a larger group of patients. Co-culture also increased BRI_58 viability under Scheme #2, indicating that the protective effect of CAF was not limited to oxaliplatin. These findings suggest that CAF may influence therapy response through multiple, potentially overlapping mechanisms, including paracrine activation of pro-survival pathways, restoration of tumour transcriptional programmes, extracellular-matrix remodelling, and secretion of cytokines such as IL-6 and IL-8, which can also contribute to the formation of physical barriers to drug penetration (Ryu et al., 2024; Lamichhane et al., 2025; Demuytere et al., 2020; Varinelli et al., 2024; Zhang et al., 2025). These findings are consistent with, and extend, previous studies showing that CAF increase the IC50 of chemotherapy drugs and reduce apoptosis in CRC-PM co-culture models (Varinelli et al., 2024). By directly comparing PDO and CAF monocultures with co-cultures our study further suggests that CAF-mediated protection cannot be explained solely by the intrinsic chemosensitivity of either the tumour or the stromal compartment. Instead, the response appears to depend on the specific interaction between each PDO and its matched CAF. In fact, although BRI_47 CAF were at least as sensitive to oxaliplatin as BRI_58 CAF, they did not confer any measurable protection to the corresponding PDO, whereas BRI_58 CAF significantly attenuated the response to treatment. These findings indicate that resistance emerging in co-culture is an interaction-dependent property of the tumour–stroma pair, rather than a simple consequence of the individual drug sensitivities of the two cell populations.

This study has several limitations. First, the limited number of PDO–CAF pairs restricts the identification of clinical or molecular features associated with stromal protection. Second, the

ATP-based CellTiter-Glo assay used in co-culture does not distinguish the contribution of PDO and CAF to residual viability, requiring future validation through compartment-specific approaches. Third, the model does not yet fully recapitulate the peritoneal tumour microenvironment, lacking immune, endothelial, and mesothelial components. Finally, the molecular mechanisms underlying CAF-mediated protection remain to be defined and will require further investigation through approaches such as secretome profiling, pathway inhibition, and transcriptomic analyses.

6. Conclusions

These findings support the rationale of including stromal components in preclinical drug-testing platforms for CRC_{PM}. While PDO monocultures remain informative and have demonstrated predictive potential in metastatic CRC (Ooft et al., 2019; Vlachogiannis et al., 2018), they may underestimate resistance mechanisms that emerge in the presence of the tumour stroma. Importantly, co-culture did not uniformly increase drug resistance; instead, it added model-specific information, highlighting the importance of tumour–stroma interactions in shaping treatment response. The observation that both CAF-mediated protection and oxaliplatin schedule dependence varied across patient-derived models further suggests that PDO–CAF co-cultures could support not only the selection of the most effective HIPEC regimen but also the optimisation of drug exposure conditions, such as perfusion time and dosing strategy, for individual patients.

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