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The High-Grade Serous Ovarian Cancer Metastasis and Chemoresistance in 3D Models --Manuscript Draft--

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Abstract:	<p>High-grade serous ovarian cancer (HGSOC) is the most frequent and aggressive type of epithelial ovarian cancer, with high recurrence rate and chemoresistance being the main issues in its clinical management. HGSOC is specifically challenging due to the metastatic dissemination via spheroids in the ascitic fluid. The HGSOC spheroids represent the invasive and chemoresistant cellular fraction, which is impossible to investigate in conventional two-dimensional (2D) monolayer cell cultures lacking critical cell-to-cell and cell-extracellular matrix interactions. Three-dimensional (3D) HGSOC cultures, where cells aggregate and exhibit relevant interactions, offer a promising in vitro model of peritoneal metastasis and multicellular drug resistance. This review summarizes recent studies of HGSOC in 3D culture conditions and highlights the role of multicellular HGSOC spheroids and ascitic environment in HGSOC metastasis and chemoresistance.</p>
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Response to Reviewers:	

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Zagreb, December 6th 2023

Dear Dr. Polyak,

Thank you for sending our manuscript BBACAN-D-23-00556 entitled “The High-Grade Serous Ovarian Cancer Metastasis and Chemoresistance in 3D Models” prepared by Vanja Tadić, Wei Zhang and Anamaria Brozovic to review. We obtained valuable and constructive suggestions, which we hopefully could address here and add to the revised version of the manuscript accordingly.

Please find our responses to the reviewer’ comments. The changes performed in the manuscript are marked in yellow.

Our responses to Reviewer #1:

Reviewer #1: This is a well-written and extensive review on high-grade serous ovarian cancer, with focus on 3D *in vitro* model systems, which imitate spheroid-like metastases in the ascitic fluid. 3D-models were described imitating the various forms of HGSOC, as well as spreading mechanisms, interaction with other cell types such as macrophages, tumor vascularization etc. It is worth to be published since it complements the existing literature.

Specific comments:

- 1. The described 3D *in vitro* models are considered to be useful for testing drug resistance, in order to avoid unnecessary treatments. Therefore, it is worth to describe in more detail which drugs can and will be tested and what the measured end points are. For instance, can apoptosis, other forms of cell death and senescence be measured in the described 3D models?**

We agree with the reviewer that it is worth providing a more detailed explanation in the main text about all the drugs tested in 3D HGSOC models. In the revised version of the manuscript, we added the section „3.1. *Treatment of High-Grade Serous Ovarian Cancer*“ in which we describe the guideline-recommended treatments for HGSOC, to provide an introduction for Table 2 and the newly added section „3.4. *Measuring Drug Resistance in 3D High-Grade Serous Ovarian Cancer Cell Models*“ (added immediately after Table 2). In section „3.4.“ we describe in more detail the strategies and measured endpoints in studies describing the testing of several approved drugs for HGSOC treatments in the various 3D models (listed in Table 2). These studies also included the measurement of cell death and we have added this to the section. We believe that this addition is a great improvement to the manuscript since it makes it more cohesive and provides better flow.

2. Fig. 2 summarizes the general mechanisms of drug resistance in HGSOC. Standard therapeutics are carboplatin and paclitaxel. The mechanism of action of these drugs is different, as the mechanisms of resistance are. I recommend to add a few words on carboplatin and paclitaxel (used alone or in combination?) to the text and legend of Fig. 2 and to indicate which of the mechanisms in Fig. 2 pertain to either one of the drugs.

We agree with the Reviewer. Following the previously-mentioned, revised section „3.1. *Treatment of High-Grade Serous Ovarian Cancer*“, a short, concise section „3.2. *Mechanisms of Action of Drugs Approved for High-Grade Serous Ovarian Cancer Treatment*“ was added. In this section, together with carboplatin and paclitaxel mechanisms of action we described in addition all the recommended therapeutics mentioned in the previous section, „3.1.“ This was a helpful way to introduce Figure 2. We have added also remarks to the legend indicating which mechanisms of drug resistance pertain to each therapeutic approach. In addition, Figure 2 was also a bit improved, in our opinion, and appears to be clearer than before.

3. Are PARP-1 inhibitors used as monotherapy or in combination with carboplatin and/or paclitaxel? How can PARP-1 sensitive cells in 3D be identified and compared with 3D resistant cells?

We agree that more information about the therapeutic strategy utilizing PARP inhibitors is a good addition to this manuscript since there are several studies listed in Table 2 investigating the PARP inhibitors sensitivity in 3D HGSOC models. In section „3.1.“ we have included information about the use of PARP inhibitors in clinical settings, and in section „3.2.“ their mechanism of action is described. In section „3.4.“ we have provided more details on one of the novel studies listed in Table 2., which describes a novel 3D model for measuring sensitivity toward PARP inhibitors olaparib and niraparib.

4. In platinum-resistant ovarian cancer, non-platinum mono-chemotherapy, including topotecan, gemcitabine, and pegylated liposomal doxorubicin (PLD) are used. I recommend to mention this and how sensitivity to these drugs can be detected in a 3D model (e.g. by extending Fig. 2).

We are thankful for this comment, as it does add to the fluidity of the manuscript. In section „3.1.“, we have addressed this comment by describing the logic behind the use of these drugs in certain HGSOC cases, and in section „3.2.“ we have described the drugs' mechanisms of action. We have also added the remarks to the legend of Figure 2 to indicate which of the depicted resistance mechanisms relate to these drugs. By doing this, Table 2, which lists studies investigating drug sensitivity in 3D models, fits the manuscript more smoothly.

5. In Fig. 3, apoptosis as a major form of cell death was even not mentioned. Does apoptotic death don't play a role in drug resistance? What is the role of senescence and can senescence (SA- β -GAL) be measured in 3D cultivated cells/spheroids?

We agree that the cell death was omitted from the main text and Figure 3, and it is, of course, the most important factor in drug response and the measured end point in many studies utilizing 3D models for investigation of HGSOC drug resistance. We have corrected this by mentioning apoptosis in the added text about drugs' mechanisms of action and in the legend of Figure 2. In the section „3.4.“ we have added information about measurement of apoptosis and necrosis in 3D models utilized in the studies listed in Table 2. We have also added the term „cell death“ to a part of Figure 3 (lower part of the Figure 3) listing measured end points in described models. As far as we know the cell senescence is not measured in 3D HGSOC model so far.

6. What is the best 3D system today in use authors may recommend for measuring chemoresistance? Is there any reference cell line/type available for comparison, e.g. a 3D cell system that is known to be highly resistant or highly sensitive?

Basic differences between organoids and spheroids are already discussed in the section „3.7. *3D Models Used for Chemotherapy Response Prediction*“. We are not aware of available reference cell line/type for comparison (highly resistant or highly sensitive) which could be used as a possible threshold/control for future use of HGSOC organoids

as a tool in the prediction of the most successful therapy in clinical settings. But this is surely one of the important points that need to be addressed in the future.

In conclusion, we sincerely hope that we have addressed all concerns and that the Reviewer and Editor will revise their opinion and accept this work for publishing in the *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*.

Thank You & best regards,



Dr. Anamaria Brozovic, in the name of the authors

The High-Grade Serous Ovarian Cancer Metastasis and Chemoresistance in 3D Models

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Abstract

High-grade serous ovarian cancer (HGSOC) is the most frequent and aggressive type of epithelial ovarian cancer, with high recurrence rate and chemoresistance being the main issues in its clinical management. HGSOC is specifically challenging due to the metastatic dissemination via spheroids in the ascitic fluid. The HGSOC spheroids represent the invasive and chemoresistant cellular fraction, which is impossible to investigate in conventional two-dimensional (2D) monolayer cell cultures lacking critical cell-to-cell and cell-extracellular matrix interactions. Three-dimensional (3D) HGSOC cultures, where cells aggregate and exhibit relevant interactions, offer a promising *in vitro* model of peritoneal metastasis and multicellular drug resistance. This review summarizes recent studies of HGSOC in 3D culture conditions and highlights the role of multicellular HGSOC spheroids and ascitic environment in HGSOC metastasis and chemoresistance.

Keywords: High-Grade Serous Ovarian Cancer, Ascites, 3D Models, Spheroids, Organoids, Metastasis, Chemoresistance

Abbreviations

2D: Two-dimensional

3D: Three-dimensional

ALDH: Aldehyde dehydrogenase

ASCs: Adult stem cells

ATCs: Ascitic tumor cells

BRD4: Bromodomain containing protein 4

CAFs: Cancer-associated fibroblasts

CA-MSCs: Carcinoma-associated mesenchymal stem/stromal cells

CBP: Carboplatin

cDDP: Cisplatin

CNVs: Copy number variations

CSCs: Cancer stem-like cells

DXR: Doxorubicin

DTX: Docetaxel

EC₅₀: Half maximal effective concentration

ECM: Extracellular matrix

EET: Epithelial-to-endothelial transition

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-to-mesenchymal transition

EOC: Epithelial ovarian cancer

EpCAM: Epithelial cell adhesion molecule

EVs: Extracellular vesicles

FAP: Fibroblast activation protein

GelMA: Gelatin methacryloyl

GEM: Gemcitabine

HGF: Hepatocyte growth factor

HGSOC: High-grade serous ovarian cancer

HIFs: Hypoxia-inducible factors

HRD: Homologous recombination deficiency

HUVECs: Human umbilical vein endothelial cells

IC₅₀: The half-maximal inhibitory concentration value

IL-6: Interleukin 6

iPSCs: Induced pluripotent stem cells
KRT14: Keratin 14
LCs: Leader cells
MDR1: Multidrug resistance gene
MET: Mesenchymal-to-epithelial transition
miR: microRNA
MMT: Mesothelial- to- mesenchymal transition
MUs: Metastatic units
NETs: Neutrophil extracellular traps
NK Cell: Natural killer cell
OC: Ovarian cancer
PANO: Panobinostat
PARP: Poly (ADP-ribose) polymerase
PARPi: PARP inhibitor
PD-1: Programmed cell death protein 1
PD-L1: PD-1/programmed death-ligand 1
PDGF: Platelet-derived growth factor
PDMS: Polydimethylsiloxane
PEG: Polyethylene glycol
PFS: Progression-free survival
PI3K: Phosphoinositide 3-kinase
PLD: PEGylated liposomal doxorubicin
poly-HEMA: Poly-2-hydroxyethyl methacrylate
ROS: Reactive oxygen species
SAHA: Suberoylanilide hydroxamic acid (vorinostat)
SMAC: second mitochondrial activator of caspase
STAT1: Signal transducer and activator of transcription-1
STIE: Systemic tumor immune environment
TAMs: Tumor-associated macrophages
TAX: Paclitaxel
T β RI: TGF- β transmembrane serine/threonine kinase receptor type I
TGF- β : Transforming growth factor beta
TIME: Tumor immune microenvironment
TME: Tumor microenvironment

TNF- α : Tumor necrosis factor alpha

TOP: Topotecan

ULA: Ultra-low adherent

VEGF: Vascular endothelial growth factor

VM: Vasculogenic mimicry

1. Background

1.1. Ovarian Cancer

Ovarian cancer (OC) is a highly aggressive malignancy that is typically identified at an advanced stage, rendering it challenging for treatment. It is the deadliest cancer of the female reproductive system, with a markedly rising incidence and mortality rate (Torre *et al.*, 2015; Bray *et al.*, 2018; Sung *et al.*, 2021). For over 25 years, the standard of care treatment for OC has consisted of cytoreductive surgery and platinum/taxane-based adjuvant chemotherapy (Raja *et al.*, 2012). Although the majority of OC patients initially respond to chemotherapy, 80-90% of patients diagnosed with advanced-stage disease will relapse and develop chemoresistance (Matulonis *et al.*, 2016). OC is a highly heterogeneous disease, with epithelial OC (EOC) accounting for over 90% of diagnoses. EOC is divided into two distinct groups: Type I EOC (endometrioid, clear cell, seromucinous, mucinous, low-grade serous carcinomas, and malignant Brenner tumors) and Type II EOC consisting of high-grade serous ovarian carcinomas (HGSOCs), carcinosarcomas and undifferentiated carcinomas (Kurman & Shih, 2016). HGSOC is responsible for more than 90% of OC deaths, is highly heterogeneous, and can be divided into many subtypes based on its molecular and morphological characteristics (Kurman & Shih, 2016). The main differences between HGSOC and Type I EOC are the rapid and aggressive progression of HGSOC, its high chromosomal instability, ubiquitous *TP53* mutations, and shared defects in the homologous recombination DNA repair pathway ('Integrated genomic analyses of ovarian carcinoma' 2011; Kurman & Shih, 2016; Köbel & Kang, 2021). Because of these fundamental differences, Type I EOCs grow slowly, *in situ*, into well-differentiated carcinomas, whereas HGSOCs proliferate and metastasize rapidly (Lengyel, 2010).

Despite therapy advances, such as the addition of anti-angiogenic drugs and targeted therapies to conventional chemotherapy, the five-year survival rate for patients with HGSOC remains low (Raja *et al.*, 2012; Maru & Hippo, 2019). The main problem with the novel targeted therapies is that they fit only a particular subgroup of patients, most of whom also develop drug resistance. For example, poly (ADP-ribose) polymerase (PARP) inhibitors, such as olaparib (LYNPARZA®), niraparib (ZEJULA®), and rucaparib (RUBRACA®), are intended for those patients with defective homologous recombination DNA repair, primarily carriers of *BRCA1/2* mutations, which is the case in approximately 15% of OC patients (Qin *et al.*, 2022). It is imperative to provide treatments for HGSOC patients who demonstrate proficiency in homologous recombination DNA repair. Additionally, most patients acquire drug resistance upon

prolonged oral administration of PARP inhibitors (Li *et al.*, 2020). To overcome the drug resistance and metastatic capability of OC, it is critical to develop preclinical models that can accurately reflect the biology of OC and can be used to evaluate the efficacy of new therapeutic approaches. Until satisfactory therapeutic strategies emerge, it is also essential to develop adequate patient-derived models as predictors of clinical outcomes of the available drug therapy. Three-dimensional (3D) cell models are the optimal choice for researching OC due to their numerous benefits.

1.2. 3D Models for Studying Ovarian Cancer

Preclinical drug development has generally employed cancer cell lines as models. These models have several shortcomings that limit their usefulness in predicting a drug's effectiveness in clinical trials (reviewed by Gillet *et al.*, 2013). Clinical trials for most drugs tested in cancer cell models frequently fail. This poor translation of preclinical data is a significant problem, and several studies emphasized the inconsistencies in therapeutic response between current *in vitro* and *in vivo* models. For EOC, 3D culturing resulted in a different proliferation rate and sensitivity to chemotherapeutics than 2D culturing of the same OC cells, as well as altered gene and protein expression patterns (Zietarska *et al.*, 2007; Myungjin Lee *et al.*, 2013; Chen *et al.*, 2014). It is rather intuitive to consider the flexible 3D cell culture models better in recapitulating primary tumors, ascitic environment, and metastasis than traditional 2D monolayer cell cultures with forced cellular polarity and cellular interactions. Since OC cells detach from the surface of the primary tumor into the peritoneum, the anchorage-independent 3D cultures and co-cultures of multicellular OC spheroids or organoids with additional cell types from the tumor microenvironment (TME) represent a promising *in vitro* model of the metastasis and drug resistance. 3D models in OC research offer a more faithful portrayal of tumors' intricate biology *in vivo*, including elements such as interactions between cells and the existence of extracellular matrix (ECM), hypoxia, and nutritional gradients (Horst *et al.*, 2021; Ciucci *et al.*, 2022). These characteristics are essential for investigating the molecular mechanisms of drug resistance in OC, which may help identify novel therapeutic targets and, in terms of increased accuracy of preclinical drug research, assess drug efficacy in a more realistic setting. 3D models most often used in studying EOC are spheroids, organoids, and co-culture models. These models have been thoroughly described in recent reviews (Dumont *et al.*, 2019; Collins *et al.*, 2020; Löhmußaar *et al.*, 2020; Semertzidou *et al.*, 2020; Horst *et al.*, 2021; Ciucci *et al.*, 2022; Qin *et al.*, 2022; Yee *et al.*, 2022), and will be briefly described in this section.

Spheroids are 3D cell aggregates generated from established cell lines or patient-derived cells. They mimic the *in vivo* tumor architecture and are frequently used in studies of drug resistance and tumor invasion. Griffon *et al.* in 1995 developed the first 3D spheroid model of EOC and used it to validate the

effects of radiation on patient-derived spheroids, but could not correlate the results with patients' responses *in vivo* because radiotherapy was not included in the majority of patients' therapeutic protocols. However, the study can be considered a milestone in the area of OC therapeutic response prediction due to the successful establishment of patient-derived 3D *in vitro* models with variable radiosensitivity (Griffon *et al.*, 1995). The *in vitro* spheroid model system is the most commonly used approach for studying EOC and its metastasis and chemoresistance, and there are several methods for its establishment, including the hanging drop method, agitation-based methods, ultra-low adherent (ULA) plates, and microfluidics (Yee *et al.*, 2022). Microfluidics chambers are an exciting type of spheroid model for studying EOC, customized to hold tumor tissue or spheroids in place and facilitate microscopic evaluation. Microfluidics "on-chip" models have been used to mimic the specific effects of the TME, e.g., fluid shear stress, and to overcome the limitations of assessing cells within spheroids using techniques that may be destructive or provide insufficient resolution (Yee *et al.*, 2022). **Organoids** are 3D structures that are derived from adult stem cells (ASCs), induced pluripotent stem cells (iPSCs), or cancer stem-like cells (CSCs), which are cultured in ECM-based media and develop multiple cell types (Qin *et al.*, 2022). EOC tumor organoids are more complicated and more expensive to culture than EOC spheroids, as they require specific stem-supporting supplements in the medium and have lower culture establishment and propagation success rates, but both platforms can be used to study the heterogeneity of EOC tumors and are valuable for patient response prediction (Hoffmann *et al.*, 2020; Qin *et al.*, 2022). Spheroids and organoids can be cultured on scaffolds or in scaffold-free systems. Scaffolds can be naturally derived ECM extracts such as Matrigel[®], collagen or alginate gels, or synthetic hydrogels such as polyethylene glycol (PEG) or gelatin methacryloyl (GelMA) gels (Yee *et al.*, 2022). ECM-derived biomaterials are most widely used and considered to provide a physiologically relevant microenvironment for cell–cell and cell–ECM interactions (reviewed by Xing *et al.*, 2020). The development of relevant personalized models is especially important for HGSOC patients suffering from aggressive disease and having only a few therapeutic options available. The 3D co-culture models involve growing EOC spheroids or organoids with other cell types, for instance, fibroblasts or immune cells. These models can be used to study interactions between different cell types in the TME and to test various therapies, including immunotherapies.

In this review, we focus explicitly on the aggressive HGSOC subtype of EOC and its metastasis and chemoresistance explored with the advent of numerous techniques of 3D cell culture, including patient-derived models, which enable the creation of more accurate *in vivo*-like tumor settings. We will disclose how the investigation of each discussed aspect of HGSOC metastasis and chemoresistance profits from specific types of 3D models and describe relevant studies that successfully utilized these

model systems. The review is divided into two chapters, the first one reviewing the characteristics of the stepwise process of HGSOC metastasis and connecting each metastatic feature to novel research performed under 3D culture conditions, including intrinsic properties of cancer cells and collaboration with biochemical and mechanical properties of the TME. The second chapter reviews the innovative research of HGSOC chemoresistance under 3D conditions focusing on drug resistance attributed to CSC properties and the TME-facilitated drug resistance, followed by a discussion on novel research utilizing patient-derived models for therapeutic response prediction. In each chapter, a table is provided summarizing related recent research. We have exclusively concentrated on studies spanning the last five years, considering the fact that 3D modeling of HGSOC is a rapidly expanding field of study with a plethora of emerging research.

2. Metastasis of High-Grade Serous Ovarian Cancer and 3D Cell Models

2.1. Process of Ovarian Cancer Metastasis

HGSOC metastasizes either directly from the primary site or indirectly from the abdominal peritoneum and omentum via the peritoneal fluid to neighboring organs (e.g., colon) (Lengyel, 2010). Although there is evidence of hematogenous and lymphatic metastasis of HGSOC (Coffman *et al.*, 2016; Yue *et al.*, 2019), here we focus on the trans-coelomic route (a route of tumor metastasis across a body cavity, such as the peritoneal cavity in the case of OC), as it is predominant and the most successful. The process of metastasis is sequential, pertaining to various intrinsic properties of cancer cells and signals from the TME (Yeung & Yang, 2017; Uno *et al.*, 2022). The main course of HGSOC peritoneal metastasis development is (i) the shedding of cancer cells into the peritoneal cavity, (ii) the formation of multicellular OC spheroids resulting in (iii) the overcoming of anoikis and the build-up of malignant ascites, followed by (iv) attachment onto the peritoneum or omentum and (v) mesenchymal-to-epithelial transition (MET), which is necessary for (vi) the formation of macrometastases (**Figure 1**) (Yeung & Yang, 2017; Teeuwssen & Fodde, 2019). It is still unknown how the tumor cells detach from the ovary, as single cells or aggregated clusters, with a strong probability of both events. Based on *in vivo* lineage tracing, Al Habyan *et al.* proposed that EOC cells detach from primary tumor sites as either single cells or clusters, with clusters having a survival advantage for being more resistant to anoikis (Al Habyan *et al.*, 2018). A recent study by Micek *et al.* presented an *in vitro* model to generate and separate the spheroids formed through aggregation of single cells or collective detachment to better study the ascitic progression

of HGSOC (Micek *et al.*, 2023). The model involves growing tumor spheroids on a substrate and then detaching them to simulate the process of collective detachment that occurs during metastasis. An interesting finding is that the ECM observed in spheroids formed through collective detachment did not originate from the surrounding environment but instead was produced by the tumor cells after they detached (Micek *et al.*, 2023). This suggests that the production of ECM by tumor spheroids contributes to metastasis by providing a supportive environment for tumor cell detachment and migration (Micek *et al.*, 2023). Recent findings suggest that the multicellular OC spheroids found in ascites are hetero-cellular and consist of tumor and non-malignant cells, such as macrophages, mesothelial cells, fibroblasts, and lymphocytes (Matte *et al.*, 2016; Gao *et al.*, 2019; Uno *et al.*, 2022). These dynamic structures are divided into proliferating, non-proliferating and hypoxic regions and are believed to contain CSCs, which are proposed to drive therapeutic resistance and metastasis (**Figure 1**) (Bapat *et al.*, 2005; Shield *et al.*, 2009; Foster *et al.*, 2013; Matte *et al.*, 2016). The utilization of 3D models enables thorough scrutiny of cellular aggregation, density, and organization in correlation with the potential for metastasis.

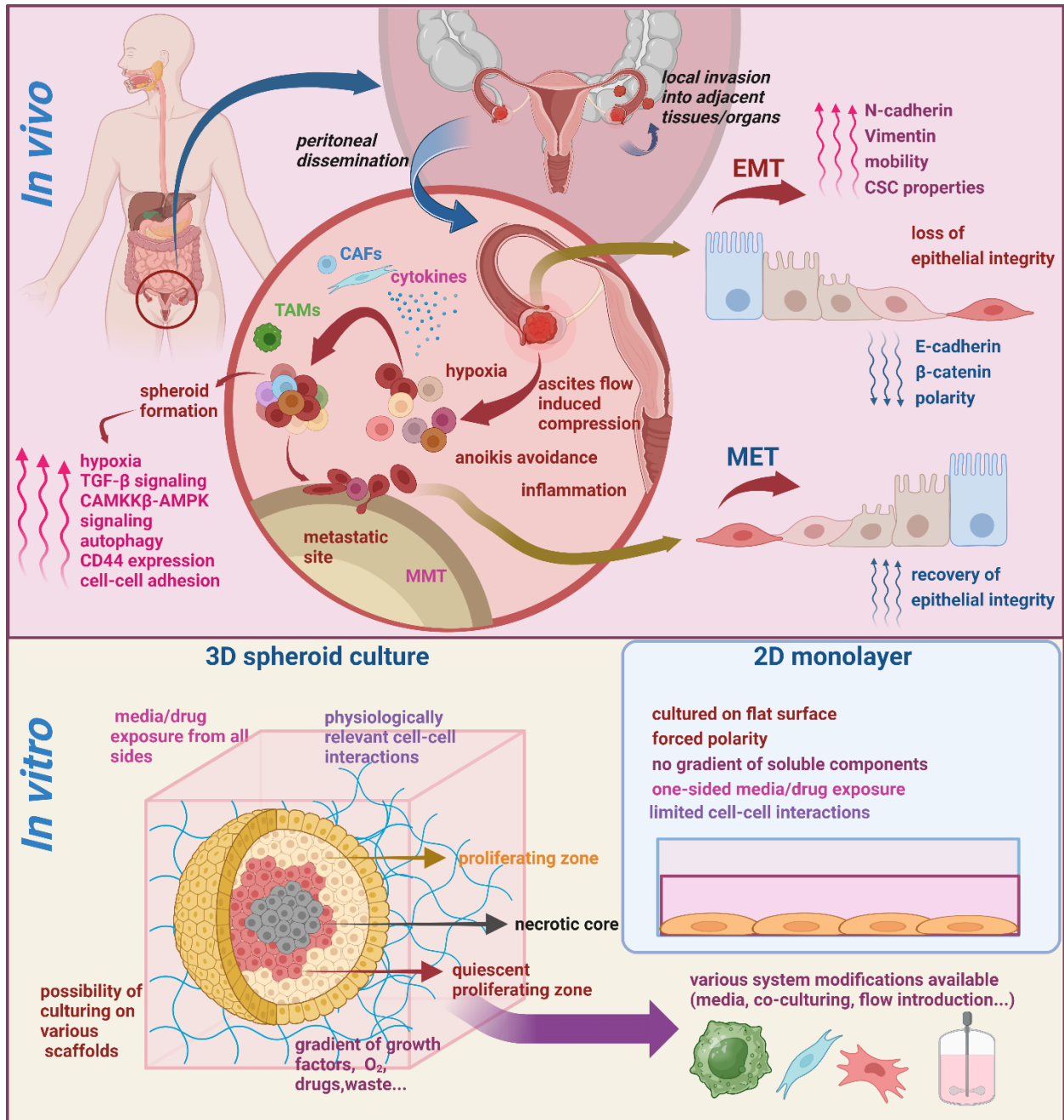


Figure 1. Peritoneal metastasis of HGSOC and basic 2D and 3D *in vitro* HGSOC cell culture models. (*In vivo*)

OC cells from the primary tumor site can invade the surrounding stroma in the process of local invasion, or they can detach and enter the peritoneal cavity in the trans-coelomic route of metastasis. In the local invasion of OC cells, blood and lymph vessels are unnecessary and rarely involved. In the process of peritoneal dissemination, the intravasation step is also omitted, as OC cells shed directly into the peritoneal fluid. Prior to dissemination, tumor cells undergo epithelial-to-mesenchymal transition (EMT), described in section 2.3. The formation of multicellular OC spheroids, which overcome anoikis, and the build-up of malignant ascites are crucial parts of this route of OC metastasis, also known as spheroid-mediated metastasis. Formed spheroids are heterogeneous aggregates of OC

cells, including cancer stem-like cells (CSCs) and cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), mesothelial cells and possibly other non-malignant cells present in ascites which support tumor growth, survival, and immune evasion. The interaction of tumor cells with other cell types is described in section 2.4. These spheroids are implanted on the mesothelial lining of the peritoneum or omentum and undergo a mesenchymal-to-epithelial transition (MET), which is necessary for the local formation of macrometastases. The hypoxic ascitic environment contributes to tumor vascularization, as described in section 2.5. The phenomenon of mesothelial- to- mesenchymal transition (MMT), leading to the conversion of peritoneal mesothelial cells into CAFs and contributing to metastasis, is described in section 2.6. (*In vitro*) The OC 3D spheroid culture is the simplest type of OC 3D model, and even in its simplest (scaffold-free) forms, it is already a better representation of the anchorage-independent growth in ascites than the conventional 2D cell culture of OC cells. Due to the physiologically relevant cell-cell interactions, aggregation capacities, 3D drug exposure, gradient components, and cellular organization, the OC 3D spheroid culture is a much better model for drug screening and investigation of multicellular drug resistance than 2D cell culture. Modifications and additional complexity in the forms of various scaffolds, media components, flow introduction, co-culturing with CAFs and/or TAMs, etc., can be applied to the 3D system for more precise/physiologically relevant OC modeling. Created with BioRender.

As we continue to describe the intricate microenvironment of malignant ascites and features mediating peritoneal metastasis (**Figure 1**), we highlight some of the most intriguing recent studies utilizing 3D models to explore different aspects of the HGSOC metastatic process, with a more extensive summary presented in **Table 1**.

Table 1. Recent studies utilizing 3D models to investigate the HGSOC metastatic process.

Investigated metastatic phenomena	Type of 3D system utilized	Main finding/model contribution	Reference
OC cell adhesion to the omentum; metastatic outgrowth; hematogenous dissemination	3D multicellular human omentum tissue model (OVCAR-8 HGSOC cells, MeT-5A mesothelial cells, MRC-5 fibroblasts, human acute monocytic leukemia cell line THP-1, EA.hy926 endothelial cells, patient-derived adipocytes, patient-derived ascites)	A novel approach to mimicking spatial distribution of five omental cell types	<i>Estermann et al. 2023</i>
Adhesive, migratory, and invasive behavior of patient-derived HGSOC cells from different disease stages and the effect of cytostatic doses	3D organotypic model composed of fibroblasts embedded in collagen I and topped with a monolayer of mesothelial cells (WI38 fibroblasts,	Cells capable of growing spontaneously as spheroids attach to a 3D organotypic model system when pre-incubated with conditioned media;	<i>Ritch et al. 2022</i>

of mifepristone on metastatic capabilities	LP9 mesothelial cells, patient-derived HGSOC cells)	mifepristone was able to cause spheroid dissociation	
Extracellular vesicles; Vasculogenic mimicry	Spheroids of CABA I cell line generated by the hanging drop method	Generated spheroids resemble <i>in vivo</i> tumors in the aspects of “inner” extracellular vesicles and tube-like structures typical of vasculogenic mimicry	<i>Giusti et al. 2022</i>
Stromal cell effects on OC cell attachment and growth; OC cell effects on vascular and mesothelial permeability in models of both early- and late-stage peritoneal metastases	Vascularized model of peritoneal omentum and OC tumor microenvironment; “Omentum-on-a-chip”	Critical cell density requirement for tumor growth in the vascularized peritoneum, enhanced by stromal adipocytes and endothelial cells; tumor growth resulted in both a physically mediated decrease and cytokine-mediated increase in microvascular permeability	<i>Ibrahim et al. 2022</i>
Characterization of tumor-infiltrating lymphocyte populations from primary OC tissue samples; evaluation of cancer immunotherapy efficacy	OC tumor model composed of patient-derived microtumors and autologous tumor-infiltrating lymphocytes	Characterization of patient-individual immune phenotypes and the assessment of responses towards immunotherapy; results generated within 3–4 weeks	<i>Anderle et al. 2022</i>
Tumor cell adhesion; the effect of tumor cells on endothelial cell-to-cell contacts	3D tissue model of the human peritoneum based on a decellularized porcine small intestinal submucosa scaffold populated with human dermal fibroblasts, LP-9 cells representing the peritoneal mesothelium and HUVECs mimicking the endothelial cell layer	An initial evaluation of the novel 3D tissue model for studying peritoneal dissemination	<i>Herbert et al. 2022</i>
CSC phenotype; connection of mesothelin expression and CSC characteristics	Spheroids of HGSOC cell lines (OVCAR-3, OVCAR-8, OVCAR-4 and BG1) cultured in round-bottom plates coated with poly-HEMA (poly(2-hydroxyethyl methacrylate)	Aggressive HGSOC behavior associated with elevated mesothelin expression is not due to mesothelin supporting the CSC phenotype	<i>Nunes et al. 2022</i>
EMT; investigation of Wnt5A association with the TGFβ1/Smad2/3 and	Spheroids derived from EOC cell lines (SK-OV-3, OVCAR-3, and CAOV-	Wnt5A is required for TGFβ1-induced migration and invasion of OC cells;	<i>Dehghani-Ghobadi et al. 2022</i>

Hippo-YAP1/TAZ-TEAD pathways implicated in EMT	4) cultured in plates coated with 1% low melt agarose	Wnt5A may be a functional predeterminant of EMT, supporting mesothelial cell activation and retraction, leading to the establishment of the first metastatic colony on the omentum/peritoneum	
Heterogeneity of metastatic HGSOc; the propagation potential of individual cells at a functional and molecular level	3D single cell-derived metastatic OC spheroids (cells from metastatic peritoneal ascites of 9 patients)	A novel method for isolation and culturing of single cells directly from patients' metastatic ascites, propagating them as 3D cultures referred to as single cell-derived metastatic OC spheroids; these spheroids retain and amplify key subpopulations from the patients' samples and recapitulate features of the original metastasis	<i>Velletri et al. 2021</i>
Tumor microenvironment; immune landscape	Customizable 3D perfused bioreactor system; patient-derived tumor specimens, independent OC cells (OVCAR-8, SK-OV-3), and OC cells co-cultured with CAFs (IHFOT-208)	A novel 3D system that can be customized by co-culturing different cell types and maintaining the immune landscape in patient-derived samples	<i>Martinez et al. 2021</i>
OC cell-tumor microenvironment interactions; effects of β -escin on OC dissemination	3D quantitative high-throughput screening platform - a multilayered culture system containing primary human fibroblasts, mesothelial cells, and ECM; 6 HGSOc cell lines (CaOV3, OVCAR-4, OVKATE, Kuramochi, Snu-119, and TYK-nu)	The natural compound β -escin has a therapeutic potential by targeting both cancer and stromal cells in the OC tumor microenvironment	<i>Kenny et al. 2021</i>
OC cell-tumor microenvironment interactions; matrisome; the effect of specific ECM protein composition and ECM stiffness on HGSOc chemoresistance	Collagen-rich 3D hydrogel cultures and laminin-rich 3D hydrogel cultures; patient-derived tumor tissue and ascitic fluid; HGSOc cell lines (OVCAR-3, OVCAR-4, OVCAR-8 and TYK-nu)	Matrix adhesion as an adaptive response, driving HGSOc aggressiveness via co-evolving extracellular matrix biochemical structure and biomechanical properties	<i>Pietilä et al. 2021</i>
Interactional dynamics of neutrophils and growing	Microfluidics-integrated 3D Tumor-immune	Neutrophils respond to the growing tumor	<i>Surendran et al. 2021</i>

tumor aggregates; the effect of neutrophils on the initiation of collective 3D invasion of OC cells	microenvironment-on-Chip system; OVCAR-3 HGSOC cell line	spheroids through both chemotaxis and generation of neutrophil extracellular traps; the formation of NETs stimulated OC cells from their aggregated state to collectively invade into the surrounding collagen matrix	
Interaction of CSCs with carcinoma-associated mesenchymal stem/stromal cells (CA-MSCs)	A model of ovarian malignant ascites in the 3D hanging drop heterospheroid array with CSCs and carcinoma-associated mesenchymal stem/stromal cells; patient-derived OC CSCs and CA-MSCs	Platelet-derived growth factor signaling increases stemness, metastatic potential, and chemoresistance of CSCs in heterospheroids	<i>Raghavan et al. 2020</i>
MicroRNA-200 family involvement in ovarian inclusion cyst formation and migration of OC cells	3D spheroid cultures in growth factor-reduced Matrigel-coated chamber slide wells and 3D spheroids in sodium hydroxide-neutralized collagen I solution; patient-derived tissue and normal human OSE and FTE primary cultures	OC spheroids with miR-200 knockdown showed changes like elevated TGF- β expression and mitotic spindle abnormalities and switched from collective to single-cell migration; migration mode probably changed due to altered TGF- β /ROCK and SRC signaling, which regulate myosin II phosphorylation	<i>Choi et al. 2020</i>
Interaction of CSCs with macrophages; immunosuppressive phenotype, chemoresistance, invasiveness, stemness	A hanging-drop heterospheroid model to bring CSCs and macrophages in close association; OVCAR-3 HGSOC cell line, CD68 ⁺ macrophages (derived from U937 or peripheral blood monocytes)	A reciprocal interaction that drives pro-tumoral macrophage activation and CSC self-renewal, CSC-derived WNT ligands drive CD206 ⁺ M2 macrophage activation, macrophage-derived WNT ligands enriched ALDH ⁺ CSC cells within hetero-spheroids	<i>Raghavan et al. 2019</i>
Interactions between non-malignant and malignant cells and the extracellular matrix of the peritoneum	Pre-cultured polycaprolactone scaffolds with mesothelial cells assembled with OC cell-seeded hydrogels; mesothelial cells Met-5A, OV-MZ-6 cells, patient-derived	Mesothelial cell microenvironment increased OC cell proliferation	<i>Loessner et al. 2019</i>

	primary HGSOC cells		
Viability and histopathological features of patient-derived OC explants propagated in agitation-based system	Long-term OC patient-derived explant cultures in orbital agitation	Successful culture of eight subtypes of OC with high cell viability levels and original tumor phenotype for at least 30 days	<i>Abreu et al. 2020</i>
Effects of reactive oxygen species on macrophage activation; interactions between OC cells, macrophages and CD3 ⁺ T cells	OC spheroids in 1% agarose substrate in the cover glass bottom of 48-well plates with subsequently (after OC spheroid formation) added macrophages; human EOC cell lines (A2780, OVCAR-3, SKOV-3), murine OC cell line ID8, human peripheral blood monocytes THP-1, PBMCs	Reactive oxygen species induce down-regulation of exosomal miR-155-5p, by which tumors modulate the tumor-promoting microenvironment through down-regulation of PD-L1 and other immunosuppressive factors	<i>Li et al. 2022</i>
The effect of ascitic fluid shear stress, mechanosensitive miR-199a-3p, and hepatocyte growth factor on OC stemness and chemoresistance	OC spheroids in a microfluidic chip; SKOV-3, A2780 and HEY A8 cells	Shear stress in the presence of hepatocyte growth factor activated specific c-Met/PI3K/Akt signaling axis through a positive feedback loop, driving OC stemness and drug resistance; miR-199a-3p expression correlates with enhanced drug resistance properties in chemoresistant OC lines	<i>Hassan et al. 2022</i>
Interaction of OC cells and tumor-associated macrophages in tumor immune microenvironment	Tumor microspheres from ascites of an untreated OC patient in 3D microfluidic chips (AIM Biotech 3-D Cell Culture Chip); murine colon carcinoma cell line CT26, leukemia cells in mouse macrophage RAW264.7, human HGSOC cell line OVCAR-4, murine OC cell line ID8, and human monocyte cell line THP-1	Bromodomain containing protein 4 inhibitor AZD5153 sensitizes OC to anti-PD-L1 therapy by changing the phenotype of tumor-associated macrophages and promoting pro-inflammatory cytokine secretion	<i>Li et al. 2020</i>
The effect of passive flow on the growth of OC organoids	A passive microfluidic platform (Mimetas 2-lane OrganoPlate [®] and medium perfusion provided by OrganoFlow [®] platform) with 3D patient-derived	Microfluidic technology outperformed static conditions in HGSOC organoid growth and the established organoids captured the histological	<i>Cavarzerani et al. 2023</i>

	ascitic OC organoids grown in Cultrex RGF BME®	characteristics of the primary tumors	
Collective detachment of HGSOC spheroids; ECM expression of spheroids in ascites	A 3D model to generate and separate spheroids formed through single cells that detached and aggregated and spheroids formed through collective detachment; OV-90, OVCAR-3, and OVCAR-8 cell lines	Fibronectin in spheroids enhances adhesion to mesothelial cells	<i>Micek et al. 2023</i>
CSC enrichment and chemoresistance	A novel engineered serially passaged 3D spheroid platform, spheroids generated on the hanging drop array; OVCAR-3 cells	CSCs enriched in serially passaged spheroids; spheroids resistant to cisplatin and sensitive to ALDH inhibitor; later passage spheroids exhibit higher tumorigenicity <i>in vivo</i> than early passage spheroids	<i>Ward Rashidi et al. 2019</i>

OC: ovarian cancer; HGSOC: high-grade serous ovarian cancer; EOC: epithelial ovarian cancer; ECM: extracellular matrix; CSC: cancer stem-like cell; ALDH: aldehyde dehydrogenase; CAFs: cancer-associated fibroblasts; TAMs: tumor-associated macrophages; PD-L1: programmed death-ligand 1; HUVEC: human umbilical vein endothelial cells; poly-HEMA: poly(2-hydroxyethyl methacrylate); NETs: neutrophil extracellular traps; OSE: ovarian surface epithelium; FTE: fallopian tube epithelium; PBMCs: peripheral blood mononuclear cells

2.2. Mimicking the Malignant Ascites Spreading

Most women diagnosed with HGSOC already have peritoneal metastasis at diagnosis (Lisio *et al.*, 2019). An excessive amount of peritoneal fluid containing cancer cells, immune cells, and various tumor-promoting soluble factors is called malignant ascites, and it provides an easy method of dissemination for HGSOC, as tumor cells shed from primary sites straight into the peritoneal cavity without any physical barrier (Ahmed & Stenvers, 2013). Malignant ascites is present in over one-third of all OC patients at initial diagnosis and in almost all cases of disease recurrence (Ford *et al.*, 2020). The accumulation of malignant ascites occurs due to peritoneal lymphatic drainage obstruction and enhanced peritoneal vascular permeability (Asem *et al.*, 2020). In addition to offering a liquid substrate filled with tumor-promoting cellular and molecular components, malignant ascites promotes metastasis mechanically through intraperitoneal compression (Asem *et al.*, 2020). Ascites-induced intraperitoneal compression increases cell adhesion to the peritoneum and induces remodeling of the peritoneal mesothelial cell surface ultrastructure via induction of tunneling nanotubes mediating mesothelial cell interactions with

tumor cells, transport of mitochondria from mesothelial cells to tumor cells and remodeling of peritoneal collagen fibers to support invasion (Asem *et al.*, 2020). Such mechanical effects of ascites on HGSOc metastasis and treatment failure have been understudied but can be elegantly investigated in 3D models incorporating flow in so-called perfusion models.

Flow-induced shear stress modulates metastasis by inducing changes in cancer morphologic, genetic, and protein profiles, including EOC (Rizvi *et al.*, 2013; Polacheck *et al.*, 2014). A recent study by Cavarzerani *et al.* developed a passive microfluidic platform for 3D culturing of HGSOc organoids in conditions mimicking ascitic flow and without ECM disruption (Cavarzerani *et al.*, 2023). Organoids were derived from either HGSOc ascites or chemo-naïve tumors and cultured in Cultrex BME from R&D Systems. Clusters of organoids were plated onto Mimetas 2-lane OrganoPlate[®] composed of 96 independent tissue culture chips with medium perfusion secured by gravity through the microfluidic channels of the OrganoFlow[®] platform from the same company. This model enabled faster growth of HGSOc organoids with higher viability in comparison with static 3D culture. Interestingly, the half-maximal inhibitory concentration (IC₅₀) values of carboplatin (CBP), paclitaxel (TAX), and doxorubicin (DXR) were lower in the passive flow system than in static conditions (Cavarzerani *et al.*, 2023). The authors discussed that the higher proliferation rate of the dynamic culture could be attributed to higher efficiencies in supplying nutrients and oxygen to organoid cores and removing cellular waste exerted by the flow. Yet, the flow could also be responsible for the lower IC₅₀ values of chemotherapeutics due to deeper penetration into organoid cores in comparison with static cultures.

Hassan and colleagues investigated the effects of ascitic fluid shear stress and specific cytokines and growth factors typically present in malignant ascites on the stemness properties of OC spheroids (Hassan *et al.*, 2022). They used a microfluidic chip coated with poly-2-hydroxyethyl methacrylate (poly-HEMA) to prevent cell attachment to the glass slide and matrix deposition. They noticed that tumor spheroids were significantly more viable under flow than in static culture. The expression of microRNA (miR) miR-199a-3p and its coding gene miR-199a-1 were significantly down-regulated by shear stress and by the addition of hepatocyte growth factor (HGF), and their strong synergistic effect was also observed. The authors further showed that shear stress can activate c-Met, an HGF receptor, and the PI3K/Akt signaling pathway, whereas c-Met and PI3K/Akt inhibition could annul the down-regulation of miR-199a-3p and miR-199a-1 under shear stress. The c-Met/PI3K/Akt/miR-199a-3p may form a positive feedback loop, as overexpression of miR-199a-3p can downregulate c-Met expression and Akt phosphorylation (Hassan *et al.*, 2022). Down-regulation of miR-199a-3p resulted in the formation of more spheroids under CSC-selective conditions, and its overexpression reduced spheroid formation. This

study provokes an interesting idea of fluid shear stress-induced increase in the CSC population, which may significantly contribute to the emergence of chemoresistance in the late stages of OC.

2.3. Mimicking Spreading by Epithelial-to-Mesenchymal Transition

Prior to dissemination, tumor cells undergo epithelial-to-mesenchymal transition (EMT), a series of cellular events known to be important in embryogenesis, wound healing, and malignant progression (Dongre & Weinberg, 2019). The process of EMT results in the loss of cell polarity and cell-cell adhesion, the acquisition of a mesenchymal phenotype, and an increase in cellular migratory capacity (Yang *et al.*, 2020). The critical event is the switching of epithelial marker epithelial (E-) cadherin expression with mesenchymal marker neural (N-) cadherin (Yang *et al.*, 2020). The role of EMT in HGSOC progression is unclear and somewhat controversial, as metastatic tumor cells still express E-cadherin, and most of the EMT phenomenon in EOC has only been revealed *in vitro* (Mei *et al.*, 2023). However, one of the hallmarks of EOCs is the heterogeneous cadherin expression, with a mixed cadherin phenotype in tumors expressing both E- and N-cadherin and a hybrid cadherin phenotype in single cells expressing both E- and N-cadherin (Yang *et al.*, 2020; Kralj *et al.*, 2022). Both circulating tumor cells and multicellular aggregates in ascites demonstrated heterogeneous cadherin expression profiles, with mesenchymal-like cells expressing N-cadherin invading much more efficiently and pointing toward the importance of EMT for successful peritoneal metastasis (Klymenko *et al.*, 2017).

The EMT has been generally associated with the acquisition of stemness properties in cancer cell populations and with acquired chemoresistance (Mani *et al.*, 2008; Papadaki *et al.*, 2014; Diepenbruck & Christofori, 2016; Yeung & Yang, 2017; Loret *et al.*, 2019; Pastushenko & Blanpain, 2019). The relationship between EMT and acquired stemness properties of cancer cells is not fully mechanistically explained. However, the altered protein expression resulting from the EMT program may activate autocrine signaling loops, which leads to the development of the CSC phenotype (Shibue & Weinberg, 2017). The pro-inflammatory and hypoxic TME drives EMT, with various immune cells and cancer-associated fibroblasts (CAFs) utilizing signaling pathways that induce transcription factors associated with EMT initiation, such as transforming growth factor beta (TGF- β), tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and hypoxia-inducible factors (HIFs) (Shibue & Weinberg, 2017). The implications of EMT in tumor progression and metastasis, as well as the interconnection of EMT and acquired stem-like properties, have been discussed in detail in many recent comprehensive reviews (Derynck & Weinberg, 2019; Dongre & Weinberg, 2019; Bakir *et al.*, 2020; Erin *et al.*, 2020; Yang *et al.*, 2020; Bayik & Lathia, 2021; Brabletz *et al.*, 2021; De Las Rivas *et al.*, 2021; Motohara *et al.*, 2021; Huang *et al.*, 2022), and are not the focus of this review. We are interested in the

exploration of EMT and CSCs in 3D models of HGSOC, for which we highlight several exciting studies and provide additional, the most recent studies in **Table 1**.

Rafehi *et al.* investigated the role of TGF- β signaling in regulating epithelial-mesenchymal plasticity in ascites-derived spheroids (Rafehi *et al.*, 2016). Due to the previously mentioned plasticity of EMT states in HGSOC and the known EMT-inducing role of TGF- β (Zavadil & Böttinger, 2005), they hypothesized that modulation of TGF- β signaling would significantly impact EMT and the malignant potential of spheroids. They used an *in vitro* 3D culture system where primary ascites-derived human EOC cells were maintained in suspension on ULA plates for three days, followed by re-introduction to standard tissue culture-treated plates, re-attachment, and dispersion. The involvement of TGF- β inhibitors seriously hindered the EMT process. The TGF- β transmembrane serine/threonine kinase receptor type I (T β RI) inhibitor, SB-431542, blocked the endogenous EMT in spheroids, and treatment with SB-431542 upon spheroid re-attachment decreased cellular motility and migration, boosting the epithelial phenotype (Rafehi *et al.*, 2016). This suggests that targeting TGF- β signaling may be a promising approach for preventing or slowing the spread of HGSOC. Ward Rashidi and colleagues developed an integrated approach to create an *in vitro* model of stemness and chemoresistance in OC, utilizing the 3D hanging drop spheroid model established by serial passaging of non-adherent spheroids (Ward Rashidi *et al.*, 2019). Spheroids were generated from the HGSOC OVCAR-3 cell line and patient-derived HGSOC abdominal metastases. They observed an increase in cellular proliferation, an enrichment of CSCs, and the parallel development of platinum resistance in the model. *In vivo*, tumor xenograft assessments revealed that spheroids from later passages had significantly greater tumorigenicity and more CSCs than those from earlier passages (Ward Rashidi *et al.*, 2019). What is particularly interesting in this study is that in contrast to the designed 3D model, under the same experimental conditions, serial passaging in the 2D model did not yield the same results, confirming the postulate that the CSC phenotype is unstable and impossible to be maintained in 2D monolayer models (Ward Rashidi *et al.*, 2019).

2.4. Mimicking the Interaction of Tumor Cells with Other Types of Cells Involved in Metastasis

The tumor stroma consists of the ECM and various cell types, such as CAFs, endothelial cells, immune cells, and adipocytes. In OC, the tumor stroma accounts for a significant portion of the tumor tissue, ranging from 7% to 83%, with a median of 50% (Labiche *et al.*, 2010). As previously stated, malignant ascites comprises a diverse array of cellular and acellular components. In addition to tumor cells expressing epithelial cell adhesion molecule (EpCAM), CAFs expressing fibroblast activation

protein (FAP), immune cells (CD45⁺), and endothelial cells (CD31⁺) are the main cell types in ascites (Erez *et al.*, 2010). Gao *et al.* investigated the aggressive nature of HGSOC ascitic tumor cells (ATCs) characterized by high integrin $\alpha 5$ (ITGA5^{high}) levels, which are prone to forming heterotypic spheroids with fibroblasts. They used the term metastatic units (MUs) for these heterotypic spheroids (Gao *et al.*, 2019). In these MUs, CAFs recruit ITGA5^{high} ATCs and maintain ATC ITGA5 expression by continuous secretion of epidermal growth factor (EGF) (Gao *et al.*, 2019). Their analysis identified fibroblasts (EpCAM⁻CD45⁻CD31⁻FAP⁺) as the cellular component showing the most considerable difference between HGSOC and low-grade serous carcinoma ascites, indicating that ascitic fibroblasts direct ATC behavior and HGSOC progression (Gao *et al.*, 2019). They established a suspension co-culture of SK-OV-3 cells and primary CAFs and found the presence of tumor cells in the form of single cells, homotypic (SK-OV-3 only) spheroids, and heterotypic spheroids made of SK-OV-3 cells and CAFs. The heterotypic spheroids displayed the most potent adhesive capacity (Gao *et al.*, 2019).

The tumor immune microenvironment (TIME) is a term specifically used to describe groups of immune cells and their interactions in the TME niche, and it needs to be systematically studied in search of successful immunotherapies (Binnewies *et al.*, 2018). The systemic tumor immune environment (STIE), the comprehensive immunity of the host, or the macro-environment of the patient's anti-tumor immunity, in coordination with the TIME, determines the patient's response to immunotherapy (Hiam-Galvez *et al.*, 2021). The OC TIME contains various immune cells, including macrophages, natural killer (NK) cells, regulatory T-cells, dendritic cells, CD8⁺ and CD4⁺ T-cells, B-cells, and myeloid-derived suppressor cells (Izar *et al.*, 2020). Surendran and colleagues developed a novel microfluidics-integrated 3D TIME-on-Chip model for studying the effect of neutrophils on the initiation of collective 3D invasion of HGSOC OVCAR-3 cells (Surendran *et al.*, 2021). They cultivated tumor spheroids on hydrogel-based multi-microwell plates immersed within a collagen matrix of defined thickness and then magnetically attached them to a microfluidic channel system made on a porous membrane containing neutrophils. Designed channels replicated the vascular structures surrounding the tumor, and the layer of collagen between tumor spheroids and channels simulated the tumor stroma. This TIME *in vitro* system modeled dynamic neutrophil migration and 3D tumor invasion, and it demonstrated the migration of neutrophils towards tumor spheroids via chemotaxis and the generation of neutrophil extracellular traps (NETs) (Surendran *et al.*, 2021). This sophisticated 3D model demonstrated how, when exposed to chemotactic stimuli from the tumor, neutrophils may extravasate from the blood vessels into the tumor tissue. OVCAR-3 cells migrated collectively upon down-regulation of E-cadherin, especially under stimulation with IL-8 and TGF- β , both secreted by activated neutrophils *in vivo*. They observed different signaling within neutrophils under flow and non-flow conditions, suggesting that fluid flow activates neutrophils

differently and could affect their tumor response (Surendran *et al.*, 2021). NETs are known to play an active role in metastasis and are found in the omentum of OC patients. This study demonstrated that tumor-associated inflammatory factors by themselves trigger naive neutrophils to produce NETs. Stromal NETs seem to excite the collective invasion of the tumor cells into the tissue and the expansion of the aggregated cell colonies (Surendran *et al.*, 2021). This is another essential complexity within T(I)ME, validated with a novel 3D system.

Tumor-associated macrophages (TAMs) are another significant fraction of cells within the ascitic microenvironment and are implicated in the recurrence and metastasis of OC. WNT signaling pathway has been shown to play a critical role in OC tumorigenesis (Barbolina *et al.*, 2011). Depending on the signals received from the TME, macrophages can display anti-tumorigenic (M1) or pro-tumorigenic (M2) phenotypes by secreting different cytokines. M1 macrophages secrete pro-inflammatory cytokines, which upregulate the local immune response, and M2 macrophages secrete anti-inflammatory cytokines, such as IL-10 and TGF- β , promoting immunosuppression and tumor progression (Malyshev & Malyshev, 2015). A recent study utilizing engineered 3D models demonstrated that HGSOC CSCs and ascitic macrophages reciprocally interact through the WNT pathway to promote pro-tumoral and malignant phenotypes (Raghavan *et al.*, 2019). Raghavan *et al.* used the hanging drop spheroid model to bring HGSOC CSCs and macrophages within heterotypic spheroids *in vitro*, simulating their presence within malignant ascites (Raghavan *et al.*, 2019). They found that HGSOC CSCs drove the up-regulation of the M2 macrophage marker CD206 within these heterotypic spheroids, resulting in immuno-suppressive properties. These heterotypic spheroids were also more resistant to CBP and demonstrated higher invasiveness as a result of an interesting loop – aldehyde dehydrogenase (ALDH) expressed by HGSOC CSCs led to the initiation of pro-tumoral cytokine IL-6 signaling by M2 macrophages, which secured and increased ALDH maintenance in HGSOC CSCs (Raghavan *et al.*, 2019). This implies that targeting the WNT pathway may be a potential therapeutic strategy for HGSOC treatment, elegantly demonstrating how 3D modeling of malignant ascites provides invaluable insights into the complex biology of HGSOC metastasis. The HGSOC spheroids and entire malignant ascites are enthralling in the context of metastasis and the development of therapeutic resistance as they form a unique interactive environment essential for the complexity and aggressiveness of HGSOC. An exciting thought is that the hypoxic and anoikis-promoting conditions in ascites drive selective pressure and allow only the fittest/most aggressive malignant cells to survive, and there are many findings correlating the formation of spheroids and the accumulation of ascites with the increasing invasiveness and chemoresistance (Cai *et al.*, 2015; Gao *et al.*, 2019; Uno *et al.*, 2022).

Apart from the previously mentioned fluid shear stress, several other biomechanical mechanisms may affect HGSOC metastasis, such as cellular elasticity, compressive and tensile stress on the cells around the tumor, and substrate stiffness stress (Sun *et al.*, 2022). Cells in the TME affect disease progression by signaling and ECM remodeling. Natarajan and colleagues demonstrated the tumor-promoting effects of collagen remodeling by mesothelial and tumor cells in the peritoneal microenvironment (Natarajan *et al.*, 2019). Hypoxic signaling promoted extracellular collagen fiber deposition by mesothelial cells and invasion of HGSOC cells in tumor-mesothelial cell 2D co-cultures (Natarajan *et al.*, 2019). The role of ECM remodeling in the metastasis of HGSOC could be better understood by studying the "mechanobiology" of the TME using scaffold-based 3D models (Winkler *et al.*, 2020). Pietilä and colleagues identified the biochemical and biomechanical properties of the ECM as crucial in determining HGSOC cell survival and resistance to chemotherapy (Pietilä *et al.*, 2021). They investigated the chemotherapy-induced alterations in the tumor stroma and the effect of the observed changes on HGSOC chemoresistance and relapse, finding that the tumor matrix co-evolved with cancer cells during disease progression (Pietilä *et al.*, 2021). They found that both matrix stiffness and specific protein structure affect the HGSOC platinum response (Pietilä *et al.*, 2021). Analysis of comprehensive changes in the ECM could aid in the early detection of cancers without available early diagnosis methods, such as HGSOC. Apart from early detection, specific ECM signatures could provide predictive value, as demonstrated for several cancer types (Winkler *et al.*, 2020).

2.5. Mimicking Tumor Vascularization

The capacity to invade and metastasize is inextricably linked to the development of a new vascular system for supplying oxygen and nutrients to the growing tumor mass, where new blood vessels originate from both endothelial and tumor cells themselves. (Ayala-Domínguez *et al.*, 2019; Wei *et al.*, 2021). Tumor vascularization is crucial for tumor growth and metastasis and the development of therapeutic resistance (van Beijnum *et al.*, 2015). In metastatic OC, in the presence of constantly active VEGF signaling in hypoxic tumor tissue, a phenomenon of vasculogenic mimicry (VM) has been observed (Ayala-Domínguez *et al.*, 2019; Lim *et al.*, 2020; Shibuya, 2008). VM implies the appearance of permeable vascular channels formed by tumor cells themselves, through which nutrients, oxygen, and soluble substances are delivered to the tumor, independent of classical angiogenesis and endothelial cells (Ayala-Domínguez *et al.*, 2019; Lim *et al.*, 2020; Wei *et al.*, 2021). Although anti-angiogenic therapies targeting VEGF have shown promising results in the treatment of some metastatic tumors, such drugs generally have only a modest impact on overall patient survival, and alternative mechanisms of tumor

vascularization, such as VM, are likely to represent a path by which tumors overcome therapy (Carbone *et al.*, 2011; Wei *et al.*, 2021; Xu *et al.*, 2012). The development of VM is associated with a pronounced hypoxic microenvironment, the presence of CSCs, the process of epithelial-to-endothelial transition (EET), and remodeling of the ECM (Fernández-Cortés *et al.*, 2019; Wei *et al.*, 2021). Several studies have shown that highly hypoxic conditions in the TME selected CSCs, promoting the expression of molecules associated with CSC characteristics such as OCT4, SOX2, and NANOG through HIF-1 α signaling (Pietras *et al.*, 2009; Mathieu *et al.*, 2011; Filatova *et al.*, 2016). Determining the causal relationship between the development of CSC characteristics and VM is difficult. However, it has been shown that the presence of CSCs, recognizable by the surface antigen CD133, promoted VM in several different types of tumors, including OC (Chiao *et al.*, 2011; Liang *et al.*, 2016; Wang *et al.*, 2016). The mechanisms of activation and maintenance of VM are still insufficiently investigated, even the process itself is still somewhat questionable in many tumors, and it is possible to investigate these exclusively in 3D culture conditions (Fernández-Cortés *et al.*, 2019; Valdivia *et al.*, 2019). In HGSOV, VM is associated with poor prognosis and poor response to anti-angiogenic therapy (Liang *et al.*, 2016; Xu *et al.*, 2012). Molecular pathways behind VM are considered attractive new therapeutic targets (Ge & Luo, 2018), and 3D models will be indispensable for future studies. Recently developed methods now model the formation of 3D cellular networks over Matrigel in OC cells *in vitro*, representing the early stages of VM (Salinas-Vera *et al.*, 2022).

2.6. Importance of Extracellular Vesicles

The phenomenon of mesothelial- to- mesenchymal transition (MMT) leading to the conversion of peritoneal mesothelial cells into CAFs has also been reported and must be considered in the complexity of peritoneal metastasis (Rynne-Vidal *et al.*, 2017). MMT is induced by TGF- β (Matte *et al.*, 2014), commonly found in ascites, and it is another excellent example of the complex interplay within a tumor-promoting ascitic environment. Another aspect worth considering in the complex ascitic environment is extracellular vesicles (EVs). It is still unclear how exosomes produced by MMT-derived CAFs can predict peritoneal tumor progression or therapeutic response in patients with advanced OC, but EVs have been shown to participate in the transmission of information between stromal cells and immune cells in the TME (reviewed by Steinbichler *et al.*, 2019). Giusti *et al.* showed that EVs can induce a CAF-like state in fibroblasts, promoting their proliferation, motility, and invasiveness (Giusti *et al.*, 2022). EVs are implicated in creating the peritoneal pre-metastatic niche by remodeling the ECM and promoting tumor cell adhesion to mesothelial cells. EVs secreted by OC cells carry proteins related to tumor progression,

such as LICAM, CD24, ADAM10, and EMMPRIN, and proteins regulating cancer signaling through ACTN4, CD44, and type-IV collagen (Tian *et al.*, 2022). These EVs have been found to inhibit the activation of dendritic cells, induce the polarization of macrophages, inhibit the cytotoxicity of NK cells, and regulate the function of T-cells (Tian *et al.*, 2022). EVs have also been found to induce angiogenesis by promoting the proliferation and migration of endothelial cells, and the combination of these effects ultimately promoted immune escape and facilitated metastasis (Tian *et al.*, 2022). Giusti *et al.* confirmed that EOC spheroids formed by the hanging drop method experienced features characteristic of *in vivo* tumors, such as inner entrapped EVs and VM (Giusti *et al.*, 2022). Investigating phenomena such as MMT and EVs in 3D models is a promising direction for future HGSOC research and could lead to a better understanding of disease progression mechanisms. EVs can be helpful in diagnosis, prognosis, therapy selection, and monitoring of therapeutic response.

3. Chemoresistance of High-Grade Serous Ovarian Cancer and 3D Cell Models

3.1. Treatment of High-Grade Serous Ovarian Cancer

High-grade serous ovarian cancer is primarily treated with surgical staging, maximal debulking, and systemic chemotherapy (Armstrong *et al.*, 2021). Patients with advanced-stage HGSOC, who, due to age, poor performance, comorbidities, or the exhaustive cancer burden, can undergo neoadjuvant chemotherapy with interval (between cycles of chemotherapy) debulking surgery. This approach has been shown to be effective in improving overall survival rates in such patients (Armstrong *et al.*, 2021). Since the 1970s, platinum-based drugs have been used as the standard treatment for ovarian cancer, with cisplatin (cDDP) followed by carboplatin-based combinations (Raja *et al.*, 2012). Intravenous administration of carboplatin and paclitaxel is now considered the mainstay of first-line treatment for HGSOC, even for stage I (Armstrong *et al.*, 2021). Other options include a combination of carboplatin with docetaxel (DTX) and carboplatin with pegylated liposomal doxorubicin (PDL), each having similar efficiency but carrying a different set of specific toxicities, so they may be an option for patients with specific risk profiles (Armstrong *et al.*, 2021).

After the initial chemotherapy treatment, patients should undergo regular clinical re-evaluation. Patients with persistent or progressing disease during initial treatment should immediately receive second-line treatments. Patients who achieve complete or partial remission have various treatment options based

on the extent of their response and the primary chemotherapy received. Recent trials have shown that maintenance therapy after postoperative platinum-based chemotherapy can have a positive impact on progression-free survival (PFS) in patients with advanced disease. As a result, the integration of maintenance therapy as part of postoperative management is becoming increasingly prevalent and important (Armstrong *et al.*, 2021). Maintenance therapy aims to prolong PFS and preserve the quality of life in patients with advanced disease. Maintenance therapy following primary chemotherapy includes the use of bevacizumab and PARP inhibitors (PARPi). Olaparib, rucaparib, and niraparib are PARPi used as maintenance monotherapy for recurrent disease, while olaparib, niraparib, and olaparib with bevacizumab are used for maintenance therapy after response to first-line chemotherapy (Armstrong *et al.*, 2021). Patients with advanced-stage HGSOc have a high chance of cancer recurrence, which is usually incurable. Recurrent HGSOc is most frequently detected by increased levels of CA125 followed by imaging, as patients are asymptomatic at the time (Matulonis *et al.*, 2016). Recurrent disease is typically categorized as platinum-sensitive or platinum-resistant based on the patient's response to platinum-based chemotherapy. Patients with recurrent platinum-sensitive disease are generally treated again with platinum-based chemotherapy, such as CBP with TAX, CBP with PDL, and CBP with gemcitabine (GEM), having a response rate of around 50%, with shorter progression-free intervals with each subsequent platinum administration (Matulonis *et al.*, 2016). Patients with platinum-resistant recurrent disease are treated with TAX, PDL, GEM, etoposide, vinorelbine, or topotecan (TOP), with or without bevacizumab (Matulonis *et al.*, 2016). As research results and new clinical trials emerge, the guidelines for optimal care will continue to be updated.

3.2. Mechanisms of Action of Drugs Approved for High-Grade Serous Ovarian Cancer Treatment

Drugs used for the treatment of HGSOc employ different mechanisms of anti-cancer activity. Due to differences in the drugs' mechanisms of action, HGSOc cells employ different strategies for resisting specific treatments. Platinum-based drugs, such as CBP and cDDP, bind to DNA to create crosslinks and induce apoptosis. CBP is a second-generation platinum agent, having a similar efficacy as cDDP but resulting in lower systemic toxicity (Zhang *et al.*, 2022). TAX and DTX are taxanes, chemotherapeutics naturally occurring in plants, and are considered mitotic inhibitors. Taxanes bind to microtubules and stabilize them, inhibiting their depolymerization and resulting in cell cycle arrest and subsequent apoptosis. TAX is also associated with activation of pro-apoptotic signaling pathways, angiogenic inhibitory activity, and induction of oxidative stress (Kampan *et al.*, 2015). Doxorubicin

(DXR) primarily inhibits DNA topoisomerase II, stabilizing it in a complex with DNA and stalling the cell cycle in the S-phase. DXR-induced topoisomerase II-DNA complexes can also induce DNA double-strand breaks, and DXR has been associated with increasing oxidative stress (Gabizon *et al.*, 2016). In PDL, the polyethylene glycol (PEG) coating on the liposome increases the longevity of the liposome and reduces toxicity compared to free DXR. The PARPi are intended for treating patients with BRCA1/2 mutations, thus harboring a homologous DNA recombination repair defect. Poly (ADP-ribose) polymerases (PARPs), especially PARP1, PARP2, and PARP3, are crucial to base excision DNA repair, the mechanism of DNA single-strand breaks repair. The rationale behind the PARPi strategy is the synergetic lethality (or “synthetic lethality”), with the inactivation of both BRCA and PARP resulting in a replication catastrophe and cell death (Li *et al.*, 2020). However, more than 40% of BRCA1/2-deficient patients who receive PARPi do not respond, and those who do eventually develop resistance (Li *et al.*, 2020). Interestingly, there is recent evidence that patients with homologous DNA recombination repair defects, excluding BRCA mutations, are also sensitive to PARPi (Li *et al.*, 2020). As previously mentioned, bevacizumab is a VEGF-A-targeting monoclonal antibody used as an angiogenesis inhibitor (Garcia *et al.*, 2020). TOP blocks the action of the enzyme topoisomerase I, which initiates the DNA relaxation by cleaving one strand, impacting replication and resulting in double-strand breaks (Thomas & Pommier, 2019). Similarly, etoposide inhibits the action of topoisomerase II (Bailly, 2023). GEM, a pyrimidine antagonist, inhibits DNA synthesis by targeting cells in S-phase (Berg *et al.*, 2019). Vinorelbine is a semisynthetic vinca alkaloid that inhibits mitosis by binding to tubulin and inhibiting microtubule assembly, preventing metaphasic cell division (Dhyani *et al.*, 2022). The subsequent sections will explore how novel 3D cell models of HGSOC are utilized to investigate resistance to the described approved drug treatments, which rely on different mechanisms of action.

3.3. Mimicking Multicellular Drug Resistance in 3D High-Grade Serous Ovarian Cancer Cell Models

Understanding the biology of HGSOC chemoresistance is crucial for overcoming the frequent recurrence in patients and improving survival rates. Chemoresistance of HGSOC can occur as a result of genetic alterations, altered signaling pathways, and changes in the TME, primarily enhanced DNA repair mechanisms, reduced drug uptake, increased drug efflux, activation of pro-survival signaling pathways, autophagy, and quiescence (McMullen *et al.*, 2021). Additionally, previously discussed processes of EMT and the acquisition of stem-like properties are interconnected with the development of drug resistance (Pan *et al.*, 2021). Known mechanisms of HGSOC chemoresistance are extensively reviewed elsewhere

(Liu *et al.*, 2020; Marchetti *et al.*, 2021; McMullen *et al.*, 2021; Ortiz *et al.*, 2022) and fall beyond the scope of this review. Here, we focus on investigating chemoresistance in 3D conditions and the benefits brought to the field by recent 3D models.

The idea of 3D multicellular drug resistance mechanisms is not new; there are studies published 30-40 years ago suggesting that the acquired anti-cancer drug resistance could result from the response of a cell population and that such responses can only be observed *in vitro* in 3D culture conditions which allow cells to aggregate (Sutherland & Durand, 1976; Kobayashi *et al.*, 1993). Cell-cell and cell-ECM interactions can be explored in the context of drug resistance in 3D spheroid models (**Figure 2**). As spheroids grow, a gradient of oxygen is established, similar to the process of tumor growth *in vivo* (Riffle & Hegde, 2017). Tumor cells in the external layer of the tumor tissue have access to oxygen, unlike those within the inner layer, which experience hypoxic conditions. This effect can be observed between the outer, proliferating layer of multicellular spheroids and the inner, quiescent viable cell zone and the necrotic core. Cells in the hypoxic microenvironment upregulate the expression of HIF family factors, opening the door for several drug resistance mechanisms indicated in **Figure 2**. Several mechanisms can be invoked to explain the effects of hypoxia on increased drug resistance; (i) up-regulation of HIF and its downstream targets (up-regulation of the multidrug resistance 1 gene, *MDRI*, and several anti-apoptotic factors and *VEGF*); (ii) inability of reactive oxygen species (ROS) formation due to lack of oxygen (which is the primary mechanism of action for many anti-cancer drugs); (iii) decrease in cell damage mediated by radiation due to limited ROS formation; (iv) acidification of the microenvironment due to metabolic reprogramming (the inhibition of oxidative phosphorylation) and lactate production by hypoxic cells which encumbers drug stability and uptake; (v) induction of a non-proliferative and quiescent state resulting in low efficacy of DNA-damaging chemotherapeutics (such as CBP) relying on rapid division of tumor cells; (vi) promotion of vascularization incurred by HIF-mediated ECM remodeling; and (vii) HIF-mediated ECM remodeling creating physical barriers (**Figure 2**) (reviewed by Riffle & Hegde, 2017; Nunes *et al.*, 2019; Winkler *et al.*, 2020). Previously described CSCs, implicated in both metastasis and chemoresistance, have been found in patient-derived spheroids, as demonstrated by Hirst *et al.*, who detected the CSC-associated markers aldehyde dehydrogenase 1A (*ALDH1A*) and CD133 and the increased expression of HIF-regulated genes in the spheroid core (**Figure 2**) (Hirst *et al.*, 2018). The dependence of drug resistance mechanisms on CSC properties can be explained by (i) increased *ALDH* levels, resulting in increased drug metabolism; (ii) improved DNA damage repair; (iii) increased expression of efflux transporters; (iv) EMT and (v) dormancy (**Figure 2**) (Hirst *et al.*, 2018; Nunes *et al.*, 2019). Autophagy, the process of breaking down and recycling components within cells to replenish energy and essential compounds, has also been implicated in the drug resistance of OC (Wang *et al.*,

2018; Škubník *et al.*, 2023). The induction of autophagy has been observed within HGSOC spheroids, as well as an increase in phosphorylated adenosine monophosphate-activated kinase (AMPK) and the autophagy marker LC3-II during spheroid formation (**Figure 2**) (Laski *et al.*, 2020). Giusti *et al.* observed the formation of entrapped EVs in EOC spheroids, which are known to contribute to drug resistance *in vivo* (**Figure 2**) (Giusti *et al.*, 2022; Steinbichler *et al.*, 2019). As mentioned previously, in the context of information transmission between OC cells and stroma in promoting metastasis, EVs also play an important role in OC drug resistance. A study by Dorayappan and colleagues demonstrated how exosomes contributed to a more aggressive and chemoresistant OC phenotype (Dorayappan *et al.*, 2018). They confirmed that hypoxic OC cells significantly increased their exosome release by up-regulating Ras-related protein Rab27a, down-regulating Ras-related protein Rab7, lysosomal-associated membrane proteins -1 and -2, LAMP1/2, neuraminidase 1, NEU-1, and also by promoting a more secretory lysosomal phenotype (Dorayappan *et al.*, 2018). Released EVs contributed to cDDP resistance by spreading the oncogenic transcription factor STAT3 (Dorayappan *et al.*, 2018). Many of the previously mentioned tumor-promoting synergistic effects of multiple cell types collaborating within heterotypic spheroids can also be investigated in the context of drug resistance in 3D models (**Figure 2**).

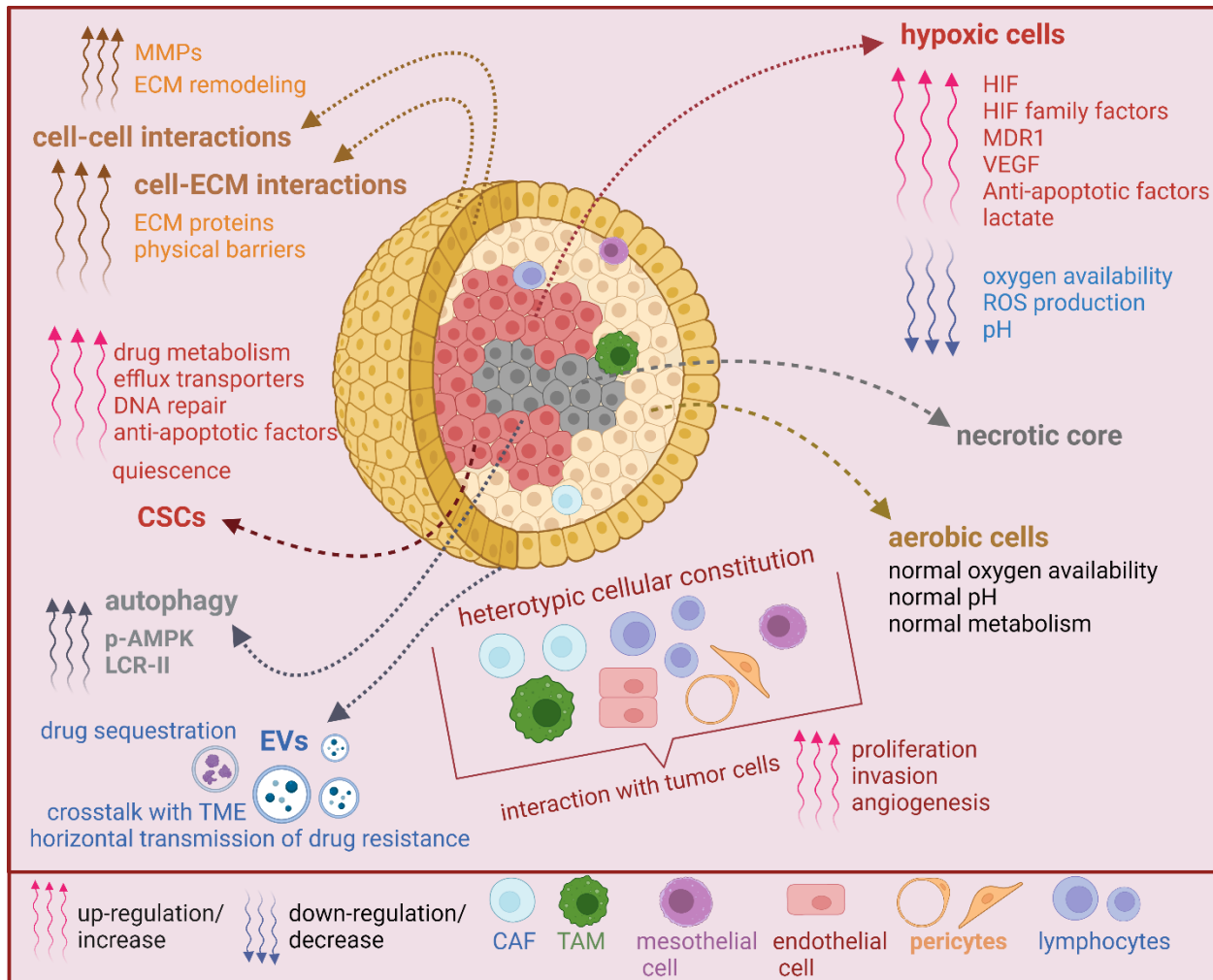


Figure 2. Mechanisms of drug resistance in 3D HGSOC spheroid model. The scheme indicates the molecular mechanisms of therapeutic resistance that can be established within 3D multicellular spheroids due to their 3D organization and flexibility in the TME. These mechanisms can result from cell-cell interactions and cell-ECM interactions, hypoxic conditions and a gradient of oxygen within the spheroid, CSC properties of inner cells, and heterotypic constitution of the spheroids in co-culture models. Different mechanisms are associated with resistance to drugs used for HGSOC treatment (the mechanisms of action described in section 3.2.), and several of them have been investigated in 3D cell models so far. Reduced drug uptake, inhibition of apoptosis-related pathways, solidification of DNA repair pathways, and metabolic reprogramming have been associated with platinum resistance. Reversion mutations and epigenetic and pharmacological modifications have been associated with PARPi resistance. ECM affects the TAX absorption of tumor cells, possibly explaining the reduced efficiency of TAX in 3D culture compared to 2D culture of the same cells. TAX resistance is associated with inhibition of apoptosis, vasculogenic mimicry, and reduction of ROS production. DXR is triggering oxidative stress, so the oxygen availability within 3D structures also affects its efficacy. Vasculogenic mimicry observed and investigated in 3D HGSOC models directly encumbers using bevacizumab as anti-angiogenic therapy. Inhibition of apoptosis and molecular mechanisms attributed to CSCs are shared by all of the above-mentioned drugs. Created with BioRender.

As stated in the introduction and the beginning of this section, for more than 25 years, the standard treatment for OC has been cytoreductive surgery and adjuvant chemotherapy based on platinum and taxane drugs (Raja *et al.*, 2012), and this has not changed since. It is important to note that currently, there are no biomarkers for EOC that have been accepted as predictors of first-line therapeutic response in the clinical setting. Platinum sensitivity is still the standard for predicting clinical prognosis (Wilson *et al.*, 2017). Platinum resistance is defined as the lack of response or relapse within six months of platinum-based chemotherapy (Wilson *et al.*, 2017; D'Amora *et al.*, 2021). Generating patient-specific indications of first-line chemotherapy response through 3D platforms in a clinically relevant time frame prior to treatment can be the best option for treatment decisions for both clinicians and patients. Upon validation in more extensive, independent cohorts, patient-derived OC spheroids and organoids hold the solid potential to become a new standard in treatment decision-making in oncology with substantial clinical value. Once authenticated and standardized, these platforms should be incorporated as a follow-up part of biopsy/debulking surgeries for newly diagnosed OC patients. Numerous recent studies have demonstrated the usefulness of 3D models for investigating the drug resistance of HGSOC (Table 2).

Table 2. Recent studies utilizing 3D models for investigation of HGSOC chemoresistance.

Investigated drug response	3D system utilized	Main finding	Reference
cDDP, Mirin	3D spheroids of platinum-sensitive and resistant OC cell lines in ULA plates; A2780, A2780cis (platinum-resistant), PE01, PE04 (BRCA2-proficient, platinum-resistant)	Mre11 depletion by gene knockdown or blockade by small molecule inhibitor (Mirin) reversed platinum resistance in OC cells and in 3D spheroid models; Mre11 inhibition was synthetically lethal in platinum-sensitive XRCC1 deficient OC cells and 3D-spheroids	<i>Alblihy et al.2022</i>
CBP, TAX, GEM, TOP, etoposide, vinorelbine, treosulfan, PLD, CBP + TAX, CBP + GEM, CBP + PLD, CBP + DTX, olaparib, erlotinib, and SAHA	Patient-derived OC spheroids	Recurrent OC characterized by high variability in druggable target expression and drug response profiling in analyzed patient cohort; interpatient tumor heterogeneity modeled on the patient-derived OC spheroid model	<i>Hoffman et al. 2022</i>
TAX, TOP, the effect of <i>ALDH1A</i> knockout on TAX- and TOP-sensitivity	3D spheroids generated in ULA plates from OC cell lines (parental W1 cell line, W1PR1 cell line resistant to TAX, W1TR cell line resistant to TOP, and <i>ALDH1A</i> knockout cell lines	The same cells were more resistant in 3D than in the 2D culture; the level of resistance in 2D culture correlated to the expression level of drug transporters; the key determinant of resistance in 3D spheroids seemed to be the presence of different cell	<i>Nowacka et al. 2022</i>

	W1PR1-C7 and W1TR-1p17)	zones, the cellular density of spheroids, and the capacity of drug diffusion into the cellular/ECM structure	
cDDP, LY-294002 (a PI3K-AKT dual kinase inhibitor), cDDP + LY-294002	3D spheroids of OC cell lines (OVCAR-4, HeyA8, SK-OV-3, A2780 and its cDDP-resistant derivative, patient-derived MCW-OSE-1, MCW-OV-SL-3 and its cDDP-resistant derivative)	Blocking of PI3K-AKT signaling by PI3K inhibitor LY294002 sensitized cells to cDDP and inhibited the cell colony formation, migration, and 3D morphogenesis in cDDP-sensitive and cDDP-resistant cell lines	<i>Parashar et al. 2022</i>
CBP, Birinapant (a bivalent SMAC mimetic compound) + CBP	3D organoid bioassay (OC cells grown as organoids embedded within Matrigel) for a panel of 7 EOC cell lines (OVCAR-3, OVCAR-4, OVCAR-8, SK-OV-3, CaOV3, Kuramochi and OAW28) and 10 platinum-resistant primary patient tumor samples	Organoid bioassay-predicted results correlated with reported platinum sensitivities for each cell line and with primary tumor samples; combining SMAC mimetics with CBP could target OCs	<i>Singh et al. 2022</i>
cDDP, methadone, cDDP + methadone	Four patient-derived HGSOc tumor-spheroid models; samples cultured in a custom-constructed, fully 3D-printed, micro-processor-controlled bioreactor system	Although it showed some apoptotic and chemosensitizing effects in preclinical tumor models, methadone demonstrated no benefits as a chemosensitizing drug in OC patients; harmful effects of methadone have been identified	<i>Fiegl et al. 2022</i>
TAX, cDDP, cDDP + TAX, PANO, cDDP + TAX + PANO, SAHA	3D spheroids generated by hanging drop method from OVCAR-3 and CaOV3 cell lines,	3D-derived OC cells demonstrated induced proliferation, migration, invasion, cancer colony formation and chemoresistance properties after a single exposure to classic chemotherapy; a combination of classic chemotherapy with epigenetic therapy was most effective for OVCAR-3 cells	<i>Bilbao et al. 2021</i>
CBP	3D spheroids generated in ULA plates from OC cell lines (OV90, OV4485, OV4453, TOV21G, TOV112D and OV1946)	Comparison of 2D monolayers, spheroids, <i>ex vivo</i> tumors, and <i>in vivo</i> models; CBP sensitivity of 2D OC cell cultures differed from the <i>in vivo</i> response; the response of 3D spheroids better correlated with the <i>in vivo</i> response than 2D cultures	<i>Brodeur et al. 2021</i>
CBP	3D spheroids from four OC cell lines (OV90, TOV3041G, TOV112D, OV866) generated by different methods: PDMS-based microfluidic systems,	The most effective 3D spheroid forming methods were microfluidic chips and Matrigel-assisted ULA plates; regardless of the spheroid forming method, CBP sensitivity inversions were noted within a given	<i>Patra et al. 2020</i>

	ultra-low attachment (ULA) plates in the presence or not of 2% Matrigel, and hanging droplets	cell line between 2D and 3D cultures	
TAX, CBP, GEM, DXR, TOP, olaparib	Primary patient tissue in 3D spheroid culture in ULA plates	Validation and initial prospective clinical validation of developed system to accurately predict patient-specific response to first-line chemotherapy in newly diagnosed OC patients prior to treatment initiation	<i>Shuford et al. 2019</i>
CBP, TAX, additional compounds combined with CBP or TAX	Patient-derived organoids (of various OC subtypes)	CBP was significantly more effective in serous than in clear cell subtypes; two lines of organoids were generated, an additional 1135 drugs were screened, and drugs with better combinatory effects with CBP than with TAX were found; certain drugs demonstrated an additive effect with CBP	<i>Ito et al. 2023</i>
cDDP	Patient-derived OC organoids (primarily HGSOC)	Metabolic signatures (measured by quantitative mass spectrometry) predicted clinical outcomes following CBP + TAX chemotherapy; specific amino acid and lipid profiles characterized a state of tumor cellular quiescence associated with immune dysfunction	<i>D'Amora et al. 2021</i>
CBP	Patient-derived HGSOC organoids	Developed six HGSOC organoid lines from tissue obtained during debulking surgery; organoid line, predicted to be CBP-resistant, correlated with a significantly shorter PFS than the rest of the subjects	<i>Gorski et al. 2021</i>
cDDP, CBP, TAX, DTX, TOP, GEM, DXR, SAHA, etoposide, olaparib, tamoxifen, eribulin, vinorelbine, trabectedin, belinostat, cediranib, pazopanib, sunitinib, everolimus, trametinib, gefitinib, lapatinib, SN-38	Patient-derived organoids (3 HGSOC, one clear cell, three endometrioid)	Developed organoids captured the characteristics of histological cancer subtypes and replicated the mutational landscape of the primary tumors; CNVs were also similar among organoids and primary tumors; HGSOC organoids with HRD shared a drug sensitivity pattern different from that of non-HRD like HGSOC organoid line which showed resistance to most of the tested drugs, except trabectedin	<i>Nanki et al. 2020</i>
CBP, TAX	Whole-genome-characterized patient-derived organoids from HGSOC patients with known clinical	Developed organoids displayed inter- and intra-patient drug response heterogeneity; genetic aberrations can partly explain drug response	<i>De Witte et al. 2020</i>

	histories	heterogeneity	
CBP, TAX, olaparib, abraxane, mocetinostat, trametinib, LY294002, AZD5363, BBI503, MK-1775, sorafenib, APR-246, CB5083, napabucasin	Short-term spheroid cultures from HGSOC malignant effusions in conditions selected to support organoid growth	Developed spheroid cultures can recapitulate the histological features of malignant ascites fluid and can be expanded for at least six days; within six days of culture, significant up-regulation of genes related to cellular proliferation, EMT, and KRAS signaling was observed	<i>Chen et al. 2020</i>
Evaluation of cancer immunotherapy efficacy	OC tumor model composed of patient-derived microtumors and autologous tumor-infiltrating lymphocytes	Characterization of patient-individual immune phenotypes and the assessment of responses towards immunotherapy; results generated within 3–4 weeks	<i>Anderle et al. 2022</i>
The effects of size and shape of the OC spheroids on drug resistance and migration; TAX, cDDP	Spheroids derived from HGSOC cell lines OVCAR-3 and OVCAR-8	Characterization of two distinct spheroid structures: loose aggregates (OVCAR-3) and compact spheroids (OVCAR-8); no differences observed in resistance to TAX and cDDP as a function of spheroid size and shape; migration capacity of compact spheroid 15-fold higher compared with that of loose aggregates	<i>Gunay et al. 2020</i>
The effect of STAT1 signaling on stemness properties in chemoresistant EOC cells; TAX	2D and 3D cultures, and tumor xenografts; spheroids of TAX-sensitive HGSOC cell line OVCAR-3 and of TAX-resistant subline OV3R-PTX	Possible regulatory mechanism of STAT1 underlying drug resistance; potential therapeutic application for EOC patients with TAX-resistant disease	<i>Wang et al. 2020</i>
Characterization of chemoresistant leader cell (LC) subpopulation within OCs; olaparib, rucaparib, TAX, TOP, CBP, cyclophosphamide, cDDP and DXR	3D spheroids derived from HGSOC and clear cell carcinoma OC cell lines (SK-OV-3, COV362.4, and OVCAR-4) generated in ULA plates; patient-derived primary cells from ascites and primary or metastatic tumors	LCs represent a transcriptionally plastic subpopulation arising independently of cell division or DNA replication, with a “stemness” profile that does not correlate with EMT, expressing chemoresistance markers ALDH1, Twist, and CD44v6; functional impairment of LCs restored chemosensitivity	<i>Karimnia et al. 2019</i>
CBP response in heterotypic spheroids	The 3D hanging drop heterospheroid array, with HGSOC CSCs and carcinoma-associated mesenchymal stem/stromal cells (CA-MSC); OVCAR-3 HGSOC cell line, human adipose-derived mesenchymal cells, patient-derived HGSOC samples	Platelet-derived growth factor (PDGF) signaling from CA-MSCs increased platinum resistance of heterospheroids; inhibition of PDGF signaling reduced platinum resistance and metastatic potential in OC CSCs	<i>Raghavan et al. 2020</i>

<p>Genomic characterization of patient-derived organoids, intratumoral heterogeneity, drug response; TAX, oxaliplatin, DXR, GEM, and 8 targeted compounds provided by AstraZeneca</p>	<p>HGSOC organoids established from patient-derived ascites (43), solid tumors (10), and patient-derived xenografts (15)</p>	<p>Analysis of CNVs and mutational signatures showed that HGSOC patient-derived organoids recapitulate the broad mutational landscape of patient samples; comparison of drug sensitivity between five organoids and their parental uncultured patient-ascites revealed a moderate to a high correlation between the drug area under the curve (AUC) of patient-derived organoids and their corresponding patient-derived ascites</p>	<p><i>Vias et al. 2023</i></p>
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CBP: carboplatin; cDDP: cisplatin; TAX: paclitaxel; TOP: topotecan; GEM: gemcitabine; DTX: docetaxel; DXR: doxorubicin; PLD: PEGylated liposomal doxorubicin; SMAC: second mitochondrial activator of caspase; PANO: panobinostat; SAHA: suberoylanilide hydroxamic acid (vorinostat); PDMS: polydimethylsiloxane; PI3K: phosphoinositide 3-kinase; CNVs: copy number variations; LCs: leader cells; HRD: homologous recombination deficiency; STAT1: signal transducer and activator of transcription-1

3.4. Measuring Drug Resistance in 3D High-Grade Serous Ovarian Cancer Cell Models

Several methods and approaches can be used to measure numerous endpoints in studies utilizing 3D HGSOC models, depending on the type of 3D model used, cultivation method and, of course, specific research questions. Generally, many commercial kits for measuring cell viability, cell proliferation, and cell death apply to 3D cell cultures, where attention should be paid to spheroid/organoid size, density, and morphology. Studies in **Table 2** used various methods to measure 3D cell cultures' growth, viability, proliferation, migration, invasion, apoptosis and necrosis. Sensitivity toward all approved treatments for HGSOC described in section **3.1.**, as well as the investigation of multiple drug candidates, has been measured in 3D HGSOC models in recent studies and summarized in **Table 2**. For example, Alblihy and colleagues investigated the platinum resistance of spheroids grown from platinum-sensitive and platinum-resistant cells in ULA plates. After cDDP treatment, they used a LIVE/DEAD Viability/Cytotoxicity Kit from Thermo Fisher Scientific to determine cellular viability, along with measurements of spheroid diameters in every treatment condition (Alblihy *et al.*, 2022). Hoffman *et al.* used a patient-derived recurrent OC spheroid model from 30 tumor samples to test the effects of platinum- and nonplatinum-based guideline-recommended therapies, as well as several drugs pending approval. For this purpose, they used a CellTiter-Glo® Luminescent Cell Viability Assay from Promega (Hoffman *et al.*, 2022). A study by Nowacka *et al.* utilized an MTT assay and phase imaging for assessing HGSOC spheroid viability following treatment with TAX and TOP (Nowacka *et al.*, 2022). Fiegl and colleagues monitored the size and morphology of spheroids cultured from ascites and tumor specimens from HGSOC patients by live-cell microscopy (Fiegl *et al.*, 2022). They used propidium iodide staining of 3D cultures to visualize cell death and CellTiter-Glo® Luminescent Cell Viability Assay from Promega to measure cell viability.

Also, they applied a human enzyme-linked immunosorbent assay (ELISA) kit to determine VEGF concentrations in both 2D and 3D cultures (Fiegl *et al.*, 2022). Bilbao and colleagues used the Invitrogen™ eBioscience™ Annexin V-FITC Apoptosis Detection Kit from Thermo Fisher Scientific to detect apoptosis and the Invitrogen™ eBioscience™ Propidium Iodide Staining Solution from the same manufacturer to detect necrosis in 3D cultures of Caov-3 and OVCAR-3 spheroids established by hanging drop method (Bilbao *et al.*, 2022). Brodeur *et al.* used flow cytometry and LIVE-DEAD™ Fixable Aqua Dead Cell Stain Kit from Thermo Fisher Scientific and immunohistochemistry with anti-Ki-67 and anti-cleaved caspase-3 antibodies to analyze the extent of apoptosis in 3D spheroids derived from cell lines (Brodeur *et al.*, 2021). Sheta and colleagues developed a 3D system for measuring sensitivity toward PARPi olaparib and niraparib in spheroids grown in agarose from ascitic HGSOc patient-derived cells (Sheta *et al.*, 2020). They measured treatment-induced cytotoxicity in 2D and 3D cultures of the same patients' cells and observed a generally stronger response to both PARPi in 2D, with niraparib being overall more cytotoxic than olaparib in both conditions. The Perfecta3D cell viability kit from Sigma Aldrich was used for measuring sensitivity towards PARPi in spheroids. They measured the γ H2AX foci formation as a marker of the homologous DNA repair pathway activity in PARPi-sensitive and resistant cultures. By comparing the gene expression patterns of PARPi-resistant and PARPi-sensitive 3D cultures, they identified 24 potential biomarkers of PARPi sensitivity, for seven of which the differential expression was subsequently confirmed in matched tumor samples. This study demonstrates how 3D models can identify predictive biomarkers for PARPi treatment (Sheta *et al.*, 2020).

3.5. 3D Models Used for Investigating Drug Resistance Mechanisms Attributed to Cancer Stem-like Cell Properties

Many cancers have been found to contain self-renewing tumorigenic CSCs with different expression profiles and phenotypes, implying that CSC-targeted therapies may not be equally effective for all types of cancer (Hirst *et al.*, 2018; Horst *et al.*, 2021). These differences likely occur due to the interactions of CSCs with other cell types in specific TMEs. Various mechanisms of CSC resistance to treatment (indicated in **Figure 2**) may be exacerbated by CSC population heterogeneity, thus increasing the likelihood of resistant variant outgrowth. Chemotherapy was associated with the induction of CSC-like traits and the migration of OC cells (Zhao *et al.*, 2020). Hirst *et al.* generated spheroids from multiple OC cell lines, most of them HGSOc, and demonstrated that the multilayered 3D architecture of spheroids comprised zones of variable gene expression, with an increase in hypoxia-regulated genes and markers of stemness in the spheroid core, which cannot be observed in monolayered cells and drives phenotypic

changes (Hirst *et al.*, 2018). The 3D spheroids expressed the CSC-associated markers *ALDH1A* and *CD133* and exhibited hypoxia and increased resistance to TAX. They investigated the effects of clinically repurposed drugs on 2D cultured cells and spheroids in search of successful candidates in 3D conditions and found that the cyclooxygenase/lipoxygenase inhibitor licofelone reversed the CSC characteristics in spheroids (Hirst *et al.*, 2018). The idea of clinical evaluation of licofelone combined with TAX-based chemotherapy resurging from this study is a good example of how utilization of spheroid culture can lead to improved chemotherapeutic response and overcoming of acquired chemoresistance (Hirst *et al.*, 2018). Wang *et al.* investigated the effect of the signal transducer and activator of transcription-1 (STAT1) signaling on stemness properties in TAX-resistant HGSOc cells (Wang *et al.*, 2020). They used the TAX-resistant cell line OV3R-PTX derived from TAX-sensitive OVCAR-3 cells and cultured them in 2D and 3D conditions. Interestingly, OV3R-PTX cells grew slower than parental cells in both 2D and 3D cultures, with OV3R-PTX spheroids having smaller micro-spheres than OVCAR-3 spheroids, but OV3R-PTX grew faster in tumor xenografts. The authors attributed this effect to the existence of heterogeneous cells in the OV3R-PTX population containing CSCs and isolated a single-cell clone that shared the characteristics of CSCs. The CSC characteristics in the monoclonal cell line were increased in the spheroids compared with the monolayer culture (Wang *et al.*, 2020). The monoclonal TAX-resistant cell line demonstrated a higher spheroid formation capacity and larger micro-spheres than the parental OVCAR-3 cell line. These findings imply that rapid tumor growth *in vivo* is a consequence of the extensive outgrowth of CSCs (Wang *et al.*, 2020). The expression of CD44, CD133, and OCT4 was higher in the monoclonal TAX-resistant cell line than in parental cells, while the STAT1 levels were significantly lower. The repression of STAT1 in TAX-resistant cells was attributed to DNA hypermethylation of the *STAT1* promoter region (Wang *et al.*, 2020). This study proposes a new therapeutic strategy for TAX-resistant patients by highlighting the correlation among strong TAX resistance, enhanced stemness markers, and down-regulation of STAT1 using 3D modeling (Wang *et al.*, 2020).

Resurfacing CSCs following chemotherapy can be studied to understand the evolution and genetics of remaining clonal populations. After various drug treatments, analyses of these remaining CSC populations can be used to identify therapeutic targets in clonal chemoresistant populations. Karimnia and colleagues investigated so-called leader cells (LCs) in the context of chemoresistance in HGSOc spheroids formed in ULA microplates (Karimnia *et al.*, 2021). LCs were characterized by keratin-14 (KRT14) expression, transcriptional plasticity, and a CSC-like phenotype but did not represent a quiescent population (Bilandzic *et al.*, 2019; Moffitt *et al.*, 2019; Karimnia *et al.*, 2021). Increased expression of KRT14 has been associated with worse progression-free survival (PFS) and a lack of

therapeutic response in OC patients (Bilandzic *et al.*, 2019). The LC sub-population is considered to drive the collective invasion in metastatic epithelial cancers by rearranging their actin filaments, facilitating membrane protrusion, and forming actin-rich "invadopodia", which direct migration and lead the packed "follower" cells. CAFs are also implicated as essential mediators of collective invasion by physically remodeling the TME to help invading cancer cells (Moffitt *et al.*, 2019; Giusti *et al.*, 2022). Karimnia *et al.* treated the LC-containing HGSOC spheroids with olaparib, rucaparib, TAX, TOP, CBP, cyclophosphamide, cDDP, and DXR. They observed an increase in the LC population under each treatment condition (Karimnia *et al.*, 2021). The LCs represented the bulk of the remaining cell population following treatment, indicating their resistance to chemotherapy, and their migration in 3D spheroid outgrowth assays remained unaffected in all tested treatments except TOP (Karimnia *et al.*, 2021).

3.6. 3D Models Used for Investigating Drug Resistance Mechanisms Attributed to Collaboration of Multiple Cell Types

Applications of immune checkpoint inhibitors are a promising strategy in the treatment of various cancer types, but for OC, the results from clinical trials to date have been disappointing, and the only available efficient immunotherapy for OC remains the VEGF-A-targeting monoclonal antibody bevacizumab (Garcia *et al.*, 2020; Leary *et al.*, 2021). The programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) signaling pathway is known to mediate tumor immune escape and is recognized as a promising target for immunotherapy in many types of cancer, with several FDA-approved PD-L1 inhibitors (durvalumab, atezolizumab, and avelumab) and PD-1 inhibitors (cemiplimab, pembrolizumab, and nivolumab) indicated for melanoma, lung cancer, gastrointestinal and hematological tumor treatment (Hargadon *et al.*, 2018; Akinboro *et al.*, 2022). Li and colleagues proposed a combinational therapeutic approach of bromodomain-containing protein 4 (BRD4) inhibition and α PD-L1 administration for targeting the TIME of HGSOC (Li *et al.*, 2020). They established models for TAMs co-culturing with T lymphocytes *in vitro* and demonstrated that the BRD4 inhibitor AZD5153 induced phenotype switching in TAMs from M2-like macrophages to M1-like macrophages, subsequently promoting the secretion of pro-inflammatory cytokines and activating CD8⁺ cytotoxic T lymphocytes. Using the 3D microfluidic model, they demonstrated that AZD5153 sensitized tumor microspheres isolated from ascites of chemo-naive patients to anti-PD-L1 therapy (Li *et al.*, 2020). This is another promising strategy for treating HGSOC, that could not be investigated without a perceptive design and a 3D co-culture model application.

Raghavan and colleagues developed a model of OC malignant ascites in a 3D hanging drop heterospheroid array, with HGSOC CSCs and carcinoma-associated mesenchymal stem/stromal cells (CA-MSCs) to investigate the effects of CA-MSC-secreted platelet-derived growth factor (PDGF) on HGSOC CSC properties (Raghavan *et al.*, 2020). They used OVCAR-3/MSCs and matched patient CSC/CA-MSC heterospheroids and found them to be platinum-resistant and enriched with ALDH⁺ cells, with significantly increased stemness, metastatic potential, and chemoresistance in the presence of PDGF signaling. PDGF signaling is known to contribute to the progression of various cancers, including breast and gastric cancer, and in OCs it has been identified as a potential key pathway implicated in poor prognosis but was not previously investigated in the context of OC CSC/CA-MSC communication and in 3D co-culture models (Raghavan *et al.*, 2020). Their results suggest that blocking stromal PDGF signaling will make HGSOC significantly more responsive to chemotherapy by abrogating CSC properties (Raghavan *et al.*, 2020). This study is another excellent example of how 3D co-cultures reveal the crucial importance of TME signaling in the drug response of HGSOC and how including the TME in the 3D modeling of HGSOC offers strategic advances in improving chemotherapeutic success.

3.7. 3D Models Used for Chemotherapy Response Prediction

Patra *et al.* recently demonstrated how challenging it is to choose the appropriate preclinical model for drug testing (Patra *et al.*, 2020). They generated spheroids from the EOC cell lines with known responsiveness to CBP using the hanging-drop method, ULA plates (with and without Matrigel[®]), and polydimethylsiloxane microfluidic chips and investigated spheroid forming ability in each system. The most effective models were microfluidic chips and Matrigel[®]-assisted ULA plates, and they were used to define the CBP sensitivity of formed spheroids and to compare responses to the 2D monolayer assays. One sensitive cell line in 2D culture exhibited resistance to CBP in 3D spheroids, and two cell lines that were previously categorized as resistant to CBP in 2D cultures were sensitive in the 3D models (Patra *et al.*, 2020). Gupta *et al.* observed, particularly for metastatic cells, that the drug response of 3D EOC models depended on the structural and biochemical composition of the model/platform (Gupta *et al.*, 2022). They compared the growth and chemotherapy response in the comparative study of primary and metastatic EOC cells in spheroids on ULA plates, synthetic PeptiGels/hydrogels of different formations, and polymeric scaffolds with various ECM layers. The three platforms supported the spheroid growth but for different culture periods, varying from 7 days to 4 weeks. An interesting notion is that tumor cells of different origins may prefer platforms with stiffness matching their tissue of origin (Gupta *et al.*, 2022).

A recent comparison study analyzed the CBP chemotherapy response of six previously characterized EOC cell lines of differing chemosensitivities (four of them HGSOE: OV90, OV1946, OV4453, and OV4485) across four different model systems including 2D monolayers, 3D spheroids, 3D *ex vivo* tumors and mouse xenograft models (Brodeur *et al.*, 2021). The 3D *ex vivo* model used in the study was a microfluidic micro-histological platform developed by Simeone *et al.* for the *ex vivo* culturing of prostate and OC micro-dissected tissue (Simeone *et al.*, 2019). Brodeur *et al.* demonstrated that the CBP response in EOC cell lines cultured in a 3D *ex vivo* model (micro-dissected tissue produced from cell line xenograft tumors) correlated best with the *in vivo* CBP response (mouse model), followed by 3D spheroids (Brodeur *et al.*, 2021). Shuford *et al.* developed a patient-derived 3D spheroid model utilizing primary tissue obtained at debulking surgery or laparoscopic biopsy from newly diagnosed OC patients that generated results within seven days of 3D culturing, which constitutes a clinically relevant time frame for treatment decisions (Shuford *et al.*, 2019). They tested 83 samples for responsiveness to CBP and TAX and observed an 89% accurately predicted response to first-line chemotherapy with patient-matched clinical outcomes of corresponding response or non-response. They examined the prognostic value of the model system and found a significant difference in median PFS from surgery for those patients who were prospectively test-predicted to respond to first-line platinum-based chemotherapy (greater than twenty months) compared with those who were test-predicted not to respond (nine months) (Kaplan-Meier analysis; HR 0.3414, 95% CI 0.08927 to 1.305, $p = 0.01$) (Shuford *et al.*, 2019). Previous 2D assays of OC patient chemo responsiveness were only effectively completed for less than 80% of enrolled patients (Rutherford *et al.*, 2013; Grendys *et al.*, 2014), and this test had a 90% success rate, demonstrating another advantage of 3D culturing (Shuford *et al.*, 2019). Patient-derived spheroid models require shorter generation duration than organoid models, one of the most critical characteristics for clinical utility, and generally have a better overall culture success rate. However, there have been patient-derived OC organoid models developed in less than three weeks (which is still a clinically relevant time frame) that successfully recapitulated histological characteristics and mutations of primary tumors (Nanki *et al.*, 2020). There have also been studies with a 100% success rate of organoid establishment (Ito *et al.*, 2022).

Nowacka *et al.* investigated the resistance to cisplatin (cDDP) and TAX in 2D and 3D culture conditions of A2780 cells and their cDDP-resistant sublines A2780CR1 and A2780CR2 and TAX-resistant sublines A2780PR1 and A2780PR2 (Nowacka *et al.*, 2021). They used ULA plates to generate multicellular spheroids of each cell line and investigated the cell lines' sensitivity towards cDDP and TAX in 2D and 3D culture conditions. The fold of resistance of all cDDP-resistant and TAX-resistant sublines compared with the parental cell line was higher in 3D conditions than in 2D. An almost 10-fold decrease in the sensitivity of parental A2780 cells in 3D compared with 2D to cDDP and a 1098-fold decrease to

TAX was observed. A similar decrease in sensitivity under 3D conditions was observed for resistant variants (the A2780CR1 cell line showed a 4.11-fold increase, and the A2780CR2 cell line showed a 2.69-fold increase in cDDP resistance; the A2780PR1 cell line presented a 217-fold increase, and A2780PR2 presented a 25-fold increase in TAX resistance) (Nowacka *et al.*, 2021). The observed greater difference in the cellular response to TAX than to cDDP in 3D compared with 2D cultures might be explained by the different modes of drug spreading, with cDDP evenly distributed within the tumor and TAX binding to cellular macromolecules (Nowacka *et al.*, 2021). For cDDP, a concentration-dependent response was observed in all examined cell lines in 3D cultures, but for TAX, the response drastically differed between 2D and 3D cultures. For the A2780 cell line, TAX treatment in 2D conditions resulted in a concentration-dependent decrease in cell viability, but in 3D conditions, a four-step response curve was observed. Increasing the TAX concentration up to 10 ng/ml did not affect cell viability, and an increase up to 50 ng/ml decreased cell viability to 70%; a further increase up to 2000 ng/ml had no effect on viability, with viability drastically dropping at concentrations higher than 2000 ng/ml (Nowacka *et al.*, 2021). It is fascinating how cell lines differed in the observed TAX response curves, implying unforeseen effects of 3D architecture that cannot be studied in monolayers. Another exciting aspect of the same study was the evaluation of genes previously shown to be implicated in drug resistance of these models in 2D (Januchowski *et al.*, 2014, 2017). The spheroids of TAX-resistant variants had an increased expression of *MDR1* and its product P-gp protein. However, there were no differences in transcript and protein levels in the cells growing in 3D compared with 2D conditions, despite decreased sensitivity to TAX of TAX-resistant sublines compared with parental cells. This implies that *MDR1* may be crucial in 2D drug resistance but not the most critical aspect in 3D (Nowacka *et al.*, 2021). This is another subtle example of how important 3D architecture is in drug response and why we must shift the focus toward 3D modeling. Nowacka *et al.* attributed the observed decrease in drug sensitivity in 3D conditions to other mechanisms, such as tissue-related features, including ECM component expression and cellular density (Nowacka *et al.*, 2021). The mentioned multi-step drug response curves in 3D conditions have also been observed in other models, unlike the typical dose-dependent responses in monolayers. Spheroids of the primary human ovarian cancer W1 cell line generated three-step response curves when treated with TAX and topotecan (TOP) in 3D cultures (Nowacka *et al.*, 2022). Low concentrations of TAX (100-500 ng/mL) significantly decreased W1 cell viability to 35% compared with non-treated cells. However, increased concentrations up to 5000 ng/mL resulted in a moderate increase in cell survival (Nowacka *et al.*, 2022). Only a very high concentration of TAX resulted in cell death. Under 2D conditions, the typical dose-dependent response was obtained (Nowacka *et al.*, 2022). To summarize, the drug response in 3D models appears to be related to the spatial and organizational properties of 3D structures, resulting in decreased sensitivity compared with monolayers.

As discussed in the introduction, HGSOCs are characterized by high heterogeneity and ubiquitous TP53 mutations, and this has been identified as one of the early events leading to chromosomal instability (CIN), which can be observed in the pre-invasive lesions and is known to drive metastasis and therapeutic resistance (Bakhoun *et al.*, 2018; Lukow *et al.*, 2021; Vias *et al.*, 2023). Vias *et al.* developed patient-derived HGSOC organoids and completed their comprehensive genomic, transcriptomic, and drug-response characterization (Vias *et al.*, 2023). They demonstrated that their models recapitulated clinically relevant CIN features observed in HGSOC patients, such as copy number-driven gene expression signatures, and the drug responses matched those of the parental tissues (Vias *et al.*, 2023). By single-cell DNA sequencing, the authors identified clonal populations with distinct copy number features. They demonstrated that the developed models recapitulated the heterogeneity of chromosomal instability of HGSOC and thus hold promise for treatment selection (Vias *et al.*, 2023).

Gorski *et al.* developed six HGSOC patient-derived organoid lines from tissue obtained during debulking surgery and used these models for clinical response prediction and searching for chemoresistance drivers in HGSOC (Gorski *et al.*, 2021). The clinical outcomes directly correlated with chemosensitivity assay results obtained from organoid cultures. The most CBP-resistant organoid line, which demonstrated a CBP EC₅₀ (half maximal effective concentration of the drug) value above the clinically achievable maximum concentration when tested in 3D culture conditions, correlated with the patient's platinum-resistant disease and significantly shorter PFS than the rest of the subjects (Gorski *et al.*, 2021). The authors performed DNA and RNA sequencing in search of the integrated genomic signature of platinum resistance, and the most commonly mutated genes were *TP53* (4/6), *FANCC*, and *NOTCH2* (both in 2/6 patient samples). The study identified 71 differentially expressed genes between CBP-resistant and CBP-sensitive organoids. The top-up-regulated genes were the ones involved in transmembrane transport, cellular differentiation, and immune response modulation, while the top-down-regulated genes were known to be involved in the regulation of cellular growth, cellular stress response, and lipid metabolism. Integrated pathway analysis suggested that CBP resistance was partly mediated by altered injury-associated pathways and the expected characteristic cancer-related pathways. Network mapping revealed that most differentially expressed pathways were connected with NF- κ B, cellular differentiation (PRDM6 activation), and the linkage of B-cell receptor signaling to the PI3K–AKT signaling pathway (Gorski *et al.*, 2021). Additional research with a greater sample size is needed for model value confirmation, but the obtained data are promising, and the number of studies is steadily growing. De Witte *et al.* analyzed 36 whole-genome-characterized patient-derived organoids from donors with known clinical backgrounds (de Witte *et al.*, 2020). They confirmed the stability of genomic features of original tumors and the precision in recapitulating patients' responses to neoadjuvant CBP/TAX

chemotherapy. Analyzing the results of multiple studies like the one from Gorski *et al.* and de Witte *et al.*, with more significant sample sizes, offers a promising way to establish an early patient chemo responsiveness stratification system and a predictive scoring system of HGSOC recurrence.

4. Perspectives

The progression and metastasis of HGSOC occur in the highly complex pro-inflammatory and tumor-promoting TME with intricate cellular, molecular, and mechanical properties, which are all interconnected and contribute in various ways to the aggressiveness of the disease. There is significant evidence that malignant ascites contributes to HGSOC metastasis (Ahmed & Stenvers, 2013; Geng *et al.*, 2022), and yet it still needs to be extensively researched. HGSOC is a multi-dimensional disease, and utilizing 3-dimensional models incorporating relevant microenvironmental features of ascites offers many opportunities for disentanglement of its pathology and progression. Several critical aspects of the TME, including cell–cell and cell–ECM interactions, ECM stiffness, oxygen and nutrient gradients, ascitic flow, etc., have been successfully recapitulated in novel 3D models (Horst *et al.*, 2021; Qin *et al.*, 2022; Yee *et al.*, 2022), as we emphasized in this review. The high inter- and intra-patient heterogeneity observed in HGSOC cases highlights the need for patient-derived personalized models for predicting the therapeutic response in a clinically relevant timeframe following debulking surgery or biopsy. There is also a clear need to utilize better preclinical models for drug development so that there is a relevant translational value in preclinical and early-phase studies. The gold standard of *in vitro* preclinical research, 2D tumor cell lines, are easily manipulated, consistent, and often respond to most therapeutic approaches but cannot model the physiologically relevant properties of the *in vivo* TME. *In vivo* animal models, the conventional next step after studies in 2D cell models, have their own drawbacks as well, being expensive, time-consuming, and introducing animal immune cells and microenvironment to human disease modeling (Tentler *et al.*, 2012). Additionally, the immunodeficient mouse strains are excellent for engraftment efficiency but cannot be utilized for TIME research and evaluation of potential immunotherapies. The validation of preclinical findings by 3D cell systems in the future should eradicate the need for animal testing. 3D models have already revealed so much of HGSOC tumorigenesis, progression, and evolution that they are genuinely indispensable for basic research, drug response assessment, and biomarker development. Sophisticated 3D co-culture systems such as those described in this review are specifically helpful for the investigation of CSC phenotypes, the development of immunotherapies, anti-metastatic and anti-angiogenic therapies due to the incorporation of T(D)ME. Even spheroids generated from the cell line CABA-1 in the simple hanging drop method exhibited inner features typical of *in vivo* tumors,

entrapped EVs, and the VM-like process (Giusti *et al.*, 2022). The use of 3D co-culture models provides another unique benefit in HGSOC research – sequential modeling of the metastatic process in the relevant TME, allowing a wholesome understanding of the HGSOC metastatic cascade and the selection of therapies depending on the phase of metastatic progression. Cellular collaboration in the TME modifies HGSOC progression and therapeutic response through signaling and ECM remodeling. As discussed by Pietilä *et al.*, the cancer cell-ECM crosstalk continues to evolve and is crucial in understanding cancer progression and therapeutic response (Pietilä *et al.*, 2021). The study by Pietilä and colleagues detected stromal pathways as potential crucial targets to improve the chemotherapeutic success against HGSOC cells that escape treatment (Pietilä *et al.*, 2021). The benefits brought to the field of HGSOC research are highlighted in **Figure 3**, together with the drawbacks of such models, which are discussed in the following section.

5. Conclusions

Although 3D models are becoming increasingly significant, they are far from becoming a standard of preclinical studies as several essential challenges still remain to be solved. The first issue is the effect of spheroid size variability on the observed drug response. Variations in size and compaction affect the delivery of nutrients and oxygen within spheroids, directly affecting proliferation, intrinsic cellular organization, and drug penetration. The effects of spheroid size and the original cell number from which the spheroid is grown must be further investigated to achieve high-throughput systems for selecting the best treatment options for each patient. Capturing tumor heterogeneity will be easier if HGSOC primary spheroid and organoid cultures can grow consistently (standardization of size and morphology with advanced culturing protocols and protocols for assaying). Also, more correlations with clinical studies are needed to set standards for data interpretation regarding drug response prediction. False-negative results from drug screenings in patient-derived models, as observed by Shuford *et al.*, should be understood and eliminated (Shuford *et al.*, 2019). Choosing a suitable preclinical model is tricky, as demonstrated in many recent studies (Patra *et al.*, 2020; Brodeur *et al.*, 2021; Gupta *et al.*, 2022), and the advantages and throwbacks of different platforms should likewise be better understood. Additionally, different phases of disease progression have diverse T(I)ME components, so models should become more sophisticated to gain clinical relevance. Patient materials are problematic owing to their limited availability (usually one opportunity to gather material from each patient; rarely more if palliative aspiration of ascites or secondary debulking surgery is done) and sometimes too scarce for successful culture establishment. There is also an issue of long-term vs. short-term expansion of patient-derived

cultures, with each approach having its good and bad sides. The long-term expansion enables multiple testing and increases reproducibility, but it induces a risk of clonal selection during prolonged culture, inevitably leading to loss of heterogeneity. Significant improvements in 3D culture manipulation are also necessary, as the culturing methods are complex and sensitive, which complicates the downstream analyses in comparison with the well-established methodology of 2D culturing. However, 3D models are being clearly beneficial in the investigation of HGSOE and already did, and will continue to, revolutionize preclinical research and lead to the discovery of novel, appropriate, and effective therapies. Emerging technologies such as CRISPR/Cas targeting of oncogenes and single-cell OMICs technologies, in combination with novel *in vitro* and *ex vivo* 3D HGSOE models, offer an integrated approach to unraveling the molecular mechanisms behind the metastasis and chemoresistance of HGSOE. Hopefully, the sophisticated 3D modeling of HGSOE will soon validate its most important potential – significant improvement of HGSOE patients' therapy outcomes.

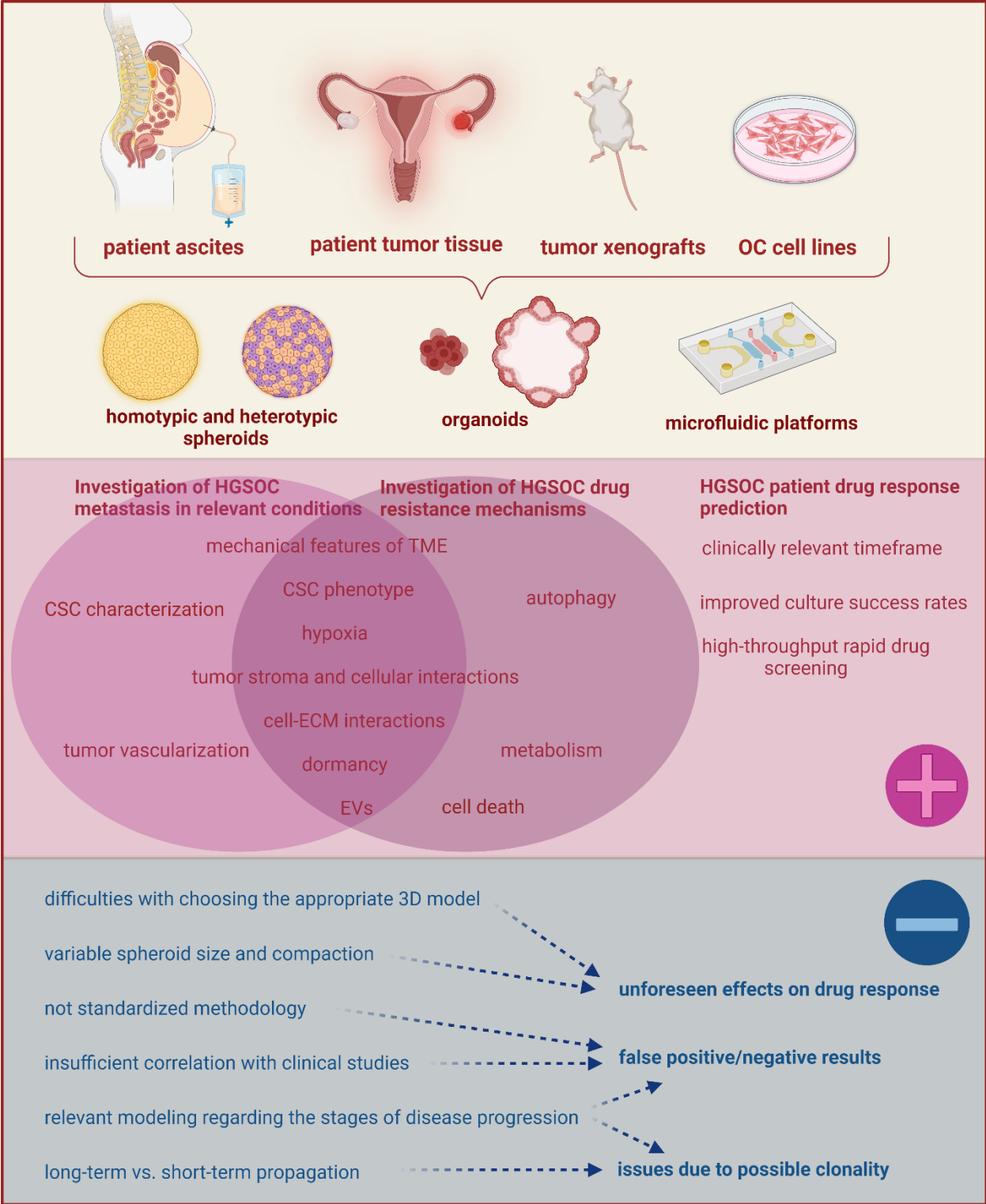


Figure 3. The benefits and challenges in 3D modeling of HGSOC. The scheme summarizes the possibilities and unresolved issues of 3D models discussed in this review. Created with BioRender.

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Credit Authorship Contribution Statement

Vanja Tadić: Investigation, Conceptualization, Writing - original draft, Visualization. Wei Zhang: Writing - review & editing. Anamaria Brozovic: Conceptualization, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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The High-Grade Serous Ovarian Cancer Metastasis and Chemoresistance in 3D Models

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Abstract

High-grade serous ovarian cancer (HGSOC) is the most frequent and aggressive type of epithelial ovarian cancer, with high recurrence rate and chemoresistance being the main issues in its clinical management. HGSOC is specifically challenging due to the metastatic dissemination via spheroids in the ascitic fluid. The HGSOC spheroids represent the invasive and chemoresistant cellular fraction, which is impossible to investigate in conventional two-dimensional (2D) monolayer cell cultures lacking critical cell-to-cell and cell-extracellular matrix interactions. Three-dimensional (3D) HGSOC cultures, where cells aggregate and exhibit relevant interactions, offer a promising *in vitro* model of peritoneal metastasis and multicellular drug resistance. This review summarizes recent studies of HGSOC in 3D culture conditions and highlights the role of multicellular HGSOC spheroids and ascitic environment in HGSOC metastasis and chemoresistance.

Keywords: High-Grade Serous Ovarian Cancer, Ascites, 3D Models, Spheroids, Organoids, Metastasis, Chemoresistance

Abbreviations

2D: Two-dimensional

3D: Three-dimensional

ALDH: Aldehyde dehydrogenase

ASCs: Adult stem cells

ATCs: Ascitic tumor cells

BRD4: Bromodomain containing protein 4

CAFs: Cancer-associated fibroblasts

CA-MSCs: Carcinoma-associated mesenchymal stem/stromal cells

CBP: Carboplatin

cDDP: Cisplatin

CNVs: Copy number variations

CSCs: Cancer stem-like cells

DXR: Doxorubicin

DTX: Docetaxel

EC₅₀: Half maximal effective concentration

ECM: Extracellular matrix

EET: Epithelial-to-endothelial transition

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-to-mesenchymal transition

EOC: Epithelial ovarian cancer

EpCAM: Epithelial cell adhesion molecule

EVs: Extracellular vesicles

FAP: Fibroblast activation protein

GelMA: Gelatin methacryloyl

GEM: Gemcitabine

HGF: Hepatocyte growth factor

HGSOC: High-grade serous ovarian cancer

HIFs: Hypoxia-inducible factors

HRD: Homologous recombination deficiency

HUVECs: Human umbilical vein endothelial cells

IC₅₀: The half-maximal inhibitory concentration value

IL-6: Interleukin 6

iPSCs: Induced pluripotent stem cells
KRT14: Keratin 14
LCs: Leader cells
MDR1: Multidrug resistance gene
MET: Mesenchymal-to-epithelial transition
miR: microRNA
MMT: Mesothelial- to- mesenchymal transition
MUs: Metastatic units
NETs: Neutrophil extracellular traps
NK Cell: Natural killer cell
OC: Ovarian cancer
PANO: Panobinostat
PARP: Poly (ADP-ribose) polymerase
PARPi: PARP inhibitor
PD-1: Programmed cell death protein 1
PD-L1: PD-1/programmed death-ligand 1
PDGF: Platelet-derived growth factor
PDMS: Polydimethylsiloxane
PEG: Polyethylene glycol
PFS: Progression-free survival
PI3K: Phosphoinositide 3-kinase
PLD: PEGylated liposomal doxorubicin
poly-HEMA: Poly-2-hydroxyethyl methacrylate
ROS: Reactive oxygen species
SAHA: Suberoylanilide hydroxamic acid (vorinostat)
SMAC: second mitochondrial activator of caspase
STAT1: Signal transducer and activator of transcription-1
STIE: Systemic tumor immune environment
TAMs: Tumor-associated macrophages
TAX: Paclitaxel
T β RI: TGF- β transmembrane serine/threonine kinase receptor type I
TGF- β : Transforming growth factor beta
TIME: Tumor immune microenvironment
TME: Tumor microenvironment

TNF- α : Tumor necrosis factor alpha

TOP: Topotecan

ULA: Ultra-low adherent

VEGF: Vascular endothelial growth factor

VM: Vasculogenic mimicry

1. Background

1.1. Ovarian Cancer

Ovarian cancer (OC) is a highly aggressive malignancy that is typically identified at an advanced stage, rendering it challenging for treatment. It is the deadliest cancer of the female reproductive system, with a markedly rising incidence and mortality rate (Torre *et al.*, 2015; Bray *et al.*, 2018; Sung *et al.*, 2021). For over 25 years, the standard of care treatment for OC has consisted of cytoreductive surgery and platinum/taxane-based adjuvant chemotherapy (Raja *et al.*, 2012). Although the majority of OC patients initially respond to chemotherapy, 80-90% of patients diagnosed with advanced-stage disease will relapse and develop chemoresistance (Matulonis *et al.*, 2016). OC is a highly heterogeneous disease, with epithelial OC (EOC) accounting for over 90% of diagnoses. EOC is divided into two distinct groups: Type I EOC (endometrioid, clear cell, seromucinous, mucinous, low-grade serous carcinomas, and malignant Brenner tumors) and Type II EOC consisting of high-grade serous ovarian carcinomas (HGSOCs), carcinosarcomas and undifferentiated carcinomas (Kurman & Shih, 2016). HGSOC is responsible for more than 90% of OC deaths, is highly heterogeneous, and can be divided into many subtypes based on its molecular and morphological characteristics (Kurman & Shih, 2016). The main differences between HGSOC and Type I EOC are the rapid and aggressive progression of HGSOC, its high chromosomal instability, ubiquitous *TP53* mutations, and shared defects in the homologous recombination DNA repair pathway ('Integrated genomic analyses of ovarian carcinoma' 2011; Kurman & Shih, 2016; Köbel & Kang, 2021). Because of these fundamental differences, Type I EOCs grow slowly, *in situ*, into well-differentiated carcinomas, whereas HGSOCs proliferate and metastasize rapidly (Lengyel, 2010).

Despite therapy advances, such as the addition of anti-angiogenic drugs and targeted therapies to conventional chemotherapy, the five-year survival rate for patients with HGSOC remains low (Raja *et al.*, 2012; Maru & Hippo, 2019). The main problem with the novel targeted therapies is that they fit only a particular subgroup of patients, most of whom also develop drug resistance. For example, poly (ADP-ribose) polymerase (PARP) inhibitors, such as olaparib (LYNPARZA[®]), niraparib (ZEJULA[®]), and rucaparib (RUBRACA[®]), are intended for those patients with defective homologous recombination DNA repair, primarily carriers of *BRCA1/2* mutations, which is the case in approximately 15% of OC patients (Qin *et al.*, 2022). It is imperative to provide treatments for HGSOC patients who demonstrate proficiency in homologous recombination DNA repair. Additionally, most patients acquire drug resistance upon

prolonged oral administration of PARP inhibitors (Li *et al.*, 2020). To overcome the drug resistance and metastatic capability of OC, it is critical to develop preclinical models that can accurately reflect the biology of OC and can be used to evaluate the efficacy of new therapeutic approaches. Until satisfactory therapeutic strategies emerge, it is also essential to develop adequate patient-derived models as predictors of clinical outcomes of the available drug therapy. Three-dimensional (3D) cell models are the optimal choice for researching OC due to their numerous benefits.

1.2. 3D Models for Studying Ovarian Cancer

Preclinical drug development has generally employed cancer cell lines as models. These models have several shortcomings that limit their usefulness in predicting a drug's effectiveness in clinical trials (reviewed by Gillet *et al.*, 2013). Clinical trials for most drugs tested in cancer cell models frequently fail. This poor translation of preclinical data is a significant problem, and several studies emphasized the inconsistencies in therapeutic response between current *in vitro* and *in vivo* models. For EOC, 3D culturing resulted in a different proliferation rate and sensitivity to chemotherapeutics than 2D culturing of the same OC cells, as well as altered gene and protein expression patterns (Zietarska *et al.*, 2007; Myungjin Lee *et al.*, 2013; Chen *et al.*, 2014). It is rather intuitive to consider the flexible 3D cell culture models better in recapitulating primary tumors, ascitic environment, and metastasis than traditional 2D monolayer cell cultures with forced cellular polarity and cellular interactions. Since OC cells detach from the surface of the primary tumor into the peritoneum, the anchorage-independent 3D cultures and co-cultures of multicellular OC spheroids or organoids with additional cell types from the tumor microenvironment (TME) represent a promising *in vitro* model of the metastasis and drug resistance. 3D models in OC research offer a more faithful portrayal of tumors' intricate biology *in vivo*, including elements such as interactions between cells and the existence of extracellular matrix (ECM), hypoxia, and nutritional gradients (Horst *et al.*, 2021; Ciucci *et al.*, 2022). These characteristics are essential for investigating the molecular mechanisms of drug resistance in OC, which may help identify novel therapeutic targets and, in terms of increased accuracy of preclinical drug research, assess drug efficacy in a more realistic setting. 3D models most often used in studying EOC are spheroids, organoids, and co-culture models. These models have been thoroughly described in recent reviews (Dumont *et al.*, 2019; Collins *et al.*, 2020; Löhmußaar *et al.*, 2020; Semertzidou *et al.*, 2020; Horst *et al.*, 2021; Ciucci *et al.*, 2022; Qin *et al.*, 2022; Yee *et al.*, 2022), and will be briefly described in this section.

Spheroids are 3D cell aggregates generated from established cell lines or patient-derived cells. They mimic the *in vivo* tumor architecture and are frequently used in studies of drug resistance and tumor invasion. Griffon *et al.* in 1995 developed the first 3D spheroid model of EOC and used it to validate the

effects of radiation on patient-derived spheroids, but could not correlate the results with patients' responses *in vivo* because radiotherapy was not included in the majority of patients' therapeutic protocols. However, the study can be considered a milestone in the area of OC therapeutic response prediction due to the successful establishment of patient-derived 3D *in vitro* models with variable radiosensitivity (Griffon *et al.*, 1995). The *in vitro* spheroid model system is the most commonly used approach for studying EOC and its metastasis and chemoresistance, and there are several methods for its establishment, including the hanging drop method, agitation-based methods, ultra-low adherent (ULA) plates, and microfluidics (Yee *et al.*, 2022). Microfluidics chambers are an exciting type of spheroid model for studying EOC, customized to hold tumor tissue or spheroids in place and facilitate microscopic evaluation. Microfluidics "on-chip" models have been used to mimic the specific effects of the TME, e.g., fluid shear stress, and to overcome the limitations of assessing cells within spheroids using techniques that may be destructive or provide insufficient resolution (Yee *et al.*, 2022). **Organoids** are 3D structures that are derived from adult stem cells (ASCs), induced pluripotent stem cells (iPSCs), or cancer stem-like cells (CSCs), which are cultured in ECM-based media and develop multiple cell types (Qin *et al.*, 2022). EOC tumor organoids are more complicated and more expensive to culture than EOC spheroids, as they require specific stem-supporting supplements in the medium and have lower culture establishment and propagation success rates, but both platforms can be used to study the heterogeneity of EOC tumors and are valuable for patient response prediction (Hoffmann *et al.*, 2020; Qin *et al.*, 2022). Spheroids and organoids can be cultured on scaffolds or in scaffold-free systems. Scaffolds can be naturally derived ECM extracts such as Matrigel[®], collagen or alginate gels, or synthetic hydrogels such as polyethylene glycol (PEG) or gelatin methacryloyl (GelMA) gels (Yee *et al.*, 2022). ECM-derived biomaterials are most widely used and considered to provide a physiologically relevant microenvironment for cell–cell and cell–ECM interactions (reviewed by Xing *et al.*, 2020). The development of relevant personalized models is especially important for HGSOC patients suffering from aggressive disease and having only a few therapeutic options available. The 3D co-culture models involve growing EOC spheroids or organoids with other cell types, for instance, fibroblasts or immune cells. These models can be used to study interactions between different cell types in the TME and to test various therapies, including immunotherapies.

In this review, we focus explicitly on the aggressive HGSOC subtype of EOC and its metastasis and chemoresistance explored with the advent of numerous techniques of 3D cell culture, including patient-derived models, which enable the creation of more accurate *in vivo*-like tumor settings. We will disclose how the investigation of each discussed aspect of HGSOC metastasis and chemoresistance profits from specific types of 3D models and describe relevant studies that successfully utilized these

model systems. The review is divided into two chapters, the first one reviewing the characteristics of the stepwise process of HGSOC metastasis and connecting each metastatic feature to novel research performed under 3D culture conditions, including intrinsic properties of cancer cells and collaboration with biochemical and mechanical properties of the TME. The second chapter reviews the innovative research of HGSOC chemoresistance under 3D conditions focusing on drug resistance attributed to CSC properties and the TME-facilitated drug resistance, followed by a discussion on novel research utilizing patient-derived models for therapeutic response prediction. In each chapter, a table is provided summarizing related recent research. We have exclusively concentrated on studies spanning the last five years, considering the fact that 3D modeling of HGSOC is a rapidly expanding field of study with a plethora of emerging research.

2. Metastasis of High-Grade Serous Ovarian Cancer and 3D Cell Models

2.1. Process of Ovarian Cancer Metastasis

HGSOC metastasizes either directly from the primary site or indirectly from the abdominal peritoneum and omentum via the peritoneal fluid to neighboring organs (e.g., colon) (Lengyel, 2010). Although there is evidence of hematogenous and lymphatic metastasis of HGSOC (Coffman *et al.*, 2016; Yue *et al.*, 2019), here we focus on the trans-coelomic route (a route of tumor metastasis across a body cavity, such as the peritoneal cavity in the case of OC), as it is predominant and the most successful. The process of metastasis is sequential, pertaining to various intrinsic properties of cancer cells and signals from the TME (Yeung & Yang, 2017; Uno *et al.*, 2022). The main course of HGSOC peritoneal metastasis development is (i) the shedding of cancer cells into the peritoneal cavity, (ii) the formation of multicellular OC spheroids resulting in (iii) the overcoming of anoikis and the build-up of malignant ascites, followed by (iv) attachment onto the peritoneum or omentum and (v) mesenchymal-to-epithelial transition (MET), which is necessary for (vi) the formation of macrometastases (**Figure 1**) (Yeung & Yang, 2017; Teeuwssen & Fodde, 2019). It is still unknown how the tumor cells detach from the ovary, as single cells or aggregated clusters, with a strong probability of both events. Based on *in vivo* lineage tracing, Al Habyan *et al.* proposed that EOC cells detach from primary tumor sites as either single cells or clusters, with clusters having a survival advantage for being more resistant to anoikis (Al Habyan *et al.*, 2018). A recent study by Micek *et al.* presented an *in vitro* model to generate and separate the spheroids formed through aggregation of single cells or collective detachment to better study the ascitic progression

of HGSOC (Micek *et al.*, 2023). The model involves growing tumor spheroids on a substrate and then detaching them to simulate the process of collective detachment that occurs during metastasis. An interesting finding is that the ECM observed in spheroids formed through collective detachment did not originate from the surrounding environment but instead was produced by the tumor cells after they detached (Micek *et al.*, 2023). This suggests that the production of ECM by tumor spheroids contributes to metastasis by providing a supportive environment for tumor cell detachment and migration (Micek *et al.*, 2023). Recent findings suggest that the multicellular OC spheroids found in ascites are hetero-cellular and consist of tumor and non-malignant cells, such as macrophages, mesothelial cells, fibroblasts, and lymphocytes (Matte *et al.*, 2016; Gao *et al.*, 2019; Uno *et al.*, 2022). These dynamic structures are divided into proliferating, non-proliferating and hypoxic regions and are believed to contain CSCs, which are proposed to drive therapeutic resistance and metastasis (**Figure 1**) (Bapat *et al.*, 2005; Shield *et al.*, 2009; Foster *et al.*, 2013; Matte *et al.*, 2016). The utilization of 3D models enables thorough scrutiny of cellular aggregation, density, and organization in correlation with the potential for metastasis.

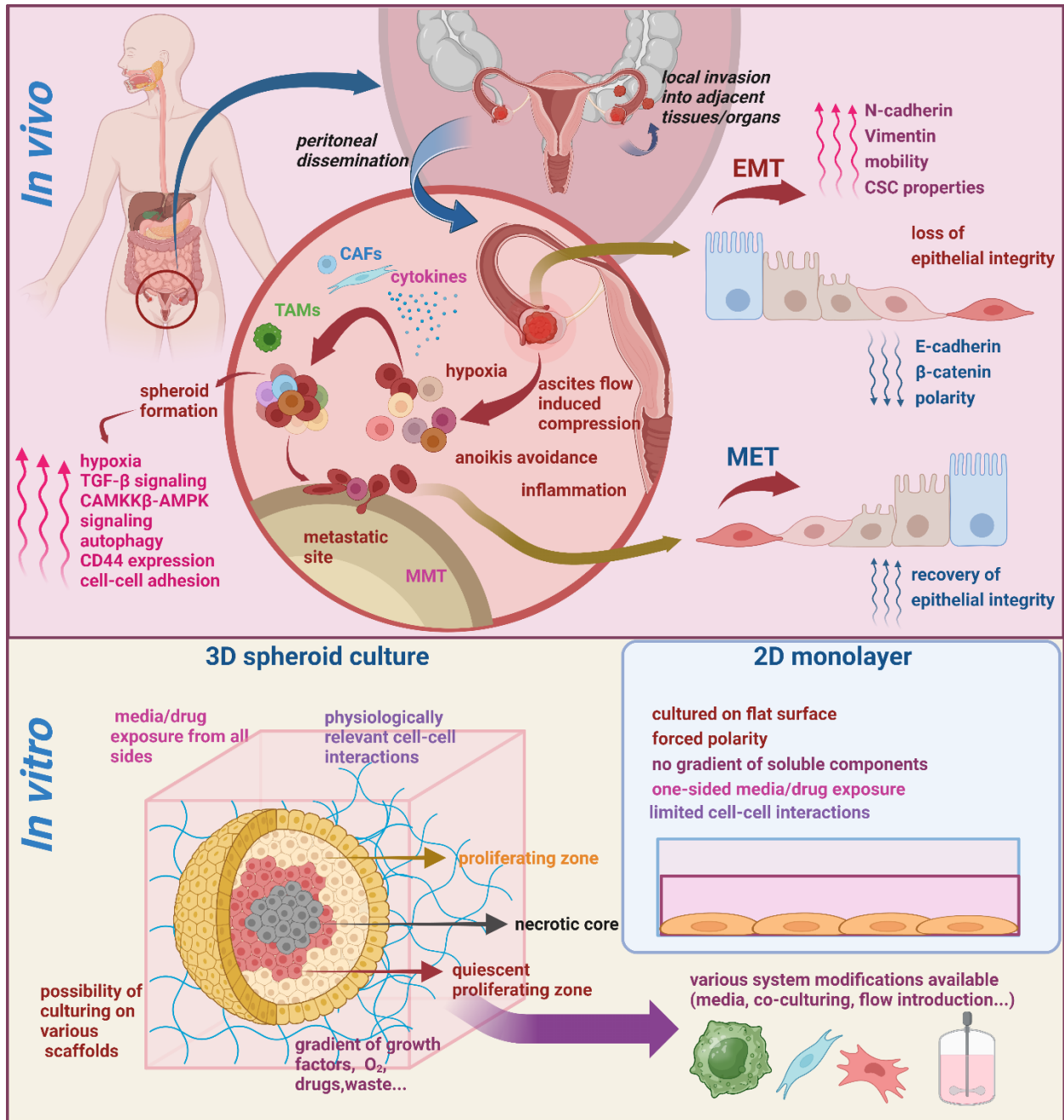


Figure 1. Peritoneal metastasis of HGSOC and basic 2D and 3D *in vitro* HGSOC cell culture models. (*In vivo*)

OC cells from the primary tumor site can invade the surrounding stroma in the process of local invasion, or they can detach and enter the peritoneal cavity in the trans-coelomic route of metastasis. In the local invasion of OC cells, blood and lymph vessels are unnecessary and rarely involved. In the process of peritoneal dissemination, the intravasation step is also omitted, as OC cells shed directly into the peritoneal fluid. Prior to dissemination, tumor cells undergo epithelial-to-mesenchymal transition (EMT), described in section 2.3. The formation of multicellular OC spheroids, which overcome anoikis, and the build-up of malignant ascites are crucial parts of this route of OC metastasis, also known as spheroid-mediated metastasis. Formed spheroids are heterogeneous aggregates of OC

cells, including cancer stem-like cells (CSCs) and cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), mesothelial cells and possibly other non-malignant cells present in ascites which support tumor growth, survival, and immune evasion. The interaction of tumor cells with other cell types is described in section 2.4. These spheroids are implanted on the mesothelial lining of the peritoneum or omentum and undergo a mesenchymal-to-epithelial transition (MET), which is necessary for the local formation of macrometastases. The hypoxic ascitic environment contributes to tumor vascularization, as described in section 2.5. The phenomenon of mesothelial- to- mesenchymal transition (MMT), leading to the conversion of peritoneal mesothelial cells into CAFs and contributing to metastasis, is described in section 2.6. (*In vitro*) The OC 3D spheroid culture is the simplest type of OC 3D model, and even in its simplest (scaffold-free) forms, it is already a better representation of the anchorage-independent growth in ascites than the conventional 2D cell culture of OC cells. Due to the physiologically relevant cell-cell interactions, aggregation capacities, 3D drug exposure, gradient components, and cellular organization, the OC 3D spheroid culture is a much better model for drug screening and investigation of multicellular drug resistance than 2D cell culture. Modifications and additional complexity in the forms of various scaffolds, media components, flow introduction, co-culturing with CAFs and/or TAMs, etc., can be applied to the 3D system for more precise/physiologically relevant OC modeling. Created with BioRender.

As we continue to describe the intricate microenvironment of malignant ascites and features mediating peritoneal metastasis (**Figure 1**), we highlight some of the most intriguing recent studies utilizing 3D models to explore different aspects of the HGSOC metastatic process, with a more extensive summary presented in **Table 1**.

Table 1. Recent studies utilizing 3D models to investigate the HGSOC metastatic process.

Investigated metastatic phenomena	Type of 3D system utilized	Main finding/model contribution	Reference
OC cell adhesion to the omentum; metastatic outgrowth; hematogenous dissemination	3D multicellular human omentum tissue model (OVCAR-8 HGSOC cells, MeT-5A mesothelial cells, MRC-5 fibroblasts, human acute monocytic leukemia cell line THP-1, EA.hy926 endothelial cells, patient-derived adipocytes, patient-derived ascites)	A novel approach to mimicking spatial distribution of five omental cell types	<i>Estermann et al. 2023</i>
Adhesive, migratory, and invasive behavior of patient-derived HGSOC cells from different disease stages and the effect of cytostatic doses	3D organotypic model composed of fibroblasts embedded in collagen I and topped with a monolayer of mesothelial cells (WI38 fibroblasts,	Cells capable of growing spontaneously as spheroids attach to a 3D organotypic model system when pre-incubated with conditioned media;	<i>Ritch et al. 2022</i>

of mifepristone on metastatic capabilities	LP9 mesothelial cells, patient-derived HGSOC cells)	mifepristone was able to cause spheroid dissociation	
Extracellular vesicles; Vasculogenic mimicry	Spheroids of CABA I cell line generated by the hanging drop method	Generated spheroids resemble <i>in vivo</i> tumors in the aspects of “inner” extracellular vesicles and tube-like structures typical of vasculogenic mimicry	<i>Giusti et al. 2022</i>
Stromal cell effects on OC cell attachment and growth; OC cell effects on vascular and mesothelial permeability in models of both early- and late-stage peritoneal metastases	Vascularized model of peritoneal omentum and OC tumor microenvironment; “Omentum-on-a-chip”	Critical cell density requirement for tumor growth in the vascularized peritoneum, enhanced by stromal adipocytes and endothelial cells; tumor growth resulted in both a physically mediated decrease and cytokine-mediated increase in microvascular permeability	<i>Ibrahim et al. 2022</i>
Characterization of tumor-infiltrating lymphocyte populations from primary OC tissue samples; evaluation of cancer immunotherapy efficacy	OC tumor model composed of patient-derived microtumors and autologous tumor-infiltrating lymphocytes	Characterization of patient-individual immune phenotypes and the assessment of responses towards immunotherapy; results generated within 3–4 weeks	<i>Anderle et al. 2022</i>
Tumor cell adhesion; the effect of tumor cells on endothelial cell-to-cell contacts	3D tissue model of the human peritoneum based on a decellularized porcine small intestinal submucosa scaffold populated with human dermal fibroblasts, LP-9 cells representing the peritoneal mesothelium and HUVECs mimicking the endothelial cell layer	An initial evaluation of the novel 3D tissue model for studying peritoneal dissemination	<i>Herbert et al. 2022</i>
CSC phenotype; connection of mesothelin expression and CSC characteristics	Spheroids of HGSOC cell lines (OVCAR-3, OVCAR-8, OVCAR-4 and BG1) cultured in round-bottom plates coated with poly-HEMA (poly(2-hydroxyethyl methacrylate)	Aggressive HGSOC behavior associated with elevated mesothelin expression is not due to mesothelin supporting the CSC phenotype	<i>Nunes et al. 2022</i>
EMT; investigation of Wnt5A association with the TGFβ1/Smad2/3 and	Spheroids derived from EOC cell lines (SK-OV-3, OVCAR-3, and CAOV-	Wnt5A is required for TGFβ1-induced migration and invasion of OC cells;	<i>Dehghani-Ghobadi et al. 2022</i>

Hippo-YAP1/TAZ-TEAD pathways implicated in EMT	4) cultured in plates coated with 1% low melt agarose	Wnt5A may be a functional predeterminant of EMT, supporting mesothelial cell activation and retraction, leading to the establishment of the first metastatic colony on the omentum/peritoneum	
Heterogeneity of metastatic HGSOc; the propagation potential of individual cells at a functional and molecular level	3D single cell-derived metastatic OC spheroids (cells from metastatic peritoneal ascites of 9 patients)	A novel method for isolation and culturing of single cells directly from patients' metastatic ascites, propagating them as 3D cultures referred to as single cell-derived metastatic OC spheroids; these spheroids retain and amplify key subpopulations from the patients' samples and recapitulate features of the original metastasis	<i>Velletri et al. 2021</i>
Tumor microenvironment; immune landscape	Customizable 3D perfused bioreactor system; patient-derived tumor specimens, independent OC cells (OVCAR-8, SK-OV-3), and OC cells co-cultured with CAFs (IHFOT-208)	A novel 3D system that can be customized by co-culturing different cell types and maintaining the immune landscape in patient-derived samples	<i>Martinez et al. 2021</i>
OC cell-tumor microenvironment interactions; effects of β -escalin on OC dissemination	3D quantitative high-throughput screening platform - a multilayered culture system containing primary human fibroblasts, mesothelial cells, and ECM; 6 HGSOc cell lines (CaOV3, OVCAR-4, OVKATE, Kuramochi, Snu-119, and TYK-nu)	The natural compound β -escalin has a therapeutic potential by targeting both cancer and stromal cells in the OC tumor microenvironment	<i>Kenny et al. 2021</i>
OC cell-tumor microenvironment interactions; matrisome; the effect of specific ECM protein composition and ECM stiffness on HGSOc chemoresistance	Collagen-rich 3D hydrogel cultures and laminin-rich 3D hydrogel cultures; patient-derived tumor tissue and ascitic fluid; HGSOc cell lines (OVCAR-3, OVCAR-4, OVCAR-8 and TYK-nu)	Matrix adhesion as an adaptive response, driving HGSOc aggressiveness via co-evolving extracellular matrix biochemical structure and biomechanical properties	<i>Pietilä et al. 2021</i>
Interactional dynamics of neutrophils and growing	Microfluidics-integrated 3D Tumor-immune	Neutrophils respond to the growing tumor	<i>Surendran et al. 2021</i>

tumor aggregates; the effect of neutrophils on the initiation of collective 3D invasion of OC cells	microenvironment-on-Chip system; OVCAR-3 HGSOC cell line	spheroids through both chemotaxis and generation of neutrophil extracellular traps; the formation of NETs stimulated OC cells from their aggregated state to collectively invade into the surrounding collagen matrix	
Interaction of CSCs with carcinoma-associated mesenchymal stem/stromal cells (CA-MSCs)	A model of ovarian malignant ascites in the 3D hanging drop heterospheroid array with CSCs and carcinoma-associated mesenchymal stem/stromal cells; patient-derived OC CSCs and CA-MSCs	Platelet-derived growth factor signaling increases stemness, metastatic potential, and chemoresistance of CSCs in heterospheroids	<i>Raghavan et al. 2020</i>
MicroRNA-200 family involvement in ovarian inclusion cyst formation and migration of OC cells	3D spheroid cultures in growth factor-reduced Matrigel-coated chamber slide wells and 3D spheroids in sodium hydroxide-neutralized collagen I solution; patient-derived tissue and normal human OSE and FTE primary cultures	OC spheroids with miR-200 knockdown showed changes like elevated TGF- β expression and mitotic spindle abnormalities and switched from collective to single-cell migration; migration mode probably changed due to altered TGF- β /ROCK and SRC signaling, which regulate myosin II phosphorylation	<i>Choi et al. 2020</i>
Interaction of CSCs with macrophages; immunosuppressive phenotype, chemoresistance, invasiveness, stemness	A hanging-drop heterospheroid model to bring CSCs and macrophages in close association; OVCAR-3 HGSOC cell line, CD68 ⁺ macrophages (derived from U937 or peripheral blood monocytes)	A reciprocal interaction that drives pro-tumoral macrophage activation and CSC self-renewal, CSC-derived WNT ligands drive CD206 ⁺ M2 macrophage activation, macrophage-derived WNT ligands enriched ALDH ⁺ CSC cells within hetero-spheroids	<i>Raghavan et al. 2019</i>
Interactions between non-malignant and malignant cells and the extracellular matrix of the peritoneum	Pre-cultured polycaprolactone scaffolds with mesothelial cells assembled with OC cell-seeded hydrogels; mesothelial cells Met-5A, OV-MZ-6 cells, patient-derived	Mesothelial cell microenvironment increased OC cell proliferation	<i>Loessner et al. 2019</i>

	primary HGSOC cells		
Viability and histopathological features of patient-derived OC explants propagated in agitation-based system	Long-term OC patient-derived explant cultures in orbital agitation	Successful culture of eight subtypes of OC with high cell viability levels and original tumor phenotype for at least 30 days	<i>Abreu et al. 2020</i>
Effects of reactive oxygen species on macrophage activation; interactions between OC cells, macrophages and CD3 ⁺ T cells	OC spheroids in 1% agarose substrate in the cover glass bottom of 48-well plates with subsequently (after OC spheroid formation) added macrophages; human EOC cell lines (A2780, OVCAR-3, SKOV-3), murine OC cell line ID8, human peripheral blood monocytes THP-1, PBMCs	Reactive oxygen species induce down-regulation of exosomal miR-155-5p, by which tumors modulate the tumor-promoting microenvironment through down-regulation of PD-L1 and other immunosuppressive factors	<i>Li et al. 2022</i>
The effect of ascitic fluid shear stress, mechanosensitive miR-199a-3p, and hepatocyte growth factor on OC stemness and chemoresistance	OC spheroids in a microfluidic chip; SKOV-3, A2780 and HEY A8 cells	Shear stress in the presence of hepatocyte growth factor activated specific c-Met/PI3K/Akt signaling axis through a positive feedback loop, driving OC stemness and drug resistance; miR-199a-3p expression correlates with enhanced drug resistance properties in chemoresistant OC lines	<i>Hassan et al. 2022</i>
Interaction of OC cells and tumor-associated macrophages in tumor immune microenvironment	Tumor microspheres from ascites of an untreated OC patient in 3D microfluidic chips (AIM Biotech 3-D Cell Culture Chip); murine colon carcinoma cell line CT26, leukemia cells in mouse macrophage RAW264.7, human HGSOC cell line OVCAR-4, murine OC cell line ID8, and human monocyte cell line THP-1	Bromodomain containing protein 4 inhibitor AZD5153 sensitizes OC to anti-PD-L1 therapy by changing the phenotype of tumor-associated macrophages and promoting pro-inflammatory cytokine secretion	<i>Li et al. 2020</i>
The effect of passive flow on the growth of OC organoids	A passive microfluidic platform (Mimetas 2-lane OrganoPlate [®] and medium perfusion provided by OrganoFlow [®] platform) with 3D patient-derived	Microfluidic technology outperformed static conditions in HGSOC organoid growth and the established organoids captured the histological	<i>Cavarzerani et al. 2023</i>

	ascitic OC organoids grown in Cultrex RGF BME®	characteristics of the primary tumors	
Collective detachment of HGSOC spheroids; ECM expression of spheroids in ascites	A 3D model to generate and separate spheroids formed through single cells that detached and aggregated and spheroids formed through collective detachment; OV-90, OVCAR-3, and OVCAR-8 cell lines	Fibronectin in spheroids enhances adhesion to mesothelial cells	<i>Micek et al. 2023</i>
CSC enrichment and chemoresistance	A novel engineered serially passaged 3D spheroid platform, spheroids generated on the hanging drop array; OVCAR-3 cells	CSCs enriched in serially passaged spheroids; spheroids resistant to cisplatin and sensitive to ALDH inhibitor; later passage spheroids exhibit higher tumorigenicity <i>in vivo</i> than early passage spheroids	<i>Ward Rashidi et al. 2019</i>

OC: ovarian cancer; HGSOC: high-grade serous ovarian cancer; EOC: epithelial ovarian cancer; ECM: extracellular matrix; CSC: cancer stem-like cell; ALDH: aldehyde dehydrogenase; CAFs: cancer-associated fibroblasts; TAMs: tumor-associated macrophages; PD-L1: programmed death-ligand 1; HUVEC: human umbilical vein endothelial cells; poly-HEMA: poly(2-hydroxyethyl methacrylate); NETs: neutrophil extracellular traps; OSE: ovarian surface epithelium; FTE: fallopian tube epithelium; PBMCs: peripheral blood mononuclear cells

2.2. Mimicking the Malignant Ascites Spreading

Most women diagnosed with HGSOC already have peritoneal metastasis at diagnosis (Lisio *et al.*, 2019). An excessive amount of peritoneal fluid containing cancer cells, immune cells, and various tumor-promoting soluble factors is called malignant ascites, and it provides an easy method of dissemination for HGSOC, as tumor cells shed from primary sites straight into the peritoneal cavity without any physical barrier (Ahmed & Stenvers, 2013). Malignant ascites is present in over one-third of all OC patients at initial diagnosis and in almost all cases of disease recurrence (Ford *et al.*, 2020). The accumulation of malignant ascites occurs due to peritoneal lymphatic drainage obstruction and enhanced peritoneal vascular permeability (Asem *et al.*, 2020). In addition to offering a liquid substrate filled with tumor-promoting cellular and molecular components, malignant ascites promotes metastasis mechanically through intraperitoneal compression (Asem *et al.*, 2020). Ascites-induced intraperitoneal compression increases cell adhesion to the peritoneum and induces remodeling of the peritoneal mesothelial cell surface ultrastructure via induction of tunneling nanotubes mediating mesothelial cell interactions with

tumor cells, transport of mitochondria from mesothelial cells to tumor cells and remodeling of peritoneal collagen fibers to support invasion (Asem *et al.*, 2020). Such mechanical effects of ascites on HGSOc metastasis and treatment failure have been understudied but can be elegantly investigated in 3D models incorporating flow in so-called perfusion models.

Flow-induced shear stress modulates metastasis by inducing changes in cancer morphologic, genetic, and protein profiles, including EOC (Rizvi *et al.*, 2013; Polacheck *et al.*, 2014). A recent study by Cavarzerani *et al.* developed a passive microfluidic platform for 3D culturing of HGSOc organoids in conditions mimicking ascitic flow and without ECM disruption (Cavarzerani *et al.*, 2023). Organoids were derived from either HGSOc ascites or chemo-naïve tumors and cultured in Cultrex BME from R&D Systems. Clusters of organoids were plated onto Mimetas 2-lane OrganoPlate® composed of 96 independent tissue culture chips with medium perfusion secured by gravity through the microfluidic channels of the OrganoFlow® platform from the same company. This model enabled faster growth of HGSOc organoids with higher viability in comparison with static 3D culture. Interestingly, the half-maximal inhibitory concentration (IC₅₀) values of carboplatin (CBP), paclitaxel (TAX), and doxorubicin (DXR) were lower in the passive flow system than in static conditions (Cavarzerani *et al.*, 2023). The authors discussed that the higher proliferation rate of the dynamic culture could be attributed to higher efficiencies in supplying nutrients and oxygen to organoid cores and removing cellular waste exerted by the flow. Yet, the flow could also be responsible for the lower IC₅₀ values of chemotherapeutics due to deeper penetration into organoid cores in comparison with static cultures.

Hassan and colleagues investigated the effects of ascitic fluid shear stress and specific cytokines and growth factors typically present in malignant ascites on the stemness properties of OC spheroids (Hassan *et al.*, 2022). They used a microfluidic chip coated with poly-2-hydroxyethyl methacrylate (poly-HEMA) to prevent cell attachment to the glass slide and matrix deposition. They noticed that tumor spheroids were significantly more viable under flow than in static culture. The expression of microRNA (miR) miR-199a-3p and its coding gene miR-199a-1 were significantly down-regulated by shear stress and by the addition of hepatocyte growth factor (HGF), and their strong synergistic effect was also observed. The authors further showed that shear stress can activate c-Met, an HGF receptor, and the PI3K/Akt signaling pathway, whereas c-Met and PI3K/Akt inhibition could annul the down-regulation of miR-199a-3p and miR-199a-1 under shear stress. The c-Met/PI3K/Akt/miR-199a-3p may form a positive feedback loop, as overexpression of miR-199a-3p can downregulate c-Met expression and Akt phosphorylation (Hassan *et al.*, 2022). Down-regulation of miR-199a-3p resulted in the formation of more spheroids under CSC-selective conditions, and its overexpression reduced spheroid formation. This

study provokes an interesting idea of fluid shear stress-induced increase in the CSC population, which may significantly contribute to the emergence of chemoresistance in the late stages of OC.

2.3. Mimicking Spreading by Epithelial-to-Mesenchymal Transition

Prior to dissemination, tumor cells undergo epithelial-to-mesenchymal transition (EMT), a series of cellular events known to be important in embryogenesis, wound healing, and malignant progression (Dongre & Weinberg, 2019). The process of EMT results in the loss of cell polarity and cell-cell adhesion, the acquisition of a mesenchymal phenotype, and an increase in cellular migratory capacity (Yang *et al.*, 2020). The critical event is the switching of epithelial marker epithelial (E-) cadherin expression with mesenchymal marker neural (N-) cadherin (Yang *et al.*, 2020). The role of EMT in HGSOC progression is unclear and somewhat controversial, as metastatic tumor cells still express E-cadherin, and most of the EMT phenomenon in EOC has only been revealed *in vitro* (Mei *et al.*, 2023). However, one of the hallmarks of EOCs is the heterogeneous cadherin expression, with a mixed cadherin phenotype in tumors expressing both E- and N-cadherin and a hybrid cadherin phenotype in single cells expressing both E- and N-cadherin (Yang *et al.*, 2020; Kralj *et al.*, 2022). Both circulating tumor cells and multicellular aggregates in ascites demonstrated heterogeneous cadherin expression profiles, with mesenchymal-like cells expressing N-cadherin invading much more efficiently and pointing toward the importance of EMT for successful peritoneal metastasis (Klymenko *et al.*, 2017).

The EMT has been generally associated with the acquisition of stemness properties in cancer cell populations and with acquired chemoresistance (Mani *et al.*, 2008; Papadaki *et al.*, 2014; Diepenbruck & Christofori, 2016; Yeung & Yang, 2017; Loret *et al.*, 2019; Pastushenko & Blanpain, 2019). The relationship between EMT and acquired stemness properties of cancer cells is not fully mechanistically explained. However, the altered protein expression resulting from the EMT program may activate autocrine signaling loops, which leads to the development of the CSC phenotype (Shibue & Weinberg, 2017). The pro-inflammatory and hypoxic TME drives EMT, with various immune cells and cancer-associated fibroblasts (CAFs) utilizing signaling pathways that induce transcription factors associated with EMT initiation, such as transforming growth factor beta (TGF- β), tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and hypoxia-inducible factors (HIFs) (Shibue & Weinberg, 2017). The implications of EMT in tumor progression and metastasis, as well as the interconnection of EMT and acquired stem-like properties, have been discussed in detail in many recent comprehensive reviews (Derynck & Weinberg, 2019; Dongre & Weinberg, 2019; Bakir *et al.*, 2020; Erin *et al.*, 2020; Yang *et al.*, 2020; Bayik & Lathia, 2021; Brabletz *et al.*, 2021; De Las Rivas *et al.*, 2021; Motohara *et al.*, 2021; Huang *et al.*, 2022), and are not the focus of this review. We are interested in the

exploration of EMT and CSCs in 3D models of HGSOC, for which we highlight several exciting studies and provide additional, the most recent studies in **Table 1**.

Rafehi *et al.* investigated the role of TGF- β signaling in regulating epithelial-mesenchymal plasticity in ascites-derived spheroids (Rafehi *et al.*, 2016). Due to the previously mentioned plasticity of EMT states in HGSOC and the known EMT-inducing role of TGF- β (Zavadil & Böttinger, 2005), they hypothesized that modulation of TGF- β signaling would significantly impact EMT and the malignant potential of spheroids. They used an *in vitro* 3D culture system where primary ascites-derived human EOC cells were maintained in suspension on ULA plates for three days, followed by re-introduction to standard tissue culture-treated plates, re-attachment, and dispersion. The involvement of TGF- β inhibitors seriously hindered the EMT process. The TGF- β transmembrane serine/threonine kinase receptor type I (T β RI) inhibitor, SB-431542, blocked the endogenous EMT in spheroids, and treatment with SB-431542 upon spheroid re-attachment decreased cellular motility and migration, boosting the epithelial phenotype (Rafehi *et al.*, 2016). This suggests that targeting TGF- β signaling may be a promising approach for preventing or slowing the spread of HGSOC. Ward Rashidi and colleagues developed an integrated approach to create an *in vitro* model of stemness and chemoresistance in OC, utilizing the 3D hanging drop spheroid model established by serial passaging of non-adherent spheroids (Ward Rashidi *et al.*, 2019). Spheroids were generated from the HGSOC OVCAR-3 cell line and patient-derived HGSOC abdominal metastases. They observed an increase in cellular proliferation, an enrichment of CSCs, and the parallel development of platinum resistance in the model. *In vivo*, tumor xenograft assessments revealed that spheroids from later passages had significantly greater tumorigenicity and more CSCs than those from earlier passages (Ward Rashidi *et al.*, 2019). What is particularly interesting in this study is that in contrast to the designed 3D model, under the same experimental conditions, serial passaging in the 2D model did not yield the same results, confirming the postulate that the CSC phenotype is unstable and impossible to be maintained in 2D monolayer models (Ward Rashidi *et al.*, 2019).

2.4. Mimicking the Interaction of Tumor Cells with Other Types of Cells Involved in Metastasis

The tumor stroma consists of the ECM and various cell types, such as CAFs, endothelial cells, immune cells, and adipocytes. In OC, the tumor stroma accounts for a significant portion of the tumor tissue, ranging from 7% to 83%, with a median of 50% (Labiche *et al.*, 2010). As previously stated, malignant ascites comprises a diverse array of cellular and acellular components. In addition to tumor cells expressing epithelial cell adhesion molecule (EpCAM), CAFs expressing fibroblast activation

protein (FAP), immune cells (CD45⁺), and endothelial cells (CD31⁺) are the main cell types in ascites (Erez *et al.*, 2010). Gao *et al.* investigated the aggressive nature of HGSOC ascitic tumor cells (ATCs) characterized by high integrin $\alpha 5$ (ITGA5^{high}) levels, which are prone to forming heterotypic spheroids with fibroblasts. They used the term metastatic units (MUs) for these heterotypic spheroids (Gao *et al.*, 2019). In these MUs, CAFs recruit ITGA5^{high} ATCs and maintain ATC ITGA5 expression by continuous secretion of epidermal growth factor (EGF) (Gao *et al.*, 2019). Their analysis identified fibroblasts (EpCAM⁻CD45⁻CD31⁻FAP⁺) as the cellular component showing the most considerable difference between HGSOC and low-grade serous carcinoma ascites, indicating that ascitic fibroblasts direct ATC behavior and HGSOC progression (Gao *et al.*, 2019). They established a suspension co-culture of SK-OV-3 cells and primary CAFs and found the presence of tumor cells in the form of single cells, homotypic (SK-OV-3 only) spheroids, and heterotypic spheroids made of SK-OV-3 cells and CAFs. The heterotypic spheroids displayed the most potent adhesive capacity (Gao *et al.*, 2019).

The tumor immune microenvironment (TIME) is a term specifically used to describe groups of immune cells and their interactions in the TME niche, and it needs to be systematically studied in search of successful immunotherapies (Binnewies *et al.*, 2018). The systemic tumor immune environment (STIE), the comprehensive immunity of the host, or the macro-environment of the patient's anti-tumor immunity, in coordination with the TIME, determines the patient's response to immunotherapy (Hiam-Galvez *et al.*, 2021). The OC TIME contains various immune cells, including macrophages, natural killer (NK) cells, regulatory T-cells, dendritic cells, CD8⁺ and CD4⁺ T-cells, B-cells, and myeloid-derived suppressor cells (Izar *et al.*, 2020). Surendran and colleagues developed a novel microfluidics-integrated 3D TIME-on-Chip model for studying the effect of neutrophils on the initiation of collective 3D invasion of HGSOC OVCAR-3 cells (Surendran *et al.*, 2021). They cultivated tumor spheroids on hydrogel-based multi-microwell plates immersed within a collagen matrix of defined thickness and then magnetically attached them to a microfluidic channel system made on a porous membrane containing neutrophils. Designed channels replicated the vascular structures surrounding the tumor, and the layer of collagen between tumor spheroids and channels simulated the tumor stroma. This TIME *in vitro* system modeled dynamic neutrophil migration and 3D tumor invasion, and it demonstrated the migration of neutrophils towards tumor spheroids via chemotaxis and the generation of neutrophil extracellular traps (NETs) (Surendran *et al.*, 2021). This sophisticated 3D model demonstrated how, when exposed to chemotactic stimuli from the tumor, neutrophils may extravasate from the blood vessels into the tumor tissue. OVCAR-3 cells migrated collectively upon down-regulation of E-cadherin, especially under stimulation with IL-8 and TGF- β , both secreted by activated neutrophils *in vivo*. They observed different signaling within neutrophils under flow and non-flow conditions, suggesting that fluid flow activates neutrophils

differently and could affect their tumor response (Surendran *et al.*, 2021). NETs are known to play an active role in metastasis and are found in the omentum of OC patients. This study demonstrated that tumor-associated inflammatory factors by themselves trigger naive neutrophils to produce NETs. Stromal NETs seem to excite the collective invasion of the tumor cells into the tissue and the expansion of the aggregated cell colonies (Surendran *et al.*, 2021). This is another essential complexity within T(I)ME, validated with a novel 3D system.

Tumor-associated macrophages (TAMs) are another significant fraction of cells within the ascitic microenvironment and are implicated in the recurrence and metastasis of OC. WNT signaling pathway has been shown to play a critical role in OC tumorigenesis (Barbolina *et al.*, 2011). Depending on the signals received from the TME, macrophages can display anti-tumorigenic (M1) or pro-tumorigenic (M2) phenotypes by secreting different cytokines. M1 macrophages secrete pro-inflammatory cytokines, which upregulate the local immune response, and M2 macrophages secrete anti-inflammatory cytokines, such as IL-10 and TGF- β , promoting immunosuppression and tumor progression (Malyshev & Malyshev, 2015). A recent study utilizing engineered 3D models demonstrated that HGSOC CSCs and ascitic macrophages reciprocally interact through the WNT pathway to promote pro-tumoral and malignant phenotypes (Raghavan *et al.*, 2019). Raghavan *et al.* used the hanging drop spheroid model to bring HGSOC CSCs and macrophages within heterotypic spheroids *in vitro*, simulating their presence within malignant ascites (Raghavan *et al.*, 2019). They found that HGSOC CSCs drove the up-regulation of the M2 macrophage marker CD206 within these heterotypic spheroids, resulting in immuno-suppressive properties. These heterotypic spheroids were also more resistant to CBP and demonstrated higher invasiveness as a result of an interesting loop – aldehyde dehydrogenase (ALDH) expressed by HGSOC CSCs led to the initiation of pro-tumoral cytokine IL-6 signaling by M2 macrophages, which secured and increased ALDH maintenance in HGSOC CSCs (Raghavan *et al.*, 2019). This implies that targeting the WNT pathway may be a potential therapeutic strategy for HGSOC treatment, elegantly demonstrating how 3D modeling of malignant ascites provides invaluable insights into the complex biology of HGSOC metastasis. The HGSOC spheroids and entire malignant ascites are enthralling in the context of metastasis and the development of therapeutic resistance as they form a unique interactive environment essential for the complexity and aggressiveness of HGSOC. An exciting thought is that the hypoxic and anoikis-promoting conditions in ascites drive selective pressure and allow only the fittest/most aggressive malignant cells to survive, and there are many findings correlating the formation of spheroids and the accumulation of ascites with the increasing invasiveness and chemoresistance (Cai *et al.*, 2015; Gao *et al.*, 2019; Uno *et al.*, 2022).

Apart from the previously mentioned fluid shear stress, several other biomechanical mechanisms may affect HGSOC metastasis, such as cellular elasticity, compressive and tensile stress on the cells around the tumor, and substrate stiffness stress (Sun *et al.*, 2022). Cells in the TME affect disease progression by signaling and ECM remodeling. Natarajan and colleagues demonstrated the tumor-promoting effects of collagen remodeling by mesothelial and tumor cells in the peritoneal microenvironment (Natarajan *et al.*, 2019). Hypoxic signaling promoted extracellular collagen fiber deposition by mesothelial cells and invasion of HGSOC cells in tumor-mesothelial cell 2D co-cultures (Natarajan *et al.*, 2019). The role of ECM remodeling in the metastasis of HGSOC could be better understood by studying the "mechanobiology" of the TME using scaffold-based 3D models (Winkler *et al.*, 2020). Pietilä and colleagues identified the biochemical and biomechanical properties of the ECM as crucial in determining HGSOC cell survival and resistance to chemotherapy (Pietilä *et al.*, 2021). They investigated the chemotherapy-induced alterations in the tumor stroma and the effect of the observed changes on HGSOC chemoresistance and relapse, finding that the tumor matrix co-evolved with cancer cells during disease progression (Pietilä *et al.*, 2021). They found that both matrix stiffness and specific protein structure affect the HGSOC platinum response (Pietilä *et al.*, 2021). Analysis of comprehensive changes in the ECM could aid in the early detection of cancers without available early diagnosis methods, such as HGSOC. Apart from early detection, specific ECM signatures could provide predictive value, as demonstrated for several cancer types (Winkler *et al.*, 2020).

2.5. Mimicking Tumor Vascularization

The capacity to invade and metastasize is inextricably linked to the development of a new vascular system for supplying oxygen and nutrients to the growing tumor mass, where new blood vessels originate from both endothelial and tumor cells themselves. (Ayala-Domínguez *et al.*, 2019; Wei *et al.*, 2021). Tumor vascularization is crucial for tumor growth and metastasis and the development of therapeutic resistance (van Beijnum *et al.*, 2015). In metastatic OC, in the presence of constantly active VEGF signaling in hypoxic tumor tissue, a phenomenon of vasculogenic mimicry (VM) has been observed (Ayala-Domínguez *et al.*, 2019; Lim *et al.*, 2020; Shibuya, 2008). VM implies the appearance of permeable vascular channels formed by tumor cells themselves, through which nutrients, oxygen, and soluble substances are delivered to the tumor, independent of classical angiogenesis and endothelial cells (Ayala-Domínguez *et al.*, 2019; Lim *et al.*, 2020; Wei *et al.*, 2021). Although anti-angiogenic therapies targeting VEGF have shown promising results in the treatment of some metastatic tumors, such drugs generally have only a modest impact on overall patient survival, and alternative mechanisms of tumor

vascularization, such as VM, are likely to represent a path by which tumors overcome therapy (Carbone *et al.*, 2011; Wei *et al.*, 2021; Xu *et al.*, 2012). The development of VM is associated with a pronounced hypoxic microenvironment, the presence of CSCs, the process of epithelial-to-endothelial transition (EET), and remodeling of the ECM (Fernández-Cortés *et al.*, 2019; Wei *et al.*, 2021). Several studies have shown that highly hypoxic conditions in the TME selected CSCs, promoting the expression of molecules associated with CSC characteristics such as OCT4, SOX2, and NANOG through HIF-1 α signaling (Pietras *et al.*, 2009; Mathieu *et al.*, 2011; Filatova *et al.*, 2016). Determining the causal relationship between the development of CSC characteristics and VM is difficult. However, it has been shown that the presence of CSCs, recognizable by the surface antigen CD133, promoted VM in several different types of tumors, including OC (Chiao *et al.*, 2011; Liang *et al.*, 2016; Wang *et al.*, 2016). The mechanisms of activation and maintenance of VM are still insufficiently investigated, even the process itself is still somewhat questionable in many tumors, and it is possible to investigate these exclusively in 3D culture conditions (Fernández-Cortés *et al.*, 2019; Valdivia *et al.*, 2019). In HGSOE, VM is associated with poor prognosis and poor response to anti-angiogenic therapy (Liang *et al.*, 2016; Xu *et al.*, 2012). Molecular pathways behind VM are considered attractive new therapeutic targets (Ge & Luo, 2018), and 3D models will be indispensable for future studies. Recently developed methods now model the formation of 3D cellular networks over Matrigel in OC cells *in vitro*, representing the early stages of VM (Salinas-Vera *et al.*, 2022).

2.6. Importance of Extracellular Vesicles

The phenomenon of mesothelial- to- mesenchymal transition (MMT) leading to the conversion of peritoneal mesothelial cells into CAFs has also been reported and must be considered in the complexity of peritoneal metastasis (Rynne-Vidal *et al.*, 2017). MMT is induced by TGF- β (Matte *et al.*, 2014), commonly found in ascites, and it is another excellent example of the complex interplay within a tumor-promoting ascitic environment. Another aspect worth considering in the complex ascitic environment is extracellular vesicles (EVs). It is still unclear how exosomes produced by MMT-derived CAFs can predict peritoneal tumor progression or therapeutic response in patients with advanced OC, but EVs have been shown to participate in the transmission of information between stromal cells and immune cells in the TME (reviewed by Steinbichler *et al.*, 2019). Giusti *et al.* showed that EVs can induce a CAF-like state in fibroblasts, promoting their proliferation, motility, and invasiveness (Giusti *et al.*, 2022). EVs are implicated in creating the peritoneal pre-metastatic niche by remodeling the ECM and promoting tumor cell adhesion to mesothelial cells. EVs secreted by OC cells carry proteins related to tumor progression,

such as L1CAM, CD24, ADAM10, and EMMPRIN, and proteins regulating cancer signaling through ACTN4, CD44, and type-IV collagen (Tian *et al.*, 2022). These EVs have been found to inhibit the activation of dendritic cells, induce the polarization of macrophages, inhibit the cytotoxicity of NK cells, and regulate the function of T-cells (Tian *et al.*, 2022). EVs have also been found to induce angiogenesis by promoting the proliferation and migration of endothelial cells, and the combination of these effects ultimately promoted immune escape and facilitated metastasis (Tian *et al.*, 2022). Giusti *et al.* confirmed that EOC spheroids formed by the hanging drop method experienced features characteristic of *in vivo* tumors, such as inner entrapped EVs and VM (Giusti *et al.*, 2022). Investigating phenomena such as MMT and EVs in 3D models is a promising direction for future HGSOC research and could lead to a better understanding of disease progression mechanisms. EVs can be helpful in diagnosis, prognosis, therapy selection, and monitoring of therapeutic response.

3. Chemoresistance of High-Grade Serous Ovarian Cancer and 3D Cell Models

3.1. Treatment of High-Grade Serous Ovarian Cancer

High-grade serous ovarian cancer is primarily treated with surgical staging, maximal debulking, and systemic chemotherapy (Armstrong *et al.*, 2021). Patients with advanced-stage HGSOC, who, due to age, poor performance, comorbidities, or the exhaustive cancer burden, can undergo neoadjuvant chemotherapy with interval (between cycles of chemotherapy) debulking surgery. This approach has been shown to be effective in improving overall survival rates in such patients (Armstrong *et al.*, 2021). Since the 1970s, platinum-based drugs have been used as the standard treatment for ovarian cancer, with cisplatin (cDDP) followed by carboplatin-based combinations (Raja *et al.*, 2012). Intravenous administration of carboplatin and paclitaxel is now considered the mainstay of first-line treatment for HGSOC, even for stage 1 (Armstrong *et al.*, 2021). Other options include a combination of carboplatin with docetaxel (DTX) and carboplatin with pegylated liposomal doxorubicin (PDL), each having similar efficiency but carrying a different set of specific toxicities, so they may be an option for patients with specific risk profiles (Armstrong *et al.*, 2021).

After the initial chemotherapy treatment, patients should undergo regular clinical re-evaluation. Patients with persistent or progressing disease during initial treatment should immediately receive second-line treatments. Patients who achieve complete or partial remission have various treatment options based

on the extent of their response and the primary chemotherapy received. Recent trials have shown that maintenance therapy after postoperative platinum-based chemotherapy can have a positive impact on progression-free survival (PFS) in patients with advanced disease. As a result, the integration of maintenance therapy as part of postoperative management is becoming increasingly prevalent and important (Armstrong *et al.*, 2021). Maintenance therapy aims to prolong PFS and preserve the quality of life in patients with advanced disease. Maintenance therapy following primary chemotherapy includes the use of bevacizumab and PARP inhibitors (PARPi). Olaparib, rucaparib, and niraparib are PARPi used as maintenance monotherapy for recurrent disease, while olaparib, niraparib, and olaparib with bevacizumab are used for maintenance therapy after response to first-line chemotherapy (Armstrong *et al.*, 2021). Patients with advanced-stage HGSOC have a high chance of cancer recurrence, which is usually incurable. Recurrent HGSOC is most frequently detected by increased levels of CA125 followed by imaging, as patients are asymptomatic at the time (Matulonis *et al.*, 2016). Recurrent disease is typically categorized as platinum-sensitive or platinum-resistant based on the patient's response to platinum-based chemotherapy. Patients with recurrent platinum-sensitive disease are generally treated again with platinum-based chemotherapy, such as CBP with TAX, CBP with PDL, and CBP with gemcitabine (GEM), having a response rate of around 50%, with shorter progression-free intervals with each subsequent platinum administration (Matulonis *et al.*, 2016). Patients with platinum-resistant recurrent disease are treated with TAX, PDL, GEM, etoposide, vinorelbine, or topotecan (TOP), with or without bevacizumab (Matulonis *et al.*, 2016).

As research results and new clinical trials emerge, the guidelines for optimal care will continue to be updated.

3.2. Mechanisms of Action of Drugs Approved for High-Grade Serous Ovarian Cancer Treatment

Drugs used for the treatment of HGSOC employ different mechanisms of anti-cancer activity. Due to differences in the drugs' mechanisms of action, HGSOC cells employ different strategies for resisting specific treatments. Platinum-based drugs, such as CBP and cDDP, bind to DNA to create crosslinks and induce apoptosis. CBP is a second-generation platinum agent, having a similar efficacy as cDDP but resulting in lower systemic toxicity (Zhang *et al.*, 2022). TAX and DTX are taxanes, chemotherapeutics naturally occurring in plants, and are considered mitotic inhibitors. Taxanes bind to microtubules and stabilize them, inhibiting their depolymerization and resulting in cell cycle arrest and subsequent apoptosis. TAX is also associated with activation of pro-apoptotic signaling pathways, angiogenic inhibitory activity, and induction of oxidative stress (Kampan *et al.*, 2015). Doxorubicin

(DXR) primarily inhibits DNA topoisomerase II, stabilizing it in a complex with DNA and stalling the cell cycle in the S-phase. DXR-induced topoisomerase II-DNA complexes can also induce DNA double-strand breaks, and DXR has been associated with increasing oxidative stress (Gabizon *et al.*, 2016). In PDL, the polyethylene glycol (PEG) coating on the liposome increases the longevity of the liposome and reduces toxicity compared to free DXR. The PARPi are intended for treating patients with BRCA1/2 mutations, thus harboring a homologous DNA recombination repair defect. Poly (ADP-ribose) polymerases (PARPs), especially PARP1, PARP2, and PARP3, are crucial to base excision DNA repair, the mechanism of DNA single-strand breaks repair. The rationale behind the PARPi strategy is the synergetic lethality (or “synthetic lethality”), with the inactivation of both BRCA and PARP resulting in a replication catastrophe and cell death (Li *et al.*, 2020). However, more than 40% of BRCA1/2-deficient patients who receive PARPi do not respond, and those who do eventually develop resistance (Li *et al.*, 2020). Interestingly, there is recent evidence that patients with homologous DNA recombination repair defects, excluding BRCA mutations, are also sensitive to PARPi (Li *et al.*, 2020). As previously mentioned, bevacizumab is a VEGF-A-targeting monoclonal antibody used as an angiogenesis inhibitor (Garcia *et al.*, 2020). TOP blocks the action of the enzyme topoisomerase I, which initiates the DNA relaxation by cleaving one strand, impacting replication and resulting in double-strand breaks (Thomas & Pommier, 2019). Similarly, etoposide inhibits the action of topoisomerase II (Bailly, 2023). GEM, a pyrimidine antagonist, inhibits DNA synthesis by targeting cells in S-phase (Berg *et al.*, 2019). Vinorelbine is a semisynthetic vinca alkaloid that inhibits mitosis by binding to tubulin and inhibiting microtubule assembly, preventing metaphasic cell division (Dhyani *et al.*, 2022). The subsequent sections will explore how novel 3D cell models of HGSOC are utilized to investigate resistance to the described approved drug treatments, which rely on different mechanisms of action.

3.3. Mimicking Multicellular Drug Resistance in 3D High-Grade Serous Ovarian Cancer Cell Models

Understanding the biology of HGSOC chemoresistance is crucial for overcoming the frequent recurrence in patients and improving survival rates. Chemoresistance of HGSOC can occur as a result of genetic alterations, altered signaling pathways, and changes in the TME, primarily enhanced DNA repair mechanisms, reduced drug uptake, increased drug efflux, activation of pro-survival signaling pathways, autophagy, and quiescence (McMullen *et al.*, 2021). Additionally, previously discussed processes of EMT and the acquisition of stem-like properties are interconnected with the development of drug resistance (Pan *et al.*, 2021). Known mechanisms of HGSOC chemoresistance are extensively reviewed elsewhere

(Liu *et al.*, 2020; Marchetti *et al.*, 2021; McMullen *et al.*, 2021; Ortiz *et al.*, 2022) and fall beyond the scope of this review. Here, we focus on investigating chemoresistance in 3D conditions and the benefits brought to the field by recent 3D models.

The idea of 3D multicellular drug resistance mechanisms is not new; there are studies published 30-40 years ago suggesting that the acquired anti-cancer drug resistance could result from the response of a cell population and that such responses can only be observed *in vitro* in 3D culture conditions which allow cells to aggregate (Sutherland & Durand, 1976; Kobayashi *et al.*, 1993). Cell-cell and cell-ECM interactions can be explored in the context of drug resistance in 3D spheroid models (**Figure 2**). As spheroids grow, a gradient of oxygen is established, similar to the process of tumor growth *in vivo* (Riffle & Hegde, 2017). Tumor cells in the external layer of the tumor tissue have access to oxygen, unlike those within the inner layer, which experience hypoxic conditions. This effect can be observed between the outer, proliferating layer of multicellular spheroids and the inner, quiescent viable cell zone and the necrotic core. Cells in the hypoxic microenvironment upregulate the expression of HIF family factors, opening the door for several drug resistance mechanisms indicated in **Figure 2**. Several mechanisms can be invoked to explain the effects of hypoxia on increased drug resistance; (i) up-regulation of HIF and its downstream targets (up-regulation of the multidrug resistance 1 gene, *MDRI*, and several anti-apoptotic factors and *VEGF*); (ii) inability of reactive oxygen species (ROS) formation due to lack of oxygen (which is the primary mechanism of action for many anti-cancer drugs); (iii) decrease in cell damage mediated by radiation due to limited ROS formation; (iv) acidification of the microenvironment due to metabolic reprogramming (the inhibition of oxidative phosphorylation) and lactate production by hypoxic cells which encumbers drug stability and uptake; (v) induction of a non-proliferative and quiescent state resulting in low efficacy of DNA-damaging chemotherapeutics (such as CBP) relying on rapid division of tumor cells; (vi) promotion of vascularization incurred by HIF-mediated ECM remodeling; and (vii) HIF-mediated ECM remodeling creating physical barriers (**Figure 2**) (reviewed by Riffle & Hegde, 2017; Nunes *et al.*, 2019; Winkler *et al.*, 2020). Previously described CSCs, implicated in both metastasis and chemoresistance, have been found in patient-derived spheroids, as demonstrated by Hirst *et al.*, who detected the CSC-associated markers aldehyde dehydrogenase 1A (*ALDH1A*) and CD133 and the increased expression of HIF-regulated genes in the spheroid core (**Figure 2**) (Hirst *et al.*, 2018). The dependence of drug resistance mechanisms on CSC properties can be explained by (i) increased *ALDH* levels, resulting in increased drug metabolism; (ii) improved DNA damage repair; (iii) increased expression of efflux transporters; (iv) EMT and (v) dormancy (**Figure 2**) (Hirst *et al.*, 2018; Nunes *et al.*, 2019). Autophagy, the process of breaking down and recycling components within cells to replenish energy and essential compounds, has also been implicated in the drug resistance of OC (Wang *et al.*,

2018; Škubník *et al.*, 2023). The induction of autophagy has been observed within HGSOC spheroids, as well as an increase in phosphorylated adenosine monophosphate-activated kinase (AMPK) and the autophagy marker LC3-II during spheroid formation (**Figure 2**) (Laski *et al.*, 2020). Giusti *et al.* observed the formation of entrapped EVs in EOC spheroids, which are known to contribute to drug resistance *in vivo* (**Figure 2**) (Giusti *et al.*, 2022; Steinbichler *et al.*, 2019). As mentioned previously, in the context of information transmission between OC cells and stroma in promoting metastasis, EVs also play an important role in OC drug resistance. A study by Dorayappan and colleagues demonstrated how exosomes contributed to a more aggressive and chemoresistant OC phenotype (Dorayappan *et al.*, 2018). They confirmed that hypoxic OC cells significantly increased their exosome release by up-regulating Ras-related protein Rab27a, down-regulating Ras-related protein Rab7, lysosomal-associated membrane proteins -1 and -2, LAMP1/2, neuraminidase 1, NEU-1, and also by promoting a more secretory lysosomal phenotype (Dorayappan *et al.*, 2018). Released EVs contributed to cDDP resistance by spreading the oncogenic transcription factor STAT3 (Dorayappan *et al.*, 2018). Many of the previously mentioned tumor-promoting synergistic effects of multiple cell types collaborating within heterotypic spheroids can also be investigated in the context of drug resistance in 3D models (**Figure 2**).

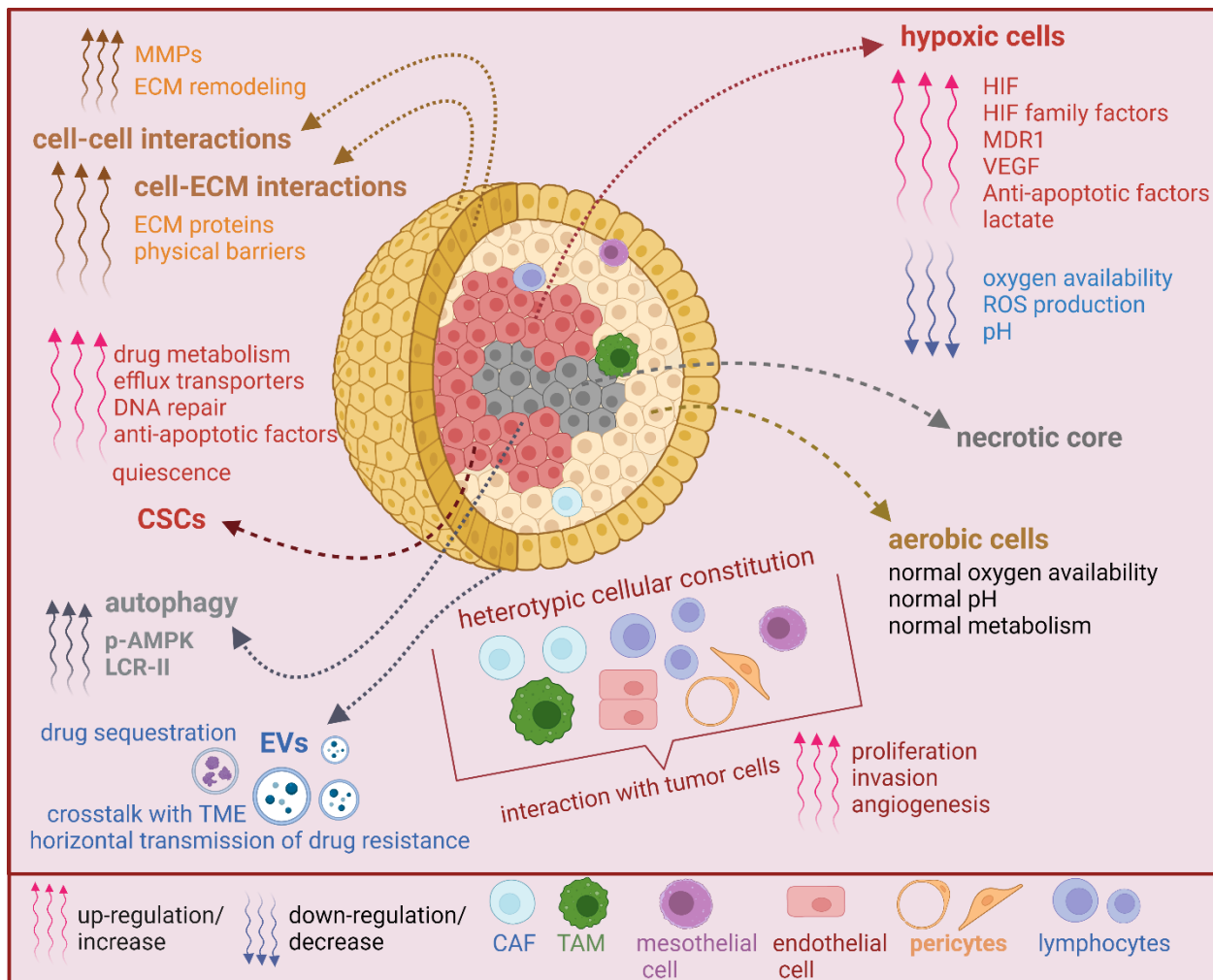


Figure 2. Mechanisms of drug resistance in 3D HGSOC spheroid model. The scheme indicates the molecular mechanisms of therapeutic resistance that can be established within 3D multicellular spheroids due to their 3D organization and flexibility in the TME. These mechanisms can result from cell-cell interactions and cell-ECM interactions, hypoxic conditions and a gradient of oxygen within the spheroid, CSC properties of inner cells, and heterotypic constitution of the spheroids in co-culture models. Different mechanisms are associated with resistance to drugs used for HGSOC treatment (the mechanisms of action described in section 3.2.), and several of them have been investigated in 3D cell models so far. Reduced drug uptake, inhibition of apoptosis-related pathways, solidification of DNA repair pathways, and metabolic reprogramming have been associated with platinum resistance. Reversion mutations and epigenetic and pharmacological modifications have been associated with PARPi resistance. ECM affects the TAX absorption of tumor cells, possibly explaining the reduced efficiency of TAX in 3D culture compared to 2D culture of the same cells. TAX resistance is associated with inhibition of apoptosis, vasculogenic mimicry, and reduction of ROS production. DXR is triggering oxidative stress, so the oxygen availability within 3D structures also affects its efficacy. Vasculogenic mimicry observed and investigated in 3D HGSOC models directly encumbers using bevacizumab as anti-angiogenic therapy. Inhibition of apoptosis and molecular mechanisms attributed to CSCs are shared by all of the above-mentioned drugs. Created with BioRender.

As stated in the introduction and the beginning of this section, for more than 25 years, the standard treatment for OC has been cytoreductive surgery and adjuvant chemotherapy based on platinum and taxane drugs (Raja *et al.*, 2012), and this has not changed since. It is important to note that currently, there are no biomarkers for EOC that have been accepted as predictors of first-line therapeutic response in the clinical setting. Platinum sensitivity is still the standard for predicting clinical prognosis (Wilson *et al.*, 2017). Platinum resistance is defined as the lack of response or relapse within six months of platinum-based chemotherapy (Wilson *et al.*, 2017; D'Amora *et al.*, 2021). Generating patient-specific indications of first-line chemotherapy response through 3D platforms in a clinically relevant time frame prior to treatment can be the best option for treatment decisions for both clinicians and patients. Upon validation in more extensive, independent cohorts, patient-derived OC spheroids and organoids hold the solid potential to become a new standard in treatment decision-making in oncology with substantial clinical value. Once authenticated and standardized, these platforms should be incorporated as a follow-up part of biopsy/debulking surgeries for newly diagnosed OC patients. Numerous recent studies have demonstrated the usefulness of 3D models for investigating the drug resistance of HGSOC (Table 2).

Table 2. Recent studies utilizing 3D models for investigation of HGSOC chemoresistance.

Investigated drug response	3D system utilized	Main finding	Reference
cDDP, Mirin	3D spheroids of platinum-sensitive and resistant OC cell lines in ULA plates; A2780, A2780cis (platinum-resistant), PE01, PE04 (BRCA2-proficient, platinum-resistant)	Mre11 depletion by gene knockdown or blockade by small molecule inhibitor (Mirin) reversed platinum resistance in OC cells and in 3D spheroid models; Mre11 inhibition was synthetically lethal in platinum-sensitive XRCC1 deficient OC cells and 3D-spheroids	<i>Alblihy et al.2022</i>
CBP, TAX, GEM, TOP, etoposide, vinorelbine, treosulfan, PLD, CBP + TAX, CBP + GEM, CBP + PLD, CBP + DTX, olaparib, erlotinib, and SAHA	Patient-derived OC spheroids	Recurrent OC characterized by high variability in druggable target expression and drug response profiling in analyzed patient cohort; interpatient tumor heterogeneity modeled on the patient-derived OC spheroid model	<i>Hoffman et al. 2022</i>
TAX, TOP, the effect of <i>ALDH1A</i> knockout on TAX- and TOP-sensitivity	3D spheroids generated in ULA plates from OC cell lines (parental W1 cell line, W1PR1 cell line resistant to TAX, W1TR cell line resistant to TOP, and <i>ALDH1A</i> knockout cell lines	The same cells were more resistant in 3D than in the 2D culture; the level of resistance in 2D culture correlated to the expression level of drug transporters; the key determinant of resistance in 3D spheroids seemed to be the presence of different cell	<i>Nowacka et al. 2022</i>

	W1PR1-C7 and W1TR-1p17)	zones, the cellular density of spheroids, and the capacity of drug diffusion into the cellular/ECM structure	
cDDP, LY-294002 (a PI3K-AKT dual kinase inhibitor), cDDP + LY-294002	3D spheroids of OC cell lines (OVCAR-4, HeyA8, SK-OV-3, A2780 and its cDDP-resistant derivative, patient-derived MCW-OSE-1, MCW-OV-SL-3 and its cDDP-resistant derivative)	Blocking of PI3K-AKT signaling by PI3K inhibitor LY294002 sensitized cells to cDDP and inhibited the cell colony formation, migration, and 3D morphogenesis in cDDP-sensitive and cDDP-resistant cell lines	<i>Parashar et al. 2022</i>
CBP, Birinapant (a bivalent SMAC mimetic compound) + CBP	3D organoid bioassay (OC cells grown as organoids embedded within Matrigel) for a panel of 7 EOC cell lines (OVCAR-3, OVCAR-4, OVCAR-8, SK-OV-3, CaOV3, Kuramochi and OAW28) and 10 platinum-resistant primary patient tumor samples	Organoid bioassay-predicted results correlated with reported platinum sensitivities for each cell line and with primary tumor samples; combining SMAC mimetics with CBP could target OCs	<i>Singh et al. 2022</i>
cDDP, methadone, cDDP + methadone	Four patient-derived HGSOc tumor-spheroid models; samples cultured in a custom-constructed, fully 3D-printed, micro-processor-controlled bioreactor system	Although it showed some apoptotic and chemosensitizing effects in preclinical tumor models, methadone demonstrated no benefits as a chemosensitizing drug in OC patients; harmful effects of methadone have been identified	<i>Fiegl et al. 2022</i>
TAX, cDDP, cDDP + TAX, PANO, cDDP + TAX + PANO, SAHA	3D spheroids generated by hanging drop method from OVCAR-3 and CaOV3 cell lines,	3D-derived OC cells demonstrated induced proliferation, migration, invasion, cancer colony formation and chemoresistance properties after a single exposure to classic chemotherapy; a combination of classic chemotherapy with epigenetic therapy was most effective for OVCAR-3 cells	<i>Bilbao et al. 2021</i>
CBP	3D spheroids generated in ULA plates from OC cell lines (OV90, OV4485, OV4453, TOV21G, TOV112D and OV1946)	Comparison of 2D monolayers, spheroids, <i>ex vivo</i> tumors, and <i>in vivo</i> models; CBP sensitivity of 2D OC cell cultures differed from the <i>in vivo</i> response; the response of 3D spheroids better correlated with the <i>in vivo</i> response than 2D cultures	<i>Brodeur et al. 2021</i>
CBP	3D spheroids from four OC cell lines (OV90, TOV3041G, TOV112D, OV866) generated by different methods: PDMS-based microfluidic systems,	The most effective 3D spheroid forming methods were microfluidic chips and Matrigel-assisted ULA plates; regardless of the spheroid forming method, CBP sensitivity inversions were noted within a given	<i>Patra et al. 2020</i>

	ultra-low attachment (ULA) plates in the presence or not of 2% Matrigel, and hanging droplets	cell line between 2D and 3D cultures	
TAX, CBP, GEM, DXR, TOP, olaparib	Primary patient tissue in 3D spheroid culture in ULA plates	Validation and initial prospective clinical validation of developed system to accurately predict patient-specific response to first-line chemotherapy in newly diagnosed OC patients prior to treatment initiation	<i>Shuford et al. 2019</i>
CBP, TAX, additional compounds combined with CBP or TAX	Patient-derived organoids (of various OC subtypes)	CBP was significantly more effective in serous than in clear cell subtypes; two lines of organoids were generated, an additional 1135 drugs were screened, and drugs with better combinatory effects with CBP than with TAX were found; certain drugs demonstrated an additive effect with CBP	<i>Ito et al. 2023</i>
cDDP	Patient-derived OC organoids (primarily HGSOC)	Metabolic signatures (measured by quantitative mass spectrometry) predicted clinical outcomes following CBP + TAX chemotherapy; specific amino acid and lipid profiles characterized a state of tumor cellular quiescence associated with immune dysfunction	<i>D'Amora et al. 2021</i>
CBP	Patient-derived HGSOC organoids	Developed six HGSOC organoid lines from tissue obtained during debulking surgery; organoid line, predicted to be CBP-resistant, correlated with a significantly shorter PFS than the rest of the subjects	<i>Gorski et al. 2021</i>
cDDP, CBP, TAX, DTX, TOP, GEM, DXR, SAHA, etoposide, olaparib, tamoxifen, eribulin, vinorelbine, trabectedin, belinostat, cediranib, pazopanib, sunitinib, everolimus, trametinib, gefitinib, lapatinib, SN-38	Patient-derived organoids (3 HGSOC, one clear cell, three endometrioid)	Developed organoids captured the characteristics of histological cancer subtypes and replicated the mutational landscape of the primary tumors; CNVs were also similar among organoids and primary tumors; HGSOC organoids with HRD shared a drug sensitivity pattern different from that of non-HRD like HGSOC organoid line which showed resistance to most of the tested drugs, except trabectedin	<i>Nanki et al. 2020</i>
CBP, TAX	Whole-genome-characterized patient-derived organoids from HGSOC patients with known clinical	Developed organoids displayed inter- and intra-patient drug response heterogeneity; genetic aberrations can partly explain drug response	<i>De Witte et al. 2020</i>

	histories	heterogeneity	
CBP, TAX, olaparib, abraxane, mocetinostat, trametinib, LY294002, AZD5363, BBI503, MK-1775, sorafenib, APR-246, CB5083, napabucasin	Short-term spheroid cultures from HGSOC malignant effusions in conditions selected to support organoid growth	Developed spheroid cultures can recapitulate the histological features of malignant ascites fluid and can be expanded for at least six days; within six days of culture, significant up-regulation of genes related to cellular proliferation, EMT, and KRAS signaling was observed	<i>Chen et al. 2020</i>
Evaluation of cancer immunotherapy efficacy	OC tumor model composed of patient-derived microtumors and autologous tumor-infiltrating lymphocytes	Characterization of patient-individual immune phenotypes and the assessment of responses towards immunotherapy; results generated within 3–4 weeks	<i>Anderle et al. 2022</i>
The effects of size and shape of the OC spheroids on drug resistance and migration; TAX, cDDP	Spheroids derived from HGSOC cell lines OVCAR-3 and OVCAR-8	Characterization of two distinct spheroid structures: loose aggregates (OVCAR-3) and compact spheroids (OVCAR-8); no differences observed in resistance to TAX and cDDP as a function of spheroid size and shape; migration capacity of compact spheroid 15-fold higher compared with that of loose aggregates	<i>Gunay et al. 2020</i>
The effect of STAT1 signaling on stemness properties in chemoresistant EOC cells; TAX	2D and 3D cultures, and tumor xenografts; spheroids of TAX-sensitive HGSOC cell line OVCAR-3 and of TAX-resistant subline OV3R-PTX	Possible regulatory mechanism of STAT1 underlying drug resistance; potential therapeutic application for EOC patients with TAX-resistant disease	<i>Wang et al. 2020</i>
Characterization of chemoresistant leader cell (LC) subpopulation within OCs; olaparib, rucaparib, TAX, TOP, CBP, cyclophosphamide, cDDP and DXR	3D spheroids derived from HGSOC and clear cell carcinoma OC cell lines (SK-OV-3, COV362.4, and OVCAR-4) generated in ULA plates; patient-derived primary cells from ascites and primary or metastatic tumors	LCs represent a transcriptionally plastic subpopulation arising independently of cell division or DNA replication, with a “stemness” profile that does not correlate with EMT, expressing chemoresistance markers ALDH1, Twist, and CD44v6; functional impairment of LCs restored chemosensitivity	<i>Karimnia et al. 2019</i>
CBP response in heterotypic spheroids	The 3D hanging drop heterospheroid array, with HGSOC CSCs and carcinoma-associated mesenchymal stem/stromal cells (CA-MSC); OVCAR-3 HGSOC cell line, human adipose-derived mesenchymal cells, patient-derived HGSOC samples	Platelet-derived growth factor (PDGF) signaling from CA-MSCs increased platinum resistance of heterospheroids; inhibition of PDGF signaling reduced platinum resistance and metastatic potential in OC CSCs	<i>Raghavan et al. 2020</i>

<p>Genomic characterization of patient-derived organoids, intratumoral heterogeneity, drug response; TAX, oxaliplatin, DXR, GEM, and 8 targeted compounds provided by AstraZeneca</p>	<p>HGSOC organoids established from patient-derived ascites (43), solid tumors (10), and patient-derived xenografts (15)</p>	<p>Analysis of CNVs and mutational signatures showed that HGSOC patient-derived organoids recapitulate the broad mutational landscape of patient samples; comparison of drug sensitivity between five organoids and their parental uncultured patient-ascites revealed a moderate to a high correlation between the drug area under the curve (AUC) of patient-derived organoids and their corresponding patient-derived ascites</p>	<p><i>Vias et al. 2023</i></p>
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CBP: carboplatin; cDDP: cisplatin; TAX: paclitaxel; TOP: topotecan; GEM: gemcitabine; DTX: docetaxel; DXR: doxorubicin; PLD: PEGylated liposomal doxorubicin; SMAC: second mitochondrial activator of caspase; PANO: panobinostat; SAHA: suberoylanilide hydroxamic acid (vorinostat); PDMS: polydimethylsiloxane; PI3K: phosphoinositide 3-kinase; CNVs: copy number variations; LCs: leader cells; HRD: homologous recombination deficiency; STAT1: signal transducer and activator of transcription-1

3.4. Measuring Drug Resistance in 3D High-Grade Serous Ovarian Cancer Cell Models

Several methods and approaches can be used to measure numerous endpoints in studies utilizing 3D HGSOC models, depending on the type of 3D model used, cultivation method and, of course, specific research questions. Generally, many commercial kits for measuring cell viability, cell proliferation, and cell death apply to 3D cell cultures, where attention should be paid to spheroid/organoid size, density, and morphology. Studies in **Table 2** used various methods to measure 3D cell cultures' growth, viability, proliferation, migration, invasion, apoptosis and necrosis. Sensitivity toward all approved treatments for HGSOC described in section **3.1.**, as well as the investigation of multiple drug candidates, has been measured in 3D HGSOC models in recent studies and summarized in **Table 2**. For example, Alblihy and colleagues investigated the platinum resistance of spheroids grown from platinum-sensitive and platinum-resistant cells in ULA plates. After cDDP treatment, they used a LIVE/DEAD Viability/Cytotoxicity Kit from Thermo Fisher Scientific to determine cellular viability, along with measurements of spheroid diameters in every treatment condition (Alblihy *et al.*, 2022). Hoffman *et al.* used a patient-derived recurrent OC spheroid model from 30 tumor samples to test the effects of platinum- and nonplatinum-based guideline-recommended therapies, as well as several drugs pending approval. For this purpose, they used a CellTiter-Glo® Luminescent Cell Viability Assay from Promega (Hoffman *et al.*, 2022). A study by Nowacka *et al.* utilized an MTT assay and phase imaging for assessing HGSOC spheroid viability following treatment with TAX and TOP (Nowacka *et al.*, 2022). Fiegl and colleagues monitored the size and morphology of spheroids cultured from ascites and tumor specimens from HGSOC patients by live-cell microscopy (Fiegl *et al.*, 2022). They used propidium iodide staining of 3D cultures to visualize cell death and CellTiter-Glo® Luminescent Cell Viability Assay from Promega to measure cell viability.

Also, they applied a human enzyme-linked immunosorbent assay (ELISA) kit to determine VEGF concentrations in both 2D and 3D cultures (Fiegl *et al.*, 2022). Bilbao and colleagues used the Invitrogen™ eBioscience™ Annexin V-FITC Apoptosis Detection Kit from Thermo Fisher Scientific to detect apoptosis and the Invitrogen™ eBioscience™ Propidium Iodide Staining Solution from the same manufacturer to detect necrosis in 3D cultures of Caov-3 and OVCAR-3 spheroids established by hanging drop method (Bilbao *et al.*, 2022). Brodeur *et al.* used flow cytometry and LIVE-DEAD™ Fixable Aqua Dead Cell Stain Kit from Thermo Fisher Scientific and immunohistochemistry with anti-Ki-67 and anti-cleaved caspase-3 antibodies to analyze the extent of apoptosis in 3D spheroids derived from cell lines (Brodeur *et al.*, 2021). Sheta and colleagues developed a 3D system for measuring sensitivity toward PARPi olaparib and niraparib in spheroids grown in agarose from ascitic HGSOc patient-derived cells (Sheta *et al.*, 2020). They measured treatment-induced cytotoxicity in 2D and 3D cultures of the same patients' cells and observed a generally stronger response to both PARPi in 2D, with niraparib being overall more cytotoxic than olaparib in both conditions. The Perfecta3D cell viability kit from Sigma Aldrich was used for measuring sensitivity towards PARPi in spheroids. They measured the γ H2AX foci formation as a marker of the homologous DNA repair pathway activity in PARPi-sensitive and resistant cultures. By comparing the gene expression patterns of PARPi-resistant and PARPi-sensitive 3D cultures, they identified 24 potential biomarkers of PARPi sensitivity, for seven of which the differential expression was subsequently confirmed in matched tumor samples. This study demonstrates how 3D models can identify predictive biomarkers for PARPi treatment (Sheta *et al.*, 2020).

3.5. 3D Models Used for Investigating Drug Resistance Mechanisms Attributed to Cancer Stem-like Cell Properties

Many cancers have been found to contain self-renewing tumorigenic CSCs with different expression profiles and phenotypes, implying that CSC-targeted therapies may not be equally effective for all types of cancer (Hirst *et al.*, 2018; Horst *et al.*, 2021). These differences likely occur due to the interactions of CSCs with other cell types in specific TMEs. Various mechanisms of CSC resistance to treatment (indicated in **Figure 2**) may be exacerbated by CSC population heterogeneity, thus increasing the likelihood of resistant variant outgrowth. Chemotherapy was associated with the induction of CSC-like traits and the migration of OC cells (Zhao *et al.*, 2020). Hirst *et al.* generated spheroids from multiple OC cell lines, most of them HGSOc, and demonstrated that the multilayered 3D architecture of spheroids comprised zones of variable gene expression, with an increase in hypoxia-regulated genes and markers of stemness in the spheroid core, which cannot be observed in monolayered cells and drives phenotypic

changes (Hirst *et al.*, 2018). The 3D spheroids expressed the CSC-associated markers *ALDH1A* and *CD133* and exhibited hypoxia and increased resistance to TAX. They investigated the effects of clinically repurposed drugs on 2D cultured cells and spheroids in search of successful candidates in 3D conditions and found that the cyclooxygenase/lipoxygenase inhibitor licofelone reversed the CSC characteristics in spheroids (Hirst *et al.*, 2018). The idea of clinical evaluation of licofelone combined with TAX-based chemotherapy resurging from this study is a good example of how utilization of spheroid culture can lead to improved chemotherapeutic response and overcoming of acquired chemoresistance (Hirst *et al.*, 2018). Wang *et al.* investigated the effect of the signal transducer and activator of transcription-1 (STAT1) signaling on stemness properties in TAX-resistant HGSOc cells (Wang *et al.*, 2020). They used the TAX-resistant cell line OV3R-PTX derived from TAX-sensitive OVCAR-3 cells and cultured them in 2D and 3D conditions. Interestingly, OV3R-PTX cells grew slower than parental cells in both 2D and 3D cultures, with OV3R-PTX spheroids having smaller micro-spheres than OVCAR-3 spheroids, but OV3R-PTX grew faster in tumor xenografts. The authors attributed this effect to the existence of heterogeneous cells in the OV3R-PTX population containing CSCs and isolated a single-cell clone that shared the characteristics of CSCs. The CSC characteristics in the monoclonal cell line were increased in the spheroids compared with the monolayer culture (Wang *et al.*, 2020). The monoclonal TAX-resistant cell line demonstrated a higher spheroid formation capacity and larger micro-spheres than the parental OVCAR-3 cell line. These findings imply that rapid tumor growth *in vivo* is a consequence of the extensive outgrowth of CSCs (Wang *et al.*, 2020). The expression of CD44, CD133, and OCT4 was higher in the monoclonal TAX-resistant cell line than in parental cells, while the STAT1 levels were significantly lower. The repression of STAT1 in TAX-resistant cells was attributed to DNA hypermethylation of the *STAT1* promoter region (Wang *et al.*, 2020). This study proposes a new therapeutic strategy for TAX-resistant patients by highlighting the correlation among strong TAX resistance, enhanced stemness markers, and down-regulation of STAT1 using 3D modeling (Wang *et al.*, 2020).

Resurfacing CSCs following chemotherapy can be studied to understand the evolution and genetics of remaining clonal populations. After various drug treatments, analyses of these remaining CSC populations can be used to identify therapeutic targets in clonal chemoresistant populations. Karimnia and colleagues investigated so-called leader cells (LCs) in the context of chemoresistance in HGSOc spheroids formed in ULA microplates (Karimnia *et al.*, 2021). LCs were characterized by keratin-14 (KRT14) expression, transcriptional plasticity, and a CSC-like phenotype but did not represent a quiescent population (Bilandzic *et al.*, 2019; Moffitt *et al.*, 2019; Karimnia *et al.*, 2021). Increased expression of KRT14 has been associated with worse progression-free survival (PFS) and a lack of

therapeutic response in OC patients (Bilandzic *et al.*, 2019). The LC sub-population is considered to drive the collective invasion in metastatic epithelial cancers by rearranging their actin filaments, facilitating membrane protrusion, and forming actin-rich "invadopodia", which direct migration and lead the packed "follower" cells. CAFs are also implicated as essential mediators of collective invasion by physically remodeling the TME to help invading cancer cells (Moffitt *et al.*, 2019; Giusti *et al.*, 2022). Karimnia *et al.* treated the LC-containing HGSOC spheroids with olaparib, rucaparib, TAX, TOP, CBP, cyclophosphamide, cDDP, and DXR. They observed an increase in the LC population under each treatment condition (Karimnia *et al.*, 2021). The LCs represented the bulk of the remaining cell population following treatment, indicating their resistance to chemotherapy, and their migration in 3D spheroid outgrowth assays remained unaffected in all tested treatments except TOP (Karimnia *et al.*, 2021).

3.6. 3D Models Used for Investigating Drug Resistance Mechanisms Attributed to Collaboration of Multiple Cell Types

Applications of immune checkpoint inhibitors are a promising strategy in the treatment of various cancer types, but for OC, the results from clinical trials to date have been disappointing, and the only available efficient immunotherapy for OC remains the VEGF-A-targeting monoclonal antibody bevacizumab (Garcia *et al.*, 2020; Leary *et al.*, 2021). The programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) signaling pathway is known to mediate tumor immune escape and is recognized as a promising target for immunotherapy in many types of cancer, with several FDA-approved PD-L1 inhibitors (durvalumab, atezolizumab, and avelumab) and PD-1 inhibitors (cemiplimab, pembrolizumab, and nivolumab) indicated for melanoma, lung cancer, gastrointestinal and hematological tumor treatment (Hargadon *et al.*, 2018; Akinboro *et al.*, 2022). Li and colleagues proposed a combinational therapeutic approach of bromodomain-containing protein 4 (BRD4) inhibition and α PD-L1 administration for targeting the TIME of HGSOC (Li *et al.*, 2020). They established models for TAMs co-culturing with T lymphocytes *in vitro* and demonstrated that the BRD4 inhibitor AZD5153 induced phenotype switching in TAMs from M2-like macrophages to M1-like macrophages, subsequently promoting the secretion of pro-inflammatory cytokines and activating CD8⁺ cytotoxic T lymphocytes. Using the 3D microfluidic model, they demonstrated that AZD5153 sensitized tumor microspheres isolated from ascites of chemo-naive patients to anti-PD-L1 therapy (Li *et al.*, 2020). This is another promising strategy for treating HGSOC, that could not be investigated without a perceptive design and a 3D co-culture model application.

Raghavan and colleagues developed a model of OC malignant ascites in a 3D hanging drop heterospheroid array, with HGSOC CSCs and carcinoma-associated mesenchymal stem/stromal cells (CA-MSCs) to investigate the effects of CA-MSC-secreted platelet-derived growth factor (PDGF) on HGSOC CSC properties (Raghavan *et al.*, 2020). They used OVCAR-3/MSCs and matched patient CSC/CA-MSC heterospheroids and found them to be platinum-resistant and enriched with ALDH⁺ cells, with significantly increased stemness, metastatic potential, and chemoresistance in the presence of PDGF signaling. PDGF signaling is known to contribute to the progression of various cancers, including breast and gastric cancer, and in OCs it has been identified as a potential key pathway implicated in poor prognosis but was not previously investigated in the context of OC CSC/CA-MSC communication and in 3D co-culture models (Raghavan *et al.*, 2020). Their results suggest that blocking stromal PDGF signaling will make HGSOC significantly more responsive to chemotherapy by abrogating CSC properties (Raghavan *et al.*, 2020). This study is another excellent example of how 3D co-cultures reveal the crucial importance of TME signaling in the drug response of HGSOC and how including the TME in the 3D modeling of HGSOC offers strategic advances in improving chemotherapeutic success.

3.7. 3D Models Used for Chemotherapy Response Prediction

Patra *et al.* recently demonstrated how challenging it is to choose the appropriate preclinical model for drug testing (Patra *et al.*, 2020). They generated spheroids from the EOC cell lines with known responsiveness to CBP using the hanging-drop method, ULA plates (with and without Matrigel[®]), and polydimethylsiloxane microfluidic chips and investigated spheroid forming ability in each system. The most effective models were microfluidic chips and Matrigel[®]-assisted ULA plates, and they were used to define the CBP sensitivity of formed spheroids and to compare responses to the 2D monolayer assays. One sensitive cell line in 2D culture exhibited resistance to CBP in 3D spheroids, and two cell lines that were previously categorized as resistant to CBP in 2D cultures were sensitive in the 3D models (Patra *et al.*, 2020). Gupta *et al.* observed, particularly for metastatic cells, that the drug response of 3D EOC models depended on the structural and biochemical composition of the model/platform (Gupta *et al.*, 2022). They compared the growth and chemotherapy response in the comparative study of primary and metastatic EOC cells in spheroids on ULA plates, synthetic PeptiGels/hydrogels of different formations, and polymeric scaffolds with various ECM layers. The three platforms supported the spheroid growth but for different culture periods, varying from 7 days to 4 weeks. An interesting notion is that tumor cells of different origins may prefer platforms with stiffness matching their tissue of origin (Gupta *et al.*, 2022).

A recent comparison study analyzed the CBP chemotherapy response of six previously characterized EOC cell lines of differing chemosensitivities (four of them HGSOE: OV90, OV1946, OV4453, and OV4485) across four different model systems including 2D monolayers, 3D spheroids, 3D *ex vivo* tumors and mouse xenograft models (Brodeur *et al.*, 2021). The 3D *ex vivo* model used in the study was a microfluidic micro-histological platform developed by Simeone *et al.* for the *ex vivo* culturing of prostate and OC micro-dissected tissue (Simeone *et al.*, 2019). Brodeur *et al.* demonstrated that the CBP response in EOC cell lines cultured in a 3D *ex vivo* model (micro-dissected tissue produced from cell line xenograft tumors) correlated best with the *in vivo* CBP response (mouse model), followed by 3D spheroids (Brodeur *et al.*, 2021). Shuford *et al.* developed a patient-derived 3D spheroid model utilizing primary tissue obtained at debulking surgery or laparoscopic biopsy from newly diagnosed OC patients that generated results within seven days of 3D culturing, which constitutes a clinically relevant time frame for treatment decisions (Shuford *et al.*, 2019). They tested 83 samples for responsiveness to CBP and TAX and observed an 89% accurately predicted response to first-line chemotherapy with patient-matched clinical outcomes of corresponding response or non-response. They examined the prognostic value of the model system and found a significant difference in median PFS from surgery for those patients who were prospectively test-predicted to respond to first-line platinum-based chemotherapy (greater than twenty months) compared with those who were test-predicted not to respond (nine months) (Kaplan-Meier analysis; HR 0.3414, 95% CI 0.08927 to 1.305, $p = 0.01$) (Shuford *et al.*, 2019). Previous 2D assays of OC patient chemo responsiveness were only effectively completed for less than 80% of enrolled patients (Rutherford *et al.*, 2013; Grendys *et al.*, 2014), and this test had a 90% success rate, demonstrating another advantage of 3D culturing (Shuford *et al.*, 2019). Patient-derived spheroid models require shorter generation duration than organoid models, one of the most critical characteristics for clinical utility, and generally have a better overall culture success rate. However, there have been patient-derived OC organoid models developed in less than three weeks (which is still a clinically relevant time frame) that successfully recapitulated histological characteristics and mutations of primary tumors (Nanki *et al.*, 2020). There have also been studies with a 100% success rate of organoid establishment (Ito *et al.*, 2022).

Nowacka *et al.* investigated the resistance to cisplatin (cDDP) and TAX in 2D and 3D culture conditions of A2780 cells and their cDDP-resistant sublines A2780CR1 and A2780CR2 and TAX-resistant sublines A2780PR1 and A2780PR2 (Nowacka *et al.*, 2021). They used ULA plates to generate multicellular spheroids of each cell line and investigated the cell lines' sensitivity towards cDDP and TAX in 2D and 3D culture conditions. The fold of resistance of all cDDP-resistant and TAX-resistant sublines compared with the parental cell line was higher in 3D conditions than in 2D. An almost 10-fold decrease in the sensitivity of parental A2780 cells in 3D compared with 2D to cDDP and a 1098-fold decrease to

TAX was observed. A similar decrease in sensitivity under 3D conditions was observed for resistant variants (the A2780CR1 cell line showed a 4.11-fold increase, and the A2780CR2 cell line showed a 2.69-fold increase in cDDP resistance; the A2780PR1 cell line presented a 217-fold increase, and A2780PR2 presented a 25-fold increase in TAX resistance) (Nowacka *et al.*, 2021). The observed greater difference in the cellular response to TAX than to cDDP in 3D compared with 2D cultures might be explained by the different modes of drug spreading, with cDDP evenly distributed within the tumor and TAX binding to cellular macromolecules (Nowacka *et al.*, 2021). For cDDP, a concentration-dependent response was observed in all examined cell lines in 3D cultures, but for TAX, the response drastically differed between 2D and 3D cultures. For the A2780 cell line, TAX treatment in 2D conditions resulted in a concentration-dependent decrease in cell viability, but in 3D conditions, a four-step response curve was observed. Increasing the TAX concentration up to 10 ng/ml did not affect cell viability, and an increase up to 50 ng/ml decreased cell viability to 70%; a further increase up to 2000 ng/ml had no effect on viability, with viability drastically dropping at concentrations higher than 2000 ng/ml (Nowacka *et al.*, 2021). It is fascinating how cell lines differed in the observed TAX response curves, implying unforeseen effects of 3D architecture that cannot be studied in monolayers. Another exciting aspect of the same study was the evaluation of genes previously shown to be implicated in drug resistance of these models in 2D (Januchowski *et al.*, 2014, 2017). The spheroids of TAX-resistant variants had an increased expression of *MDR1* and its product P-gp protein. However, there were no differences in transcript and protein levels in the cells growing in 3D compared with 2D conditions, despite decreased sensitivity to TAX of TAX-resistant sublines compared with parental cells. This implies that *MDR1* may be crucial in 2D drug resistance but not the most critical aspect in 3D (Nowacka *et al.*, 2021). This is another subtle example of how important 3D architecture is in drug response and why we must shift the focus toward 3D modeling. Nowacka *et al.* attributed the observed decrease in drug sensitivity in 3D conditions to other mechanisms, such as tissue-related features, including ECM component expression and cellular density (Nowacka *et al.*, 2021). The mentioned multi-step drug response curves in 3D conditions have also been observed in other models, unlike the typical dose-dependent responses in monolayers. Spheroids of the primary human ovarian cancer W1 cell line generated three-step response curves when treated with TAX and topotecan (TOP) in 3D cultures (Nowacka *et al.*, 2022). Low concentrations of TAX (100-500 ng/mL) significantly decreased W1 cell viability to 35% compared with non-treated cells. However, increased concentrations up to 5000 ng/mL resulted in a moderate increase in cell survival (Nowacka *et al.*, 2022). Only a very high concentration of TAX resulted in cell death. Under 2D conditions, the typical dose-dependent response was obtained (Nowacka *et al.*, 2022). To summarize, the drug response in 3D models appears to be related to the spatial and organizational properties of 3D structures, resulting in decreased sensitivity compared with monolayers.

As discussed in the introduction, HGSOCs are characterized by high heterogeneity and ubiquitous TP53 mutations, and this has been identified as one of the early events leading to chromosomal instability (CIN), which can be observed in the pre-invasive lesions and is known to drive metastasis and therapeutic resistance (Bakhoun *et al.*, 2018; Lukow *et al.*, 2021; Vias *et al.*, 2023). Vias *et al.* developed patient-derived HGSOC organoids and completed their comprehensive genomic, transcriptomic, and drug-response characterization (Vias *et al.*, 2023). They demonstrated that their models recapitulated clinically relevant CIN features observed in HGSOC patients, such as copy number-driven gene expression signatures, and the drug responses matched those of the parental tissues (Vias *et al.*, 2023). By single-cell DNA sequencing, the authors identified clonal populations with distinct copy number features. They demonstrated that the developed models recapitulated the heterogeneity of chromosomal instability of HGSOC and thus hold promise for treatment selection (Vias *et al.*, 2023).

Gorski *et al.* developed six HGSOC patient-derived organoid lines from tissue obtained during debulking surgery and used these models for clinical response prediction and searching for chemoresistance drivers in HGSOC (Gorski *et al.*, 2021). The clinical outcomes directly correlated with chemosensitivity assay results obtained from organoid cultures. The most CBP-resistant organoid line, which demonstrated a CBP EC₅₀ (half maximal effective concentration of the drug) value above the clinically achievable maximum concentration when tested in 3D culture conditions, correlated with the patient's platinum-resistant disease and significantly shorter PFS than the rest of the subjects (Gorski *et al.*, 2021). The authors performed DNA and RNA sequencing in search of the integrated genomic signature of platinum resistance, and the most commonly mutated genes were *TP53* (4/6), *FANCC*, and *NOTCH2* (both in 2/6 patient samples). The study identified 71 differentially expressed genes between CBP-resistant and CBP-sensitive organoids. The top-up-regulated genes were the ones involved in transmembrane transport, cellular differentiation, and immune response modulation, while the top-down-regulated genes were known to be involved in the regulation of cellular growth, cellular stress response, and lipid metabolism. Integrated pathway analysis suggested that CBP resistance was partly mediated by altered injury-associated pathways and the expected characteristic cancer-related pathways. Network mapping revealed that most differentially expressed pathways were connected with NF- κ B, cellular differentiation (PRDM6 activation), and the linkage of B-cell receptor signaling to the PI3K–AKT signaling pathway (Gorski *et al.*, 2021). Additional research with a greater sample size is needed for model value confirmation, but the obtained data are promising, and the number of studies is steadily growing. De Witte *et al.* analyzed 36 whole-genome-characterized patient-derived organoids from donors with known clinical backgrounds (de Witte *et al.*, 2020). They confirmed the stability of genomic features of original tumors and the precision in recapitulating patients' responses to neoadjuvant CBP/TAX

chemotherapy. Analyzing the results of multiple studies like the one from Gorski *et al.* and de Witte *et al.*, with more significant sample sizes, offers a promising way to establish an early patient chemo responsiveness stratification system and a predictive scoring system of HGSOC recurrence.

4. Perspectives

The progression and metastasis of HGSOC occur in the highly complex pro-inflammatory and tumor-promoting TME with intricate cellular, molecular, and mechanical properties, which are all interconnected and contribute in various ways to the aggressiveness of the disease. There is significant evidence that malignant ascites contributes to HGSOC metastasis (Ahmed & Stenvers, 2013; Geng *et al.*, 2022), and yet it still needs to be extensively researched. HGSOC is a multi-dimensional disease, and utilizing 3-dimensional models incorporating relevant microenvironmental features of ascites offers many opportunities for disentanglement of its pathology and progression. Several critical aspects of the TME, including cell–cell and cell–ECM interactions, ECM stiffness, oxygen and nutrient gradients, ascitic flow, etc., have been successfully recapitulated in novel 3D models (Horst *et al.*, 2021; Qin *et al.*, 2022; Yee *et al.*, 2022), as we emphasized in this review. The high inter- and intra-patient heterogeneity observed in HGSOC cases highlights the need for patient-derived personalized models for predicting the therapeutic response in a clinically relevant timeframe following debulking surgery or biopsy. There is also a clear need to utilize better preclinical models for drug development so that there is a relevant translational value in preclinical and early-phase studies. The gold standard of *in vitro* preclinical research, 2D tumor cell lines, are easily manipulated, consistent, and often respond to most therapeutic approaches but cannot model the physiologically relevant properties of the *in vivo* TME. *In vivo* animal models, the conventional next step after studies in 2D cell models, have their own drawbacks as well, being expensive, time-consuming, and introducing animal immune cells and microenvironment to human disease modeling (Tentler *et al.*, 2012). Additionally, the immunodeficient mouse strains are excellent for engraftment efficiency but cannot be utilized for TIME research and evaluation of potential immunotherapies. The validation of preclinical findings by 3D cell systems in the future should eradicate the need for animal testing. 3D models have already revealed so much of HGSOC tumorigenesis, progression, and evolution that they are genuinely indispensable for basic research, drug response assessment, and biomarker development. Sophisticated 3D co-culture systems such as those described in this review are specifically helpful for the investigation of CSC phenotypes, the development of immunotherapies, anti-metastatic and anti-angiogenic therapies due to the incorporation of T(D)ME. Even spheroids generated from the cell line CABA-1 in the simple hanging drop method exhibited inner features typical of *in vivo* tumors,

entrapped EVs, and the VM-like process (Giusti *et al.*, 2022). The use of 3D co-culture models provides another unique benefit in HGSOC research – sequential modeling of the metastatic process in the relevant TME, allowing a wholesome understanding of the HGSOC metastatic cascade and the selection of therapies depending on the phase of metastatic progression. Cellular collaboration in the TME modifies HGSOC progression and therapeutic response through signaling and ECM remodeling. As discussed by Pietilä *et al.*, the cancer cell-ECM crosstalk continues to evolve and is crucial in understanding cancer progression and therapeutic response (Pietilä *et al.*, 2021). The study by Pietilä and colleagues detected stromal pathways as potential crucial targets to improve the chemotherapeutic success against HGSOC cells that escape treatment (Pietilä *et al.*, 2021). The benefits brought to the field of HGSOC research are highlighted in **Figure 3**, together with the drawbacks of such models, which are discussed in the following section.

5. Conclusions

Although 3D models are becoming increasingly significant, they are far from becoming a standard of preclinical studies as several essential challenges still remain to be solved. The first issue is the effect of spheroid size variability on the observed drug response. Variations in size and compaction affect the delivery of nutrients and oxygen within spheroids, directly affecting proliferation, intrinsic cellular organization, and drug penetration. The effects of spheroid size and the original cell number from which the spheroid is grown must be further investigated to achieve high-throughput systems for selecting the best treatment options for each patient. Capturing tumor heterogeneity will be easier if HGSOC primary spheroid and organoid cultures can grow consistently (standardization of size and morphology with advanced culturing protocols and protocols for assaying). Also, more correlations with clinical studies are needed to set standards for data interpretation regarding drug response prediction. False-negative results from drug screenings in patient-derived models, as observed by Shuford *et al.*, should be understood and eliminated (Shuford *et al.*, 2019). Choosing a suitable preclinical model is tricky, as demonstrated in many recent studies (Patra *et al.*, 2020; Brodeur *et al.*, 2021; Gupta *et al.*, 2022), and the advantages and throwbacks of different platforms should likewise be better understood. Additionally, different phases of disease progression have diverse T(I)ME components, so models should become more sophisticated to gain clinical relevance. Patient materials are problematic owing to their limited availability (usually one opportunity to gather material from each patient; rarely more if palliative aspiration of ascites or secondary debulking surgery is done) and sometimes too scarce for successful culture establishment. There is also an issue of long-term vs. short-term expansion of patient-derived

cultures, with each approach having its good and bad sides. The long-term expansion enables multiple testing and increases reproducibility, but it induces a risk of clonal selection during prolonged culture, inevitably leading to loss of heterogeneity. Significant improvements in 3D culture manipulation are also necessary, as the culturing methods are complex and sensitive, which complicates the downstream analyses in comparison with the well-established methodology of 2D culturing. However, 3D models are being clearly beneficial in the investigation of HGSOC and already did, and will continue to, revolutionize preclinical research and lead to the discovery of novel, appropriate, and effective therapies. Emerging technologies such as CRISPR/Cas targeting of oncogenes and single-cell OMICs technologies, in combination with novel *in vitro* and *ex vivo* 3D HGSOC models, offer an integrated approach to unraveling the molecular mechanisms behind the metastasis and chemoresistance of HGSOC. Hopefully, the sophisticated 3D modeling of HGSOC will soon validate its most important potential – significant improvement of HGSOC patients' therapy outcomes.

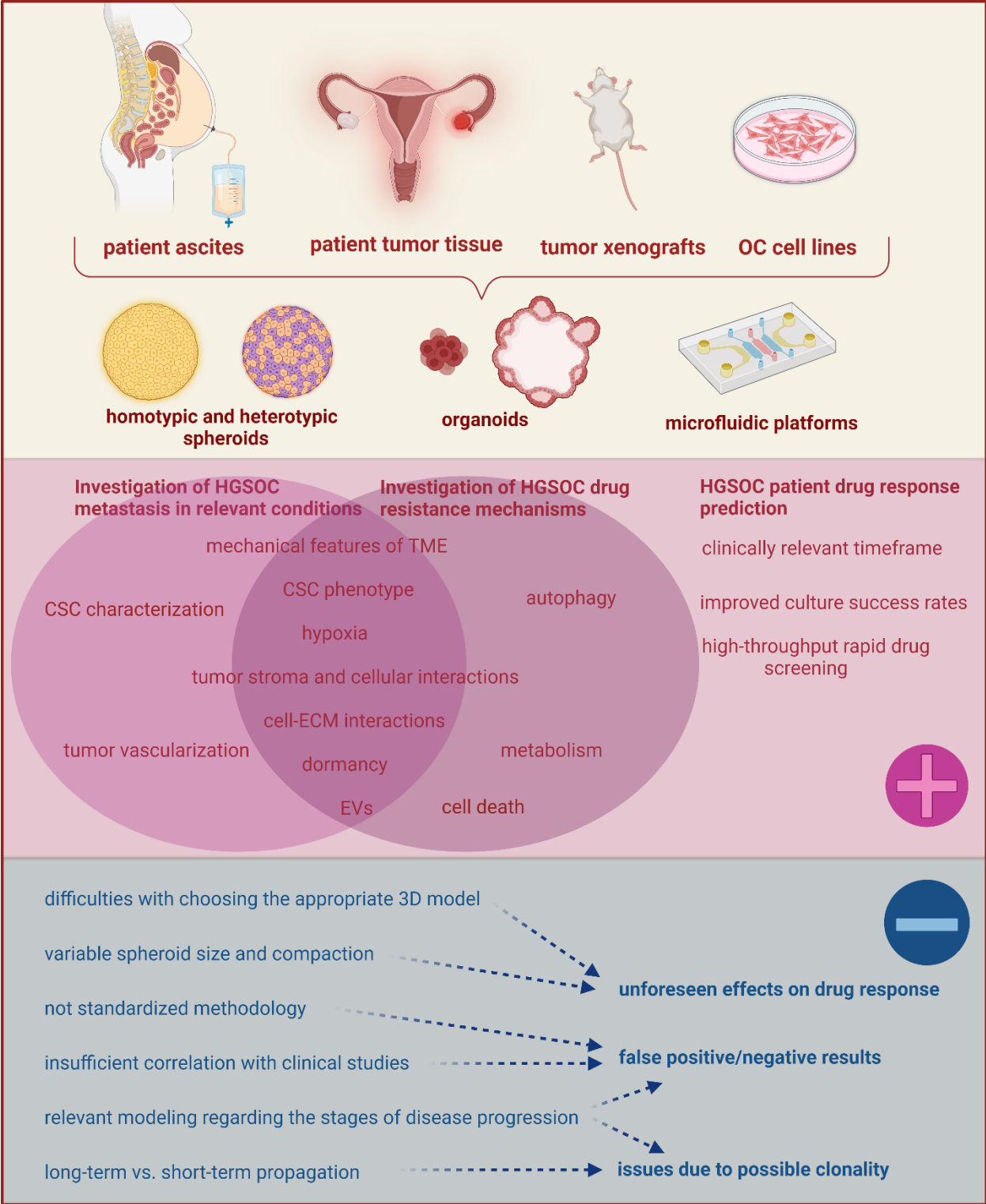


Figure 3. The benefits and challenges in 3D modeling of HGSOC. The scheme summarizes the possibilities and unresolved issues of 3D models discussed in this review. Created with BioRender.

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Credit Authorship Contribution Statement

Vanja Tadić: Investigation, Conceptualization, Writing - original draft, Visualization. Wei Zhang: Writing - review & editing. Anamaria Brozovic: Conceptualization, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: