Research Article
3D Fluid-dynamic Ovarian Cancer Model Resembling Systemic Drug Administration for Efficacy Assay

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Abstract
Recently, 3D in vitro cancer models have become important alternatives to animal tests for establishing the efficacy of anticancer treatments.

In this work, 3D SKOV-3 cell-laden alginate hydrogels were established as ovarian tumor models and cultured within a fluid-dynamic bioreactor (MIVO®) device able to mimic the capillary flow dynamics feeding the tumor. Cisplatin efficacy tests were performed within the device over time and compared with (i) the in vitro culture under static conditions and (ii) a xenograft mouse model with SKOV-3 cells, by monitoring and measuring cell proliferation or tumor regression, respectively, over time.

After one week of treatment with 10 μM cisplatin, viability of cells within the 3D hydrogels cultured under static conditions remained above 80%. In contrast, the viability of cells within the 3D hydrogels cultured within dynamic MIVO® decreased by up to 50%, and very few proliferating Ki67-positive cells were observed through immunostaining. Analysis of drug diffusion, confirmed by computational analysis, explained that these results are due to different cisplatin diffusion mechanisms in the two culture conditions. Interestingly, the outcome of the drug efficacy test in the xenograft model was about 44% of tumor regression after 5 weeks, as predicted in a shorter time just in the fluid-dynamic in vitro tests carried out within MIVO® device.

These results indicate that the in vivo-like dynamic environment provided by the MIVO® device allows to better model the 3D tumor environment and predict in vivo drug efficacy than a static in vitro model.

1 Introduction
Ovarian cancer is one of the main causes of death in female cancer patients and has one of the highest gynecological mortality rates (Sankaranarayanan and Ferlay, 2006). The poor survival rate is mainly due to chemoresistance to established drug protocols, as also happens in many other cancer cases (Lowe et al., 2013). In this context, the increasing prevalence of drug-resistant cancers necessitates further research and treatment development. Currently, an anticancer drug candidate that enters Phase I trials will successfully proceed further with a probability of only 8%, highlighting the urgent need for new physiologically relevant in vitro tumor models better resembling the in vivo conditions to test novel drugs and therapies (Suggitt and Bibby, 2005).

Based on current regulatory guidelines, the screening of new cancer drugs is carried out by using high-throughput assays, where in vitro toxicity and efficacy tests are performed on cells grown as monolayers over planar plastic surfaces; then, in the preclinical development, in vivo toxicological and ADME (adsorption, distribution, metabolism, excretion) studies are performed in animal models. However, it is now widely demonstrated that 2D cell cultures are oversimplified and poorly resemble the complex 3D tumor microenvironment (Abbott, 2003; Loessner et al., 2010; Marrella et al., 2019). On the other hand, animal models commonly fail to predict human safety and efficacy in clinical studies, besides being expensive and associated with ethical issues (Liu et al., 2013). Therefore, in the last years, novel 3D human in vitro culture systems have increasingly gained attention as potential compromises between traditional 2D cultures and in vivo models (Hourau-Véchot et al., 2018). They aim to combine the advantages of the former (better control of the experimental conditions, relative ease of manipulation and analysis, species-specificity) and approach the latter by better representing in vivo physiology.

In this scenario, 3D tumor spheroids have been proposed as in vitro human cancer models (Raghavan et al., 2015; Herter et al., 2017). Spheroids are scaffold-free aggregations of cells suitable for prolonged in vitro culture and high-throughput drug testing. They have been shown to resemble many physiological aspects better than cells grown in monolayers (e.g.

Received March 13, 2020; Accepted July 8, 2020;
Epub August 3, 2020; © The Authors, 2020.
ALTEX 37(4), ###-###. doi:10.14573/altex.2003131

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physical borders, chemical gradients (Thoma et al., 2014). In particular, cells exhibit cell-cell interactions in all spatial directions, finally resembling shape and phenotypes close to those observed in vivo. Moreover, the cancer cell proliferation rate within spheroids has been found to be comparable with that observed in vivo, which is substantially lower than that of cells cultured in 2D culture conditions (Longati et al., 2013). Depending on the tumor cell type and cell packing densities, tumor spheroids with diameters of up to 600 μm can be generated (Xu et al., 2014). Specifically, they are particularly suitable to reproduce ovarian cancer, since ovarian cancer cells grow as spheroids in some patients (Burleson et al., 2004).

Although these 3D culture systems widely improved the reliability of in vitro tests in different oncologic areas, they still have some limitations, particularly due to a lack of surrounding extracellular matrix (ECM). For example, cell-based models are susceptible to physical disintegration during manipulation; moreover, microenvironmental conditions, for example cell-ECM interactions and matrix mechanical rigidity, cannot be finely tuned (Markovitz-Bishitz et al., 2010). Furthermore, the mass transport and the kinetics of the release of anticancer drugs in solid tumors cannot be accurately replicated due to the non-uniform secretion of endogenous extracellular matrix by the spheroids.

Therefore, 3D scaffold-based cancer models able to resemble the function of the ECM have been investigated and recently developed. Tumor-associated stromal ECM is in fact an important component of the tumor microenvironment, playing crucial roles in cancer progression and invasion (Weaver and Roskelley, 1997; Dutta and Dutta, 2009). Tumor cells can be either encapsulated into hydrogels, bioprinted in 3D matrices or cultured embedded in artificial membranes/scaffolds. The association of cancer cells with an artificial ECM allows to more closely recapitulate pathophysiological features of native tumor tissues (Hutmacher et al., 2010), improving the understanding of the reciprocal interactions between the tumor cells and their surrounding microenvironment, which physiologically includes the stromal ECM molecules, immune cells, stromal cells, as well as growth factors and cytokines (LaBarbera et al., 2012).

Polymeric materials (mainly hydrogels) are used as artificial ECM (Nyga et al., 2011; Fang and Eglen, 2017; Marrella et al., 2018). They are matrices in which tumor cells can be encapsulated to then proliferate, migrate and arrange (Nicolademus and Bryant, 2008; Marrella et al., 2017). These hydrogel-based models have provided new methods for accelerating cancer research, in particular improving the quality of preclinical cancer research (Jiguet Jiglare et al., 2014). In fact, these 3D matrix-assisted cancer models support more complex cell-cell and cell-ECM interactions, leading to biochemical signals and mechanical forces that can influence cell motility, proliferation and gene expression (Huber et al., 2016).

Matrix-assisted tumor models also can be derived from patient biopsies or explants, named organoids, allowing the ex vivo propagation of tumors from individual patients; the organoids are also able to self-organize to resemble organ function (Clevers, 2016). Although standard protocols for their maintenance in culture are not yet available (Weeber et al., 2017) and their manipulation requires a specific expertise (patient cell isolation can be challenging in terms of sterility and cell expansion), these systems are valuable for the in vitro analysis of those types of cancers for which there are no immortalized cell lines (Branca et al., 2020).

If originally the term organoid referred to primary cultures of tissue fragments separated from the stroma within 3D gels to form organ-like structures (Simian and Bissell, 2017), now the term organoid refers to a wide range of techniques for the in vitro culture of self-organizing and self-renewing 3D cultures obtained from primary tissue, embryonic or induced pluripotent stem cells, whose functionality recapitulates that of the tissue from which they have been extracted (Lancaster and Knoblisch, 2014; Shamir and Ewald, 2014; Fatehullah et al., 2016; Kretzschmar and Clevers, 2016; Simian and Bissell, 2017).

However, current 3D tumor models are still quite far from recapitulating the whole in vivo scenario, since they do not resemble the fluid-dynamic stimuli at the cancer microenvironment level, and consequently, the drug transport mechanisms across the vascular endothelium structure. Recently, micro-fluidic systems (so called lab-on-chip) have been developed with the ambition to overcome some of these disadvantages (Trujillo-de Santiago et al., 2019). They enable easy manipulation of liquid with microliter volumes, the generation of fluid flow induced forces and dynamic control of tumor-ECM interaction. However, in most of the cases the fluid flow is not regulated by a peristaltic or syringe pump but guided by gravity-driven flows, thus limiting the possibility to tune and set the desired fluidic parameters (velocity, shear stress) within the circuit.

Moreover, the small dimensions of these devices limit preclinical studies, since the small cell number used (typically <1000) poorly resembles the phenotypic and cellular heterogeneity as well as microenvironmental features (Shin et al., 2012).

Moreover, the solid tumor microenvironment is highly complex and its over-miniaturization is problematic. The 3D extracellular matrix and the blood vessel walls represent physical barriers for drug transport, which determine the kinetics of drug delivery. In fact, many anticancer drugs that reach the clinic are potent enough to kill cancer cells in petri dishes (2D culture), but fail in clinical trials since they are not able to reach cancer cells in an amount that is sufficient to kill them without causing severe side effects (Das et al., 2015; Marrella et al., 2019). Therefore, a reliable in vitro culture system should emulate the drugs’ systemic administration and their route through the vascular system to reach the tumor site and penetrate to kill cancer cells.

To this aim, we here show a successful combination use of 3D cell-laden hydrogel and a fluidic culture system that more closely mimics the in vivo drug administration via the systemic circulation and the drug transport mechanisms across the vascular wall to the ovarian tumor mass. In detail, 3D cancer cell-laden hydrogels as ovarian cancer models (OCM) were cultured within a fluid-dynamic Multi In vitro Organ-MIVO® device in comparison to static conditions; as a proof-of-principle, drug efficacy of cisplatin on cell proliferation was assessed in comparison to its efficacy in a xenograft mouse model. This system may represent a suitable in vitro tool to predict the efficacy of anticancer drugs and favor their clinical translation.

2 Materials and Methods

2.1 3D ovarian cancer model

The human ovarian cancer SKOV-3 cell line (ATCC, Manassas, VA, USA) was maintained in Dulbecco’s modified Eagle’s medium high glucose (HyClone - vWt) supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine (Sigma Aldrich) and 1% penicillin/streptomycin solution (Sigma Aldrich) and plated at a density of 1x10⁵ cells/cm². The

2
cells were incubated in a humidified, 5% CO₂ atmosphere at 37°C. Medium was changed 2 days after the original plating and then twice a week. When culture dishes were near confluent, cells were detached with 1X trypsin (EuroClone) after two washes in D-PBS 1X and replated until the next confluence. After two passages, cells were used for the in vitro and in vivo experiments.

Alginate (Alg) powder (Manugel GMB, FMC Biopolymer) was dissolved in physiologic solution (0.9% NaCl solution) at 1% w/v and the solution was then filtered under sterile conditions. SKOV-3 were detached from plastic tissue culture flasks with 0.05% trypsin and resuspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The SKOV-3 suspension then was mixed with the sterile Alg solution to obtain a final Alg concentration of 0.5% w/v. The SKOV-3/Alg suspension was dripped into a sterile 0.5M CaCl₂ (Sigma Aldrich) gelling bath to form alginate spheres with a final concentration of cells of 1.3 × 10⁶ cells/mL.

After washing the spheres with DI water to remove the excess of Ca, the OCM were gently moved into 6 well-plates and cultured with 4.5 ml DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and CaCl₂ (5 mM) in a humidified environment (5% CO₂) at 37°C.

2.2 Ovarian cancer model viability and proliferation

SKOV-3 viability within OCM was evaluated qualitatively through a live/dead assay (Sigma Aldrich). Briefly, after 24 h, OCM were washed with PBS and incubated in 2 mM calcein-AM and in 4 mM EthD-1 in PBS for 15 min at 37°C in a dark environment to detect live and dead cells, respectively. OCM were washed three times in PBS and then observed by means of fluorescence microscopy (Nikon H550L).

SKOV-3 proliferation within tumor models was quantitatively assessed by Alamar Blue Assay (Thermo Fisher Scientific). SKOV-3 cultured in monolayers were used as 2D control. In particular, 15 × 10⁵ cells were cultured over a glass slide placed in a 6-well plate with the same volume of medium as the 3D models (4.5 ml). For 3D proliferation rate analysis, after 3 hours (T0), 2 (T2), 4 (T4), 7 days (T7) of culture, the OCM were placed in 96 well-plates containing 0.2 ml of 1% v/v of Alamar Blue solution, as indicated by the manufacturer. Samples were incubated at 37°C for 4 h in the dark. The supernatants containing Alamar blue were collected and absorbance readings assessed spectrophotometrically. A calibration curve was derived by seeding known number of cells in 96-well plates to find the correspondence between the number of cells and the output of the absorbance readings. The proliferation rate was calculated as the ratio between the number of cells detected at each time point respect the number of cells after 3 hours of culture (T0). Then, OCM were washed with physiologic solution and placed in 6-well plates containing the original culture medium. (N=3 biological replicates; n= 2 technical replicates)

2.3 In vitro drug efficacy tests

In vitro drug efficacy tests were performed by using a compartmental fluidic device (commercialized as MIVO® by React4life S.r.l., IT). The system design is shown schematically in Figure 1.

24-well Transwell inserts (Corning) containing an OCM were placed and cultured within the bioreactor, forming two fluidically independent chambers: the tissue culture chamber, which was filled with culture medium (0.3 ml), and the circulatory chamber, connected to a closed loop fluidic circuit containing 4.2 ml medium where cisplatin (purchased from Sigma Aldrich srl.) was allowed to circulate at a rate of 0.3 cm/s, simulating the capillary flow rate. Four hours after their formation, OCM were placed into the bioreactor chamber and cisplatin was added into the bioreactor circuit connected with the receiver chamber (Fig. 1).

3D hydrogels cultured within 6-well plates with 4.5 ml medium with or without cisplatin were used as static controls.

Cell viability of cisplatin-treated SKOV-3 was assessed quantitatively by Alamar Blue Assay at different time points. Briefly, the samples were placed in 96-well plates and incubated with fresh medium containing 0.2 ml of 1% v/v Alamar Blue solution, at 37°C for 4 h in the dark. Cell viability was derived as % of live cells normalized to the untreated controls. Student’s paired t-test between each dynamic condition and the respective static one was performed for each time point and statistical significance was set at *P < 0.05, (N=3 biological replicates; n= 2 technical replicates).
2.4 In vivo xenograft model

A total of twelve 5-week-old female nude mice (Mice Hsd: Athyemic Nude Foxn1 nu female) were purchased from Envigo RMS srl, San Pietro al Natisone, Italy. The mice had a body weight of 21.1/20.1-24.3 g (median/interquartile range) at the beginning of the experiment.

Animals were delivered to the animal facility 10 days prior to the beginning of the study for acclimatization. The mice were housed in SealSafe Plus GM500 plastic cages (Tecniplast Spa, Buguggia, Italy) with a light dark cycle of 12h/12h at a temperature of 21 ± 2°C (dawn: 6:30-7:00 am) and a relative humidity of 60 ± 20%. Food (pellets, 10 mm, 2018 Envigio RMS Srl, San Pietro al Natisone, Italy) and sterilized water were provided ad libitum. Enrichment was supplied by Mouse house (Tecniplast Spa, Buguggia, Italy). The animals in each group were divided into 3 animals per cage. Cages were clearly labelled with an ID card indicating study number, group, gender and treatment schedule. All animals were subjected to the same environmental conditions. The study was carried out according to the guidelines enforced in Italy and in compliance with the Guide for the Care and Use of Laboratory Animals, 8th Edition, 2011.

SKOV-3 derived tumors were established via subcutaneous injection of 1 x 10⁶ cells into the right flank of mice. The cells were resuspended in phosphate buffered saline (PBS) for s.c. injection. Tumor size was monitored over time. After 10 days, when tumor volumes had increased to 50 mm³, mice were randomized into two treatment groups, i.e., a control group (sham treated, N=6) and an experimental group (treated, N=6). The mice were administered PBS (control group) or cisplatin (6 mg/kg) intravenously once every seven days for 3 weeks. Tumor growth was quantified three times a week using a digital caliper. The tumor volume was calculated as follows: 0.5 x length x width².

The results are expressed as tumor growth inhibition (%TGI), which was calculated as the percentage of reduction of tumor volume compared to the control:

\[ \%TGI = 100 \times \left(1 - \frac{TumorVolume_{treated}}{TumorVolume_{control}}\right) \]

For comparison with the in vitro cell viability data, the reciprocal trendline %TGI values (100-%TGI) were used.

The effect size f and total sample size were calculated using the G*Power software. The input data for the analysis were extrapolated from the published literature (Faul et al., 2007).

The in vivo experiments were authorized by the Ministry of Health for in vivo studies and by the Body for the Protection of Animals (OPBA).

2.5 Immunostaining

Hydrogels were fixed with 4% para-formaldehyde in PBS (PFA; pH 7.4) for 1 h and incubated for 1 h in blocking buffer (0.5% Triton X-100, BSA 2% w/v, CaCl₂ 5mM in physiologic saline solution). Subsequently, hydrogels were incubated with primary antibody for 1 h at room temperature. Cell proliferation was detected by staining cells with a rabbit anti-Ki67 antibody (Abcam, USA; 1:400 dilution in blocking buffer); while apoptotic cells were stained by using rabbit Anti-Cleaved Caspase-3 antibody (Abcam, USA; 1:100 dilution in blocking buffer). Samples were then washed three times with PBS and incubated for 1 h with Alexa Fluor 488-conjugated goat antirabbit secondary antibody (Abcam, USA; 1:200 dilution in blocking buffer) for anti-Ki67 and with Alexa Fluor 555-conjugated goat antirabbit secondary antibody (Abcam, USA; 1:200 dilution in blocking buffer) for anti-Caspase-3. Nuclei were counter-labeled with DAPI (Sigma-Aldrich). Imaging was performed with a fluorescence microscope (Nikon H550L). Images obtained by fluorescence microscopy were analyzed using ImageJ software.

2.6 Drug diffusion within the 3D tumor hydrogel

Drug diffusion into the 3D tumor hydrogel was determined via HPLC. Alginate hydrogels were cultured either under static conditions or within the MIVO for 7 days with cisplatin 10 μM. The hydrogels were then left in the incubator for 3 days in 5 mM CaCl₂ physiologic solution to allow the cisplatin to diffuse from the hydrogel to the solution. Then the samples were analyzed by HPLC. The HPLC system consisted of a pump, column compartment and RS variable wavelength detector (all UltiMate 3000, Thermo Fisher Scientific). The injection valve fitted with a 20 μL sample loop and an Accucore 150-C18 (Dimensions = 150 x 3 cm and particle size 2.6μm) was purchased from Thermo Fisher Scientific. The mobile phase consisted of methanol-water (80:20, v/v). The UV detector was adjusted at 254 nm. The flow rate was set at 0.2 mL/min (isocratic flux) and the column temperature at 40°C (Kaushik et al., 2010; Tezcan et al., 2013) (N=3 biological replicates).

2.7 Computational fluid-dynamic (CFD) simulations

Fluid dynamic and mass transport simulations were performed both in static and dynamic conditions to simulate the concentration of cisplatin within the cell-laden hydrogels over time.

The 3D domain, the related size and dimensions were calculated based on the real dimensions of the microfluidic circuit used during the test. As shown in Figure S1. Domain 1 represents the circulatory chamber of the bioreactor and the fluidic pattern of the circuit, Domain 2 is the tissue culture chamber, which is represented by a well filled with medium, and Domain 3 represents the alginate-based hydrogel sphere. The obtained geometry, the fluid dynamics within the circuit, and the mass transport of cisplatin through the entire system was modeled using Comsol Multiphysics 5.3a. For the numerical solution of the physics involved in this system, the Laminar Fluid Flow module and the Transport of Diluted Species module were used.

The first physical phenomenon involved in this system was represented by the fluid dynamics from the circulatory chamber of the bioreactor (i.e. Domain 1) to the tissue culture chamber (Domain 2) where the cell-laden hydrogel is cultured (Domain 3). The fluid was supposed to be laminar, incompressible, and not turbulent. The velocity and pressure field profiles were calculated according to Navier-Stokes and the continuity equation (Equation 1):

\[ \text{Equation 1} \]

1 doi:10.14573/altex.2003131s
\[
\begin{align*}
\rho \left( u \cdot \nabla u \right) &= -\nabla p + \mu \nabla^2 u \\
\rho (\nabla \cdot u) &= 0
\end{align*}
\] (1)

where \( u, \rho \) and \( \mu \) are the velocity, the density \((10^3 \text{ kg/m}^3)\) and the viscosity \((10^{-3} \text{ Pa} \cdot \text{s})\) of the fluid, and \( p \) is the pressure, respectively. The flow rate was set to \( Q = 3 \text{ ml/min} \) to generate velocity resembling the capillary blood flow. An iterative geometric multigrid (GMRES) algorithm was used to solve the equations. Discretization was chosen P2+P1 for velocity and pressure field, respectively. In the outlet, the pressure was set equal to zero with no backflow. A no-slip condition was fixed on the boundary of the geometry. As the initial value, the velocity was set equal to zero in the entire system.

The general mass transport equation was used to describe cisplatin mass transport through the system:

\[
\frac{\partial c}{\partial t} + \nabla (-D \nabla c) + u \nabla c = R
\] (2)

where \( c \) is the cisplatin concentration in the system, \( D \) is the diffusivity of cisplatin, \( u \) is the velocity field, and \( R \) is the reaction term. The diffusivity of cisplatin \( D \) in the medium (approximated as water) at 37°C \((D_{Cis-Pt}^{64})\), included in the equation for domains 1 and 2, is set equal to 1.034 \( \times 10^{-5} \text{ cm}^2/\text{s} \), as reported in the literature (Modok et al., 2007; Panczyk et al., 2013).

On the contrary, the diffusion constant of cisplatin within the alginate hydrogels \((D_{Alg}^{64})\) was calculated using the empirical Ogston model, as shown in equation 3. The model takes into account several parameters including the polymer volume fraction \( \phi \), the solute (cisplatin) radius \( r_s \), the alginate fiber radius \( r_f \) and the diffusion value of cisplatin in water \( D_{w}^{64} \). In particular, the value was calculated using the following expression:

\[
D_{Alg}^{64} = \exp \left( -\frac{r_s + r_f}{r_f} \phi^{0.5} \right)
\] (3)

where the polymer volume fraction was approximated to 0.01 while the radius of cisplatin and alginate fiber were 4 \( \times 10^{-10} \text{ m} \) and 8 \( \times 10^{-10} \text{ m} \), respectively (Amsden, 1998; Modok et al., 2007).

Moreover, it should be considered that the diffusivity value of cisplatin in the alginate hydrogels is affected by the presence of dead cells, which decrease the mass transport of cisplatin within the polymer.

Firstly, the Michaelis-Menten parameters were calculated by considering that 50.000 molecules of cisplatin are enough to kill one cell (Amsden, 1998), as reported in the following reaction (equation 4):

\[
50000 \text{ molecules of cisplatin} + 1 \text{ cell} \rightarrow 1 \text{ dead cell}
\] (4)

Based on the best fitting of the experimental data (Fig. S31), the obtained values were \( V_{max} = 1.66 \times 10^{-12} \text{[mol} \cdot \text{m}^{-3} \cdot \text{s}^{-1}] \) and \( K_m = 6.64 \times 10^{-8} \text{[mol} \cdot \text{m}^{-3}] \).

In this case, the Ogston model considers an added corrective term to describe the mass transport in the system. Therefore, the diffusion of cisplatin within the hydrogel could be described by the following equation:

\[
D_{Alg}^{64} = D_{w}^{64} \cdot \frac{k}{t + b}
\] (5)

where \( D_{Alg}^{64} \) is the diffusivity of cisplatin in water, \( a \) is the ratio calculated according to the Ogston model (equal to 0.86 in (3)), and the exponential term defines the decrease of the diffusion during the time \( t \) due to the presence of dead cells.

Parameters \( k \) and \( b \) (Tab. 1) depend on the velocity field (either in static or dynamic conditions) and were calculated considering the amount of cisplatin within the alginate hydrogels experimentally measured through HPLC analysis after 7 days of culture.

**Tab. 1: k and b values for the cisplatin diffusivity in static and dynamic conditions**

<table>
<thead>
<tr>
<th></th>
<th>Static</th>
<th>Dynamic</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k )</td>
<td>9.5±0.05</td>
<td>6±0.05</td>
</tr>
<tr>
<td>( b(s) )</td>
<td>4x10^2±100</td>
<td>4x10^2±100</td>
</tr>
</tbody>
</table>

The initial concentration of cisplatin \((C_o)\) in the bioreactor was set at 100 μM or 10 μM. Danckwerts conditions were selected in the inlet, while in correspondence of the boundary surfaces no-flux condition was considered. For the outlet, the diffusion term was considered equal to zero.

A direct backward differentiation formula (BDF) algorithm was required for the transient study. Linear discretization was chosen for the concentration field. The reaction term was defined according to the Michaelis-Menten kinetics:

\[
R = -\frac{V_{max} \cdot c}{K_m + c}
\] (6)

where \( V_{max} \) is the maximum consumption rate and \( K_m \) is the concentration of cisplatin when the rate is equal to \( V_{max}/2 \). The reaction term takes into account the deactivation of cisplatin molecules bound to the DNA of cancer cells and no longer available during the experimental tests. This term was considered as a consumption rate of cisplatin.

The average concentration profile of cisplatin within the hydrogels was calculated as follows:

\[
C = \frac{1}{V_{sphere}} \int_{Sphere} C(x,y,z) dV
\] (7)
3 Results

3.1 3D cancer cell viability and proliferation
Firstly, ovarian cancer cell viability and growth within the OCM were investigated and visualized by calcein-AM staining. Four h after OCM generation (day 0), most of the cells were alive, indicating the suitability of the procedure for cellular embedment within the alginate hydrogels. Cell viability was evaluated also after 7 days of culture. A higher cell density was observed at day 7, proving the cells’ ability to proliferate within the hydrogels (Fig. 2A).

To quantify this result, the cell proliferation rate was measured by Alamar Blue assay after 2, 4 and 7 days of culture. Figure 2B shows that cancer cells cultured within the OCM proliferate more slowly than cells grown in 2D monolayers, in agreement with other reports (Chitcholtan et al., 2013).

![Fig. 2: Cell viability and proliferation of OCM in culture](image)

(A) Cell viability represented by live/dead images of OCM after 4 hours (DAY 0) and 7 days (DAY 7) of culture. Scale bar is 500 µm. (B) Quantitative analysis of the proliferation rate of ovarian cancer cells embedded within alginate hydrogels (3D) or cultured in monolayers (2D) assessed by Alamar Blue assay. Values are reported as mean ± SD. (N=3 biological replicates; n= 2 technical replicates).

3.2 In vitro drug efficacy test
The viability of SKOV-3 OCM cultured within MIVO® and treated with cisplatin (10 µM or 100 µM) was measured over time. In particular, while in static conditions the drug was directly added to the medium surrounding the OCM, in dynamic conditions (i.e. MIVO®) it was injected into the fluidic circuit beneath the membrane from which it could reach the tumor tissue cultured in the upper chamber of the bioreactor by diffusion, resembling extravasation of the drug.

After two days of culture in the presence of 100 µM cisplatin, cell viability in static and in dynamic conditions was significantly reduced and was even lower after 4 and 7 days with no significant differences observed between static and dynamic conditions (Fig. 3).

Viability was weakly but not significantly reduced after two days culture in the presence of 10 µM cisplatin under static conditions. Interestingly, significantly decreased cell viability to 67.81% ±0.62 and 50.44% ±0.25 after 4 and 7 days respectively at 10 µM cisplatin was observed only under dynamic conditions within MIVO®. OCM cultured under static conditions remained above 80% cell viability for the overall time of observation (Fig. 3).

![Fig. 3: Cell viability of SKOV-3 culture assessed through Alamar blue assay within OCM in static and in dynamic conditions treated with the indicated concentration of cisplatin](image)

Cell viability was derived as % of alive cells normalized respect the untreated controls. Values are reported as mean ± SD. Student’s paired t-test between each experimental dynamic condition and the respective the static one for each time point was performed and statistical significance was set at *P < 0.05. (N=3 biological replicates; n= 2 technical replicates).
3.3 In vivo drug efficacy test

The in vivo efficacy of cisplatin against ovarian cancer SKOV-3 cells was evaluated in a xenograft model. Treatment of six nude mice with 6 mg/kg cisplatin versus six control mice started 10 days after tumor induction, defined as Day 0. The experiment was stopped on Day 35 when the tumor volume in the control group reached 2000 ± 270 mm³ and was more than double compared to the treated group (see Fig. S2 for tumor volume data). Figure 4A shows the inhibitory effect of cisplatin on tumor growth over time. The %TGI was 7.3%, 31.1% and 56.9% on Days 12, 21 and 35, respectively, as shown in Fig. 4A.

Interestingly, comparing the endpoints of the in vitro (static treatment), in MIVO® (dynamic treatment) and in vivo (xenograft model), an excellent overlap of drug efficacy data is observed only between MIVO® and in vivo data, although on a different time scale (Fig. 4B); comparable results are obtained after 2, 4 and 7 days of treatment in MIVO® and after 2, 11 and 25 days of treatment in mice. Due to the differences in drug distribution and metabolism (ADME profiles) between these two different experimental conditions, the time needs to be rescaled to obtain comparable results (~3 times faster in MIVO®). On the contrary, static in vitro results do not resemble data obtained in vivo.

3.4 CFD simulations and mass transport within MIVO®

The concentration fields of 10 µM cisplatin within the OCM cultured in static and dynamic conditions are shown in Figure 6 based on the geometry and the model set-up defined in Materials and Methods.

After 12 h, the amount of cisplatin diffused within the alginate spheres seemed to be higher in the static than in the dynamic condition, i.e., values of 0.0053 mol/m² and 0.00707 mol/m² were detected for the dynamic and the static conditions, respectively (Fig. 5A-B). Interestingly, after 4 days of culture the trend was opposite, with higher amounts of cisplatin in dynamic conditions than in static (Fig. 5C-D). The same results were also obtained after 7 days. Specifically, during the dynamic culture, the alginate hydrogel was completely filled with cisplatin, while in static conditions concentration gradients through the sphere were still evident (Fig. S5-E-F).

Simulations were performed at the two concentrations tested in vitro (10 µM and 100 µM). No main differences between the amount of cisplatin simulated in the model and that experimentally measured were detected (Fig. 6), proving the reliability of the mathematical model.

The average concentration profiles of cisplatin within the hydrogels showed a significant difference among the different cases tested over 7 days, as shown in Figure 7.

The diffusion of the drug within the hydrogels in static conditions was faster at short time scales at both concentrations of 100 µM and 10 µM than in dynamic conditions. It is likely that when the hydrogel was placed in the well plate dipped in the cisplatin solution, the concentration gradients of the drug were high enough to allow a fast diffusion through the hydrogel. It should be noted that in static conditions, the corresponding convection term is missing.

On the contrary, in dynamic conditions the cisplatin concentration within the hydrogels increased slowly and reached the same concentration of the static condition after 18 hours both at 100 and 10 µM. Under these conditions, the drug needed to move from domain 1 to domain 2, finally reaching domain 3 (Fig. S1I). However, the concentration of cisplatin within the hydrogels became higher than in static conditions after one day of culture. After 4 days of culture in dynamic conditions, the cisplatin concentration reached a plateau, and a clear difference of cisplatin concentration between the two culture conditions was detected at both tested concentrations (i.e.100 and 10 µM), as shown in Figure 7.

In summary, in static conditions, the absence of fluid motion generates a greater cisplatin resistance within the hydrogel due to the accumulation of dead cells, which limit the mass transport within the polymer. In contrast, in dynamic conditions, the flow allows a continuous removal of dead cells, thus leading to less hydrogel resistance to the cisplatin diffusion.
Fig. 5: Distribution of cisplatin in dynamic and static conditions at 10 μM and different time points A) dynamic; 12h, B) static; 12h, C) dynamic; 4 days, D) static; 4 days, E) dynamic; 7 days, F) static; 7 days. While dynamic condition shows a complete drug distribution within the 3D volume after 7 days of culture, under static condition a strong gradient is still present.

Fig. 6: Concentration of cisplatin in OCM after 7 days culture in dynamic or static condition
Comparison between the amount estimated by simulation and measured by HPLC in the experimental tests. Cisplatin concentration: 10 μM. Values are reported as mean ± SD. (N=3 biological replicates).

Fig. 7: Concentration profile of cisplatin within OCM over time for the four different experimental conditions.
For each drug concentration (10 or 100 μM) the dynamic condition shows a slower diffusion over time that reaches an higher plateau value.

3.5 Immunostaining
Immunostaining of OCM collected at different time points was carried out to determine the expression of Ki67 to identify proliferating cells, and of Caspase-3 to mark the cells undergoing apoptosis in response to treatment with 10 μM and 100 μM cisplatin (Fig. 8 and Fig. S4 respectively).

Ki67 positive cells were detected in untreated OCM cultured in static (Fig. 8, Fig. S4) or dynamic conditions (Fig. S5A) after 2 days of culture and numbers increased further after 7 days.

Interestingly, cell-laden hydrogels treated with 10 μM cisplatin under dynamic conditions displayed an overall positive staining for proliferating cells comparable to untreated hydrogels after 2 days, however the Ki67 staining was drastically reduced after 7 days (Fig. 8A). This behavior was even more evident for the high dose of the drug (i.e. 100 μM) (Fig. S4).

On the other side, when the tumor tissue was treated under static conditions, there was less Ki67 staining than in untreated hydrogels or under dynamic conditions after 2 days and Ki67 staining was still well evident after 7 days of 10μM.
Fig. 8: Fluorescence images showing immunostaining of Ki67 (green) as index of proliferation and Caspase-3 (red) as marker of apoptosis of SKOV-3 cultured within alginate hydrogels treated with 10 µM cisplatin in static or dynamic conditions (MIVO). The untreated controls were cultured in static conditions. Cells were stained after 2 or 7 days and counter-labeled with DAPI (blue). Scale bar is 500 µm. (N=3 biological replicates; n=2 technical replicates).

drug treatment, especially in the inner part of the hydrogel (Fig. 8A). When the treatment was carried out at a higher drug concentration, Ki67 staining was reduced compared to the 10µM dose (Fig. S4`). These data are in line with the cell viability reduction shown in Figure 3.

The expression of caspase-3 was weak in the untreated control after 2 days and almost undetectable after 7 days in static and dynamic conditions (Fig. 8, Fig. S4 and Fig. S5A`). Positive caspase staining was observed in both static and dynamic conditions after 2 days of 10 µM drug treatment (Fig. 8B); this was much more evident for samples treated with 100 µM (Fig. S4`); interestingly, after 7 days of treatment within the MIVO® device, cell-laden hydrogels displayed a homogeneous spatial distribution of apoptotic cells, while samples treated in static conditions displayed caspase-positive staining mainly in the outer
rim of the OCM, indicating an accumulation of dead cells that could form a physical barrier hindering cisplatin diffusion (Fig. 8B). Again, this was much more evident for samples treated with 100 µM (Fig. S4).

4 Discussion

The ability to rapidly and efficiently screen drugs with a more accurate preclinical tumor model is of great importance in drug development, because currently used assays still have severe limitations and poor predictivity. Considering the limitations of animal experiments to predict human response and the high need for novel drugs, it is necessary to include highly reproducible human systemic tumor models in preclinical analyses to validate more accurately the efficiency of drug candidates.

Recently, regulatory authorities have voiced a common desire to standardize the preclinical tests of drugs by replacing/reducing animal experimentation during early product development with specific in vitro systems, summarized in Table 2 (Daniel et al., 2018). This can be achieved only by strictly linking academic research outcomes with key industry entities to foster the implementation and optimization of relevant in vitro models on a large scale. The production of scientific data supporting the high reliability of alternative animal models is the first step to support industries in reducing time and costs of preclinical research.

<table>
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<th>Tab. 2: Main advantages and limitations of the different tumor models available for drug tests</th>
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<td><strong>Model</strong></td>
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<td>2D culture</td>
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<td>3D scaffold-free spheroid</td>
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<td>3D matrix-assisted tumor model</td>
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<td>3D fluid-dynamic tissue culture (e.g. MIVO® device)</td>
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<td><em>In vivo</em> model</td>
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In our previous work, a fluidic device was adopted to culture a breast cancer tumor model *in vitro*, resembling some crucial steps of tumor growth: cell migration within the 3D hydrogel, cell evasion from the hydrogel and their intravasation into the fluid circuit (Cavo et al., 2018). Likewise, oral administration and the subsequent intestinal passage of other kinds of molecules and drugs can be modeled with the MIVO® chamber, as already reported (Marrella et al., 2020). Starting from these promising results, we have here combined the use of a 3D cell-laden hydrogel as an ovarian tumor model of clinically relevant size with the MIVO® fluidic device to resemble the human circulation and drug extravasation to reach the tumor mass. To test this technological approach, a drug efficacy assay was carried out as a proof-of-principle in parallel to a xenograft model, which represents the current gold standard. Moreover, the 3D tumor models were also cultured under static conditions, resembling the traditional assays using organoids/spheroids.

In order to further decrease the use of animals in pre-clinical research, serum-free medium can be a valuable option to perform *in vitro* cell culture. Along this line, also the use of recombinant antibodies can be desirable, respect to animal-derived ones. In this work, the bovine-derived serum and animal-derived antibodies were used to validate this novel technological approach by comparing these results with data present in the literature and internal to our laboratory practice. In the next future we will move towards the evaluation of the use of alternative animal-free strategies for cellular assays, accordingly with the 3R principles.

Here the ovarian tumor was selected since it has the highest mortality rate of all the gynecological cancers worldwide (Siegel et al., 2012). However, this approach can be extended to many other solid tumors. SKOV-3 were embedded within 3D hydrogels or injected into mice, as previously reported (Cavo et al., 2018; Marrella et al., 2019).

The tumor model is composed of a cell-laden hydrogel. Among polymers, alginate was selected for its well-known advantages like inertness, chemical stability and no intrinsic bioactivity (Khurana and Godugu, 2018), to better focus on the drug penetration mechanisms and compare the *in vitro* model with the scaffold-free xenograft model. Further experiments could investigate increasing the level of complexity of the tissue model by combining different biopolymers and by incorporating multiple cell types associated with the ovarian tumor niche (e.g. fibroblasts, myofibroblasts, pericytes, vascular or lymphatic endothelial cells, and undifferentiated mesenchymal stem cells) to better model tumor microenvironment, which
is in fact a complex, heterogeneous and multi-cellular environment involving dynamic interactions between malignant cells and their surrounding stroma, including both cellular and acellular components.

Cisplatin was selected since it represents, together with its analogs, the first-line chemotherapeutic agent for the treatment of human ovarian cancer, exerting its cytotoxicity by forming DNA-links, which trigger cell apoptosis (Tiwari et al., 2005). The drug concentration tested in vivo (6 mg/kg) was adopted because it represents the "maximum tolerated dose", i.e. the most efficient dose without toxic effect (Aston et al., 2017). Two concentrations were employed in vitro to investigate a concentration-dependent cytotoxic effect and also to enable a comparison with in vivo data. For the higher drug concentration tested (100 μM), we observed that most of the cells died after a few days of culture both in the static and the dynamic conditions indicating excessive toxicity. Differently, with the lower drug concentration (10 μM), the decrease in tumor cell proliferation was greater in the dynamic conditions than in the static ones. Moreover, this drug concentration, which is commonly used to perform in vitro drug efficacy tests (Gao et al., 2015; Tang et al., 2015), enabled to observe within one week the cytotoxic effects of the drug over time comparable with those observed in vivo within three weeks.

It is an important aspect to determine at what concentration and for what duration a drug should be administered in vitro to allow an in vitro-in vivo comparison so that in the future the use of animal models in the preclinical phase can be reduced.

The MIVO® platform allows to drastically reduce the overall experimental time, since there is no need to wait for the tumor tissue to grow in mice after cancer cell injection. Moreover, the platform can cut out two highly time-consuming stages of (i) modeling a xenograft model by growing hydrogels in static conditions in the core volume of the OCM chamber) can induce ovarian cancer progression (Ip et al., 2016).

This is supported by immunostaining against proliferating cells (i.e. Ki67) and apoptotic cells (i.e. caspase-3) in both tissue culture conditions (Fig. 8 and S4). As expected, after 7 days of drug treatment, proliferating cells were observed under static conditions in the core volume of the OCM, while very few cells positive for Ki67 were found within the 3D hydrogels cultured in MIVO®. Moreover, an external barrier of apoptotic cells was stained in static conditions, while cell-laden hydrogels within MIVO® displayed a homogeneous spatial staining of caspase-3. These results were observed at both drug concentrations and were more evident at the higher concentration.

The experimental measurements of cisplatin concentration within the hydrogels through HPLC confirmed the different drug diffusion kinetics within the polymeric matrix: after 7 days of drug treatment, the drug nominal concentration (i.e. 10 μM) was found within the hydrogels that had been cultured in dynamic conditions, while a lower concentration (7 μM) was measured in the hydrogels that had been cultured under static conditions.

These results confirm the key role of the fluid-dynamic environment in resembling the physiological 3D tumor tissue mass and drug transport through a fluid dynamic stream of the blood circulation in vitro. Under these conditions, obtained by

ALTEX preprint Published August 3, 2020
doi:10.14573/altex.2003131
using the MIVO® tissue culture device, the in vitro tumor regression curve resembles the tumor mass reduction measured in vivo though in a shorter time. In fact, the same drug efficacy results were obtained in vitro in just 1 week instead of 5 weeks as in vivo. This is mainly due to a different clearance and distribution of the drug in the two different experimental conditions. These results highlight that the in vivo-like dynamic environment provided by the MIVO® device allows to better resemble 3D tumor tissue perfusion and its culture under a capillary circulation and the systemic drug transport mechanisms, suggesting its potential as a relevant platform for preclinical drug efficacy tests.

In conclusion, the combination of 3D tumor models with MIVO® fluidic device may have great potential as a novel and human-relevant preclinical drug efficacy assay. This could allow improvement of the drug development process and lead to more effective, standardized, fast and ethical in vitro outcomes, finally leading towards significant patients benefits.

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Conflict of interest
S.S and M.A are cofounders and shareholders of React4life S.r.l., C.D and M.M are cofounders and shareholders of MTTlab S.r.l.

Acknowledgements
The authors want to thank Paolo Buratti for cell culture support and Francesca Cella for microscopy analysis. This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 801159.