Patient-derived organoids as individual patient models for chemoradiation response prediction in gastrointestinal malignancies

ABSTRACT

Chemoradiotherapy (CRT) is an important treatment modality for specific gastrointestinal (GI) cancers, as it has been shown to improve clinical outcomes. Recent developments in the neoadjuvant setting such as wait-and-see strategies for rectal as well as for esophageal cancers have even proven that CRT might be an effective organ-sparing treatment. However, due to molecular heterogeneity, only a subset of patients will show a complete response to CRT, which addresses the need for an individualized treatment approach. In recent years, the demand for more physiologically relevant predictive in vitro models has fostered the development of patient-derived tumor organoids.

In this review, we describe the current treatment options for patients with gastrointestinal cancers who are treated with (neo)adjuvant CRT. Furthermore, we provide an in depth-discussion of the organoid technology in the context of predicting CRT response for GI cancers as well as possible challenges for clinical implementation.

1. Introduction

The mucosal lineage of the human gastrointestinal (GI) tract is susceptible to oncogenic transformations due to a high cell division activity, large surface, influence of intraluminal contents and (epi)genetic predisposition. Therefore, GI cancers are highly prevalent and cause significant health burden. Unfortunately, GI tumors frequently become symptomatic at a late stage and therefore, it is not uncommon that complications already exist at diagnosis due to size and relation to nearby anatomical structures. Radical surgical resection is an important treatment for most GI cancers. However, it is often hampered by the stage at diagnosis. Preoperative downsizing by neoadjuvant treatment has become an important strategy enabling curative surgery for patients who otherwise would be at risk for irradicality. An important and effective modality to downsize a tumor is external beam radiotherapy. Advances in radiotherapy techniques have resulted in effective radiation delivery, allowing maximal tumor response with limited damage to surrounding tissues. The addition of chemotherapy radiosensitizes the target tissue, hence consolidating the ionizing radiation-induced DNA damage. Chemoradiotherapy (CRT) is now firmly embedded as a neoadjuvant modality in the curative approach for locally advanced rectal cancer (LARC) and for almost all stages (≥T1bN0) of esophageal adenocarcinoma (EAC) as well as esophageal squamous cell cancers (ESCC). While pathological complete responses are observed in about 20% of LARC patients, 25% of EAC and 50% of ESCC, some patients do not benefit from the preoperative CRT at all. Reliable prediction of the response to CRT, i.e. by using molecular biomarkers, would greatly contribute to an individualized therapy approach for these patients. However, the predictive accuracy of current epi(genetic) biomarkers is hampered by the extensive molecular heterogeneity resulting in multiple subgroups with subsequent misclassification of a considerable percentage of patients. Furthermore, these biomarkers or genetic (expression) profiles are often surrogates and not directly related to the response mechanism itself. The concept of using patients-specific *in vitro* or *ex vivo* models to classify future responders and non-responders allows a one-on-one comparison and subsequently overcomes the issue of molecular heterogeneity. This review aims to give a brief overview of current treatment options for patients with esophageal and rectal malignancies. Furthermore, the usefulness of individual patient models in predicting CRT response as well as the possible advantages, limitations and clinical challenges of tumor organoids as individual patient platforms will be discussed in depth.

2. Clinical perspective

This paper focuses on GI malignancies of which CRT forms an element of the standard multimodality curative treatment schedule. In general, external beam irradiation can only be applied to organs that have a somewhat fixed anatomical position and therefore the small bowel and colon are relatively unsuitable due to mobility of the mesentery as well as peristalsis. There is evidence that shows benefit of neoadjuvant CRT in gastric cancer although this is not advocated in European treatment guidelines [1]. No survival improvement was shown of adjuvant CRT over chemotherapy in gastric cancer in a large randomized trial and a follow up study currently investigates the optimal neoadjuvant schedule in gastric cancer comparing CRT and chemotherapy [2, 3]. We therefore now further discuss esophageal and rectal cancer here in more detail as CRT is widely established for these malignancies.

Esophageal Malignancies

There is a large variability in esophageal cancer diagnosis worldwide. In general, in the Western world, the most prevalent diagnosis is an EAC of the gastroesophageal junction or distal third of the esophagus. Acidic reflux disease, due to elevated intra-abdominal pressure in a patient with visceral obesity, is an important risk factor leading to a rising incidence of this EAC subtype. In Asian countries, the most common type is a squamous cell carcinoma, localized in the upper two-third of the esophagus and associated with smoking habits, but also with consumption of alcoholic beverages, pickled vegetables and hot foods [4]. The treatment strategies for these two histological subtypes are not the same, mainly driven by the markedly higher radiosensitivity of the squamous cell subtype. This is reflected by a roughly two-fold higher complete histopathological response rate after neoadjuvant therapy in ESCC (around 50-55%) as compared to distal EAC (around 20-25%). The oncological benefit of neoadjuvant CRT for EAC as well as ESCC has been established in various studies and was adopted in many treatment guidelines. The CROSS trial showed that preoperative CRT (23 x 1.8 Gray (Gy) with weekly administration of carboplatin and paclitaxel) was superior in terms of overall survival as compared to surgery alone [5-7]. Nevertheless, for distal EAC, evidence also exists for perioperative triplet chemotherapy (i.e. epirubicin, cisplatin and infused 5-FU based on the MAGIC trial data) and recently improved complete response rates (up to 17%) were reported by using the FLOT regimen (5-FU, leukovorin, oxaliplatin, docetaxel) [8-10]. This demonstrates that multiple effective and clinically safe regimens exist and that individual patient models could have a significant role in selecting the most optimal choice. When patients are deemed resistant to both chemo and radiotherapy, upfront surgery could also be considered.

The esophagus measures about 30 centimeters and crosses many important anatomical structures (i.e. trachea, pulmonary vein, recurrent nerves, aorta). Therefore, the mass effect of lesions at various levels can cause various symptoms. From a surgical point of view, patients with ESCC require a complete esophagectomy with 3 field (abdominal, chest and neck) lymphadenectomy. Continuity is preferably restored with a gastric conduit which is sewn to the short esophageal remnant in the neck. For a distal EAC, the upper third of the esophagus can be spared and a two-field (abdomen and chest) lymphadenectomy is performed with the gastric conduit connection in the chest at the level of the azygos vein. Surgery for esophageal cancer is extensive and has considerable perioperative morbidity and long-term functional problems. Organ-sparing strategies to avoid surgery after CRT is an attractive option for patients with a complete response to CRT. However, less is known about long-term oncological and functional outcomes and clinical studies are evaluating the safety of an organ-sparing, so-called “surgery-as-needed” or “watch-and-wait” approach for EAC and ESCC [11, 12]. For ESCC this approach is more widely accepted because of the relatively high complete response rate and the increased burden of surgery as described above. However, the challenge is to reliably assess a complete response without performing surgical resection. Up to now, all available diagnostic modalities (endoscopic ultrasonography, (bite-on-bite) endoscopic biopsies, (PET-)CT)) either individually applied or combined have limited sensitivity/specificity to allow clinical implementation. Therefore, individualized patient models could also have an important additional value in guiding organ-sparing treatment for esophageal cancer.

Rectal Cancer

The incidence of rectal cancer in the European Union is 125 000 per year, which is 35% of the total colorectal cancer incidence, and is predicted to increase in both genders [13]. Incidence

rates vary according to age and, while decreasing in older patients, currently about one-third of rectal cancer patients is younger than 55 years [14]. Similar to esophageal cancer management, rectal cancer treatment concerns a multimodality approach consisting of neoadjuvant radiotherapy or CRT followed by radical resection, being a total mesorectal excision. Based on risk factors, adjuvant chemotherapy can be considered.
It has been shown that neoadjuvant radiotherapy followed by a total mesorectal excision reduces local recurrence rates for both early stages and LARC [15-17]. However, there is no consensus on the most effective radiotherapy regimen. The two most commonly used neoadjuvant treatment protocols are short course radiotherapy (SCRT) and long course CRT.
In the SCRT regimen, patients are irradiated with 25 Gy in five fractions, typically followed by total mesorectal excision within one week. Long course CRT typically consists of 45 to 50 Gy in 25 fractions, combined with a fluoropyrimidine based chemotherapy as a radiosensitizer. For patients with resectable disease, no difference between SCRT and long course CRT in local recurrence rates could be demonstrated [18, 19].
To date, CRT is the neoadjuvant treatment of choice for rectal tumors threatening the surgical resection margin, due to its capacity to induce volume reduction and / or tumoral downstaging. Moreover, in many countries CRT is the preferred neoadjuvant treatment for LARC (stage II and III). Since the incorporation of restaging examinations performed after CRT, it has been shown that a pathological complete response occurs in 15 to 30 % of patients [20, 21]. This has, similar as in ESCC, led to the development of an organ preserving treatment in which the patient is not operated on but is subjected to meticulous follow-up by means of MRI and endoscopy and digital rectal examination [20, 22].

Total mesorectal excision is associated with a six-months mortality of approximately 5 % for patients aged 65 to 74, rising to about 13 % for patients aged 75 to 84 [23]. Anastomotic leakage rates, leading to pelvic sepsis, are as high as 15 to 20 % and often require reintervention [24, 25]. The so-called “low anterior resection syndrome”, defined as a “disordered bowel function leading to a detriment in quality of life (QoL)”, occurs in about 90 % of patients after total mesorectal excision [26, 27]. Autonomic nerve damage can also lead to urinary and sexual dysfunction in 30 % of patients, further reducing QoL [28, 29]. Due to these morbidity rates, the watch and wait strategy has gained popularity, prompting research on optimizing neoadjuvant protocols. The “Stockholm 3-trial” randomized patients with resectable rectal cancer by 3 different neoadjuvant protocols, being SCRT followed by total mesorectal excision after 1 week, SCRT followed by total mesorectal excision after 4-8 weeks and CRT followed by total mesorectal excision after 4-8 weeks. This trial demonstrated that SCRT with total mesorectal excision after a 4 to 8 weeks delay has similar oncological outcome with fewer postoperative complications. Moreover, pathological complete responses were observed in 11% of the patients [30]. In an attempt to avoid surgery-related morbidity, several trials have been performed on the implementation of neoadjuvant treatment protocols for early stage rectal tumors that are primarily treated by surgery. Examples are the “CARTS-study”, studying outcome of CRT followed by local excision instead of total mesorectal excision, and the “TREC-trial”, comparing total mesorectal excision with SCRT followed by local excision [31, 32]. However, the optimal neoadjuvant approach for organ-preservation in early rectal cancer remains unknown and is currently being investigated in the STAR-TREC trial. Other trials evaluate the effect of adjuvant treatment protocols. The TESAR-trial randomizes patients after radical local excision of an early stage rectal carcinoma between “salvage total mesorectal excision” and adjuvant CRT [33].

The aforementioned studies aim to evaluate a standardized, i.e. a non-individualized, rectal cancer treatment. The tumor biology, expressed for example as molecular subtype, is not taken into account. Guinney et al. have established 4 consensus molecular subtypes, each having their own clinicopathological characteristics [30]. They found that about 13% of the tumors had mixed features, possibly representing a transition phenotype or intra-tumoral heterogeneity. The same group has stated that the complexity of the cancer genome and the interactions between tumor cells and the different treatment modalities warrants a 'multi-gene, multi-drug' model instead of a 'one gene, one drug' approach when considering chemotherapy for colorectal cancer [34]. A similar statement can be made about radiotherapy or CRT.

The variability in treatment outcome of rectal cancer patients can most likely be explained by the tumor heterogeneity. This heterogeneity implies that some rectal cancer patients are under-, and some are overtreated. Therefore, rectal cancer patients, similar to esophageal cancer patients, would greatly benefit from a tailored treatment approach based on *in vitro* testing on an individual patient model.

3. The organoid technology in the evolving era of personalized oncology

Until recently, traditional treatment strategies have long relied on the one-size-fits all-approach. However, nowadays, clinical evidence has shown that tailoring cancer treatment to individual molecular profiles (e.g. genetic alterations), significantly improves the QoL and survival [35, 36]. But even though this therapeutic paradigm shift is gaining popularity in various malignancies, current predictive (molecular) biomarkers fail to prospectively distinguish CRT-sensitive from CRT-resistant patients [37-40]. Therefore, over the past years, considerable efforts have been made in developing physiologically relevant and individualized preclinical models that can accurately predict treatment response [41]. The use of traditional two-dimensional (2D) cancer cell lines is still considered as the gold standard to study the underlying mechanisms of cancer growth. On the other hand, it is widely recognized that 2D culture models fail to recreate the complex cell-cell interactions and heterogeneous environment present in the original tumor [42]. To overcome these limitations, patient-derived xenografts (PDX) emerged as attractive translational models because of their preservation of the *in vivo* cellular architecture (e.g. tumor vasculature) [43, 44]. Moreover, besides promising applications in drug development and biomarker discovery, recent evidence has suggested that PDX models also hold great potential to predict CRT response [45]. Nevertheless, the clinical implementation of PDX models as avatars for personalized medicine is currently hampered by the time consuming (4-8 months) engraftment process and limited success rate. Additionally, in the setting of predicting CRT response, it should be addressed that the original (human) tumor-associated stroma can be replaced by murine stroma, which can affect the physiological properties and treatment response [46].

The urgent need for alternative predictive *in vitro* models to foster the concept of personalized oncology has led to establishment of patient-derived tumor organoids (PDO). Although there is no consensus definition of 'tumor organoids', they can be described as three-dimensional (3D), self- organizing multicellular constructs that can be derived from both tumor tissues (e.g. peri-operative endoscopic biopsies and surgical resections) and stem cells [47, 48]. The basis for organoid growth relies on the stimulation and propagation of cancer stem cells (CSC). Accordingly, niche-recapitulating and tissue specific growth factors are added to the culture medium to either stimulate or inhibit numerous signaling pathways involved in sustaining the self-renewal capabilities of the CSC. For instance, the addition of Wnt, R-spondin-1 and

CHIR99021 orchestrates stem cell renewal by stimulating the Wnt/β-catenin signaling. Conversely, noggin supplementation represses the Bone Morphogenetic Protein (BMP) signaling pathway, which is commonly involved in restricting self-renewal and promoting proliferation, differentiation and migration (i.e. epithelial-to-mesenchymal transition). Other essential media components to culture GI tumor organoids include B27, nicotinamide epidermal growth factor (EGF), transforming growth factor-β (TGFβ) inhibitor (A83-01), p38 inhibitor (SB202190), fibroblast growth factor 10 (FGF10), gastrin and prostaglandin E_2 (PGE_2). However, it is noteworthy that the niche factor requirements are tissue specific and largely depend on the mutational status (e.g. rectal tumor organoids propagate in the absence of exogenous Wnt due to activating mutations in the Wnt/β-catenin signaling) and tumor stage [49, 50].

So far, the organoid technology has been applied to model a wide variety of GI malignancies such as esophageal, colorectal, liver, gastric and pancreatic cancer [51]. The major advantage of using these 'patients in the lab' is that they closely recapitulate the histological architecture, cellular heterogeneity, and mutational landscape of the parental tumor tissue they originate from [52, 53]. Combining these auspicious features with their relatively short generation time (compared to PDX models) and high-throughput screening compatibility, it is unsurprising that this model has the potential to become the foundation for guiding multimodal treatment decisions in the future.

4. Recent advances of gastrointestinal PDOs as predictors of chemoradiation response

Esophageal cancer PDOs

Since the introduction of the organoid technology in basic and translational oncological research, only a few attempts have been made in developing patient-derived esophageal tumor organoids. However, despite our increased knowledge in terms of generating and cultivating 3D *in vitro* models, the success rate of establishing representative esophageal tumor organoid cultures varies greatly (Table 1) [54]. Karakasheva et al. attribute their success for generating EAC PDOs to the addition of Wnt signaling potentiators (gastrin and CHIR99021), Y-27632 (Rho-kinase inhibitor) and N-2 supplement to their medium, providing them with a success rate of 80% [55]. By contrast, Li et al. report a success rate of 31% for generating EAC PDOs [56]. For the latter, it should be taken into account that they included neoadjuvant treated patients, having frequently less (viable) tumor material available for organoid derivation. In the same study, Karakasheva et al. report a success rate of 60% for ESCC organoids using a more basic medium compared to EAC (Table 1), corresponding with the success rate of an earlier study by the same group [57]. Altogether, esophageal cancer PDOs can be generated with an acceptable efficiency for their use in a clinical setting.

Li et al. examined whether the *in vitro* sensitivity to the ECF (epirubicin, cisplatin and 5-fluorouracil) chemotherapy regimen matched the *in vivo* response [56]. In general, the majority (6/8) of the PDOs were insensitive to the administered chemotherapy regimen, which was consistent with the poor *in vivo* response observed in the clinic. In contrast, two PDOs derived from pre-treated (resistant) patients showed an unforeseen treatment sensitivity to these agents. However, the authors suggested that this conflicting outcome could be a result of the clonal selection process that inevitably occurs during organoid culturing and eventually may have influenced therapy response (see section 5; challenges).

The abovementioned findings illustrate the potential of PDOs as predictors of chemotherapy response. For many patients, neoadjuvant CRT (CROSS regimen) is considered as the backbone of locally advanced esophageal cancer treatment. Unfortunately, so far, only a few organoid-based studies have been published that integrated both chemotherapy and radiotherapy. However, one research group managed to model a multimodal approach *in vitro* by treating EAC organoids with one dose of paclitaxel/carboplatin and subsequently irradiating them with seven fractions of 1 Gy [58]. Interestingly, the authors also used an unconventional and rather basic growth medium composition to culture the PDOs. As it has been previously described that different components of the traditional organoid medium could conceivably alter treatment response *in vitro*, this growth factor reduced medium might be an evidential solution to circumvent this issue. Either way, it should be considered that they used a PDX graft expansion approach to establish the PDOs, which could have influenced the organoid growth in the absence of the traditional self-renewal promoting compounds. Altogether, first evidence has been provided that esophageal PDOs are capable of modeling chemo(radiation) response *in vitro*.

Rectal cancer PDOs

Presently, rectal PDOs have been successfully propagated from patients with primary, metastatic or recurrent disease. Remarkably, compared to the overall low success rate of upper GI organoids, the organoid-forming efficiency of rectal PDOs has shown to be substantially higher (Table 1) [59]. Yao and colleagues succeeded in expanding a library of 96 rectal PDO lines derived from treatment naïve patients with LARC, with a success rate of 85.7%, [60]. Interestingly, the authors also validated the use of rectal PDOs as predictive models of CRT response. They showed a strong correlation, with an accuracy of 84.43% and an AUC of 0.88, between the clinical outcome and the *in vitro* sensitivity to at least one of the treatments (irradiation, 5-FU and Irinotecan). Interestingly, they also used an innovative approach to determine the *in vitro* threshold for response. Generally, based on Youden's index and bootstrap samples, they calculated the mean size recovery ratio after treatment (using bright-field imaging) to empirically determine the optimal cut-off value for response. Furthermore, they retrospectively correlated the *in vitro* sensitivity values with the clinical tumor regression grade data of the patient after treatment to validate the reliability of the established PDO platform. A similar study by Ganesh et al. implemented another approach to compare the PDO response with clinical outcome [61]. Basically, they obtained a significant correlation between 5-FU or FOLFOX *in vitro* chemosensitivity (normalized AUC) and a patient's progression free survival. On the other hand, the *in vitro* radiosensitivity ($\geq 75^{th}$ percentile of AUC is considered as resistant, $\leq 25^{th}$ percentile is sensitive) was correlated with a clinical endoscopic assessment that has been conducted before and after radiation. Even though both studies highlighted the potential of rectal PDOs as promising preclinical predictive tools, their treatment schedules were not consistent with standard clinical practice. For instance, single radiation doses were used to treat the PDOs instead of fractionated regimens. Notwithstanding, Janakiraman and colleagues designed a treatment strategy combining a 5 x 2 Gy fractionated radiotherapy regimen (representative for the 1.8 Gy used in clinic) with a single dose of 5-FU [62]. They also managed to assess the *in vitro* sensitivity to this CRT regimen within a short period of time, which eventually opens the door for clinical implementation.

Ref.	Type	Success rate	R-spondin-1	Noggin	Wnt	EGF	FGF10	Gastrin	PGE_2	A83-01	SB 202190	B27	N2	Nicotinamide	NAC	Y-27632**	Additional supplements	Remarks
Li et al.	EAC	10/34 (31 %)	●	●	●	●	●	-	-	●	●	●	-	●	●	-		PDO cultures were generated from surgical resected EAC tissue samples from chemotherapy resistant patients or treatment naïve patients.
Karakasheva et al. *	EAC	5/6 (83 %)	●	●	●	●	●	●	-	●	●	●	●	●	●	●	CHIR99021 (GSK-3 inhibitor)	PDO cultures were established from surgical resected tissue samples or biopsies.
Ebbing et al.	EAC	2/2 (100 %)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	heparin, insulin, β-mercaptoethanol, trace elements B and C	PDO cultures were established from PDXs from biopsies from treatment-naïve EAC patients.
Kijima et al. *	ESSC	11/16 (68 %)	●	●	●	●	-	●	-	●	●	●	●	●	●	●		PDO cultures were established from endoscopic biopsies from treatment-naïve ESCC (or OPSCC) patients. PDOs from adjacent normal mucosa were generated with a success rate of 66.7% (12/18). The majority of tumor biopsies produced predominantly non-neoplastic over neoplastic structures, indicating that the medium is not selective for tumor organoids. Successful formation of organoids was significantly associated with poor therapy response in the patient.
Karakasheva et al. *	ESSC	15/25 (60 %)	●	●	-	●	-	-	-	-	-	●	●	-	●	●		PDO cultures were established from surgical resected tissue samples or biopsies. Only ~10% of primary ESCC PDO can be passages ≥5 times.
Yao et al.	LARC	96/112 (85 %)	●	●	-	●	-	●	●	●	●	●	●	-	●	●	Niacinamide	PDO cultures were established from tissue biopsies (1 - 5 mm) from treatment-naive LARC patients.
Ganesh et al.	LARC	65/84 (77 %)	-	-	-	●	-	●	-	●	●	●	●	●	●	-	Wnt, noggin, R-Spondin-1 withdrawn after passaging. Supplements required for normal PDO growth.	PDO cultures were established from surgical resected tissue samples or biopsies from treatment-naïve (n = 22) and pre-treated (n = 43) patients. Normal rectal PDOs were generated from normal adjacent tissue from 51 patients.
Janakiraman et al.	LARC	4/4 from biopsy (100 %) 9/10 from PDX (90 %)	-	-	-	●	-	●	●	●	●	●	-	●	●	●		PDO cultures (n = 4) were established directly from biopsies from treatment-naïve patients. Other PDO cultures (n = 9) were established from low passaged PDX cancer cells.

Table 1. Generation of 3D organoids. Medium supplementation for each tumor type. Note: Basic components (advanced Dulbecco's modified Eagle medium (DMEM)/F12, antibiotics, GlutaMAX, HEPES) are not included in this table. A83-01 (TGFβ kinase/activin receptor-like kinase (ALK 5) inhibitor); SB20190 (p38 Mitogen-activated Protein Kinase inhibitor); Y-27632 (Rho-associated Protein Kinase (ROCK) inhibitor); EGF (epidermal growth factor); PGE_2 (prostaglandin E_2); NAC (N-acetylcysteine); FGF10 (fibroblast growth factor 10); PDO (patient-derived organoid). * From same research group. **Only added to the culture medium when starting from single cells to promote organoid formation.

Ref.	Type	Therapy	Treatment schedule	Readout	TAT	Correlation with clinical response			Remarks
						Ex vivo	In vivo	Correlation	
Li et al.	EAC	**Chemotherapy**: ECF regime (4µM cisplatin 10µM 5-FU and a 7-point half log dilution series of maximum 10µM epirubicin)	**Chemotherapy:** 6 days drug incubation.	**Endpoint:** CellTiter-Glo viability assay	NS	AUC	TRG following chemotherapy (ECX, ECF,CF)	Ex vivo response matched clinical outcome in 6/8 PDOs.	Most PDO cultures were established from neoadjuvant treated patients with TRG > 2 classified as resistant to therapy.
Yao et al.	LARC	**Radiotherapy**: 8 Gy single dose **Chemotherapy**: Fixed dose 5-FU (10µM) and CPT-11 (10µM)	**Radiotherapy:** Follow-up 24 days after treatment **Chemotherapy:** 3 days drug incubation, follow-up until day 24.	**Kinetic and endpoint**: Size of organoids as a measure of organoid survival (microscopy)	NS (> 4 weeks)	PDO size change (cutoff 36.42 % on day 24)	TRG following NACR (5-FU with without CPT-11; cutoff TRG 2)	Matched clinical outcome with an accuracy of 85 % (68/80), sensitivity of 78 % and specificity of 92 %.	Patients achieved a good clinical response when their tumor organoids were sensitive to at least one of the three treatment components tested ex vivo (RT, 5-FU and CPT-11).
Ganesh et al.	LARC	**Radiotherapy:** 0-8 Gy (2 Gy interval dose response) **Chemotherapy**: 5-FU (0-50µM) and FOLFOX (25:5:1)	**Radiotherapy:** Cell viability assessed 8-13 days after treatment. **Chemotherapy:** 3 days drug incubation, cell viability was assessed at day 6.	**Endpoint:** CellTiter-Glo viability assay	6-12 weeks (includes *in vivo* PDX sensitivity)	AUC	**Chemotherapy:** PFS (from 5-FU-based start date) **Radiotherapy:** Endoscopic tumor assessment (percent circumference) immediately before and after radiation.	**Radiotherapy:** Ex vivo sensitivity corresponds to clinical radiotherapy responses. **Chemotherapy:** Strong correlation (spearman r = 0.86, P = 0.024, n = 7).	Endoluminal rectal cancer xenograft (PDO implantation) showed that the patterns of metastasis also correlated with the metastatic sites seen in the patient and in vivo chemosensitivity corresponded with the patient's clinical course.
Janakiraman et al.	LARC	**Chemoradiation**: 2 Gy fraction dose/ 5-FU (1µM)	**Chemoradiaton**: Overnight 5-FU treatment followed by 2 Gy RT daily for 5 days. Cell viability assessed 48h after RT.	**Endpoint:** CellTiter-Blue viability assay	NS (> 4 weeks)	% Cell viability	TRG following NACR (5-FU)	Treatment outcomes in PDO models replicated the clinical 5-FU/RT response in the corresponding patient tumors ($p < 0.05$).	Single 5-FU or RT treatment did not correspond with the clinical response in the patient.

Table 2. Correlation of *ex vivo* standard of care therapy response with *in vivo* response. PDO: Patient-derived organoid; TRG: Tumor regression grading; NACR: neoadjuvant chemoradiation; pCR: pathological complete response; TAT: turnaround time from biopsy until ex vivo treatment response; NS: not specified; PFS: progression free survival; 5-FU: 5-fluorouracil; FOLFOX: folonic acid, 5-FU and oxaliplatin; ECF: epirubicin, cisplatin and 5-FU; ECX: epirubicin, oxaliplatin and capecitabine; CPT-11: irinotecan.

5. Challenges for clinical implementation of PDOs in diagnostic processes and clinical decision making

Emerging role of the tumor microenvironment in modulating therapy response

Although previous studies have shown that PDOs are able to successfully predict patient response, it should be noted that in 10-30% of the cases, the *in vitro* response did not match clinical outcome. From a clinical perspective, it is paramount to obtain the highest negative predictive value, sensitivity and specificity possible in order to guarantee optimal treatment decisions for each patient. However, up to now, PDO studies have not been able to achieve this predictive performance. The most conceivable explanation for this limited predictive value (in a subset of patients) is that current studies do not take the potential effects of the tumor microenvironment (TME) and immune system on therapy response into account (Figure 1). For instance, it has been known since 1969 that radiotherapy might interact with the immune system, leading to both local and systemic (abscopal effect) antitumor responses [63]. Therefore, it would be of great interest to integrate the immune compartment into the organoid platform hence enabling a more accurate prediction of clinical outcome. Notably, in 2018, Neal and colleagues succeeded in establishing an advanced organoid platform that can model the native cellular diversity and architecture of the TME [64]. By using the innovative air-liquid interface technology, they were able to preserve the endogenous immune compartment and stromal elements of the original tumor. Considering that certain cell types of the TME (e.g. regulatory T cells and tumor-associated macrophages) are known to modulate CRT response, such an organoid platform provides an opportunity to more reliably assess individualized treatment responses. However, this remains to be explored for esophageal and rectal cancer [65, 66]. Even though this study is a commendable step towards establishing more physiological relevant *in vitro* models, the development of such an advanced organoid co-culture platform would be rather time consuming and labor-intensive, eventually limiting its clinical applications.

Another dynamic component of the TME is the cancer associated fibroblast (CAF). Although it is still under debate whether CAFs have tumor-promoting or suppressive properties, it has been demonstrated that the presence of CAFs is associated with CRT resistance and poor clinical outcome in esophageal and rectal cancer patients [67-70]. In accordance with these clinical observations, a recent study has demonstrated that patient-derived EAC-associated fibroblasts confer CRT resistance in esophageal tumor organoids through the paracrine secretion of IL-6 [58]. Of note, in certain circumstances (e.g. small biopsies), it is difficult to isolate a sufficient amount of CAFs from an individual patient. However, it has previously been shown that a co-culture setting with unmatched (or commercially available) CAFs is still more physiologically relevant compared to a monoculture set-up [71-73]. Even though this partially diminishes the autologous power of the co-culture setting, it would be more feasible and practically relevant to integrate widely available unmatched CAFs into the organoid platform. Taken together, the abovementioned findings indicate that different cellular components of the TME could impact the therapeutic response *in vitro.* This emphasizes the need to further explore the feasibility, translatability and clinical relevance of organoid co-cultures as more physiologically relevant predictive platforms. Additionally, apart from the cellular components, the TME is also characterized by the presence of intratumoral sub-regions with deprived oxygen levels, referred to as hypoxic regions [74]. Moreover, it has been shown that the adaptive response (mediated by O_2-sensitive hypoxia inducible factors) to these deprived oxygen conditions may provide the tumor cells a survival advantage, leading to chemotherapy

and radiotherapy resistance [75, 76]. This dynamic and adaptive feature has also been observed *in vitro* where esophageal cancer cells, cultured under hypoxic conditions, were significantly more radioresistant compared to the control group (normoxic conditions) [77, 78]. This observation could eventually raise the question whether the organoid culturing conditions (hypoxic vs normoxic) should be tailored to the individual intratumoral oxygenation levels of the patient. In line with this, a recent study by Fujii and colleagues has shown that a small subset (5/40) of their patient-derived colorectal tumor organoid panels required a hypoxic environment to successfully grow, which underlines the inter-individual variance in niche dependency [79]. However, whether the inclusion of hypoxia strengthens the predictive power of the PDO platform remains to be verified.

Effects of growth medium

To date, different media formulations have been described to establish GI organoids (Table 1). Nonetheless, there are some drawbacks regarding this variance in organoid media composition. The main concern is that removal/addition of certain growth factors could dramatically alter therapy response *in vitro* (Figure 1). For example, it has been shown that an increased Wnt signaling drives chemotherapy and radiotherapy resistance in various GI cancers [80-83]. In line with this, an intriguing study by Zhao and colleagues has provided evidence that Wnt1 pretreated esophageal cancer cells were more radioresistant compared to the untreated cells [84]. Furthermore, a recent study has demonstrated that inhibition (e.g. by noggin) of the BMP signaling pathway attenuates radiosensitivity of colorectal CSCs [85]. Conversely, stimulation of the BMP signaling cascade has shown to sensitize both naïve and chemoresistant colorectal CSCs to oxaliplatin/5-FU treatment [86]. It is further noteworthy

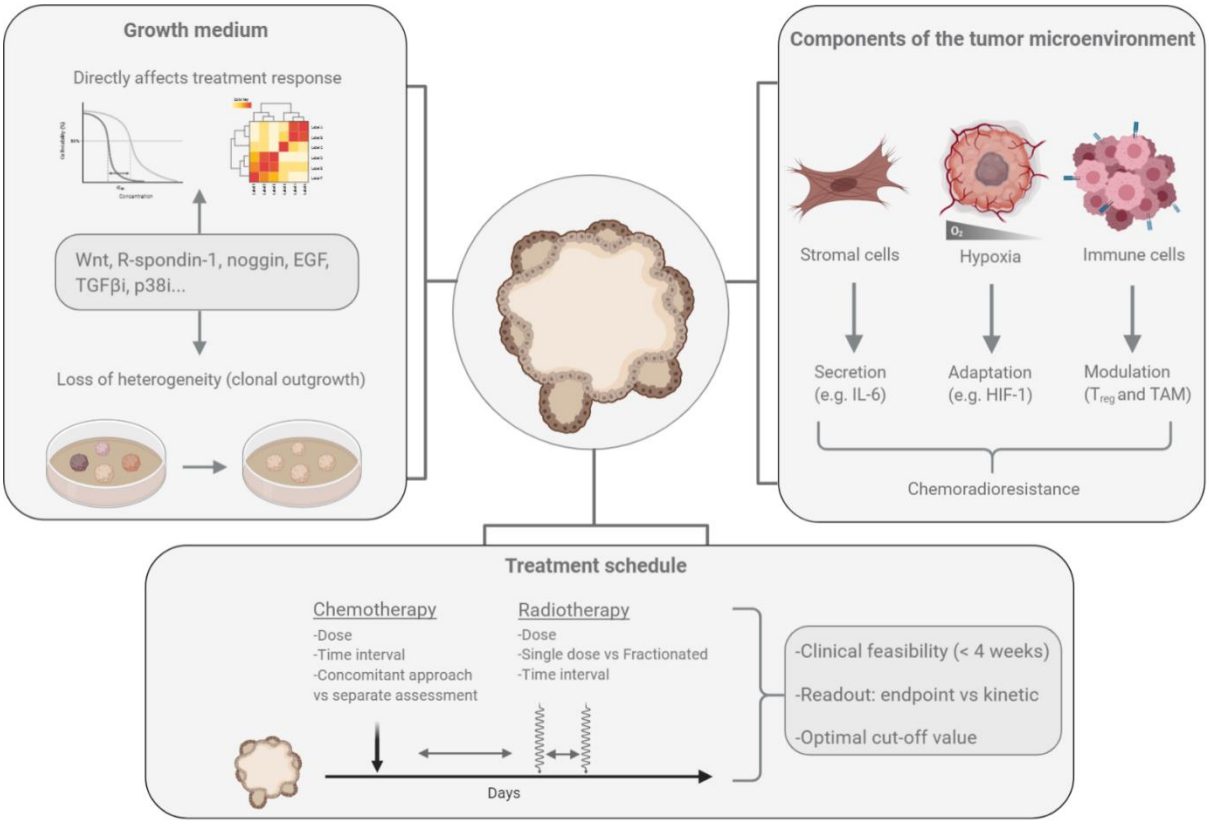

Figure 1. Challenges for clinical implementation of patient-derived organoids in diagnostic processes and clinical decision making.

that, in the two pioneering studies related to predicting CRT in rectal cancer organoids, Yao et al. have used noggin supplemented medium whereas Ganesh et al. have withdrawn this compound after passaging [60, 61]. Considering the striking effects of the BMP signaling on influencing organoid growth and CRT response, it should be highlighted that noggin addition/withdrawal could conceivably affect the experimental outcome. Nevertheless, further studies are needed to fully elucidate the underlying mechanisms.

In addition, besides the direct effects of the different growth factors on therapy response, it is also suggested that the media composition passively selects for subclones that harbor specific oncogenic driver mutations. For example, the use of EGF-depleted medium has shown to stimulate the outgrowth of KRAS mutant clonal subpopulations within the organoid culture [87]. Over time, this growth factor-mediated clonal selection could partially diminish the original intratumoral heterogeneity, which might influence treatment response *in vitro*. Taken together, these findings underscore the need for caution when comparing therapy response in different studies and different tumor types. Therefore, standardized organoid growth media formulations need to be implemented to empower the reproducibility and translatability of PDOs as predictive *in vitro* models.

Assay limitations

One of the major challenges in establishing a predictive organoid platform is to integrate an appropriate readout that enables an accurate translation of the *in vitro* findings to the clinic. Since the introduction of organoids in oncological research, several assays have been developed to study these multicellular constructs [88-91]. The current gold standard for organoid research is the bioluminescent-based CellTiter-Glo 3D endpoint assay, which quantifies the extracellular ATP levels following lysis as an indicator of cell viability [92]. However, despite the reproducibility and sensitivity of this assay, it lacks the ability to distinct a cytostatic from a cytotoxic response [93]. In terms of clinical translatability, this could be a limiting factor because chemotherapy and radiotherapy are known to induce cell cycle arrest and cellular senescence, which cannot be distinguished from a cell death response [94, 95]. Moreover, it is known that metabolic (e.g. senescent vs non-senescent cells) and growth-rate variations could further confound the experimental outcome of this readout [96, 97]. However, this biological variability can partially be bypassed through corrections such as the growth rate inhibition and the normalized drug response metrics [98, 99].

Another commonly used method to assess treatment response is to quantify the number/size of the organoids over time using a bright field microscopy. Even though this image-based approach could be helpful to dynamically monitor organoid growth, it has been demonstrated that a reduced viability upon treatment does not always correlate with a reduction in organoid size [100]. Considering that current readouts do not always extract enough information and therefore fail to fully exploit the potential of the organoid model, there is a strong interest in developing more advanced organoid-based high-throughput assays. Furthermore, aside from the readout, it has been shown that the treatment schedule itself could substantially influence CRT sensitivity *in vitro* [101, 102] (Figure 1). So far, the majority of the organoid-based studies integrated single-dose radiation treatment schemes to predict patient response. However, the disadvantage of this treatment set-up is that single-dose radiation schedules disregard the effects of repopulation, redistribution and repair (three of the 6Rs of radiotherapy) on therapy response, which are subsequently known to occur *in vivo* [103]. Moreover, most of the

predictive *in vitro* studies also independently assessed radiotherapy and chemotherapy sensitivity (Table 2). Regrettably, this separate assessment does not take the synergistic effects (i.e. radiosensitization) of both treatments into account [104]. In line with this, a study by Driehuis et al. has shown that the addition of cisplatin improved the *in vitro* response to radiotherapy in head and neck squamous cell carcinoma PDOs [105]. Accordingly, the implementation of multiple treatment modalities and fractionated radiation regimens might be more applicable to the clinical setting. Nevertheless, it remains to be elucidated whether the integration of a more clinically relevant treatment approach will improve the predictive value and reliability of the organoid platform. Lastly, another issue is that for clinical purposes, the *in vitro* sensitivity to the treatment must be assessed within a certain frame (usually treatment starts within 4-6 weeks after diagnosis). Considering that some studies integrated time-consuming approaches to examine the *in vitro* sensitivity of the PDOs, further protocol refinements (e.g. shortened assays and decreasing the time for organoid outgrowth) are required. Therefore, alongside with establishing the predictive value, streamlining the diagnostic process for PDO therapy-response assays should be taken into account to ensure future clinical application.

Conclusive remarks

It has become increasingly clear that the rapidly evolving landscape of personalized cancer medicine needs to be accompanied by the development of individualized tumor models. Especially for EAC, ESCC and LARC a tailored treatment could lead to large improvements and possible omission of high-risk surgery for several patients. This unequivocal need of a more personalized approach combined with the current limitations of traditional preclinical tumor models have eventually fostered the development of PDOs. To date, it is beyond question that the organoid technology has revolutionized basic and translational oncological research in terms of modeling tumor growth and unraveling novel drug targets. Moreover, recent studies demonstrated the enormous potential of PDOs in predicting individual treatment responses for patients diagnosed with esophageal or rectal cancer. However, despite the numerous applications and promising preliminary results, the clinical implementation of PDOs for CRT response prediction is still hampered by several issues, which we have addressed in this review. Nevertheless, it is self-evident that with further fine-tuning, we will be able to fully unlock the potential of PDOs as preclinical predictive platforms.

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