Exploiting a human intestinal organoid platform for development of personalised medicine for colon cancer

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Abstract

The colonic epithelium forms a vital barrier between harmful luminal contents and the underlying host tissues. It is comprised of millions of invaginations called crypts, at the base of which resides a stem cell population which proliferates and gives rise to all cell types found in the gut. The intestinal epithelium is the most rapidly renewing tissue in the human body, and unsurprisingly it is the site of initiation for a number of diseases including colorectal cancer and Inflammatory Bowel Diseases. Notwithstanding advances in chemotherapeutic strategies, patients with colorectal cancer currently have less than a 50% survival rate after 5 years. Further, the current treatment strategies are highly cytotoxic for all rapidly renewing tissues, and therefore have dose-limiting side effects. This highlights a grave need for the development of novel and better therapies and treatments. Developing a new model system for which these therapies are tested on, is another vital step in the fight for colorectal cancer treatment. The Williams' Laboratory, have developed a patient-matched organoid and tumouroid personalised medicine pipeline to be used for the investigation of novel chemotherapeutic strategies for CRC. The aim of this thesis was to assess the efficacy of a personalised medicine pipeline, and study the implications that inhibitors have on key pathways that are highlighted by the pipeline.

Using whole exome sequencing the mutational status of key homeostatic genes were determined in four patient matched tumouroid lines (UEA003, UEA005, UEA006 and UEA007). Using cell viability assays the effect of chemotherapy (5-FU) and these key pathway inhibitors were determined on the patient-matched organoid and tumouroids.

We observed patient-specific differential sensitivity to standard of care chemotherapy drugs, for example tumouroids derived from patient UEA005 were highly sensitive to low concentrations of SOC chemotherapy, whereas at low concentrations the organoids retained viability. Other tumouroid lines showed less sensitivity to low concentrations of SOC chemotherapy, suggesting that adjuvant or different therapies might benefit these patients more. Taking these factors into consideration holds promise to benefit patient care, in that patients who show little sensitivity to a specific chemotherapy drug could potentially be spared treatment and side effects in the clinic.

The patient-matched organoid and tumouroid system showed promise for the testing of standard of care chemotherapy and inhibitors based upon their mutational status. However further investigation to determine if findings in this thesis matched that of the clinic would be needed. The findings in this thesis could help identify promising next steps for the development of personalised medicine.

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Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that is has not been previously included in a thesis, dissertation or report submitted to the university or any other institution for degree, diploma, or other qualifications.

Jordan Champion
Master by Research candidate

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Preface

There are over 40,000 new cases of colorectal cancer (CRC) and 16,000 related deaths per year in the UK. Notwithstanding advances in surgical and chemotherapeutic interventions, only 50% of individuals diagnosed with CRC will live for more than 5 years. Therefore, there remains a grave need to advance our understanding of this disease and improve both chemotherapy and chemoprevention strategies. The healthy gut is the most rapidly renewing tissue in the human body, and this has proven to be a major limitation of current chemotherapeutic strategies because they target highly mitotic cells. Unfortunately, they are unable to distinguish between healthy and diseased tissue, leading to dose limiting side effects such as mucositis. Chemotherapy induced mucositis is a significant burden for patients undergoing treatment. Mucositis results in inflammation and ulceration of the epithelial lining, including the gut and the mouth (Thomsen and Vitetta, 2018). Promising findings in CRC cell lines indicate that specific genetic mutations are able to dictate response to chemotherapy (Barretina *et al.*, 2012). However, translating this to the clinic proves difficult due to the cell lines being isogenic, meaning the cell lines harbour the unique genetic mutations that are harboured by the specific patient model they are derived from (Torrance *et al.*, 2001). Whereas when patients present in the clinic the patient's tumour will already harbour multiple mutations that might not fit the model used in the lab. This highlights the need for model systems with different mutational profiles mimicking what is observed *in vivo*. Seminal work carried out by Van De Wetering *et al.*, (2015) indicated, using a 3D biobank of cancer organoids, that the mutational profile of tumour-derived organoids conferred differential sensitivity to chemotherapeutic agents. Mutations were commonly observed in the APC gene, which has been detected in ~80% of CRC cases. These were the first steps needed in the field to bridge the gap needed between 2D models and what was observed in clinic (Van De Wetering *et al.*, 2015).

The Williams Laboratory has developed a biobank of patient-matched organoids and tumouroids; healthy organoids are a derivative of the patient's non-involved mucosa whilst tumouroids are from the tumour tissue. This model can be used to further develop a personalised medicine pipeline for CRC treatment.

Adenomatous polyposis coli (APC), a key component in the WNT signalling pathway, along with axin, GSK3-ß and CK1-α, forms a destruction complex which targets ß-catenin for degradation resulting in controlled WNT signalling. APC has been identified as a key molecular change occuring in ~80% of all CRC cases both sporadic and herdiatary, resulting in hyperactivation of the WNT signalling pathway. Developing novel therapeutics to target this pathway could prove promising in the treatment of CRC (Dow *et al.*, 2015). Recent findings in mouse models highlighted this signalling pathway as a useful target in the

treatment of colorectal cancer. Tankyrase inhibitors (TNKSi) have been shown to restore normal regulation of the WNT signalling pathway. However work carried out by Tanaka *et al.*, (2017) and Schatoff *et al.*, (2019) both show that the APC mutations dictate tumour response to a TNKS inhibitor. The work was carried out on isogenic CRC cells line, primary cell lines and mouse tumour organoids respectively (Tanaka *et al.*, 2017; Schatoff *et al.*, 2019). This work aims to translate these findings into our model system of patient-matched healthy and tumour organoids by targeting the WNT signalling pathway using a TNKSi as an adjuvant therapy for CRC, as well as seeing if other inhibitors influence the efficacy of CRC treatment working in our model system. Moreover we hope that exploiting a patient-matched organoid and tumouroid model system could be beneficial for predicting patient response in the clinic.

1.0 Introduction

1.1 Epidemiology of Colorectal cancer

In 2015 alone CRC accounted for 9.7% of all deaths worldwide making it the 4th most common cause of cancer mortality. There were 814,000 new cases in men and 664,000 in women making it the 3rd most common and 4th most frequent cancer respectively (Cancer Research UK, 2022). In the UK there are ~42,000 new cases of CRC every year and ~16,000 new deaths. Statistics show that despite patients undergoing rigorous courses of treatments and initially showing remission the 5–10-year survival rate is still only 50%. (Cancer Research UK, 2022). These statistics make CRC a very prominent topic of research, including research into novel therapies that can be used in combination with current chemotherapies or stand-alone treatments.

1.2 Understanding the structure and function of the healthy gastrointestinal tract

To understand the cellular and molecular mechanisms that underpin CRC we first must understand how the colon is regulated during homeostasis. The colon is comprised of five main compartments, the cecum, ascending colon, transverse colon, descending colon, and the sigmoid colon (**Figure 1**). The colon is where final water absorption takes place and waste is excreted (Reviewed by Azzouz and Sharma, 2018; Ogobuiro and Tuma, 2019). The inner lining of the gut is composed of a monolayer of epithelial cells that form millions of invaginations known as crypts (**Figure 1**). The epithelial monolayer is separated from the underlying vascular network by the the lamina propria. The lamina propria contains a diverse population of immune cells that all work in a uniform manner that helps preserve barrier function (Gerbe and Jay, 2016).

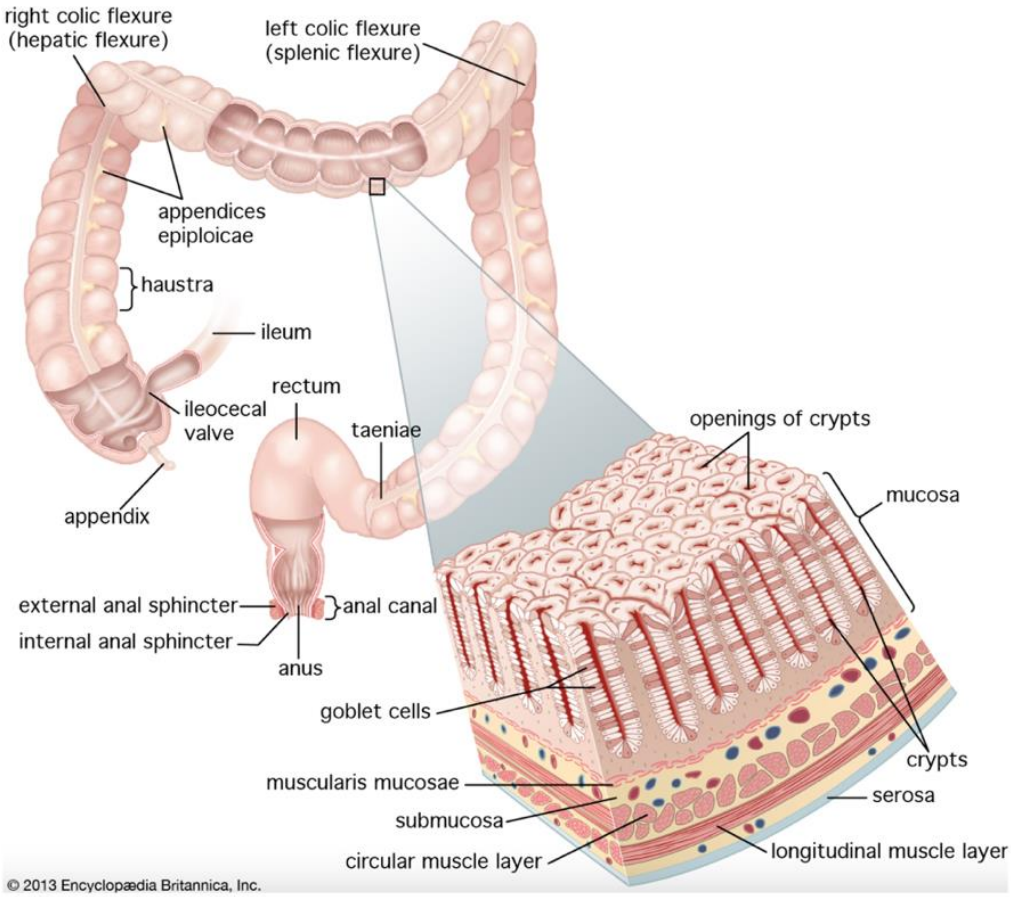


Figure 1. Anatomy of colon structure.

Schematic diagram adapted from (The Editors of Encyclopaedia Britannica, 2020) displaying the anatomy of the colon and a zoomed cross sectional area view of the tissue complex organisation.

Colonic crypts are composed of multiple different cell types that work together to maintain homeostasis within the gut, including goblet cells, enteroendocrine cells, enterocytes, tuft cells and stem cells.

Maintenance of gut homeostasis is highly reliant on the secretion of mucus layers that prevents harmful microbial metabolites in the lumen from coming into contact with the epithelium; this is maintained by **goblet cells**. These cells are a specialised type of epithelial cells that secret mucins (**Figure 2**). These mucins protect the colonic epithelium by providing a thick layer of mucus which physically prevents bacteria and other pathogens from coming into contact with the mucosal lining. A defective mucus barrier has been associated with the development of diseases such as IBD (Birchenough *et al.*, 2015; Knoop and Newberry, 2018).

Enteroendocrine cells are chemosensory meaning that they secret hormones based upon hormonal and dietary changes (**Figure 2**) (Gunawardene, Corfe and Staton, 2011; Gribble and Reimann, 2016). These cells have recently been of interest as they can possibly be a therapeutic target for the treatment of obesity and diabetes (Gribble and Reimann, 2016).

Hyperpolarised epithelial cells known as **enterocytes** are found in colonic crypts and have vital roles in the uptake of antigens from the luminal contents through fluid-phase endocytosis (low efficacy uptake of fluids)(**Figure 2**) (Snoeck, Goddeeris and Cox, 2005). Enterocytes are joined via gap junctions and other proteins which help to establish and then maintain cell polarity which is vital for homeostasis (Snoeck, Goddeeris and Cox, 2005).

Tuft cells were first discovered in the trachea and GI tract over 6 decades ago and the functional role of these cells remained to be elucidated until recently. Tuft cells are distinct from other cells by having a brush border at the apical pole of the cell (**Figure 2**). Tuft cells are classed as chemosensory, meaning they use these villi on the apical pole of the cell to 'taste' the luminal contents and respond to the luminal environment (Gerbe and Jay, 2016; Banerjee *et al.*, 2018).

The final cell type found in colonic crypts are intestinal **stem cells**, which reside in a relatively safe harbour at the base of crypts (**Figure 2**), these cells are easily distinguished from other cell types that are found within crypts due to their slender morphology. Stem cells divide daily and give rise to transit amplifying cells (**Figure 2**), which go on to migrate and differentiate up the crypt axis giving rise to the previously mentioned cell types (Barker *et al.*, 2007; Beumer and Clevers, 2016).

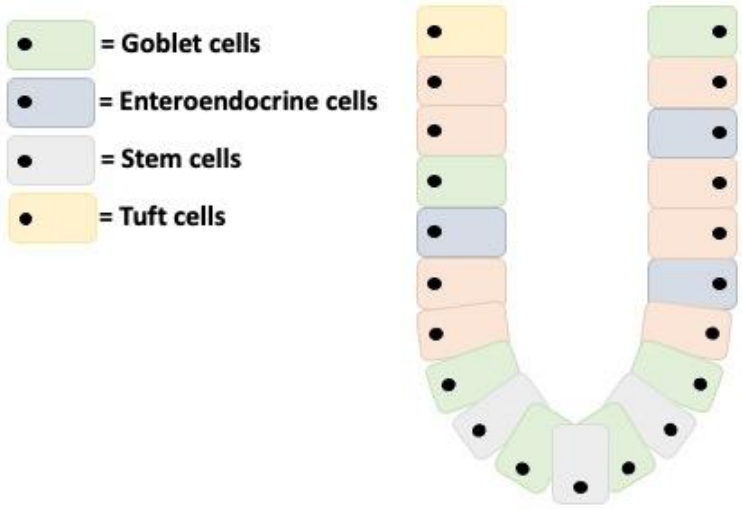


Figure 2. Colonic crypt structure.

Schematic diagram showing colonic crypt structure and the primary cell types that are found in colonic crypts including goblet cells, tuft cells, enterocytes, stem cells and enteroendocrine cells.

1.3 Intestinal tissue renewal

The lining of the intestine is one of the most rapidly renewing tissues found in the body, renewing every 5-7 days (Janes, Lowell and Hutter, 2002). Tissue renewal is regulated by stem cells which reside at the base of crypts (Vermeulen and Snippert, 2014; Gerbe and Jay, 2016). Following proliferation, cells migrate up the crypt axis and differentiate into one of the four major cell types described above; goblet cells, tuft cells, enterocytes and enteroendocrine cells. These cells migrate up the crypt axis until reaching the lumen of the gut where cells undergo apoptosis/anokis and are shed into the lumen. This process of shedding cells into the lumen helps to maintain the homeostasis of the gut (Grossmann *et al.*, 2002).

This process is tightly regulated by the epithelium and mesenchymal cells that surround colonic crypts. Mesenchymal cells and the surrounding environment including fibroblasts, immune cells, enteric neurons and capillaries secret vital cytokines and growth factors which influence stem cell proliferation(Beumer and Clevers, 2016). Signalling pathways including the WNT signalling pathways are vital for stem cell maintenance, as demonstrated by high expression in the intestinal stem cell niche and a gradient of up the crypt axis (**Figure 3**). In contrast, the BMP (Bone Morphogenic Protein) signalling pathway is vital for the

differentiation of the intestinal stem cells. Mesenchymal cells at the base of the crypt secrete inhibitors resulting in a low BMP signalling environment whilst cells further up the crypt axis create a higher BMP signalling environment resulting in a gradient of BMP signalling (**Figure 3**) (Kikuchi, Kishida and Yamamoto, 2006; Reynolds *et al.*, 2014; Meran, Baulies and Li, 2017). Other signalling pathways including the Notch and EGF also influence the proliferation, migration and differentiation of the intestinal stem cells residing in the stem cell niche (Beumer and Clevers, 2016).

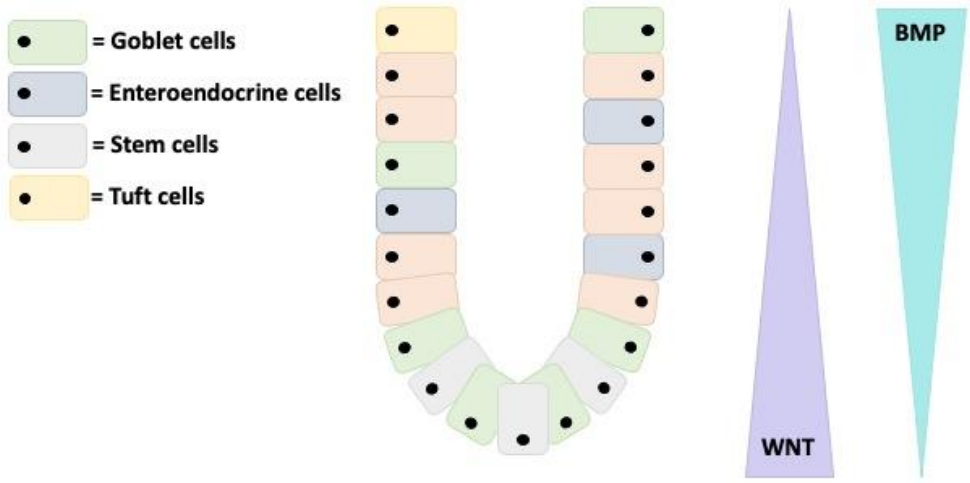


Figure 3. Colonic crypt proliferation, migration, and differentiation.

Schematic diagram showing the proliferative zone of the colonic crypt and the differentiation and migration zone. These are the different processes that stem cells undergo to give rise to their progeny in crypts. The diagram further shows the gradient of the BMP signalling pathway that aid the migration and differentiation of stem cells.

To study intestinal tissue renewal it was important to have a marker of intestinal stem cells. Seminal work by Barker *et al.*, (2007) discovered the stem cell marker LGR5 via *in vivo* lineage tracing. Through the use of *in situ* hybridisation the group discovered that a population of LGR5+ cells resided at the base of crypts and through the use of proliferation markers found they were highly proliferative. Moreover, through the use of genetically modified mice the group were able to trace the lineage of differentiated cells that are located further up the crypt axis. The group found that these cells originated from the base of crypts and could give rise to the different cell types that are found in colonic crypts. Importantly the findings suggested a single LGR5 positive stem cell was sufficient to generate intestinal organoids (Barker *et al.*, 2007).

Due to the rapid renewal of intestinal stem cells, these cells have been implicated as the origin of CRC. Work carried out by (Barker *et al.*, 2009) highlighted that loss of function of

tumour suppressor gene APC results in the a rapid migration of cells with increased WNT signalling. This excessive cell growth went onto form adenomas and subsequent carcinomas in mice (Barker *et al.*, 2009).

1.4 Signalling pathways in health and colon cancer

Different signalling pathways are vital in maintaining homeostasis in the gut including the BMP signalling pathway, EGFR signalling pathway, Notch signalling pathway and the WNT signalling pathway. These signalling pathways all have vital roles aiding the proliferation and differentiation of cell types in colonic crypts.

1.4.1 Wnt signalling

The WNT signalling pathway is critical in both development and the maintenance of homeostasis of tissue through their regulation of stem cells (Duchartre, Kim and Kahn, 2016). WNT proteins are a family of glycoproteins with a N-terminus signal sequence allowing their targeting for secretion. The WNT proteins undergo many post-translational modifications including glycosylation and lipidation in the endoplasmic reticulum before they are secreted. There are 13 WNT genes, 1-11, 16 and WNTA which all play vital roles within the body (Takada *et al.*, 2017; Routledge and Scholpp, 2019).

There are two different forms of WNT signalling pathways, canonical WNT signalling and non-canonical WNT signalling. The non-canonical WNT signalling pathway, signals through receptor tyrosine kinases which activate the PI3K-AKT signalling pathway inducing an increase in intracellular calcium from the endoplasmic reticulum (Katoh, 2017). The canonical WNT signalling pathway signals through the frizzled lipoprotein-related-protein (LRP) 5/6. In the absence of WNT, ß-catenin is targeted for degradation via the destruction complex. The destruction complex is composed of APC, a tumour suppressor, scaffolding protein Axin and two different kinases. These kinases are casein kinase 1-α (CK1-α) and Glycogen synthase kinase 3-ß (GSK3-ß). Both these kinases phosphorylate ß-catenin on multiple serine and threonine residues of the N-terminus. This results in ß-transducin recognising phosphorylated ß-catenin leading to its polyubiquitination and proteasomal degradation (**Figure 4**) (Duchartre, Kim and Kahn, 2016; Tran and Zheng, 2017). In the presence WNT, the WNT protein binds to Frizzled, a seven transmembrane receptor and LRP5/6, this binding results in the activation of Dishevelled. When dishevelled is activated it recruits which deconstructs the degradation complex. This in turn allows cytoplasmic levels of ß-catenin to increase. ß-catenin can translocate to the nucleus where it activates WNT target protein T-Cell Factor (TEF) and Lymphoid Enhance Factor (LEF). This activation leads to the transcription of WNT target genes (**Figure 4**) (Voronkov and Krauss, 2012).

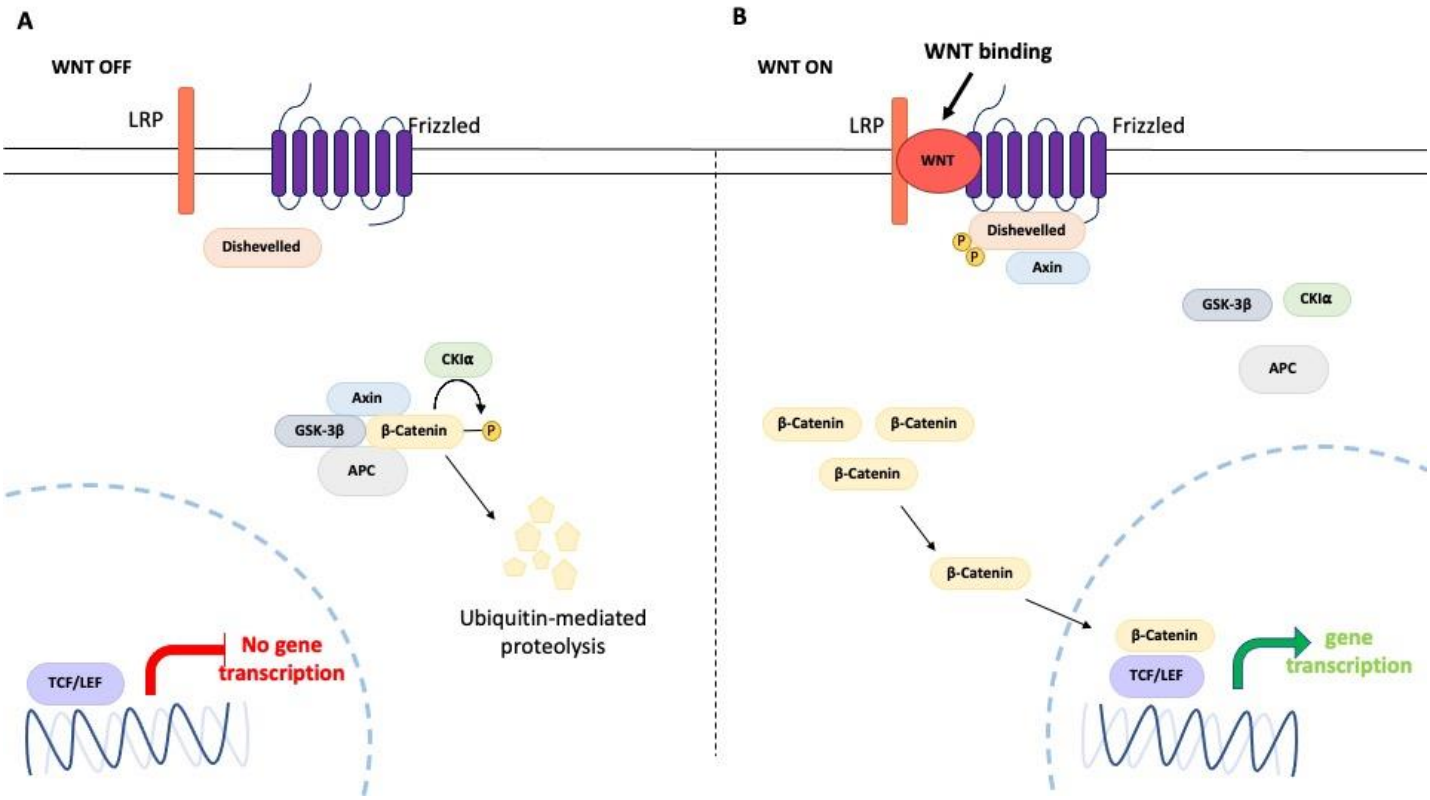


Figure 4. WNT signalling pathway.

A) schematic diagram showing the degradation of ß-catenin by the destruction complex. In the absence of WNT, the destruction complex is formed, causing the phosphorylation of ß-catenin and its subsequent ubiquitination and degradation. **B)** schematic diagram showing the accumulation of ß-catenin in the cytoplasm in the presence of WNT protein. WNT activates Dishevelled which recruits Axin, impairing the formation of the destruction complex. ß-catenin accumulates in the cytoplasm and translocates to the nucleus where it activates WNT target genes such as TCF and LEF.

Importantly the WNT signalling pathway has been implicated in CRC due to mutations in APC resulting in hyperproliferative cells (Dow *et al.*, 2015). Different components of these signalling pathways can become mutant resulting in hyperproliferative cells causing adenoma formation which can lead to CRC. Therefore targeting these pathways with different inhibitors could prove promising in the treatment of cancer (Van De Wetering *et al.*, 2015).

The WNT signalling pathway has been implicated in CRC through mutations in APC which is found in 80% of CRC cases (Siraj *et al.*, 2020). APC is described as the gatekeeper gene and is the mutation that activates the adenoma-carcinoma sequence (Armaghany *et al.*, 2012; Siraj *et al.*, 2020). These genomic changes commonly result in activation of proto-oncogenes including KRAS and inactivation of tumour suppressor genes such as APC (Armaghany *et al.*, 2012). APC is a vital component for the formation of the destruction

complex, however mutations in this tumour suppressor gene have been linked to sporadic and hereditary forms of CRC (Polakis, 1995, 1999).

The APC gene is composed of multiple different domains which have roles in the formation of the destruction complex. The oligomerisation domain allows the APC protein to form oligomers with other APC proteins (**Figure 5**). The Armadillo repeat region is highly conserved and binds to the regulator B56 subunit of protein phosphatase 2A (PP2A) which is an enzyme involved in the binding of axin (**Figure 5A**). The 15-aminoacid and 20-aminoacid repeats provide a binding site for ß-catenin (**Figure 5A**). However, when ß-catenin binds to these repeats it results in ß-catenin phosphorylation and its subsequent degradation. The basic region contains multiple arginine, lysine and proline domains which are likely to bind microtubules (**Figure 5**). Finally, the EB1 domain is important in cell cycle check points, however this is not thought to be involved in tumorigenesis (**Figure 5**) (Fearnhead, Britton and Bodmer, 2001; Schneikert and Behrens, 2007).

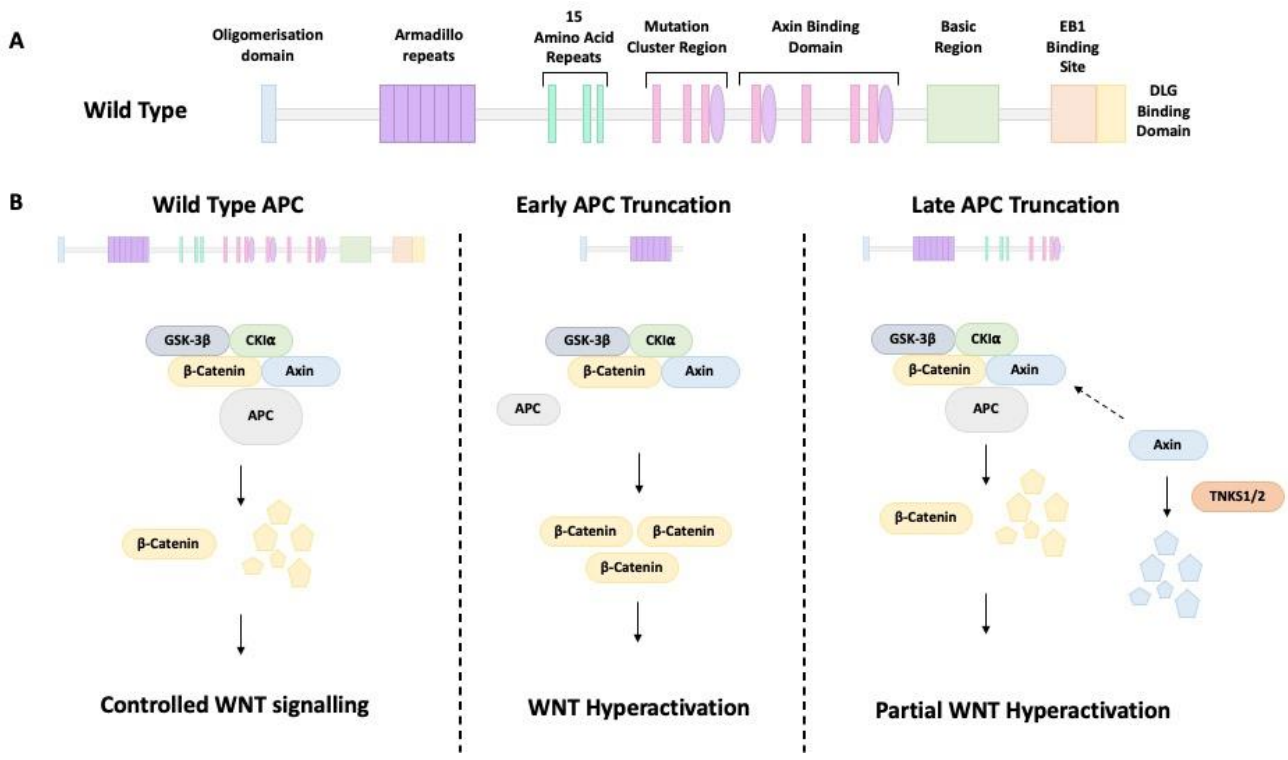


Figure 5. APC gene and its mutations.

A) Schematic diagram showing the structure of the APC gene and the different domains that are found within it. **B)** Schematic diagrams showing (left) wild type APC interacting in the destruction complex resulting in controlled WNT signalling. The middle image shows an early truncation in the APC gene which prevents APC from interacting with the destruction complex resulting in WNT signalling hyperactivation and the image on the right shows a late truncation in the gene resulting in APC being able to interact with the destruction complex only partially, in limited WNT signalling hyperactivation. It also shows the degradation of axin via the enzyme Tankyrase which takes place in all three circumstances.

The 15-amino-acid repeats and 20-amino-acid repeats are vital in ß-catenin binding as previously mentioned. Mutations that result in loss of these repeats prevent APC from forming the destruction complex meaning there is an increase in cytoplasmic ß-catenin which causes WNT hyperactivation (**Figure 5**). However if mutations arise in some of the amino-acid repeats remaining, APC is able to form the destruction complex, resulting in controlled WNT signalling (**Figure 5**) (Fearnhead, Britton and Bodmer, 2001). Targeting this pathway could prove promising in the treatment of CRC.

1.4.2 EGF signalling

The EGF receptor (EGFR)is part of the family of ERbB receptors (ERbB-1). The EGFR is a 1186 amino acid transmembrane protein and formed of three domains. The extracellular domain is composed of 621 amino acids and split into four domains (Wee and Wang, 2017). Domains 1-3 are leucine rich and participate in the ligand binding, domain 2 forms homo and heterodimers with domains of its family members. Domain 2 and 4 are both cysteine rich domains and form disulphide bonds between one another (Wee and Wang, 2017). The transmembrane domain is composed of 23 amino acids and is vital in anchoring the receptor into the membrane. Finally, the intracellular domain is composed of 542 amino acids and has three key domains. The flexible juxta-membrane segment, a tyrosine kinase domain and a C-terminus (Wee and Wang, 2017). The tyrosine kinase domain is divided into two key lobes the N-lobe and C-lobe which has an ATP binding pocket between them. The trans-autophosphorylation is reliant on the C-lobe of one of the receptors and the N-lobe of the other. Finally the C-terminus tail has various tyrosine residues that once phosphorylated anchors intracellular proteins to activate receptors (Wee and Wang, 2017).
The EGF signalling pathway is vital for in the regulation of intestinal epithelial cell growth and differentiation and therefore is highly implicated in CRC. The overexpression and mutations in the receptors have been associated with the over growth of the epithelial tissue (Abud, Watson and Heath, 2005). The EGF signalling pathway is activated when growth factors bind to the receptor (**Figure 6**), resulting in the receptors forming homo or hetero-dimers that trans-phosphorylate one another. This process takes place on the cytoplasmic tail at specific tyrosine residues (**Figure 6**)(Normanno *et al.*, 2006; Wee and Wang, 2017). This phosphorylation recruits adaptor proteins such as SHC and Growth factor receptor bound protein 2 (GRB2) (Normanno *et al.*, 2006). The exchange of GDP for GTP results int he activation of RAS, RAF as well as PI3K which in turn activates downstream signalling pathways including those involved in cellular proliferation, growth and survival (**Figure 4**) (Normanno *et al.*, 2006; Tomas, Futter and Eden, 2014).

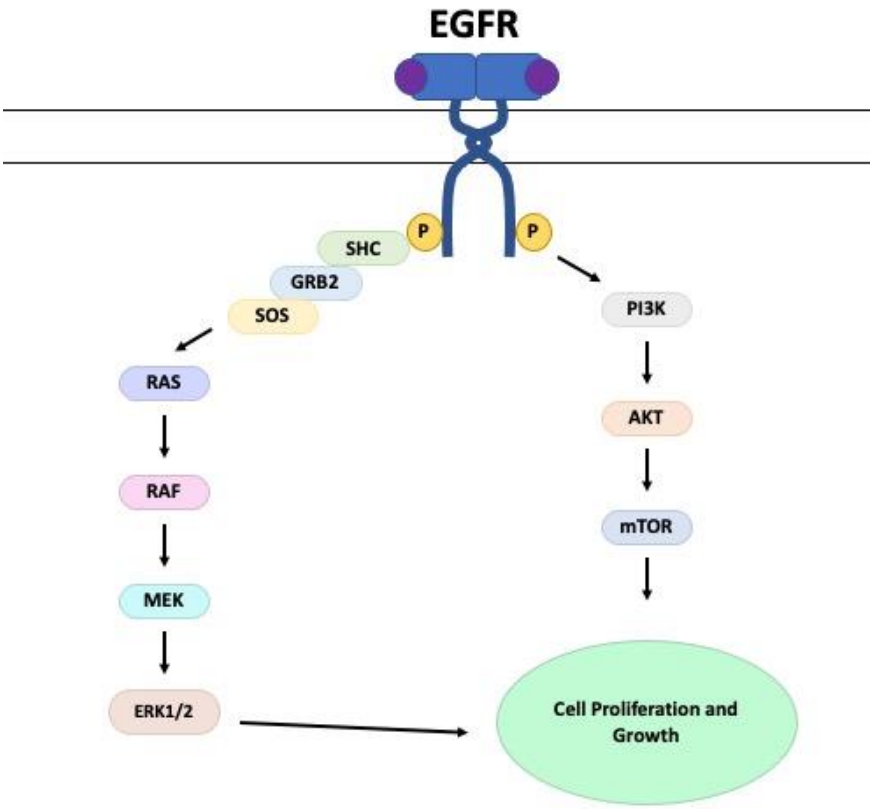


Figure 6. The EGF signalling pathway.

Schematic diagram showing the activation of the EGF signalling pathway via ligand binding. This results in the recruitment of adaptor proteins (SHC, GRB2) activating downstream targets such as the RAS-MAP kinase signalling pathway. This pathway is implicated in the proliferation, survival, and growth of cells.

This pathway plays a key role in gut homeostasis and has been a gene of interest in the study of CRC. Overexpression or mutations in the EGFR can result in spontaneous dimerization of the receptor and thus lead to activation of its downstream targets (Tomas, Futter and Eden, 2014). Anti-EGFR therapies have been studied as treatment for CRC, in particular metastatic CRC (mCRC), as 49-82% of this cancer harbours EGFR mutations. However, previous studies have shown that EGFR status is unable to predict the tumour response to the therapies (Cunningham *et al.*, 2009; Miyamoto, Suyama and Baba, 2017). Recent studies suggest targeting downstream of the EGFR such as RAS might prove promising (Tomas, Futter and Eden, 2014).

1.4.3 TP53 signalling

The TP53 protein was first discovered in 1979, and the tumour suppressive function was alluded to by Eliyahu and colleagues that showed the induction of WT P53 in cancer cells prevent the growth (Eliyahu *et al.*, 1989; Baker *et al.*, 1990). TP53 signalling pathway is a network of multiple genes which respond to both external and internal stress signals that might impact homeostatic functions in the body. When cells are put under stress from stressors such as UV damage, heat or cold shock, genotoxic drugs, it results in the activation of p53. MDM-2 is a product of p53 activation meaning if p53 becomes upregulated so does MDM-2. MDM-2 is a E-3 ubiquitin ligase meaning it attaches ubiquitin to p53 which results in its degradation via the proteosome. Therefore MDM-2 plays a vital role is maintaining the levels of p53 in cells (Harris and Levine, 2005). 31 genes are thought to be regulated by p53, depending on their function and role they contribute to many homeostatic roles within the body including growth arrest, apoptosis, inhibition of angiogenesis and finally *DNA* repair (Aubrey *et al.*, 2017).

Due to p53 having such a vital role in cellular homeostasis, it has also been implicated in cancer. The TP53 protein plays a vital role as a tumour suppressor gene in the function of cancer. However due to its multifunctional role the exact molecular mechanism is unknown. Mutations in TP53 are found in about half of all cancers, and result in a single amino acid change in the protein. This results in minimal control of the TP53 proteins preventing the key homeostatic processes such as apoptosis becoming activated. This results in a population of cells with an accumulation of *DNA* damage resulting in hyperproliferative cells and tumour growth (Aubrey, Strasser and Kelly, 2016).

1.5 Adenoma carcinoma sequence

The adenoma-carcinoma sequence, also known as the chromosomal instability pathway, highlights genetic and histological changes that gastrointestinal tissue undergo during the development of CRC from an adenoma to a carcinoma (Pino and Chung, 2010; Armaghany *et al.*, 2012). An adenoma is a lesion in the colon that is not malignant meaning it will not evade surrounding tissues. However, when an adenoma becomes a carcinoma, it means the lesion has become malignant and can spread to surrounding tissues (Carvalho *et al.*, 2012). Work conducted by (Fearon and Vogelstein, 1990) proposed specific pathways that are required for the development of CRC, which have since been shown to mutate during the progression of CRC. The group quantified the number of genetic changes in a single CRC tumour in addition to quantifying specific oncogenes such as RAS in the three adenoma stages, early, intermediate, and late as well as the carcinoma stage (Fearon and Vogelstein, 1990). This work meant Fearon, and Vogelstein were the first group to propose the idea of a

multistep genetic model by which CRC develops. The multistep model includes the transformation of the gastrointestinal epithelium, the formation of an adenoma and finally the formation of the adenoma-carcinoma (Fearon and Vogelstein, 1990; Pino and Chung, 2010).

Genomic instability plays an important role in the development of CRC and refers to the tendency of *DNA* to become mutated and other genetic changes to occur. This is commonly caused by defects in certain processes that control the division of cells. This can primarily occur due to mutations in protein in signalling pathways vital for homeostasis (Porru *et al.*, 2018). An example of this is the receptor tyrosine kinase, EGFR, which is mutated in around 35-49% of all CRC. The mutations regularly occur in hotspots located in the extracellular region, the kinase domains, and the c-terminus (**see section 1.4.2**) (Wee and Wang, 2017).

Downstream from EFGR other proteins are commonly mutated in CRC including KRAS and BRAF. These proteins are part of the MAP kinase signalling pathway and are mutated in ~30% of all CRC cases. Mutation in these proteins results in the MAPK pathway being constitutively active resulting in upregulation of cell proliferation and differentiation (Oliveira *et al.*, 2007).

Like previously stated, *DNA* damage, hypoxia and nutrient depletion are all conditions that result in the activation of the P53 signalling pathway leading to cell cycle arrest and apoptosis. P53 is a tumour suppressor gene and has been identified in 50-60% of all CRC cases. Mutations in P53 results in loss of function of this signalling pathway and is thought to promote tumorigenesis (**see section 1.4.3**)(Nakayama and Oshima, 2019).

Adenomatous polyposis coli (APC) is the known as the gate keeper gene, meaning loss of function of this gene is what initiates the adenoma-carcinoma sequence. Seminal work carried out by (Dow *et al.*, 2015) used shAPC animals treated with doxycycline resulting in hyperproliferative cells up the crypt villus axis. This finding was in line with other research that suggested that loss of APC resulted in hyperproliferation due to constitutive WNT activation (Cheadle *et al.*, 2002; Dow *et al.*, 2015). However the group went onto show that restoration of APC function by reversion of the mutation meant that the adenoma or even the carcinoma reverted back to normal epithelium (**Figure 7**) (Dow *et al.*, 2015);further supporting the hypothesis that APC is the gate keeper gene for CRC. The role of APC in the formation of the destruction complex has been well established; Therefore, targeting the WNT signalling pathway via APC could prove a promising treatment for CRC.

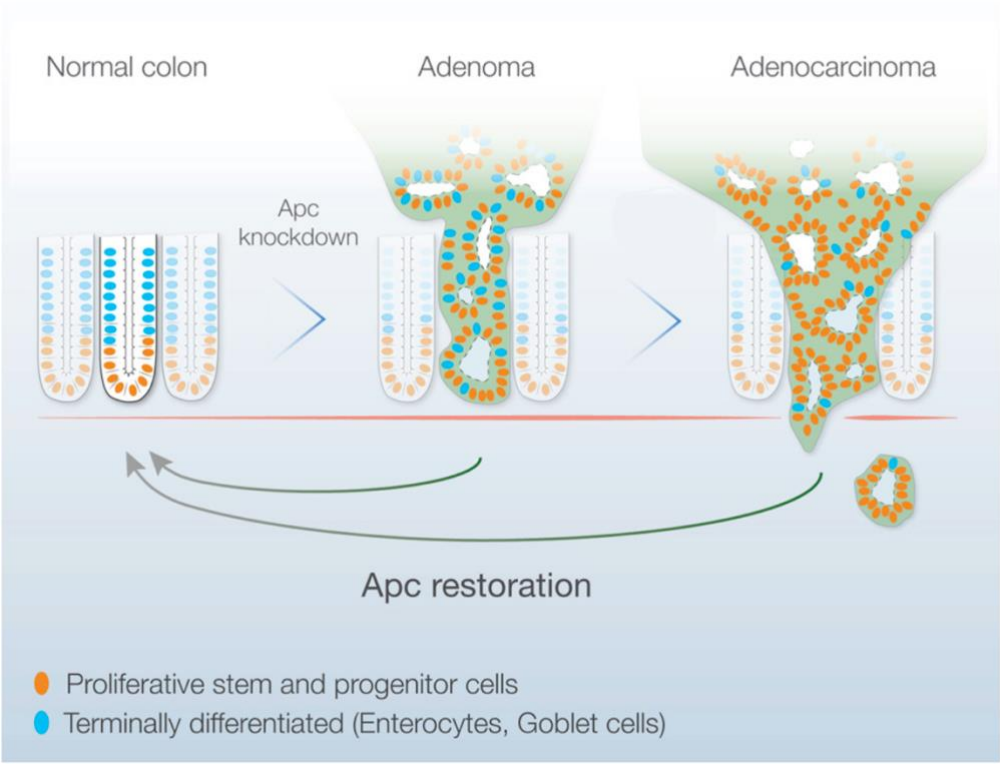


Figure 7. APC restoration in colonic crypts.

Schematic diagram showing when APC is knocked down it results in adenoma and then adenoma carcinoma formation. However, if APC is restored at either stage it results in the tissue reverting back to healthy mucosa. (Dow et al., 2015).

1.5.1 The hallmarks of cancer

Initially there were six proposed hallmarks of cancer, these are producing their own growth signals for survival, resistance to anti-growth signals, evading apoptosis, rapid replication of cancer cells, ability to form their own blood vessels and the ability to invade and metastasis to surrounding and distant tissues (Hanahan and Weinberg, 2000). Following the discovery of the six hallmarks of cancer there were a following four proposed in 2011. This discovery included cancer's ability to avoid immune destruction, a tumours ability to promote inflammation, genome instability and the potential for further mutation and the dysregulation of cell energetics. These findings furthered researcher understandings of how cancers avoid treatment and highlighted novel areas to be targeted (Hanahan and Weinberg, 2011). Finally in 2022 there were another four emerging hallmarks of cancer proposed. These are senescent cells, non-mutational reprogramming, phenotypic plasticity, and polymorphic microbiomes. Although these hallmarks are new further research into these areas could highlight vital new targets for cancer treatment (Hanahan, 2022). All these hallmarks give cancer a growth and survival advantage and therefore highlights key areas that could be targeted for treatment in cancer.

1.6 Current gold standard treatment for colorectal cancer

The staging and prognosis of a patient at diagnosis is vital in determining the treatment course. The staging of the cancer at diagnosis is commonly via the tumour-node-metastasis (TNM) system. This system uses the invasion into the bowel, the amount of lymph node involvement and the presence of metastasis as a way to stage a patient's tumour. As the stage of the tumour increases, the overall 5-year survival of the patient decreases from 90% (lower stage tumours) to 10% (highest stage tumour) (Van Laethem, 2001).

Stage 1 CRC will mean that cancer cells have invaded both the mucosal and muscle layer of the colon but has failed to spread to neighbouring tissue and lymph nodes. Patients diagnosed with stage 1 CRC will normally only receive surgery as their treatment and have a 5-year survival rate of ~90%. Stage 2 cancer refers to CRC that have spread through the colon wall and now spread to the surrounding tissue, these patients will have surgery as well as chemotherapy as their treatment. The patients diagnosed with stage 2 CRC have an ~80% 5-year survival rate. Stage 3 CRC indicated lymph node involvement as well as a one-two metastasis surrounding tissues however there will be no metastases to distant organs in the body. Patients with stage 3 CRC again will receive both a surgery along with chemotherapy treatment to tackle the disease. Patients who receive treatment will have ~70% 5-year survival rate. Finally, patients diagnosed with stage 4 CRC will now have spread to more distant organs including the liver and lungs as well as having lymph node evolvement. This stage of CRC is known as advanced CRC. Normally patients aren't able to have surgery due to the size and spread of the cancer. Instead, patients are offered treatments that are used to treat the symptoms including chemotherapy, radiotherapy, and immunotherapy. Due to the aggressive nature of stage 4 CRC the 5-year survival rate is 10% (Cancer Research UK, 2022; Cancer.Net Editorial Board, 2022).

The front-line treatment for CRC is surgery. This normally results in patients having large parts of their bowels resected which will commonly result in a colostomy bag being placed. The resection rate for CRC is around 60% with a high curative rate. However, some patients will need to go on and receive further treatment; therefore, the development of novel therapies to replace these invasive treatments are vital.

If surgery appears unsuccessful a patient will then undergo a course of chemotherapy treatment. The standard of care chemotherapy (SOC) for CRC is 5-fluorouracil (5-FU). This drug is an antimetabolite which prevents cellular proliferation. These compounds work by mimicking molecules that are required for cellular proliferation. In the case of 5-FU the agent works by mimicking Thymidine, primarily inhibiting the enzyme thymidylate synthase which then blocks the synthesis of Thymidine formation which is vital for the synthesis of *DNA* (Parker, 2009; Wigmore *et al.*, 2010). 5-FU has been SOC for patients with metastatic

colorectal for many years and was the only treatment available to patients with CRC until 1985. Although still the main treatment for CRC since the 2000, 5-FU is commonly used in combination with another chemotherapy agent such as oxaliplatin. Oxaliplatin, is a platinum-*based chemotherapy which results in the formation of DNA adducts. The DNA adducts* prevents *DNA* replication resulting in cell cycle arrest and apoptosis (Di Francesco, Ruggiero and Riccardi, 2002). These combinations have been vital in improving the response rate of tumours to 20%, from 50% (Venook, 2005). Moreover, another combination chemotherapy commonly used in the treatment of CRC is FOLFOX. This is a chemotherapy containing three components, 5-FU, folinic acid and oxaliplatin. Studies have shown even though this chemotherapy can be effective in killing CRC this line of treatment has the potential to leave a population of cells that can result in high reoccurrence rates (Yu *et al.*, 2009; Cho *et al.*, 2020).

Chemoresistance is a common downfall of patients that undergo chemotherapy treatment for CRC (**Figure 8**). Tumours undergoing treatment can result in residual chemoresistant tumour cells once treatment has ended. This can result in tumour reoccurrence from this population chemoresistant cells meaning the tumour is harder to treat (**Figure 8**).

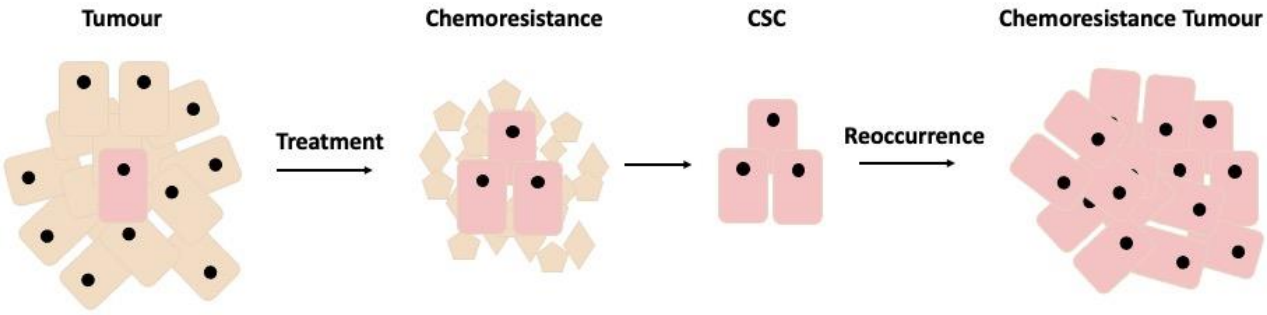


Figure 8. Chemoresistance resulting from cancer stem cells.

Timeline showing that there is a population of cancer stem cells (CSCs) within a tumour that after treatment with chemotherapy is not killed. This then allows the tumour to reoccur from the remaining population of CSC.

Although 5-FU and other chemotherapies have proven promising in the treatment for CRC there are many side effects that patients suffer for these treatments. Chemotherapy targets highly mitotic cells such as cancer cells but will also target healthy highly mitotic cells such as the healthy mucosa in the gut. This commonly results in a side effect known as mucositis which is when the lining of the gut is damaged. Therefore, establishing novel adjuvant therapies that can lower the dosages of chemotherapy in CRC treatment whilst maintaining killing of the tumour is imperative.

Although chemotherapy is the frontline treatment for patients with CRC, adjuvant therapies have been recently discovered which can improve the efficacy of the treatment (Arnold and Seufferlein, 2010).

As previously mentioned, there are multiple different signalling pathways that are involved in colonic homeostasis and importantly when these are dysregulated, they can result in disease. One pathway that has been extensively investigated as a target for adjuvant therapy is the EGF signalling pathway (**Figure 6**) and the role that inhibitors can play in the treatment of CRC (**see section 1.4.2**). EGFR is a transmembrane glycoprotein and when activated initiates a cascade of signalling pathways including the RAS-MAP kinase signalling pathway and the PI3K-AKT signalling pathway. These pathways go on to activate transcription of genes involved in cellular proliferation and survival and due to this role in proliferation and survival, the EGF signalling pathway has also been implicated in tumorigenesis (Arnold and Seufferlein, 2010; You and Chen, 2012). EGFR mutations are found in 22-77% of all CRC cases and therefore have been a potential target as an adjuvant therapy to chemotherapy. The use of Cetuximab, an EGFR inhibitor, which has been approved to be used in patients with CRC have shown some efficacy (Wolpin *et al.*, 2007; Arnold and Seufferlein, 2010). Although promising effects have been shown with EGFR inhibitor (EGFRi), there have been some rate limiting factors associated with this treatment. If a patient has a mutation KRAS or BRAF, both proteins down stream of EGFR, patient tumours appear to have very little to no response to the therapy (You and Chen, 2012). Therefore, the discovery of other small molecule inhibitors for other vital signalling pathways is vital.

As previously mentioned, another valuable pathway to target would be the WNT signalling pathway, due to APC being mutant in 80% of all CRC cases. Novel Tankyrase inhibitors (TNKSi) have sensitised CRC cell lines and mouse models to chemotherapy (**see section 1.4.1**) (Tanaka *et al.*, 2017; Schatoff *et al.*, 2019). A recent phase 1 clinical trial looked at a PARP and TNKS inhibitor E7449, with two patients showing partial responses to the oral dose of TNKSi and 13 showing a stable disease. The trial concluded that the drug had a good tolerability level and also promising antitumour activity so moved onto the next phase (Plummer *et al.*, 2020).

1.6.1 Tankyrase inhibitors

Tankyrase (TNKS) is a subpopulation of an enzyme super family known as Diphtheria toxin-like ADP-ribosyltransferase (ARTD) and are also known as poly (ADP-ribose) polymerases (PARPs). These enzymes have a vital role in catalysing the transfer of ADP-ribose from its co-substrate NAD^+(Otto *et al.*, 2005). TNKS1 and TNKS2 differ from other enzymes in this

superfamily and form their own distinct subpopulation. One distinguishing factor of the enzymes is the SAM domain. This domain is prone to oligomerisation and forms homo and heterooligomers. An oligomer is a polymer composed of molecules with very few repeats (Haikarainen, Krauss and Lehtio, 2014).

Human TNKS is a multidomain protein; TNKS1 is composed of 1327 residues whilst TNKS2 is composed of 1166. The C-terminus of the proteins contain a catalytic ARTD domain (**Figure 9**) which has been characterised resulting in the development of inhibitors for these proteins (Wahlberg *et al.*, 2012; Haikarainen, Krauss and Lehtio, 2014). The N-terminus contains a SAM domain (**Figure 9**), which is known to oligomerise and form homo and heterooligomers (De Rycker and Price, 2004). The main components of TNKS1 and TNKS2 protein are the five ankyrin repeats (**Figure 9**) located in the middle. These domains are involved in protein-protein interactions (Haikarainen, Krauss and Lehtio, 2014). A primary distinguishing factor between TNKS1 and TNKS2 is the HPS domain found in TNKS1 which is a stretch of histidine, proline and serine residues (**Figure 9**) (Kim, 2018).

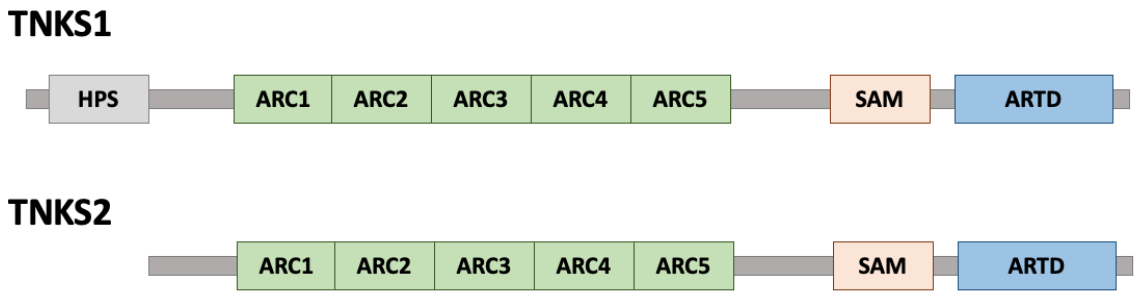


Figure 9. TNKS1/2 gene structure.

Schematic diagram showing the multidomain structure of TNKS1 and TNKS2. The key domains include ARTD domain at the C-terminus, the SAM domain at the N-terminus and the 5 ankyrin repeats located in the centre of the protein.

TNKS have been implicated in many cellular functions including mitotic progression, glucose metabolism, stress granule formation and most importantly WNT signalling (Haikarainen, Krauss and Lehtio, 2014). TNKS1 and TNKS2 have been shown to regulate the cytoplasmic levels of axin which is a rate limiting factor in the formation of the destruction complex which is vital for WNT signalling regulation (Huang *et al.*, 2009). Important work carried out by (Huang *et al.*, 2009) showed that the ankyrin repeat domains of the TNKS protein is vital for its interaction with axin. The group further showed that TNKS inhibitors (TNKSi) work through stabilising axin by preventing its polyubiquitination. This results in an increase of cytoplasmic levels allowing it to form the destruction complex (Huang *et al.*, 2009). TNKS1

causes axin PARylation, which is a post translational modification process by which polymers of ADP ribose are attached to proteins through covalent bonds by a PAR polymerase enzyme. This results in the degradation of axin through the ubiquitination proteosome pathway (Morrone *et al.*, 2012; Kim, 2018).

The WNT signalling pathway has already been implicated in CRC, therefore the targeting of TNKS using a TNKSi could pose a promising new therapy in the treatment of cancer. A TNKSi works by preventing TNKS1/2 degrading axin, this causes axin accumulation in the cytoplasm and allowing it to freely form the destruction complex. Moreover, using a TNKSi can restore normal WNT function through increasing cytoplasmic levels of axin. This then allows the axin to freely interact with the destruction complex. (**Figure 10**) (Schatoff et al., 2019).

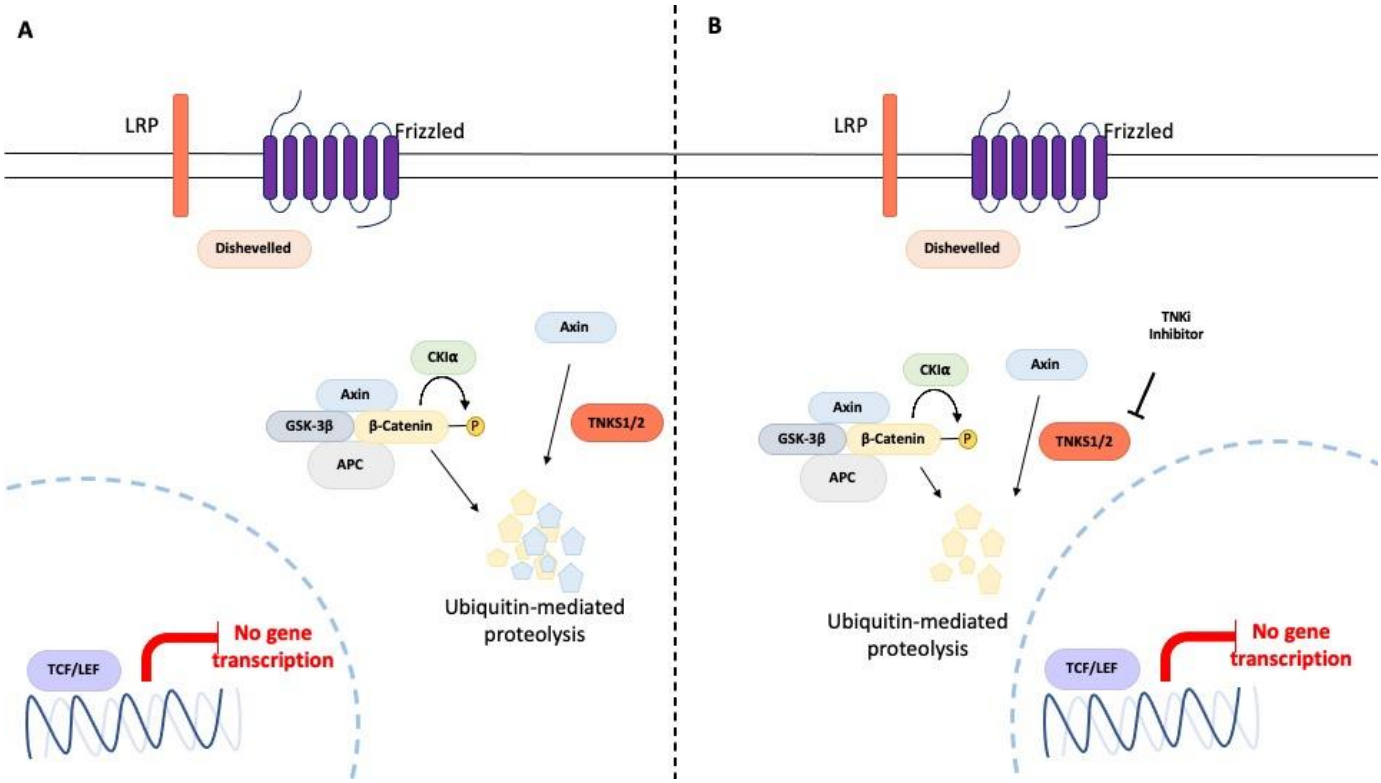


Figure 10. WNT signalling with TNKS1/2.

A) schematic diagram showing the degradation of axin as a rate limiting factor of the destruction complex via TNKS1/2. **B)** schematic diagram showing the inhibition of TNKS1/2 using a TNKSi causing an increase in cytoplasmic axin meaning it can form the destruction complex.

Moreover, findings by Badder *et al.*, (2019) found that novel TNKSi reduced the growth of mouse CRC tumouroids *in vitro*. The group further characterised the different levels of destruction complex components and TNKS-axin complex and found when treated with a TNKSi there is a reduction in TNKS1, TNKS2 and ß-catenin but an increase in cytoplasmic levels of axin. The decrease in ß-catenin and increase in axin allowed the group to conclude that the increased levels of axin allowed the destruction complex to form resulting in ß-catenin degradation (Badder *et al.*, 2019). Recent work carried out by (Schatoff *et al.*, 2019) and (Tanaka *et al.*, 2017) suggest that there may be differential sensitivity to TNKSi when using this as an adjuvant therapy alongside chemotherapy in CRC cell lines, primary cell lines and organoids derived from mouse intestine. However work carried out by Tanaka *et al.*, (2017) suggested that early APC truncations should be sensitive to a TNKSi whilst late APC truncations should be insensitive. Furthermore studies carried out by (Schatoff *et al.*, 2019) contradicts this suggesting that early APC truncations will be insensitive to a TNKSi whilst late APC truncations will be sensitive (**Figure 11**). Therefore, further work to elucidate the mechanism of action is required.

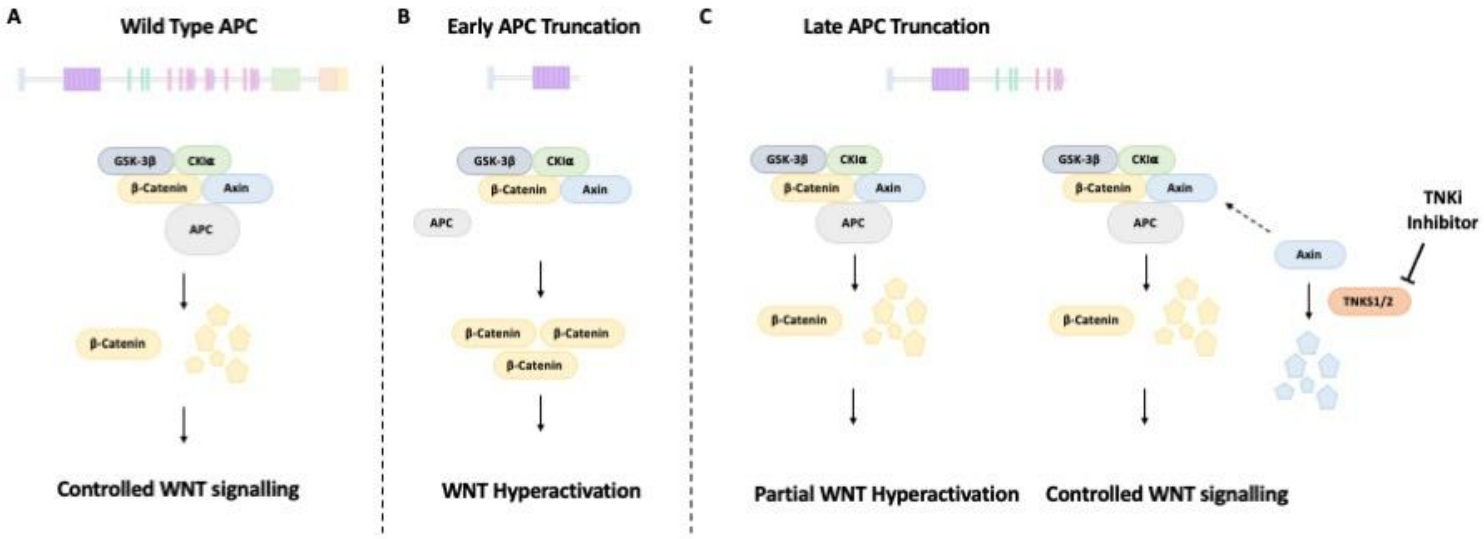


Figure 11. TNKSi effect on APC mutants.

Schematic diagram showing the effect of a TNKSi on an early APC truncation, a TNKSi will likely have no effect due to APC being unable to form the destruction complex and causing WNT hyperactivation and the effect of a TNKSi on a late APC truncation when there should be controlled WNT signalling due to the residual APC being able to form the destruction complex and degrade ß-catenin.

1.6.2 EGFR, MEK and BRAF inhibitors

MEK, EGFR and BRAF inhibitors could be used to target another important pathway in the treatment of CRC. The RAS-MAP kinase signalling pathway plays pivotal in cancer cell growth and survival (Guo *et al.*, 2020). Under homeostatic conditions this pathway activates target genes that aid cellular proliferation, growth, and survival. However when these key gene become mutated in CRC these homeostatic processes are activated continuously resulting in hyperproliferative cells and cancer survival (Wee and Wang, 2017). EGFR is frequently mutated in cancers and has been the target of multiple cancer therapies. Cetuximab is an EGFR inhibitor (EGFRi) and shown to increase the efficacy of chemotherapy treatment by 10-20% in metastatic CRC. This lead to the FDA approving this as a treatment for patients with CRC in February 2004 (Sigismund, Avanzato and Lanzetti, 2018). Although the use of an EGFRi might prove promising in the treatment of CRC, research has also shown that CRC has the ability to acquire resistance to this treatment or be resistance to this treatment due to other mutations that they harbour. Studies has shown when CRC also has an activating mutation is Kras, resulting in EGFR independent activation of the MAP kinase signalling pathway, blocking the EGFR doesn't prevent the over expression of this signalling pathway (Sjoerd Rodenhuis *et al.*,1988; Ciardiello and Tortora, 2008).

Due to mutations in Kras influencing the response of cancers to and EGFRi, researchers have proposed targeting tumours downstream of the EGFR might prove promising. Although until recently there were no direct inhibitors of Kras, many studies have shown that using MEK inhibitor, MEK is activated downstream of Kras, proves a promising adjuvant therapy alongside chemotherapy (Hatzivassiliou *et al.*, 2010). Similar to EGFR mutations Kras mutations result in constitutive activation of the RAS MAP kinase signalling pathway. However, when a MEK inhibitor is present is stops the activation of downstream homeostatic processes, cellular proliferation, and survival, that aids the cancer cell growth and survival (Porru *et al.*, 2018).

Similar to Kras BRAF is also a common component of the RAS MAP signalling pathway that results in cancer cell growth and survival. Due to this mutation being downstream of EGF again MEK inhibitors can be used to target these mutations. However, BRAF inhibitors have been shown to be highly effective in targeting mutations such as BRAF V600E that is commonly found in melanoma. However determining exactly how these treatments might effect patients need to be further determined (Yao *et al.*, 2017).

1.7 Model system

To assess the effect of these drugs via to their use in humans *in vitro* models such as cell lines and mouse models might be used. The ideal *in vitro* model would have key characteristics including these five points.

Having the **same histopathologic features** of the human tumour, this would allow direct comparisons to be drawn between the model being used and the human tumour. The ability for the cancer to **progress and undertake similar stages** like what is seen in human cancer, this would then hopefully result in the same physiological and systemic effects. The same **genes and biochemical** pathways to be involved, this would allow researchers to assess the effects of drugs that target both these genes and biochemical pathways. It would be vital that the model would be able to **reflect the response** of the human tumour to the therapies of choice. This would allow researchers to decide the most effective course of treatment for the patient. Finally, the model would allow researchers to **predict the therapeutic efficacy** of different treatments in human clinical assays. This would prevent the need of patients to undergo multiple treatment plans whilst trying to find the most effective treatment for the tumour (Céspedes *et al.*, 2006).

1.7.1 Limitations of current model systems

Mouse models are the most commonly used model system to research the tumorigenic process. Due to extensive research on mouse models the genomic and biochemical processes are highly understood, as well as being able to easily manipulate the model (Céspedes *et al.*, 2006). This model has allowed many insights into colon cancer and including the mechanisms, progression, metastasis, maintenance and chemoresistance that all results in the development and progression of cancer. Important studies found disruption of tumour suppressor genes and/or oncogenes results in embryo lethality or development of abnormal tissue phenotypes which again would result in death of the embryo. They also discovered disruption to vital developmental pathways such as the WNT signalling pathway were imperative in the progression and survival of cancer (Gowen *et al.*, 1996; Hakem *et al.*, 1996). Moreover, mouse models highlighted pathways that cancer activates to survive including angiogenic, hypoxic and metabolic pathways. This allowed further research into the chemoresistance, for example an increase in VEGF independent signalling when anti-VEGF therapy is used to treat a cancer (Casanovas *et al.*, 2005). Mouse models allowed researchers to study vital genes that are involved in cancer initiation. For example APC is the most common gene that is mutated in sporadic colorectal cancer as well as being the cause of Familial Adenomatous Poly-posis (FAP) which results in multiple polys forming in the colon (Jackstadt and Sansom, 2016). Mouse models were development to mimic this

loss of function and this resulted in the formation of colon cancer in the mouse models (Jackstadt and Sansom, 2016).

1.7.2 Personalised medicine pipeline

The organoid and tumouroid culture system were an important step in advancing research into treatments for CRC. These systems have helped bridge a gap between the use of single cell 2D culture to the use of 3D systems that mimic the environment of a human gut. High throughput screening of cancer cell lines to determine drug sensitivity related to their genomic alterations (Barretina *et al.*, 2012). However, these cell lines would be unable to dictate patient specific responses. In 2011 the Clevers laboratory released data that suggest that murine LGR5 stem cells have the ability to autonomously grow into crypt like structures. This was only possible when put into Matrigel culture that contained R-spondin, EGF and Noggin. This is to mimic normal homeostatic conditions that are seen in the gut (Sato *et al.*, 2011). Although these were murine stem cells it was still a big step towards the organoid culture system that is used today.

Moreover, seminal work by Van De Wetering *et al.*, (2015) used patient derived tumour organoids to develop a biobank and test SOC. The group obtained tissue that had been resected during surgy, from which they derived tumouroids and organoids from the surround tissue. This can then be cultured to form a living biobank that can then be sequenced, and high throughput drug screening can be carried out on them. This can be used to determine the best possible treatment for the patient (Van De Wetering *et al.*, 2015).

The Williams Laboratory have also been at the forefront of the progress of 3D culture systems to develop our understanding of CRC. The laboratory has been able to develop both a crypt culture system as well as a patient-matched organoid and tumouroid system. This type of 3D model is distinguished from other 3D models that are used due to the organoids and tumouroids that are culture being patient matched. This allows drugs to be tested on both the healthy and diseased tissue and allows the group to see the effects on the healthy tissue. This should lead to a decrease in the harsh side effects observed with chemotherapy treatment due to patients being put on the lowest possible dosage with the best effect. This low dose of chemotherapy can be in combination with other drugs that are used to target different signalling pathways that are dysregulated during cancer.

Work carried out by (Ooft *et al.*, 2019) and (Vlachogiannis *et al.*, 2018) showed that cancer organoids, colorectal organoids that have been derived from patient tumours, can inform the response to cancer treatment in the clinic. The information that are obtained from these experiments have the ability to implemented in the clinic for personalised medicine programmes (Vlachogiannis *et al.*, 2018; Ooft *et al.*, 2019). However, there are some

limitations associated with the work carried out by Ooft et al., 2019, the work carried out by this group was only performed of patient derived tumouroids thus meaning they didn't assess the effect of cancer treatment on the healthy tissue as well as the diseased tissue. Moreover, they showed that this model was only effective at determining patient response to irinotecan chemotherapy treatment, meaning this model could only be used for this treatment if translated to the clinic (Ooft *et al.*, 2019).

Moreover, work carried out by Badder *et al.*,(2019) showed the effect of novel TNKSi on growth of cancer organoids. The group showed that when colonic cancer organoids are treated with TNKSi there is a reduction in the cancer organoid growth. This work showed promising effects of TNKSi as an adjuvant therapy (Badder *et al.*, 2019).

These groups have all shown that tumouroids show promise in the laboratory in dictating the response to chemotherapies and adjuvant therapies. However, unlike the model system that will be used in this project, these groups did not have the healthy organoids to match the patient tumouroids. A patient-matched organoid and tumouroid system allows you to see the effects on the tumour but also the healthy counterpart. In a personalised medicine setting it will allow you to give the patient the best treatment for their cancer whilst minimising the effect on the healthy surround tissue.

1.8 Project aims and objectives

Previous work carried out by (Tanaka *et al.*, 2017; Schatoff *et al.*, 2019) suggests differential sensitivity to a TNKSi is dependent on the APC mutation the tumour harbours. An APC mutation that occurs before the mutational cluster region will leave a small amount of the APC gene (long truncating mutations) and would be insensitive to a TNKSI (Schatoff *et al.*, 2019). Whilst a mutation in or after the mutation cluster region leaves a larger amount of residual APC (short truncating mutations) and would be expected to be sensitive to a TNKSi (Schatoff *et al.*, 2019).

From WES data, we were able to deduce that two of the tumouroid lines would likely be sensitive to a TNKSi due to late APC truncations (unpublished, William's lab). One of the tumouroid lines has an early APC truncation and therefore would most likely be insensitive, following the hypothesis that (Schatoff *et al.*, 2019) proposed. Due to the conflicting data suggesting sensitivity to TNKSi the purpose of this project is to target the WNT signalling pathways with a TNKSi. Also, to identify if there is differential sensitivity of patient-matched organoids and tumouroids to TNKSi (G007LK) as an adjuvant therapy alongside chemotherapy. This points to the hypothesis that:

Mutational profiling of patient-matched organoids and tumouroids can predict response to novel adjuvant therapies for colorectal cancer.

This hypothesis will be tested using the following aims:

- Determine the sensitivity of normal organoids and tumouroids to SOC
- Use whole exome sequencing data to determine the genotype of patient-matched organoids and tumouroids
- Use morphological and metabolic assays to quantify tumouroid and organoid killing and survival in the presence of SOC and/or a TNKSi or other novel adjuvant therapies.

2.0 Methodology

Table 1. List of chemicals and reagents

Chemical or Reagent	Supplier
Paraformaldehyde	Sigma
Ammonium Chloride (NH_4Cl_2)	Fisher Scientific
Sodium Dodecyl Sulfate (SDS)	Melford
Triton-X-100	Roche
Click-IT reaction kit	Fisher Scientific
Bovine Serum Albumin	Sigma
Donkey Serum	Sigma
Goat Serum	Abcam
Hoescht	Life Technologies
Vectashield	Vector laboratories
Dimethyl Sulfoxide (DMSO)	Sigma
Advanced DMEM	Invitrogen
Ethanol	Sigma
HEPES	Fisher Scientific
Matrigel	BD Biosciences
Phosphate buffered saline	OXOID
EDTA	Sigma
Iso-pentane	Fisher Scientific
IMDM	Invitrogen
Cell Titer Glo 3D	Promega

2.1 Organoid and tumouroid tissue culture

Colonic tissue is collected from patients undergoing treatment at the Norfolk and Norwich University Hospital (NNUH) and has been approved by the East of England research ethics committee [2013/2014–62 HT (ongoing approval)]. Human colonic crypts are isolated, tissue samples are obtained from patients at the NNUH and placed into ice cold PBS. Once transported to the laboratory the tissue is placed into HBS (Table 1) and supplemented with EDTA for one hour. Crypts are obtained through rounds of shaking, sedimentation, and collection. Crypts are embedded in a 20μl droplet of Matrigel that are placed onto glass coverslips in 12 well plates and left to polymerise for 10 minutes. The wells are then flooded with human colonic crypt culture medium (hCCCM): advanced F12/DMEM containing B27,

N2, n- acetylcysteine (1mM), hepes (10mM), pen/strep (100U/ml) L-Glutamine (2mM), Wnt-3A (100ng/ml), IGF-1 (50ng/ml), Noggin (100ng/ml) or Gremlin-1 (200ng/ml), RSPO-1 (500ng/ml), and the ALK 4/5/7 inhibitor A83-01-01 (0.5μM) (Reynolds *et al.*, 2014).

Organoids were obtained from crypts that have been allowed to grow for seven days. These organoids were generated by fragmenting the tissue into smaller pieces using a pipette and then centrifuged at 4°C and the supernatant was removed. Media is added to the pellet that remains and the fragments are resuspended. These fragments are embedded into Matrigel and flooded with human colonic crypt culture media (hCCCM). Organoids were cultured at 37°C and 5% CO2, fed every 3 days and passaged every 5-7 days.

2.2 Whole exome sequencing

Whole exome sequencing was carried out on four patient-matched organoid and tumouroid lines using a pipeline developed by Ryan Cardenas and Dr Dan Brewer. Single nucleotide polymorphisms (SNPs) and indels were identified using the Sanger Mutect2 and cgpWXS. The cgpWXS pipeline used caveman to identify SNPs whilst pindel was used to identify indels. The research presented in this paper was carried out on the High-Performance Computing Cluster supported by the Research and Specialist Computing Support service at the University of East Anglia (https://github.com/R-Cardenas/MED-Pipelines).

2.3 Immunocytochemistry

The tissue is fixed for five minutes at -20ºC in Methanol and then washed in ice cold PBS every 10 minutes for one hour. The tissue is then fixed again for one hour in 4% PFA on ice. All processing takes place on ice unless stated otherwise. The tissue is then put in 100mM ammonium chloride for 13 minutes removing the excess aldehyde groups. The plates are removed from the ice and placed in 1% SDS for five minutes, permeabilising the membrane and causing protein unfolding. The plates are placed back on ice and treated with 1% Triton X for 30 minutes permeabilising the membranes further. The samples were blocked with 10% Donkey serum and 1% BSA for two hours to prevent non-specific binding of the primary antibodies. Primary antibodies (**Table 2**) are added to samples over night at 4ºC.

Table 2. Primary antibodies

Antibody	Species	Clonality	Working concentration	Supplier
Anti-E-cadherin	Goat	Polyclonal	1:100	R&D System
Anti-Active Beta Catenin	Mouse	Monoclonal	1:50	Millipore
Anti-AxinII	Rabbit	Polyclonal	1:100	Abcam
Anti-OLFM4	Rabbit	Polyclonal	1:100	Abcam
Anti-CHAT	Goat	Monoclonal	1:100	Cell Signalling Technology
Anti-ChromagraninA	Rabbit	Polyclonal	1:100	Abcam
Anti-E-Cadherin	Mouse	Monoclonal	1:100	Abcam

The samples were washed twice with PBS and incubated with the correspondent secondary antibodies (**Table 3**) for two hours in the dark. The samples are mounted onto microscope slides using Vectashield containing 1μg/ml of the *DNA* stain Hoeschst.

Table 3. Secondary antibodies

Antibody	Species	Working concentration	Supplier
Anti-Mouse Alexa Fluor 488	Donkey	1:100	Invitrogen
Anti-Rabbit Alexa Fluor 488	Donkey	1:200	Invitrogen
Anti-Rabbit Alexa Fluor 568	Donkey	1:200	Invitrogen
Anti-Goat Alexa Fluor 647	Donkey	1:200	Invitrogen

2.4 Cell Proliferation Assays

Cell proliferation assays were carried out using the EdU Click-iT kit. EdU is a Thymidine analogue that is incorporated into the *DNA* of proliferating cells. The fluorescently labelled EdU molecule allows detection of stained nuclei using microscopy imaging. Human colonic organoids and tumouroids were incubated with EdU (10μM) for two hours, and then fixed and permeabilised as previously described. The tissue is taken off ice and washed in 3% BSA and placed into a Click-iT reaction using Invitrogen's EdU assay kit as per the manufacturer's instructions. The samples are then blocked as previously described.

2.5 Cell Viability Assays

Chemotherapy concentrations for this set experiments were determined by work carried out by Victoria Jones in the Williams laboratory prior to the start of my project. Organoids were treated with a combination of SOC (5-FU) and/or a TNKSi at different concentrations for 7 days with treatment taking place on day 1 and day 4 (**Figure 12**). On day 7 organoids and tumouroids are processed using Promega Cell Titer Glo kit as per the manufacturer's instructions for 30 minutes. The samples are then moved into a domino plate and read using a FLUOstar Omega plate reader.

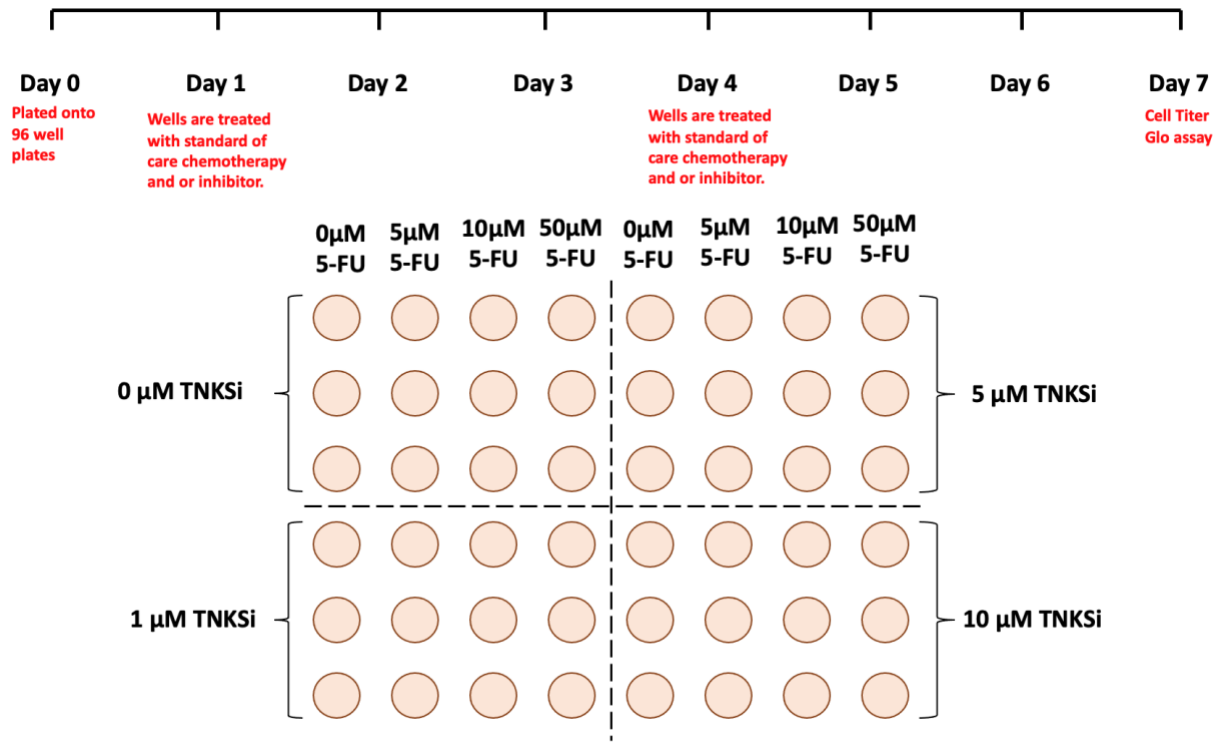


Figure 12. 96 well plate layout and timeline of treatment

Schematic diagram of 96 well layout for Cell Titer Glo experiments with ranging concentrations of TNKSi and SOC (5-FU) as well as a timeline showing when the organoids and tumouroids are treated and when the assay is run.

Cell Titer Glo quantifies the amount of metabolically active ATP in a sample which indicates the number of live cells present after a 7-day treatment with a TNKSi and/or chemotherapy drug. In the presence of magnesium, oxygen, and intracellular ATP. Luciferin is converted to Oxyluciferin and light (**Figure 13**). The light is then measured by a plate reader, and this is converted into a numerical value.

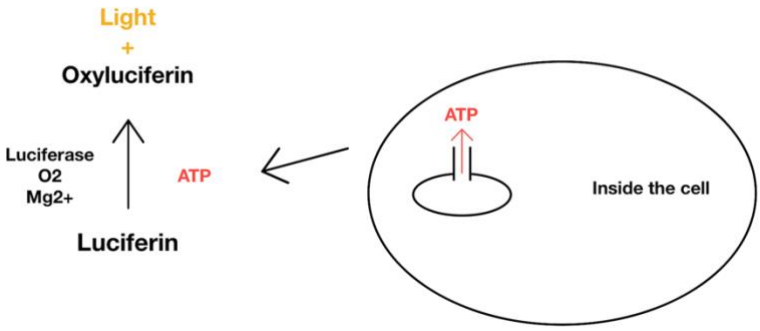


Figure 13. Cell Titer Glo chemical reaction.

Schematic diagram showing the Luciferin to Oxyluciferin reaction required for Cell Titer Glo, this is catalysed by the enzyme luciferase. When Oxyluciferin is converted, light is emitted to which is recorded by a plate reader and quantified.

2.6 Confocal microscopy

High resolution images were obtained using Zeiss LSM 980 confocal laser scanner microscope. Immunolabelling of organoids and tumouroids were visualised using a x40 oil immersion lens. When required, stacks of 2-3µm intervals were used to cover the Z axis of the area of interest. The images are then analysed and processed on Fiji ImageJ.

2.7 Data analysis

Fluorescence intensity analysis was used to compare the active beta-catenin present in the nucleus of organoids and tumouroids when treated with a TNKSi. The images were analysed using Fiji ImageJ (**Figure 14**), all data is normalised to control. Using this software, regions of interest (ROIs) were drawn around 10 random nuclei of individual organoids and tumouroids (**Figure 14**). Images are taken of tumouroids and organoids in different conditions using the same exposure settings.

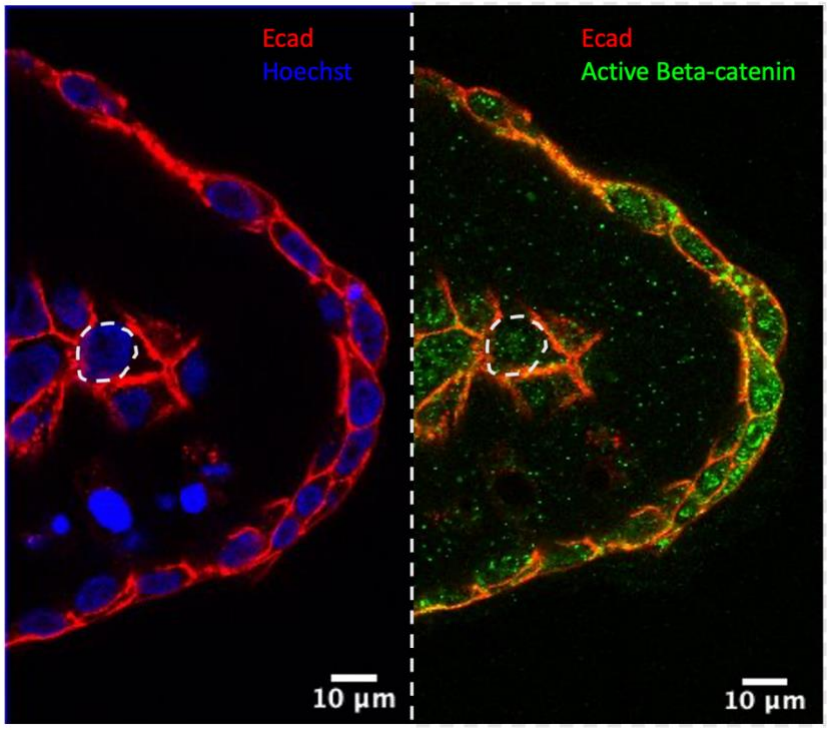


Figure 14. Fluorescence intensity analysis.

Colonic tumouroids and organoids were imaged on the Zeiss LSM 980 confocal microscope. The image shows a colonic tumouroid and how fluorescence intensity analysis was carried out. The white outline highlights how nucleic active beta catenin was measured. Scale bar 10µM.

2.8 Validation of data analysis

To validate the data analysis from the Cell Titer Glo, live dead counts and area counts were used. Live dead counts compared the number of live tumouroids and organoids vs the number of dead tumouroids and organoids on Day 7 after treatment with standard of care chemotherapy and the chosen candidate drug. Area counts analyse the effects of living tumouroids after a 7 Day treatment with standard of care chemotherapy and the candidate drug of choice. The change in cross sectional area is measured on Day 1, Day 3, and Day 7. The data is then averaged and normalised to the control. R was used to run two-way ANOVAs to test for significance between the data sets.

2.9 Statistical analysis

Statistical analysis was carried out in a programme written in R, a 2-way ANOVA followed by a post hoc Tukey test. This was used to determine statistical significance in experimental data sets. Experiments were conducted in biological replicates (N) of two where possible. Within each biological replicate there were techincal replicates (n) of three.

3.0 Results

In order to investigate the opportunities a patient-matched organoid and tumouroid personalised medicine pipeline has to advance research into new therapies for colon cancer, a number of Cell Titer Glo assays, viability assays, were carried out. Cell Titer Glo assays quantify the amount of metabolically active ATP present under certain condition, therefore we were able to determine the different effects of 5-FU and inhibitors. These assays were carried out on patient-matched organoids and tumouroids, in turn allowing us to determine the most effective treatments for individual patient lines.

3.1 Tumouroids show a differential response to standard of care chemotherapy (5-FU).

It was imperative to characterise the efficacy of 5-FU chemotherapy on patient-matched organoids and tumouroids. Patients undergoing chemotherapy treatment for CRC in the clinic will all exhibit a differential sensitivity to their treatment. Thus determining the sensitivity of the tumouroid lines to SOC (5-FU) (Venook, 2005). To establish this effect a seven-day cell viability assay was run where UEA005 and UEA005 tumouroids are treated with 5-FU at 4 different concentrations (0μM, 5μM, 10μM and 50μM).

Strikingly, when UEA005 was treated with four different concentrations of 5-FU there was a significant reduction in cell viability compared to the control which had no treatment with 5-FU (**Figure 15i, Figure 15iii**). Moreover, the analysis of tumouroid cross-sectional area further confirmed the findings, tumouroid growth reduced until Day 2 and Day 3 where they stopped growing and began to die therefore size of the tumouroids began to decrease (**Figure 1i**).

Although both these tumouroid lines are sensitive to SOC, 5-FU, there is an apparent difference in the two tumouroid lines. UEA005 appear to be highly sensitive to SOC even at low concentrations such as 5μM showing an 80% reduction in the viability of the cells when compared to control (**Figure 15iii**). Whereas the UEA007 line only showed a 60-70% reduction in the viability of the cells (**Figure 15ii**). This difference in sensitivity to 5-FU alone can be influenced by many things including the genetics factors and the stage at which the cancer was diagnosed. The cell viability assay for UEA007 also appears to indicate an increase in the viability as the concentration of 5-FU increase however this data is not conferred in the cross-sectional area analysis (**Figure 15ii**).

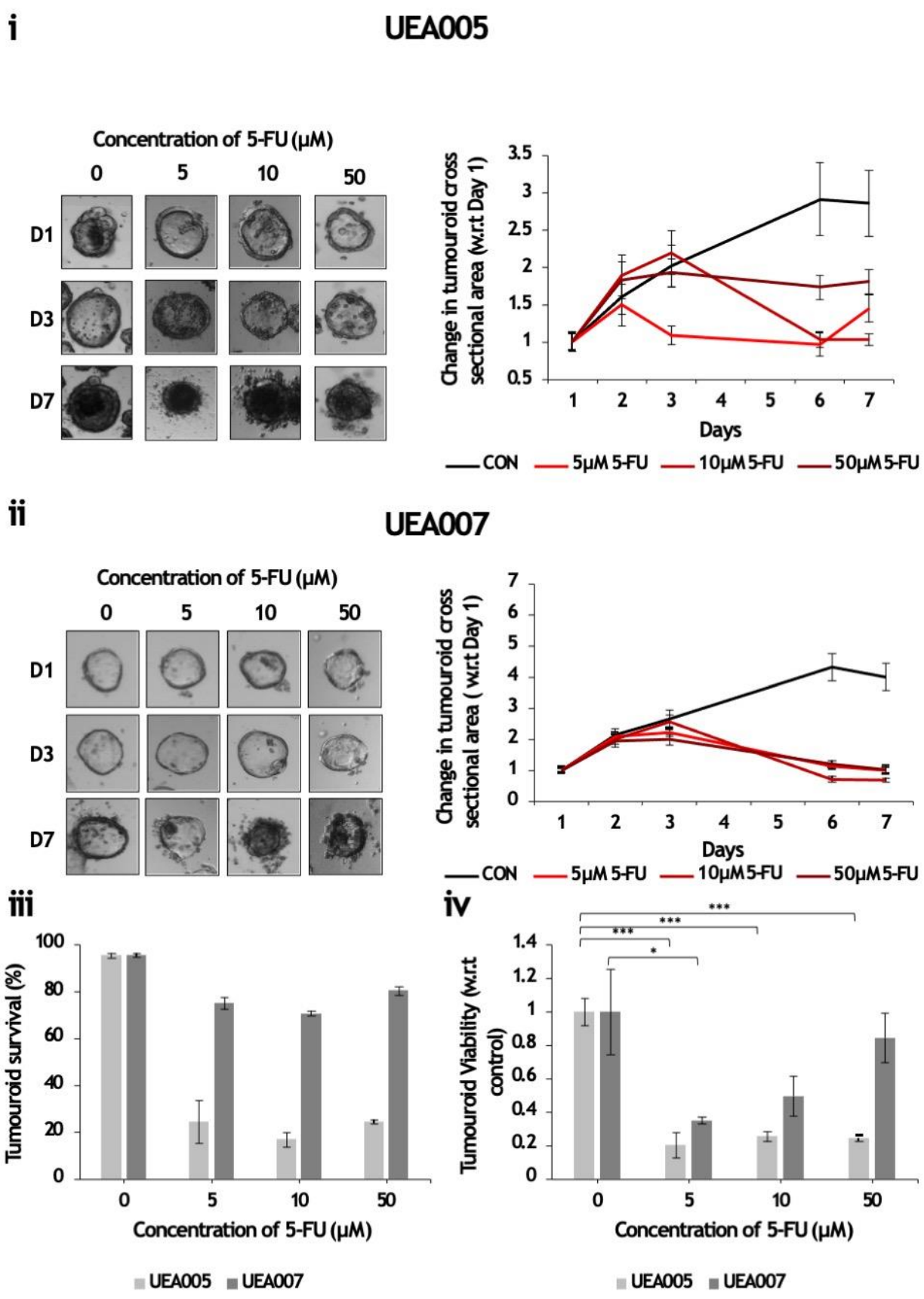

i
UEA005
Concentration of 5-FU (μM)
0
5
10
50
D1
D3
D7
Change in tumouroid cross sectional area (w.r.t Day 1)
Days
CON
5μM 5-FU
10μM 5-FU
50μM 5-FU
ii
UEA007
Concentration of 5-FU (μM)
Change in tumouroid cross sectional area (w.r.t Day 1)
Days
CON
5μM 5-FU
10μM 5-FU
50μM 5-FU
iii
Tumouroid survival (%)
Concentration of 5-FU (μM)
UEA005
UEA007
iv
Tumouroid Viability (w.r.t control)
Concentration of 5-FU (μM)
UEA005
UEA007

Figure 15. Sensitivity of UEA005 and UEA006 Tumouroids to standard of care chemotherapy 5-FU.

i) UEA005 representative images showing tumouroids that have been treated with a range of concentrations of 5-FU and the change in tumouroid cross sectional area showing a decrease in the area over a seven-day course. **ii)** UEA007 representative images showing tumouroid lines treated with a range in concentrations of 5-FU and the change in tumouroid cross sectional area over a seven-day course of treatment. **iii)** Live dead analysis of UEA005 and UEA007 tumouroids on day seven, this analysis quantifies the number of living tumouroids after the seven-day treatment with 5-FU. **iv)** Cell viability analysis of UEA005 and UEA007 tumouroids after seven-day chemotherapy treatment showing UEA005 are highly sensitive to 5-FU whilst UEA007 are not as sensitive. Experiments are performed in biological repeats (N=2) and technical repeats (n=6) and error bar indicative of S.E.M (*** $p<0.001$, * $p<0.05$, Two Way ANOVA followed by a post hoc Tukey test).

3.2 Patient matched organoids and tumouroids have a diverse mutational profile.

Having shown the differential sensitivity of tumouroids, it was important to determine the suite of mutations that influence the response to 5-FU. Previous studies indicate the mutational profiles of the tumouroids can dictate their response to chemotherapy (Ooft *et al.*, 2019). Therefore, patient derived tumouroids were analysed using whole exome sequencing (WES) to determine the individual mutational signatures they have. The sequencing analysis highlighted a variety of TIER ONE cancer driver gene mutations in multiple different oncogenes and tumour suppressor genes (**Figure 16i**). The WES also highlights if the mutations are nonsense, missense or indel. Nonsense mutations results in a change in *DNA* causing the protein to terminate and stop the translation of *DNA*. Missense mutations result in different amino acids being encoded which could alter protein function. Finally, Indels is used to describe insertions or deletions of bases into the protein code which again has the ability to alter protein function (Iranzo, Martincorena and Koonin, 2018). The tumouroid lines show distinct patterns and harbour individual mutations that are specific to the tumour. Importantly the whole exome sequencing showed that all three of the tumouroid lines have a mutation in APC (**Figure 16i**). From the mutational profiles exhibited, UEA005 have an early APC truncation whilst UEA006 and UEA007 have a late APC truncation. Therefore, determining the sensitivities of the tumouroids to SOC treatment and TNKSi could prove promising (**Figure 16i**).

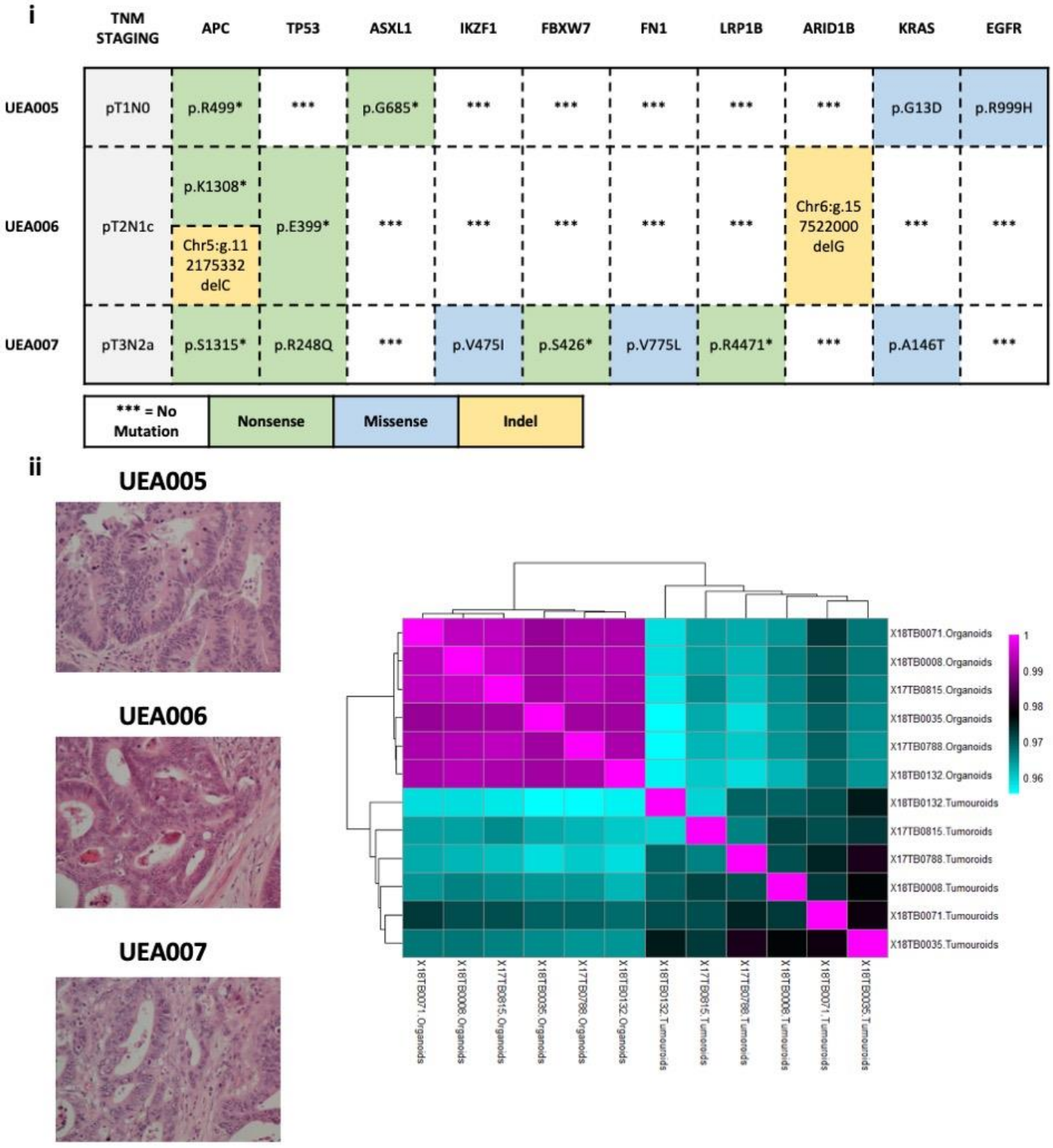


Figure 16. Mutational profile of patient-matched tumouroids.

i) Mutational classification of three patient tumouroid lines UEA005, UEA006 and UEA007 and the tier one cancer driver gene mutational profiles. As well as identifying if the mutations they harbour are missense, nonsense or indels. **ii)** Tissue histology of patient-matched tumouroids UEA005, UEA006 and UEA007. **iii**) correlation matrix showing how transcriptomically distinct the organoids are compared to the tumouroids.

Moreover, TP53 might also prove a promising biomarker in the research for sensitivities of a tumour to SOC chemotherapy. Research suggests that TP53 might be able to predict the response of a tumour to SOC chemotherapy. The studies suggest tumours with wild type (WT) TP53 would be highly sensitive to treatment with 5-FU. However when the tumour harbours a TP53 mutation they would be less sensitive (Oden-Gangloff *et al.*, 2009). WES sequencing data predicts that UEA005 tumouroids would be sensitive to SOC chemotherapy, whilst UEA006 and UEA007 tumouroids would be less sensitive (**Figure 16i**). Another gene highlighted by the WES was KRAS. In UEA005 and UEA007 tumouroids there is a gain of function mutation resulting in the MAP kinase signalling pathway being constitutively active. Activation of this pathway results in the tumours growth and survival (**Figure 16i**). It might prove beneficial when selecting adjuvant therapies to be used alongside chemotherapy. KRAS mutations are thought to be sensitive to MEK inhibitors, as§ this is a gene that is activated downstream of KRAS in the EGF signalling pathway (**Figure 6**) (Guo *et al.*, 2020). Moreover, KRAS can dictate a tumours response to an EGFR inhibitor. When tumours harbour both an EGFR and a KRAS gain of function mutation, studies have shown that using an EGFR inhibitor alone does not increase the killing effect of the treatment and it is insensitive to the inhibitor. This is due to the KRAS mutation that is functioning downstream of the initial EGFR mutation (Phadke *et al.*, 2018). Moreover, studies suggest using both an EGFR inhibitor and a MEK inhibitor in combination might be the most beneficial. From the WES data using a MEK and EGFR inhibitor in combination with 5-FU on the UEA005 and UEA007 tumouroid line could be beneficial (**Figure 16i**). patient-matched organoids and tumouroids were also compared transcriptomically, Figure 16ii shows a correlation matrix indicating the transcriptomes of the organoids and tumouroids are genetically distinct (**Figure 16ii**). Thus, indicating different factors could drive the tumours survival compared to the organoids.

3.3 APC mutational status of patient-matched organoids and tumouroids do not dictate the response to a TNKSi alone or in combination with 5-FU.

After establishing the mutational profiles of patients matched organoids and tumoroids interestingly all tumouroids harbour an APC mutation. Work carried out by (Tanaka *et al.*, 2017; Schatoff *et al.*, 2019) both suggest the length of the APC truncation can dictate the response of the tumour to a Tankyrase inhibitor.

3.3.1 Patient-matched UEA005 organoids and tumouroids

UEA005 tumouroids have an early APC truncation and therefore would be expected to be insensitive to a TNKSi. This is due to the minimal amount of APC protein remaining meaning it is unable to interact with the destruction complex (Schatoff *et al.*, 2019). In order to investigate the role that TNKSi may play in improving the efficacy of 5-FU treatment in this line patient-matched organoids and tumouroids were treated with a range in concentrations of 5-FU and TNKSi.

The outcome of these experiments suggests that UEA005 tumouroids when treated with SOC chemotherapy appear highly sensitive even when treated with the lowest concentration of 5-FU, 0.5μM showing a highly significant difference from the control (**Figure 17 A, Bi**). The heat map of tumouroid organoid and tumouroid viability in Figure 17A suggests that when this tumouroid line is treated with SOC chemotherapy they become highly sensitive and begin to die. This sensitivity to standard of 5-FU does not appear to increase when the concentration of 5-FU is increased meaning for this tumouroid line a low dose of chemotherapy might be beneficial. From the data in Figure 17 Bii, Biii, Biv it suggests that a TNKSi does not improve the efficacy of the 5-FU treatment. However due to the sensitivity of the tumouroids to treatment it can be challenging to deduce any patterns that might appear when using a TNKSi. This data presented so far fits within the hypothesis laid out in the paper by Schatoff and colleagues which suggests that early APC truncations will be insensitive to a TNKSi.

When UEA005 organoids are treated with 5-FU there appears to be some sensitivity to the treatment. However, the treatment doesn't appear to be as effective at killing the healthy organoids when compared to the tumouroids (**Figure 17A, Bi**). For example, when the tumouroids were treated with 5-FU alone there was ~70-80% death (**Figure 17Bi**) whilst when the organoids are treated with 5-FU alone only ~40-50% death is seen (**Figure 17Bi**). This finding could be crucial as this might mirror what was observed in the clinic. This patient might have responded to treatment better and also had minimal side effects to their treatment due to the tumour being so sensitive to treatment. Once the organoids begin to be treated with a TNKSi the organoids appear more sensitive to not only 5-FU but the TNKSi too. When treated with the TNKSi there is around 60-70% organoid death (**Figure 17B ii, iii, iv**). However, although there is an increase in the death of the organoids there is also a minimal increase in the death of tumouroids (**Figure 17**). When these tumouroids are treated with increasing concentrations of 5-FU and TNKSi there is 80-90% tumouroid death.

Similar to what was observed when UEA005 organoids and tumouroids are treated with 5-FU, UEA005 organoids are less sensitive to 5-FU used in combination with a TNKSi compared to tumouroids (**Figure 17**).

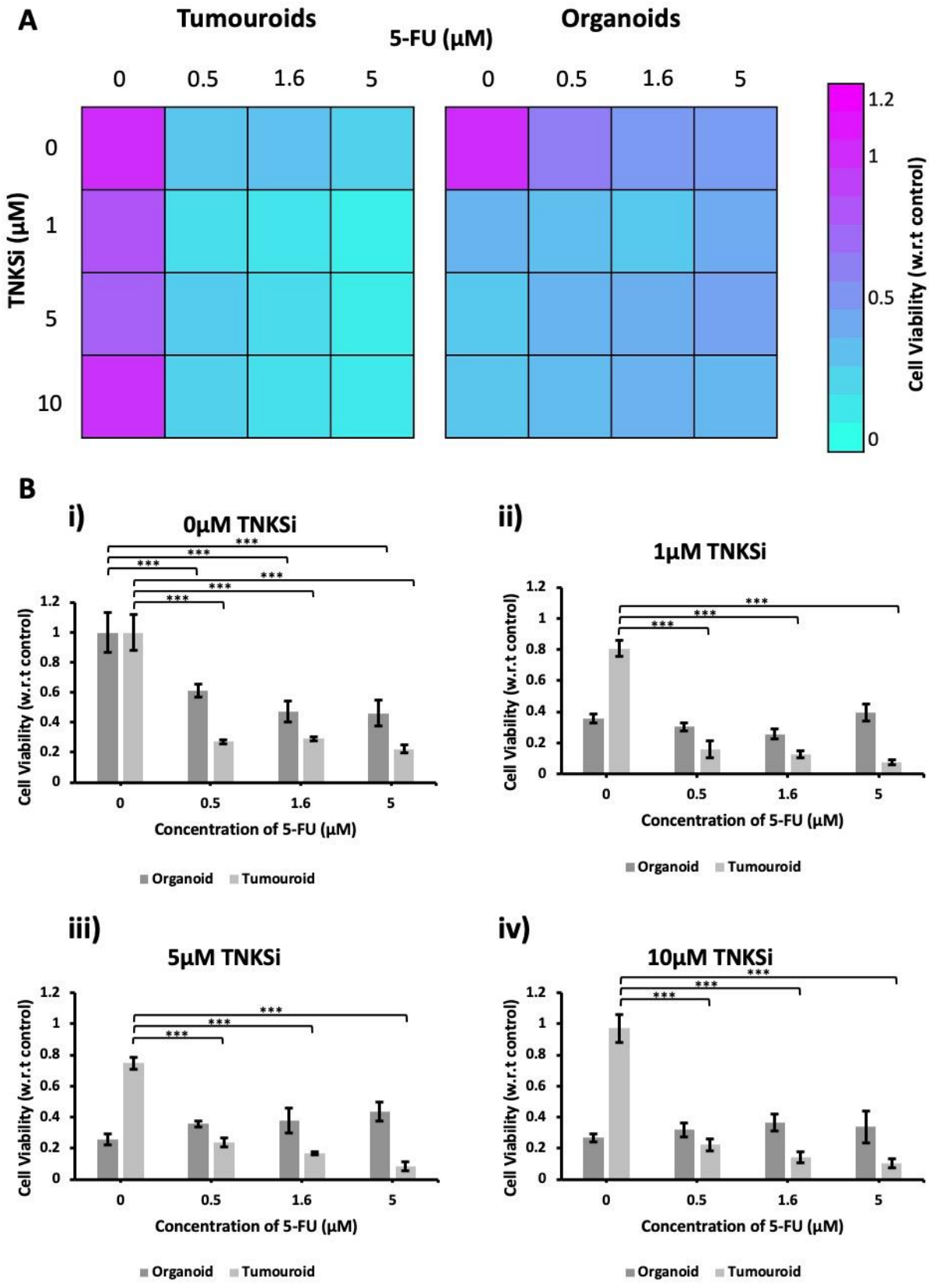

A
Tumouroids
Organoids
5-FU (µM)
0
0.5
1.6
5
TNKSi (µM)
0
1
5
10
Cell Viability (w.r.t control)
1.2
1
0.5
0
B
i)
0µM TNKSi
ii)
1µM TNKSi
iii)
5µM TNKSi
iv)
10µM TNKSi
***
Cell Viability (w.r.t control)
Concentration of 5-FU (µM)
Organoid
Tumouroid

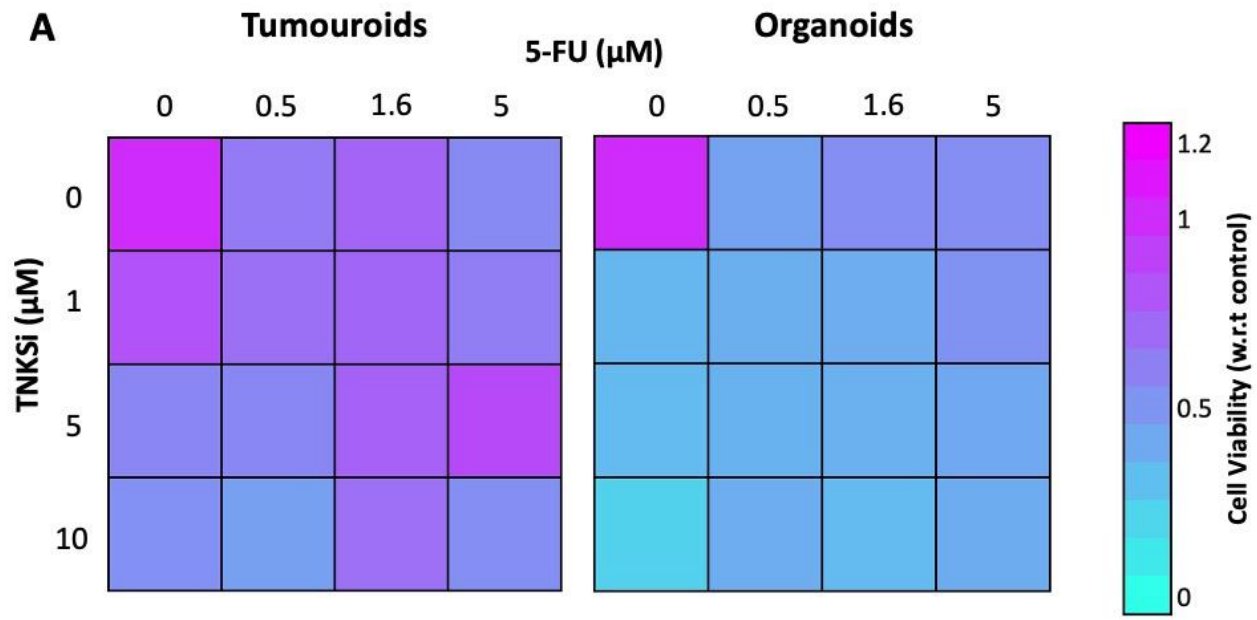
Figure 17. UEA005 patient-matched organoid and tumouroid sensitivity to a TNKSi alone or in combination with standard of care chemotherapy.

A) Heat maps comparing the sensitivity to patient-matched organoid and tumouroids when treated with 5-FU alone and/or a TNKSi. Bi) Comparison of UEA005 patient-matched organoids and tumouroids treated with 5-FU alone. Bii) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 1μM of TNKSi. Biii) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 5μM of TNKSi. Biv) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 10μM of TNKSi. Experiments are performed in biological repeats (N=2) and technical repeats (n=6) and error bar indicative of S.E.M (*** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.3.2 Patient-matched UEA006 organoids and tumouroids

UEA006 patient-matched tumouroids harbour a late APC truncation resulting in a longer residual APC protein being present. In theory this would allow APC to still be able to interact within the destruction complex and lead to some activation of the WNT signalling pathway and destruction of beta catenin (Schatoff *et al.*, 2019). Therefore, when a TNKSi is used we would expect the tumouroids to be further sensitised to standard of care chemotherapy compared to when chemotherapy is used alone.

To analyse the effect of standard of care chemotherapy and/or TNKSi cell viability assays were conducted. The organoids and tumouroids are treated with a range of different concentrations of 5-FU and/or TNKSi (**Figure 18**). The results suggest UEA006 tumouroids are not as sensitive to standard of care chemotherapy alone compared to UEA005 tumouroids (**Figure 18A, Bi**). When a TNKSi is introduced UEA006 tumouroids appear more sensitive when treated with a TNKSi alone with 20% more death observed compared to when treated with no TNKSi (**Figure 18Bi,ii**). Moreover, as the concentration of TNKSi is increased to 5μM and 10μM TNKSi there is an increase in the death of the tumouroids. Compared to the 20% death observed at 1μM there is between 40-50% death at 5μM and 10μM. When the TNKSi is used in combination with 5-FU, the tumouroids do not appear to be further sensitised (**Figure 18**).


A
Tumouroids
Organoids
5-FU (µM)
0
0.5
1.6
5
TNKSi (µM)
0
1
5
10
Cell Viability (w.r.t control)
1.2
1
0.5
0

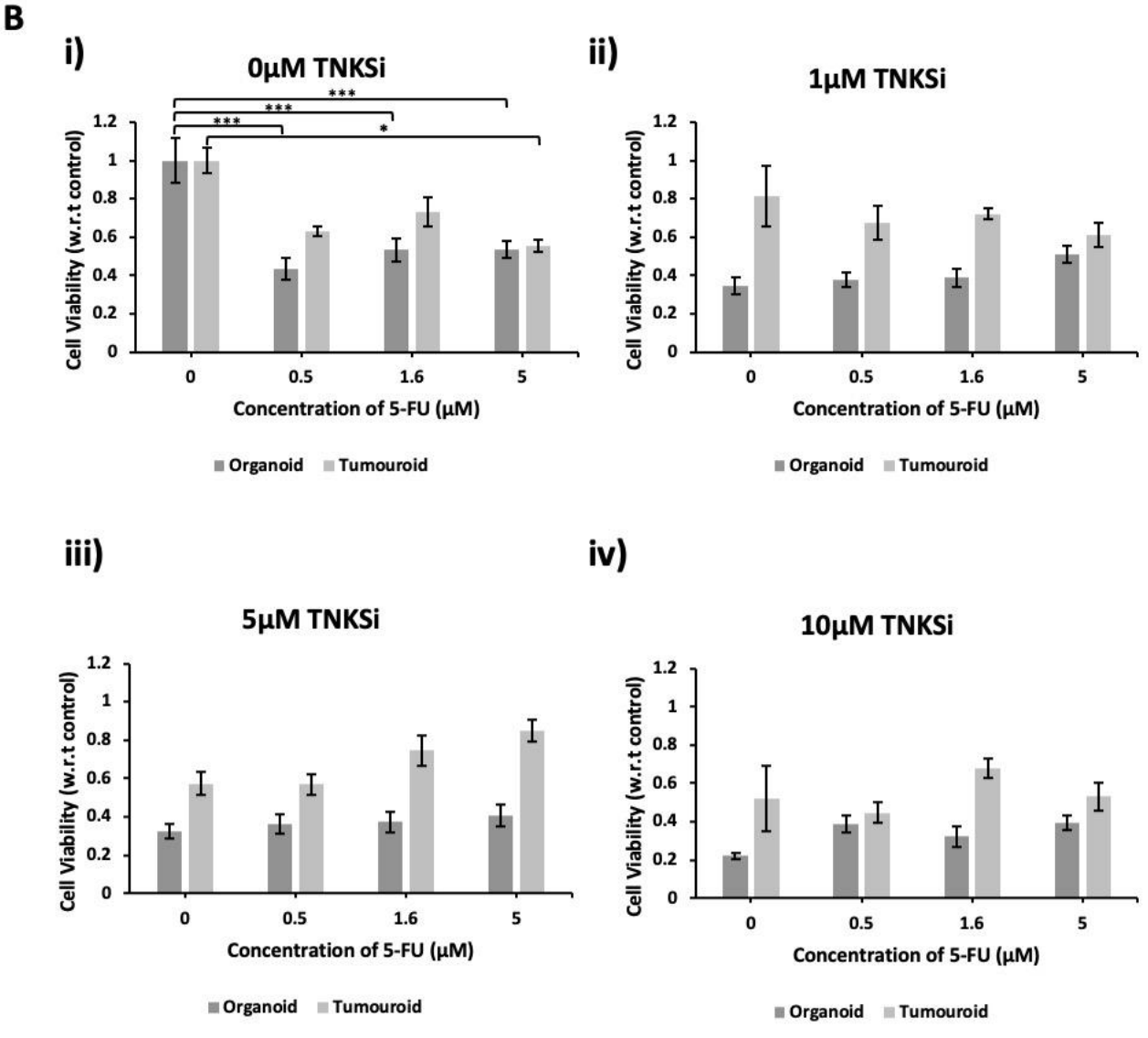

B
i)
0µM TNKSi
ii)
1µM TNKSi
iii)
5µM TNKSi
iv)
10µM TNKSi
Cell Viability (w.r.t control)
Concentration of 5-FU (µM)
Organoid
Tumouroid
***
***
***
*

Figure 18. Patient-matched UEA006 organoid and tumouroid sensitivity to a TNKSi alone or in combination with standard of care chemotherapy.

A) Heat maps comparing the sensitivity to patient-matched organoid and tumouroids when treated with 5-FU alone and/or a TNKSi. Bi) Comparison of UEA006 patient-matched organoids and tumouroids treated with 5-FU alone. Bii) Comparison of Patient-matched organoids and tumouroids treated with standard of care chemotherapy and 1μM of TNKSi. Biii) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 5μM of TNKSi. Biv) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 10μM of TNKSi. Experiments are performed in biological replicates (N=2) technical repeats (n=6) and error bar indicative of S.E.M (* P<0.05, *** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.3.3 UEA007 Tumouroids

UEA007 tumouroids have a late APC truncation resulting in more residual APC protein being present. This would allow APC to still be able to interact within the destruction complex and lead to some activation of the WNT signalling pathway and destruction of beta catenin (Schatoff *et al.*, 2019). Therefore, when a TNKSi is used we would expect the tumouroids to be further sensitised to standard of care chemotherapy compared to when chemotherapy is used alone similar to UEA006 tumouroids.

To further analyse the effect of standard of care chemotherapy alone and in combination with a TNKSi. UEA007 tumouroids were treated with 5-FU or a TNKSi alone. This tumouroid line appeared to be the least sensitive to 5-FU alone, which could be linked to the mutational burden of the tumouroid line (**Figure 16, Figure 19**). Although tumouroid death increases as the concentration of 5-FU increases, introducing a TNKSi does not aid the efficacy of the chemotherapy (**Figure 19A**).

From the results it appears a TNKSi does not sensitise the tumouroids further to 5-FU, when used in combination or alone. Therefore, for this tumouroid line it would be most effective to use 1.6μM 5-FU alone (**Figure 19Bi**). This would hopefully minimise the effect on the healthy tissue as the highest concentration of 5-FU results in 50% tumouroid death which is the same that is seen for 1.6μM 5-FU. UEA007 organoids were not analysed and therefore meant a comparison of the organoids and tumouroids were unable to be drawn.

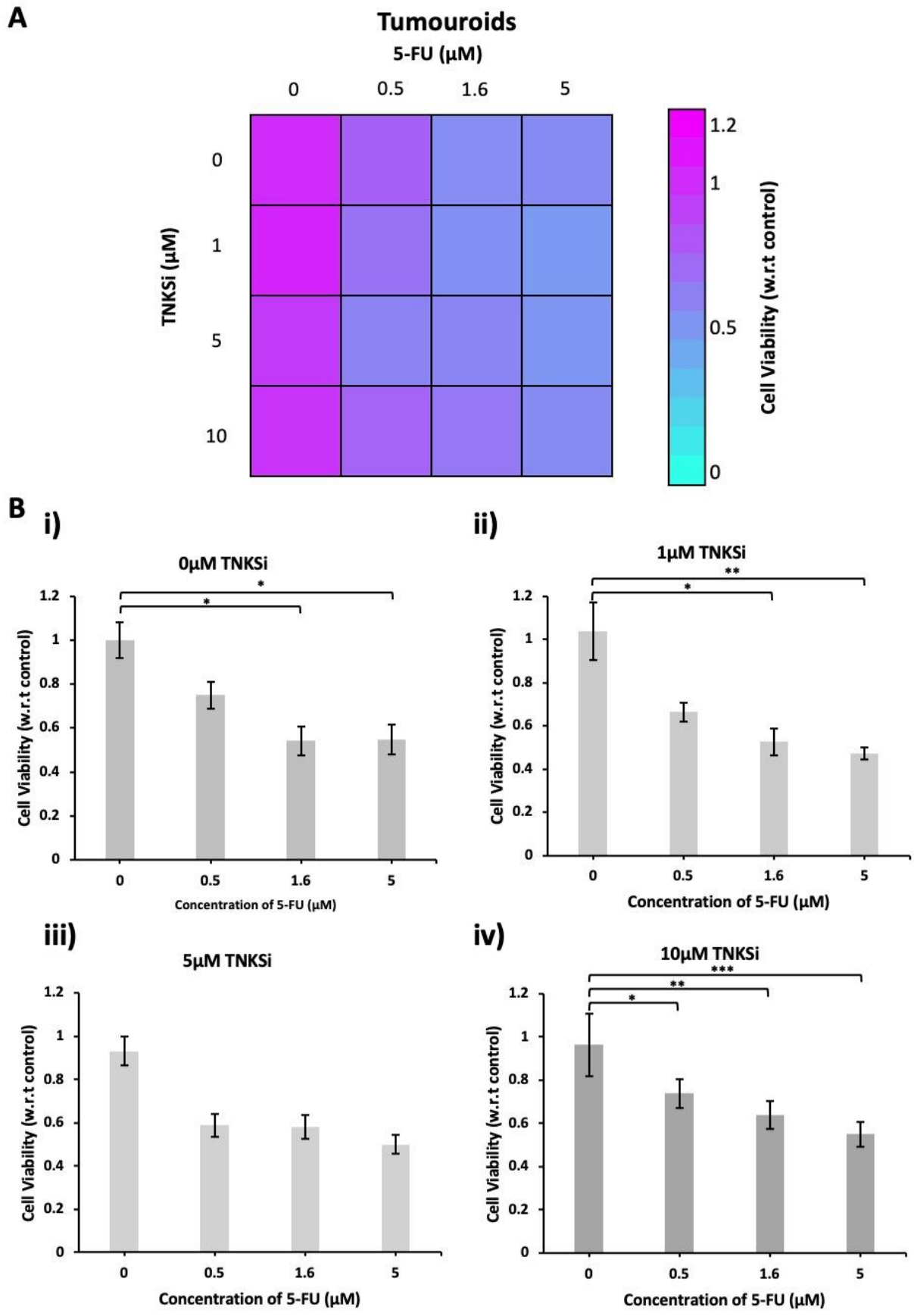

A
Tumouroids
5-FU (µM)
0
0.5
1.6
5
TNKSi (µM)
0
1
5
10
Cell Viability (w.r.t control)
1.2
1
0.5
0
B
i)
0µM TNKSi
ii)
1µM TNKSi
iii)
5µM TNKSi
iv)
10µM TNKSi
Cell Viability (w.r.t control)
Concentration of 5-FU (µM)

Figure 19. Sensitivity of UEA007 tumouroids when treated with TNKSi alone or in combination with standard of care chemotherapy.

A) Heat map showing the sensitivity of UEA007 tumouroids to a TNKSi alone or in combination with standard of care chemotherapy. Bi) Sensitivity of UEA007 tumouroids to chemotherapy alone. Bii) Tumouroids treated with 1μM TNKSi and standard of care chemotherapy. Biii) Tumouroids treated with 5μM TNKSi and standard of care chemotherapy. Biv) Tumouroids treated with 10μM TNKSi and standard of care chemotherapy. Experiments are performed in biological replicates (N=2) technical repeats (n=6) and error bar indicative of S.E.M (* P<0.05, ** P<0.01, *** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.3.4 Patient-matched UEA003 organoids and tumouroids

UEA003 tumouroids have an early APC truncation and therefore would be expected to be insensitive to a TNKSi. Therefore, when a TNKSi is used we would expect the tumouroids to be insensitive to the inhibitor.

When UEA003 organoids were treated with 5-FU alone the treatment elicited 50% organoid death. However, when the tumouroids are treated with 5-FU alone there was only 10-20% tumouroid death (**Figure 20 A, Bi**).

From work carried out by Schatoff and colleagues we would expect the tumouroids to be insensitive to a TNKSi when used alone and in combination with 5-FU. From the results it can be concluded that the UEA003 are insensitive to a TNKSi when used at all three concentrations, with tumouroid death ranging from 20-50% (**Figure 20**).

Moreover, due to the organoids being more sensitive to a TNKSi when used in combination with 5-FU, using 5μM TNKSi with 0.5μM 5-FU could be the most effective. This resulted in 50% tumouroid and organoid death (**Figure 20Biii**). Compared to 0.5μM 5-FU alone which resulted in 50% organoid death and 40% tumouroid death (**Figure 20Bi**). These results suggest a TNKSi might have a prohibitive effect on the organoids compared to the tumouroids.

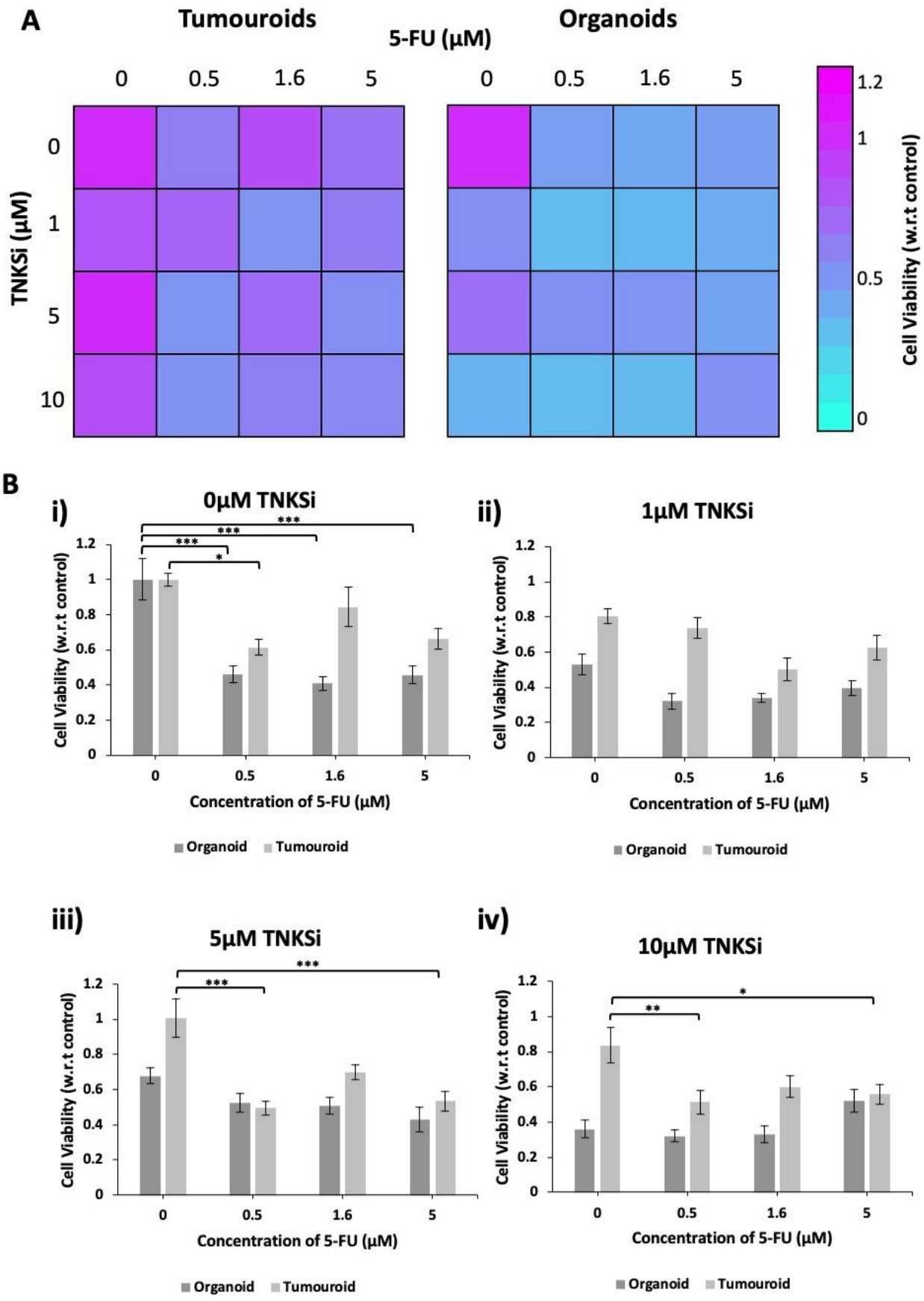

A
Tumouroids
Organoids
5-FU (μM)
0
0.5
1.6
5
TNKSi (μM)
0
1
5
10
Cell Viability (w.r.t control)
1.2
1
0.5
0
B
i)
0μM TNKSi
ii)
1μM TNKSi
iii)
5μM TNKSi
iv)
10μM TNKSi
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)
Organoid
Tumouroid
***
**
*

Figure 20. Patient-matched UEA003 organoid and tumouroids after treatment with a TNKSi alone or in combination with standard of care chemotherapy.

A) Heat maps comparing the sensitivity to patient-matched organoid and tumouroids when treated with 5-FU alone and/or a TNKSi. Bi) Comparison of UEA003 patient-matched organoids and tumouroids treated with 5-FU alone. Bii) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 1μM of TNKSi. Biii) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 5μM of TNKSi. Biv) Comparison of patient-matched organoids and tumouroids treated with standard of care chemotherapy and 10μM of TNKSi. Experiments are performed in biological replicates (N=2) and technical repeats (n=6) and error bar indicative of S.E.M (* P<0.05, **<0.01, *** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.4 KRAS mutational status of patient-matched organoids and tumouroids might dictate the response to an EGFR and MEK inhibitors alone or in combination with 5-FU.

KRAS mutations result in the constitutive activation of the RAS-MAP kinase signalling pathway and results in cellular proliferation and survival of tumours. Therefore, targeting this signalling pathway with an EGFR inhibitor (Afatinib) or a MEK inhibitor (Selumetinib) may prove a promising adjuvant therapy in targeting CRC (**Figure 6**).

3.4.1 Patient-matched UEA005 organoids and tumouroids

To analyse the effect of a KRAS inhibitor or an EGFR inhibitor alone or in combination with 5-FU. UEA005 tumouroids and organoids were treated for 7 days after which a cell viability assay was assessed. UEA005 tumouroids harbour a KRAS and EGFR mutation so it is therefore hypothesised that they would be sensitive to use of Selumetinib and Afatinib when used in combination with 5-FU.

UEA005 tumouroids are highly sensitive to 5-FU alone showing 50-60% tumouroid death compared to organoids that show between 30-40% organoid death (**Figure 21Bi**). Therefore, using chemotherapy alone might be an effective enough treatment for this line. However, UEA005 tumouroids appear highly sensitive to Selumetinib alone showing 60% tumouroid death whilst the organoids exhibit survival advantage when treated with Selumetinib alone (**Figure 21Bii**). This therefore might make this an effective treatment for tumouroids without affecting the organoids. The organoids being highly sensitive to these inhibitors is not unexpected, due to Selumetinib and Afatinib blocking both the RAS map kinase and PI3K signalling pathway, which are both vital in homeostasis.

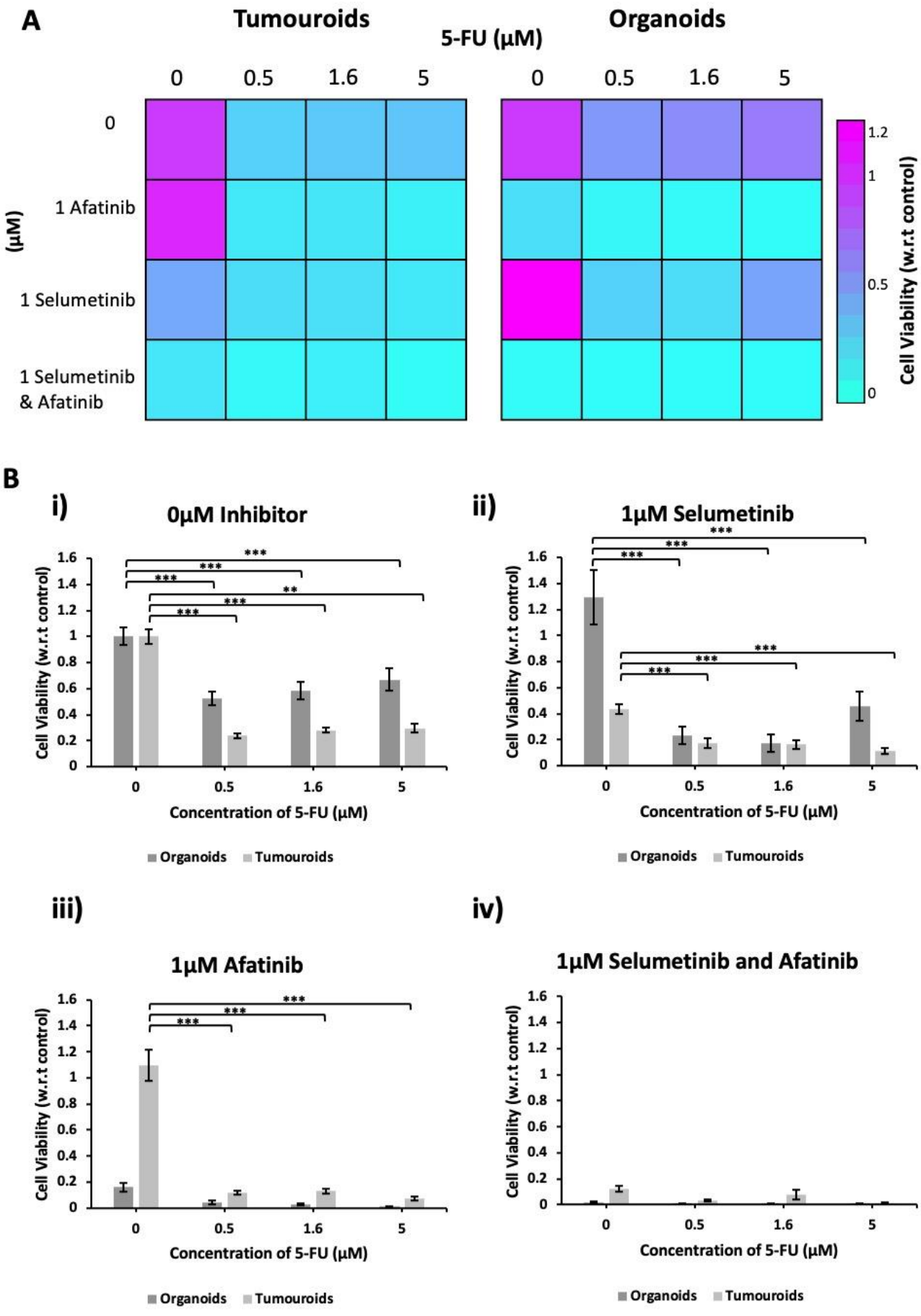

A
Tumouroids
Organoids
5-FU (µM)
0
0.5
1.6
5
(µM)
0
1 Afatinib
1 Selumetinib
1 Selumetinib & Afatinib
1.2
1
0.5
0
Cell Viability (w.r.t control)
B
i)
0µM Inhibitor
ii)
1µM Selumetinib
iii)
1µM Afatinib
iv)
1µM Selumetinib and Afatinib
Cell Viability (w.r.t control)
Concentration of 5-FU (µM)
Organoids
Tumouroids

Figure 21. Patient-matched UEA005 organoid and tumouroid sensitivity to EGF and KRAS inhibitors alone or in combination with standard of care chemotherapy.

A) Heat map showing a comparison UEA005 organoids and tumouroids when treated with 5-FU, Afatinib and Selumetinib alone or a combination of the two inhibitors. Bi) Comparison of organoids and tumouroids treated with 5-FU. Bii) Comparison of organoids and tumouroids treated with Afatinib alone or in combination with 5-FU. Biii) Comparison of organoids treated with Selumetinib alone or in combination with 5-FU. Biv) Comparison of organoids treated with Selumetinib and Afatinib alone or in combination with 5-FU. Experiments are performed in biological replicates (N=2) and technical repeats (n=6) and error bar indicative of S.E.M (*** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.4.2 UEA006 Organoids

UEA006 organoids are expected to be sensitive to both Selumetinib and Afatinib due to the role of EGF and KRAS in homeostatic processes. Preventing this signalling pathway from functioning would prevent the proliferation and differentiation of cells. This was observed in the Cell Titer Glow results.

UEA006 organoids appear relatively sensitive to 5-FU alone where we observed 40-50% organoid death (**Figure 22Bi**). These results are similar to what was seen when UEA006 organoids were treated with a TNKSi alone or in combination with 5-FU (**Figure 18**). However, when the organoids are treated with the inhibitors alone or in combination the organoids become highly sensitive to the treatment.

When the organoids are treated with Selumetinib alone or in combination with 5-FU there was 40-70% organoid death. Moreover, when the organoids are treated with Afatinib alone the organoids exhibited 70-90% death (**Figure 22 Biii**). Finally, when used in combination the organoids showed 90-95% organoid death. These results suggest that UEA006 organoids are highly sensitive to these inhibitors and when choosing a treatment for UEA006 tumouroids and 5-FU based treatment only would be most effective (**Figure 22**). From these results it can be concluded that the effects of Selumetinib and Afatinib in combination with 5-FU were highly toxic.

Due to lack of tumouroid availability UEA006 tumouroids were not tested against these drugs. If these drugs were tested against this tumouroid line, it would be hypothesised that they would not be sensitive to both inhibitors. This is due to the tumouroid not harbouring a KRAS or EGFR mutation.

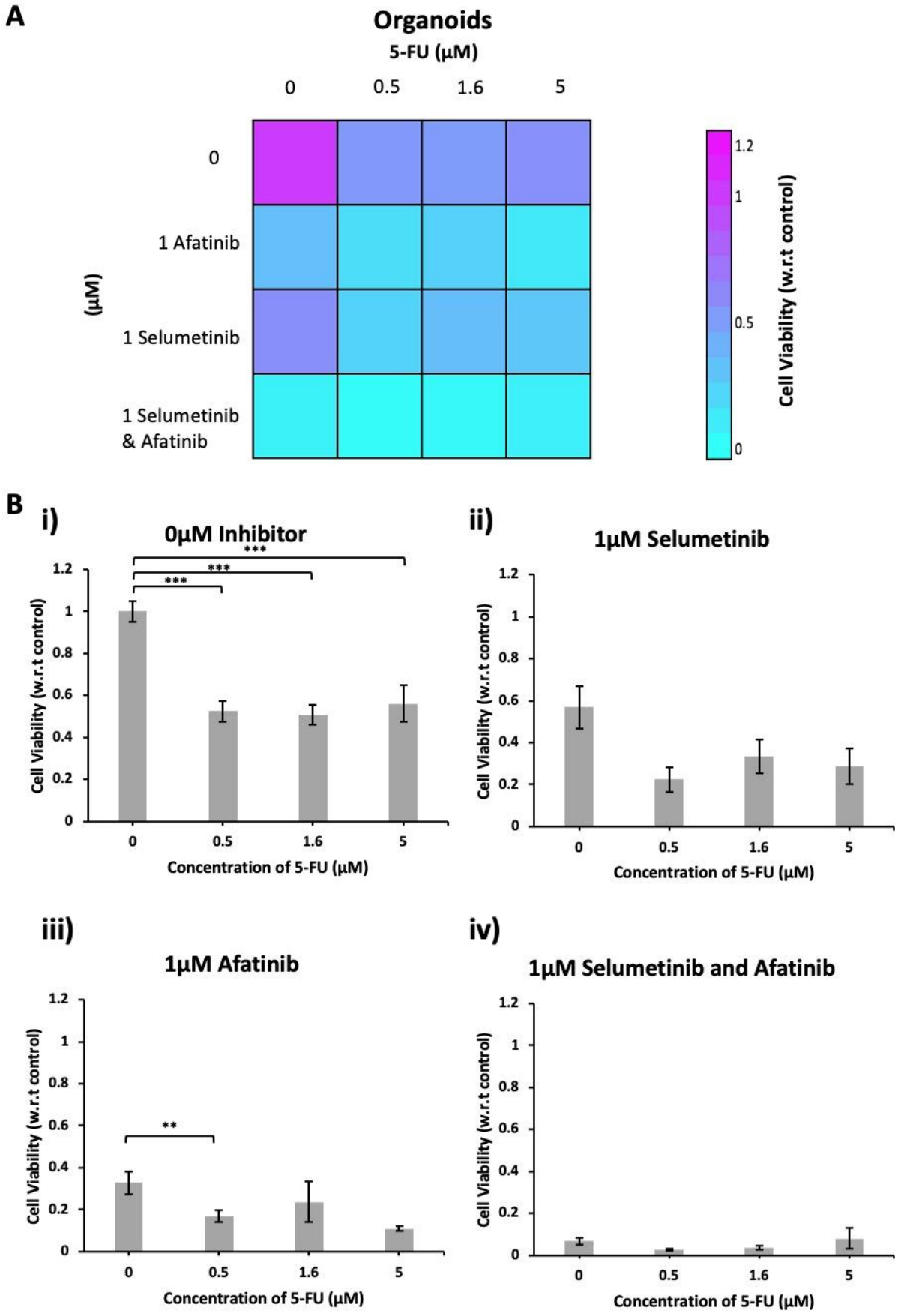

A
Organoids
5-FU (μM)
0
0.5
1.6
5
(μM)
0
1 Afatinib
1 Selumetinib
1 Selumetinib & Afatinib
Cell Viability (w.r.t control)
1.2
1
0.5
0
B
i)
0μM Inhibitor
***
***
***
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)
ii)
1μM Selumetinib
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)
iii)
1μM Afatinib
**
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)
iv)
1μM Selumetinib and Afatinib
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)

Figure 22. Sensitivity of UEA006 organoids to EGF and KRAS inhibitors alone or in combination with standard of care chemotherapy.

A) Heat map showing UEA006 organoids treated with 5-FU, Afatinib and Selumetinib alone or a combination of the two inhibitors. Bi) Organoids treated with 5-FU. Bii) Organoids treated with Afatinib alone or in combination with 5-FU. Biii) Organoids treated with Selumetinib alone or in combination with 5-FU. Biv) Organoids treated with Selumetinib and Afatinib alone or in combination with 5-FU. Experiments are performed in biological replicates (N=2) and technical repeats (n=6) and error bar indicative of S.E.M (**P<0.01, *** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.4.3 UEA007 Tumouroids

To analyse the effect of a KRAS inhibitor or an EGFR inhibitor alone or in combination with 5-FU. UEA007 tumouroids were treated for 7 days after which a cell viability assay was run to see the amount of viable tissue left. Due to the tumouroid line having a KRAS mutation the line would be expected to be sensitive to Selumetinib (KRAS inhibitor) but not Afatinib (EGFR inhibitor). This is due to KRAS being down stream of EGFR and results in the MAP kinase signalling pathway becoming constitutively active.

Previous experiments showed that UEA007 tumouroids are fairly insensitive to standard of care chemotherapy alone, again this was seen in this set of experiments. The Cell Titer Glo results indicated that when UEA007 tumouroids were only treated with Afatinib alone or in combination there was no drastic increase in the tumouroid death (**Figure 23Bii**) with only 50% tumouroid death. Similar to this when UEA007 tumouroids are treated with Selumetinib there appears to be between 40-50% tumouroid death (**Figure 23Biii**). However, when these inhibitors were used in combination along with chemotherapy there was an increased in tumouroid death (**Figure 23Biv**) resulting in 60-70% tumouroid death. Therefore, the optimum treatment for this tumouroid line would be 1μM Afatinib and Selumetinib in combination with 0.5μM 5-FU resulting in 70% tumouroid death (**Figure 23Biv**).

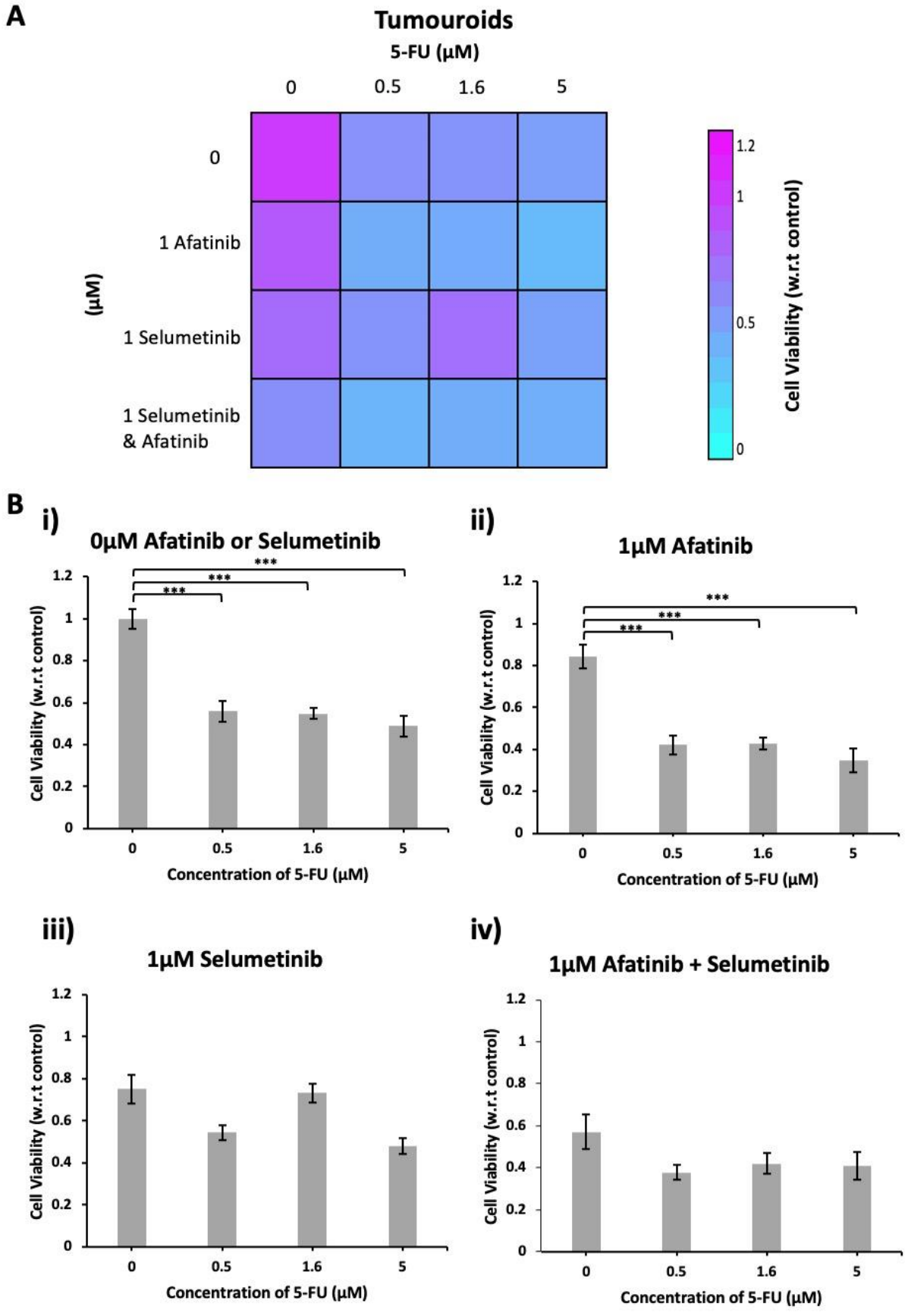

A
Tumouroids
5-FU (μM)
0
0.5
1.6
5
(μM)
0
1 Afatinib
1 Selumetinib
1 Selumetinib & Afatinib
1.2
1
0.5
0
Cell Viability (w.r.t control)
B
i)
0μM Afatinib or Selumetinib
ii)
1μM Afatinib
iii)
1μM Selumetinib
iv)
1μM Afatinib + Selumetinib
Cell Viability (w.r.t control)
Concentration of 5-FU (μM)
***

Figure 23. Sensitivity of UEA007 tumouroids to EGF and KRAS inhibitors alone or in combination with standard of care chemotherapy.

A) Heat map showing UEA007 tumouroids treated with 5-FU, Afatinib and Selumetinib alone or a combination of the two inhibitors. Bi) Tumouroids treated with 5-FU alone. Bii) Tumouroids treated with Afatinib alone or in combination with 5-FU. Biii) Tumouroids treated with Selumetinib alone or in combination with 5-FU. Biv) Tumouroids treated with Selumetinib and Afatinib alone or in combination with 5-FU. Experiments are performed in biological replicates (N=3) and technical repeats (n=9) and error bar indicative of S.E.M (*** P<0.001, Two Way ANOVA followed by a post hoc Tukey test).

3.5 Pramlintide, an antidiabetic drug, does not further sensitise patient-matched organoids and tumouroids to 5-FU.

Pramlintide is an antidiabetic drug which is currently approved as a therapy in the treatment of type I and type II diabetes. Pramlintide is thought to exert its anticancer effect on tumours through enhancing the cytotoxic effects of chemotherapy. The anticancer effect of pramlintide has only been studied two other groups (Venkatanarayan *et al.*, 2015). Venkatanarayan and colleagues found pramlintide's effect on cancer was related to the TP53 mutational status of the tumour. Furthermore Al-Keilani and colleagues found that pramlintide further enhanced the cytotoxic effects of chemotherapy treatment on CRC cell lines (Al-Keilani *et al.*, 2018).

3.5.1 UEA007 Tumouroids

UEA007 Tumouroids harbour a TP53 mutation suggesting this tumour could be targeted using Pramlintide. Work carried out by Al-Keilani and colleagues would suggest that when these tumouroids are treated with a combination of both Pramlintide and 5-FU the cytotoxic effects of the chemotherapy would be enhanced.

To investigate this hypothesis tumouroids were treated with 5-FU alone or in combination with Pramlintide. After 7 days a cell titer glo assay was run to assess cell viability after this treatment. From these results it was concluded that Pramlintide did not enhance the cytotoxic effect of 5-FU.

When the tumouroids were treated with 5μM 5-FU alone there was approximately 60-70% tumouroid death. When this concentration was used in combination with pramlintide at all concentrations a similar amount of tumouroid death was observed (**Figure 24Bi,ii,iii,iv**).

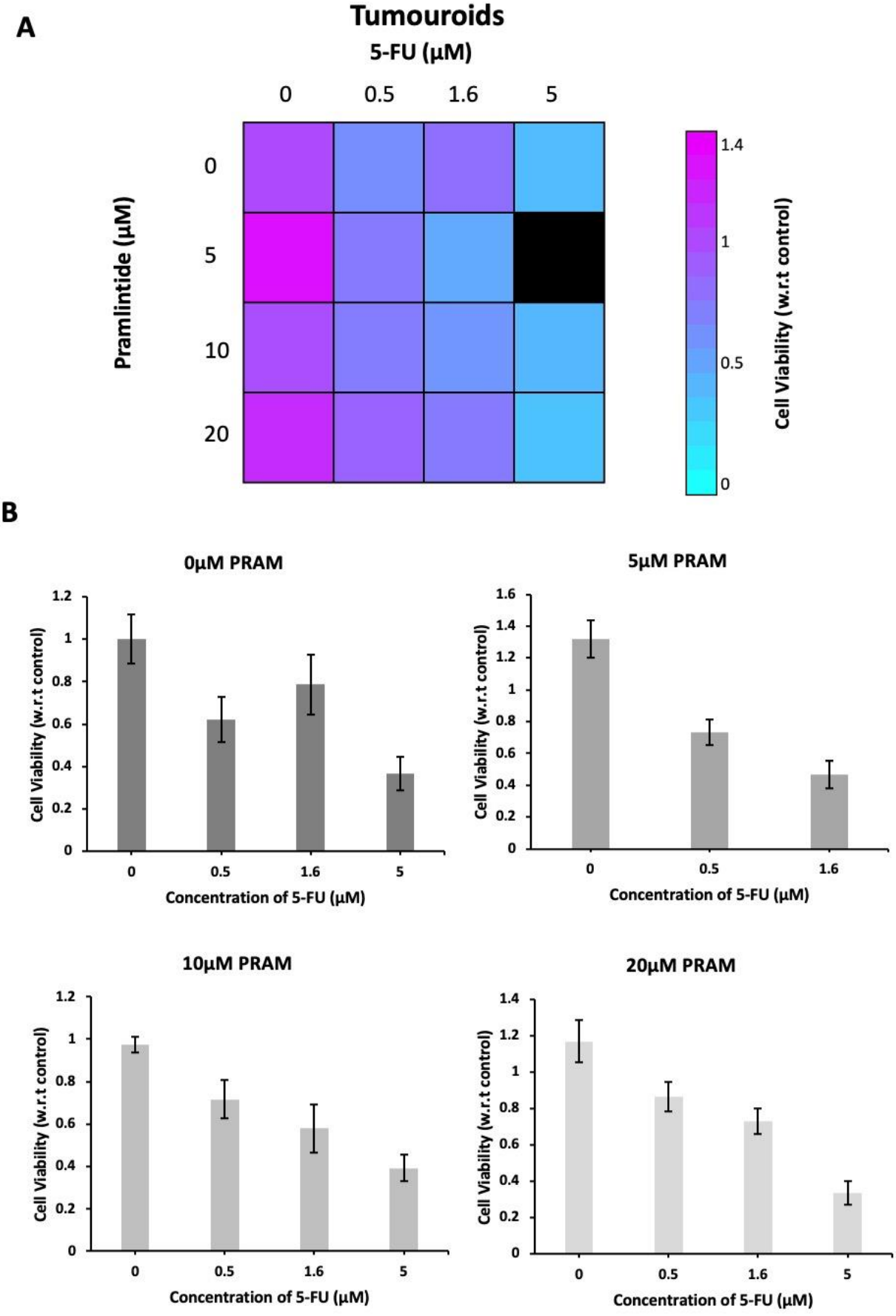

A
Tumouroids
5-FU (µM)
0
0.5
1.6
5
Pramlintide (µM)
0
5
10
20
1.4
1
0.5
0
Cell Viability (w.r.t control)
B
0µM PRAM
5µM PRAM
10µM PRAM
20µM PRAM
Cell Viability (w.r.t control)
Concentration of 5-FU (µM)

Figure 24. Sensitivity of UEA007 tumouroids to standard of care chemotherapy alone or in combination with Pramlintide.

A) Heat map showing the effect of pramlintide and 5-FU on UEA007 tumouroids, the black square shows that this combination of 5-FU and Pramlintide was not tested. Bi) Effect of 5-FU on tumouroids. Bii) Effect of 5-FU and/or 5μM Pramlintide on tumouroids. Biii) Effect of 5-FU and/or 10μM Pramlintide on tumouroids. Biv) Effect of 5-FU and/or 20μM Pramlintide on tumouroids. Experiments are performed in biological replicates (N=1) technical repeats (n=3) and error bar indicative of S.E.M.

3.6 Summary table

Overall, from this set of experiments it was determined that a patient-matched organoid and tumouroid system could be beneficial for the development and testing of novel therapies. The table below (**Table 4**) summarises the findings of these experiments.

Like previously stated, UEA005 tumouroids are highly sensitive to all therapies tested, although the exact reason for the sensitivity was not determined. It could be linked to the tumour's mutational burden (**Figure 16Bi**). This tumouroid line had one of the lowest numbers of TIER1 cancer driver genes mutations. Therefore, for this line, using 5-FU alone would be the most beneficial treatment. Moreover, it was determined that UEA005 organoids are comparably less sensitive to 5-FU treatment, which could be indicative of the healthy tissue being less damaged during treatment (**Figure 16Bi**).

In comparison, UEA006 and UEA007 tumouroids are not as sensitive to 5-FU treatment alone and therefore might benefit from treatment with 5-FU and an adjuvant inhibitor. The most effective treatment test for UEA006 tumouroids was a TNKSi inhibitor (**Figure 17Bi**). However, this didn't improve the efficacy of treatment any more than chemotherapy alone. This might indicate there were better inhibitors that could improve the efficacy of 5-FU due to the other mutations that the tumouroid harbours. However, UEA006 organoids were highly sensitive to this treatment, meaning that more of the healthy tissue was damaged compared to the tumouroids during treatment (**Figure 17Bi**).

The most beneficial treatment for UEA007 tumouroids were the combination treatment of Selumetinib and Afatinib used in combination with the lowest concentration of 5-FU (**Figure 23Biv**). This combination of drugs might prove promising in clinic. However, this was not tested on UEA007 patient-matched organoids meaning the cytotoxic effects of these drugs were not determined on the healthy tissue for this line.

Table 4. Summary table of patient-matched organoid and tumouroid sensitivity to inhibitors and chemotherapy drugs

	Tier 1 Cancer Driver Genes																							
	Tumour Suppressor Gene										Oncogenes													
UEA005	TP53		APC		FBXW7	NRG1		BRAF		EGFR						KRAS								
Target Drug	5-FU		TNKSi	TNKSi + 5-FU	---	---		Dab	Dab + 5-FU	S	S + 5-FU	A	A+5-FU	S+A	S+A + 5-FU	p.G13D	S	S + 5-FU	A	A+5-FU	S+A	S+A + 5-FU
Overall sensitivity																								
UEA006	p.E339*		p.K1308* Chr5:g.112175332 delC		---	INTRON VARIENT		---		---						---								
Target Drug	5-FU		TNKSi	TNKSi + 5-FU		A	A + 5-FU	Dab	Dab + 5-FU															
Overall sensitivity																								
UEA007	p.R248Q		p.S1315*		p.S426*	---		---		---						p.A146T								
Target Drug	5-FU	PRAM + 5-FU	TNKSi	TNKSi + 5-FU	Rapamycin			Dab	Dab + 5-FU								S	S + 5-FU	A	A+5-FU	S+A	S+A + 5-FU
Overall sensitivity																								
UEA005 ORG	---		---		---	---		---		---						---								
Target Drug	5-FU		TNKSi	TNKSi + 5-FU																				
Overall sensitivity			TBC	TBC																				
UEA006 ORG	---		---		---	---		---		---						---								
Target Drug	5-FU		TNKSi	TNKSi + 5-FU		A	A + 5-FU			S	S + 5-FU	A	A + 5-FU	S+A	S+A + 5-FU									
Overall sensitivity																								

Insensitive | Partial sensitivity | Sensitive

4.0 Discussion

4.1 Key findings

The patient-matched organoid and tumouroid culture system has potential to be a pivotal tool in the research and development of novel CRC therapeutic treatments. The findings above suggest that using a patient-matched organoids and tumouroid culture system to assess the effect of cancer treatment might prove promising over long term studies of different drugs. Overall, these results suggest a more in-depth analysis is needed into the effect of chemotherapy (5-FU) and other signalling pathway inhibitors on patient-matched organoids and tumouroids.

4.1.1 The effect of Tankyrase inhibitor

APC is mutated in ~80% of all CRC, therefore this gene is known as the gate keeper gene for the initiation of the adenoma carcinoma sequence. Therefore, targeting APC status of CRC can prove a promising in the development of new cancer therapeutic targets.
Although targeting APC directly could prove promising, previous work has suggested that using a TNKSi which prevents the degradation of AXIN II. AXIN II is another key protein in the WNT signalling pathway which could regulate the hyperactive WNT signalling. Regulation of the WNT signalling would in turn result in controlled cellular proliferation and preventing tumour growth. Although targeting this signalling pathway would be beneficial for treating CRC, there could be some adverse effects of targeting this pathway on the healthy tissue. For example, inhibiting key homeostatic pathways can result in damage to the healthy tissue resulting in side effects to the patient including mucositis.
Work carried out by Schatoff, and colleagues suggested using TNKSi would be beneficial in the treatment of CRC with tumours harbouring an early APC truncation, resulting in more protein being present, would be sensitive to a TNKSi and tumours with a later APC truncation, resulting in less protein being present, would insensitive. Suggesting if there was a late APC truncation, APC would still interact with the destruction complex resulting in controlled WNT signalling.
Therefore, from this work we hypothesised that UEA005 tumouroids and UEA003 tumouroids should be insensitive to a TNKSi whilst UEA006 and UEA007 would be sensitive (**Figure 5**). We found that, due to the high sensitivity of UEA005 tumouroids to 5-FU, it was hard to determine the sensitivity to a TNKSi, therefore in the future lowering the concentration further could allow us to discern if the tumouroids fit this hypothesis and are insensitive to a TNKSi.

However, when evaluating the sensitivity of the patient-matched organoids to this treatment the organoids were not as sensitive to 5-FU alone compared the tumouroids. When considering the sensitivity of UEA005 organoids and tumouroids it would be optimum to use 0.5μM of 5-FU alone. UEA005 organoids are less sensitive to 5-FU alone compared to UEA005 tumouroids which are exquisitely sensitive.

Similar to UEA005 tumouroids, we predicted that UEA003 tumouroids would also be insensitive to standard of care chemotherapy and TNKSi treatment. From the results UEA003 tumouroids appear insensitive when treated with 5-FU alone and in combination with a TNKSi, this result is in line with the hypothesis that tumouroids with an early APC truncation will be insensitive to a TNKSi. Moreover, when organoids were treated with standard of care chemotherapy or with a TNKSi they appeared far more sensitive to the treatment than the tumouroids. This might suggest that the patient when undergoing treatment might suffer from more side effects due to the healthy mucosa being affected more than the tumour.

From the hypothesis, that a late APC truncation would be sensitive to a TNKSi we would expect UEA006 tumouroids to be sensitive to a TNKSi when used alone and also in combination with chemotherapy. From the results UEA006 tumouroids appeared sensitive to a TNKSi at higher concentrations (5μM and 10μM). Approximately 40-50% tumouroid death was observed when 5μM TNKSi was used in combination with 0.5μM 5-FU. This result was promising. However, when these concentrations were also tested on the patient-matched organoids, they were found to be highly sensitive. Therefore, although the optimum treatment for tumouroids would be 5μM TNKSi with 0.5μm 5-FU, this resulted in 60% organoid death. Meaning the patient would suffer from side effects due to the healthy mucosa being killed more than the tumouroid.

Finally, UEA007 tumouroids appear fairly insensitive to a TNKSi at all concentrations of TNKSi and chemotherapy this might could be linked to the mutational burden of the tumouroids. This tumouroid line had the most TIER1 cancer driver gene mutations, this implies that other pathways might play a more pivotal role in the cancers growth and survival compared to APC. Moreover, this tumouroid line didn't fit the hypothesis that we predicted, expecting this line to be sensitive to a TNKSi due to the APC status. Unfortunately, UEA007 organoids were not tested due this line being unavailable at the time of testing. Therefore, future work to assess the effect of this inhibitor on the healthy organoids would be beneficial.

Overall, not all the tumouroid lines fit the hypothesis that the sensitivity of the line is dictated by the position of the APC mutation. The reason our findings might not be in line with previous work by schatoff could be down to the model system used by this research team. Schatoff and colleagues worked on organoids derived from mouse models with APC

mutations only. This does not consider other mutations that tumours harbour, which could have more of an influence of the tumour's survival.

4.1.2 The effect of Selumetinib and Afatinib

KRAS and EGFR are a highly mutated gene in CRC, targeting these genes alone or in combination could be beneficial for patient treatment. Like APC, KRAS and EGFR undergo mutations in the adenoma carcinoma sequence and can help indicate treatments that would be best for the patient. Until this year there have been no inhibitors that directly target KRAS therefore key research has gone into the effect of MEK inhibitors on KRAS mutations due to this protein being downstream in the MAP kinase signalling pathway (**Figure 6**).
Furthermore, the effect of EGFR inhibitors has been extensively research and shown to have a beneficial role in the treatment for cancer. As well as research into using these individual inhibitors alone, work has also been conducted to establish the effect of these inhibitors used in combination with chemotherapy on cancer treatment.
Work carried out by Cunningham and colleagues have established tumours harbouring both a KRAS and EGFR mutation are insensitive to an EGFR inhibitor alone (Cunningham *et al.*, 2009). This is due to the KRAS mutation being down stream of the EGFR mutation and therefore continuing to promote the proliferation and metastasis of the cancer. Moreover, there are clinical trials carried out that have shown that MEK inhibitors have proved a promising treatment for CRC (Corcoran *et al.*, 2018).
From this work it was hypothesised that UEA005 tumouroids should be highly sensitive to Selumetinib (KRAS inhibitor) and Afatinib (EGFR inhibitor) when used in combination, due to the KRAS and EGFR mutation they harbour. UEA007 tumouroids should also be sensitive to a KRAS and EGFR inhibitor used in combination, however it would be expected to not be sensitive to and EGFR inhibitor alone due to the KRAS mutation being down stream of EGFR.
Similar to what was observed with UEA005 tumouroids the tumouroids were highly sensitive to 5-FU treatment with compared to the organoids. However, promising results were observed when UEA005 organoids are treated with Selumetinib alone, where there was no death observed, compared to approximately 60% death observed by the tumouroids. This finding is in line with previous work suggesting that tumouroids that harbour a KRAS mutation will be sensitive to a MEK inhibitor (Corcoran *et al.*, 2018). Afatinib used alone or in combination with Selumetinib are highly toxic for both the organoids and tumouroids resulting in almost 100% death for both tissues. This can be explained due to Afatinib inhibiting the RAS map kinase and PI3K signalling pathway which are key homeostatic pathways in healthy tissue. Therefore, this would not be a viable treatment for these lines.

The most optimum treatment for this line would be 1μM Selumetinib alone, as it was highly effective on the tumouroids and had no effect on the organoids.
UEA007 tumouroids were treated with Selumetinib and Afatinib. From the hypothesis we predicted that this line should be highly sensitive to a MEK inhibitor, Selumetinib, and even more sensitive when used in combination with and EGFR inhibitor Afatinib. This is due to the tumouroid harbouring both a KRAS and EGFR mutation. However, when these experiments were conducted, we found very little sensitivity to Selumetinib and Afatinib alone. But when used in combination with Afatinib there was a slight increase in the sensitivity of the tumouroids. Therefore, the optimum concentration for treatment of this line would be 1μM Afatinib and Selumetinib and 0.5μM 5-FU, due to this therapy enhancing the killing effect seen on the tumouroids.
Due to UEA007 organoids not being treated, to further assess the effect of this treatment on organoids UEA006 organoids were assessed. When treated with all three different inhibitor conditions it was found that the organoids were highly sensitive. This is due to Selumetinib and Afatinib inhibiting key homeostatic pathways within the healthy tissue. Both the RAS map kinase and PI3K signalling pathways play key roles in the proliferation and survival of healthy tissue.
Overall, we hypothesised that UEA005 and UEA007 tumouroids would be sensitive to treatment with Afatinib and Selumetinib alone or in combination with one another. We observed an exquisite sensitivity of UEA005 tumouroids to this treatment and a partial sensitivity of UEA007 tumouroids. Although a beneficial effect was observed in the tumouroids a highly toxic effect was also observed on the organoids treated. Therefore, suggesting the effects on the healthy tissue might outweigh the effect on the tumouroids.

4.1.3 The effect of pramlintide

Targeting the P53 status of tumours might prove promising, as previous studies have linked P53 status to the sensitivity of the tumour to 5-FU. Using inhibitors to restore the homeostatic P53 status of the tumour will help regulate key processes including apoptosis (Iacopetta, 2003).
To the best of my knowledge the effect of Pramlintide on cancer has been studied by two other groups. Work carried out by Venkatanarayan and colleagues showed that the cytotoxic effects of pramlintide was based on the P53 status of the tumour (Venkatanarayan *et al.*, 2015). This lead to another group to further assess if Pramlintide is able to increase the cytotoxic effect of chemotherapy, again they linked this to the P53 status and showed again that Pramlintide was able to increase the effect of chemotherapy on tumour cells (Al-Keilani *et al.*, 2018).

Therefore, from this work it is hypothesised that UEA007 tumouroids would be further sensitised to chemotherapy when pramlintide is used in combination with 5-FU due to the P53 status. However, from the experiment conducted it was clear that Pramlintide alone was unable to enhance the cytotoxic effects of 5-FU. Again, this can be linked to the mutational burden of this tumouroid line, indicating that the P53 status of the tumouroid might not be the key driver in this tumouroid lines growth and survival. Moreover, the model system used by Al-Keilani and colleagues was on primary and isogenic cell lines with TP53 mutations, therefore not taking into account other mutations a tumour might harbour (Al-Keilani *et al.*, 2018)

4.2 Limitations and further work

Although the model system has proved promising in determining the effect of different inhibitors and chemotherapy on different patient-matched organoid and tumouroid lines there have been some limitations to this project too.

Even though we have been able to determine effects of different treatments on the line we have been unable to translate this to the patient outcome from which the patient-matched organoids and tumouroids have been derived. If we are able to draw a comparison from the cell titer glo output (viability of the tumouroid) we could compare it to what worked for the patient and what their outcome was, including the cytotoxic effect that the treatments had on the healthy mucosa.

Moreover, the mutational burden of the tumouroids were never considered. For example, all mutations were assessed individually rather than in combination with one another. However, one mutation will not solely function alone, but instead will influence different response and play pivotal roles in different signalling pathways.

If time permitted assessing the effect of different treatments in a co-culture of organoids and tumouroids might provide a more well-rounded analysis of the different treatments on different lines. Work carried out by Jacquemin and colleagues has shown that tumouroids exhibit a survival advantage by secreting WNT inhibitors into the environment preventing the healthy tissue from surviving (Jacquemin *et al.*, 2022). Moreover, since finishing the experiment a group have published findings on an inhibitor that directly targets KRAS. It would be interesting to determine if the effects seen when using an MEK inhibitor are similar to that of the KRAS inhibitor (Bar-Sagi, Knelson and Sequist, 2020). Although these treatments could be a promising next step in the fight against CRC, chemoresistance is also an important topic that needs to be addressed. Therefore, further studying the remaining tumouroid population at the end of a cell viability assay could allude to mechanisms that is

aiding the cancer cell growth and survival. These studies could help address key drug targets for the development of novel therapies for CRC.
Finally, determining optimal concentrations of 5-FU for all tumouroid lines, in particular UEA005 tumouroids would be vital due to the exquisite sensitivity of this tumouroid line to concentrations that were used. Due to UEA005 exquisite sensitivity to 5-FU it would be vital to determine the optimum concentration for this line. This would reduce the effect on the healthy tissue whilst achieving the optimal killing of the tumour.

4.3 Summary and conclusions

In summary a patient-matched organoid and tumouroid personalised medicine pipeline has been used to assess the effect of key pathway inhibitors that have been highlighted by WES. The contributions to this field have highlighted the beneficial status of determining both the effect on the healthy and diseased tissue. The personalised medicine pipeline has helped identify key treatments that could benefit induvial patients, potentially improving patient outcome. Moreover, this work has highlighted the need to consider multiple pathways which could influence one another when determining the optimum treatment for a given patient. The novel findings in this thesis show the benefit of a patient-matched organoid and tumouroid personalised pipeline to help aiding the testing and development of novel therapies in CRC.

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