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Personalised Treatment for Cystic Fibrosis Patients with Rare CFTR Mutations

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Summary

Cystic fibrosis (CF) is a life-threatening disease caused by variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene encodes a transmembrane protein that is involved in the regulation of chloride (Cl^-) and bicarbonate (HCO_3^-) transport across the plasma membrane of epithelial cells. CFTR variants are grouped into seven functional classes based on their molecular/cellular defect.

Recently, small-molecule drugs have been approved for the treatment of people with CF (pwCF) carrying specific CFTR variants. These include VX-770, or ivacaftor (Kalydeco®), a CFTR potentiator that can be combined with VX-661 (tezacaftor), known as Symkevi® (or Symdeko®). The more recent approved triple combination, Kaftrio® (or Trikafta®) consists of ivacaftor and the two correctors tezacaftor and elexacaftor (VX-445).

The goal of this MSc project was to help bring personalised therapies to portuguese pwCF carrying rare CFTR variants that are not eligible to receive the currently approved modulator treatments. For this, the Forskolin Induced Swelling (FIS) assay was used to assess the CFTR function in 3D intestinal organoids from those pwCF and to further evaluate the effect of the currently approved CFTR treatments to find the best therapy for each individual. Organoids from eight individuals carrying the following variants: p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu I, p.Ala561Glu/p.Ala561Glu II, c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro, p.Gln1100Pro/p.Ser4Ter, p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter, were analysed. The results of the FIS assays show that CFTR function was rescued in six of the organoids (p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu I, p.Ala561Glu/p.Ala561Glu II, c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro and p.Gln1100Pro/p.Ser4Ter), while two (p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter) did not present any significant responses. Thus, these six portuguese individuals may potentially have clinical benefit from the currently approved CFTR modulators, which can improve their quality of life. In summary, the results obtained here confirm the use of functional CFTR assessment in 3D intestinal organoids to predict the efficacy of these modulators in a personalised approach.

Key-words: Cystic Fibrosis; CFTR; CFTR modulators; FIS; Intestinal organoids.

Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva mais comum em caucasianos. É causada por variantes no gene CFTR (*Cystic Fibrosis Transmembrane conductance Regulator*). Este gene codifica uma proteína transmembranar, a CFTR, que está envolvida no transporte de cloreto (Cl^-) e bicarbonato (HCO_3^-) através da membrana plasmática apical das células epiteliais. A CFTR disfuncional provoca uma anomalia no transporte destes aniões, que por sua vez afetam a secreção de fluidos. Isto leva a um desequilíbrio na homeostase dos fluidos em vários órgãos e tecidos epiteliais, tais como as vias respiratórias, o sistema digestivo, as glândulas sudoríparas e o aparelho reprodutivo (sobretudo o masculino). No entanto, a doença das vias respiratórias é a principal causa de morbilidade e mortalidade nos indivíduos com FQ. Esta é marcada por uma insuficiente limpeza mucociliar que resulta em ciclos recorrentes de infeção, inflamação e cicatrização, conduzindo gradualmente ao declínio da função pulmonar. A proteína CFTR pertence à superfamília dos transportadores ABC (*ATP binding cassette*), cujos membros são responsáveis pelo transporte de substratos através das membranas celulares, normalmente contra um gradiente de concentração, utilizando para tal a hidrólise do ATP. A CFTR é constituída por cinco domínios: dois domínios transmembranares (TMD1 e TMD2), dois domínios de ligação ao nucleótido (NBD1 e NBD2) e um domínio regulador (RD). Os domínios TMD1 e TMD2 criam o canal da proteína CFTR e os domínios NBD1 e NBD2 ligam-se ao ATP e regulam a abertura e o fecho do canal. Como o canal da CFTR é dependente do ATP, requer a fosforilação do RD pela proteína cinase A (PKA) e ligação do ATP aos domínios NBD, o que causa a sua dimerização e permite o fluxo dos iões Cl^-/HCO_3^- através da membrana apical das células epiteliais. O canal fecha quando ocorre a hidrólise do ATP, o que resulta na separação do dímero NBD e na recuperação da conformação dos domínios TMD. Mais de 2.000 variantes foram já identificadas até ao momento no gene CFTR e agrupadas em sete classes com base no seu impacto na expressão/função da proteína CFTR. As variantes que pertencem à classe I são *nonsense* resultando assim num defeito na produção de proteína, causado pela introdução de um codão *stop* prematuro. A classe II contém variantes que causam um processamento e tráfego anormal da proteína CFTR, o que leva a uma proteína com um *folding* incorreto que é degradada prematuramente. A classe III é constituída por variantes que influenciam a regulação da abertura do canal da CFTR, afetando a sua ativação por ATP. As variantes da classe IV diminuem o fluxo de iões no canal, reduzindo assim a sua condutância. A classe V é composta por variantes que reduzem a quantidade de canais da CFTR na membrana plasmática, principalmente por causa de *splicings* alternativos, mas ainda apresentam alguma função residual da CFTR. A classe VI está relacionada com uma desestabilização da CFTR na membrana plasmática, reduzindo a quantidade de proteína na membrana. As variantes da classe VII não produzem RNA mensageiro (mRNA) funcional da CFTR e não se conseguem recuperar farmacologicamente. Variantes pertencentes às classes I, II, III e VII normalmente resultam em fenótipos mais graves enquanto que variantes das classes IV, V e VI permitem alguma função residual, originando formas atípicas de FQ. As variantes que são raras também se chamam variantes órfãs. Estas apresentam uma previsão difícil da doença porque os seus defeitos funcionais não foram definidos. Por serem raras, o desenvolvimento de novas terapias não é direcionado para a sua correção. Na última década, foi aprovado o uso de pequenas moléculas na clínica para o tratamento de indivíduos com FQ com variantes da CFTR específicas. Estes compostos incluem o VX-770, também conhecido como ivacaftor (Kalydeco®), que atua como potenciador do canal CFTR. Além deste fármaco, existem outros que são combinações deste potenciador com corretores (como o VX-661 e o VX-445) concebidos para tratar os defeitos de *folding* e de tráfego associados à variante mais comum, p.Phe508del. O VX-661 (tezacaftor) combinado com o ivacaftor é conhecido como Symkevi® (Symdeko® nos EUA). A combinação tripla, aprovada mais recentemente e conhecida como Kaftrio® (Trikafta® nos EUA), consiste no potenciador ivacaftor (VX-770) com

dois corretores, tezacaftor (VX-661) e elexacaftor (VX-445). No entanto, estas terapias altamente eficazes como moduladoras da CFTR foram desenvolvidas para indivíduos com FQ com as variantes mais comuns da CFTR. Por isso, os indivíduos com FQ portadores de variantes raras (ou órfãs) não são elegíveis para estes tratamentos, apesar de poderem beneficiar clinicamente dos mesmos. Por este motivo, é necessário disponibilizar terapias personalizadas para indivíduos com FQ com variantes raras (órfãs) da CFTR. Para tal, têm sido criados alguns modelos que ajudam a prever a eficácia dos moduladores, como os organoides intestinais 3D, que são obtidos através do isolamento de criptas de biópsias retais. Estes modelos de organoides 3D são fundamentais para o desenvolvimento de tratamentos personalizados, pois permitem que muitas pessoas possam usufruir das terapias que já se encontram no mercado, mas que não foram originalmente desenhadas para os seus genótipos. As criptas intestinais contêm vários tipos de células, incluindo células estaminais. Estas encontram-se usualmente na base das criptas e têm a capacidade de se diferenciarem noutras células. Quando as criptas intestinais são mantidas em cultura nas condições apropriadas, podem gerar organoides intestinais, estruturas 3D que imitam a composição e o comportamento do intestino. Quando os organoides intestinais são gerados a partir de biópsias de indivíduos com variantes raras da CFTR, podem ser usados para testar compostos num contexto personalizado para esse indivíduo. As respostas clínicas às terapias com moduladores podem ser previstas em organoides intestinais derivados de indivíduos com FQ utilizando o ensaio FIS (*Forskolin Induced Swelling*), que mede a função da CFTR através do inchamento dos organoides quando estimulados por forscolina, que é um agonista da CFTR. Os corretores, VX-445 e VX-661, são adicionados no dia anterior à experiência enquanto que o potenciador, VX-770, é adicionado no próprio dia. A forscolina aumenta o cAMP intracelular, pois ativa a PKA, que fosforila a CFTR, abrindo o canal e permitindo a passagem de água e de iões, ativando assim a CFTR, que estimula a secreção de fluido para o lúmen do organoide. Consequentemente, os organoides incham pela entrada de fluido. Quando a CFTR está ausente ou disfuncional não induz o inchamento dos organoides, pois este é dependente da atividade da CFTR. Este teste pode ser usado para testar o efeito dos moduladores nas variantes CFTR.

Este projeto de Mestrado teve como objetivo ajudar a trazer terapias personalizadas para indivíduos portugueses com variantes raras da FQ, que não são elegíveis para receber os medicamentos moduladores da CFTR atualmente aprovados. Para tal, a atividade intrínseca da CFTR de organoides intestinais previamente gerados a partir de biópsias retais desses indivíduos com FQ com variantes órfãs foi estudada. Os organoides intestinais são um ótimo material para este tipo de estudos porque conseguem replicar a morfologia e a fisiologia do intestino, incluindo os vários tipos de células e as interações entre elas. De modo a se poder prever a resposta clínica dos moduladores aprovados, estes foram testados em organoides intestinais de indivíduos com variantes raras utilizando o ensaio FIS para determinar a melhor terapia para cada indivíduo, numa abordagem de medicina personalizada. Para isto, organoides de oito indivíduos com as variantes p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu I, p.Ala561Glu/p.Ala561Glu II, c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro, p.Gln1100Pro/p.Ser4Ter, p.Tyr1092Ter/p.Tyr1092Ter e p.Gly542Ter/p.Gln220Ter foram analisados. Os resultados dos ensaios FIS demonstraram que a função da CFTR foi recuperada em seis dos organoides (p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu I, p.Ala561Glu/p.Ala561Glu II, c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro e p.Gln1100Pro/p.Ser4Ter) enquanto que dois dos organoides (p.Tyr1092Ter/p.Tyr1092Ter e p.Gly542Ter/p.Gln220Ter) não apresentaram resposta significativa aos moduladores testados. Assim, conseguimos prever que estes seis indivíduos portugueses poderão obter benefício clínico dos moduladores aprovados atualmente, o que irá certamente melhorar significativamente a sua qualidade de vida. Em suma, os resultados obtidos confirmam a utilização da

quantificação da CFTR funcional em organoides intestinais 3D para a previsão da eficácia destes moduladores, numa abordagem personalizada.

Palavras-chave: Fibrose Quística; CFTR; Moduladores da CFTR; FIS; Organoides Intestinais.

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List of Acronyms and Abbreviations

3D	Three-dimensional
ABC	ATP-binding cassette
ANOVA	Analysis of variance
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AUC	Area under the curve
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
Cl^-	Chloride ion
DMEM	Dulbeco's Modified Eagles Medium
EMA	European Medicines Agency
ENaC	Epithelial sodium channel
$F12^{ghp}$	Advanced DMEM/F12 + 1% Pen/Strep + 1% GlutaMax + 1% HEPES
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIS	Forskolin-induced swelling
Fsk	Forskolin
HCO_3^-	Bicarbonate
LGR5	Leucine rich repeat-containing G protein coupled receptor 5
mEGF	Mouse epidermal growth factor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NBD	Nucleotide binding domain
NCM	Noggin conditioned medium
NMD	Nonsense-mediated decay
PBS	Phosphate-buffered saline
Pen/Strep	Penicillin/Streptomycin
PI	Pancreatic insufficiency
PKA	Protein kinase A
PM	Plasma membrane
PTC	Premature stop codon
pwCF	People with Cystic Fibrosis
RCM	Rspondin-1 conditioned medium
RD	Regulatory domain
t	Time
TMD	Transmembrane domain
WCM	Wnt-3a conditioned medium

1. Introduction

1.1. Cystic Fibrosis

Cystic fibrosis (CF) is the principal autosomal recessive disease among Caucasians[1]. It is caused by cystic fibrosis transmembrane conductance regulator (CFTR) gene variants[1]. The CFTR gene encodes the CFTR protein, a chloride (Cl^-) and bicarbonate (HCO_3^-) channel that can be found on epithelial cells, at the apical plasma membrane (PM). It keeps the salt and water balance on the surface epithelia, regulating the flux of ions[2]. The epithelial sodium (Na^+) channel (ENaC) is one of the channels that is also regulated by CFTR[3]. CF is a disease that affects multiple organs with epithelial cells and mucus, such as the lungs, pancreas, digestive system, liver, and reproductive system[4]. Despite advances in treatment, pulmonary disease continues to be the main cause of morbidity and mortality in people with CF (pwCF)[5]. Defective CFTR causes a decrease in the airway surface liquid (ASL), leading to the buildup of thick, sticky mucus, impaired mucociliary clearance, inflammation and infection[6,7].

CF also affects the pancreas leading to pancreatic insufficiency (PI) which results in inadequate absorption and incomplete digestion of nutrients and fat-soluble vitamins[8]. pwCF with PI need to take pancreatic enzyme supplements[8]. Some CF manifestations on the intestine include meconium ileus, a condition that primarily affects newborns which consists in distal small bowel obstruction, and distal intestinal obstruction syndrome, which also affects the distal small bowel due to the accumulation of thick and sticky mucus in this region[9].

CF was a fatal disease for children when it was discovered[10]. Therapies that focus on the symptoms have increased life expectancy for pwCF to over 40 years in developed countries[11]. Early and accurate diagnosis, based on a better understanding of the disease, can detect mild and atypical forms of CF, leading to significantly improved disease outcomes[12]. However, these therapies focus at the level of the symptoms and not correcting the basic defect leading to the disease pathophysiology. In addition, the expensive treatments can be a lifelong financial burden on patients and their families[13]. In order to increase the lifespan and the life quality of pwCF, therapies that are designed to correct the underlying molecular CFTR defects that cause CF are of utmost importance.

1.2. CFTR

The CFTR gene is located on the long arm of chromosome 7 (7q31.2)[14]. It consists of 27 exons and produces the CFTR glycoprotein which is composed of 1480 amino acids. CFTR belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily, which is responsible for transporting substrates across cellular membranes, often against a concentration gradient, using ATP hydrolysis. CFTR is the only known member of this family that acts as an ion channel[15]. It has a complex architecture that permits the exit of Cl^- ions from epithelial cells. Its structure (Figure 1.1) consists of five domains: two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2), and a regulatory domain (RD)[16]. The TMDs create the channel of the CFTR protein, while the NBDs regulate its opening and closing. Since the CFTR channel is an ATP-dependent ion channel, it requires RD phosphorylation by protein kinase A (PKA) and ATP binding at the NBDs, which causes their dimerization, enabling Cl^- ions to leave the epithelial cells[3]. The closure of the channel is triggered by ATP hydrolysis, which results in the separation of the NBD dimer and the restoration of the TMD conformation[3].

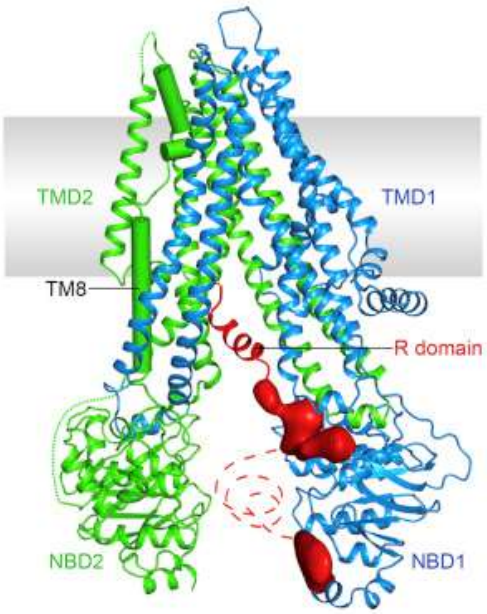


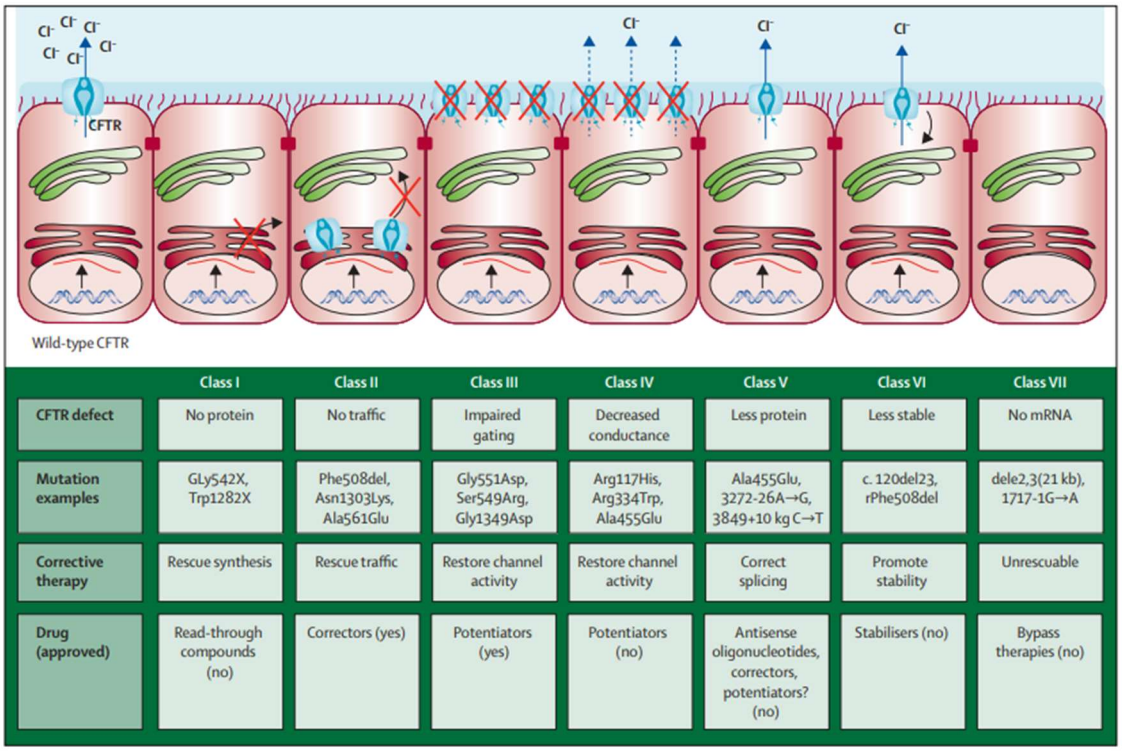
Figure 1.1 - Cryo-electron microscopy structure of CFTR in its dephosphorylated, ATP-free conformation. CFTR is constituted by two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and one regulatory domain (RD) which contains the protein kinase A (PKA) phosphorylation sites. CFTR channel opening is promoted by the binding of ATP and phosphorylation by PKA, while channel closing is facilitated by the hydrolysis of ATP. The transmembrane helix 8 (TM8), a recently identified feature different from all other ABC transporters, is thought to form the structural basis of CFTR's function as an ion channel. From Liu *et al*. (2017)[16].

1.3. Classification of CFTR Variants

Over 2.000 genetic variants have been identified, which exert different effects on the synthesis, functionality and stability of the CFTR protein at the PM[17]. The deletion of the amino acid phenylalanine at position 508 of the CFTR protein, p.Phe508del, is the most common CFTR variant among pwCF[18]. It leads to a defective processing and trafficking of the CFTR protein and pwCF homozygous for p.Phe508del have very little protein at the apical PM[19].

CF-causing variants are classified into seven classes, based on their effect on the production, trafficking, functioning or stability of the CFTR channel (Figure 1.2)[1,20]. Class I variants are often nonsense variants and result in a defect in protein production, which is often caused by a premature termination codon (PTC) that triggers the nonsense-mediated decay (NMD) system[21]. These variants lead to a reduction or absence of CFTR channels. Class II variants cause abnormal processing and trafficking of the CFTR protein, resulting in misfolded protein that is degraded prematurely. These variants reduce the amount of CFTR protein in the PM[21]. The p.Phe508del variant belongs to class II[1]. Class III variants affect the gating of the CFTR channel, impairing its ATP-activation[22]. Class IV variants decrease the ion flow through the channel by reducing the conductance of the CFTR. Class V variants reduce the amount of CFTR channels at the PM due to splicing abnormalities, but some residual CFTR function is still present[23]. Class VI variants destabilize the CFTR protein at the plasma membrane, leading to increased endocytosis or decreased recycling. Class VII variants result in no production of CFTR messenger RNA (mRNA) and they cannot be rescued pharmacologically[1].

Variants under class I, II, III and VII result in severe clinical outcomes, while those under class IV, V and VI are linked to milder phenotypes[23]. This classification system has some limitations as certain variants may cause multiple defects and therefore fit into more than one class[24].



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	Gly542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A
Corrective therapy	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability	Unrescuable
Drug (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)	Bypass therapies (no)

Figure 1.2 - Classification of CFTR variants and current therapies. CFTR variants were divided in seven functional classes. From De Boeck & Amaral (2016)[1].

1.3.1. Orphan Variants

Rare CFTR variants are classified as orphan due to their low prevalence. Predicting disease caused by these rare variants is challenging as their functional defects have not been defined[25]. Some of them may lead to severe forms of CF, others to milder forms of CF with partial CFTR function[25]. Due to their rarity, therapeutic efforts are typically not considered for their correction[26]. Even though they could potentially benefit from approved CFTR modulator therapies, individuals with rare CFTR variants are not eligible for these treatments because their variants are not listed in the drugs targets list. Therefore, to try to help every person with CF, it is necessary to identify and analyse these rare variants to determine their response to modulators for variant-based therapies[27].

1.3.2. CFTR Modulators

CFTR modulators are drugs that tackle the underlying cause of CF. They are categorized as either correctors or potentiators and have been approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for treating pwCF with specific variants[28]. Correctors aid the CFTR protein by ensuring it folds accurately and promoting its traffic to the cell surface[29]. Potentiators increase the CFTR channel open probability which lets Cl^- flow through[29].

Ivacaftor (VX-770, Vertex Pharmaceuticals) can enhance the function of CFTR channels in a way that does not require ATP but does depend on phosphorylation[30]. In 2012, both FDA and EMA approved the use of ivacaftor (Kalydeco®, Vertex Pharmaceuticals)[31]. Tezacaftor (VX-661, Vertex Pharmaceuticals) is a corrector that enhances p.Phe508del-CFTR trafficking and increases p.Phe508del-CFTR protein activity *in vitro*[32]. In 2018, FDA and EMA approved the co-treatment with tezacaftor/ivacaftor (Symdeko® or Symkevi®, Vertex Pharmaceuticals) for patients aged ≥ 12 years who are p.Phe508del-homozygous or p.Phe508del-heterozygous with a residual function variant in trans[31]. Elexacaftor (VX-445, Vertex Pharmaceuticals) is a corrector that acts on a different binding site than tezacaftor to increase CFTR function[33]. Used together with tezacaftor and ivacaftor, elexacaftor provides additional benefits to the treatment. The triple combination elexacaftor/tezacaftor/ivacaftor was also approved by the FDA and the EMA (Trikafta® or Kaftrio®, Vertex Pharmaceuticals) for pwCF homozygous for the p.Phe508del variant or presenting a p.Phe508del variant associated with a minimal function, gating or residual function variant[34]. This combination is expected to benefit 80-85% of CF patients worldwide since they carry the p.Phe508del variant[35], but those with rare variants are not eligible for this treatment, highlighting the importance of personalised testing.

1.4. Personalised Therapies

1.4.1. 3D Intestinal Organoids

Different cell types can be found in intestinal crypts, including $LGR5^+$ stem cells[36]. LGR5 is a marker for intestinal stem cells. These are usually located at the base of the crypts and can differentiate into other cells with distinct functions in the intestine[36]. The intestinal crypts can be isolated from rectal biopsy tissue and when cultured under appropriate conditions, they can generate intestinal organoids - 3D structures that mimic the composition and behaviour of the intestine (Figure 1.3)[37]. Intestinal organoids generated from individuals with rare CFTR variants have been shown to be fundamental for personalised drug testing in CF[38].

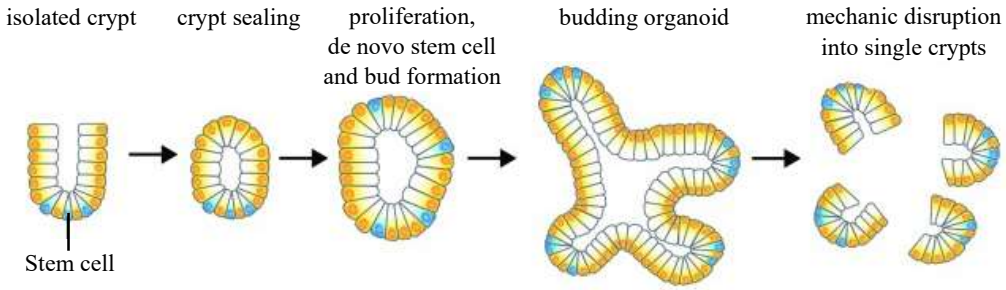


Figure 1.3 - Representation of organoid generation from isolated crypts. Isolated crypts from rectal biopsies seal and develop for 7-10 days. Then they are passaged into single smaller organoids. Adapted from Dekkers, van der Ent & Beekman (2013)[37].

As described above, isolating crypts from rectal biopsies that contain stem cells allows for the development of human 3D intestinal organoids. In order to generate and culture 3D organoids, a complete medium is used to provide the optimal conditions for organoids to develop. This medium is composed of Wnt-3a conditioned medium (WCM), Noggin conditioned medium (NCM), Rspondin-1

conditioned medium (RCM), harvest medium F12^{ghp}, B27 supplement, N-acetylcysteine, mouse epidermal growth factor (mEGF), Nicotinamide, A83-01 and P38 inhibitor.

WCM activates the Wnt pathway where Wnt proteins act on target cells through their connection to the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to intracellular proteins like β-catenin, a transcriptional regulator[39]. Wnt signalling is fundamental for early development, maintenance and regeneration of stem cells[39]. NCM is used to stimulate crypt development. Noggin is a bone morphogenic protein (BMP) antagonist. When NCM is added to exogenous Wnt, the stem cells can preserve and proliferate[40]. Therefore, Noggin is essential for the culture of intestinal organoids[41]. RCM is another key component of the complete medium. It is a ligand for LGR5 and influences the stem cells self-renewal[42]. B27 supplement is used to induce growth and viability of cells without differentiation. A good reductive capacity for the cell is ensured by high concentrations of glutathione. As such, N-acetylcysteine is added to serve as a substrate for glutathione synthesis[43]. mEGF induces proliferation, differentiation and survival of the cells. Nicotinamide enhances intestinal stem cells maintenance[40]. A83-01 inhibits TGFβ pathway[44] and P38 inhibitor (SB202190) is used as a secretory differentiation inhibitor[40].

1.4.2. Forskolin Induced Swelling (FIS) Assay

3D organoids can be used in the forskolin-induced swelling (FIS) assay, which uses forskolin (Fsk) as a CFTR agonist[45]. Forskolin raises intracellular cyclic adenosine monophosphate (cAMP) and thereby activates CFTR[45]. This stimulus activates PKA, which phosphorylates CFTR leading to the opening of the channel and allowing ion and water to flow through. Consequently, organoids swell due to the accumulation of fluid in the organoid lumen, a process that is fully CFTR-dependent. The organoid swelling is impaired when CFTR is dysfunctional or absent. Thus, the swelling can then be correlated with CFTR activity to test CFTR modulators or other compounds[45].

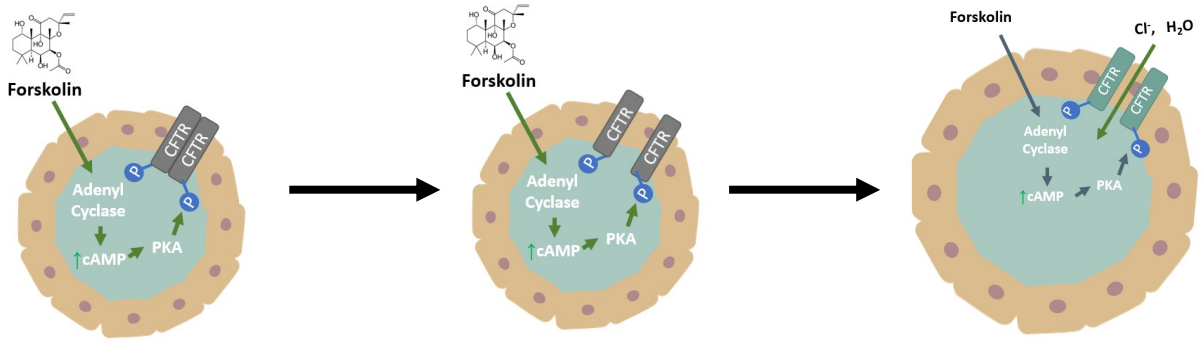


Figure 1.4 - Schematic representation of the Forskolin Induced Swelling (FIS) assay on an intestinal organoid. Fsk increases cAMP levels which activates PKA which phosphorylates CFTR, opening the channel and letting water and ions enter. This causes swelling of the organoid. Images created and provided by Cláudia Rodrigues (PhD student in the Amaral Lab).

2. Objectives

The aim of the present MSc work was to help bring personalised therapies to Portuguese CF individuals carrying rare CFTR variants, for which the currently available CFTR modulators are not approved.

1. **To assess CFTR function in intestinal organoids from CF individuals carrying rare CFTR variants**

   Intrinsic CFTR activity from intestinal organoids previously generated from rectal biopsies from CF individuals with orphan variants were assessed through the FIS assay.

2. **To evaluate approved CFTR modulator drugs on those organoids**

   Existing CFTR modulator therapeutics were tested in intestinal organoids from individuals carrying rare CFTR variants using the FIS assay to determine the best therapy for each individual, in a personalised-medicine approach.

3. **To biobank the intestinal organoids**

   All organoids were biobanked (labelled and stored in appropriate conditions for future use).

3. Materials and Methods

All experiments within this project were performed at Professor Margarida Amaral's lab (Functional Genomics and Proteostasis (FunGP), Biosystems and Integrative Sciences Institute (BioISI), Faculty of Sciences, University of Lisbon, Portugal).

3.1. Cell Culture

All cells were cultured at 37ºC in a 5% CO_2 and 95% air-humidified water saturated atmosphere.

3.1.1. Wnt-3a Conditioned Medium (WCM)

L-Wnt3a cells were thawed in DMEM +/+ (DMEM (Invitrogen) with 10% FBS (Gibco) and 1% pen/strep, (Invitrogen)) in a Falcon tube that was then centrifuged for 5 minutes at 1100 rpm and 4ºC. After removing the supernatant, the pellet was resuspended in 1 mL of DMEM +/+. 25 mL of DMEM +/+, the resuspended pellet and 75 µL of zeocin (100mg/mL, Invitrogen) were added to a T175 flask. The cells were grown until they reached 80-90% confluency. The medium was removed and cells were washed with 5 mL of PBS. Next, cells were trypsinized. 5 mL of DMEM +/+ were added to resuspend the cells. The resuspended cells were centrifuged for 5 minutes at 1100 rpm and 4ºC. The supernatant was removed and the pellet was resuspended in 1 mL of DMEM +/+. The resuspended pellet was divided to each of 3 x T175 flasks with 25 mL of DMEM +/+. 50 µL of the resuspended pellet was transferred to a T75 flask with 10 mL of DMEM +/+ and 30 µL of zeocin (this was the base for a new batch). The cells were grown until they reached 80-90% confluency. The cells were then split into 145 mm petridishes and grown for 8 days (1,5 x 106 cells in 22 mL of DMEM +/+ per 145 mm tissue culture petridish). After this, the medium was harvested from the petridishes, centrifuged, filtered through a 0,22 µm filter and stored at 4ºC.

3.1.2. Noggin Conditioned Medium (NCM)

HEK293-Noggin-hFc cells were thawed in DMEM +/+ and G418 (dilution 1/200). When confluent, the cells were split into 6 x T175 flasks. For this, the medium was removed from the T175 flask, cells were washed with 10 mL of PBS and trypsinized. DMEM +/+ was added to resuspend the cells which was followed by a centrifugation step. The supernatant was removed and the pellet was resuspended in 5 mL of DMEM +/+. The resuspended pellet was then transferred to 6 x T175 flasks with 40 mL of DMEM +/+ with G418. When confluent, the medium was removed, the cells were washed and trypsinized and 5 x 106 cells were added to 5 x T175 flasks with 40 mL of DMEM +/+ without G418 and to one T175 flask with G418 (for a new batch). When cells that were in DMEM +/+ without G418 were confluent, the DMEM +/+ was removed and 50 mL of $F12^{ghp}$ was added to each T175 flask. After 8-10 days, the medium was harvested, centrifuged for 5 minutes at 1100 rpm and 4ºC, filtered through a 0,22 µm filter and stored.

3.1.3. Rspondin-1 Conditioned Medium (RCM)

HEK293-HA-Rspo1-mFc cells were thawed in 3 mL of DMEM in a 15 mL Falcon tube that was then centrifuged for 5 minutes at 1100 rpm and 4ºC. After removing the supernatant, the pellet was resuspended in 1 mL of DMEM +/- (DMEM with 10% FBS). 25 mL of DMEM +/+ (DMEM with 10% FBS and 1% pen/strep), 900 µL of the resuspended pellet and 75 µL of zeocin was added to a T175 flask. Then the cells were incubated. When confluent, the cells were split in 6 x T175 flasks. For this, the medium was removed from the T175 flask, the cells were washed with 10 mL of PBS and trypsinized. Then they were incubated at 37ºC for 7-10 minutes and DMEM +/+ was added to resuspend the cells. They were centrifuged for 5 minutes at 1100 rpm and 4ºC, the supernatant was removed and the pellet was resuspended in 5 mL of DMEM +/+. The resuspended pellet was then transferred to 6 x T175 flasks with 25 mL of DMEM +/+ with zeocin. When confluent, the medium was removed, the cells were washed and trypsinized and 5 x 106 cells were added to 5 x T175 flasks with 25 mL of DMEM +/+ without zeocin and to one T175 flask with zeocin (for a new batch). When the cells that were in DMEM +/+ without zeocin were confluent, the DMEM +/+ was removed and 50 mL of $F12^{ghp}$ was added to each T175 flask. After 8-10 days, the medium was harvested, centrifuged for 5 minutes at 1100 rpm and 4ºC, filtered through a 0,22 µm filter and stored in 50 mL tubes or 0,5-1L containers at -20ºC.

3.1.4. Complete Medium

Conditioned media contain components that are secreted by cells and that are needed by other cells. For organoid culture, Wnt-3a, Noggin and Rspondin-1 conditioned media are required and were previously prepared in the lab by PhD students Cláudia Rodrigues and Violeta Railean. To prepare 500 mL of complete medium, 50 mL of $F12^{ghp}$ medium, 150 mL of RCM, 50 mL of NCM, 10 mL of B27 supplement (Invitrogen), 1,250 mL of N-acetylcysteine (Sigma-Aldrich), 0,05 mL of mEGF (Invitrogen), 5 mL of Nicotinamide (Sigma-Aldrich), 0,05 mL of A83-01 (Tocris), 0,165 mL of P38 inhibitor (Sigma-Aldrich) and 1 mL of primocin (InvivoGen) were added. To this medium, WCM was then added in a 1:1 ratio. This complete medium was used to culture the 3D organoids.

3.1.5. Culture and Passaging of Intestinal Organoids

Organoids that were previously generated and stored at a -80ºC freezer by other lab members were thawed. For this, cryovials containing organoids were obtained from the -80 freezer, 2 mL of $F12^{ghp}$ were added per cryovial to Falcon tubes, the 1 mL content of the cryovials was transferred to the Falcon tubes as soon as it thawed, the Falcon tubes were centrifuged for 5 minutes at 1100 rpm and 4ºC, then the supernatant was removed and the organoids were resuspended in 30 µL of matrigel per well and plated to a pre-warmed 24-well culture plate (approximately 3 drops of 10 µL per well). After incubation of the culture plate for 30 minutes, 500 µL of culture medium were added to each well and the plate was incubated. To passage the intestinal organoids, the medium was removed from the wells of the 24-well culture plate. Then, the drops of matrigel were loosened by resuspending them in 1 mL of $F12^{ghp}$. The content was transferred to a 15 mL Falcon tube where the organoids were resuspended around 20 times with a P1000 pipette with a 1000 µL tip and a 200 µL tip on top. Next, 2 mL of $F12^{ghp}$ were added and cells were centrifuged for 5 minutes at 1100 rpm and 4ºC. After removing the supernatant, 30 µL of matrigel per well were added to the Falcon tube. The organoids were plated to another pre-warmed 24-well culture plate (approximately 3 drops of 10 µL per well).

The culture plate was incubated for 30 minutes and then 500 µL of culture medium were added to each well before incubating again.

3.2. FIS Assay

The organoids were plated in 4 µL drops of matrigel containing 20-50 organoids in a pre-warmed 96-well culture plate and treated for 24 h with CFTR correctors, VX-445 (3µM) and VX-661 (5µM). The FIS assay was performed based on the protocol from Beekman's lab, Utrecht, The Netherlands[38]. After incubation with calcein green (Gibco), a cell-permeant dye that shows fluorescence, (3µM), serial dilutions of 0.02, 0.128, 0.8 and 5 µM of forskolin (Sigma-Aldrich, USA) solutions or of forskolin combined with VX-770 (3,33 µM) were added to the organoids. A confocal microscope (Leica SP8, Leica Microsystems, Germany) was used for live cell imaging at 37ºC and 5% CO_2 and images (fluorescence and brightfield) were taken for 60 min in 10 min intervals (7 timepoints).

3.3. FIS Quantification

Imaging data obtained through the FIS assay were analysed as described in Hagemeijer *et al*[46]. From the FIS assay, fluorescence microscopy images were obtained every 10 minutes, producing 7 timepoints (t = 0, 10, 20, 30, 40, 50, 60 min) per well. Then they were analysed by FIJI, a software that allows data visualization and image processing and by Cell Profiler, another software that measures and analyses cell images. Organoid Analyst was also used to normalize and export the data in a table format[46].

The data exported from Organoid Analyst was then used in GraphPad Prism software where the increase of the surface area relative to t = 0 was quantified at different concentrations of Fsk for each treatment and averaged from two different wells for each assay. Results were expressed as the absolute area under the curve (AUC) which was calculated from the normalized surface area increase (baseline = 100%, t = 60 min), averaged from a minimum of three independent assays (n=4 for p.Gly85Glu/p.Gly85Glu, n=5 for p.Ala561Glu/p.Ala561Glu I, n=6 for p.Ala561Glu/p.Ala561Glu II, n=4 for c.3140-26A>G/p.Tyr1014ThrfsTer9, n=5 for c.3140-26A>G/p.Gln1100Pro, n=5 for p.Gln1100Pro/p.Ser4Ter, n=4 for p.Tyr1092Ter/p.Tyr1092Ter and n=3 for p.Gly542Ter/p.Gln220Ter). CFTR rescue was assessed by comparing the average AUC values between control (Fsk only) and treatments (Fsk + VX-770; Fsk + VX-661/770; Fsk + VX-445/661/770), for the forskolin concentration of 0.128 µM, the standard for *in vivo* treatment efficacy prediction. Reference values refer to data obtained from FIS results from organoids homozygous for the p.Phe508del variant, which served as control.

3.4. Statistical Analyses

Statistical differences were calculated using analysis of variance (ANOVA) to assess statistical differences among the different treatments. Data are represented as mean ± standard error of the mean (SEM). Significance was considered for p-value ≤ 0.05.

4. Results

The main goal within this project was to evaluate currently approved CFTR modulator drugs for their capacity to rescue CFTR function in CF individuals carrying rare variants. These variants with such low incidence are not well, if at all, characterized. Therefore, they are not eligible for the available CFTR modulator therapies. However, these individuals might benefit from those treatments, reflecting the importance of personalised medicine for CF. It has been shown that 3D intestinal organoids are a good model to predict clinical benefit of CFTR modulators treatment[45]. The FIS assay is used to assess CFTR function in intestinal organoids using confocal live-cell microscopy. This technique uses forskolin, a CFTR agonist, to promote the opening of the CFTR channel which, due to ion and water uptake, results in organoid swelling. In the presence of non-functional CFTR, no organoid swelling occurs.

In this project, the effect of CFTR correctors and potentiator (VX-445, VX-661 and VX-770) on CFTR function was assessed using the FIS assay. Intestinal organoids from eight individuals with the following CFTR genotypes: p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu (two individuals), c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro, p.Gln1100Pro/p.Ser4Ter, p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter were analysed. To this end, intestinal organoids were pre-incubated for 24h with the correctors VX-661 or VX-445/661 and then stimulated with the potentiator VX-770 and different Fsk concentrations (0.02, 0.128, 0.8 and 5 μM). Confocal live-cell microscopy was used to monitor the swelling of organoids over time.

4.1. p.Gly85Glu/p.Gly85Glu

Intestinal organoids from an individual carrying the p.Gly85Glu variant in homozygosity were analysed. This specific variant is categorized as a class II CFTR variant[47]. The missense variant p.Gly85Glu results in the replacement of glycine with negatively charged glutamic acid at position 85 within TMD1 of CFTR[48]. It causes severely impaired processing and trafficking, resulting in the complete elimination of CFTR-mediated ion transport[47]. The following figure shows the results for these organoids which were analysed by the previously described FIS assay (Figure 4.1).

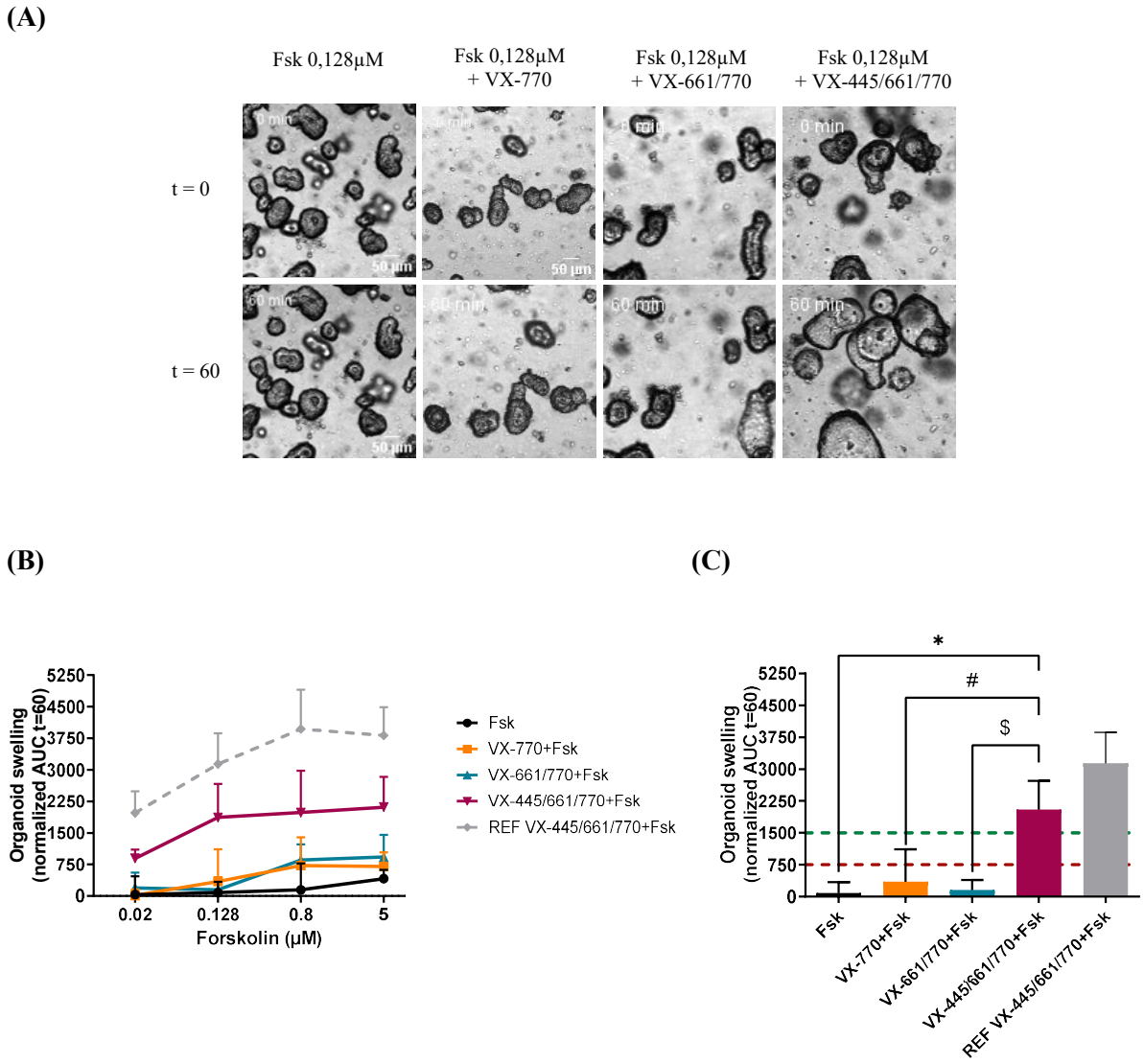


Figure 4.1 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Gly85Glu/p.Gly85Glu genotype. (A) Representative original confocal microscopy images of p.Gly85Glu/p.Gly85Glu intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=4. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbols represent significance vs. * - stimulation with Fsk alone; # - stimulation with VX-770 + Fsk; $ - stimulation with VX-661/770 + Fsk.

The results in Figure 4.1 A and B show no organoid swelling after stimulation with all different Fsk concentrations tested, which indicates no residual CFTR function. Confocal microscopy images in Figure 4.1 A and the corresponding analysis in Figure 4.1 C show that the treatment with VX-770 alone or in combination with VX-661 (together with Fsk concentration 0,128 μM) fails to induce any organoid swelling. However, in the presence of the triple combination, VX-445/661/770, a significant swelling could be detected, being above the threshold for high clinical benefit. Nevertheless, the observed swelling did not reach the level of p.Phe508del/p.Phe508del organoids swelling treated with VX-445/661/770, which is used as a reference for maximal CFTR rescue.

4.2. p.Ala561Glu/p.Ala561Glu

The next organoids which were analysed by the FIS assay are from two individuals with the same genotype. Both are homozygous for variant p.Ala561Glu (Figures 4.2 and 4.3). Variant p.Ala561Glu is also a class II missense mutation located in NBD1 of CFTR and is associated with severe disease[49].

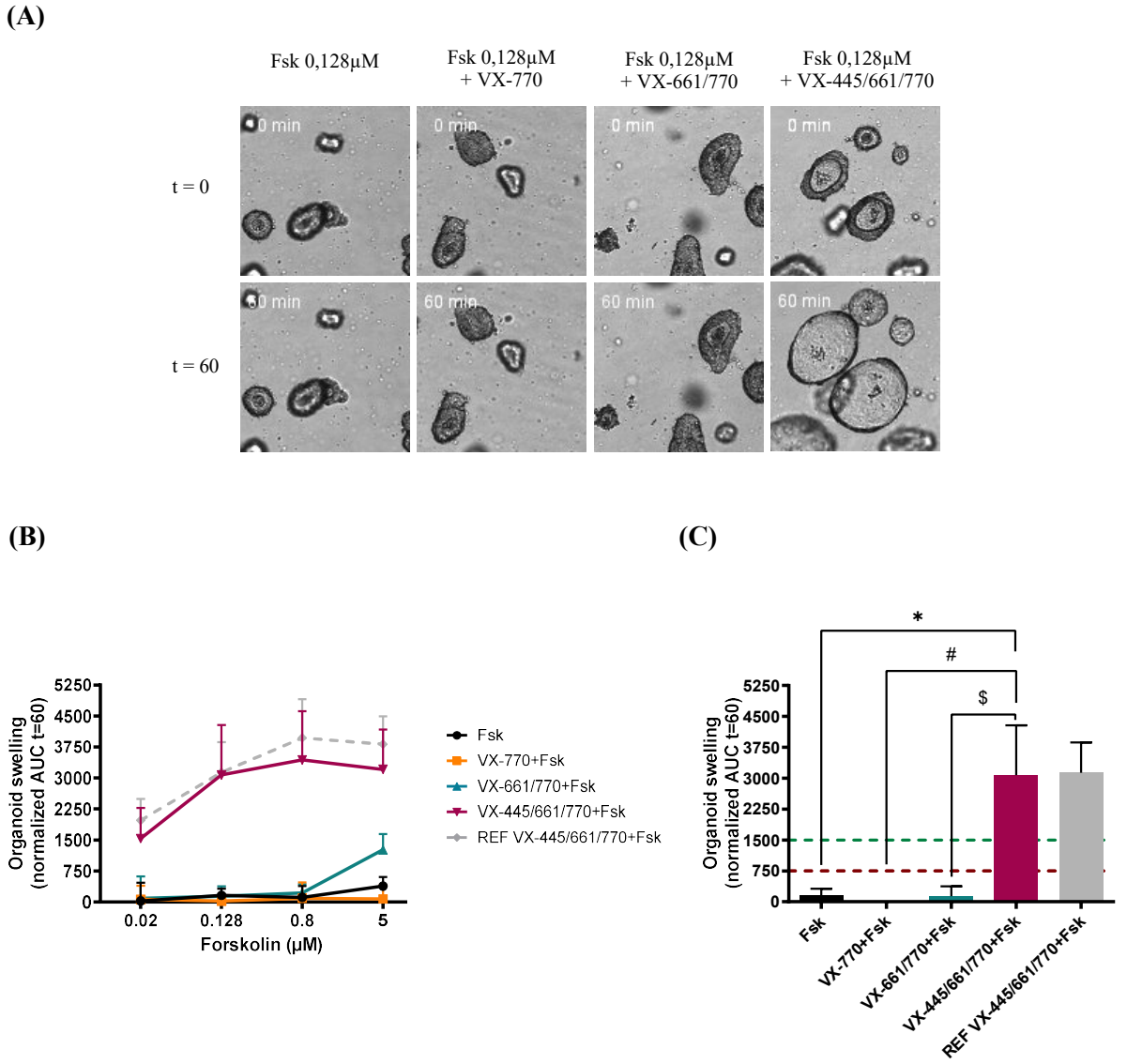


Figure 4.2 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Ala561Glu/p.Ala561Glu genotype (I). (A) Representative original confocal microscopy images of p.Ala561Glu/p.Ala561Glu intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=5. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbols represent significance vs. * - stimulation with Fsk alone; # - stimulation with VX-770 + Fsk; $ - stimulation with VX-661/770 + Fsk.

As seen in Figure 4.2 B, no organoid swelling could be observed upon stimulation with different Fsk concentrations, suggesting no residual CFTR function for variant p.Ala561Glu. Figure 4.2 A and C show that the treatment with VX-770 alone or in combination with VX-661 also did not induce any organoid swelling. Nonetheless, a significant increase in swelling, above the threshold for high clinical benefit, was seen in the presence of the triple combination VX-445/661/770, even reaching the level of reference p.Phe508del/p.Phe508del organoids treated with VX-445/661/770.

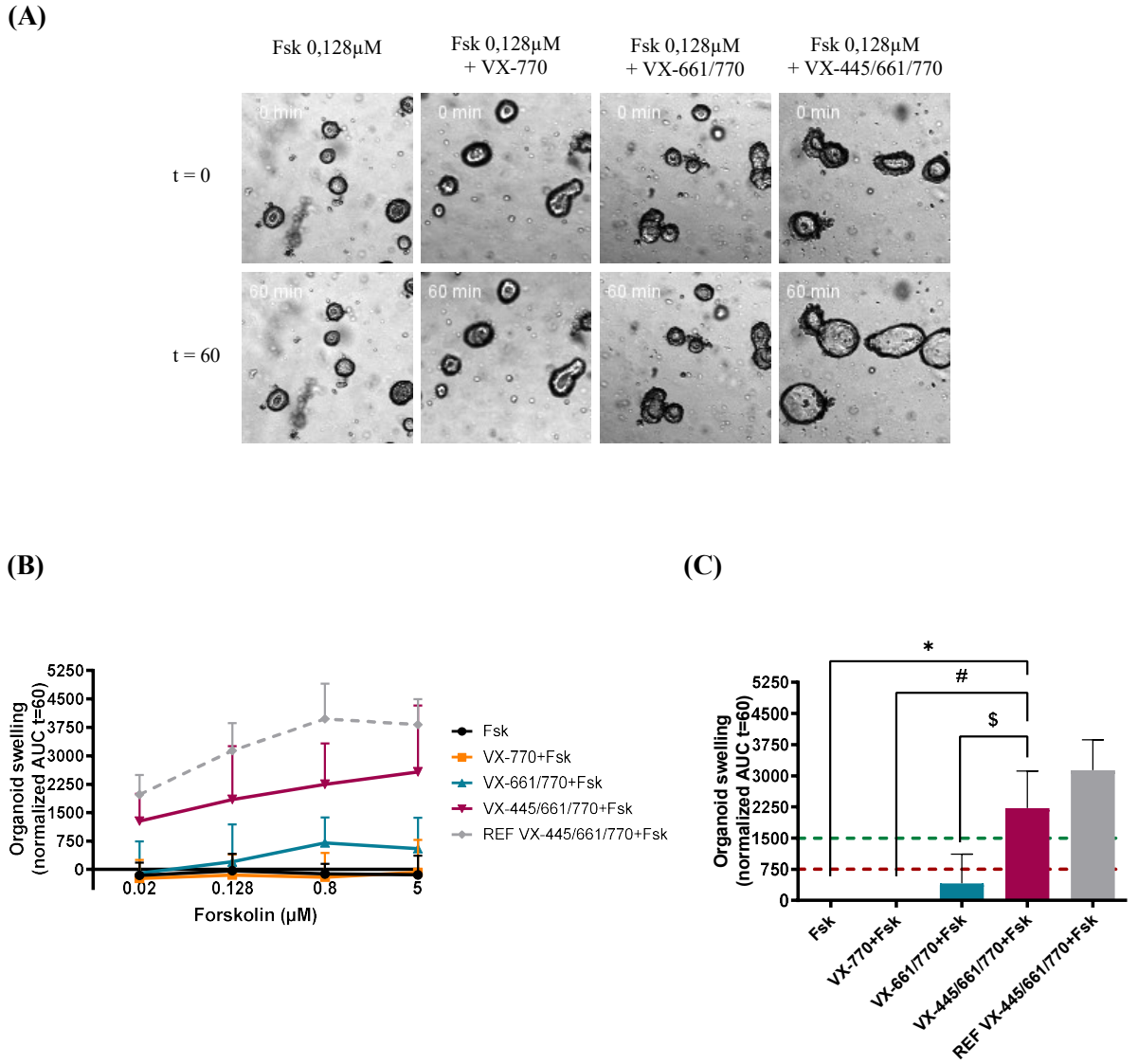


Figure 4.3 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Ala561Glu/p.Ala561Glu genotype (II). (A) Representative original confocal microscopy images of p.Ala561Glu/p.Ala561Glu intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=6. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbols represent significance vs. * - stimulation with Fsk alone; # - stimulation with VX-770 + Fsk; $ - stimulation with VX-661/770 + Fsk.

For the organoids from the second individual bearing the p.Ala561Glu CFTR variant in homozygosity, the treatment with different Fsk concentrations did also not promote any organoid swelling and as such suggests no residual CFTR function (Figure 4.3). Analysis in Figure 4.3 C and confocal microscopy images in Figure 4.3 A show that the addition of VX-770 alone or in combination with VX-661 did not lead to any organoid swelling. However, similar as for the organoids from the first individual, treatment with VX-445/661/770 led to a significant swelling which was above the threshold for high clinical benefit, but did not reach the reference level (p.Phe508del/p.Phe508del organoids swelling treated with VX-445/661/770).

4.3. c.3140-26A>G/p.Tyr1014ThrfsTer9

The following section describes the analysis of organoids from an individual carrying the two variants c.3140-26A>G and p.Tyr1014ThrfsTer9. The c.3140-26A>G variant belongs to class V and alters the correct splicing of the CFTR gene[50]. Variant p.Tyr1014ThrfsTer9 causes a frameshift resulting in premature termination[51].

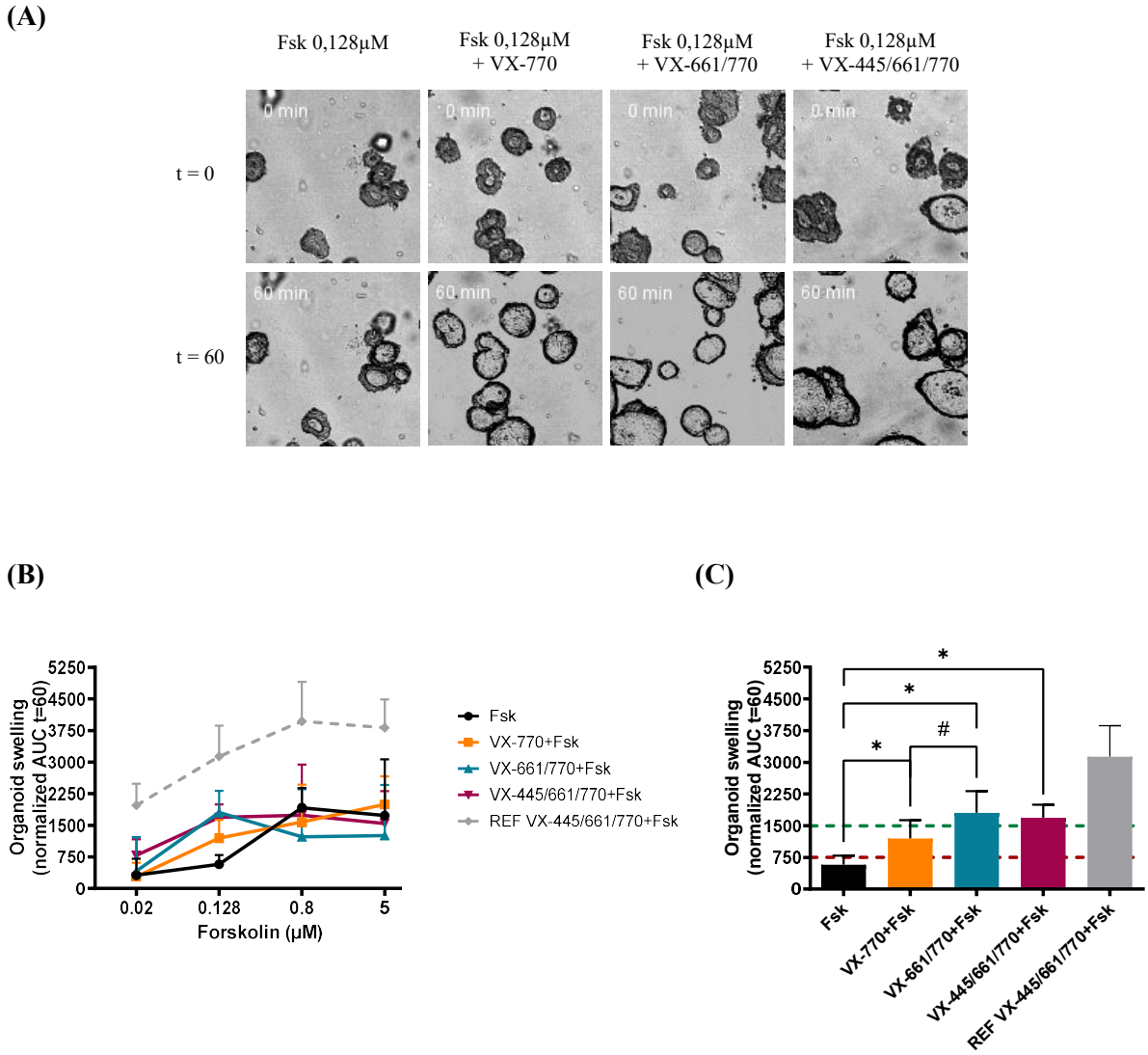


Figure 4.4 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a c.3140-26A>G/p.Tyr1014ThrfsTer9 genotype. (A) Representative original confocal microscopy images of c.3140-26A>G/p.Tyr1014ThrfsTer9 intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=4. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbols represent significance vs. * - stimulation with Fsk alone; # - stimulation with VX-770 + Fsk.

As it can be seen in the confocal microscopy images in Figure 4.4 A, a pre-swelling of organoids can be observed in the presence of Fsk concentration 0.128 μM in combination with VX-661/770 and VX-445/661/770 (Figure 4.4 A, t = 0 min). Furthermore, a pre-swelling was also detected at higher Fsk concentrations (0.8 and 5 μM), suggesting a residual function of CFTR (Figure 4.4 B, black line). Moreover, at timepoint 60, an increase in organoid swelling in the presence 0.128 μM Fsk in combination with of VX-770, VX-661/770 and VX-445/661/770 (Figure 4.4) was detected. Importantly, the swelling caused by VX-661/770 or the triple combination VX-445/661/770 surpassed the threshold for high potential clinical benefit for treatments (Figure 4.4 C), although the level of reference for maximal CFTR rescue was not reached.

4.4. c.3140-26A>G/p.Gln1100Pro

Intestinal organoids which were next analysed using the FIS assay carried variants c.3140-26A>G and p.Gln1100Pro. Variant c.3140-26A>G belongs to class V and causes less functional CFTR[50], while p.Gln1100Pro is a class II missense variant[52].

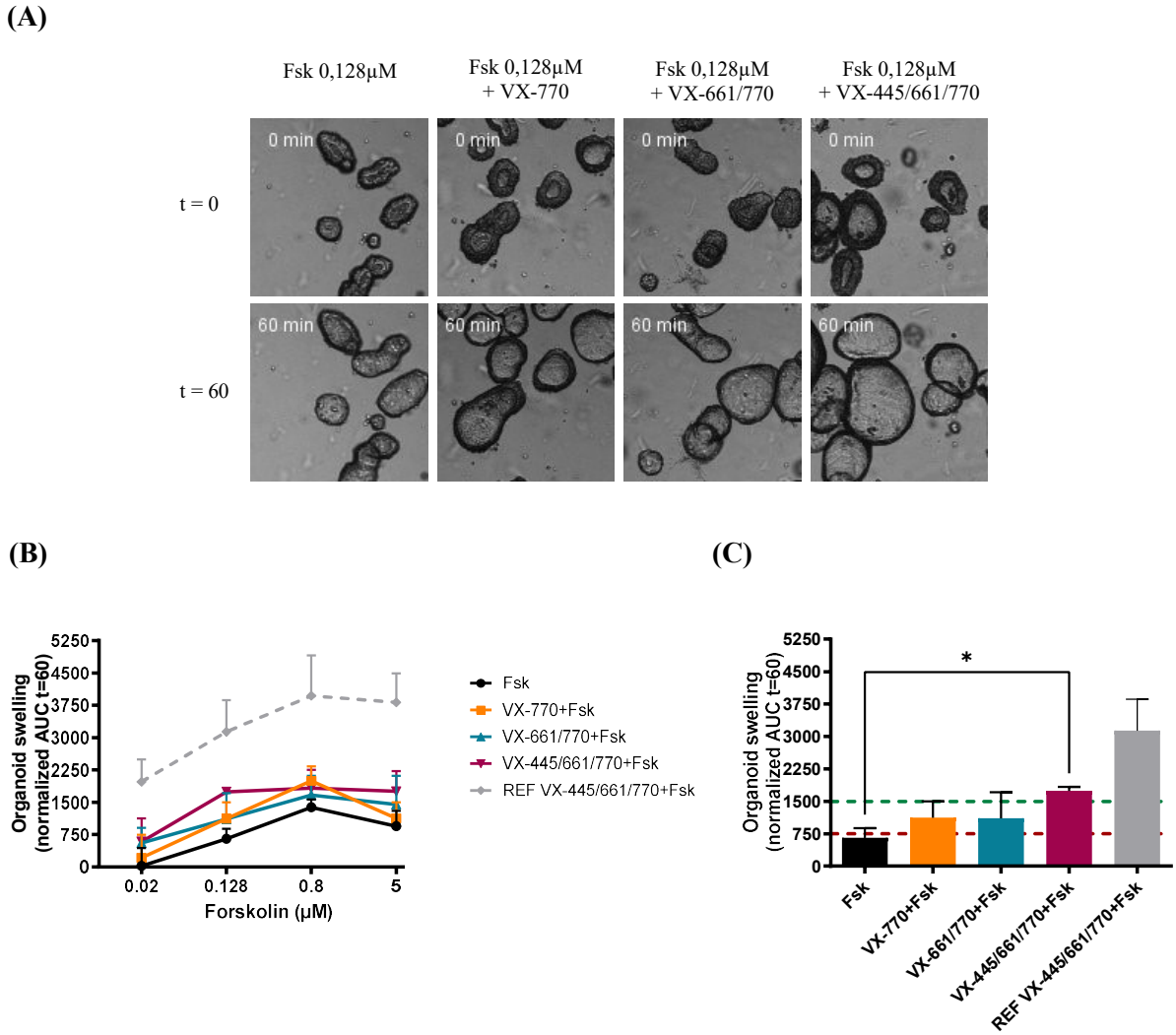


Figure 4.5 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a c.3140-26A>G/p.Gln1100Pro genotype. (A) Representative original confocal microscopy images of c.3140-26A>G/p.Gln1100Pro intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=5. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbol represents significance vs. * - stimulation with Fsk alone.

As presented in Figure 4.5 A, confocal microscopy images show an increased swelling of the organoids when treated with VX-770, VX-661/770 or VX-445/661/770 at Fsk concentration 0.128 µM. Swelling of organoids at Fsk concentration 0.128 µM could be observed in control conditions and in combination with VX-770 or VX-661/770 (Figure 4.5 B and C). The treatment with VX-445/661/770 significantly increased swelling compared to control conditions and reached the

threshold for high potential clinical benefit for treatments (Figure 4.5 C). Residual CFTR function was seen at Fsk concentrations of 0.128, 0.8 and 5 μM (Figure 4.5 B), indicating the presence of functional CFTR.

4.5. p.Gln1100Pro/p.Ser4Ter

The next organoids analysed carried CFTR variants p.Gln1100Pro and p.Ser4Ter. The p.Gln1100Pro variant is a class II missense variant[52]. The p.Ser4Ter is a nonsense variant[53].

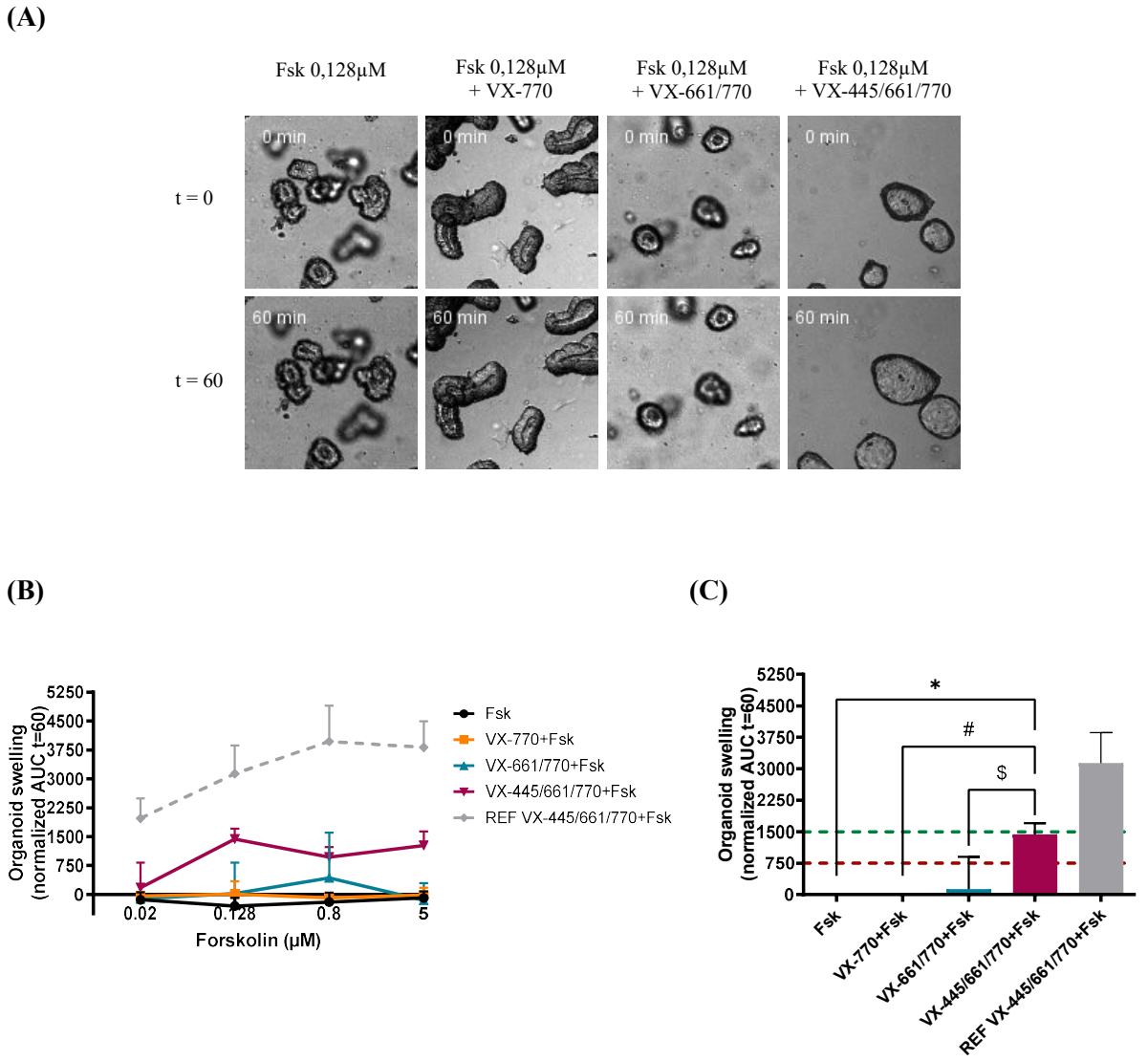


Figure 4.6 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Gln1100Pro/p.Ser4Ter genotype. (A) Representative original confocal microscopy images of p.Gln1100Pro/p.Ser4Ter intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=5. Significance was considered for p-value ≤ 0.05 (ANOVA) and symbols represent significance vs. * - stimulation with Fsk alone; # - stimulation with VX-770 + Fsk; $ - stimulation with VX-661/770 + Fsk.

The results in Figure 4.6 A and B indicate no residual CFTR function since none of the tested Fsk concentrations resulted in any organoid swelling. As the confocal microscopy images in Figure 4.6 A show, neither VX-770 alone nor in combination with VX-661 were able to cause swelling of the

organoids. Nevertheless, the treatment with the triple combination of VX-445/661/770 led to a significant organoid swelling, just below the threshold for high clinical benefit.

4.6. p.Tyr1092Ter/p.Tyr1092Ter

The following organoids originate from an individual homozygous for CFTR variant p.Tyr1092Ter which results in a premature stop codon (PTC) in the CFTR gene resulting in an absent or disrupted protein product[54].

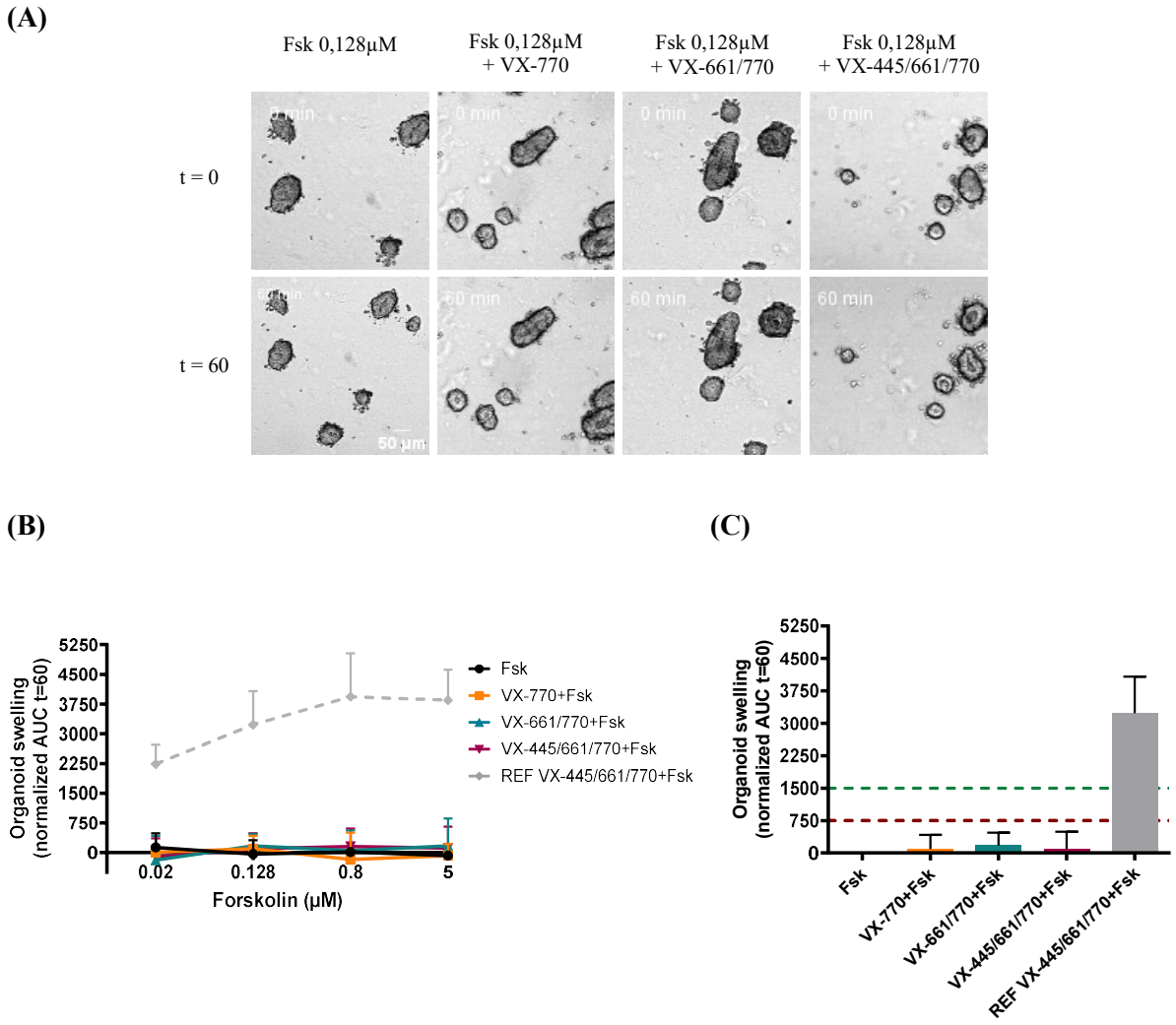


Figure 4.7 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Tyr1092Ter/p.Tyr1092Ter genotype. (A) Representative original confocal microscopy images of p.Tyr1092Ter/p.Tyr1092Ter intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=4. Significance was considered for p-value ≤ 0.05 (ANOVA).

The results in Figure 4.7 A and B show no organoid swelling following stimulation with varying concentrations of Fsk, indicating a lack of residual CFTR function. The confocal microscopy images

in Figure 4.7 A clearly show that no swelling occurred in the presence of any treatment, not even the triple CFTR modulator combination, implying no CFTR rescue for variant p.Tyr1092Ter.

4.7. p.Gly542Ter/p.Gln220Ter

The next organoids belong to an individual carrying the p.Gly542Ter and p.Gln220Ter variants. Both lead to a PTC thus preventing the synthesis of CFTR protein[55,56].

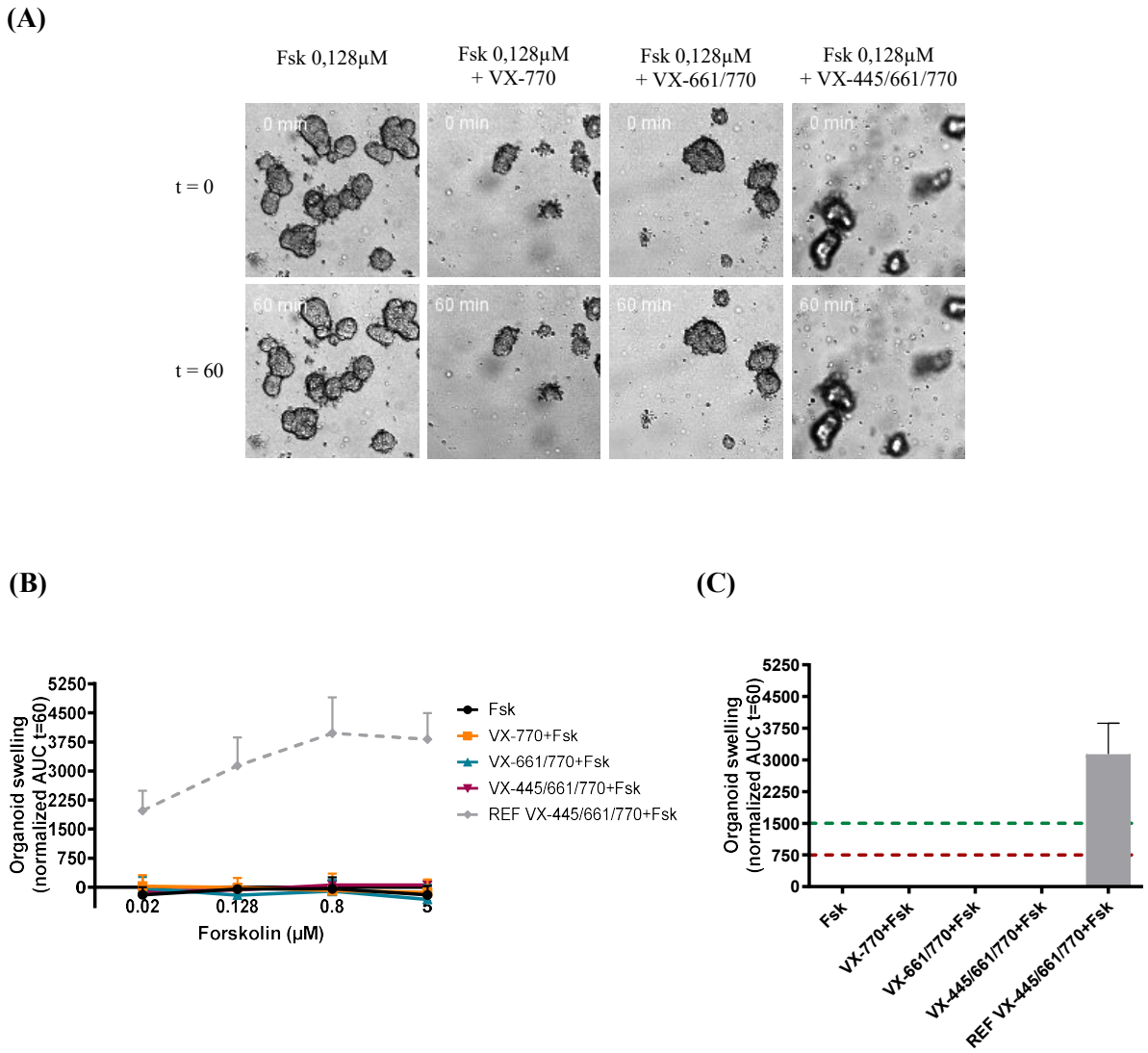


Figure 4.8 - Forskolin-induced swelling (FIS) assay on 3D intestinal organoids from an individual with a p.Gly542Ter/p.Gln220Ter genotype. (A) Representative original confocal microscopy images of p.Gly542Ter/p.Gln220Ter intestinal organoids at times 0 and 60 min of incubation with the following treatments: Forskolin (Fsk) alone at the concentration of 0.128 µM, VX-770 + Fsk, VX-661/770 + Fsk and VX-445/661/770 + Fsk. **(B)** FIS quantification of organoids treated with different Fsk concentrations (0.02, 0.128, 0.8 and 5 µM), expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). **(C)** Quantification of organoid swelling at t = 60 min for stimulation with 0.128 µM Fsk alone or in combination with CFTR modulators. Dashed red and green lines represent the established thresholds for medium and high potential clinical benefit for treatments, respectively. p.Phe508del/p.Phe508del organoids treated with VX-445/661/770 were used as a reference for maximal CFTR rescue. Data represent the mean ± SEM; n=3. Significance was considered for p-value ≤ 0.05 (ANOVA).

Figure 4.8 B clearly shows that the stimulation with different concentrations of Fsk did not induce any organoid swelling. Thus, there is no residual CFTR function for variants p.Gly542Ter and p.Gln220Ter (Figure 4.8 A and B). It is also evident from the confocal microscopy images and

corresponding analysis (Figure 4.8 A and C) that there was no swelling of the organoids with any treatment, including the triple CFTR modulator combination, indicating no CFTR rescue.

5. Discussion

Recently, a new treatment for cystic fibrosis (CF) has been approved. This treatment, known as CFTR triple modulator therapy (comprising elexacaftor, tezacaftor, and ivacaftor), is approved for individuals with CF who carry at least one copy of the most common p.Phe508del class II trafficking variant. It is providing additional benefits to the previously approved combination consisting of only tezacaftor and ivacaftor. In addition to this, CFTR potentiator therapy using ivacaftor has been shown to improve CFTR protein function in individuals with CF who have a class III gating mutation[57]. Despite these advancements, there is still a significant unmet need for many people with CF who are not eligible for CFTR modulator therapy, as they carry rare CFTR variants. These individuals have so far only access to symptomatic treatments.

In this study, 3D intestinal organoids from eight individuals with CFTR variants p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu (two individuals), c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro, p.Gln1100Pro/p.Ser4Ter, p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter were analysed for potential CFTR modulator treatment in a personalised approach. Here, the FIS assay was performed using confocal live-cell microscopy to assess the swelling of intestinal organoids which is proportional to CFTR function.

Intestinal organoids were analysed from an individual homozygous for p.Gly85Glu. This variant belongs to class II of CFTR mutations. p.Gly85Glu is a missense variant which causes a substitution of glycine to negatively charged glutamic acid at position 85 in TMD1 in CFTR[48]. It has been described that even though individuals with this variant present different clinical manifestations, it is associated with a severe phenotype[58]. Within this study, intestinal organoids bearing this variant showed no residual function at all different concentrations of Fsk (Figure 4.1) which confirms its classification to class II CFTR variants. Class II variants lead to CFTR misfolding and premature degradation and thus result in the near-absence of mature CFTR reaching the PM[21]. Furthermore, only the treatment with the triple combination (VX-445/661/770) showed a rescue of CFTR with clinical benefit. These findings are in line with a recent report on organoids carrying p.Gly85Glu in heterozygosity with the stop mutation p.Trp1282X or p.Phe508del, which also showed rescue well above the threshold considered relevant (1600 AUC after 60 min)[47]. The rescue of CFTR-p.Gly85Glu has also been reported by another group in primary human nasal epithelial cells as well as in a CFTR overexpressing human embryonic kidney (HEK) cell model[59]. The CFTR rescue of this variant by VX-445/661/770 indicates that those modulators act on the level of CFTR folding and/or trafficking and gating. In conclusion, the results suggest that this individual is likely to benefit from the combination treatment Trikafta® (or Kaftrio®).

Organoids from two individuals homozygous for p.Ala561Glu were further analysed. This variant is also a class II missense mutation located in NBD1 of CFTR and is associated with severe disease[49]. It is caused by the replacement of alanine by glutamic acid at position 561 of the CFTR polypeptide, which consequently leads to impaired folding and processing, originating a trafficking defect to the PM, similar to the most common CFTR variant, p.Phe508del[60]. Moreover, p.Ala561Glu is the second most frequent CF variant in Portugal[61]. Within this study, intestinal organoids from both individuals responded in the same way to the Fsk and CFTR modulator treatments. Although data has shown that VX-770 can partially restore p.Ala561Glu-CFTR function[60], this was not observed in the current study (Figures 4.2 and 4.3). The triple combination caused a significant increase in organoid swelling for both individuals, surpassing the threshold for high potential clinical benefit (Figures 4.2 C and 4.3 C). For organoids from the first individual analysed, the swelling reached similar values as the

p.Phe508del/p.Phe508del treated organoids used as reference (Figure 4.2 C), while for the second individual the levels were lower. Anyway, it is very likely that these two individuals could benefit from the treatment with Trikafta® (or Kaftrio®).

Next, organoids bearing heterozygous CFTR variants c.3140-26A>G and p.Tyr1014ThrfsTer9 were investigated. c.3140-26A>G is a class V variant[62]. It is usually associated with a milder form of CF[62] and individuals are diagnosed at an older age[50]. It alters the correct splicing of the CFTR as it creates a new acceptor splice site, competing with the normal one during RNA processing and causes an abnormal inclusion of 25 nucleotides within exon 20. This leads to a frameshift in the mRNA of CFTR, which creates a PTC and consequently an expression of a truncated non-functional CFTR protein[63]. As c.3140-26A>G leads to the production of an alternatively spliced CFTR transcript, it causes a reduction in the amount of functional CFTR at the PM[50,52,64]. The other more rare variant, p.Tyr1014ThrfsTer9, causes a frameshift resulting in premature termination[51]. The organoids from the individual carrying those two mentioned CFTR variants presented pre-swelling at Fsk concentration 0.128 µM alone and in combination with VX-661/770 and VX-445/661/770 (Figure 4.4 A, t = 0 min). The lumen of the organoids is already more visible at time point 0 (t = 0) if pre-swelling occurs. There was not a significant difference between VX-661/770 and VX-445/661/770 treatments at Fsk concentration 0.128 µM (Figure 4.4 C, blue and magenta bars), which means that the treatment with Symdeko® (or Symkevi®) (VX-661/770) would be enough to rescue CFTR function for this individual. As for the residual function found for these organoids, in a previous study from our lab and colleagues[62], it was found that the alternative splicing site within intron 17a of the CFTR gene competes with the regular splicing process for variant c.3140-26A>G. As a result, the unaffected CFTR mRNA undergoes normal processing, ultimately producing some functional CFTR protein on the cell membrane, which could here be the reason for the pre-swelling of the organoids. No functional data has yet been reported for p.Tyr1014ThrfsTer9.

In this study, organoids from another individual carrying the previously discussed variant c.3140-26A>G in heterozygosity with p.Gln1100Pro were analysed. p.Gln1100Pro is a very rare missense variant which is not reported in the CFTR2 database[65]. It belongs to class II of CFTR variants[52]. The analysis for these organoids also revealed residual function of CFTR at Fsk concentrations 0.128, 0.8 and 5 µM (Figure 4.5 B), most likely also due to variant c.3140-26A>G, as described above. Furthermore, significant organoid swelling with the triple combination was also observed, showing the rescue of CFTR trafficking and function. Thus, this individual could also benefit from taking Trikafta® (or Kaftrio®). These results are in line with data reported by our own group[52] and data from the organoids tested here bearing p.Gln1100Pro together with a stop mutation, p.Ser4Ter. The p.Ser4Ter variant is a nonsense variant located in exon 1 of the CFTR gene. It is thought to cause the termination of translation and as such it is likely associated with severe pancreatic disease[53] and thus less likely to respond to correctors and/or potentiators. Previous reports show that organoids carrying p.Gln1100Pro/p.Ser4Ter significantly rescue CFTR activity in the presence of the triple combination of modulators whereas VX-661 alone did not improve CFTR function in intestinal organoids[52]. This was also observed in the results within this study (Figure 4.6). Since p.Ser4Ter causes the introduction of a stop codon at amino acid position 4[53], it is less likely to respond to correctors or potentiators and therefore, the observed response is most likely associated with variant p.Gln1100Pro[52]. As such, this individual could also benefit from the Trikafta® (or Kaftrio®) treatment.

The organoids from an individual homozygous for p.Tyr1092Ter did not respond to any of the treatments (Figure 4.7). p.Tyr1092Ter leads to a change of C to A at nucleotide position 3408 of CFTR cDNA which causes a premature stop codon at position 1092 of the CFTR protein[54]. As such,

no functional protein is produced to be rescued, as the results confirm. Therefore, this individual is not likely to benefit from any of the currently approved modulator treatments.

The organoids from an individual with variants p.Gly542Ter and p.Gln220Ter did not show any swelling for any treatment (Figure 4.8). Being the most common class I variant, p.Gly542Ter results in a stop codon in the mRNA, which prevents normal synthesis of CFTR protein[55]. Consequently, a G to T substitution occurs in the first nucleotide of the glycine codon (GGA) at position 542 of the protein[66]. p.Gly542Ter is a nonsense variant[67,68], it produces a PTC triggering the surveillance pathway and causing mRNA NMD, which leads to the absence of CFTR protein production[66]. The p.Gln220Ter variant also belongs to class I and it causes a premature termination of CFTR synthesis[56]. It results in a C + T substitution at base 790, leading to a stop mutation at amino acid 220[56]. Since both variants do not produce any protein, it was unlikely to see a correction or potentiation for these organoids reaching clinical benefits for this individual.

6. Conclusion and Future Perspectives

In this project, rare CFTR variants from eight individuals were studied using the FIS assay on intestinal organoids in order to test their response to currently available CFTR modulator drugs. Out of the eight organoids tested, six (p.Gly85Glu/p.Gly85Glu, p.Ala561Glu/p.Ala561Glu I, p.Ala561Glu/p.Ala561Glu II, c.3140-26A>G/p.Tyr1014ThrfsTer9, c.3140-26A>G/p.Gln1100Pro and p.Gln1100Pro/p.Ser4Ter) showed a significant potential clinical benefit for modulator treatments that are currently available (Kalydeco®, Symdeko® or Symkevi® and Trikafta® or Kaftrio®), enabling the repurpose of these drugs for these variants that were previously not considered. Two organoids (p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter) showed no improvement with these modulators and thus, these individuals would not clinically benefit from the treatment.

Since therapeutic efforts are not directed at developing new compounds for rare CFTR variants, mostly for cost reasons, a personalised approach to extend the labels of the currently approved drugs so that more pwCF can benefit from them is urgently needed. The FIS assay has been shown to be a robust assay to predict clinical benefit for CFTR modulators. It uses human 3D intestinal organoids which mimic the structure and functions of the intestine, including the various types of cells and the interactions between them. It provides a personalised method for testing modulators on organoids derived from individuals with CF, which may then benefit from those approved therapies that were not initially designed for them.

Here, we were able to show clinical benefit for six pwCF. However, there are still many pwCF carrying rare variants for which their organoids should be tested. In regard to the individuals that did not show any organoid swelling in our assay, biobanking of organoids is crucial for future testing of novel drugs that are still to be approved or identified with the goal that all CF patients can be reached.

Individuals with p.Tyr1092Ter/p.Tyr1092Ter and p.Gly542Ter/p.Gln220Ter variants could not benefit from any of the currently approved treatments. This is important because with this information people can avoid expensive treatments with these drugs. Personalised therapy helps pwCF to know the best therapy for each individual and allows for the expansion of the labels of the currently approved therapies so that more people can benefit from possibly life-changing treatments.

7. References

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