2022

European Cystic Fibrosis Society

17th ECFS Basic Science Conference

Conference Programme & Abstract Book

Albufeira, Portugal

Chairpersons

Carlos Farinha, Nicoletta Pedemonte and Jeff Brodsky

30 March – 02 April 2022

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CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

WELCOME FROM THE ECFS PRESIDENT

Dear Friends and Colleagues,

It is a great pleasure to welcome you in Portugal at the 17th ECFS Basic Science Conference. After two hectic years, we are particularly happy to be able to gather again for this much awaited ECFS event.

We are delighted to welcome Carlos Farinha as the conference Chairperson who will be supported by Nicoletta Pedemonte and Jeff Brodsky as co-chairpersons.

Basic scientific discovery is critical to our understanding of CF, and already we can see new therapies coming through from the hard work of this endeavour.

The ECFS is committed to providing you with a platform to discuss your ideas with your colleagues during this important conference which will comprise a series of symposia with invited European and International guest speakers. It is a high priority for us to support the work of scientists in CF and we are proud that this conference is now established as a key event in the ECFS programme.

Your active participation will contribute to a productive exchange of information, and I hope fruitful collaborations.

I extend a very warm welcome to this exciting conference.

Isabelle Fajac
President
European Cystic Fibrosis Society

WELCOME FROM THE CONFERENCE CHAIRPERSONS

Located in the south of Portugal, in the centre of the Algarve, Albufeira is an exceptional place where you will discover a landscape full of contrasts.

Albufeira is one of the oldest cities in the Algarve. Its name derives from Arabic and means "Sea Castle", probably due to the proximity of the village to the sea or lagoon that had been formed in the lower part of the area.

In older times, it was a fishing village and artists, painters, poets and writers fell in love with it. Albufeira has become one of the most popular and much-visited tourist centres in the south of Europe. It is said that it was here that Paul McCartney wrote "Yesterday", one of the most beautiful songs of the Beatles, while looking at the intensity of the blue sea.

But let's come back to today and to the exciting next few days! Needless to say, we are really happy to see you all again in person.

Over the last months, many of you expressed the need to network, exchange ideas and experiences, to have live discussions and to meet again. We are confident you will have a lot of opportunities to do so in the coming days.

During this ECFS conference, you will hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment. A key characteristic of the conference is the high attendance of young participants, both PhD students and post-docs, who contribute enormously to the success of this annual event with their enthusiasm and vivid discussions. This group in particular will have excellent opportunities to discuss your data and interact in a great atmosphere with the best experts in this field. This combination of younger and more experienced researchers has been extremely successful in the past and the conference offers a forum for informal brainstorm-type discussions.

This year's programme also includes a number of symposia with international speakers covering topical aspects of high-quality basic research in cystic fibrosis, together with invited talks from submitted abstracts. There are also two keynote lectures and flash poster sessions.

We welcome all scientists, not only from the field of CF research but also from a diverse range of related fields, and wish you an inspiring conference!

Carlos Farinha
University of Lisbon
Portugal

Nicoletta Pedemonte
Istituto Giannina Gaslini, Genoa
Italy

Jeff Brodsky
University of Pittsburgh
United States

2022 ECFS Conference

New Frontiers in Basic Science of Cystic Fibrosis

30 March – 02 April 2022, Albufeira, Portugal

Programme

Chairpersons:

Carlos Farinha (Lisbon, PT), Nicoletta Pedemonte (Genoa, IT) Jeffrey L. Brodsky (Pittsburgh, US)

Wednesday, 30 March 2022 (Day 1)	
13:30-17:00	**Pre-Conference Seminar (organised by the ECFS and the Patient organisations)**
	Antimicrobial resistance in cystic fibrosis
	Chairs: Pavel Drevinek (CZ) - Jane Davies (UK)
13:30-14:45	**Part 1: The phenomenon of antimicrobial resistance in cystic fibrosis**
	AMR, epidemiology, current strategy of antibiotic therapy in CF - Pavel Drevinek (CZ)
	How to determine and interpret antimicrobial susceptibility of CF pathogens - Rafael Canton (ES)
	AMR and *P. aeruginosa*: towards the MDR phenotype - Helle Krogh Johansen (DK)
	Does the CF resistome matter? Lucas Hoffman (US)
14:45-15:15	*Coffee break*
15:15-17:00	**Part 2: Novel antimicrobial approaches to fight CF infections**
	Does the CF resistome matter? - Clinical view - Lucas Hoffman (US)
	Preclinical development of new anti-biofilm therapies - Tom Coenye (BE)
	Phage therapy: The Belgian experience - Sarah Djebara (BE)
	What is in the clinical pipeline - Pierre-Régis Burgel (FR)
	General discussion and wrap-up - Jane Davies (UK)
17:30-18:00	**Official Opening of the Conference by the Conference Chairpersons**
18:00-19:00	**Opening Keynote Lecture**
	Mucins, CFTR and their Intimate connection - Camille Ehre (US)
19:00-19:45	**Welcome Reception**
19:45-21:30	*Dinner*

Thursday, 31 March 2022 (Day 2)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Gene expression and RNA processing**
	Chairs: Margarida Amaral (PT) - Uta Griesenbach (UK)
08:45-09:10	Splicing modulation as a therapeutic approach for CF patients carrying rare CFTR mutations – Batsheva Kerem (IL)
09:10-09:35	Modulation of CFTR exon 22/23 splicing and/or intron 22 alternative polyadenylation (ApA) usage may have therapeutic potential for the treatment of certain CFTR PTC variants - Normand Allaire (US)
09:35-10:00	MicroRNA-dependent regulation of CFTR and its therapeutic potential - Chiara De Santi (IE)
10:00-10:10	Abstract 01 - LncRNAs: emerging players in CFTR gene regulation - Jessica Varilh (FR)
10:10-10:20	Abstract 02 - Development of a new microRNA therapeutic approach for the treatment of all patients with Cystic Fibrosis - Christie Mitri (FR)
10:20-10:30	Abstract 03 - Transcriptomic and proteomic analysis identifies changes associated with several prototypical cystic fibrosis-causing mutations - Lucia Santos (PT)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 2 – Personalized medicine approaches**
	Chairs: Nicoletta Pedemonte (IT) - Agnieszka Swiatecka-Urban (US)
11:00-11:25	Rare mutations in Cystic Fibrosis: from molecular diagnosis to clinical applications - Felice Amato (IT)
11:25-11:50	Predicting pharmacological rescue of CFTR misfolding mutations in human translational models - Martina Gentzsch (US)
11:50-12:15	Rectal organoids as a tool for personalized medicine - Anabela Ramalho (BE)
12:15-12:25	Abstract 10 - Development of a High Throughput Functional Screen Allows Drug Repurposing and Reveals Novel Drug Candidates for rescuing CFTR function in Patient-Derived Organoids with Nonsense Mutations - Sacha Spelier (NL)
12:25-12:35	Abstract 12 - Identification of novel small molecule modulators for PTC mutations in CFTR - Luka Clarke (PT)
12:35-12:45	Abstract 14 - Proof of concept of ionocytes' CFTR content as a novel biomarker for cystic fibrosis diagnosis and follow up - Floriana Guida (IT)
12:45-14:30	*Lunch*

14:30-15:30	**Flash Poster Session (even numbers)**
	Chair: Alexandre Hinzpeter (FR)
15:30-16:00	*Coffee break* & **Poster viewing**
16:00-17:45	**Symposium 3 – Rare mutations: molecular defects and protein correction**
	Chairs: Carlos Farinha (PT) – Ineke Braakman (NL)
16:00-16:25	CFTR modulation: insight from 3D structures - Isabelle Callebaut (FR)
16:25-16:50	Perspectives on precision therapeutics for rare CF genotypes - Eric Sorscher (US)
16:50-17:15	Rescue of mutant CFTR chloride channels by a mimetic peptide targeting the AKAP function of PI3Kgamma – Alessandra Ghigo (IT)
17:15-17:25	Abstract 26 - Structural plasticity of the Nucleotide Binding Domain 1 (NBD1) of CFTR is linked to pathogenesis of cystic fibrosis. - Rafael Colomer Martinez (BE)
17:25-17:35	Abstract 30 - Characterization of the [1898+3A>G;186-13C>G] complex allele by means of patient-derived nasal epithelial cells: molecular and functional analysis of CFTR mRNA and protein - Cristina Pastorino (IT)
17:35-17:45	Abstract 29 - Rescue of rare CFTR trafficking mutants highlights a structural location-dependent pattern for correction - Sónia Zacarias (PT)
19:45-21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Even numbers

Friday, 01 April 2022 (Day 3)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4 – CFTR folding and trafficking**
	Chairs: Jeff Brodsky (US) – Marianne Carlon (BE)
08:45-09:10	Requirements for CFTR folding and transport - Ineke Braakman (NL)
09:10-09:35	CFTR's Site 1: Degenerate but not disabled - T.C. Hwang (US)
09:35-10:00	Cif: a therapeutic target in persistent airway infections – Dean R. Madden (US)
10:00-10:10	Abstract 36 - Characterization of corrector ARN23765 mechanism of action via Photo-Affinity Labeling (PAL) approach - Fabio Bertozzi (IT)
10:10-10:20	Abstract 37 - New kinase therapeutic targets for cystic fibrosis from a global functional genomics screen - Hugo Botelho (PT)
10:20-10:30	Abstract 38 - Rescue F508del-CFTR with nanobodies - Marie Overtus (BE)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 5 – CFTR: beyond the airway**
	Chairs: Mike Gray (UK) - Pascale Fanen (FR)
11:00-11:25	CFTR: a new horizon in pancreatitis - Peter Hegyi (HU)
11:25-11:50	The "CF gut", its abnormalities directly and indirectly related to CFTR dysfunction, and strategies to improve gut fluidity and alkalinity in the CF gut beyond CFTR rescue - Ursula Seidler (DE) [Pre-recorded talk]
11:50-12:15	Physiology and pathology of CFTR in the kidney - Karl Kunzelmann (DE)
12:15-12:25	Abstract 54 - CFTR activity is determined by the store-independent activation of SPCA2/STIM1/ORAI1 complex in secretory epithelial cells - Arpad Varga (HU)
12:25-12:35	Abstract 56 - Cystic Fibrosis-related Bone Disease: CFTR class II mutations deregulate osteoclast formation and favor RANK+MCSFR+ circulating pre-osteoclasts - Johan Sergheraert (FR)
12:35-12:45	Abstract 60 - Integrative analysis of vascular impairment in models of cystic fibrosis - Lucas Treps (FR)
12:45-14:00	*Lunch*
14:00-18:30	**Free Afternoon**
18:30-19:30	**Flash Poster Session (odd numbers)**
	Chair: Felice Amato (IT)
19:45 -21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Odd numbers

Saturday, 02 April 2022 (Day 4)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 6 – Restoring epithelial homeostasis**
	Chairs: Marcus Mall (DE) – Dean R.Madden (US)
08:45-09:10	Novel mechanisms of TGF-beta signaling in CF - Agnieszka Swiatecka-Urban (US)
09:10-09:35	Investigating the therapeutic potential of phages as antibacterials and immunomodulators- Anna Pistocchi (IT)
09:35-10:00	Apical hydration protects the CF airway epithelium from *P. aeruginosa* by restoring junctional networks - Marc Chanson (CH)
10:00-10:10	Abstract 63 - Identification of drugs activating CFTR-independent fluid secretion in nasal organoids based on a high-content screening assay - Lisa Rodenburg (NL)
10:10-10:20	Abstract 64 - ATP12A upregulation in airway epithelial cells by inflammatory stimuli - Daniela Guidone (IT)
10:20-10:30	Abstract 65 - SLC26A4 but not TMEM16A directly regulates ASL pH under inflamed conditions in nasal epithelia derived from donors with rare class I mutations - Livia Delpiano (UK)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 7 – Mucus and mucins**
	Chairs: Camille Ehre (US) - Karl Kunzelmann (DE)
11:00-11:25	Role of submucosal glands and mucous strands in airway host defense - Lynda S. Ostedgaard (US) [Pre-recorded talk]
11:25-11:50	What is wrong with the CF lung mucus and how can we fix it? - Gunnar Hansson (SE)
11:50-12:15	The *Xenopus tropicalis* tadpole as a model system to define mechanisms of mucus function - David Thornton (UK)
12:15-12:25	Abstract Abstract 84 - Extracellular vesicle lncRNA MALAT1 drives HDAC11-dependent chronic inflammation in cystic fibrosis airway neutrophils - Brian Dobosh (US)
12:25-12:35	Abstract 85 - HiPSC-derived AECs as a novel platform to study the role of ionocytes in mucociliary clearance - Marta Vila Gonzalez (UK)
12:35-12:45	Abstract 83 - Hydrogel-encapsulated niclosamide for topical treatment of inflammatory airway diseases - Raquel Centeio (DE)
12:45-14:15	*Lunch*

14:15-16:00	**Symposium 8 – Gene-based therapeutic approaches**
	Chairs: Jeff Beekman (NL) - Martina Gentzsch (US)
14:15-14:40	CFTR gene editing – recent highlights, future goals and therapeutic opportunities - Patrick Harrison (IE)
14:40-15:05	Development of suppressor tRNA gene therapies targeting nonsense associated CF- John Lueck (US)
15:05-15:30	Progression towards a first-in-man lentiviral vector trial - Uta Griesenbach (UK)
15:30-15:40	Abstract 89 - Development of *in vitro* transcribed mRNA therapeutics for cystic fibrosis - Ruhina Maeshima (UK)
15:40-15:50	Abstract 91 - CFTR super exon splice site and polyA signal affect CFTR expression and function - Hillary Valley (US)
15:50-16:00	Abstract 94 - Correction of the CFTR 1717-1G>A splicing mutation through CRISPR based technology - Alessandro Umbach (IT)
16:00-16:30	*Coffee Break*
16:30-17:30	**Closing Keynote lecture**
	How to tackle what is still missing: striking CF by mechanistic approaches – Margarida Amaral (PT)
20:00	*Dinner / Social Event*

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P7 Linked-read sequencing identifies the complex variation at the chromosome 7 trypsin locus contributing to cystic fibrosis comorbidities
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Daniela Guidone, Martina Buccirossi, Paolo Scudieri, Vito Terlizzi, Federico Cresta, Isabelle Sermet-Gaudelus, Elise Dreano, Gabrielle Planelles, Rossella De Cegli, Sergio Sarnataro, Gilles Crambert, Luis J.V. Galietta

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Marco Cafora, Noemi Poerio, Francesca Forti, Nicoletta Loberto, Federica Briani, Massimo Aureli, Maurizio Fraziano, Anna Pistocchi

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Brian Dobosh, Diego Moncada Giraldo, Rabindra Tirouvanziam

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Karen Mention, Kader Cavusoglu-Doran, Lúcia Santos, David J. Sanz, Anya T. Joynt, Carlos M. Farinha, Neeraj Sharma, Patrick T. Harrison

P89 Development of in vitro transcribed mRNA therapeutics for cystic fibrosis
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Joseph Porter, John Lueck

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Nathalie François, Itishri Sahu, Sean Mc Cafferty, Nicoletta Pedemonte, A.K.M. Ashiqul Haque, Niek N. Sanders

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Alessandro Umbach, Simone Amistadi, Giulia Maule, Anna Cereseto

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Simone Amistadi, Giulia Maule, Marjolein Ensinck, Liesbeth De Keersmaecker, Marianne Carlon, Anna Cereseto

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Carina Graham, Stephen Hart, Amy Walker, Deborah Baines, Ahad Rahim

P101 Harnessing CRISPR-Cas9 technology to revert F508del-CFTR defect
Giulia Maule, Irene Carrozzo, Simone Amistadi, Eyemen Kheir, Marta Stancampiano, Gianluca Petris, Daniele Arosio, Anna Cereseto

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ECFS Young Fellows Travel Award

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Vaincre La Mucoviscidose Grant

Solenne Bleuse

30 March — 18:00–19:00

Opening Keynote Lecture

Mucins, CFTR and their intimate connection

Camille Ehre[1,2]

[1]Marsico Lung Institute, University of North Carolina, Chapel Hill, United States, [2]Department of Pediatrics, University of North Carolina, Chapel Hill, United States

CF is a multi-organ disease primarily affecting mucin-producing tissues, such as the lungs and gastrointestinal tract. Loss of CFTR function alters ion fluxes (decreased Cl- and HCO3 - secretion and increased Na+ absorption) and results in the production of a thick mucus that adheres to cell surfaces. Several hypotheses regarding the role of CFTR on mucus properties invoking airway surface liquid (ASL) acidification, low [HCO3 -], and dehydration have been explored in recent years (1). Impaired CFTR-mediated HCO3 - secretion reduces polycation chelation and pH. Inside goblet cells, mucins are tightly packed within granules as a result of a Ca2+ - and H+ -rich environment to ensure nematic arrangement and polyionic charge shielding (2). Upon secretion, electrolyte concentrations are adjusted to physiological levels, which prompts mucin expansion. In CF, chronic HCO3 - deficiency produces inadequate Ca2+ sequestration, causing incomplete unfolding of the mucins (3). In addition, the acidic pH inside the mucin granules generates non-covalent, hydrogen bonding to prevent electrostatic repulsion from negatively charged sugars. Once released in the more alkaline ASL environment, hydrogen bonds break, allowing the mucin network to relax. In a low pH environment, side chains are maintained in a protonated state, which can compromise the relaxation of the polymeric network. A mild-acidification, like in CF airways (pH 6.5), should allow the deprotonation of most carboxyl side chains; and although, increased electrostatic interactions have been described in CF ASL mucus (4), the effects of lowering pH were less significant (2.5-fold increase) than the effects of hyperconcentration (logarithmic scale change) on both GI and airway mucus (5). Defective CFTR reduces Cl- secretion and upregulates ENaC-mediated Na+ absorption, leading to subsequent water hyperabsorption and ASL dehydration (6). Moreover, goblet cell and submucosal gland hyperplasia are responsible for increased mucus production and sputum concentration. The combination of mucus overproduction and dehydration increases the solidity of CF airway secretions measured at 8-10 % solids (7). Concentration-dependent polymeric gel behavior is governed by the distance between mucin monomers/dimers (8). As water content decreases, mucin concentrations (cm) transition from an overlap concentration (c* at ~1mg/ml) to an entanglement concentration (c e >25 mg/ml), which affects mucus rheology by orders of magnitude. In a semi-dilute untangled regime (c*3% solids affect the relaxation time and diffusion of the mucin chains, hindering motion and overall dynamics of the network. The recent development of effective modulator therapies has highlighted the role of CFTR and showed that restoration of CFTR function can "reverse" mucus defects. Using genetically modified cell lines and human bronchial epithelial cells from subjects with G551D or F508del mutations, we examined the three prevailing hypotheses (i.e., low pH, low [HCO3 -], and dehydration) in response to Ivacaftor and Elexacaftor-Tezacaftor-Ivacaftor (9). Our data indicate that airway dehydration, not acidic pH and/or low [HCO3 -], dominates the biochemical properties of CF mucus and that CFTR modulation predominantly reduces mucin entanglement.

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31 March — 08:45–10:30

Symposium 1 – Gene expression and RNA processing

S1.1 Splicing modulation as a therapeutic approach for CF patients carrying rare CFTR mutations

Batsheva Kerem

Hebrew University, Jerusalem, Israel

CFTR mutations are divided to various classes according to their effect on the protein function. Patients carrying the class II mutation, F508del, are eligible for TRIKAFTA. Patients carrying Class III mutations are eligible for KALYDECO. However, 10-15% of the patients carry mutations that are not responsive to these modulators and thus remain with an unmet need. Our approach for development therapies for these patients is based on antisense oligonucleotides (ASOs), which are RNA-like short sequences that can bind and modulate their target RNA.

Our first project is focusing on class V splicing mutations which affect non-canonical splicing motives and generate both aberrantly and correctly spliced transcripts. We focus on an ASO-based drug for patients carrying the 3849+10kb C-T mutation. The mutation leads to inclusion of 84bp intronic sequences in the mature mRNA. We have identified a highly potent lead ASO, efficiently delivered by free uptake, that was able to significantly increase the level of correctly spliced mRNA and completely restore the CFTR function to wild type levels in human nasal epithelial (HNE) cells from a homozygote patient. This ASO led to CFTR function with an average of 43% of wild type levels in cells from various heterozygote patients. We developed a protocol for free delivery of ASOs to intestinal organoids and found that our lead ASO significantly decreased the aberrant splicing of the 3849 allele and restored the CFTR function in these cells. All these results demonstrate the ASO therapeutic potential benefit for CF patients carrying splicing mutations and is aimed to serve as the basis for our current clinical development. Our second project is aimed to develop ASO-based drug for patients carrying the class I nonsense mutation W1282X that is located in exon 23. Our approach is to generate skipping over exon 23, to eliminate the W1282X nonsense mutation and avoid RNA degradation induced by the nonsense mediated mRNA decay mechanism, allowing production of partially active CFTR proteins lacking exon 23. We identified several ASOs that significantly decrease the level of CFTR transcripts including exon 23 in HBE16ge W1282X. The ASOs resulted in significant levels of mature CFTR protein and together with modulators restore the channel function following free uptake into these cells. Importantly, our lead ASO was able to increase the level of transcripts lacking exon 23 and restore the CFTR function in cells from a W1282X homozygote patient. Our results demonstrate the ASO therapeutic potential benefit for CF patients carrying the W1282X mutation with the objective to advance the lead candidate to proof-of-concept clinical study.

S1.2 Modulation of CFTR exon 22/23 splicing and/or intron 22 alternative polyadenylation (ApA) usage may have therapeutic potential for the treatment of certain CFTR PTC variants.

Allaire, N., Yoon, JS., Armstrong, M., Valley, H., Bukis, K., Sivachenko, A., Wilson, E., LaPan, A., Conte, J., Harrington, J., Tabak, B., Bihler, H., Cheng, Y., Coote, K., Cotton, C., Mense, M.

CFFT Lab, Cystic Fibrosis Foundation, Lexington, MA, USA

A focus of the CFF research lab is the discovery of agents that promote readthrough for premature termination codons (PTC). In support of these efforts, we previously showed that certain pathogenic CFTR PTC variants are associated with aberrant mRNA isoforms including: 1) Exon 12 skipped (ΔE12)-associated isoforms (R553X or G542X) and 2) increased usage of the first of 11 putative alternative polyadenylation sites (ApA) in intron 22 which results in a CFTR Exon 22 (E22) 3' truncated mRNA (R1162X or W1282X). Consequently, these aberrant isoforms reduce full-length (FL) CFTR mRNA template available for PTC readthrough beyond the impact from Non-sense Mediated mRNA Decay (NMD). Furthermore, these changes originally observed in 16HBEge cell lines have been validated in fully differentiated human bronchial epithelial (hBE) cells, and intestinal organoids (IOs) derived from people with CF.

Here, we propose a model where intron 22 ApA usage and E22/23 splicing are competing events and have designed experiments to explore the therapeutic potential of (1) suppressing ApA usage to promote FL-CFTR mRNA expression, or (2) suppressing exon 22/23 splicing to promote CFTR mRNA truncated after exon 22.

To test the effect of suppression of ApA usage the first ApA site in intron 22 was removed by gene editing CFTR in 16HBEge-R1162X cells and 9 edited clonal lines were evaluated for CFTR mRNA expression. 8 of 9 lines showed a modest increase in FL-CFTR mRNA (~45.6% +/-38.9%; n=9) and concomitant decrease in CFTR E22 3' truncated mRNA (~-34.9+/-15.0%) compared to the parental cell line. Additionally, in 5/9 edited clones also showed small but statistically significant increases in CFTR function as measured by Cl^- current. CFTR mRNA analysis of the primary ApA gene edited cell lines revealed that one or more downstream ApA sites in intron 22 were recruited. Further experiments are underway to assess the effect of deleting all 11 putative intron 22 ApA sites.

Conversely, we predicted the suppression of E22/23 splicing would favor intron 22 ApA usage and in W1282X CFTR, increase the expression of a stable CFTR E22 3' truncated mRNA, not subject to NMD. To test this, we deleted the intron 22 splice acceptor site (SAd) via gene editing in the 16HBEge-W1282X cell line. This deletion resulted in a ~4-fold increase in CFTR E22 3' truncated mRNA compared to the parental line (~60% of WT CFTR mRNA). Furthermore, the half-life of CFTR E22 3' truncated mRNA is ~4 hrs. compared to << 1hr. for W1282X FL-CFTR mRNA. To test the therapeutic utility of the resultant CFTR E22 C-terminal truncated protein, it was heterologously expressed in FRT cells. After treatment with VX-661, VX-445, and VX-770 these cells yielded ~15% of the Cl^- current obtained from matching cells expressing CFTR WT. Cell lines in which CFTR E23-27 were deleted by gene-editing are being studied to assess the maximal therapeutic potential of CFTR E22 3' truncated mRNA.

Our data suggest modulation of E22-intron 22 ApA usage and/or E22/23 splicing may have therapeutic potential for certain CFTR PTCs.

S1.3 MicroRNA-dependent regulation of CFTR and its therapeutic potential

Chiara De Santi

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MicroRNAs (miRNAs) are small non-coding RNAs involved in regulation of gene expression. They bind in a sequence-specific manner to miRNA recognition elements (MREs) located in the 3' untranslated region (UTR) of target mRNAs and prevent mRNA translation. MiRNA expression is dysregulated in cystic fibrosis (CF) bronchial epithelium, a disease where expression of the cystic fibrosis transmembrane conductance regulator gene *CFTR* is altered. We have investigated the role of up regulated miRNAs on *CFTR*. miR-145, miR-223 and miR-494 were up regulated in CF versus non-CF bronchial brushings and bronchial epithelial cell (BEC) lines; in Phe508del CFTR homo vs. heterozygotes; in subjects positive for *P. aeruginosa* and; in cells treated with a CFTR inhibitor or IL-1β. Reciprocal down or up regulation of CFTR gene/protein expression was observed following miRNA manipulation and direct miRNA/target relationships were demonstrated via a CFTR 3'-UTR reporter plasmid. Increased expression of miR-145, miR-223 and miR-494 *in vivo* in bronchial epithelium of individuals carrying the Phe508del CFTR mutation correlated with decreased CFTR expression. We also identified two predicted binding sites for miR-143-5p on the *CFTR* mRNA, (starting at residues 558 and 644) and assessed whether *CFTR* is a true molecular target of miR-143-5p. Expression of miR-143-5p was found to be up-regulated in a panel of CF vs non-CF bronchial epithelial cell lines and its levels were increased *in vitro* after treatment with bronchoalveolar lavage fluid from CF patients compared to vehicle-treated cells. CFTR 3'-UTR luciferase assays identified that the site beginning at 558 was the actual active binding site and that miR-143-5p modestly but significantly inhibits CFTR, improving the knowledge on functional MREs within the *CFTR* 3'UTR. Taken together these studies revealed that defective CFTR function, *Pseudomonas* colonization and inflammation may affect miRNA expression and contribute to the regulation of Phe508del CFTR.

Importantly the miRNAs that are overexpressed in CF BEC and negatively regulate *CFTR* could potentially nullify the beneficial effects of CFTR modulators. We hypothesized that it is possible to reverse miRNA-mediated inhibition of CFTR using CFTR-specific target site blockers (TSBs) and developed a proof-of-concept drug-device combination inhalation therapy for CF. Lead miRNA expression was quantified in a series of human CF and non-CF samples and in vitro models. A panel of *CFTR* 3'-UTR -specific locked nucleic acid antisense oligonucleotide TSBs was assessed for their ability to increase CFTR expression. Their effects on CFTR activity alone or in combination with CFTR modulators were measured in CF BEC models. TSB encapsulation within poly-lactic-co-glycolic acid (PLGA) nanoparticles was assessed as a proof of principle of delivery into CF BECs. TSBs targeting the *CFTR* 3'UTR 298-305:miR-145-5p or 166-173:miR-223-3p sites increased CFTR expression and anion channel activity, and enhanced the effects of Ivacaftor/Lumacaftor or Ivacaftor/Tezacaftor in CF BECs. Biocompatible PLGA-TSB nanoparticles promoted CFTR expression in primary BECs and retained desirable biophysical characteristics following nebulization. Alone or in combination with CFTR modulators, aerosolized CFTR-targeting TSBs encapsulated in PLGA nanoparticles could represent a promising drug-device combination therapy for the treatment for CFTR dysfunction in the lung.

P1

S1.4 LncRNAs: emerging players in CFTR gene regulation

Jessica Varilh[1], Solenne Bleuse[1], Karine Delétang[1], Arnaud Bourdin[1,2], Magali Taulan-Cadars[1]

[1]UMR 9014 CNRS-INSERM 1046, PhyMedExp, Montpellier, France, [2]CHRU Montpellier, Montpellier, France

Objectives:*CFTR* gene displays a tightly tissue specific and temporal expression pattern that still remain incompletely understood. Previously, we showed that transcription factors and miRNAs act in synchrony to explain the weak *CFTR* mRNA level in mature lung cells. Other key regulatory players, long non-coding RNAs (lncRNAs), participate to the control of gene expression by influencing mainly the regulation of nearby genes. Here, we identified the presence and the expression of nine lncRNAs within the *CFTR* locus and focused our investigation on the role of two of them on *CFTR* gene expression.

Materials and methods: LncRNAs have been quantified by RT-qPCR in 20 different human tissues. LncRNA silencing or overexpression was performed in bronchial cells, with antisense oligonucleotides (GapmeRs) or overexpression with expression vectors, respectively. LncRNAs and *CFTR* mRNA level were assessed by RT-qPCR; CFTR protein level by immunoblot. LncRNA role and lncRNA/DNA interactions have been investigated by using luciferase reporter assays, through co-transfection of lncRNAs and vectors containing different length of *CFTR* promoter. Mutagenesis of potential lncRNAs binding sites on *CFTR* promoter were produced and Chromatin immunoprecipitation assays were also conducted.

Results: Examination of the *CFTR* locus and adjacent regulatory regions by using databases collecting human annotated lncRNAs led to the identification of 9 lncRNAs harboring signatures of active transcription. Quantitative analysis showed that 6 lncRNAs displayed a detectable and tissue specific expression. We focused on CF003 and CF006, which are downregulated in bronchial cells taken from CF patients. To assess whether these lncRNA exert a cis-acting role on CFTR, we used gapmeRs to inhibit their expression. Silencing of CF003 and CF006 induced a decrease of CFTR at mRNA and protein level in 16HBE; data confirmed by overexpression assays, depicting a positive role of lncRNAs on CFTR expression. Using *in silico* tools, we predicted binding sites for these lncRNAs on the *CFTR* promoter. Using luciferase reporter assay with different length of *CFTR* promoter, we showed that CF003 and CF006 increase the luciferase activity under the control of *CFTR*-1kb-promoter. Mutagenesis of their potential binding sites on *CFTR* promoter confirmed that these lncRNAs act directly on *CFTR* promoter. As the binding of these lncRNAs on *CFTR* promoter could affect local binding of transcription factors, we performed chromatin immunoprecipitation assays and showed that overexpression of CF003 and CF006 induced the enrichment of transcription factors (E2F1, E47, CREB1 and SOX17) on *CFTR* promoter in bronchial cells. By luciferase reporter assays with *CFTR*-1kb-promoter, we characterize the positive impact of E2F1, E47 and CREB1 and the negative impact of SOX17 on *CFTR* promoter. Furthermore, *in silico* tools highlighted a miR-145 motif on the CF003 and CF006 sequences; a miRNA strongly deregulated in CF cells. We showed that the overexpression of miR-145 decreases CF003 and CF006 level.

Conclusion: In this study, we characterized new lncRNAs transcribed within the *CFTR* locus and highlighted the role of CF003 and CF006 as activating cis-lncRNAs on CFTR expression. Together, these results reveal new promising targets to increase *CFTR* mRNA rate in addition to current CF therapy.

Supported by: AFM-Téléthon-Vaincre-La-Mucoviscidose

P2

S1.5 Development of a new microRNA therapeutic approach for the treatment of all patients with Cystic Fibrosis.

C. Mitri[1], N. Rousselet[2], H. Corvol[2], O. Tabary[2]

[1]Centre de Recherche Saint-Antoine, CRSA, Sorbonne Université, Inserm, Paris, France, [2]Département de Pédiatrie Respiratoire, Hôpital Trousseau, AP-HP, Paris, France

Objectives: Cystic fibrosis (CF), an autosomal recessive genetic multiorgan disease, is caused by a dysfunctional CF Transmembrane conductance Regulator (CFTR). CF is a complex pathology due to the multiplicity of mutations found. Correctors and potentiators have demonstrated good clinical outcomes for patients with specific Cftr gene mutations; however, there are still patients for whom those treatments are not suitable and require alternative CFTR-independent strategies. Although CFTR is the main chloride channel in the lungs, anoctamin 1 (TMEM16A) could compensate for CFTR deficiency. Interestingly, we showed that the expression and activity of TMEM16A were decreased in CF patients and that this deregulation was due to overexpression of microRNA-9 (miR-9) (Sonneville et al., 2017). To this end, we have developed an antisense oligonucleotide (ASO TMEM16A) that prevents microRNA-9 from binding to TMEM16A mRNA, thereby increasing its expression and activity. Here, we have investigated the effects of TMEM16A potentiation *in vitro* and *in vivo* and prepared for preclinical studies by assessing the best administration route and the ASO TMEM16A's toxicity and specificity.

Methods: The experiments are performed on cell lines and primary cells with different mutations. The CF mouse model is used to study the different administration routes, complete survival data, and study acute toxicity and long-term effects.

Results: The first results show that the effects of ASO TMEM16A are very specific to the target, which is the 3'UTR of TMEM16A. Thus, ASO TMEM16A does not induce inflammatory cytokines expression, does not alter either intracellular calcium mobilization or cell proliferation. These results were confirmed by "In vitro pharmaceutical profiling", a standard method used by the industry. While studying different administration routes on mice, we showed that ASO TMEM16A is detectable 30 days after subcutaneous injection or intranasal instillation. A first experiment on CF mice, which normally die of intestinal obstruction upon weaning, shows that injection of ASO TMEM16A significantly increases the lifespan of the mice up to 200 days. Acute administration of 50 times the effective dose did not show behavioural changes in the mouse, nor macroscopic or pathological changes. In addition, all of the blood markers were found to be normal. Pharmacokinetic and pharmacodynamic experiments are currently underway to confirm these results. In addition, our preliminary results show an improvement in male CF mice fertility by restoring vas deferent abnormalities, also described in CF male patients.

Conclusion: This strategy could apply to all patients with CF, regardless of their mutations, to correct chloride efflux and mucociliary clearance without inducing inflammation or toxicity.

P3

S1.6 Transcriptomic and proteomic analysis identifies changes associated with several prototypical cystic fibrosis-causing mutations

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Cystic Fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population, and it is caused by mutations in both alleles of the *CFTR* gene. The phenotypic heterogeneity observed in CF patients, even among those sharing the same *CFTR* genotype, suggests the involvement of other genes contributing to this phenotypic variability. Omics studies can greatly contribute to the identification of such genes. To better understand the global gene expression patterns associated with *CFTR* mutations, combined transcriptome and proteome analysis were performed in isogenic human bronchial epithelial (16HBE) cell lines each homozygous for one of five different CFTR mutations classes; this included four existing lines containing G542X, F508del, N1303K and G551D, and one additional isogenic line we made using CRISPR gene editing with I1234V. Changes in the expression profile of about 13,800 genes and 830 proteins were identified in each mutant cell line, and selected genes and proteins were used to validate RNA-Seq and mass spectrometry results by RT-qPCR and western blot, respectively. Evaluation of differentially expressed genes (DEGs) and proteins (DEPs) identified in each CFTR mutant cell line revealed a subset of DEGs and DEPs that are common to all mutations, and others that are unique to particular mutations. Functional enrichment analysis clustered DEGs and DEPs into biological processes relevant in CF pathophysiology, including actin cytoskeleton organization, cell differentiation, wound healing, protein targeting to organelles such as the ER and plasma membrane, protein glycosylation, and protein folding. Combination of both datasets revealed that approximately 97% of the proteins identified have matched transcripts, and that the resulting mutation-specific detected translated-transcripts (Dtts) have a high level of consistency in expression patterns. The results of the present study represent the first combined transcriptomic and proteomic study focusing on prototypical *CFTR* mutations. Analysis of both common and unique Dtts provide novel insight into the pathophysiology of CF, and on the mechanisms through which each mutation causes disease. Finally, this new dataset will likely contribute to the identification of new therapeutic targets and/or biomarkers for CF.

Work supported by UIDB/04046/2020 and UIDP/04046/2020 research unit grants from FCT, Portugal (to BioISI) and Cystic Fibrosis Foundation grant HARRIS17G0. LS is the recipient of a fellowship from BioSys PhD programme (Ref. PD/BD/130969/2017) from FCT (Portugal).

31 March — 11:00–12:45

Symposium 2 - Personalized medicine approaches

S2.1 Rare mutations in Cystic Fibrosis: from molecular diagnosis to clinical applications

Immacolata Zollo, Antonella Miriam Di Lullo, Monica Gelzo, Gustavo Cernera, Filippo Scialò, Federica Zarrilli, Giuseppe Castaldo, Felice Amato

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To date, about 2000 genetic variants have been reported in the *CFTR* gene but the disease-liability of all these variants has been completed only for □400 of the most common ones. The diagnosis of Cystic Fibrosis (CF) is supported by clinical symptoms, abnormal sweat chloride test and by the identification of two *CFTR* disease-causing mutations. The use of next generation sequencing (NGS) in molecular diagnostics has allowed incredible progress in the identification of CFTR genetic variants with high accuracy and a significant cost reduction. However, the interpretation of genetic variations represents one of the limiting phases of NGS technologies, also because thousands of rare variants are without pathogenic effects. In addition, the advent of new drugs, which are able to correct the basic defect of the CFTR protein, is changing the fate of many patients, but not all of them. Indeed, these new drugs are currently available for patients with a specific subset of mutations, and again, patients with rare or ultra-rare mutations are the most penalized.

Thus, despite the great contribution of NGS in the Cystic Fibrosis molecular diagnosis, a common effort is needed, through appropriate methodologies, to accurately and quickly define the effect of these rare mutations and their possible response to drugs.

We developed a functional studies platform, based on an ex vivo analysis of human nasal epithelial cells (hNECs), to unveil both the physio-pathological effect of novel/rare mutations and the responsiveness of patients to approved drugs or putative novel ones. In particular, this functional studies platform is composed of an interdisciplinary approach that takes advantage of different expertises in the field of cell and molecular biology, biochemistry, genetics and that is based on a continuous interaction with clinicians of our Regional Cystic Fibrosis Reference Center in Campania Region.

The hNECs can be easily obtained from patients, including those in early pediatric age and can be either used to study the effect of mutations of uncertain significance and to measure the responsiveness of CF Patients to therapy. Here I report the data obtained from the application of this type of approach on some patients with rare mutations and how this has made possible the treatment of these patients with the current therapies, for which they had been excluded based on their genotype.

S2.2 Predicting pharmacological rescue of CFTR misfolding mutations in human translational models.

Deborah M. Cholon, Nancy L. Quinney, Susan E. Boyles, Timothy J. Jensen, Luba A. Aleksandrov, Andrei A. Aleksandrov, Carla M. Ribeiro, Martina Gentzsch

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Cystic fibrosis (CF) is caused by mutations in the protein channel CFTR that perturb anion transport across the epithelia of the airways and other organs. The most common CFTR mutation, F508del, results in a misfolding defect. To cure CF, treatments that target CFTR have been developed such as correctors that enhance transfer of misfolded CFTR to the apical membrane, and potentiators that increase CFTR channel activity. A triple combination using correctors tezacaftor (VX-661) and elexacaftor (VX-445) and potentiator ivacaftor (VX-770) resulted in impressive clinical responses in CF patients with at least one copy of F508del. While this triple treatment has been expanded to several other CFTR mutations, around 10% of CF patients have rare mutations that are still without an approved CFTR-targeting drug. To identify relevant therapies for these patients and improve therapies for all patients, culture models using nasal, bronchial and rectal tissue from individual patients allow functional, biochemical and cellular detection of drug-rescued CFTR.

We utilized 2D and 3D airway cultures as a platform to characterize new CFTR mutations of uncertain significance to guide therapy optimization, illustrating the power of ex vivo and in vitro biochemical, physiological and molecular techniques to quantitate pathophysiologic derangements to support diagnosis and treatment. Our studies characterize novel molecular abnormalities caused by rare mutations of CFTR in individual CF patients and correlate these in vitro data with in vivo clinical presentation, allowing a more precise physiological summary of the effects of each CFTR mutation on CFTR function and subsequent theratyping of rare CFTR mutations.

To optimize CFTR modulator usage, it is critical to understand their mechanisms. Some modulator actions remain unclear, such as corrector VX-445, which may also act as a potentiator, and we found that the potentiator GLPG1837 may also exhibit corrector activity. Our electrophysiological and biochemical studies revealed that acute VX-445 did not drastically change CFTR single-channel conductance, but enhanced short-circuit current. VX-445 substantially improved the stability of F508del and affected its phosphorylation state. Chronic VX-770 abrogated correction of F508del by modulators, including VX-445 and VX-661. Interestingly, treatment with GLPG1387 or another potentiator, icenticaftor (QBW251) was not detrimental to the stability of rescued mature F508del as was observed with VX-770.

Currently, little is known about the impact of the CF airway environment on CFTR modulator processes. Therefore, we conducted studies that examine the impact of infection and inflammation on CFTR rescue. Interestingly, we found that infection and inflammation enhance the biochemical and functional rescue of F508del by CFTR modulator therapies and that inflammation overcomes abrogation of CFTR correction by chronic VX-770 treatment in vitro.

Mechanistic insights into modulator actions and its enhancements will not only be applicable for CFTR-targeting treatments, but may also be relevant to enhance newly developed gene therapies and read-though therapeutics that may be utilized in combination with CFTR modulators. Thus, the presented data and assays may have immediate translational relevance in providing methods and knowledge for accurate elucidation of CF drug effects, which will allow optimal therapies for all CF patients. (Supported by CFF and NIH).

S2.3 Rectal organoids as a tool for personalized medicine

Senne Cuyx[1,2], Mieke Boon[1,2], Marijke Proesmans[1,2], Lieven Dupont[3,4], Kris De Boeck[1,2], François Vermeulen[1,2], Anabela S. Ramalho[1]

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Treatment perspectives for patients with cystic fibrosis (CF) have dramatically changed over the last few years. A major breakthrough in the treatment of CF came with the advent of CFTR modulators aiming to directly improve CFTR function. The recent approval of the 'triple combination' associating ivacaftor, tezacaftor and elexacaftor, for people with CF (pWCF) carrying a F508del mutation hugely increase the number of patients with an approved treatment (around 90%). The therapeutic arsenal is expected to grow further over the next years when more CF therapies in development progress to approval. Despite this fast progression of the modulator family, there is still an unmet need for patients that do not carry the F508del CFTR mutation or an already mutation approved for treatment with those compounds. Also, some patients with common mutations seem to have a weak response to the currently available modulators. Another challenge will be the selection of the optimal CF therapy combination for each patient when more become available. The rectal organoid model allows to predictive response to CF therapies as a tool for 'precision medicine', which can easy up access to a treatment for pwCF, and giving a physiologic base to choose between different therapeutic options.

Our group is using this tool, testing the efficacy of CFTR modulators in pwCF. In a Belgian multicentre pre-clinical trial using rectal organoids from pwCF, we established a biobank collection currently with more than 300 different organoid cultures many with rare mutations. We explored the responsiveness of organoids in the biobank to the approved CF modulators (lumacaftor, tezacaftor, ivacaftor and elexacaftor alone or in combination) using the forskolin induced swelling (FIS) assay (developed by the Beekman lab). This analysis allowed to stratify pwCF for modulators efficacy based on the responses to the therapies. Stratifications of the patients by disease severity can also be done based on organoid residual function. We have found clear responses to the approved modulators on organoids from some patients with rare CFTR mutations. For the organoids showing no responses or low responses to the approved modulators we are testing combinations with new CFTR compounds in development.

In our lab, we have designed a new approach to help CF diagnosis based on the rectal organoid morphology: the rectal organoid morphology analysis (ROMA) assay. This assay was proven to be able to fully discriminate between organoids from subjects with clear CF diagnosis and from non-CF subjects. Ongoing work is now being done on milder CF cases and unclear CF diagnosis. The utility of this assay to test CF therapies efficacy in rectal organoids is currently being explored in our lab.

A personalized medicine tool, assessing the effect of CFTR modulators in patients with rare mutations in a patient-derived model such as rectal organoids could allow further label extension and grant access to modulators for responsive patients. Assessment of modulators in development in this personalized model could guide in a more precise manner future drug development strategies.

This work was funded by the Belgian CF patient Association "Mucoverening".

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S2.4 Development of a high throughput functional screen allows drug repurposing and reveals novel drug candidates for rescuing CFTR function in patient-derived organoids with nonsense mutations

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Recent therapies enable effective restoration of CFTR function of the most prevalent F508del CFTR mutation, and shift the clinical unmet need largely towards people with rare CFTR mutations such as nonsense mutations. Approximately 10% of the worldwide CF population carry premature termination codons (PTC) or nonsense mutations, resulting in production of truncated CFTR protein.

Previous research identified compounds with read-through (RT) activity that result, via various mechanisms, in introduction of an amino acid at the PTC site. At present-day only one RT compound has reached clinical trials, a chemically-engineered aminoglycosides derivative termed ELX-02 (NB124; Eloxx Pharmaceuticals). Recent results from ELX-02 clinical trials however show only a minor decrease in sweat chloride levels in CF patients homozygous for the G542X PTC mutations. Altogether, this underlines the need for continuing the search for novel CF therapeutics. Drug repurposing of FDA approved compounds could potentially aid in characterizing novel therapeutic options for individuals with CF. Drug repurposing is a strategy to identify new indications for approved or investigational drugs that are outside the scope of the original medical indication. It is particularly relevant for individuals with rare diseases for who limited treatments are available due to the economic and technical complexities of drug development for small populations.

A prerequisite for drug repurposing, is that the exploited assay is robust as well as clinically relevant. CFTR function measurements in patient-derived intestinal organoids associate with clinical features of cystic fibrosis and may enable drug repurposing in a personalized setting. We upscaled the previously described forskolin induced swelling (FIS) assay to a 384 WP format, allowing medium-to-high throughput assays. We exploited patient-derived organoid lines with one of the most prevalent nonsense mutations, W1282X/W1282X, to screen a library of FDA-approved compounds. We first characterized toxicity of 1443 FDA-approved compounds, after which we assessed the effect of the non-toxic compounds on CFTR mediated fluid secretion. We show that the 384-wells FIS-assay is reproducible, has a large wide dynamic range and comparable responses were obtained as with the conventional 96-well based setup. Similarly to CFTR function, compound-induced toxicity was quantified in a robust way and 43-1440 were excluded for future experiments. Results of the drug repurposing FIS assay identified 19 potential inducers of organoid fluid secretion. The top 5 hits were further characterized in a secondary screen and identified members of the statin family as compounds that can rescue CFTR function. Statins are commonly used medicines to lower blood pressure by inhibiting HMG CoA reductase and have not elaborately been described to affect CFTR function. Additionally, they are reported as inhibitors of AKT. We are currently performing experiments to further clarify the mode of action of statin family members in the aspect of increase of CFTR function in PTC organoid lines.

In brief, our study exemplifies the feasibility of large-scale compound screening with a functional read-out using patient derived organoids. Furthermore we show a not earlier described interaction between CFTR and members of the statin family in patient-derived organoids harbouring nonsense mutations.

P12

S2.5 Identification of novel small molecule modulators for PTC mutations in CFTR

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Background: Premature termination codons (PTCs) are introduced into CFTR mRNA by nonsense mutations, which account for a significant proportion of total known CFTR variants (8.4%), and for which there are still no approved CFTR modulator therapies. The effect of such mutations is generally two-fold: *i)* the presence of the PTC triggers mRNA transcript degradation by nonsense-mediated decay (NMD) of the mRNA transcript [1]; and *ii)* any resulting translated protein is truncated and almost always non-functional. Such mutations are therefore associated with severe CF phenotypes, and there is thus an urgent need to find novel therapeutic strategies for individuals with CF with PTC mutations, which could include both PTC read-through agents and NMD inhibitors.

Aim: To identify novel small molecules to correct the defective processing of CFTR transcripts bearing PTCs, within the scope of the *PTSense* project.

Methods: A microscopy screen of compounds was performed in HEK Flp-in cells stably expressing a triple-tagged CFTR PTC mini-gene construct (mCherry G542X-Flag-CFTR-GFP cDNA with 3 introns between exons 13-15), including mCherry (red) at the N-terminus, eGFP (green) at the C-terminus, and a Flag-tag for detection of plasma membrane (PM) localization by immunofluorescence with anti-Flag antibody in unpermeabilized cells. In this microscopy assay, NMD suppression was assessed by increased red fluorescence from a basal level, and if PTC read-through occurred, enhanced green fluorescence was also detected. Two series of novel compounds (a total of 50 compounds) were synthesized[2][3] and tested alongside the SMG1i NMD inhibitor and the G418 aminoglycoside readthrough compound as positive controls.

Results: In total, among the 50 novel compounds tested, 2 enhanced PTC readthrough alone, 11 had an inhibitory effect on NMD alone, and 7 compounds had a dual effect inhibiting NMD and inducing read-through. The best 10 compounds are the subject of ongoing validation experiments in human intestinal organoids and other PTC cell models expressing a variety of CFTR genotypes.

Conclusion: We synthesized and identified a number of novel small molecules as potential PTC/read-through modulators in a unique cellular model of CFTR PTC mutations. The novel compounds promoted PTC readthrough and/or inhibition of the associated NMD. Ongoing studies are focused on their validation, their specificity and applicability to other PTC mutations in other cell models of CF.

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[1] Clarke LA, *et al* (2019) *Hum Mutat* **40**: 326-334

[2] Vieira P, *et al* (2020) *Chem Eur J* **26** : 888-899

[3] Moiteiro C, *et al* (2022) *Eur J Org Chem* Accepted Author Manuscript

P14

S2.6 Proof of concept of ionocytes' CFTR content as a novel biomarker for cystic fibrosis diagnosis and follow up

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Despite the huge progresses made in cystic fibrosis (CF) knowledge and care, the exact relationships between primary defects and different manifestations of the disease are still poorly understood. Newborn bloodspot screening (NBS) is an effective strategy for the early recognition of infants with CF. However, increasing number of infants with a positive NBS result have an inconclusive diagnosis (CRMS/CFSPID). Nevertheless, CF still is a diagnostic and therapeutic challenge in the case of rare variants that are associated with varying symptoms and are unlikely to enter clinical trials. Therefore, it is important to identify precise and disease-relevant biomarkers allowing a better understanding of CFTR mutations effects in vivo, and also useful as outcome parameters to accurately quantify the rescue of CFTR by novel modulators.

In this context, the recent identification of the CFTR-rich airways ionocytes could highlight novel possibilities to monitor CFTR in CF patients' airways. Despite their low quantity, ionocytes express the highest levels of CFTR per cell and are relatively abundant in the upper airways. These findings suggest that quantitative analysis of CFTR could be efficiently done in these cells, that can be collected by the minimal invasive nasal brushing procedure.

As a proof of concept, we investigated the genotype-phenotype-ionocytes' CFTR content relationships in a small cohort of non-CF, CF, and CRMS/CFSPID subjects. Moreover, we collected nasal cells from CF patients treated with ELX/TEZ/IVA (pre- and 30 days post-treatment). Ionocytes' CFTR content was evaluated by immunofluorescence detection of CFTR and FOXI1 (as a ionocytes marker) combined with confocal imaging and analysis of the intensity of the CFTR signal in the apical membrane relative to that in the intracellular compartments. In accordance with genotypephenotype, we found increasing levels of ionocytes' CFTR content in the following groups: CF patients with severe mutations < CF patients with mild mutation/CRMS/CFSPID < CF patients treated with ELX/TEZ/IVA < non-CF individuals. This finding suggests that this type of analysis could be useful to investigate genotype--phenotype correlation and, possibly, the efficacy of CFTR pharmacotherapies. In addition, analyzing CFTR activity in airways ionocytes will be an important future step to better clarify their role in airways epithelium.

31 March — 16:00–17:45

Symposium 3 – Rare mutations: molecular defects and protein correction

S3.1 CFTR modulation: insight from 3D structures

Ahmad Elbahnsi[1], Nesrine Baatallah[2], Jean-Paul Mornon[1], Alexandre Hinzpeter[2], Isabelle Callebaut[1]

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3D structure and dynamics provide an essential knowledge for understanding the molecular basis of perturbation by CF mutations and rescue by CF modulators. In particular, a comprehensive description at the molecular level of the possible binding sites for small molecules and analysis of the effect of their occupancy on the whole properties of the protein provide unvaluable support for designing better modulators, as well as novel ones for rare mutations which are not responsive to existing modulators.

Here, I will illustrate how the identification of corrector binding sites allows to understand the effect of such molecules on different types of class II mutations, and how this information leads in turn to increase our basic knowledge of the CFTR protein folding and function.

This work is supported by the French Association Vaincre La Mucoviscidose

S3.2 Perspectives on precision therapeutics for rare CF genotypes

Eric Sorscher

Emory University School of Medicine

Substantial progress has been made regarding personalized strategies for treatment of cystic fibrosis (CF). While highly effective modulator therapies are available to a sizeable majority of individuals with the disease, significant challenges to CF precision medicine remain. These include improving health of patients carrying variants not responsive to available compounds, cost and patient access to modulator treatment, molecular complexity involving refractory CFTR mutations, and addressing ultra-rare or private CFTR abnormalities not suited for conventional, double-blind placebo-controlled clinical analysis. In this presentation, we consider barriers to cystic fibrosis "personalized" therapy and limitations to drug access among individuals with rare forms of CF. We describe basic research directed towards predicting treatment efficacy, novel tools for mechanistic and therapeutic analysis, and use of high throughput phenomic technology to advance treatment for individuals otherwise at risk of being "left behind" during the current modulator era. In particular, we discuss use of iPSC-derived airway epithelia as a means to evaluate "theratype" in a manner that: 1) accounts for complex CFTR alleles, 2) incorporates native CFTR regulatory elements impacting mRNA expression, and 3) may be less influenced by epigenetic factors associated with chronic tissue infection or inflammation. An open-label clinical trial is enrolling subjects aged 12 and older diagnosed with CF-related pulmonary disease and evidence of partial CFTR function (sweat chloride < 80 mEq/L, pancreatic sufficiency), but without access to FDA-approved CFTR corrector or potentiator compounds. The clinical study also investigates ability of iPSC-based systems to identify patients most likely to benefit from modulator treatment.

S3.3 Rescue of mutant CFTR chloride channels by a mimetic peptide targeting the AKAP function of PI3Kgamma

Alessandra Ghigo[1,2], Alessandra Murabito[1], Valentina Sala[1,2], Anna Rita Pisano[3], Serena Bertolini[3], Ambra Gianotti[4], Emanuela Caci[4], Alessio Montresor[5], Aiswarya Premchandar[6], Flora Pirozzi[1,7], Kai Ren[1], Angela Della Sala[1], Marco Mergiotti[1], Wito Richter[8], Eyleen de Poel[9], Michaela Matthey[10], Sara Caldrer[5], Rosa A Cardone[11], Federica Civiletti[1], Andrea Costamagna[1], Nancy L. Quinney[12], Cosmin Butnarasu[1], Sonja Visentin[1], Maria Rosaria Ruggiero[1], Simona Baroni[1], Simonetta Geninatti-Crich[1], Carlo G. Tocchetti[7], Marco Conti[13], Paola Melotti[14], Claudio Sorio[5], Virgina De Rose[1], Fabrizio Facchinetti[3], Vito Fanelli[1], Daniela Wenzel[10,15], Bernd K. Fleischmann[15], Marcus A. Mall[16], Jeffrey Beekman[9], Carlo Laudanna[5], Martina Gentzsch[12], Gergely L. Lukacs[6], Nicoletta Pedemonte[4], Emilio Hirsch[1,2]

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Background and rationale: Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride (Cl^-) channel whose dysfunction leads to airway mucus obstruction, inflammation and, ultimately, respiratory failure. For patients carrying the most common F508del-CFTR mutation in at least one allele, FDA approved elexacaftor/tezacaftor/ivacaftor (ETI), a combination therapy able to restore both the folding and the Cl^- secretion of the mutant channel. Despite being highly effective in improving lung function, these modulators rescue the activity of F508del-CFTR only up to 60% of physiological values, underlying the need of new therapeutic approaches.

Hypothesis and objectives: We previously identified phosphoinositide 3-kinase γ (PI3Kγ) as a PKA-anchoring protein that tethers PKA to the cAMP hydrolysing enzymes, PDE3 and PDE4, favouring their PKA-mediated activation and in turn cAMP reduction. Here, we hypothesize that targeting this function of PI3Kγ could be exploited to achieve therapeutic cAMP elevation in the airways triggering bronchodilation, reduced inflammation and CFTR modulation.

Materials and methods: We explored the ability of a cell-permeable mimetic peptide, namely PI3Kγ MP (WO/2016/103176), targeting the PKA-anchoring activity of PI3Kγ to function as a (i) bronchodilator, (ii) anti-inflammatory agent and (iii) CFTR modulator. A mouse model of chronic lung inflammation was used to assess the bronchodilator and anti-inflammatory effects of PI3Kγ MP. Biochemical assays were performed in immortalized cell lines (HEK293T, 16HBE14o- and CFBE41o-), while CFTR activity was measured in Ussing chamber in primary cells derived from healthy or CF subjects.

Results: Intra-tracheal instillation of PI3Kγ MP in mice induced a significant and dose-dependent increase in cAMP in both tracheas and lungs, which persisted up to 24 hours without affecting cAMP in other organs. Furthermore, in a mouse model of chronic lung inflammation, PI3Kγ MP limited methacholine-induced airway hyper-responsiveness and reduced infiltration of neutrophils, the major leukocyte population responsible for lung structural damage in CF. Notably, in bronchial epithelial cells the peptide induced a compartmentalized cAMP elevation in the proximity of the CFTR, triggering PKA-mediated phosphorylation of the activating residue S737, correlating with a dose-dependent increase in Cl^- secretion in primary bronchial epithelia cells of healthy subjects. Intriguingly, the peptide retained the ability to partially increase short circuit currents even in the presence of a CFTR inhibitor, suggesting the ability of the compound to trigger Cl- secretion not only through a direct action on the CFTR, but also indirectly, by activating channels that enhance the electrochemical driving force. Finally, in primary bronchial epithelial cells from F508del patients, PI3Kγ MP doubled the effects of gold standard CFTR modulators, like ETI, on Cl^- secretion.

Conclusions: These results unveil PI3Kg as the orchestrator of a β_2-AR/cAMP microdomain central to smooth muscle contraction, immune cell activation and epithelial fluid secretion in the airways, eventually indicating the use of an inhaled PI3Kg MP for compartment-restricted, therapeutic cAMP elevation in CF and other chronic obstructive airway diseases.

This work was supported by grants from Italian Cystic Fibrosis Research Foundation (FFC#25/2014, 23/2015, 8/2018, 4/2016 and 11/2017) and Telethon Foundation (GGP20079).

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S3.4 Structural plasticity of the Nucleotide Binding Domain 1 (NBD1) of CFTR is linked to pathogenesis of cystic fibrosis

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Cystic fibrosis (CF) is the most common lethal genetic disease in Western countries. It is due to mutations in the gene coding for the cystic fibrosis transmembrane regulator (CFTR), a chloride channel required for proper fluid balance in many tissues (lungs, digestive organs, etc.). In over 80% of the CF cases, the disease-causing mutation is the deletion of the phenylalanine 508 (F508del), located in the nucleotide binding domain 1 (NBD1) [1]. In our recent published study, a novel conformation of NBD1 (called the b-SS conformation) was captured using a conformation-specific nanobody (an antibody fragment recognizing specific conformations). This b-SS conformation and the canonical conformation of NBD1 (the conformation previously described in the literature) are ruled by an equilibrium tightly correlated to stability and function (destabilizing conditions such as F508del mutation favour the b-SS conformation). This study indicated that the transition between the conformations is an Achille's heel in the structural landscape of the protein and enables protein unfolding by the prevalent pathological mutation F508del [2].

The present research focused on investigating if this correlation between plasticity and pathology is unique for F508del or also observed for other disease-causing mutations of NBD1 as this observation could lead to the development of therapies common to several untreated NBD1 mutations.

To measure if rare mutants of NBD1 shifted the equilibrium towards the b-SS states, we followed the conformational landscape of NBD1 by single molecule fluorescence (smFRET), specifically the transitions between the b-SS and the canonical conformation. In addition, we used Thermal Shift Assay (TSA) to test the thermostability of those mutants with different conditions known to affect the equilibrium and stability of F508del such as ATP, mutations, or a promising stabilizing nanobody. Finally, we crystalised some mutants to look for any structural changes.

We show that all mutations tested diminished the canonical population in FRET measurements. TSA analysis showed that all the mutants are thermally destabilized and can be stabilized with the same conditions as for F508del. Using a series of DRI variants we showed by X-ray crystallography that the mutations do not prevent folding of the domain. Therefore, we conclude that, mutations studied in NBD1, enable the premature unfolding of the protein not by disrupting structural elements but rather by perturbing structural transitions between the canonical and the b-SS states. This work provides a new framework to design conformation-based therapeutics for several mutations lacking any treatment so far.

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S3.5 Characterization of the [1898+3A>G;186-13C>G] complex allele by means of patient-derived nasal epithelial cells: molecular and functional analysis of CFTR mRNA and protein

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Cystic fibrosis (CF) is due to loss of function mutations of the CFTR gene. More than 2000 variants have been identified, of which 382 have been clearly defined as CF causing alleles with F508del as the most common. CF mutations can be grouped into seven classes according to the functional defect they cause. Small molecules called correctors and potentiators (CFTR modulators) have been developed to rescue the basic functional defect(s) of different variants. However, many CF patients carry poorly characterized variants (in Italy about 30%), whose response to drug treatment is yet to be defined. Moreover, the existence of complex alleles, with the presence of more than a variant in cis may significantly affect the behaviour of mutated protein thus impairing or modifying the effect of modulators.

In this work, we present preliminary data regarding a patient recruited within the frame of a wide research project aimed at defining/providing a therapeutic approach for cystic fibrosis patients with rare and poorly characterized variants.

The patient is compound heterozygous for the CFTRdele1 mutation and a complex allele [1898+3A>G;186-13C>G]. CFTRdele1 is a well-known CF-causing allele; the 1898+3A>G substitution is already reported as a splicing mutation that may generate a defective protein. However, the variant has never been studied in ex-vivo models and its possible response to CFTR modulators, never investigated in disease-relevant cells such as nasal epithelial cells derived from patients. The second 186-13C>G substitution maps in the first intron, close to the acceptor splicing site of exon 2, and is indicated as a VUS4.

Patient's cells from nasal brushing were cultured, expanded and reprogrammed to generate differentiated epithelia. Then CFTR function was evaluated in Ussing chamber upon treatment with different CFTR modulators. Cells were then harvested for RNA extraction to allow study of the CFTR mRNA.

Short-circuit current analysis highlighted a nearly absent CFTR activity under basal condition and a very small, but significant, CFTR function rescue in patient's epithelia treated with VX-661/VX-445/VX-770 combination. CFTRdele 1 is expected to behave as a null-allele, therefore we supposed that the obtained results were attributable to the protein encoded by the complex allele, which apparently could not be rescued up to obtain an acceptable level of functional correction. Further experiments to complete this characterization are presently ongoing. We are carrying on the qualitative analysis of CFTR mRNA obtained by patient's epithelia, to define the composition of the detectable transcripts, thus inform about the structure of the resulting mutated protein that will be further characterized in heterologous models. Moreover, we are also applying a minigene approach to isolate and study the intron 1 variant, to define its impact, if any, on CFTR mRNA splicing.

This work was supported by the Italian Cystic Fibrosis Foundation (grants FFC #9/2019 and FFC #10/2021), by Cystic Fibrosis Foundation (grant PEDEMO20G0) and by the Italian Ministry of Health (grant GR-2018-12367126). The authors thank the DINOGMI, Department of Excellence of MIUR 2018-2022 (legge 232 del 2016) for general support.

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S3.6 Rescue of rare CFTR trafficking mutants highlights a structural location-dependent pattern for correction

Sónia Zacarias, Carlos M. Farinha

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Among the more than 2,100 variants in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, more than 1,000 are very rare being present in less than 5 individuals worldwide. Approval of modulators that act on mutant CFTR protein, correcting its molecular defect thus alleviating disease burden, revolutionized the CF field. However, these drugs do not apply to all CF patients, especially those harboring rare mutations -- for which there is a lack of knowledge on its molecular mechanisms of disease and response to modulators.

Our aim here was to assess the impact of several rare putative class II mutations on CFTR expression, processing and response to modulators. Furthermore, we aimed at analyzing if mutation location would somehow influence/predict the response to the correctors.

We virally transduced the CFBE41o- parental cell line [1] to generate novel cell lines with heterologous expression of CFTR bearing the mutation of interest (generated by in vitro mutagenesis), as before [2]. We analysed a total of 11 mutations located at either Transmembrane Domain 1 (TMD1) - R75G, H139R, I148T, D192G, G194R, H199Y, V201M, and W361R - or very close to the signature motif of Nucleotide Binding Domain 1 (NBD1) - A550T, L558S, and L571S. Expression and processing were assessed by Western Blot, with or without treatment with the double corrector combination VX-661/VX-445. Mutations were also mapped to the human CFTR structure determined by cryoEM [3], grouped them as responders or non-responders to the modulators.

Results show that, for the mutations analysed, all of them impair CFTR processing (lack or severe reduction of band C -- mature form), confirming them as class II. Furthermore, considering their localization in CFTR structure, mutations located in the TMD1 respond to modulators, particularly to the combination VX-661 and VX-445, whereas those localized in NBD1 do not respond.

As we are increasing the number of variants analyzed, our study confirms the relevance of assessing the response of rare mutations to modulators (in a personalized medicine approach) and suggest the existence of a location-dependent pattern of response to modulators that may contribute to further clarify the mechanism of action of correctors.

Acknowledgments: Work supported by UIDB/04046/2020 and UIDP/04046/2020 centre grants from FCT, Portugal (to BioISI) and DysMut2 grant (Ref. FARINH19I0) from CFF (USA).

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01 April — 08:45–10:30

Symposium 4 – CFTR folding and trafficking

S4.1 Requirements for CFTR folding and transport

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Programme note: the details are not authorised for inclusion

S4.2 CFTR's Site 1: Degenerate but not disabled

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CFTR, an ATP-gated chloride channel, inherits all the structural motifs in a prototypical exporter member of the ABC Transporter Superfamily: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) assembled in a pseudo two-fold symmetry of a TMD1/NBD1-TMD2/NBD2 complex. Unique in CFTR is a disordered R (regulatory) domain wedged in between the two halves of the whole molecule. Phosphorylation of the R domain is a prerequisite for ATP to serve as a ligand to open the gate of CFTR through dimerization of the two NBDs with two ATP molecules sandwiched at the NBD dimer interface. Biochemical studies and sequence analyses indicate that only site 2 (composed of the head subdomain of NBD2 and the tail subdomain of NBD1) can hydrolyze ATP, whereas site 1 is degenerate (or catalysis-incompetent). Unlike traditional ligand-gated channels, the ATP at site 2 is hydrolyzed and the free energy hence harvested powers the separation of the two NBDs and subsequent closure of the channel. This sole action of ATP binding/hydrolysis at site 2 in controlling opening/closing of CFTR negates any role of the ATP bound at the degenerate site 1. In this presentation, we will summarize previous studies and present unpublished data to support an active role of site 1 in controlling both opening and closing processes. Using a hydrolyzable high-affinity ATP analog (N^6-phenylethyl ATP, P-ATP), we showed that P-ATP increases the opening rate and decreases the closing rate of wildtype CFTR with two distinct EC50, suggesting an involvement of two different ATP binding sites in CFTR gating: besides a known low-affinity site 2 controlling gate opening, the high-affinity site 1 can modulate gate closure triggered by ATP hydrolysis at site 2. This idea of the presence of a main gating site 2 and a modulating site 1 is further supported by the observation that ADP not only competitively inhibits channel opening, but also accelerates channel closing. This effect of site 1 on gate closure may be directly attributed to the tight binding of ATP at site 1 to stabilize the NBD dimer structure. Indeed, in hydrolysis-deficient backgrounds (e.g., K1250A or E1371S), P-ATP can slow gate closure whereas mutations that destabilize site 1 ATP binding expedite channel closure. However, using 2'-deoxy-ATP (dATP) as a tool, we found that this hydrolyzable ATP analog affects CFTR gating through a long distance effect: when site 2 is occupied by ATP or dATP, binding of dATP at site 1 drastically slows down channel closing, in contrast to ATP at site 1 which allows speedy closure of the channel. These unpublished results thus suggest that the structural changes at site 1 by dATP can alter the catalysis function of site 2. Moreover, our latest publication showed that a depletion of ATP at site 1 could dramatically perturb gate opening by ATP binding at site 2 possibly due to a complete separation of the two NBDs upon nucleotide dissociation from both sites. We therefore conclude that site 1, despite its defective catalysis, is evolved to play an active role in CFTR function.

S4.3 Cif: a therapeutic target in persistent airway infections

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The era of highly effective modulator therapies (HEMTs) requires a comprehensive reassessment of therapeutic strategies for people with CF. While HEMTs have provided substantial improvements in lung function and reductions in pulmonary exacerbations, a surprising observation in follow-up studies is the resilience of chronic airway infections in many patients. Several factors likely contribute to the persistence of infections, including heterogeneity of bacterial populations and adaptive virulence strategies. One of these strategies utilized by *Pseudomonas aeruginosa* involves the sabotage of corrector drugs and the concomitant dysregulation of immune regulatory signals, leading to an on-going hyperinflammatory response.

Cif (CFTR Inhibitory Factor) inhibits the post-endocytic deubiquitination of mature CFTR, accelerating its degradation and reducing CFTR abundance at the apical membrane. This effect is mediated by inhibition of the deubiquitinase USP10 through an interaction with G3BP1, an RNA-binding protein involved in stress-granule formation. New data suggest that the USP10:G3BP1 interaction occurs in the context of droplets formed by liquid-liquid phase separation (LLPS). The interaction, based on recognition of peptide motifs, may sequester USP10 from interaction with CFTR and may also involve competition with Caprin-1, adding complexity to efforts to protect or restore post-endocytic recycling of CFTR. Cif also hydrolyzes 14,15-EET, an epoxidated polyunsaturated fatty acid (epoxy-PUFA) that initiates pro-resolving signals in the airway. High levels of Cif are thus associated with elevated IL-8 levels in patient samples and *in vivo* infection models. Finally, Cif triggers the degradation of the transporter associated with antigen processing (TAP)-1. Taken together, these data confirm that Cif can mediate wide-ranging effects on the host immune system. Homologs of Cif are expressed in *Acinetobacter nosocomialis*, *Burkholderia cenocepacia*, and *Inquinulus limosus*, suggesting that this approach may be shared among opportunistic pathogens.

Cif is a secreted dimeric a/b epoxide hydrolase virulence factor that catalyzes the conversion of epoxy-PUFAs, including 14,15-EET, to cognate vicinal diols. This enzyme activity is strictly required for Cif's virulence activities. Structurally conservative active-site mutations block Cif's ability to accelerate degradation of CFTR or to dysregulate immune responses, and we are therefore interested in understanding and targeting Cif's enzyme activity. Proof-of-concept inhibitor screens have identified candidate small molecules that prevent virulence effects *in vitro*. However, conformational cross-talk between substrate and inhibitor binding sites can complicate the development of drugs. We have recently identified a panel of Cif-specific camelid nanobodies that can detect Cif expression and facilitate screening for Cif inhibitors. Crystallographic structures of Cif:nanobody complexes may help us to understand the conformational basis for cooperative and allosteric ligand interactions and thus to develop more effective inhibitors. Overall, a deeper mechanistic understanding of Cif's effects on host immune responses may lead to therapeutic strategies to reverse its pernicious virulence effects and thus help to address chronic post-HEMT airway infections in CF.

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S4.4 Characterization of corrector ARN23765 mechanism of action via Photo-Affinity Labeling (PAL) approach

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Cystic Fibrosis (CF) is a rare genetic disease characterized by deficiencies in the synthesis or function of the CF transmembrane conductance regulator (CFTR) anion channel, caused by mutations in the *CFTR* gene. Small-molecule compounds addressing the basic defect of the disease have been described, and are referred to as CFTR modulators. Among these, *ARN23765*, a potent F508del-CFTR corrector discovered by our group, showed high potency in rescuing the function of mutant CFTR in primary human bronchial epithelial cells from F508del/F508del CF patients.[1] CFTR correctors can act either directly by binding to CFTR or by interacting with the protein machinery responsible for protein synthesis and maturation. Indications of correctors binding to CFTR come from either indirect proofs or by experiments with purified wild type (wt) full-length protein or single domains.[2-3] No data are so far available disclosing the interaction of modulators with CFTR (either wt- or F508del mutant) in a native cellular environment.

We envisioned the *Photo-Affinity Labeling (PAL)*[4-6] approach as a suited methodology to investigate the target(s) of CFTR correctors in living cells. In this technology, a photo-affinity probe (PAP) is synthesized by incorporating in the structure of the compound under evaluation a small photo-reactive moiety and a reporter/purification tag or a chemical handle suitable for conjugation to such a tag.[7]

The photo-reactive moieties, inert under standard chemical and biological conditions, can be activated by UV light to generate highly reactive transient chemical species that crosslink in a covalent manner to bio-molecules in close proximity.[5] Probe photo-crosslinked targets can be identified with different approaches, including electrophoresis experiments, western blot and mass spectrometry studies.

In this work, we synthesized several *ARN23765*-derived PAPs and, after a preliminary evaluation of their activity in rescuing F508del-CFTR function, we used them in both cell lysates and intact cells demonstrating the binding of one of our corrector probes to wild type and mutant F508del-CFTR in living CFBE41o- cells. To the best of our knowledge, our study is the first to disclose the interaction of a corrector probe to wild type and mutant F508del-CFTR in an integral cellular setting.

This work was supported by the Italian Foundation for Cystic Fibrosis (FFC) as part of the "FFC#4-2020" project.

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S4.5 New kinase therapeutic targets for cystic fibrosis from a global functional genomics screen

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Introduction: The pharmacological management of Cystic Fibrosis (CF) has dramatically changed over the last years with the introduction of CFTR-targeting modulators. Rescue of the most common CFTR mutant (F508del) requires addressing not only its folding, traffic and stability defects, but also channel gating. In a clinical setting, these molecular detects are addressed with the combination of corrector (lumacaftor/VX-809, tezacaftor/VX-661, elexacaftor/VX-445) and potentiator (ivacaftor/VX-770) molecules. Nevertheless, these drugs do not restore clinical efficacy endpoints of CF individuals to carrier levels. We hypothesize that functional F508del-CFTR expression at the epithelial apical plasma membrane (PM) is hampered by still unknown proteins which retain it at the endoplasmic reticulum (ER). These retention factors are potential novel drug targets for CF.

Objective: To identify proteins retaining F508del-CFTR in the ER through a high-content siRNA-based microscopy screen.

Methods: A microscopy-based F508del-CFTR traffic assay [1] was used to screen Ambion's Extended Druggable Genome siRNA library (27,312 siRNAs targeting 9,128 human genes). The efficacy of the genetic knock-down (KD) of hit genes (alone or in combination with correctors) was validated by a secondary (validation) screen and by additional assays: Western blot (WB), halide-sensitive YFP (HS-YFP) quenching, forskolin-induced swelling (FIS) in intestinal organoids, Ussing chamber and patch-clamp. Gene expression was determined with RT-PCR.

Results: The primary siRNA screen pinpointed 227 genes whose KD significantly increased F508del-CFTR PM levels, of which 35 were confirmed by re-screening with stringent criteria. Knocking down 33 of the 35 hit genes significantly increased F508del-CFTR post-ER maturation, as seen in WB assays. We selected a set of 5 kinase hit genes for additional validation and mechanistic analyses. Functional F508del-CFTR rescue could be obtained by KD several of the kinase genes using multiple cellular and biochemical assays: WB to monitor CFTR maturation, patch-clamp, Ussing chamber and HS-YFP quenching to quantify ion transport, and FIS to measure ion and fluid transport in primary intestinal organoids. Rescue by kinases KD was often additive to VX-809, VX-661 and VX-445, indicating that gene KD and chemical correctors may be combined for maximal CFTR rescue. Hit kinases are not part of the CFTR interactome and have not been reported as F508del-CFTR traffic regulators before. Moreover, our data also show robust F508del-CFTR rescue through chemical inhibition of one of the kinases (1 μM) regarding both PM traffic and function. Rescue could be further increased by simultaneously adding VX-661 or VX-445.

Conclusion: Through a high-content functional genomics screen, we identified a kinase which can be genetically or pharmacologically inhibited to rescue F508del-CFTR PM traffic and function, thus emerging as a novel attractive potential novel drug target for CF.

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S4.6 Rescue F508del-CFTR with nanobodies

Marie Overtus[1], Marjolein Ensinck[2], Rafael Colomer Martinez[1], Yiting Wang[3], Luise Franz[4], Christian Hackenberger[4], Marianne Carlon[2], David Sheppard[3], Cédric Govaerts[1]

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F508del mutation causes thermal destabilization and misfolding of CFTR that leads to its degradation and subsequent lack of cell-surface expression. The approved modulators, even the most recent Trikafta, do not improve thermal stability of CFTR and show limited effect on pulmonary function. Obviously new therapies that address the molecular origin of the pathology are required. In this context, we have developed a collection of nanobodies against NBD1 and identified a serie of high affinity binders capable to thermally stabilize full-length CFTR. These nanobodies have been extensively characterized from a biochemical and biophysical standpoint and their binding mode to NBD1 was resolved by X-ray crystallography. Single-channel patch-clamp experiments not only demonstrate that binding of stabilizing nanobodies to CFTR is compatible with channel activity but moreover that the presence of the nanobodies stabilizes F508del-CFTR over time, leading to a dramatically sustained function. When transfected in HEK293 cells, our stabilizing nanobodies promote maturation (detected by Western blot) and cell-surface expression of F508del-CFTR (detected by flow cytometry and fluorescence microscopy). This effect is highly synergistic with that of approved correctors (such as VX809, VX661 and VX445), indicating that their modes of correction are different. Using the Halide-Sensitive-YFP assay, we have investigated whether this increase in protein rescue leads to functional improvement. As for protein expression, the presence of stabilizing nanobodies leads to recovery of F508del-CFTR activity and this response is synergistic when cells are treated with approved correctors, leading to wt-like behavior. The functional effect of nanobodies was subsequently tested in a clinically-relevant model, patient-derived organoids. Using lentiviral-transduction of organoids we evaluated the effect of the nanobody expression on the recovery of CFTR activity using the forskolin-induced swelling assay and observed a robust recovery of channel function. The combination of corrector and nanobodies show a sustained recovery that far excelled the effect of the corrector or the nanobodies alone. This indicates that NBD1-stabilizing nanobodies may provide a promising route to improve the current CF drugs and reach normal-like level of CFTR activity. However, in order to rescue F508del-CFTR during biogenesis, our NBD1 nanobodies must act intracellularly thus requiring the development of methods to deliver nanobodies inside the cells. Several strategies will be followed in parallel. The first approach will make use modified nanobodies with cell-penetrating peptides (CPP) developed to allow direct protein delivery inside the cytoplasm. In a second approach the nanobodies will be encapsulated in liposomes by using an innovative microfluidic technique. In therapeutic perspective, CPP-bound and encapsulated nanobodies could be directly delivered to the lung by nebulization, a method that has been successfully used for nanobodies developed for different pulmonary pathologies. Our work will provide novel therapeutic routes to treat cystic fibrosis.

01 April — 11:00–12:45

Symposium 5 – CFTR: beyond the airway

S5.1 CFTR: a new horizon in pancreatitis

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It is well documented that mutations in the CFTR gene diminish the ion channel function and lead to impaired epithelial fluid transport in multiple organs such as the lung and the pancreas resulting in cystic fibrosis. Heterozygous carriers of CFTR mutations do not develop cystic fibrosis but exhibit increased risk for pancreatitis and associated pancreatic damage characterized by elevated mucus levels, fibrosis, and cyst formation. Importantly, recent studies demonstrated that pancreatitis causing insults, such as alcohol, smoking, or bile acids, strongly inhibit CFTR function. Furthermore, human studies showed reduced levels of CFTR expression and function in all forms of pancreatitis. These findings indicate that impairment of CFTR is critical in the development of pancreatitis; therefore, correcting CFTR function could be the first specific therapy in pancreatitis. In this presentation, I summarize recent advances in the field and introduce new possibilities for the treatment of pancreatitis.

S5.2 The “CF gut”, its abnormalities directly and indirectly related to CFTR dysfunction, and strategies to improve gut fluidity and alkalinity in the CF gut beyond CFTR rescue

Ursula Seidler

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The intestinal epithelium expresses the CFTR with high abundance and preferential expression in the stem cells, nondifferentiated progenitor cells, the villus-located small intestinal "high CFTR expressor" cells, to some extent in the differentiated goblet cells, and very little in the absorptive cells. The loss of CFTR function in these cells causes defective opening of the CFTR anion channels, leading to decreased cryptal fluid and alkaline secretion, and an increase in intracellular Cl^- concentration and pH, which are linked to a plethora of dysfunctions, including epithelial hyperproliferation and mucin synthetic alterations and secretory defects. The manifestations of the "CF gut" include delayed intestinal transit, dysbiosis, inflammation and intestinal obstructive episodes. Pancreatic dysfunction (not present in CFTR-deficient mice) may both alleviate constipation because malabsorption and steathorrea result in osmotic fluid retention, but may aggravate obstructive episodes because of insufficient protein (including endogenous mucus) digestion.

While susceptible CF patients may experience benefits from corrector and potentiator therapy, a group of patients will not respond, and the incidence DIOS-related surgeries is increasing despite the broad use of these therapies in developed countries. Because gut luminal fluidity and alkalinity is achieved by a balance of electrolyte and fluid absorptive and secretory processes, pharmacological strategies have been searched for to either stimulate anion and fluid secretion by activating alternative apical anion conductances (which have not met with success in the intestine), or to inhibit electrolyte and fluid absorptive processes. Since the CFTR-deleted or the F508del mutant mouse shows all features of intestinal CF-related disease, but does not develop pancreatic and pulmonary insufficiency, it is considered a good animal model to study pharmacological strategies to prevent obstructive episodes and promote gut health. CFTR null mice continuously receive PEG-containing electrolyte (often containing high HCO_3^- concentrations) drinking fluids to prevent the otherwise very high death rate by intestinal obstruction. A previous study showed that replacing the PEG drinking fluid by the anti-inflammatory drug talniflumate, which inhibits both TMEM16a channels and the apical Cl^- (and fluid) absorptive anion exchanger Slc26a3 (DRA), decreased obstructive episodes and increased survival. We tested the effect of three intestine-specific drugs that are currently approved for the treatment of constipation-prone irritable bowel disease, namely the guanylate cyclase C agonist linaclotide, the selective NHE3 inhibitor tenapanor, and the prostaglandin E1 analogue lubiprostone, for their ability to increase luminal fluidity and alkalinity during single pass perfusion of a jejunal and colonic segments in anesthetized, acid/base- and blood pressure-controlled CFTR KO, F508del mutant, and WT mice. Net fluid balance before and during drug application was assessed gravimetrically, and the net alkaline output into the perfusate was determined by pH-stat backtitration. While all three drugs were able to decrease fluid absorption and increase alkaline output in the jejunum, only the latter two drugs also did so in the colon. We are currently performing three week oral gavage experiments to determine the ability of these drugs to prevent obstruction and to study their effect on the hallmarks of the CF gut. The first results will be presented at the meeting.

S5.3 Physiology and pathology of CFTR in the kidney

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CFTR is an epithelial Cl^- channel that is expressed in the human kidney. For long scientists tried to identify the renal function of CFTR and searched for renal defects related to cystic fibrosis (CF) and CFTR. An early study suggested abnormal fractional Na^+ absorption via the amiloride-sensitive sodium channel ENaC in F508del-CFTR mice under salt restriction (1). Bretscher et al (2) detected decreased fractional Na^+ excretion in CF patients with administration of the hormone secretin. Even more impressive, HCO_3^- excretion was reduced in CF patients, a finding that triggered subsequent studies (3, 4). A detailed analysis in mice with knockout of CFTR or the HCO_3^- transporter SLC26A4 (pendrin) demonstrated the underlying mechanism: CFTR serves as a Cl^- recycling channel that drives urinary HCO_3^- excretion by SLC26A4 in ß-intercalated cells of the renal collecting duct (4, 5). In addition, HCO_3^- may be excreted into the urine directly through CFTR channels. Because CFTR is not functional in cystic fibrosis, HCO_3^- is not adequately excreted when plasma HCO_3^- or secretin levels increase. This leads to metabolic alkalosis occasionally observed in CF patients. Excitingly, the team by Leipziger and coworkers developed a simple drinking test to assess the function of CFTR *in vivo*, which demonstrated the efficacy of CFTR-correctors in patients with CF (4). Because in ß-intercalated cells CFTR is coexpressed with the Ca^{2+} activated Cl^- channel TMEM16A that is required for CFTR to operate properly (6), one may speculate that volunteers currently treated in a phase one clinical trial with the TMEM16A-activator ETD002 may present enhanced urinary HCO_3^- excretion. Defective renal HCO_3^- excretion in CF may even lead to suppressed alveolar ventilation (7), which could be a factor contributing to CF lung disease.

Expression of CFTR has also been detected in renal proximal tubules and was implicated in the regulation of protein reabsorption by receptor mediated endocytosis (8). Dysfunctional CFTR was proposed to lead to reduced acidification of endosomes, thereby leading to low molecular weight proteinuria. Notably, loss of function of CFTR-interacting TMEM16A in TMEM16A knockout mice also caused a lack of endosomal acidification and proteinuria (9). Finally, CFTR was claimed to play a major role as a secretory Cl^- channel in autosomal dominant polycystic kidney disease, a very common severe renal disease (10). However, we recently identified TMEM16A as the essential Cl^- channel in ADPKD (11). While CFTR was not required for cyst formation in mice, knockout or inhibition of TMEM16A almost abolished cysts growth in ADPKD *in vivo* (12). Collectively, these studies demonstrate the importance of CFTR and TMEM16A in renal disease and cystic fibrosis.

1.Kibble et al. 2000;526:27-34;

2.Bretscher et al. 1974;8:899 (Abstract);

3.Kunzelmann et al. 2017;16:653-62;

4.Berg et al. 2020;31:1711-27;

5.Berg et al. 2021;231:e13591;

6.Park et al. 2020;58:247-53;

7.Javaheri and Kazemi. 1987;136:1011-6;

8.Jouret et al. 2007;18:707-18;

9.Faria et al. *Kindey Int.* 2014;85:1369-81;

10.Hanaoka et al. 1996;270:C389-C99;

11.Cabrita et al. 2020;11:4320;

12.Talbi et al. 2021;35:e21897.

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S5.4 CFTR activity is determined by the store-independent activation of SPCA2/STIM1/ORAI1 complex in secretory epithelial cells

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Introduction: Cystic fibrosis transmembrane conductance regulator (CFTR) regulates the composition of bodily fluids secreted by epithelial cells. For CFTR activation the cAMP/PKA signaling is crucial. However, several studies suggest that the intracellular Ca^{2+} signaling, which strongly depends on the store operated Ca^{2+} entry (SOCE) in epithelial cells, is also able to alter the activity of CFTR through an unknown mechanism.

Therefore, we aimed to clarify the molecular background of Ca^{2+} signaling-dependent CFTR regulation in primary, polarized epithelial cells.

Methods: Mouse and human pancreas, liver and airway organoid cultures and isolated pancreatic ductal fragments were used for RNA-seq, immunostaining and fluorescent Cl^-, Ca^{2+} and fluid secretion measurements. Protein-protein interactions were investigated by direct stochastic optical reconstruction microscopy (dSTORM) and cluster analysis while cAMP signaling was investigated with FRET.

Results: We demonstrated that the plasma membrane (PM) Ca^{2+} entry channel Orai1, which is activated during SOCE, mediates constitutively active extracellular Ca^{2+} entry in primary polarized epithelial cells (derived from the pancreas, liver and lung). This store independent activity is maintained by Secretory Pathway Ca^{2+}-ATPase 2 (SPCA2), which increases the Stim1-Orai1 interaction and Orai1 current in unstimulated cells. We found that Orai1 is co-expressed with CFTR in nanodomains of the apical PM and determines the activity of CFTR in epithelial cells from human and mice pancreas, liver and lung. Finally, we demonstrated that the regulation of CFTR by Orai1 involved Ca^{2+}/Calmodulin stimulated AC1, 3 and 8, which are located in the same protein nanodomain accompanied by CFTR and Orai1.

Conclusion: Our results suggest that the SPCA2 regulated, store-independent extracellular Ca^{2+} influx via Orai1 determines the activity CFTR in polarized epithelia, which is a novel form of regulation and have major physiological relevance.

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S5.5 Cystic fibrosis-related bone disease: CFTR class II mutations deregulate osteoclast formation and favor RANK+MCSFR+ circulating pre-osteoclasts

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Introduction: Cystic fibrosis-related bone disease (CFBD) is one of many comorbidities associated to CFTR mutations. CFBD leads to early decreased bone mineral density and increased fracture risk (Sermet-Gaudelus *et al.*, 2011). Bone resorption is under the control of osteoclast (cells able to resorb mineralized matrix) differentiated from circulating monocytes. We previously demonstrated an increased RANK+MCSFR+ circulating osteoclast precursors in G551D-CFTR patients (Velard *et al.*, 2018). In human CFTR-F508del primary human osteoclast culture, we showed a reduction in osteoclast number associated with a higher size and reduced resorption abilities (Jourdain *et al.*, 2021).

Purpose: Our aim is to identify putative impact of CFTR class II mutations on osteoclastogenesis.

Methods: Circulating pre-osteoclast monocytes were isolated from 16 CFTR class II mutations bearing patients (Reims Cystic Fibrosis Resource and Competence Centre - NCT04877223)(n=9 F508del homozygous, n=1 N1303K homozygous, n=3 F508del/class I mutation, n=3 F508del/class II mutation)(aged 19 to 51 years) and 13 healthy controls (EFS Grand Est - ALC/PIL/DIR/AJR/FO/606)(ages 22 to 39 years). PBMCs were labelled for membrane RANK and MCSFR receptors analysis on monocytes using flow cytometry. After 21 days of differentiation culture, osteoclasts were stained (Phalloïdine-AlexaFluor488®, Vinculine immunofluorescent staining, DAPI) to determine osteoclasts number per field, size and evaluate focal adhesion plate.

Results: We evidenced that class II mutations bearing patients exhibit increased double positive $RANK^+/MCSFR^+$ circulating monocytes compared to healthy donors (58 *vs* 28%, $p<0.05$). Osteoclasts exhibiting CFTR mutations (n=16) were 33% less numerous ($p<0.05$) and 104% larger ($p<0.05$) than healthy culture (n=13). No variation in osteoclasts shapes was demonstrated between healthy and CF cells, indicating a similar osteoclasts maturation. However, vinculin staining, evidencing the sealing zone in osteoclasts, was thinner and less co-located with actin ring in osteoclast bearing-class II CFTR mutation than healthy controls. None of the parameter studied was modified according to whether the patients bore a homozygous (n=9) or heterozygous (n=6) mutation ($p>0.05$ between CFTR homozygous and heterozygous patients).

Conclusion: In our study, we demonstrated an increased osteoclast differentiation potential in F508del-like patients by a rise of RANK+/MSCFR+ circulating osteoclasts precursors. Surprisingly, *in vitro*, we noticed a reduction in cell number and a higher size in culture for CF osteoclasts, without alteration of their shape. Also, F508del mutation could cause modification in cytoskeleton that decreased sealing zone formation and osteoclast function (Blangy *et al.*, 2020). These results could explain the lack of resorption activity observed in F508del-CFTR osteoclasts culture (Jourdain *et al.*, 2021), then causing dysregulation of bone homeostasis in CFBD-suffering patients.

Keywords: CFBD, F508del, Osteoclast, Monocytes

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S5.6 Integrative analysis of vascular impairment in models of cystic fibrosis

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Background: Cystic fibrosis (CF) is a multisystemic disease primordially affecting lungs but also associated with broad systemic complications. While CFTR is mainly studied in epithelial cells, it has an important (and overlooked) role in endothelial cells (ECs). Several evidences point out a vascular dysfunction in CF and link it to the development of pulmonary hypertension and CF-associated liver diseases (CFLD). However, the precise mechanism involving blood vessels in these life-threatening disorders is not fully understood.

Aims: We aimed to characterize, using meta-analysis, bulk and single cell RNA-sequencing (scRNA-seq), how CFTR-impairment impacts EC biology and could thus contribute to CF pathogenesis.

Material and methods: We used an unbiased transcriptomic approach in two complementary models of CFTR deficiency ($CFTR_{inh-172}$ & shRNAs) in primary human ECs. We extensively characterized and validated the changes in EC properties occurring upon CFTR dysfunction *in vitro*, *in vivo* & *ex vivo*. Additionally, we performed a meta-analysis to compare our results to CFTR-impaired epithelial models. Finally, we isolated ECs from human CF liver explants and performed scRNA-seq to capture endothelial heterogeneity and transcriptomic changes occurring at the single cell level.

Results: With this study we demonstrated that CF models of ECs display impaired proliferation, migration, mesenchymal state and autophagy. Remarkably, we also uncovered a profound pro-inflammatory state leading to increased leukocyte adhesion and extravasation. Finally, our scRNA-seq data revealed a distinct population of ECs, unique to samples from patients with CF, upregulating genes involved in coagulation and the complement cascade.

Conclusion: Our integrative data thus suggests that ECs are no innocent bystander in CF pathology, but rather may be central mediators of the exaggerated inflammatory phenotype. Besides, our work showed novel aspects of human liver ECs at single-cell level thereby supporting endothelial involvement in CFLD.

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02 April — 08:45–10:30

Symposium 6 – Restoring epithelial homeostasis

S6.1 Novel mechanisms of TGF-beta signaling in CF

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The transformative corrector/potentiator therapy, culminating decades of research, has been recently benefiting most CF patients. However, some biological effects of the most common disease-causing *CFTR* gene mutation are still incompletely understood. As an example, the role of Transforming Growth Factor (TGF)-β1 in the pathogenesis of CF lung disease have not been fully elucidated.

The *TGF-β1* gene is a modifier of the lung disease severity in patients homozygous for *F508del*. High TGF-β1 levels are seen in approximately 40% of these patients due to polymorphisms in the *TGF-β1* gene. Air pollutants, including cigarette smoke exposure also increase TGF-β1 levels. Higher TGF-β1 concentration in turn increases the severity of CF lung disease in patients. In addition to genetic variants, other factors increase TGF-β1 levels in *F508del* patients, including *P. aeruginosa* infection or poor nutrition. Independent of the underlying cause, high TGF-β1 levels strongly associate with poor outcomes and may compromise therapies aimed at boosting F508del-CFTR activity.

TGF-β1 inhibits ancillary ion channels, ANO1 (TMEM16A; Ca^{+2}-activated Cl^- channel) and BK (the large conductance, Ca^{+2}-activated, voltage-dependent K^+ channel) that remain functional in CF and hydrate the airway surface. Inhibition of ANO1 and BK may explain why TGF-β1 increases severity of CF lung disease.

TGF-β1 inhibits F508del-CFTR mRNA and blocks corrector-mediated rescue of the mutant CFTR channel function and compromises the ASL volume regulation in primary differentiated human bronchial epithelial cells. The effects are specifically observed in cells from CF lungs, compared to controls from lungs without known lung disease. TGF-β1 inhibits F508del-CFTR mRNA post-transcriptionally, by increasing degradation of CFTR mRNA. The beneficial effects of CFTR amplifier therapies affecting proteostasis lends support to the notion that CFTR correction is augmented by the available substrate. Thus TGF-β1 may represent a prevalent ASL inhibitor and an antagonist limiting residual and corrected CFTR activity.

TGF-β1 exerts its effects in the CF bronchial epithelium through multiple mechanisms, including regulation of micro(mi)RNA.s Specifically, TGF-β1 increases the active pool of selected miRNAs validated as CFTR inhibitors, recruiting them to the RNA-induced silencing complex (RISC). *F508del* mutation also globally modulates TGF-β1-induced changes in the miRNA landscape, creating a permissive environment required for degradation of F508del-CFTR mRNA.

Although our understanding of the role of TGF-β1 in CF airway disease has been growing, many aspects of this complex function remain to be answered, including how TGF-β1 modulates expression and function of SARS-CoV2 receptors in CF bronchial epithelium.

Our work has been supported by the National Institutes of Health R01HL144539.

S6.2 Investigating the therapeutic potential of phages as antibacterials and immunomodulators

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Pseudomonas aeruginosa is the main cause of chronic lung infections and function decline in individuals with cystic fibrosis (CF). The alarming diffusion of isolates of *P. aeruginosa* multi-resistant to the antibiotics currently in use makes urgently need to develop new antibacterial therapies. In this context phage therapy, the use of phages to kill bacteria, has gained renewed interest. This therapy has been used for decades in Eastern Europe to treat a variety of infections and thus it is considered generally safe. However, there are aspects such as the interaction among phages and host immune system, that deserve to be further studied to make phage therapy a realistic therapeutic option for curing infections in patients with CF. Therefore, we investigated the immunomodulatory effects of a cocktail made of four phages, previously tested for their efficacy in eradicating *P. aeruginosa* infection. We found that phages mitigate the hyper-inflammation presented by *CFTR* loss-of-function zebrafish embryos not infected by bacteria. This situation mimics the constitutive inflammation present in individuals with CF, even in the absence of bacterial infection. We are also investigating the mechanism through which phages act as anti-inflammatory agents using the *CFTR* loss-of-function zebrafish model and immortalized human bronchial epithelial cells homozygous for the *CFTR* mutation F508del. Our data will solve some of the still open questions about phage therapy, like what happens when phages get in touch with human cells or are injected into an animal model of CF and what can be done to make phage therapy effective and robust towards resistance onset. The research of new anti-inflammatory agents in a cheap and easy-of-use *CFTR* loss-of-function zebrafish model, together with the studies on the effects of phages on CF human cells could speed-up the translational potential of this research and the introduction of bacteriophages into clinics.

S6.3 Apical hydration protects the CF airway epithelium from *P. aeruginosa* by restoring junctional networks

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Airway epithelial homeostasis is dramatically altered in CF. CFTR dysfunction is associated with airway surface liquid (ASL) dehydration, chronic lung infection with *Pseudomonas aeruginosa*, excessive innate immune response and altered regeneration of the airway epithelium.

Several hypotheses, among which increased mucus clogging by neutrophil extracellular traps, reduced mucociliary clearance due to increased mucus viscosity, decreased sphingosine-dependent bacteria killing caused by ceramide accumulation or ASL acidification that inactivates antimicrobial peptides, have been reported to explain the links between CFTR dysfunction and the infection-prone airway phenotype.

We recently showed that overexpression of the guanine exchange factor Vav3, a Rho GTPase activator, creates bacterial docking stations on the CF airway epithelium surface, promoting *P. aeruginosa* adhesion, which may represent an irreversible step for airway colonization by these bacteria.

The integrity of the airway epithelial barrier is determinant for efficient protection against infection while tight- and adherens junctions are targets of bacteria. How CFTR dysfunction affects this balance toward infection-prone epithelial damages remains poorly understood. CFTR drives ion and water movement to the apical surface to generate an ASL. It remains unclear whether the presence of an ASL, beyond its composition, is sufficient to maintain airway epithelium integrity and protection against infection. The low ASL volume phenotype was reproduced by CRISPR-Cas9 CFTR knockdown (CFTR-KD) in an airway epithelium cell line (Calu-3) polarized at the air liquid interface. Using this model, we studied the impacts of infection by wild type and mutants with attenuated virulence of the laboratory strain PAO1 on airway epithelium integrity. We observed enhanced damages of the CF airway epithelium infected with ΔfliC and ΔlasR PAO1 mutant strains, while the normal CFTR-expressing epithelium remains intact. However, these damages were prevented by apical hydration, both with ASL collected from normal cultures or a simple physiological saline. These manipulations did not modulate bacterial growth nor immune gene expression as evaluated by multiplex NanoString analysis. We further show that both apical manipulations promoted expression of tight- and adherens junction proteins. These observations indicate that an epithelial integrity gatekeeper is subordinated to the apical hydration, beyond liquid's composition or expression of a functional CFTR at the apical membrane. They also support rehydration therapy strategies to restore epithelial homeostasis in CF airway disease.

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S6.4 Identification of drugs activating CFTR-independent fluid secretion in nasal organoids based on a high-content screening assay

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Introduction: Alternative chloride channels are an interesting target to restore the epithelial fluid homeostasis in cystic fibrosis (CF) airway epithelia. As this approach bypasses cystic fibrosis transmembrane conductance regulator (CFTR), it might help a large proportion of people with CF, independent of their mutation type. A high-content screening assay with nasal organoids from individuals with CF was used to identify drugs that induced epithelial fluid secretion via alternative ion channels.

Methods: Nasal organoids from individuals with CF (n=4 donors) were cultured in 384-well plate format. A drug library containing 1400 FDA-approved drugs was screened on their ability to induce fluid secretion, based on organoid swelling. The primary screen was performed in 384-well plate format, with 2 compounds per well. Compounds which induced swelling were subsequently tested in a secondary screen in the more conventional 96 well-plate format in order to make a selection of hits. These hits were then tested in nasal cells from CFTR-null donors to confirm their CFTR-independency. To determine if the drugs activated the alternative chloride channel TMEM16A, gene knockouts were created using CRISPR-Cas9 gene editing in cells derived from individuals with CFTR-null mutations.

Results: Ninety compounds which induced fluid secretion in CF nasal organoids were identified in the primary screening assay. After the secondary screen, 13 hits were chosen based on the amount of swelling and their working mechanism. Most of the compounds also induced swelling in nasal organoids from CFTR-null donors, suggesting CFTR-independency. In order to test if the compounds activate TMEM16A, TMEM16A knockout nasal cells from CFTR-null mutations were created with a efficiency of 87-95%, which was determined by Sanger sequencing. Western blot showed reduced TMEM16A protein expression in the TMEM16A knockout cells and Ussing chamber experiments showed reduced UTP-induced current in the genetic knockout cells, indicating less TMEM16A activity.

Conclusion: We have identified and validated multiple FDA-approved drugs activating CFTR-independent swelling in nasal organoids. Using TMEM16A knockout nasal organoids, we will further determine the contribution of this specific alternative ion channel.

Funding: CF Trust grant SRC013

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S6.5 ATP12A upregulation in airway epithelial cells by inflammatory stimuli

Daniela Guidone[1], Martina Buccirossi[1], Paolo Scudieri[2,3], Vito Terlizzi[4], Federico Cresta[2], Isabelle Sermet-Gaudelus[5], Elise Dreano[5], Gabrielle Planelles[6], Rossella De Cegli[1], Sergio Sarnataro[1], Gilles Crambert[6], Luis J.V. Galietta[1]

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In a previous study, we found that expression of ATP12A proton pump is increased in the bronchi of cystic fibrosis (CF) patients undergoing lung transplant (Scudieri et al., JCI Insight 2018). We asked whether ATP12A upregulation was due to the inflammation at an advanced stage of lung disease or a direct consequence of the CFTR basic defect. Therefore, we investigated ATP12A expression in nasal brushings of CF patients and control individuals. We found significantly higher ATP12A expression in the nasal mucosa of CF patients, even of very young age. In cultured sterile conditions, we found no difference in ATP12A expression between CF and non-CF airway epithelial cells, thus suggesting a role of inflammation in the upregulation of ATP12A in vivo. We treated CF and non-CF epithelia with a panel of pro-inflammatory cytokines that have been reported to be involved in CF. We found that the combination of IL-17 plus TNF-α was the most effective stimulus increasing ATP12A at the protein and functional level. We evaluated ATP12A function by measuring pH on the apical surface of bronchial epithelia under bicarbonate-free conditions. Non-CF cells under control conditions showed a relatively small but significant effect of ouabain and bafilomycin A1, which inhibit ATP12A and V-ATPase, respectively. The effect of the two agents was additive. The bafilomycin-sensitive component was absent in CF cells. Treatment of CF and non-CF cells with IL-17 plus TNF-α induced a marked acidification that was exclusively sensitive to ouabain. We analyzed the effect of IL-17 plus TNF-α at the transcriptome level using RNAseq. For comparison, we also investigated the individual effects of IL-17, TNF-α, and IL-4. This analysis revealed a profound change elicited by IL-17 plus TNF-α combination, including genes involved in transepithelial transport (e.g. SLC26A4, ATP12A), cytokine/chemokine signaling (CSF3, IL-19, CCL20, CXCL6, CXCL5, CXCL1), modulation of immune response and antimicrobial activity (IDO1, NOS2, DEFB4A, LTF, DEFB4B), and mucins (MUC5B). ATP12A upregulation at the mRNA levels was modest but significant. We performed also single cell RNA sequencing (scRNAseq) to further analyze the effects of IL-17 plus TNF-α treatment. The treatment caused the appearance of a cluster enriched with cells expressing ATP12A and SLC26A4. In conclusion, we found that IL-17 plus TNF-α treatment induces a complex program that involves profound changes in gene expression profile, probably to boost the ability of the airway epithelium to respond to inflammatory stimuli. ATP12A upregulation by pro-inflammatory condition may be detrimental, causing acidification of ASL that in CF cannot be rescued by CFTR-dependent bicarbonate secretion.

This study is supported by Cystic Fibrosis Foundation, Telethon Foundation, and Italian Foundation for Cystic Fibrosis (FFC).

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S6.6 SLC26A4 but not TMEM16A directly regulates ASL pH under inflamed conditions in nasal epithelia derived from donors with rare class I mutations

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Introduction: Airway surface liquid (ASL) pH is more acidic in CF due to faulty CFTR, which contributes to mucus stasis and bacterial infection. ASL pH is therefore a potential factor that could be targeted for future CF therapies. However, we lack a complete understanding of the complex mechanisms underlying ASL pH regulation. In addition to CFTR, possible targets, such as Na^+/H^+-exchanger, K^+H^+-ATPase, SLC26 ion transporters and TMEM16A, a calcium activated chloride channel, have all been suggested to regulate ASL pH, as well as the paracellular ion shunt. Our aim here was to investigate the potential role of the Cl^-/HCO_3^- exchanger, SLC26A4, and TMEM16A, in acid-base homeostasis in fully differentiated nasal CF airway cultures derived from donors with class I mutations, under both normal and inflammatory conditions.

Methods: SLC26A4 (26A4) and TMEM16A (T16A) knock-out (KO) cells were produced by CRISPR-Cas9 gene-editing of CF-null basal cells from 3 donors with class I mutations. The cells were then differentiated at an air-liquid interface (ALI) for 4 weeks. ASL pH was measured in real-time using a fluorescent plate-reader under thin film conditions (Saint-Criq et al., JOVE, 2019). To induce a pro-inflammatory phenotype, and increase the expression of 26A4 and T16A, cell cultures were treated with IL-4 [10ng/mL] for 48hrs.

Results: The baseline ASL pH in CF nasal epithelial cell cultures was 6.63±0.29 (n=27) and was unaffected by IL-4 treatment (6.56±0.48, n=27). However, in 26A4 KO epithelia, although baseline ASL pH was unaltered under normal conditions (6.64±0.30, n=12), it was markedly acidified (6.06±0.42, $p<0.0001$, n=12) in IL-4 treated cultures. In contrast, the baseline ASL pH for T16A KO epithelia, under both normal and IL-4 conditions, was significantly increased (6.82±0.26 pH, $p<0.05$ and, 6.88±0.53, $p<0.001$, respectively, n=15). The addition of the calcium agonist, carbachol (CCh) caused a small, but not significant, acidification of ASL pH under all the conditions tested. In contrast, the subsequent addition of the cAMP-agonist, forskolin (FSK), alkalinized ASL pH (ΔASLpH 0.27±0.37), a response that was significantly enhanced in IL-4 treated epithelia (ΔASLpH 1.01±0.44, $p<0.0001$). In T16A KO epithelia, with or without IL-4, the FSK-induced alkalinisation was unaffected. In 26A4 KO epithelia under normal conditions, although the FSK response was unaltered (ΔASLpH 0.18±0.21), it was significantly attenuated in 26A KO epithelia treated with IL-4 (ΔASLpH 0.44±0.24, $p<0.0001$).

Conclusion: Genetic KO of TMEM16A increased baseline ASL pH under all conditions but did not affect the response to calcium or cAMP agonists. This suggests that TMEM16A indirectly contributes to steady-state ASL pH homeostasis. Genetic KO showed that SLC26A4 plays a minor role in ASL pH homeostasis and response to agonists under non-inflammatory conditions. However, under IL-4 treatment, SLC26A4 KO significantly impacted ASL pH, leading to a more acidic pH, as well as a reduced response to the cAMP agonist. Overall, this study shows that TMEM16A indirectly contributes to ASL pH homeostasis, but highlights an important direct role of the non-CFTR transporter, SLC26A4, which suggests this transporter could be an important alternative target for ASL pH therapy in CF.

Funding: CF Trust grant SRC013

02 April — 11:00–12:45

Symposium 7 – Mucus and mucins

S7.1 Role of submucosal glands and mucous strands in airway host defense

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To understand mucins' role in airway host defense, we studied the closely related gel-forming airway mucins, Muc5AC and Muc5B, in newborn pigs. These mucins have similar domains, but are localized in different parts of the airway and show different morphologies. Muc5AC is secreted from surface epithelia goblet cells as threads. Muc5B is secreted from submucosal glands (SMG) as strands that are released from SMG ducts onto the airway surface.

In CF, mucus strands secreted from the SMG fail to release from the SMG ducts: they remain attached and form aggregates of mucus on the airway surface. This aberrant behavior is due to changes in the mucus strands caused by decreased pH and volume in the CF airway. When we blocked Cl^- secretion and removed HCO_3^- in wild-type tracheas to mimick CF, strand release was similarly blocked. Thus, the CF strand defect is due to loss of anion transport through CFTR.

Mucociliary clearance (MCT) is one form of airway defense. MCT is the movement of mucus strands propelled by beating cilia over the airway surface to clear the mucus and attached bacteria out of the airway. In CF, the SMG strands are not released from the SMG ducts, MCT is impaired, bacteria are not expelled and disease worsens. These studies and others point to the importance of SMG secretions as strands for optimal airway clearance.

The second arm of airway defense is antimicrobials. Both the surface epithelium and the SMG secrete antimicrobials and thus contribute to airway defense. Thus, it has been difficult to assess their relative contribution to host defense. We directly tested the relative contributions by making a pig lacking SMG.

The gene EDA encodes ectodysplasin, a TNF-like ligand that is the initiating step for formation of gland ducts and acini. Mice, cows and dogs that have EDA mutations lack some or all glands and may exhibit respiratory problems. EDA mutations lead to the disease hypohidroitic ectodermal dysplasia (HED) in humans, some of whom exhibit respiratory symptoms.

We used CRISPR to disrupt the pig EDA, and generated pigs that lack SMG throughout the conducting airway. Using these EDA-KO pigs, we were able to determine how airway defense differed when the contributions of SMG were missing.

Following bacterial challenge, EDA-KO pigs showed reduced bacterial killing, consistent with lack of SMG antimicrobials and suggesting that glands are required for optimal host defense.

Furthermore, MCT was disrupted in EDA-KO pigs under both basal and stimulated conditions, indicating glands and mucus strands are necessary for optimal MCT.

These *in vivo* functional studies show that SMG are necessary for antimicrobial activity and mucociliary transport, key host defense mechanisms in the lung.

S7.2 What is wrong with the CF lung mucus and how can we fix it?

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When the CFTR gene and its main CF mutation, F508del, was discovered 1989, many with me thought it should be easier to fix the lung mucus problem than to correct the CFTR channel. We were wrong, as understanding lung mucus proved much more challenging than expected. A major reason for this is our poor understanding of mucus and the healthy lung clearance system and how this is dramatically changed upon infection and disease as in CF.

In recent years, we and others, have by studies on newborn piglets started to understand the contribution from the submucosal glands by their formed mucus bundles. These are normally sweeping and cleaning the upper respiratory tract, but in CF these are immobile from birth. The healthy respiratory epithelial surface has a liquid layer, is not covered by mucus, and not protected from contact with inhaled bacteria. The lower airways lacking glands, are like the mouse respiratory tract cleared by linear MUC5B strands from the surface goblet cells.

Upon infection and immune stimulation, an attached dense mucus layer is formed with components and properties similar to the protective mucus layer of colon. In healthy individuals, this mucus can be coughed up, but in the absence of a functional CFTR channel the mucus remain in the lung together with trapped bacteria.

Recent understanding on the relation between CFTR and mucus secretion as obtained from intestinal studies will be explained as well current misunderstandings around the effect of ENaC inhibitors. Recent insight in mucus organization by structural studies of mucins and related proteins forming the attached mucus layer will be presented.

Today, early introduction by efficient CFTR correctors is the only way to restore CF mucus.

S7.3 The *Xenopus tropicalis* tadpole as a model system to define mechanisms of mucus function

David J Thornton

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The secreted mucus matrix is a complex biological hydrogel which controls access of microbes and harmful substances to the underlying epithelial cells and is therefore critical for host defence. The gel-forming mucins (MUC5AC and MUC5B) are the key components of the mucus matrix and provide its structural integrity allowing mucus transport out of the airways by mucociliary clearance and cough. However, accumulation of mucus, with non-optimal transport properties, is a pathologic feature of cystic fibrosis. Currently, we lack a clear understanding of how dynamic changes in mucus composition and properties underpin the multiple roles which mucus plays in maintaining the healthy lung. To address these gaps in our knowledge, we are developing an *in vivo* model system, the *Xenopus tropicalis* tadpole, specifically to investigate mucin, mucus and mucociliary biology. The unique combination of conserved cell types, genetic tractability, fast generation time and, unlike mammalian models, ease of access to mucin producing tissues makes the tadpole model ideally suited for the study of mucus biology.

We have shown that the tadpole has mucin producing cells in the skin surface which produce the mucin, MucXS, which has structural similarity to mammalian gel-forming mucins[1]. Functionally, we have shown that MucXS underpins a host protective mucus barrier that overlays the tadpole skin surface and knockdown of MucXS causes a marked reduction in barrier thickness and loss of protection against infection[1]. We have now discovered that the *X. tropicalis* tadpole model produces a distinct mucin, Muc5j, exclusively from a glandular structure (the cement gland) at the head end, which also has characteristics of mammalian gel-forming mucins. We have shown that the Muc5j-based mucus strands are adherent along their length and can bind and trap particles, this is analogous to the function of mucus strands produced by submucosal glands in the upper airways of the pig and human that bind bacteria and debris to "clean" the upper airways[2]. Thus, this model system produces mucus from different tissue sites (layers produced by the skin and strands produced from the cement gland) with striking structural and functional similarity to the morphologically distinct forms of mucus (layers and bundles/strands) produced by the mucosal tissue of the human (and pig) respiratory tract[2-5].

In related work, we have found that the MucXS producing skin secretory cells express the calcium-dependent chloride channel, TMEM16A. We have shown that *X. tropicalis* TMEM16A is functionally equivalent to human TMEM16A in its sensitivity to calcium and voltage, and its response to specific inhibitors. Depletion of TMEM16A affects the biophysical properties of secreted MucXS, which may affect mucociliary function.

The structural and functional similarities of the different types of mucin-based matrices produced by the *X. tropicalis* tadpole to those encountered on mammalian airway surfaces demonstrate the utility of this model system to discover new mucus biology relevant to the human lung.

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S7.4 Extracellular vesicle lncRNA MALAT1 drives HDAC11-dependent chronic inflammation in cystic fibrosis airway neutrophils

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Objectives: We previously showed that polymorphonuclear neutrophils (PMNs) recruited to the airway lumen in people with CF undergo rapid transcriptional reprogramming, resulting in hyperexocytosis yet reduced bacterial killing. Together, this promotes chronic infections, feed-forward inflammation and structural tissue damage. These PMNs also secrete a large number of extracellular vesicles (EVs). Here, we hypothesized that EVs secreted by reprogrammed PMNs cause newly recruited PMNs to undergo similar pathological changes, thereby explaining the chronicity of CF airway inflammation.

Methods: EVs were characterized from CF sputum and conditioned media from airway-like neutrophil cultures derived from a lung transmigration model. EVs were first purified by differential centrifugation followed by fractionation on a 300 kDa MWCO and downstream analysis by RNA-seq, nanoflow cytometry and nanoparticle tracking analysis. To study recursive signaling, EVs from transmigrated CF-like and healthy neutrophils were applied to a naïve population of neutrophils recruited in a second round of transmigration. This secondary set of transmigrated neutrophils were characterized by RNA-seq and flow cytometry to assess activation and inflammatory poise. As a functional validation, secondary transmigrated neutrophils were incubated with *P. aeruginosa* (PA01) to assess bacterial killing capacity.

Results: Exposure of PMNs to EVs from CF sputum in an airway transmigration model induced hyperexocytosis and reduced bacterial killing, recapitulating in vivo findings. Removal of PMN-derived (CD66b+) EVs from CF sputum prevented hyperexocytosis and increased bacterial killing by recruited PMNs. Moreover, EVs from PMNs recruited to CF sputum in vitro induced hyperexocytosis and reduced bacterial killing by a second wave of recruited PMNs.

RNA-seq analysis identified lncRNA MALAT1 as differentially expressed in EVs from CF- or healthy-conditioned PMNs. Transfection of naïve PMNs with MALAT1 induced hyperexocytosis and reduced bacterial killing. The regulatory enzyme HDAC11 was rapidly upregulated in CF sputum exposed and MALAT-1 transfected PMNs. Transfection of naïve PMNs with HDAC11 resulted in hyperexocytosis, decreased bacteria killing and lead to the localization of MALAT1 to secreted EVs, while inhibiting HDAC11 blocked these processes. Finally, MALAT1 levels in CD66b+ EVs from adult CF patients correlated negatively with %FEV1 and increased during acute pulmonary exacerbations.

Conclusion: CF patient sputum contains a high concentration of EVs, particularly those originating from prior waves of inflammatory PMNs. PMNs recruited to the CF airway lumen are exposed to these EVs leading to MALAT1-dependent HDAC11 induction and subsequent packaging of MALAT1 into EVs. These PMNs exocytose primary granules and fail to clear invading pathogens. The released MALAT1+ CD66b+ EVs reprogram naïve PMNs to exhibit a similar phenotype resulting in feed forward reprogramming of PMNs and chronic airway disease. These findings identify MALAT1 and HDAC11 as potential targets for intervention to lessen inflammation and improve pathogen killing in CF airways and other diseases dominated by PMNs.

Funding: CFF (TIROUV19A0), Emory I3 Program

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S7.5 HiPSC-derived AECs as a novel platform to study the role of ionocytes in mucociliary clearance

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Cystic Fibrosis originates from biallelic mutations of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene. These result in increased mucus viscosity which reduces clearance in the airway epithelium and predisposes to bacterial infection and biofilm formation. The main cell type involved in this process are ciliated cells of the airway epithelium. These specialised cells orchestrate mucus movement upwards and outside the airways to keep them free of pathogens and external particles. Based on this essential function, ciliated cells have been a major focus for CF research. More recently, ionocytes have been identified as the cell type which expresses the highest levels of CFTR and they have been hypothesised to have a key role in CF. However, their exact function in the airway epithelium remains to be elucidated.

The study of ionocytes in the human airway and their role in disease remains challenging because of their rarity, the lack of availability of fresh tissue/brushings and the patient burden that their obtention implies. Furthermore, the comparison between patient and healthy donor samples has limitations due to genetic background-derived variability. For all these reasons, our group has decided to use of human induced pluripotent stem cells (hiPSCs) for disease modelling and for studying the airway epithelium. This model system allows the reprogramming of somatic cells from patients into pluripotent cells that can be differentiated into virtually any cell type, including AECs. However, production of ionocytes from hiPSCs remains to be demonstrated.

To address this question, we have developed a differentiation protocol to produce AECs from hiPSCs. This method includes a sorting step at day 16 to enrich for lung progenitors ($NKX2.1^{+}$), after which the cells can be expanded as 3D organoids before they are further matured. Importantly, the resulting cells can also be cryopreserved, allowing to re-start cultures from lung progenitors rather than having to differentiate hiPSCs every time, thereby increasing the practicality of the protocol. Maturation of the cells in air-liquid interface using an in-house chemically defined medium leads to the formation of a polarised pseudostratified epithelium that includes basal cells (p63, CK5), secretory cells (SCGB3A2, MUC5AC), ciliated cells (FOXJ1) with motile cilia and more importantly ionocytes (FOXI1), as shown by qPCR and immunocytochemistry analyses. To further investigate the role of ionocytes, we generated hiPSCs knock out for FOXI1, a transcription factor essential for their differentiation. $FOXI1^{KO}$ hiPSCs were differentiated into AECs and, when compared to their isogenic wildtype control, we observed a decrease in number of ciliated cells both at mRNA and protein level. In terms of functionality, the cells showed no significant difference in epithelial barrier properties (TEER) while ciliary function remains to be fully characterised.

Our approach constitutes a unique platform to study the importance of human ionocytes in cilia motility in health and in disease paving the way to more targeted treatments. Furthermore, the model has the potential to be useful for the study of other lung diseases and to bring us a step closer to regenerative therapy.

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S7.6 Hydrogel-encapsulated niclosamide for topical treatment of inflammatory airway diseases

Jiraporn Ousingsawat[1], Raquel Centeio[1], Inês Cabrita[1], Khaoula Talbi[1], Oliver Zimmer[2], Achim Göpferich[2], Rainer Schreiber[1], Karl Kunzelmann[1]

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Background: Previous work showed that the Ca^{2+}-activated Cl^- channel TMEM16A is upregulated in cystic fibrosis and asthma and accompanies goblet cell hyperplasia/metaplasia and mucus hypersecretion, being predominantly expressed in mucus-producing cells in the airway submucosal glands. Repurposing of the anthelminthic drug niclosamide -- a potent TMEM16A inhibitor -- was proposed as an effective treatment for inflammatory airway diseases. Niclosamide showed a potent inhibition of mucus production and mucus secretion in the airways, inhibition of the release of the CF-typical inflammatory mediator IL-8, and a pronounced induction of bronchorelaxation, shown both *in vivo* and *ex vivo*. While systemic application of niclosamide may lead to unwanted side effects, local application to the lungs may circumvent these problems, particularly when the drug is encapsulated into hydrogels.

Methods/results: In the present study, we achieved the encapsulation of niclosamide in polyethylene glycol hydrospheres of micrometer and nanometer size (Niclo-spheres). When applied to ovalbumin (OVA)-sensitized and challenged -- asthmatic -- mice via intratracheal instillation to the lungs, free niclosamide and Niclo-spheres strongly attenuated the overproduction of mucus, measured by mucins-specific alcian blue staining of paraffin-embedded lung tissue. All niclosamide formulations were also shown by immunohistochemistry to inhibit the secretion of the major proinflammatory mediator CLCA1 in the airways, and to improve mucociliary clearance in *ex vivo* asthmatic tracheas, roughly back to healthy control levels. Niclo-spheres were confirmed by whole-cell Patch-Clamp measurements to strongly inhibit TMEM16A, and by immunocytochemistry to inhibit mucus production, in CFBE and Calu-3 human airway epithelial cells, which is due to a pronounced inhibition of intracellular Ca^{2+} signals. Empty/unloaded particles were shown to have no effect in any parameter analysed.

Conclusions/future perspectives: The data suggests that poorly dissolvable drugs such as niclosamide can be deposited on the airway epithelium as hydrogel-encapsulated formulations for the resolution of inflammatory airway disease. This local application may present several advantages over systemic administration by limiting off-target effects and the need for repetitive dosing due to slow long-lasting release of the hydrogel-cargo. Conjugation of the hydrogel-particles with unique ligands designed to target airway cell type-specific surface receptors and prompt cell-specific internalization/uptake is the next step to achieve an enhanced specificity of niclosamide delivery to inflamed airways.

Keywords: Niclosamide, TMEM16A, cystic fibrosis, inflammatory airway disease

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02 April — 14:15–16:00

Symposium 8 – Gene-based therapeutic approaches

S8.1 CFTR gene editing – recent highlights, future goals and therapeutic opportunities

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Small molecule drugs have revolutionised the treatment of CF over the last decade, and can potentially treat about 90% of people depending on their CFTR genotype. But what about a treatment for the remaining 10%, and potentially a cure for everyone with CF, regardless of their genotype?

Here, I will discuss progress towards these goals using gene editing. Building on early studies that provided proof-of-principle that editing of F508del was feasible, many groups have used both CRISPR and non-CRISPR systems to correct many different individual CF-causing mutations in cell, organoid and animal models within increasing efficiency. As potential limitations of certain editing strategies have been identified, solutions have swiftly emerged, and CF researchers have been quick to adopt these new techniques. As a community, we have a full range of editing tools and the drive now is towards editing strategies that are amenable to clinical development, especially for mutations in the remaining 10% category. The recent focus of our lab has been to compare different methods to correct W1282X, the second most common mutation in this category.

Using homology-directed repair, we have shown precise repair in ≥20% of alleles in a population of cells, but this was accompanied by a similar level of on-target insertions/deletions (indels), a level comparable to many other studies. To minimise indels, we have used adenine base editing to correct W1282X with a similar level of editing but negligible indel formation. However, a different limitation was detected, the so-called bystander editing of adjacent adenine residues which in this case change the neighbouring codon, R1283 to G1283, a variant that has been reported to be CF-causing. Clonal cells containing the corrected W1282 codon and bystander G1283 codon show production of stable CFTR mRNA, production of full length CFTR protein with a reasonable proportion of band C representing cell surface expression, and ~15% WT levels of short circuit current. A third method, the homology-independent targeted integration (HITI) of a superexon comprising exons 23-27 resulted in a slightly lower level of editing, but without on-target indels or bystander effect seen with HDR and base editing. Analysis of mRNA in an HITI-edited clonal cell line showed ~70% of transcripts included the superexon, with cells showing ~15% WT levels of short circuit current. Strategies to edit other mutations in the 10% category by Prime editing and adenine base editing will also be described.

Finally, a discussion of some of the hurdles to be clear on the path to clinical development of gene editing for CF; it may be the same approach for everyone, or possibly a number of different strategies tailored to particular mutations or classes/groups of mutations, such that we have a treatment for everyone with CF

S8.2 Development of suppressor tRNA gene therapies targeting nonsense associated CF

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10-15% of cystic fibrosis (CF) results from nonsense mutations. Nonsense mutations occur when a single nucleotide change converts an amino acid encoding codon to premature termination codon (PTC; TGA, TAG or TAA), resulting in a truncated protein often with complete loss of function and degradation of the mRNA transcript by nonsense mediated decay (NMD). Because all nonsense-associated diseases coalesce around a central mechanism, there has been a concerted effort to identify PTC readthrough therapeutics. We have recently developed a library of Anti-Codon Edited (ACE)-tRNAs that recognize and promote read-through of all 'in-frame' premature termination codons (PTCs) that result in CF. Unlike small molecule readthrough agents, ACE-tRNAs suppress PTCs with the correct amino acid, resulting in WT full-length protein. Further, using ribosomal profiling, we have shown that ACE-tRNAs 'prefer' PTCs over natural termination codons (NTCs), providing a possible therapeutic window for their use *in vivo*. While much of the previous suppressor tRNAs studies by us and others have been performed with PTC targets encoded in cDNAs, our current work focuses on suppression of PTCs encoded in the endogenous *cftr* gene where transcriptional regulation and post-transcriptional processing likely impacts the ability of ACE-tRNAs to rescue therapeutically meaningful levels of CFTR channel expression. Using CRISPR gene-edited human bronchial epithelial cell lines (16HBEge) with G542X, R1162X and W1282X CF mutations, we have found that delivery of ACE-tRNAs significantly inhibits NMD and promotes significant expression of cftr mRNA. Further, using the whole-cell patch-clamp technique, we found that transfection of ACE-tRNA cDNAs rescues near 100% of CFTR-W1282X channel function. These findings are exciting as they demonstrate ACE-tRNAs, to our knowledge, are the most potent readthrough technology for rescue of both mRNA and protein expression encoded from the endogenous cftr gene to date. Expecting that therapeutically meaningful levels of CFTR channel rescue will be limited by ACE-tRNA delivery methods, we performed a 'hit-to-lead' optimization of ACE-tRNA sequences to further increase PTC suppression activity 200-400%. We propose that the 'enhanced' ACE-tRNA sequences can be delivered as RNA or DNA and paired with burgeoning nucleotide delivery technologies to effectively lower the delivery hurdle. Moving forward we look to combine our research efforts for effective delivery of ACE-tRNAs to lungs of mice, pigs and cultured primary airway epithelia from CF patients to determine PTC suppression ability and toxicity.

S8.3 Progression towards a first-in-man lentiviral vector trial

Uta Griesenbach

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The UK CF Gene Therapy Consortium has previously demonstrated that repeated delivery of a CFTR-liposome (GL67A) complex can stabilise lung function in a double-blind placebo-controlled Phase IIb trial. However, the magnitude of benefit did not warrant continued progression in the context of the welcome benefit provided by small molecule modulators. In parallel, we have developed a lentiviral vector pseudotyped with the Sendai-virus envelope glycoproteins (F/HN) (rSIV.F/HN). In preclinical studies we have shown that the vector efficiently and persistently transduces airway epithelial cells and in contrast to adenoviral and adeno associated virus-based vectors retains efficacy on repeated administration. We have recently completed a licensing deal with Boehringer Ingelheim and are now preparing for a first-in-human clinical trial. The initial trial population will predominantly consist of patients not eligible for CFTR modulator therapies.

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S8.4 Development of in vitro transcribed mRNA therapeutics for cystic fibrosis

Ruhina Maeshima[1], Martin Attwood[1], Amy Walker[1], Aristides Tagalakis[1], Drew Weissman[2], Robin McAnulty[3], Chris O' Callaghan[1], Deborah Baines[4], Stephen Hart[1]

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Cystic fibrosis (CF) is a recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), affecting approximately 10,000 people in the UK. Absent/non-functional CFTR leads to thick, sticky mucus in the lung which results in chronic bacterial infection and inflammation. Gene replacement therapy with viral/non-viral vectors has been explored in the last 25 years but all failed to show significant clinical efficacy. *In vitro* transcribed (IVT) mRNA has emerged in the last few years as a new approach for protein replacement.

Our aim is to develop CFTR IVT mRNA therapy for CF to replace the missing wild type CFTR protein using receptor-targeted nanocomplex (RTN) formulations. We have developed RTN formulations that consist of liposomes epithelial receptor targeting peptides and the nucleic acid. Our approach was to first optimise RTN nanoparticles for transfection of primary cystic fibrosis bronchial epithelial (CFBE) cells, at submerged culture and air-liquid interface (ALI) cultures, as well as in mouse lung, using reporter IVT mRNAs. We then assessed delivery efficiency of CFTR mRNA in CFBE cells at submerged culture and ALI culture by immunostaining of protein. Functional testing for restoration of ion transport by Ussing chamber analysis is in progress and will be reported.

We first optimised the RTN formulations, comparing combinations of three different cationic liposomes and five peptides for their biophysical properties and transfection efficiency. We identified a novel formulation (size 130 nm, PDI 0.28, charge 38 mV) for mRNA delivery that achieved almost 100% cellular uptake efficiency and 90% transfection efficiency. There were no differences in transfection efficiency between primary normal human bronchial epithelial (NHBE) cells and CFBE cells. The same RTN formulation was able to deliver the mRNAs in ALI cultured cells and mouse lungs where luciferase expression in mouse lungs was ~200-fold higher than plasmid DNA encoded luciferase. CFTR mRNA was successfully delivered to NHBE and CFBE cells. In addition, we co-packaged the CFTR corrector VX-809 with CFTR mRNA into RTNs and found that this improved the expression of CFTR protein in CFBE cells in submerged culture by approximately 2 to 2.5-fold. CFTR protein translated from the IVT mRNA was detected by immunoblotting from 4-hour to at least 48-hour after the CFBE mRNA transfections.

In conclusion, CFTR IVT mRNA delivery is a promising novel therapeutic for cystic fibrosis. In addition, the flexibility of the RTN formulation allows co-delivery of CFTR mRNA with VX-809, which significantly improved CFTR expression.

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S8.5 CFTR super exon splice site and polyA signal affect CFTR expression and function

Hillary Valley, Katherine Bukis, Rebecca Seymour, Alisa Bell, Yi Cheng, Joshua Conte, Emily Wilson, Andrey Sivachenko, Normand Allaire, Kevin Coote, Martin Mense, Calvin Cotton

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Gene editing and gene therapy are promising therapeutic approaches for cystic fibrosis (CF). Strategies to correct individual *CFTR* variants are not feasible for most CF-causing genetic variants as more than 1,700 genetic variants of the *CFTR* gene have been suggested to cause CF. Insertion of a partial cDNA (also known as a super exon) into the native *CFTR* genomic locus can restore the *CFTR* gene to a wildtype coding sequence for all mutations downstream of the insertion site while retaining the endogenous *CFTR* promoter and chromatin architecture. Several groups have successfully inserted *CFTR* super exons of various sizes, but much super exon biology remains unexplored. There are many variables for super exon insertion, such as insertion site, codon optimization, splice site, and polyadenylation signal sequence. We inserted three super exons with the same coding sequence into the same location of *CFTR* and found that both the super exon splice site and polyadenylation signal sequence can have significant consequences on super exon expression and function.

We inserted a super exon into intron 22 of the *CFTR* gene with the W1282X or N1303K CF-causing mutation. To separate super exon expression and function from the efficiency of super exon insertion in a population of cells, we isolated super exon-positive clonal cell lines. Super exon 1 consisted of the native exon 23 splice acceptor, native CFTR coding sequence for exons 23-27, and the BGH polyA signal. Surprisingly, this super exon resulted in poor (5-10% of WT) restoration of CFTR function. Analysis of *CFTR* mRNA isoforms revealed that many transcripts were splicing to the native exon 23 and bypassing the super exon. To address this issue, we created super exon 2, which has the same sequence as super exon 1, but with a stronger splice site. This super exon resulted in increased CFTR Function (~30% of WT) but was still not able to completely rescue CFTR expression and function. The third super exon had both a strong splice site as well as a different polyA signal sequence (SV40 polyA). This super exon resulted in robust CFTR functional rescue (50-100% of WT).

In summary, we have demonstrated successful targeted insertion of three *CFTR* super exons with the same coding but different flanking sequences, resulting in variable functional rescue of CFTR. These studies demonstrate that simple recapitulation of WT DNA coding sequence does not necessarily produce normal levels of WT mRNA transcript and complete functional rescue. Additionally, analysis of super exon function in a population of cells may mask inefficiencies in the super exon construct. Work is ongoing to evaluate these super exons in other CFTR cell types and in vivo, and to apply these improved super exon features (splice site and polyA signal sequence) to creating better, larger super exons that could help more people with CF.

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S8.6 Correction of the CFTR 1717-1G>A splicing mutation through CRISPR based technology

Alessandro Umbach, Simone Amistadi, Giulia Maule, Anna Cereseto

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Almost 13% of mutations causing cystic fibrosis alter the correct splicing of the CFTR gene. Among these, the Class 1 1717-1G>A substitution is one of the most common mutation affecting approximately 1% of cystic fibrosis (CF) patients. This mutation consists in a G to A conversion in intron 11 which results in the inactivation of a conserved AG dinucleotide at the 3' end acceptor splice site leading to exon 12 skipping or recognition of an alternative cryptic splice site. Both altered splicing products generate a stop codon and consequent lack of CFTR protein synthesis which is associated with CF clinical development not treatable with any currently available pharmacological agent. Gene therapy, in particular CRISPR-Cas derived systems, is an emerging opportunity to permanently cure CF caused by any class type mutations. The recently developed adenine base-editing (ABE) and prime-editing (PE) technologies are capable of promoting precise genome editing in the absence of double-strand breaks (DSB) as opposed to the original CRISPR-Cas strategies thus preventing deleterious genotoxic effects. With this study we aim at developing a genome editing strategy to correct the 1717-1G>A mutation through the most recent CRISPR-Cas technology, DSB-free.

We have set up the correction strategy in 1717-1G>A HEK293 models that we have developed by using minigene constructs mimicking the splicing defect. We obtained up to 42% of correction using NG-ABE8e and NG-ABE8.20m base-editors through plasmid transfection. Nevertheless, both ABEs were associated with high levels of bystander edits, which resulted in the unwanted modifications near the mutated nucleotide with potential detrimental effects in CFTR protein synthesis. As alternative strategy we tested the most recent prime-editing approach which produced encouraging preliminary results with over 10% scarless correction and no detected indels.

Overall, we obtained results showing that the recent prime-editor CRISPR-Cas approach can be exploited to repair the 1717-1G>A providing precise modification over the bystander unwanted mutations introduced by the base-editors. Further optimization of the prime-editor strategies is ongoing to produce a more efficient correction of the 1717-1G>A splicing mutation.

02 April — 16:30–17:30

Closing Keynote Lecture

How to tackle what is still missing: striking CF by mechanistic approaches

Margarida D. Amaral

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Despite the tremendous success in approval of CFTR modulator drugs for the most common mutations, still 15-20% of all individuals with CF worldwide have rare mutations which are not targeted by those drugs.

There is thus an unmet need to promptly find feasible and efficient therapeutic strategies tackling the basic CF defect in individuals with such rare mutations. Notwithstanding, these mutations bring about very diverse molecular defects in the cell. Importantly, we still miss a global view of mechanisms and pathways involved in CF pathophysiology. Indeed, although CF is caused by a defective anion channel, CFTR has also been implicated in other cellular processes, namely epithelial cell differentiation [1] and, when dysfunctional, cancer.

We developed several cell-based high-throughput (HT) assays and used them either in functional genomics approaches (siRNA screens) or compound screens for both a global mechanistic characterization of defects underlying CFTR mutations in different functional classes and the identification of putative drug targets and compounds. Approaches were designed for: class I (stop mutations); class II (trafficking defect); and class VII ('unrescuable' CFTR).

For class I mutations, we introduced G542X into a CFTR mini-gene assay [2] to identify novel genes and compounds suppressing premature termination codon (PTC) mutations and inhibiting nonsense-mediated decay (NMD) of PTC-mRNAs.

For class II mutations, we applied a HT microscopy F508del-CFTR traffic assay in human bronchial epithelial cells [3] to screen ~27,000 siRNAs targeting about half of the human genome. We found key regulator genes (kinases) with additive effect to current CFTR correctors.

For class VII mutations, we performed a HT microscopy-based traffic assay for TMEM16A/Anoctamin 1 [4] to screen a library of siRNAs of traffic regulators [5]. The activators of this non-CFTR Cl^- channel are potential drug targets to compensate for the absence of functional CFTR in all individuals with CF.

In parallel, we have tackled the CF differentiation defect by designing a HT wound-healing assay and applied it to CF cells, having identified key transcriptional factors linking CFTR to epithelial differentiation [5] and epithelial-mesenchymal transition (EMT) [6].

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P1

LncRNAs: emerging players in CFTR gene regulation

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Objectives:*CFTR* gene displays a tightly tissue specific and temporal expression pattern that still remain incompletely understood. Previously, we showed that transcription factors and miRNAs act in synchrony to explain the weak *CFTR* mRNA level in mature lung cells. Other key regulatory players, long non-coding RNAs (lncRNAs), participate to the control of gene expression by influencing mainly the regulation of nearby genes. Here, we identified the presence and the expression of nine lncRNAs within the *CFTR* locus and focused our investigation on the role of two of them on *CFTR* gene expression.

Materials and methods: LncRNAs have been quantified by RT-qPCR in 20 different human tissues. LncRNA silencing or overexpression was performed in bronchial cells, with antisense oligonucleotides (GapmeRs) or overexpression with expression vectors, respectively. LncRNAs and *CFTR* mRNA level were assessed by RT-qPCR; CFTR protein level by immunoblot. LncRNA role and lncRNA/DNA interactions have been investigated by using luciferase reporter assays, through co-transfection of lncRNAs and vectors containing different length of *CFTR* promoter. Mutagenesis of potential lncRNAs binding sites on *CFTR* promoter were produced and Chromatin immunoprecipitation assays were also conducted.

Results: Examination of the *CFTR* locus and adjacent regulatory regions by using databases collecting human annotated lncRNAs led to the identification of 9 lncRNAs harboring signatures of active transcription. Quantitative analysis showed that 6 lncRNAs displayed a detectable and tissue specific expression. We focused on CF003 and CF006, which are downregulated in bronchial cells taken from CF patients. To assess whether these lncRNA exert a cis-acting role on CFTR, we used gapmeRs to inhibit their expression. Silencing of CF003 and CF006 induced a decrease of CFTR at mRNA and protein level in 16HBE; data confirmed by overexpression assays, depicting a positive role of lncRNAs on CFTR expression. Using *in silico* tools, we predicted binding sites for these lncRNAs on the *CFTR* promoter. Using luciferase reporter assay with different length of *CFTR* promoter, we showed that CF003 and CF006 increase the luciferase activity under the control of *CFTR*-1kb-promoter. Mutagenesis of their potential binding sites on *CFTR* promoter confirmed that these lncRNAs act directly on *CFTR* promoter. As the binding of these lncRNAs on *CFTR* promoter could affect local binding of transcription factors, we performed chromatin immunoprecipitation assays and showed that overexpression of CF003 and CF006 induced the enrichment of transcription factors (E2F1, E47, CREB1 and SOX17) on *CFTR* promoter in bronchial cells. By luciferase reporter assays with *CFTR*-1kb-promoter, we characterize the positive impact of E2F1, E47 and CREB1 and the negative impact of SOX17 on *CFTR* promoter. Furthermore, *in silico* tools highlighted a miR-145 motif on the CF003 and CF006 sequences; a miRNA strongly deregulated in CF cells. We showed that the overexpression of miR-145 decreases CF003 and CF006 level.

Conclusion: In this study, we characterized new lncRNAs transcribed within the *CFTR* locus and highlighted the role of CF003 and CF006 as activating cis-lncRNAs on CFTR expression. Together, these results reveal new promising targets to increase *CFTR* mRNA rate in addition to current CF therapy.

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P2

Development of a new microRNA therapeutic approach for the treatment of all patients with Cystic Fibrosis.

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Objectives: Cystic fibrosis (CF), an autosomal recessive genetic multiorgan disease, is caused by a dysfunctional CF Transmembrane conductance Regulator (CFTR). CF is a complex pathology due to the multiplicity of mutations found. Correctors and potentiators have demonstrated good clinical outcomes for patients with specific Cftr gene mutations; however, there are still patients for whom those treatments are not suitable and require alternative CFTR-independent strategies. Although CFTR is the main chloride channel in the lungs, anoctamin 1 (TMEM16A) could compensate for CFTR deficiency. Interestingly, we showed that the expression and activity of TMEM16A were decreased in CF patients and that this deregulation was due to overexpression of microRNA-9 (miR-9) (Sonneville et al., 2017). To this end, we have developed an antisense oligonucleotide (ASO TMEM16A) that prevents microRNA-9 from binding to TMEM16A mRNA, thereby increasing its expression and activity. Here, we have investigated the effects of TMEM16A potentiation *in vitro* and *in vivo* and prepared for preclinical studies by assessing the best administration route and the ASO TMEM16A's toxicity and specificity.

Methods: The experiments are performed on cell lines and primary cells with different mutations. The CF mouse model is used to study the different administration routes, complete survival data, and study acute toxicity and long-term effects.

Results: The first results show that the effects of ASO TMEM16A are very specific to the target, which is the 3'UTR of TMEM16A. Thus, ASO TMEM16A does not induce inflammatory cytokines expression, does not alter either intracellular calcium mobilization or cell proliferation. These results were confirmed by "In vitro pharmaceutical profiling", a standard method used by the industry. While studying different administration routes on mice, we showed that ASO TMEM16A is detectable 30 days after subcutaneous injection or intranasal instillation. A first experiment on CF mice, which normally die of intestinal obstruction upon weaning, shows that injection of ASO TMEM16A significantly increases the lifespan of the mice up to 200 days. Acute administration of 50 times the effective dose did not show behavioural changes in the mouse, nor macroscopic or pathological changes. In addition, all of the blood markers were found to be normal. Pharmacokinetic and pharmacodynamic experiments are currently underway to confirm these results. In addition, our preliminary results show an improvement in male CF mice fertility by restoring vas deferent abnormalities, also described in CF male patients.

Conclusion: This strategy could apply to all patients with CF, regardless of their mutations, to correct chloride efflux and mucociliary clearance without inducing inflammation or toxicity.

P3

Transcriptomic and proteomic analysis identifies changes associated with several prototypical cystic fibrosis-causing mutations

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Cystic Fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population, and it is caused by mutations in both alleles of the *CFTR* gene. The phenotypic heterogeneity observed in CF patients, even among those sharing the same *CFTR* genotype, suggests the involvement of other genes contributing to this phenotypic variability. Omics studies can greatly contribute to the identification of such genes. To better understand the global gene expression patterns associated with *CFTR* mutations, combined transcriptome and proteome analysis were performed in isogenic human bronchial epithelial (16HBE) cell lines each homozygous for one of five different CFTR mutations classes; this included four existing lines containing G542X, F508del, N1303K and G551D, and one additional isogenic line we made using CRISPR gene editing with I1234V. Changes in the expression profile of about 13,800 genes and 830 proteins were identified in each mutant cell line, and selected genes and proteins were used to validate RNA-Seq and mass spectrometry results by RT-qPCR and western blot, respectively. Evaluation of differentially expressed genes (DEGs) and proteins (DEPs) identified in each CFTR mutant cell line revealed a subset of DEGs and DEPs that are common to all mutations, and others that are unique to particular mutations. Functional enrichment analysis clustered DEGs and DEPs into biological processes relevant in CF pathophysiology, including actin cytoskeleton organization, cell differentiation, wound healing, protein targeting to organelles such as the ER and plasma membrane, protein glycosylation, and protein folding. Combination of both datasets revealed that approximately 97% of the proteins identified have matched transcripts, and that the resulting mutation-specific detected translated-transcripts (Dtts) have a high level of consistency in expression patterns. The results of the present study represent the first combined transcriptomic and proteomic study focusing on prototypical *CFTR* mutations. Analysis of both common and unique Dtts provide novel insight into the pathophysiology of CF, and on the mechanisms through which each mutation causes disease. Finally, this new dataset will likely contribute to the identification of new therapeutic targets and/or biomarkers for CF.

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P4

Cis-regulatory elements of the *CFTR* gene

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Chromatin is a dynamic structure that determine gene expression. Long-range interactions between promoter and *cis*-regulatory elements (CREs) regulate this expression. Dysregulation of gene expression can lead to diseases, called "enhanceropathies". In recent years, chromatin conformation techniques allowed to study long-range interactions. Some patients with cystic fibrosis or *CFTR-related disorders* (*CFTR-RD*) have incomplete genotypes or present extreme phenotypes. This project aims to explain these unresolved cases by first identifying CREs in *CFTR* locus and in a second part by highlighting dysfunctions in the latter.

In order to study the regulation of the *CFTR* gene, reporter gene activity and chromatin immunoprecipitation tests in intestinal cells (Caco-2) allowed us to characterize new CREs. To validate the purpose, invalidation assays with CRISPR/dCas9 are developed. Then, the chromatin interactions (DNA loops) have to be confirmed by using chromatin study techniques (4C). A second part is dedicated to the detection of variants within CREs by NGS sequencing of the *CFTR* locus in *CFTR-RD* patients.

A bioinformatics prediction tool (GWAS3D score) has identified four introns that are considered important in *CFTR* regulation. Introns 1 (185 + 10 kb), 12 (1811 + 0,8 kb) have already been described as CREs in intestinal cells but introns 24 (4095 + 7,2 kb) and 26 (4374 + 1,3 kb) were newly identify. These two CREs have an important cooperative enhancer activity according to activity tests. Transcription factors (TFs) binding analyses by chromatin immunoprecipitation demonstrated enrichment of TFs HNF1a, p300, FOXA1/2, and CDX2 on the enhancers of introns 24 and 26. These factors are known to be involved in the regulation of the *CFTR* gene in intestinal cells as their binding to the enhancers of introns 1 and 12 have already been described. These results allowed us to propose a three-dimensional model of *CFTR* gene regulation within the locus.

By sequencing Congenital bilateral absence of the vas deferens (CBAVD) patients, eight potential regulatory variants have been highlighted due to their frequencies compare to the European population. To understand their impact on the three-dimensional regulation of the CFTR locus, functional tests are performed. Next to mutagenesis, activity tests shows that variant within introns 1, 12 and 24 lead to decrease the expression of the CFTR gene and disturb cooperative enhancer activities. This can be explained by the presence of FTs binding motifs on these regions. Thus, this work allows a better understanding of the three-dimensional organization of the CFTR locus in order to improve the care of patients.

P5

Combined treatment of VX-770 and ELX-02 enhances CFTR activity in various PTC mutation backgrounds - Implications for clinical trials

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Background: ELX-02 is in Phase 2 of clinical trials for treating CF patients with nonsense-mutations who currently do not have any treatment options. ELX-02 is a eukaryotic ribosomal selective glycoside (ESRG) that co-translationally enables the readthrough of various CFTR premature termination codon (PTC) mutations allowing the recruitment of near-cognate amino acids. The ability of ELX-02 to induce the expression of full-length functional CFTR protein has been robustly demonstrated in multiple *in vitro* and *in vivo* preclinical CF models. Here we optimize the assay conditions for assessing the readthrough activity of ELX-02, as these assays were originally used to determine the activity of CFTR correctors and potentiators. Furthermore, it was previously demonstrated that VX-770 increases wild type CFTR activity by increasing the open-probability of the channel. We therefore hypothesized that upon inducing PTC readthrough with ELX-02, CFTR activity could be enhanced in the presence of VX-770.

Methods: CFTR activity was measured in Fischer Rat Thyroid (FRT) cell monolayers (CFTR-G542X and -R1162X) and in Human Bronchial Epithelial (HBE) cells (F508del/G542X) using the TECC24 and Ussing chamber assays respectively and in human intestinal epithelial cells using the Forskolin Induced organoid Swelling (FIS) assay (G542X/G542X, W1282X/W1282X, G542X/W1282X, F508del/R11162X, R1066C/G542X, F508del/G542X).

Results: Significant ELX-02-dependent readthrough was demonstrated in multiple mutation backgrounds using electrophysiological and organoid swelling readouts.

In the presence of VX-770, prolonging ELX-02 incubation periods from 2 to 4 days enhances CFTR response by 2-fold in Ussing chamber assays (F508del/G542X HBE cells), and by 3.8-fold in organoid swelling assays (G542X/G542X).

Co-treatment of ELX-02 with VX-770 increases CFTR response by 2-fold as demonstrated by electrophysiological (F508del/G542X HBE cells) and organoid swelling assays (G542X/G542X) after 2 days of treatment.

Conclusions: Progressive enhancement of CFTR activity upon longer ELX-02 incubation periods indicates that lower doses can be administered in patients to achieve the same desired effect and will assist in determining dosing strategies for future clinical studies.

The superior CFTR response observed upon co-treatment of ELX-02 and VX-770 relative to ELX-02 single agent treatment is a strong indicator that coadministration of these compounds should have a more clinically beneficial response in patients.

P6

Targeted locus amplification reveals allelic complexity of the CFTR gene and association with functional response

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The impact of allelic variation within the CFTR gene on baseline function and response to CFTR modulators has been described but remains still mostly uncharacterised. Targeted Locus Amplification (TLA), in combination with next-generation sequencing technology has recently been developed to haplotype alleles from targeted genomic loci. Based on 4C chromosome confirmation capture techniques, TLA enables for the targeted DNA analysis of a complete gene or region of interest and the definition of haplotypes. This facilitates the analysis of single nucleotide variants and structural changes in both the coding and non-coding regions of the DNA in an allele-specific manner without prior locus information such as parental sequences. Here, we aim to use TLA to study heterogeneity within the CFTR gene and study the individual haplotypes in the context CFTR modulator response and CFTR mRNA quantity in patient-derived intestinal organoids.

For this study, multiple groups of intestinal organoids are used, including F508del/Class I and F508del/S1251N organoids. CFTR function is established in the presence and absence of modulator compounds using the forskolin induced swelling (FIS) assay. FIS measurements are performed at three moments, whilst organoid lysates are harvested for RNA extraction and analysis at the same time as organoid seeding for the FIS assay, allowing for the generation of paired samples. Haplotypes are generated from the same intestinal organoids cultures using targeted locus amplification (TLA) technology for the in depth DNA analysis.

Here, the TLA protocol optimised for the studying of the CFTR gene from intestinal organoid samples is presented, showing the ability to map the complete >200 kb-sized CFTR gene in an allele specific manner using only 0.5 -- 2 $x10^6$ cells as starting material. Our first results reveal significant diversity between CFTR alleles, in addition to associations between TLA-defined CFTR haplotypes and mRNA expression and functional response. This data shows that TLA-based sequencing can be used to for detailed characterisation of CFTR haplotypes that can help increase understanding of genotype-phenotype relations in people with CF.

P7

Linked-read sequencing identifies the complex variation at the chromosome 7 trypsin locus contributing to cystic fibrosis comorbidities

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Conventional whole genome sequencing applications ignore the phase of heterozygous alleles. Phase is critical for interpretation of *cis*-effects of rare disease-causing mutations and of more common regulatory variants. Here we investigate the quality of read-based phasing achievable with different sequencing technologies, prioritize linked-read technology as the most informative and assemble the highest quality phase set for the commonly used public benchmark genome HG002. Using the linked-read technology we then sequenced 477 individuals with Cystic Fibrosis (CF) and leverage the phase information (phase block N50=4.5Mb) to fine map *CFTR* and the chr7q35 genome-wide association study (GWAS) modifier gene locus for meconium ileus in CF near the trypsinogen genes *PRSS1* and *PRSS2*. This region is challenging for standard high-throughput sequencing analysis due to the presence of five highly similar trypsinogen paralogs, a common 20kb deletion and assembly differences between human reference genome versions. Phasing and fine mapping demonstrate that the common deletion haplotype and a missense variant p.Thr8Ile (rs62473563) both associate with increased meconium ileus risk in CF and *PRSS2* expression, explaining the GWAS locus. Linked-read sequencing provides for improved fine-mapping and phasing, enabling understanding of causal variation at GWAS loci where multiple variants impact complex traits, such as in CF. This work will have implications for the genetic basis of CF-related diabetes and non-CF pancreatitis to which this locus has also been reported to contribute.

P9

Study of genetic variants influencing CF penetrance using hIPSCs

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More than half of the lung phenotype is not due to mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene but to the environment and genetic modifiers. The latter has recently gained interest as they could explain in part the variability of responses to treatments observed between CF patients carrying the same CFTR mutations. Genome-Wide Association Studies (GWAS) have identified a diversity of Single Nucleotide Polymorphisms (SNPs) likely to impact epithelial cell function, disease penetrance, and patient responsiveness to therapeutics. However, the mechanisms by which these genetic variants can alter the functioning of the lung epithelium remain to be fully uncovered. We decided to explore specifically an epithelium-specific gene of one of the outlined regions, region 11q13, named ETS Homologous Factor (EHF). Here, we used human induced Pluripotent Stem Cells (hiPSC)- derived Airway Epithelial Cells (AECs) to look at the role of EHF in the airway epithelium.

Briefly, hiPSCs were differentiated into definitive endoderm, anterior foregut endoderm, and lung stem cells. On Day16, $CD47^{HI}/CD26^{-}$ cells were sorted and cultured as organoids in 3D culture conditions to obtain mature basal cells. NGFR+ cells were then sorted and seeded in Air-Liquid Interface (ALI) conditions. This condition allows the generation of a polarized pseudostratified epithelium containing basal cells (TP63, NGFR), goblet cells (MUC5AC, MUC5B), club cells (SCBG1A1, SCBG3A2), and ciliated cells (FOXJ1, CFTR) with motile cilia.

Using this model, we first assessed the expression of EHF among other candidate genes. These experiments revealed high and airway-specific expression of EHF, thereby suggesting an important function for this transcription factor in the airway epithelium. We proceeded to knock out EHF in hiPSCs using CRISPR/Cas9. Knock-out efficiency was confirmed both at mRNA and protein levels. Interestingly, EHF-/- AECs had lower goblets cells markers (MUC5AC, SPDEF) and higher expression of the proliferation marker Ki67. In addition, cilia motility and synchronism were affected in the absence of EHF assessed by microscopy. Moreover, preliminary data indicated that HIF-dependant response to hypoxia was decreased in EHF-/- cells. Taken together these results suggest a key function for EHF in the functioning of lung epithelium, especially in stress-response.

To conclude, we developed a new platform to investigate the importance of genes potentially associated with genetic variants influencing CF lung disease. Of particular interest, we showed that EHF could be involved in the regulation of goblet cells production, cell proliferation, cilia motility, and response to stress. These findings could pave the way to finding new approaches for personalized treatments.

P10

Development of a high throughput functional screen allows drug repurposing and reveals novel drug candidates for rescuing CFTR function in patient-derived organoids with nonsense mutations

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Recent therapies enable effective restoration of CFTR function of the most prevalent F508del CFTR mutation, and shift the clinical unmet need largely towards people with rare CFTR mutations such as nonsense mutations. Approximately 10% of the worldwide CF population carry premature termination codons (PTC) or nonsense mutations, resulting in production of truncated CFTR protein.

Previous research identified compounds with read-through (RT) activity that result, via various mechanisms, in introduction of an amino acid at the PTC site. At present-day only one RT compound has reached clinical trials, a chemically-engineered aminoglycosides derivative termed ELX-02 (NB124; Eloxx Pharmaceuticals). Recent results from ELX-02 clinical trials however show only a minor decrease in sweat chloride levels in CF patients homozygous for the G542X PTC mutations. Altogether, this underlines the need for continuing the search for novel CF therapeutics. Drug repurposing of FDA approved compounds could potentially aid in characterizing novel therapeutic options for individuals with CF. Drug repurposing is a strategy to identify new indications for approved or investigational drugs that are outside the scope of the original medical indication. It is particularly relevant for individuals with rare diseases for who limited treatments are available due to the economic and technical complexities of drug development for small populations.

A prerequisite for drug repurposing, is that the exploited assay is robust as well as clinically relevant. CFTR function measurements in patient-derived intestinal organoids associate with clinical features of cystic fibrosis and may enable drug repurposing in a personalized setting. We upscaled the previously described forskolin induced swelling (FIS) assay to a 384 WP format, allowing medium-to-high throughput assays. We exploited patient-derived organoid lines with one of the most prevalent nonsense mutations, W1282X/W1282X, to screen a library of FDA-approved compounds. We first characterized toxicity of 1443 FDA-approved compounds, after which we assessed the effect of the non-toxic compounds on CFTR mediated fluid secretion. We show that the 384-wells FIS-assay is reproducible, has a large wide dynamic range and comparable responses were obtained as with the conventional 96-well based setup. Similarly to CFTR function, compound-induced toxicity was quantified in a robust way and 43-1440 were excluded for future experiments. Results of the drug repurposing FIS assay identified 19 potential inducers of organoid fluid secretion. The top 5 hits were further characterized in a secondary screen and identified members of the statin family as compounds that can rescue CFTR function. Statins are commonly used medicines to lower blood pressure by inhibiting HMG CoA reductase and have not elaborately been described to affect CFTR function. Additionally, they are reported as inhibitors of AKT. We are currently performing experiments to further clarify the mode of action of statin family members in the aspect of increase of CFTR function in PTC organoid lines.

In brief, our study exemplifies the feasibility of large-scale compound screening with a functional read-out using patient derived organoids. Furthermore we show a not earlier described interaction between CFTR and members of the statin family in patient-derived organoids harbouring nonsense mutations.

P11

Functional characterization of rare CFTR mutations and response to modulators is best assessed in both cellular and patient-derived models

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Background: Among the ~2,100 CFTR mutations so far described, most are presumed to cause CF. However, the respective functional defect is still uncharacterized for a large proportion, thus posing considerable challenges in the establishment of a CF diagnosis/prognosis. Elucidation of the molecular and cellular defects associated with these mutations in patient-derived materials complemented by data from physiologically-relevant cellular models is of high relevance for prediction of disease outcomes, and also to predict effectiveness of CFTR modulator therapies.

Aim: To assess the molecular/functional CFTR defect associated with six rare mutations both in patient-derived materials and cellular models, and to determine their responsiveness to CFTR highly effective modulators.

Methods: We produced novel CFBE-based cell lines stably expressing six different CFTR variants (P5L, P205S, R334W, S945L, D1152H and M1137R) and used them to assess CFTR expression and maturation by Western blot after treatment with VX-661 alone or in combination with VX-445. CFTR function was assessed by Ussing chamber. Additionally, we performed the forskolin-induced swelling (FIS) assay in intestinal organoids from individuals bearing these variants.

Results: In the absence of any compound, P205S-, P5L- and M1137R-CFTR fail to produce the fully-glycosylated mature form, being only detected the core-glycosylated immature form, being thus classified as class II mutants. In contrast, the mature form of R334W-, S945L- and D1152H-CFTR could be detected, albeit at low levels, suggesting the presence of some CFTR at the plasma membrane (PM). Traffic of P5L, P205S, and S945L CFTR mutants was rescued at low levels by VX-661 (tezacaftor) but at higher levels by VX-445/VX-661 (elexacaftor/tezacaftor). M1137R showed a significant increase in maturation only after VX-445/VX-661 treatment. Although not clearly evidencing a traffic defect, D1152H-CFTR showed an increase in maturation also only after VX-445/VX-661 incubation. Ussing chamber measurements confirmed that this rescue was associated with an increase in CFTR function. Functional assessment of R334W-, S945L- and D1152H-CFTR showed reduced function which was rescued by treatment with the CFTR potentiator genistein alone. Data from FIS analysis of organoids showed that CFTR function in D1152H/F508del organoids was rescued by VX-770 (ivacaftor) alone and that its rescue in R334W/F508del organoids was variable among the different individuals: in some, rescue was observed by VX-770 alone while in others only by VX-661/VX-770 or VX-445/VX-661/VX-770 combinations. Organoids with P5L/F508del and P205S/Y1092X genotypes were rescued by VX-661/VX-770 although a more robust rescue was observed with the VX-445/VX-661/VX-770 combination. In contrast, in S945L/F508del and M1137R/ 2789+5 G>A organoids CFTR function was only rescued by VX-445/VX-661/VX-770.

Conclusion: Studies in cell lines are relevant to determine responsiveness of CFTR modulators to rare mutations. However, distinct levels of response were observed among organoids from individuals with the same genotype. Accordingly, characterization of rare CFTR mutants in best determined by combined data from cellular and patient-derived models towards personalized therapy.

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P12

Identification of novel small molecule modulators for PTC mutations in CFTR

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Background: Premature termination codons (PTCs) are introduced into CFTR mRNA by nonsense mutations, which account for a significant proportion of total known CFTR variants (8.4%), and for which there are still no approved CFTR modulator therapies. The effect of such mutations is generally two-fold: *i)* the presence of the PTC triggers mRNA transcript degradation by nonsense-mediated decay (NMD) of the mRNA transcript [1]; and *ii)* any resulting translated protein is truncated and almost always non-functional. Such mutations are therefore associated with severe CF phenotypes, and there is thus an urgent need to find novel therapeutic strategies for individuals with CF with PTC mutations, which could include both PTC read-through agents and NMD inhibitors.

Aim: To identify novel small molecules to correct the defective processing of CFTR transcripts bearing PTCs, within the scope of the *PTSense* project.

Methods: A microscopy screen of compounds was performed in HEK Flp-in cells stably expressing a triple-tagged CFTR PTC mini-gene construct (mCherry G542X-Flag-CFTR-GFP cDNA with 3 introns between exons 13-15), including mCherry (red) at the N-terminus, eGFP (green) at the C-terminus, and a Flag-tag for detection of plasma membrane (PM) localization by immunofluorescence with anti-Flag antibody in unpermeabilized cells. In this microscopy assay, NMD suppression was assessed by increased red fluorescence from a basal level, and if PTC read-through occurred, enhanced green fluorescence was also detected. Two series of novel compounds (a total of 50 compounds) were synthesized[2][3] and tested alongside the SMG1i NMD inhibitor and the G418 aminoglycoside readthrough compound as positive controls.

Results: In total, among the 50 novel compounds tested, 2 enhanced PTC readthrough alone, 11 had an inhibitory effect on NMD alone, and 7 compounds had a dual effect inhibiting NMD and inducing read-through. The best 10 compounds are the subject of ongoing validation experiments in human intestinal organoids and other PTC cell models expressing a variety of CFTR genotypes.

Conclusion: We synthesized and identified a number of novel small molecules as potential PTC/read-through modulators in a unique cellular model of CFTR PTC mutations. The novel compounds promoted PTC readthrough and/or inhibition of the associated NMD. Ongoing studies are focused on their validation, their specificity and applicability to other PTC mutations in other cell models of CF.

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P13

Effects of elexacaftor/tezacaftor/ivacaftor therapy on CFTR function in patients with cystic fibrosis and one or two F508del alleles

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Objectives: The CFTR modulator combination elexacaftor/tezacaftor/ivacaftor (ELX/TEZ/IVA) was shown to improve clinical outcomes and sweat chloride concentration (SCC) in patients with cystic fibrosis (CF) and one or two F508del alleles. However, the effect of ELX/TEZ/IVA on CFTR function in the airways and intestine has not been studied. We, therefore, performed a real-world multicenter study to assess the effect of ELX/TEZ/IVA on CFTR function in airway and intestinal epithelia in patients with CF and one or two F508del alleles aged 12 years and older.

Methods: This prospective observational multicenter study assessed clinical outcomes including FEV_1 %predicted and body mass index, and the CFTR biomarkers SCC, nasal potential difference (NPD) and intestinal current measurement (ICM) before and 8-16 weeks after initiation of ELX/TEZ/IVA.

Results: A total of 107 patients with CF including 55 patients with one F508del and a minimal function mutation and 52 F508del homozygous patients were enrolled in this study. In patients with one F508del allele, NPD and ICM showed that ELX/TEZ/IVA improved CFTR function in nasal epithelia to a level of 46.5% (IQR, 27.5-72.4; P<0.001) and in intestinal epithelia to 41.8% of normal (IQR, 25.1-57.6; P<0.001). In F508del homozygous patients, ELX/TEZ/IVA exceeded improvement of CFTR function observed with TEZ/IVA and increased CFTR-mediated Cl^- secretion to a level of 47.4% of normal (IQR, 19.3-69.2; P<0.001) in nasal and to 45.9% (IQR, 19.7-66.6; P<0.001) in intestinal epithelia.

Conclusion: Treatment with ELX/TEZ/IVA results in effective improvement of CFTR function in airway and intestinal epithelia in patients with CF and one or two *F508del* alleles.

P14

Proof of concept of ionocytes' CFTR content as a novel biomarker for cystic fibrosis diagnosis and follow up

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Despite the huge progresses made in cystic fibrosis (CF) knowledge and care, the exact relationships between primary defects and different manifestations of the disease are still poorly understood. Newborn bloodspot screening (NBS) is an effective strategy for the early recognition of infants with CF. However, increasing number of infants with a positive NBS result have an inconclusive diagnosis (CRMS/CFSPID). Nevertheless, CF still is a diagnostic and therapeutic challenge in the case of rare variants that are associated with varying symptoms and are unlikely to enter clinical trials. Therefore, it is important to identify precise and disease-relevant biomarkers allowing a better understanding of CFTR mutations effects in vivo, and also useful as outcome parameters to accurately quantify the rescue of CFTR by novel modulators.

In this context, the recent identification of the CFTR-rich airways ionocytes could highlight novel possibilities to monitor CFTR in CF patients' airways. Despite their low quantity, ionocytes express the highest levels of CFTR per cell and are relatively abundant in the upper airways. These findings suggest that quantitative analysis of CFTR could be efficiently done in these cells, that can be collected by the minimal invasive nasal brushing procedure.

As a proof of concept, we investigated the genotype-phenotype-ionocytes' CFTR content relationships in a small cohort of non-CF, CF, and CRMS/CFSPID subjects. Moreover, we collected nasal cells from CF patients treated with ELX/TEZ/IVA (pre- and 30 days post-treatment). Ionocytes' CFTR content was evaluated by immunofluorescence detection of CFTR and FOXI1 (as a ionocytes marker) combined with confocal imaging and analysis of the intensity of the CFTR signal in the apical membrane relative to that in the intracellular compartments. In accordance with genotypephenotype, we found increasing levels of ionocytes' CFTR content in the following groups: CF patients with severe mutations < CF patients with mild mutation/CRMS/CFSPID < CF patients treated with ELX/TEZ/IVA < non-CF individuals. This finding suggests that this type of analysis could be useful to investigate genotype--phenotype correlation and, possibly, the efficacy of CFTR pharmacotherapies. In addition, analyzing CFTR activity in airways ionocytes will be an important future step to better clarify their role in airways epithelium.

P15

Large-scale production of 3D airway organoids derived from submerged differentiated nasal epithelia and validation of CFTR modulator responses

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Introduction: Airway organoids may potentially act as cystic fibrosis (CF) disease model in which novel therapies can be identified in high-content screening assays. However, a major limitation is the lack of a cost-efficient way to produce airway organoids at a large scale. For this reason, we have developed a procedure that enables bulk generation of airway organoids derived from 2D submerged differentiated airway epithelial monolayers.

Methods: Nasal brushing-derived airway basal stem cells from healthy control subjects and individuals with CF (F508del homozygous) were first expanded in conventional cell cultures using feeder-free and growth factor defined culture conditions. Next, stem cells were cultured in plastic culture dishes as confluent monolayers and differentiated at submerged conditions. To generate airway organoids, epithelial fragments from differentiated monolayers were subsequently cultured in matrigel droplets or matrigel suspension cultures using spinner flasks. Airway organoids transferred into 96 well plates were used to validate CFTR function and modulator responses in a forskolin-induced swelling assay.

Results: Nasal brushing-derived airway basal stem cells were well differentiated in submerged cultures, based on the expression of ciliated and secretory cell markers. This was determined by immunofluorescence, quantitative PCR, and RNA sequencing. Airway organoids derived from epithelial monolayers, cultured either in matrigel droplets or suspension cultures, displayed a persistent differentiated phenotype based on immunofluorescence staining and quantitative PCR of epithelial differentiation markers. From a single epithelial monolayer we were able to generate organoids to test 288 independent conditions in a 96 well plate format. CFTR function in organoids from healthy control subjects and CFTR modulator responses (VX-809/VX-770, VX-661/VX-770, and VX-661/VX-445/VX-770) in organoids from individuals with CF could be quantitated in forskolin-induced swelling assays.

Conclusion: We provide a scalable method of generating mucociliary differentiated airway organoids from nasal brushing-derived epithelial cells. Large-scale produced airway organoids may potentially be further applied in high content screening assays to characterize CFTR targeting drugs. In follow up studies this will be validated in forskolin-induced swelling assays conducted in a 384 well plate format.

P16

Trikafta™ corrects function of H1085R-, N1303K- and R334W-CFTR and improves clinical status of patients

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Introduction: The next generation CFTR modulator TrikaftaTM is the combination of correctors (elexacaftor and tezacaftor) with potentiator (ivacaftor). So far TrikaftaTM has been approved in France for patients carrying at least one F508del-CFTR allele (p.Phe508del). We aimed to study the efficacy of Trikafta™ to correct activity of non-F508del-CFTR variants with processing defects in the human nasal epithelial (HNE) primary cell model.

Methods: CF patients with severe respiratory insufficiency were recruited for HNE sampling with following genotypes (N1303K/N1303K, M1T/R334W N1303K/H1085R (n=2)). HNE were amplified with conditional reprogramming and differentiated in air-liquid interface (ALI) conditions. To assess CFTR function, the short-circuit current measures were performed on ALI HNE cultures treated with DMSO or Trikafta (elexacaftor and tezacaftor at 3 µM and ivacaftor at 100 nM) for 48h.

Results: Trikafta™ significantly corrected N1303K-CFTR and R334W-CFTR chloride secretion activity up to 5.2% (range 4.7% - 5.5%) and up to 10% (range 8.5% - 11.8%) of the WT-CFTR function. The most spectacular, significant increase of CFTR-dependent chloride secretion was measured in N1303K/H1085R cells, which reached the level over 63% (range 57% - 69%) of WT-CFTR activity. Bicarbonate transport in the M1T/R334W patient's HNE cells was improved up to 13% and in N1303K/H1085R cells up to 60% (range 40% - 70%) of WT-CFTR level.

All these patients improved at short time FEV1 by a mean of 40% (range 20% - 67%), and body weight by a mean of 4.3 kg (range 1.5 kg - 7.4 kg). Sweat chloride was significantly decreased by a mean of 65 mmol/l (range 56 - 74 mmol/l) apart the N1303K homozygous patient.

Conclusion: Trikafta™ significantly improves CFTR activity of N1303K, H1085R and R334W variants.

P17

Novel CFTR modulator combinations maximize functional rescue of G85E and N1303K in rectal organoids

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Objectives: CFTR modulators (correctors/potentiators) have been developed to overcome molecular defects associated with *CFTR* mutations. These have been divided into different types to reflect the distinct mechanisms of action of different modulators. The well-established forskolin induced swelling (FIS) assay in patient-derived rectal organoids allows to assess responses of CFTR mutants to the different types of CFTR modulators. By combining investigational and market approved CFTR modulators, we aimed to maximize functional rescue in G85E or N1303K organoids, refractory to iva-, luma- and tezacaftor treatment.

Methods: FIS analysis was performed with brightfield microscopy, allowing both 1h and 24h follow-up. Rectal organoids from patients with G85E, N1303K, and F508del mutations were treated with combinations of type I/II/III correctors tezacaftor (3 µM)/corr4a (5 µM)/elexacaftor (3 µM) or DMSO control for 24h prior to analysis. Type I/II potentiators ivacaftor (3 µM)/apigenin (50 µM) were added together with forskolin (0.8 µM). When potentiator activity of elexacaftor was investigated, it was also added together with forskolin. Area under the curve (AUC) of relative organoid area was determined to quantify CFTR function. Corrector activity of elexacaftor (and other correctors) was evaluated by determination of expression of 3HA-tagged CFTR variants at the plasma membrane by flow cytometry. Mean±SD, n=3.

Results: Corrector types I/II/III and potentiator types I/II were combined to maximize functional rescue of G85E and N1303K in patient-derived rectal organoids. For G85E, maximal rescue was observed by a combination of elexacaftor and corr4a (AUC G85E/W1282X at 1h: 3003±206). For N1303K, the quadruple combination teza-elexa-ivacaftor with apigenin was required to obtain a rescue similar to that of orkambi™-rescued F508del (AUC N1303K/N1303K at 1h: 1832±155). Elexacaftor rescued G85E and N1303K by different mechanisms, with chronic corrector effects on G85E and acute potentiation of N1303K only in the presence of ivacaftor. Synergy in N1303K rescue for iva-elexacaftor and apigenin suggests at least three potentiator mechanisms for this mutant. 24h FIS is more sensitive for the detection of small improvements in CFTR function due to the accumulation of fluid inside organoids caused by CFTR mediated ion transport. Using 24h FIS we identified ivacaftor as the main CFTR modulator for N1303K and elexacaftor and apigenin as co-potentiators.

Conclusions: Combining CFTR modulators with complementary mechanisms, such as the novel combinations tested, can further improve functional rescue of G85E and N1303K in rectal organoids, although for the latter more effective CFTR modulators are still needed. Currently, in an attempt to further enhance functional rescue of N1303K, we are testing several hits from a repurposing library screen in the 24h FIS assay.

P18

Development of molecular imaging biomarkers for assessment of CFTR localization *in vivo*

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Nuclear molecular imaging techniques as positron emission tomography (PET) and single photon emission computed tomography (SPECT) are the most sensitive imaging modalities currently available and are used clinically for the diagnosis and therapeutic follow-up in several diseases. In the field of CF, several PET and SPECT studies have been performed, mainly for the assessment of secondary pathological manifestations of lung disease and aerosol delivery, proving their applicability to CF patients.

We aim to establish an innovative molecular imaging approach to CF. This challenging objective will be accomplished through the development, followed by *in vitro* and *in vivo* biological evaluation of noninvasive molecular imaging probes for plasma membrane CFTR. Within this goal, we have previously developed a noninvasive small molecule--based radioprobe targeting plasma membrane CFTR through radiolabeling of a CFTR inhibitor with ^{99m}Tc. *In vitro* assessment of the probe's binding to wild type versus mutant CFTR showed promising results [Ferreira *et al*., 2018 ChemMedChem].

More recently, we expanded these studies with antibody-based probes to further improve the ability to target CFTR. Here we report the ongoing development of a probe for plasma membrane CFTR through the isolation of CFTR-specific antibody fragments selected by phage display technology and subsequent labeling with a useful radionuclide. First a human naïve phagemid library of single-chain variable fragments (scFvs) was panned for the isolation of plasma membrane CFTR-binding clones. After selection, expression and purification of positive clones, validation was performed through a flow cytometry study, but scFvs were not able to detect CFTR efficiently at the cell surface.

We then explored an alternative strategy for selecting improved CFTR-specific antibody fragments through the development of an immune library. Rabbits were successfully immunized with a CFTR epitope, and an antibody fragment library produced. The antibody fragment library was screened using a cell-based panning strategy, followed the analysis of the positive clones using cell-phage Elisa and final validation by cell-phage flow cytometry. These validated clones are currently being cloned in an appropriate expression vector. Next, the antibody fragments will be radiolabeled with ^{99m}Tc and their ability to detect CFTR at the cell surface, both wt and rescued F508del, will be assessed first in human bronchial epithelial cells and then in animal models. These noninvasive molecular imaging probes will have the potential to be a useful imaging biomarker in the assessment of early therapy response in drug evaluation.

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P19

Assessment of the triple CFTR modulator combination in rectal organoids

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Background: Clinical trials have shown superiority of teza-/elexa-/ivacaftor (Kaftrio) in patients with CF (pwCF) with ≥1 F508del mutation, leading to EMA approval. Rectal organoids have seen increasing use as a patient-based *in vitro* model for modulator response, and showed promising correlation with clinical response. This personalised medicine approach could guide decisions about CF treatment, making it more cost-efficient and preventing unnecessary treatment.

Aims: To compare responses to different modulator treatments in rectal organoids obtained from F508del homozygous (F/F) pwCF.

Methods: A forskolin-induced swelling (FIS) assay was used to quantify responses in organoids from 43 F/F pwCF to the following combinations: luma-/ivacaftor (Orkambi), teza-/ivacaftor (Symkevi), and teza-/elexa-/ivacaftor (Kaftrio). Organoids were incubated for 18-24h with correctors (luma-, teza-, elexacaftor), while the potentiator ivacaftor was added acutely. Mean swelling responses with 0.8 µM forskolin were compared between patients.

Results: The FIS response to Orkambi was correlated with the response to Symkevi but consistently higher. The response to Kaftrio was also correlated with the response to Orkambi and Symkevi, but consistently higher. Patients were thus either high or low responders to modulator combinations. Organoids incubated with elexacaftor showed swelling before addition of forskolin and ivacaftor. Each condition was tested twice in each assay, and three assays were performed for every patient.

Conclusions: Rectal organoids of F/F pwCF showed increased response to Kaftrio when compared to Orkambi and Symkevi. Orkambi was superior to Symkevi. Patients were either high or low responders, which might be explained by other variants in the *CFTR* gene or modulator genes. This will be further explored through DNA and RNA analysis, western blotting, and functional CFTR assays. The swelling seen with elexacaftor before addition of forskolin or ivacaftor is probably due to its potentiator effect, and will be further examined with rectal organoid morphology analysis (ROMA).

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P20

Effects of ivacaftor therapy confirm the results of theratyping using rectal and nasal epithelial cells of a CF patient carrying the ultra-rare CFTR genotype W57G/A234D

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Background: The effects of CFTR modulators on the rare cystic fibrosis (CF) causing variant W57G (c.169T>G), *in trans* with A234D (c.701C>A) is unknown. We characterized in rectal organoids (colonoids) CFTR translation and function and the *in vitro* and *in vivo* response to CFTR modulators.

Methods: Colonoids were developed from rectal biopsies and CFTR function was measured by forskolin-induced swelling (FIS) assay (3D) and by short-circuit current (Isc) in 2 dimensional (2D) rectal organoids. Nasal epithelial cells derived from nasal brushing. CFTR expression was analyzed by Western blotting. The patient was treated with ivacaftor, off-label. CFTR dependent sweat was tested by optical beta adrenergic test and chloride sweat concentration was determined following the Gibson and Cooke method.

Results: FIS and short-circuit current (Isc) measurements indicated the presence of a residual CFTR function. FIS detected a strong increase by treatment with the CFTR potentiator ivacaftor (VX-770, Vertex Pharmaceuticals) and the corrector tezacaftor (VX-661, Vertex Pharmaceuticals). The combination with elexacaftor (VX-445, Vertex Pharmaceuticals) increased the swelling, also in the absence of forskolin treatment. CFTR-mediated chloride secretion assay of colonoid monolayers indicated a residual function of 7% of wild-type (WT) CFTR, a relative enhancement of the forskolin-induced, CFTR-specific current response to VX-661 of 20% of WT CFTR reaching 28% with VX-661/VX-445 and 32% with VX-661/VX-445/VX770 combination. Increased CFTR protein levels was detected in both intestinal organoids and nasal brushing samples cultures. Patient treatment with Ivacaftor reduces sweat chloride concentration from 102 mM to 36 and 43 mM, increases the number of CFTR dependent sweat droplets from 0 to 29 and 81 after 6 and 28 days of treatment, respectively, with a 6% absolute increase of predicted FEV1% after 6 days.

Conclusion: The successful targeting of this CFTR genotype by VX-770 identifies this potentiator as a therapeutic candidate, with treatments corresponding to VX-661/VX-445±VX-770 combinations providing the maximum effect on both CFTR expression and function. Clinical data confirm the predicted response to Ivacaftor.

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P21

Functional restoration of complex CFTR allele (p.[R74W;R1070W;D1270N]) in trans with CFTR dele22_24 by CFTR modulators in colonoids

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Background: To date, CFTR modulators have been approved for therapy for the most prevalent CF-causing genotypes. Other pathogenic CFTR variants can be addressed for targeted therapies thanks to the use of in vitro models such as organoids, which have been used lately as a valuable tool for personalized medicine. In the present study, we demonstrated the effect of Ivacaftor+Lumacaftor (IVA/LUMA), Ivacaftor+Tezacaftor (IVA/TEZA) and Ivacaftor+Tezacaftor+Elexacaftor (IVA/TEZA/ELE) on rectal organoids carrying the complex allele p.[R74W;R1070W;D1270N] in trans with a type 1 mutation (CFTR dele22-24) without predicting the contribution of each mutation to the phenotypic expression. Our aim was to characterize these variants using patient-derived materials (rectal biopsies and intestinal organoids) from one CF individual and assess the functional and biochemical response to CFTR modulators.

Material and methods: This study focused on an Italian woman diagnosed as CF at age 46, carrying p.[R74W;R1070W;D1270N] / CFTR dele22-24 variants. This patient presented borderline values of sweat chloride: 43 mmol/L (Gibson & Cooke method); sweat Na:Cl ratio 0.96; compromised pulmonary function: FEV1 around 62% of predicted value; Pseudomonas aeruginosa colonization; optical ratiometric beta adrenergic sweat test in the CF range: average CFTR dependent sweat rate /methacholine induced sweat rate ratio 0.003 (± 0.003). Intestinal organoids were obtained from rectal biopsies and the rescue of mutated CFTR activity was evaluated by two complementary functional assays: 1) Forskolin Induced Swelling (FIS) assay and 2) electrophysiology measurements, using chloride-rich solution to determine the magnitude of FSK-induced short-circuit current (Isc) change in the presence and absence of modulators. CFTR correctors were pre-incubated for 24h whereas the CFTR potentiator was added in acute. Increased level of mature form of CFTR protein after treatments was detected by Western blotting.

Results: Our results show that the correction of the CFTR defect is achieved using one of the drugs combination: IVA/LUMA, IVA/TEZA and IVA/TEZA/ELE obtained with two different functional assays. Of note, FIS rate in response to IVA/TEZA/ELE was underestimated due to increase of steady-state lumen area of organoids prior to the addition of forskolin, preventing further swelling. CFTR activity measured by Isc analysis indicate a 2 to 4 times increase (DMSO-Δ_{Isc} 3±3 μA/cm^2; IVA/LUMA-Δ_{Isc} 9±5 μA/cm^2; IVA/TEZA-Δ_{Isc} 13±10 μA/cm^2 and IVA/TEZA/ELE-Δ_{Isc} 13±11 μA/cm^2), with a recovery of 9-13% of the activity of the CFTR-WT protein. The CFTR correctors also increased the production of mature form of mutated protein, with maximal expression detected by TEZA/ELE combination.

Conclusion: Altogether, these results predict a potential, significant, efficacy of treatment with IVA/LUMA, IVA/TEZA and IVA/TEZA/ELE, with the latter combination being more effective, based on the recovery of CFTR function on intestinal organoids derived from this patient.

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P22

Complex CFTR allele p.(Phe508del;Leu467Phe) and the altered in vitro response to CFTR modulators

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Routine CF diagnostic testing aims to identify two CF causing *CFTR* variants, each present in *trans* on both parental alleles. The most common mutation is p.Phe508del and if found in homozygosity, the testing is regarded as finished; thus, other *CFTR* variants which in theory might be present in position *cis* can be left undetected.

We hypothesize that full understanding of intra-*CFTR* genetic background is becoming increasingly important for CFTR modulators (CFTRm) therapies since additional variants beyond p.Phe508del / p.Phe508del genotype might modify basal treatment response, predicted simply on the presence of both copies of p.Phe508del. In this context, we present *in vitro* treatment response to CFTRm using forskolin induced swelling (FIS) assay in intestinal organoids of two p.Phe508del homozygous patients, carrying the complex allele p.(Phe508del;Leu467Phe). The rescue of the CFTR function is expressed as AUC at 0.128 μM concentration of forskolin; for both patients we observed minimal rescue of the CFTR function by TEZ/IVA (AUC 184 and 373), and the significant CFTR rescue by ELX/TEZ/IVA (AUC 2619 and 3362).

These data document that the CFTR variants beyond p.Phe508del on the same allele might affect the efficacy of individual CFTRm therapies. While p.Phe508del / p.Phe508del shall be the genotype that is in principle responsive to TEZ/IVA, the additional variant (p.Leu467Phe in our case) minimized its *in vitro* effect. We underline the importance of establishing the intra-*CFTR* background in patients indicated for CFTRm based on their conventional genotype, either *a priori* or at the moment when a patient does not response to the CFTRm therapy as expected.

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P23

Theratyping of the CFTR variant L227R (+/+) in rectal organoids predicts marginal response to CFTR modulators in vivo

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Background: Stem cell-based organoids permit to expand treatment eligibility for patients with rare or very rare CFTR variants by predicting in vitro patient's response to drugs. Here we compared the effects of therapy with CFTR targeted drugs with *in vitro* modulation of CFTR function in L227R+/+ rectal organoids (colonoids).

Material and methods: Colonoids were developed from rectal biopsies of a 14 years old male CF patient of Tunisian origin homozygous with the CFTR variant L227R. He had abnormal sweat Cl concentration at diagnosis: 104 mmol/L and later in 2020: 89 mmol/L; pancreatic insufficiency; chronic lung infection (Staphylococcus aureus, Escherichia coli); impaired lung function: FEV1: 37 % and severe malnutrition: BMI 13.9 kg/m2. This variant belongs to class II, that leads to a severe structural conformation defect of the CFTR protein. CFTR function assays were performed in 2D undifferentiated colonoids cells grown in Transwell® and mounted in Ussing chambers, in order to determine the magnitude of forskolin-induced short-circuit current (Isc) changes. The effects of CFTR modulators (correctors: tezacaftor, VX-665; lumacaftor, VX-809; elexacaftor, VX-445;potentiator: ivacaftor, VX-770) in 2D CF colonoids were compared with those obtained in 3D organoids using forskolin induced swelling (FIS) and immunoblotting assays.

Results: Measurement of transepithelial current performed in colonoids (L227R +/+) indicated thepresence of minimal function of the mutated CFTR protein. The pre-incubation with CFTR modulators 3μM VX-809 or 3μM VX-661/3μM VX-445 enhanced CFTR-dependent currents up to 4 times (from 0.5 ± 0.33 μA/cm2 recorded for untreated filters up to 2.4 ± 0.87 μA/cm2 seen on treated filters). FIS assays showed similar results with VX-809 or VX-661/VX-445, both used in combination with the CFTR potentiator VX-770 (Ivacaftor), producing a slight but significant increase (16 to 28% maximum) in the lumen area of the organoids in response to forskolin (5μM) in comparison to control (DMSO). The recovery of mutated CFTR, however, represents less than 5% of WT protein activity compared to a non-FC sample based on Isc analysis. Protein expression analysis indicates that this genotype expresses a low level of CFTR protein, which was slightly increased by treatments with VX-809 and VX-661/VX-445. After treatment for 3 months of the patient with ivacaftor+lumacaftor sweat chloride concentration was 75 mmol/L, BMI 14.09 kg/m2 and FEV1 33%.

Conclusions: Data obtained from 2D colonoids in Ussing chambers, overlapping with 3D rectal organoid FIS assay, predicted marginal benefit of the treatment that was confirmed in vivo.

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P24

Functional characterization of the rare V317A CFTR variant using ex vivo models

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Mutations of the *CFTR* gene affect the encoded protein through a variety of molecular mechanisms, leading to different functional defects. Until now,more than 2000 mutations have been identified. Some variants are well studied and may act by a promiscuous mechanism, as in the case of F508del, the most frequent in cystic fibrosis (CF) patients, causing both defective maturation and gating. On the contrary, more than a hundred have not been yet characterized, also due to their low frequency.

Understanding the functional consequence of rare CFTRvariantsis essential for the adoption of precision therapeutic approaches for CF.

In the frame of a project aimed to characterize rare CFTR mutations, we focused on a patient compound heterozygous forthe V317A/G542X variants. This provides the ideal setting to perform a functional studyof the V317A variant, never characterized before, sincethe G542X mutation is essentially a null-allele.

V317A is not present in the CFTR2 database and is reported in ClinVaras variant of uncertain significance, due to the lack of information. By using the anti-SMAD protocol, we cultured patient's nasal epithelial cells derived from nasal brushing and we developed differentiated epithelia in air-liquid interface. We then performed short-circuit current measurements by Ussingchamber to evaluate CFTR-dependent Cl- secretion in patient-derived nasal epithelia. In parallel, we also exploited heterologous expression systems to further investigate V317A-CFTR expression pattern and activity. To this aim, CFBE41o- cells were transiently transfected with vectors carrying CFTR-WT or CFTR-V317A cDNAs. Mutant CFTR function was measured byfluorimetric assay based on the halide-sensitive Yellow Fluorescent Protein (YFP), and expression pattern was evaluated biochemically, by SDS-PAGE/Western Blot analysis.

The electrophysiological analysis by Ussing chamber showed that, in nasal epithelial cells treated with vehicle alone (DMSO), there is a marked cAMP-dependent, CFTR-mediated current, further increased by the addition of VX-770 or other potentiators.Treatment with CFTR correctors such as VX-809 or VX-661 did not increase the total current elicited in the presence of a cAMP agonist plus VX-770. However, and quite interestingly, in epithelia treated for 24 h with the VX-445/VX-661 combination, the CFTR-dependent current elicited by the cAMP agonist was significantly increased, with no further effect following addition of a potentiator. Total CFTR-mediated activity measured following acute treatment with VX-770 or 24 h treatment withVX-445/VX-661 was similar to that measured in nasal epithelia derived from non-CF subjects.

Functional and biochemical analysis performed on transiently transfected CFBE41o- cells confirmed that V317A-CFTR is normally expressed as the mature, fully glycosylated form, but it displays defective activity that can be rescue by a potentiator.

Taken together, our results suggest that patients carrying this gating variant might benefit from treatment with a potentiator drug.

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P26

Structural plasticity of the Nucleotide Binding Domain 1 (NBD1) of CFTR is linked to pathogenesis of cystic fibrosis

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Cystic fibrosis (CF) is the most common lethal genetic disease in Western countries. It is due to mutations in the gene coding for the cystic fibrosis transmembrane regulator (CFTR), a chloride channel required for proper fluid balance in many tissues (lungs, digestive organs, etc.). In over 80% of the CF cases, the disease-causing mutation is the deletion of the phenylalanine 508 (F508del), located in the nucleotide binding domain 1 (NBD1) [1]. In our recent published study, a novel conformation of NBD1 (called the b-SS conformation) was captured using a conformation-specific nanobody (an antibody fragment recognizing specific conformations). This b-SS conformation and the canonical conformation of NBD1 (the conformation previously described in the literature) are ruled by an equilibrium tightly correlated to stability and function (destabilizing conditions such as F508del mutation favour the b-SS conformation). This study indicated that the transition between the conformations is an Achille's heel in the structural landscape of the protein and enables protein unfolding by the prevalent pathological mutation F508del [2].

The present research focused on investigating if this correlation between plasticity and pathology is unique for F508del or also observed for other disease-causing mutations of NBD1 as this observation could lead to the development of therapies common to several untreated NBD1 mutations.

To measure if rare mutants of NBD1 shifted the equilibrium towards the b-SS states, we followed the conformational landscape of NBD1 by single molecule fluorescence (smFRET), specifically the transitions between the b-SS and the canonical conformation. In addition, we used Thermal Shift Assay (TSA) to test the thermostability of those mutants with different conditions known to affect the equilibrium and stability of F508del such as ATP, mutations, or a promising stabilizing nanobody. Finally, we crystalised some mutants to look for any structural changes.

We show that all mutations tested diminished the canonical population in FRET measurements. TSA analysis showed that all the mutants are thermally destabilized and can be stabilized with the same conditions as for F508del. Using a series of DRI variants we showed by X-ray crystallography that the mutations do not prevent folding of the domain. Therefore, we conclude that, mutations studied in NBD1, enable the premature unfolding of the protein not by disrupting structural elements but rather by perturbing structural transitions between the canonical and the b-SS states. This work provides a new framework to design conformation-based therapeutics for several mutations lacking any treatment so far.

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P27

A rare mutation found in Portuguese individuals with cystic fibrosis (I148N) responds to approved CFTR modulators

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Background: To date, about 2,100 genetic variants were reported in the *CFTR* gene, most presumed to cause Cystic Fibrosis (CF). Elucidation of the molecular and cellular effects caused by CFTR mutations is relevant to understand disease pathophysiology, but also for genetic counselling, to predict disease severity and to decide on the most adequate CFTR-modulator therapeutic strategy for individuals with CF.

Aim: The aim of this work was to confirm/exclude a diagnosis of CF in individuals that: 1) have a diagnosis of CF but do not have both CFTR mutations identified; 2) have one or two CFTR mutations of unknown disease liability; 3) have a 'non-classical' presentation of CF; or 4) are asymptomatic but have a positive result in newborn screen. In case new mutations were identified, our goal was to characterize their molecular and functional effect on CFTR expression/function.

Methods: In total, 34 individuals with a suspicion of CF were recruited for this study, of which 30 were selected for *CFTR* gene next generation sequencing (NGS). In cases with a strong suspicion of CF, when NGS failed to identify a second *CFTR* mutation, CFTR function was assessed by Ussing chamber in rectal biopsies. To study novel CFTR mutations, CFBE cell lines stably expressing such CFTR variants were generated. Intestinal organoids were also generated and analysed by forskolin-induced swelling (FIS) assay with and without the following CFTR modulators: potentiator VX-770 alone or with correctors VX-445 and/or VX-661.

Results: So far, this project already established a diagnosis of CF in five individuals and excluded in eight. NGS identified the following mutations: I148N, c.2470dupA, c.3321dupT, M1137V, E528E, A120T, G576A and R668C. A previously uncharacterized mutation, I148N, was identified in five individuals (14.7% of our cohort) who had CF symptoms. CFBE cells expressing the I148N-CFTR mutant show that it causes a traffic defect and reduces CFTR function to 5% of wt-CFTR. Incubation with CFTR modulators, VX-661 or VX-445/VX-661, rescued I148N-CFTR plasma membrane (PM) traffic and function to ~50% of wt-CFTR, after both treatments. In rectal biopsies from 3 individuals with I148N (two twins P205S/I148N, and one with F508del/I148N), we detected residual function of 12%-33% of wt-CFTR, leading to a diagnosis of "atypical" CF, consistently with their clinical phenotype. For the F508del/I148N individual, FIS confirmed the results in the CFBE cell line, by showing that VX-661/VX-770 and VX-445/VX-661/VX-770 rescued CFTR, indicating that this individual could get potential clinical benefit from both the double and the triple modulator combinations.

Conclusion: Our results show that I148N is a CF-causing mutation impairing both CFTR PM traffic and function, albeit partially and that it is rescuable by approved CFTR modulators. These results also reinforce the importance of testing approved CFTR drugs in rare mutations.

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P28

Antisense oligonucleotide splicing modulation as a novel cystic fibrosis therapeutic approach for the W1282X nonsense mutation

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Objective: An important goal of the CF research and care community is to provide CFTR-based therapies to every individual with CF. Despite enormous progress, CFTR modulators cannot assist all patients with CF and are suitable only for some subgroups of them. Therefore, further strategies of drug development are essential to address the unmet needs of other patients with non-responsive mutations. These mutations include stop mutations as well as different missense mutations leading to folding defects. SpliSense aims to address the unmet need of a specific sub-group of CF patients carrying the W1282X CFTR mutation, a stop mutation located at exon23 of the CFTR gene associated with severe CF phenotype.

SpliSense develops SPL23-2 as an ASO treatment for Cystic Fibrosis patients carrying the W1282X CFTR mutation by inducing skipping over exon 23 of the CFTR transcript. Skipping over exon 23 eliminates the Premature Termination Codon generated by the W1282X mutation and avoids RNA degradation induced by the Nonsense Mediated Decay (NMD) mechanism, allowing the production of active CFTR proteins lacking exon 23.

Results: Cumulative results indicate that SPL23-2 MoA, leads to the production of mature CFTR proteins. These proteins, missing exon 23, are the molecular basis for the functional response measured by the Ussing Chamber experiments following the addition of the CFTR modulators. Further analysis in HNE cells from several W1282X patients on different genetic backgrounds (heterozygous and homozygous) revealed that treatment with SPL23-2 leads to significant levels of CFTR activation reaching ~40% of WT. This level of activity in primary respiratory cells predicts a clinical benefit to patients.

Conclusion: These results highlight the potential therapeutic and clinical benefit of ASO-based exon 23 skipping SPL23--2, supplemented by CFTR modulators, for CF patients carrying the W1282X nonsense mutation aiming to enable the initiation of clinical study by next year.

P29

Rescue of rare CFTR trafficking mutants highlights a structural location-dependent pattern for correction

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Among the more than 2,100 variants in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, more than 1,000 are very rare being present in less than 5 individuals worldwide. Approval of modulators that act on mutant CFTR protein, correcting its molecular defect thus alleviating disease burden, revolutionized the CF field. However, these drugs do not apply to all CF patients, especially those harboring rare mutations -- for which there is a lack of knowledge on its molecular mechanisms of disease and response to modulators.

Our aim here was to assess the impact of several rare putative class II mutations on CFTR expression, processing and response to modulators. Furthermore, we aimed at analyzing if mutation location would somehow influence/predict the response to the correctors.

We virally transduced the CFBE41o- parental cell line [1] to generate novel cell lines with heterologous expression of CFTR bearing the mutation of interest (generated by in vitro mutagenesis), as before [2]. We analysed a total of 11 mutations located at either Transmembrane Domain 1 (TMD1) - R75G, H139R, I148T, D192G, G194R, H199Y, V201M, and W361R - or very close to the signature motif of Nucleotide Binding Domain 1 (NBD1) - A550T, L558S, and L571S. Expression and processing were assessed by Western Blot, with or without treatment with the double corrector combination VX-661/VX-445. Mutations were also mapped to the human CFTR structure determined by cryoEM [3], grouped them as responders or non-responders to the modulators.

Results show that, for the mutations analysed, all of them impair CFTR processing (lack or severe reduction of band C -- mature form), confirming them as class II. Furthermore, considering their localization in CFTR structure, mutations located in the TMD1 respond to modulators, particularly to the combination VX-661 and VX-445, whereas those localized in NBD1 do not respond.

As we are increasing the number of variants analyzed, our study confirms the relevance of assessing the response of rare mutations to modulators (in a personalized medicine approach) and suggest the existence of a location-dependent pattern of response to modulators that may contribute to further clarify the mechanism of action of correctors.

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P30

Characterization of the [1898+3A>G;186-13C>G] complex allele by means of patient-derived nasal epithelial cells: molecular and functional analysis of CFTR mRNA and protein

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Cystic fibrosis (CF) is due to loss of function mutations of the CFTR gene. More than 2000 variants have been identified, of which 382 have been clearly defined as CF causing alleles with F508del as the most common. CF mutations can be grouped into seven classes according to the functional defect they cause. Small molecules called correctors and potentiators (CFTR modulators) have been developed to rescue the basic functional defect(s) of different variants. However, many CF patients carry poorly characterized variants (in Italy about 30%), whose response to drug treatment is yet to be defined. Moreover, the existence of complex alleles, with the presence of more than a variant in cis may significantly affect the behaviour of mutated protein thus impairing or modifying the effect of modulators.

In this work, we present preliminary data regarding a patient recruited within the frame of a wide research project aimed at defining/providing a therapeutic approach for cystic fibrosis patients with rare and poorly characterized variants.

The patient is compound heterozygous for the CFTRdele1 mutation and a complex allele [1898+3A>G;186-13C>G]. CFTRdele1 is a well-known CF-causing allele; the 1898+3A>G substitution is already reported as a splicing mutation that may generate a defective protein. However, the variant has never been studied in ex-vivo models and its possible response to CFTR modulators, never investigated in disease-relevant cells such as nasal epithelial cells derived from patients. The second 186-13C>G substitution maps in the first intron, close to the acceptor splicing site of exon 2, and is indicated as a VUS4.

Patient's cells from nasal brushing were cultured, expanded and reprogrammed to generate differentiated epithelia. Then CFTR function was evaluated in Ussing chamber upon treatment with different CFTR modulators. Cells were then harvested for RNA extraction to allow study of the CFTR mRNA.

Short-circuit current analysis highlighted a nearly absent CFTR activity under basal condition and a very small, but significant, CFTR function rescue in patient's epithelia treated with VX-661/VX-445/VX-770 combination. CFTRdele 1 is expected to behave as a null-allele, therefore we supposed that the obtained results were attributable to the protein encoded by the complex allele, which apparently could not be rescued up to obtain an acceptable level of functional correction. Further experiments to complete this characterization are presently ongoing. We are carrying on the qualitative analysis of CFTR mRNA obtained by patient's epithelia, to define the composition of the detectable transcripts, thus inform about the structure of the resulting mutated protein that will be further characterized in heterologous models. Moreover, we are also applying a minigene approach to isolate and study the intron 1 variant, to define its impact, if any, on CFTR mRNA splicing.

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P31

An optimized and protease - resistant PI3Kγ - competing peptide for inhaled therapy in cystic fibrosis

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Background and rationale: Cystic fibrosis (CF) is caused by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP (cAMP)-stimulated chloride channel. Hypofunction of this chloride channel drives airway surface dehydration causing airway obstruction and neutrophilic inflammation. Chronic neutrophilia in turn leads to high levels of proteases, in particular neutrophil elastase (NE) that, combined with mucus thickness, reduces the effectiveness of drug delivery to the lungs. The clinical approval of a number of CFTR modulators, rescuing the trafficking and gating defect of the channel, highlighted the possibility of targeting the basic molecular defect of CF, although their efficacy is unsatisfactory and 30% of total CF patients with rare mutations are not eligible for these treatments.

Hypothesis and objectives: Previously, we have demonstrated the ability of a cell-permeable PI3Kγ-derived mimetic peptide (PI3Kγ MP, patent #WO/2016/103176) to enhance cAMP levels and rescue CFTR function in F508del lung epithelial cells by interrupting the interaction between PI3Kγ and PKA. The aim of this project is to improve the stability and effectiveness of the lead molecule for inhalation therapy. Furthermore, we hypothesize that pharmacological targeting of PI3Kγ scaffold activity could be exploited to rescue the activity of CFTR in different subpopulations of CF patients, including those carrying rare mutations.

Methods: We have predicted the neutrophil elastase cutting sites within the PI3Kγ MP sequence and generated a library of modified peptides with aa substitutions in the corresponding sites. The ability of the compounds to raise cAMP levels was evaluated using the cAMP Glo Assay in human bronchial epithelial cells (HBE). Among them, one compound was selected as it showed to significantly raise cAMP levels in HBE with improved stability and effectiveness, compared to the parental peptide, PI3Kγ MP.

Results: We found that the optimized PI3Kγ-competing peptide showed improved resistance, retaining 100% of its biological function after incubation with clinically relevant concentrations of NE, and improved permeability as assessed by Parallel Artificial Membrane Permeability assay in presence of mucus of CF patients, compared to the parental PI3Kγ MP.

Moreover, the new peptide selectively disrupted the interaction between the catalytic subunit of PI3Kγ, p110γ, and PKA-RII with higher affinity than the parental compound. The optimized peptide displayed a tight binding affinity for PKA with Kd in the nM range, as assessed by steady-state fluorescence and stopped flow techniques. Moreover, the effects of new peptide on Cl- conductance were validated by short-circuit currents (ISC) measurements in primary HBE cells, where the peptide increased CFTR currents in dose-dependent manner.

Conclusions: Overall, the results of this study demonstrate that the new peptide targeting PI3Kγ represents an optimized compound that may offer unique advantages over current existing therapies for CF. Ongoing work will clarify how this peptide might be exploited to restore CFTR conductance in orphan patients who cannot benefit from approved modulators.

Exogenous overexpression systems will be exploited to evaluate PI3Kγ peptide-mediated rescue of rare class III/IV mutants exhibiting gating defects or reduced conductance, respectively.

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P32

Pharmacological rescue of nonsense mutation in CF patients: a cell-based screening strategy to identify novel compounds modulators of the NMD machinery

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There are numerous mutations that cause cystic fibrosis (CF) by impairing CFTR protein synthesis, maturation, trafficking, and/or function. There is still a substantial number of CF patients who cannot benefit from the present drugs based on CFTR correctors and potentiator. Most of these patients carry nonsense mutations (a.k.a. premature termination codons, PTCs) in the CFTR protein sequence, leading to the production of a severely truncated and non-functional protein. Pharmacological agents, such as G418 and ELX-02, act on the translation machinery of the cell inducing the read-through of the PTC. However, the efficacy of read-through agents is markedly limited by the nonsense-mediated decay (NMD) phenomenon, a cell surveillance mechanism that causes the degradation of mRNAs carrying nonsense mutations. Our work has the aim to find novel NMD inhibitors for the rescue of PTCs. For this purpose, we have stably introduced the halide-sensitive yellow fluorescent protein (HS-YFP) in the 16HBE14o- cell line carrying the W1282X mutation (kindly provided by CFF). Using this cell line and the HS-YFP functional assay, we have been able to rapidly screen a panel of pharmacological compounds including read through agents (G418, ELX-02, clitocine), NMD inhibitors (SMG1i, amlexanox, NMDI14, cardiac glycosides), CFTR amplifier, and correctors. The most effective treatment was the combination of SMG1i plus VX-445 and VX-809. The functional rescue was in agreement with rescue of CFTR protein as detected by immunoblot and immunofluorescence. We are now looking for other NMD inhibitors since SMG1i may not be suitable for applications in vivo. For this purpose, we are screening a commercially available 9,351 compounds chemical library composed by approved drugs, investigational drugs tested in clinical trials, and other small molecules active on known biological targets (mechanistic probes). The goal is to identify novel NMD inhibitors acting with a MOA different from of SMG1i. In this respect, we consider that the complex NMD machinery could be pharmacologically modulated to obtain only a partial inhibition of the NMD event, thus supporting PTC rescue but avoiding undesired effects due to the total inhibition of SMG1. Active compounds identified in the screening will be validated in secondary assays to confirm the activity and define the MOA. Finally, most promising compounds will be tested on native airway epithelial cells from CF patients.

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Detection and functional characterization of an ALU insertion in the *CFTR* gene

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Background: Rare mutations in the *CFTR* gene have been poorly studied making it difficult to determine a genotype-phenotype correlation, understand the effect of these mutations on CFTR activity, and choose possible therapeutic approaches. Furthermore, some of these mutations such as large insertion/deletions are challenging to be identified even by Next Generation Sequencing and different approaches need to be used for a correct genetic analysis. Here we report the identification and functional characterization of a rare event of an ALU insertion in the exon 15 of the *CFTR* gene.

Materials and methods: MiSeq (Illumina), Nanopore, and Sanger sequencing methods and MLPA assay were used to identify CFTR mutations. WB analysis, Fluorescence-activated cell sorting and Fluorescence based assay were used to assess CFTR processing and function.

Results:*CFTR* gene sequencing performed by MiSeq revealed any SNVs of relevant clinical significance, whereas the CNV analysis showed a warning at amplicon 2 level of exon 15 of the gene, thus suggesting a likely deletion event, not confirmed by MLPA analysis, the gold standard for gene deletions/duplications detection. At this point, the patient's diagnosis would have been closed as negative. However, a deep analysis by Sanger and Nanopore sequencing showed the insertion of an ALU sequence in the exon 15. We hypothesize that this ALU insertion caused the amplification of one of the three amplicons of exon 15 to fail, resulting in a deletion signal.

In order to evaluate the effect of the insertion on the protein structure we performed an *in silico* analysis that showed a partial missed folding of the first alpha-helix of TMD2, site of the Alu insertion. This prediction was later validated by Western Blot analysis on the CFTR recombinant protein containing the Alu element. Lastly, the functional analysis, based on halide sensitive YFP assay, was consistent with WB results as it revealed that CFTR-ALU protein is unable to function as chloride channel and it is not responsive to CFTR modulators.

Conclusion: With these data, we want to emphasize that NGS results need to be carefully interpreted and corroborated by using different approaches to have a correct genetic analysis. Finally, this study confirms the importance of functional studies in the cystic fibrosis theratyping and personalized medicine.

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Evaluation of the functional consequences of the rare I556V CFTR variant using in vitro and ex vivo models

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Cystic Fibrosis is caused by mutations in the CFTR gene encoding the CFTR channel. More than 2000 CFTR variants have been described, but pathogenicity has been demonstrated only for <400 of them. The majority of these mutations have been identified in European and North American populations, but the spectrum of these variants has not been well described in Asian populations.

In the frame of a theratyping project we recruited two subjects of Asian origin with a referred mild CF phenotype and carrying the CFTR substitution I556V (NM_000492.3: c.1666A>G), very rare in Europeans and more frequent in the Asian population. The first individual is compound heterozygous for the I566V/T1036I variants while the second for the I556V/N1303K variants. Isoleucine 556 maps in the NBD1 domain of CFTR channel. In ClinVar this mutation is predicted to be of uncertain significance with conflicting interpretations of pathogenicity. I556V has been identified in Japanese patients with infertility, in Chinese CBAVD patients, but also in French patients with asthma-like bronchopathy and chronic diarrhea. Since the molecular consequences of the I556V missense variant are still unknown, we aimed to characterize I556V-CFTR function.

Methods: Nasal epithelial cells were collected by nasal brushing, cultured using a proliferative medium, and then differentiated in air-liquid interface. Short-circuit current measurements by Ussing Chamber were performed to assess CFTR-dependent activity in the presence of cAMP agonists (resembling physiological stimuli) or following treatment with CFTR modulators. CFBE41o- cells expressing the halide-sensitive YFP were transiently transfected with vectors carrying wildtype or I556V-CFTR and CFTR function was evaluated using the YFP-based assay.

Results: The analysis of CFTR activity performed on nasal epithelia derived from the I556V/N1303K donor allowed us to study the specific contribution of I556V variant, since N1303K mutation causes severe folding and gating defects. Nasal epithelia from the I556V/N1303K patient stimulated with a cAMP agonist displayed a large CFTR-mediated current that was approximately 50% of that observed in non-CF nasal epithelia. Subsequent addition of VX-770 caused only a very modest increase in the current. Treatment with correctors for 24 hr did not change total CFTR-mediated activity. Similarly, in vitro experiments performed on CFBE41o- cells transiently transfected with I556V-CFTR showed an activity comparable to that of wild-type CFTR.

Short-circuit current analysis performed on nasal epithelia derived from the I566V/T1036I subject revealed that the cAMP agonist elicited a large CFTR-mediated current (approximately 50% of that observed in non-CF nasal epithelia). This current was further increased by VX-770 and by treatment with correctors up to 80% of the activity observed in non-CF nasal epithelia.

Conclusions: Taken together, our in vitro and ex vivo analyses suggest that the I556V CFTR variant displays an activity comparable to that of wt CFTR protein and therefore it may be considered a benign variant.

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The corrector Lumacaftor restores wild type folding of mutant CFTR helical hairpins in native-like membranes

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Recent breakthroughs in the development and approval of cystic fibrosis transmembrane conductance regulator (CFTR) modulators made causal therapy available for the majority of cystic fibrosis (CF) patients. However, occurrence of hundreds of rare mutations renders appropriate care inaccessible for many patients. With respect to precision medicine, it is of high importance to examine the impact of rare CF-phenotypic mutations on CFTR folding and processing. Investigation of the interaction of approved or novel pharmacological correctors with these individual mutations could also lead to drug repurposing. To investigate the structural effects of patient-derived mutations in different lipid environments, we scrutinized an α-helical hairpin construct derived from CFTR's membrane-spanning domain 1, encompassing transmembrane (TM) helices 3 and 4 (TM3/4) and their connecting extracellular loop. In conjunction with single-molecule FRET, circular dichroism spectroscopy and tryptophan fluorescence quenching, we probed the folding and membrane insertion behavior of mutant and wild type (WT) hairpins reconstituted in phospholipid vesicles. Hereby we focused on their interplay with cholesterol and the small-molecule corrector Lumacaftor (VX-809). We found that addition of 30 mol% cholesterol induced a modest-to-strong increase in helicity and depth of membrane insertion in all TM3/4 hairpins vs. pure phosphatidylcholine vesicles. When this set of experiments was repeated with the addition of VX-809, whether in presence or absence of cholesterol, all mutants displayed folding and helicity largely indistinguishable from the WT hairpin. Thus, VX-809 shields the protein from the lipid environment in a mutant-independent manner such that the WT scaffold prevails. Such 'normalization' to WT conformation is consistent with the action of VX-809 as a protein-folding chaperone and implies that it could efficiently aid the maturation of a large set of CFTR mutants.

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Characterization of corrector ARN23765 mechanism of action via Photo-Affinity Labeling (PAL) approach

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Cystic Fibrosis (CF) is a rare genetic disease characterized by deficiencies in the synthesis or function of the CF transmembrane conductance regulator (CFTR) anion channel, caused by mutations in the *CFTR* gene. Small-molecule compounds addressing the basic defect of the disease have been described, and are referred to as CFTR modulators. Among these, *ARN23765*, a potent F508del-CFTR corrector discovered by our group, showed high potency in rescuing the function of mutant CFTR in primary human bronchial epithelial cells from F508del/F508del CF patients.[1] CFTR correctors can act either directly by binding to CFTR or by interacting with the protein machinery responsible for protein synthesis and maturation. Indications of correctors binding to CFTR come from either indirect proofs or by experiments with purified wild type (wt) full-length protein or single domains.[2-3] No data are so far available disclosing the interaction of modulators with CFTR (either wt- or F508del mutant) in a native cellular environment.

We envisioned the *Photo-Affinity Labeling (PAL)*[4-6] approach as a suited methodology to investigate the target(s) of CFTR correctors in living cells. In this technology, a photo-affinity probe (PAP) is synthesized by incorporating in the structure of the compound under evaluation a small photo-reactive moiety and a reporter/purification tag or a chemical handle suitable for conjugation to such a tag.[7]

The photo-reactive moieties, inert under standard chemical and biological conditions, can be activated by UV light to generate highly reactive transient chemical species that crosslink in a covalent manner to bio-molecules in close proximity.[5] Probe photo-crosslinked targets can be identified with different approaches, including electrophoresis experiments, western blot and mass spectrometry studies.

In this work, we synthesized several *ARN23765*-derived PAPs and, after a preliminary evaluation of their activity in rescuing F508del-CFTR function, we used them in both cell lysates and intact cells demonstrating the binding of one of our corrector probes to wild type and mutant F508del-CFTR in living CFBE41o- cells. To the best of our knowledge, our study is the first to disclose the interaction of a corrector probe to wild type and mutant F508del-CFTR in an integral cellular setting.

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New kinase therapeutic targets for cystic fibrosis from a global functional genomics screen

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Introduction: The pharmacological management of Cystic Fibrosis (CF) has dramatically changed over the last years with the introduction of CFTR-targeting modulators. Rescue of the most common CFTR mutant (F508del) requires addressing not only its folding, traffic and stability defects, but also channel gating. In a clinical setting, these molecular detects are addressed with the combination of corrector (lumacaftor/VX-809, tezacaftor/VX-661, elexacaftor/VX-445) and potentiator (ivacaftor/VX-770) molecules. Nevertheless, these drugs do not restore clinical efficacy endpoints of CF individuals to carrier levels. We hypothesize that functional F508del-CFTR expression at the epithelial apical plasma membrane (PM) is hampered by still unknown proteins which retain it at the endoplasmic reticulum (ER). These retention factors are potential novel drug targets for CF.

Objective: To identify proteins retaining F508del-CFTR in the ER through a high-content siRNA-based microscopy screen.

Methods: A microscopy-based F508del-CFTR traffic assay [1] was used to screen Ambion's Extended Druggable Genome siRNA library (27,312 siRNAs targeting 9,128 human genes). The efficacy of the genetic knock-down (KD) of hit genes (alone or in combination with correctors) was validated by a secondary (validation) screen and by additional assays: Western blot (WB), halide-sensitive YFP (HS-YFP) quenching, forskolin-induced swelling (FIS) in intestinal organoids, Ussing chamber and patch-clamp. Gene expression was determined with RT-PCR.

Results: The primary siRNA screen pinpointed 227 genes whose KD significantly increased F508del-CFTR PM levels, of which 35 were confirmed by re-screening with stringent criteria. Knocking down 33 of the 35 hit genes significantly increased F508del-CFTR post-ER maturation, as seen in WB assays. We selected a set of 5 kinase hit genes for additional validation and mechanistic analyses. Functional F508del-CFTR rescue could be obtained by KD several of the kinase genes using multiple cellular and biochemical assays: WB to monitor CFTR maturation, patch-clamp, Ussing chamber and HS-YFP quenching to quantify ion transport, and FIS to measure ion and fluid transport in primary intestinal organoids. Rescue by kinases KD was often additive to VX-809, VX-661 and VX-445, indicating that gene KD and chemical correctors may be combined for maximal CFTR rescue. Hit kinases are not part of the CFTR interactome and have not been reported as F508del-CFTR traffic regulators before. Moreover, our data also show robust F508del-CFTR rescue through chemical inhibition of one of the kinases (1 μM) regarding both PM traffic and function. Rescue could be further increased by simultaneously adding VX-661 or VX-445.

Conclusion: Through a high-content functional genomics screen, we identified a kinase which can be genetically or pharmacologically inhibited to rescue F508del-CFTR PM traffic and function, thus emerging as a novel attractive potential novel drug target for CF.

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Rescue F508del-CFTR with nanobodies

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F508del mutation causes thermal destabilization and misfolding of CFTR that leads to its degradation and subsequent lack of cell-surface expression. The approved modulators, even the most recent Trikafta, do not improve thermal stability of CFTR and show limited effect on pulmonary function. Obviously new therapies that address the molecular origin of the pathology are required. In this context, we have developed a collection of nanobodies against NBD1 and identified a serie of high affinity binders capable to thermally stabilize full-length CFTR. These nanobodies have been extensively characterized from a biochemical and biophysical standpoint and their binding mode to NBD1 was resolved by X-ray crystallography. Single-channel patch-clamp experiments not only demonstrate that binding of stabilizing nanobodies to CFTR is compatible with channel activity but moreover that the presence of the nanobodies stabilizes F508del-CFTR over time, leading to a dramatically sustained function. When transfected in HEK293 cells, our stabilizing nanobodies promote maturation (detected by Western blot) and cell-surface expression of F508del-CFTR (detected by flow cytometry and fluorescence microscopy). This effect is highly synergistic with that of approved correctors (such as VX809, VX661 and VX445), indicating that their modes of correction are different. Using the Halide-Sensitive-YFP assay, we have investigated whether this increase in protein rescue leads to functional improvement. As for protein expression, the presence of stabilizing nanobodies leads to recovery of F508del-CFTR activity and this response is synergistic when cells are treated with approved correctors, leading to wt-like behavior. The functional effect of nanobodies was subsequently tested in a clinically-relevant model, patient-derived organoids. Using lentiviral-transduction of organoids we evaluated the effect of the nanobody expression on the recovery of CFTR activity using the forskolin-induced swelling assay and observed a robust recovery of channel function. The combination of corrector and nanobodies show a sustained recovery that far excelled the effect of the corrector or the nanobodies alone. This indicates that NBD1-stabilizing nanobodies may provide a promising route to improve the current CF drugs and reach normal-like level of CFTR activity. However, in order to rescue F508del-CFTR during biogenesis, our NBD1 nanobodies must act intracellularly thus requiring the development of methods to deliver nanobodies inside the cells. Several strategies will be followed in parallel. The first approach will make use modified nanobodies with cell-penetrating peptides (CPP) developed to allow direct protein delivery inside the cytoplasm. In a second approach the nanobodies will be encapsulated in liposomes by using an innovative microfluidic technique. In therapeutic perspective, CPP-bound and encapsulated nanobodies could be directly delivered to the lung by nebulization, a method that has been successfully used for nanobodies developed for different pulmonary pathologies. Our work will provide novel therapeutic routes to treat cystic fibrosis.

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A PI3Kγ mimetic peptide promotes plasma membrane CFTR stabilization through a cAMP-independent mechanism

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Background and rationale: Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most frequent mutation in patients (F508del-CFTR) results in a trafficking defect of the channel, that is retained in the endoplasmic reticulum (ER) and consequently degraded by activation of the ERAD pathway. Recently developed CFTR correctors have been shown to only partially rescue the trafficking of F508del-CFTR, underlying the need for additional approaches to enhance CFTR localization at the plasma membrane (PM).

Hypothesis and objectives: Previous work from our group demonstrated that phosphoinositide 3-kinase γ (PI3Kγ) anchors PKA to the cAMP-degrading enzymes, phosphodiesterases (PDEs) 3 and 4, favoring their activation and the consequent reduction of cAMP levels in the vicinity of the CFTR. Because cAMP has been shown to promote PM CFTR stabilization (Lobo et al., 2016), we anticipated that disrupting the scaffold function of PI3Kγ with a previously described mimetic peptide (PI3Kγ MP; Patent n° WO/2016/103176) could enhance CFTR PM density. Preliminary data revealed that PI3Kγ MP significantly increases CFTR amount at the PM in 16HBE14o-. Here, we intend to explore the molecular mechanisms underlying this CFTR-stabilizing effect of the peptide.

Methods: HEK293T, 16HBE14o- and CFBE41o- cell lines were treated with PI3Kγ MP (or a control peptide) and CFTR PM density was analyzed through biochemical (cell-surface protein biotinylation) and microscopy-based (immunogold electron microscopy) assays.

Results: Both biochemical and microscopy approaches demonstrated that PI3Kγ MP increases the stabilization at the PM of both wt- and F508del-CFTR. Intriguingly, this effect was independent from the ability of the peptide to modulate the cAMP/PKA signaling, since PKA and PDE inhibitors failed to block the effect of PI3Kγ MP on CFTR stability, suggesting the existence of alternative mechanism(s). An unbiased approach based on phosphoproteomics revealed protein kinase D 1 (PKD1), a well-established orchestrator of protein trafficking, as being specifically activated by the peptide. In agreement, PI3Kγ MP failed to promote wt- and F508del-CFTR stabilization at the PM in cells treated with PKD1 inhibitors, revealing PKD1 as the key mediator of the effect of PI3Kγ MP on CFTR stability. Finally, PI3Kγ MP increased the PM amount of F508del-CFTR in cells exposed to the approved cocktail of CFTR modulators Trikafta®.

Conclusion: Overall, this study identifies PI3Kγ and PKD1 as pivotal regulators of CFTR stability at the PM and suggests exploiting the ability of PI3Kγ MP to stabilize the CFTR at the PM to eventually augment the therapeutic effect of CFTR modulators, such as Trikafta®.

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New small nitrogen heterocycles as correctors of mutant CFTR protein

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Pharmacological correction of mutant CFTR defects is an effective therapeutic strategy for cystic fibrosis (CF). In particular, combinations of correctors with complementary mechanisms can be used to maximize the rescue of F508del-CFTR protein. Class 1 correctors need to be combined with the class 3 correctors that are believed to interact with NBD1 (Veit et al., 2018). The present standard-of-care of patients with F508del is Trikafta, the Vertex drug that combines the correctors VX-661 and VX-445 with the potentiator VX_770 (Veit et al., 2020; Keating et al., 2018). We have recently identified a very promising class of small molecules, PP compounds, in the rescue of F508del-CFTR in cell lines and in primary airway epithelial cells, particularly in combination with type 1 correctors such as VX-809. In particular, compound PP28 shows activity as F508del-corrector with strong synergism when combined with VX-809 (EC_{50} = 0.47 μM and 1.1 μM in the presence and absence of VX-809, respectively). The obtained effect is nearly four-fold larger than that measured with either compound alone and eliciting high levels of F508del-CFTR function in native airway (bronchial and nasal) epithelial cells from CF patients. To generate more effective and potent analogues, iterative cycles of chemical synthesis and evaluation of corrector activity have been done so far. In this way, more than 200 compounds have been synthetized and tested with the cell-based HS-YFP functional assay. Many active candidates have been identified based on the chemical scaffold of PP028, which was also selected for mechanistic studies. Treatment of CFBE41o- cells with VX-809 plus PP028 caused the appearance of signal at the cell periphery, consistent with improved trafficking to the plasma membrane. Furthermore, the evaluation of F508del-CFTR protein maturation by immunoblot showed an increased abundance of the mature form of the protein. Interestingly, PP compounds induced synergistic effect when combined with class 1 (VX-809) and 2 (3151) correctors but not with class 3 (4172), thus leading to the hypothesis that they could belong to the latter class of correctors. Further studies will be required to better understand the mechanism of PP compounds. The optimization process of ADME profile is ongoing, in order to improve medchem properties preserving the activity. We aim at obtaining the best trade-off between potency/efficacy and "drug-likeness" in order to develop a drug candidate.

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Rescue of F508del-CFTR traffic and function by FDA-approved drugs

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Background: Despite the remarkable progress in developing CFTR modulators to target the primary defect in CF, individuals carrying the F508del mutation and taking these drugs may still face several disease-related symptoms and complications, including a progressive decline of lung function. This suggests that there is still scope for additional or better correctors to further enhance the rescue of F508del-CFTR.

Objectives: To evaluate FDA-approved drugs in combination with the approved CFTR correctors VX-661 and/or VX-445 to identify combinations that further rescue F508del-CFTR traffic and function.

Methods: A microscopy-based high-throughput (HT) CFTR traffic assay was used to screen an FDA-approved Drug Library in CF bronchial epithelial (CFBE) cells stably expressing F508del-CFTR and a mCherry-Flag-CFTR traffic reporter. CFTR plasma membrane (PM) expression was quantified from immunofluorescence images and drug hits were identified as partially rescuing F508del-CFTR PM traffic. The effect of top hits alone or in combination with VX-661 and/or VX-445 in rescuing F508del-CFTR function was thus measured by the HT halide sensitive (HS)-YFP quenching rate assay in CFBE cells co-expressing F508del-CFTR and HS-YFP. Western Blot (WB) was performed to further assess the rescue of F508del-CFTR processing by the most significant hits. Finally, the mechanism of action (MoA) of selected hits was investigated by evaluating their additive effects to low temperature incubation and to CFTR genetic revertants (DD/AA- and F508del/4RK-CFTR).

Results and discussion: In preliminary data from the HT screening of 1,612 FDA-approved drugs, 114 demonstrated to rescue F508del PM traffic. Among these compounds, 40 top hits were selected for further validation by determining CFTR function using the HS-YFP assay alone or in combination with VX-661/VX-445. Sixteen compounds were additive to at least one of the approved correctors in rescuing F508del-CFTR function. Six of these compounds were able to further rescue F508del-CFTR function with additive effects when combined with both VX□661 and VX-445. In WB analysis, eleven compounds were additive to at least one of the approved correctors in increasing F508del-CFTR processing and nine of these compounds were able to further increase F508del-CFTR processing when in a triple combination with both VX-445 and VX-661. The MoA of these selected hits was assessed by evaluating their additivity to low temperature and genetic revertants of F508del. Under low temperature incubation, one compound significantly increased the amount of fully-glycosylated form of CFTR (Band C), while six compounds increased the amount of core-glycosylated form of CFTR (Band B). Two compounds were additive to genetic revertant 4RK and increased both Band C and Band B levels. However, no compound rescued DD/AA trafficking. In conclusion, these preliminary findings suggest that eleven FDA-approved drugs can rescue both F508del-CFTR PM traffic and function. Furthermore, the additive effects of these drugs and VX-661/VX-445, low temperature and CFTR genetic revertants indicate that they may rescue F508del-CFTR by distinct mechanisms of action.

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Exploring the endoplasmic reticulum retention mechanisms to rescue the rare CFTR mutation N1303K

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Background: Most of the ~2,100 CFTR variants so far reported are very rare and still uncharacterized regarding their Cystic Fibrosis (CF) disease liability. Over 1,000 variants exist in less than five patients worldwide therefore, prediction of disease outcome is difficult. CFTR variants have been grouped into seven classes according to their cellular defect [1]. The most common CFTR mutation -- F508del -- belongs to class II, which includes variants that affect CFTR folding, causing endoplasmic reticulum (ER) retention, premature degradation, and failure to reach the plasma membrane (PM). Multiple interactome and proteome studies have been carried out to find therapeutic targets that rescue CFTR mutants not responding to current modulators. Furthermore, second-site mutations - such as 4RK -- allow F508del-CFTR to escape from the ER quality control (ERQC) to the PM. However, there is still an unmet need to functionally characterize mutations that do not respond to current therapies.

Aim: To characterize the mechanisms involved in the ER retention of N1303K-CFTR using site-directed mutagenesis and available proteomics data for global protein expression and ERQC interactions.

Methods: Generation of CFBE cells stably expressing N1303K- and N1303K-4RK-CFTR (with genetic revertant 4RK in *cis* with N1303K), and CFTR protein analysis by Western blot (WB). Usage of available data on F508del-CFTR interacting proteins (F508del-CIPs) regulating its ER exit upon introduction of 4RK [2] and comparison of total protein expression in 16HBE cells expressing wt-, F508del- or N1303K (Santos LA, personal communication). Bioinformatic analyses were used to reveal the proteins upregulated in each cell line and to compare and cross them with those involved in regulating CFTR ER exit. A siRNA-based traffic assay was performed to assess possible rescue of N1303K and F508del by knocking-down targets identified by mass spectrometry (MS).

Results and discussion: Results obtained in a novel CFBE cell line stably expressing N1303K-CFTR show that this mutation leads to total absence of mature CFTR, confirming previous results classifying it as a class II mutation. Interestingly, the introduction of 4RK *in cis* with N1303K rescued the mature form of CFTR. MS analysis resulted in the identification of 5 proteins upregulated only in F508del cells, 10 proteins upregulated only in N1303K-cells and 8 proteins upregulated in both F508del and N1303K. Preliminary results from the siRNA assay after knocking-down these selected proteins in 16HBE expressing N1303K or F508del show that of N1303K-CFTR processing defect was rescued for some proteins. Ongoing comparisons with the interactomics data will allow the identification of critical factors involved in the retention of this "hard-to-rescue" mutant. Altogether, these results highlight the importance of studying the mechanisms underlying the defect of N1303K and the need to continuously find novel protein targets that correct its defect.

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Impact of the F508del mutation on pig and sheep cystic fibrosis transmembrane conductance regulator (CFTR), Cl^- channels with enhanced conductance and ATP-dependent channel gating

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To understand the pathophysiology of cystic fibrosis (CF) and test new therapeutics, large animal models have been developed using pigs and sheep. Because cystic fibrosis transmembrane conductance regulator (CFTR) function varies across species, the aim of this study was to investigate the single-channel behaviour of pig CFTR and the impact on it of the F508del mutation compared with human and sheep CFTR. Using the patch-clamp technique and the experimental conditions described in Ref. (1), we studied CFTR Cl^- channels in excised inside-out membrane patches from CHO cells transiently transfected with CFTR constructs. Like other CFTR orthologues, pig CFTR formed Cl^--selective channels regulated by protein kinase A-dependent phosphorylation and intracellular ATP. However, when compared with human CFTR, pig and sheep CFTR differed in two important ways. First, the single-channel conductance of pig (9.9 ± 0.5 pS; *n* = 7; means ± SD) and sheep CFTR (9.9 ± 0.5 pS; *n* = 13) were greater than human CFTR (9.2 ± 0.2 pS; *n* = 5). Second, distinct differences were observed in ATP-dependent channel gating. At 1 mM ATP, channel openings of pig and sheep CFTR were noticeably longer than those of human CFTR. However, the long closures separating channel openings of pig CFTR resembled those of human CFTR, while those of sheep CFTR were shorter. Consequently, the open probability (P_o) of pig (0.68 ± 0.12; *n* = 13) and sheep CFTR (0.60 ± 0.08; *n* = 24) were greater than that of human CFTR (0.39 ± 0.07; *n* = 15). To explore these gating differences, we examined the ATP dependence of P_o between 0.03 mM and 3 mM ATP. By fitting mean P_o data with Michaelis-Menten functions, we found that ATP regulated pig and sheep CFTR with increased apparent affinity and efficacy (human: K_D = 180 µM, $P_{o(max)}$ = 0.61, r^2 = 0.96, *n* = 4 -- 16; pig: K_D = 25 µM, $P_{o(max)}$ = 0.76, r^2 = 0.78, *n* = 4 -- 7; sheep: K_D = 77 µM, $P_{o(max)}$ = 0.70, r^2 = 0.93, *n* = 5 -- 13). Consistent with previous studies (1 -- 3), the severity of the F508del mutation varied across species. When compared to human CFTR, the F508del mutation had reduced impact on pig and sheep CFTR, with sheep F508del-CFTR retaining greater channel activity than pig and especially human F508del-CFTR, but pig F508del-CFTR greater thermostability than sheep and particularly human F508del-CFTR at 37 °C. We conclude that *i)* pig and sheep CFTR form regulated Cl^- channels with enhanced conductance and ATP-dependent channel gating, and *ii)* the F508del mutation has distinct consequences in human, pig and sheep CFTR. Thus, these data provide insights into species-specific differences that illuminate analyses of CFTR structure-function relationships and animal models of CF.

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P44

Nanomechanics combined with HDX reveal allosteric drug binding sites of CFTR NBD1

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Mutations in the gene of the CFTR/ABCC7 chloride channel, a member of the ATP-Binding Cassette (ABC) protein superfamily, cause a sever disease, cystic fibrosis (CF). Many alterations are localized in the first nucleotide binding domain (NBD1) of CFTR, including the most frequent mutation, the F508 deletion (ΔF508). CF-causing mutations can disrupt the chloride-conducting function by altering protein folding, stability, domain-domain assembly, or trafficking. The currently used CF drugs mainly stabilize the mature protein. Since correction of folding during the early step of maturation may result in more efficient therapies, a detailed knowledge of the folding pathway at the atomic resolution is required. Our goal was to characterize the NBD1 folding/unfolding pathway of the wild type and its clinically most relevant mutant with deleted Phe508 by using computational and experimental methods. We performed steered molecular dynamics simulations using constant velocity pulling to compare the mechanical unfolding of the wild type and ΔF508 NBD1. To validate the *in silico* results, we performed atomic force microscopy experiments. An NBD1 region, including the α-subdomain, was identified as a potentially important participant of the first folding steps, characterized by non-native interactions of F508, thus destabilized in the deletion mutant. The instability was counteracted by the low-potency corrector 5-bromoindole-3-acetic acid, increasing the mechanical resistance of the ΔF508-NBD1 α-subdomain, which was confirmed as a binding site by computational modeling and HDX experiments. Our results underline the complementarity of computational and experimental methods and provide a possible strategy to improve folding correctors.

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Correction of F508del-CFTR by Trikafta/Kaftrio® (Elexacaftor/Ivacaftor/Tezacaftor): mechanism of action

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Trikafta, currently the leading therapeutic in cystic fibrosis (CF), has demonstrated a real clinical benefit. This treatment is the triple combination therapy of two folding correctors elexacaftor/tezacaftor (VX445/ VX661) and the gating potentiator ivacaftor (VX770). In this study, our aim is to compare the properties of F508del-CFTR (cystic fibrosis transmembrane conductance regulator) in airway epithelial cells treated with either tezacaftor, elexacaftor, elexacaftor/tezacaftor with or without ivacaftor.

We study F508del-CFTR interaction and maturation by immunoprecipitation, PLA assay, western blot and immunolocalisation and record the activity of F508del-CFTR by Ussing chamber and whole cell patch clamp.

We found that, whereas the combination elexacaftor/tezacaftor/ivacaftor was efficient in rescuing F508del-CFTR abnormal maturation, apical membrane location and function, the presence of ivacaftor limits these effects (Becq et al. 2021). Elexacaftor/tezacaftor combination reveals a synergy in their action but the addition of ivacaftor affects the membrane stability of the F508del-CFTR at plasma membrane. Corrected F508del-CFTR interaction with multiple molecular chaperones such as HSC70 and HSP90 was modulated regarding the F508del-CFTR.

These findings show that ivacaftor reduces the correction efficacy of Trikafta. Thus, combining elexacaftor/tezacaftor with a different potentiator might improve the therapeutic efficacy for treating CF patients. Moreover, the differential mechanism of action of elexacaftor and tezacaftor regarding the maturation of the F508-CFTR to plasma membrane could explain the synergy mechanism. The following objectives are to study all the molecular actors of the F508del targeting pathway in the presence of Trikafta from maturation of F508del-CFTR to plasma membrane to recycling pathway.

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P46

Exploring the role of EPAC1 to stabilize CFTR in intestinal cells

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Introduction: Regulation of CFTR at the plasma membrane (PM) is a complex process involving multiple interaction partners, signalling pathways and the actin cytoskeleton. Using bronchial epithelial cells, we have shown previously that, although low concentrations of cAMP activate PKA to regulate CFTR function, higher levels of this second messenger lead to the activation of the cAMP sensor EPAC1, promoting CFTR:NHERF1 interaction to increase CFTR PM levels by decreasing endocytosis [1]. Furthermore, this process involves the recruitment of several cytoskeleton regulators to CFTR vicinity [2].

Aim: To study the EPAC1-mediated stabilization pathway of PM CFTR in intestinal cells -- including intestinal organoids as a more physiologically relevant system in comparison with immortalized cell lines.

Methods: To assess whether EPAC1 activation affected CFTR function in intestinal organoids from individuals with CF, we performed the forskolin-induced swelling (FIS) assay after treatment with adenyly cyclase activator forskolin and EPAC1 agonist 007-AM in the presence/absence of CFTR corrector VX-661.

EPAC1 expression levels were assessed by Western blot (WB) after protein extraction from intestinal organoids of individuals with CF and in intestinal cell lines (NCM460 and Caco-2) and bronchial CFBE cells stably expressing wt-CFTR as a control. To assess the effect of EPAC1 activation upon CFTR at the PM, Caco-2 cell lines were treated with 007-AM and cell surface biotinylation assay was performed. To assess the relocation of EPAC1 to the PM after activation with 007-AM, live-cell imaging was used.

Results: Activation of EPAC1 with 007-AM showed no increase in intestinal organoid swelling, and consequently no impact in CFTR function vs control conditions, with or without CFTR rescue by VX-661. Live-cell imaging of CFBE cells showed that 007-AM treatment was able to induce EPAC1 relocation to the PM. The lack of effect in the FIS assay was due to the absence of expression of EPAC1 protein in intestinal organoids, independently of CFTR mutation, as observed by WB. Taken together, these results suggest that the known stabilizing effect of EPAC1 activation in CFTR at the PM does not apply to the intestinal organoid model. Despite the results in intestinal organoids, EPAC1 expression was observed by WB in intestinal cell lines, with expression levels similar to CFBE cells. EPAC1 activation in Caco-2 cells resulted in an increase in mature CFTR PM levels, as previously observed in bronchial cells [1], suggesting that the EPAC1 stabilization pathway is active in these cells.

Conclusion: Our results show that the EPAC1 activation is a relevant pathway in stabilizing PM CFTR, but it does not apply to the intestinal organoid model due to the lack of expression of the cAMP sensor. Thus, despite being a more physiologically relevant system, intestinal organoids are not suited to explore the impact of the EPAC1 pathway in CFTR traffic, PM insertion and/or stabilization.

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"Accessory" mechanisms of action of TRIKAFTA™: its effect on the sphingolipid metabolism

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The recent pharmacological therapies for the treatment of the Cystic Fibrosis (CF) are aimed to the correction of folding defects of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein and to ameliorate its functionality as channel, once reached the plasma membrane. For CF patients carrying the most frequent CFTR mutation, the F508 deletion, the first therapeutic approach was based on the use of ORKAMBI® formulation. Unfortunately, this treatment showed a time limited effect on the amelioration of the disease phenotype. Several studies indicated a possible side effect of the potentiator ivacaftor on the stability of rescued CFTR.

The recently approved pharmacological treatment is based on the triple combination of two correctors, elexacaftor (VX-445) and tezacaftor (VX-661), and the potentiator ivacaftor (VX-770) and it is known by the name of TRIKAFTA™. It has been demonstrated that this formulation improves the therapeutic efficacy on CF patients carrying at least one allele of CFTR with the mutation F508del. In spite of the presence of the potentiator ivacaftor in the new formulation, in cellular models TRIKAFTA™ does not seem to show the same instability effect on the corrected protein at the plasma membrane level observed with the ORKAMBI®.

It has been demonstrated that VX-770 exerts its destabilizing effect interfering with the membrane surrounding CFTR. Indeed, it is known to what extent the lipid composition and organization at the plasma membrane plays a fundamental role in stabilizing the channel. For this reason, we focused our attention on the effect of TRIKAFTA™ on the lipid metabolism. What we observed in treated CFBE cells is a clear modulation of lipid composition. In particular, we observed an increase in the GM1 ganglioside, lipid interactor of CFTR, and a reduction in the cholesterol content. Moreover, the activities of glycohydrolytic and biosynthetic enzymes involved in the sphingolipid metabolism resulted altered by the TRIKAFTA™ treatment. Interestingly, in both CFBE and CuFi-1 cells, the activity of non-lysosomal β-glucocerebroside (GBA2) showed a two-fold increase after treating the cells with TRIKAFTA™, whereas the activities of β-hexosaminidase and β-galactosidase were reduced. Furthermore, the pharmacological treatment induced an increased activity of the sialyl-transferase, enzyme involved in the biosynthesis of the gangliosides, and a decrease in the activity of the sialidase, responsible for the gangliosides catabolism, both results in accordance to what observed as changes in the lipid pattern.

These experimental evidences open an interesting scenario in the comprehension of secondary mechanisms of action of TRIKAFTA™ treatment and in the identification of characteristics of lipid and protein microenvironment which could favor the correction and functionality of CFTR.

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Rescue of F508del-CFTR traffic and function by novel triazole compounds

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Background: Although some therapeutic progress has been achieved in developing small molecules that correct F508del-CFTR defects, alternative CFTR modulators are still needed to identify even more potent compound combinations. Triazoles are well-known synthetic heterocycle compounds widely used in both industry and academic researchers. However, the use of 1,2,3-triazole as a chemical scaffold for F508del-CFTR correctors remains poorly explored.

Objectives: This study aims to explore the biological activity and mechanism of action (MoA) of 1,2,3-triazole derivative compounds to rescue F508del-CFTR traffic and function.

Methods: CF bronchial epithelial (CFBE) cell lines stably co-expressing F508del-CFTR and Halide Sensitive (HS)-YFP were used to test a library of triazole compounds in a small-scale screen in the HS-YFP assay on a plate reader. After primary screening, validation of hits was performed by biochemical, immunofluorescence and functional assays. Thereafter, the MoA of the most promising compounds were investigated by assessing their additive effects to the FDA-approved correctors VX-661 and VX-445, low temperature and to previously characterized CFTR genetic revertants of F508del (G550E, R1070W, 4RK and DD/AA).

Results and discussion: From the 45 compounds tested in the primary screening, four were capable of rescuing F508del-CFTR at the two concentrations tested (3 and 10 μM): LSO-18, LSO-24, LSO-28 and LSO-39. After validation, LSO-18, LSO-24 and LSO-39 showed comparable effects of that achieved by VX-661, while LSO-28 and VX-445 exhibited greater rescue of F508del-CFTR protein membrane expression. LSO-18, LSO-24 and LSO-28, but not LSO-39, showed additive rescue of F508del-CFTR processing and function in combination with VX-661. On the other hand, LSO-28 and LSO-39, but not LSO-18 and LSO-24, presented additive effect in combination with VX-445. All these compounds were additive to low temperature, but only LSO-28 and VX-445 presented a greater rescued of F508del-CFTR processing and function compared to VX-661. F508del/G550E-CFTR processing and function were increased in cells incubated with any of these compounds. In cells expressing F508del/R1070W-CFTR, LSO-18, LSO-24, LSO-28 and VX-445, but not LSO-39 and VX-661, increased its processing and function. Incubation of cells with any of these compounds increased F508del/4RK-CFTR processing and function, with a further increase in processing observed for LSO-18, LSO-24 and VX-445, while these three compounds in addition to LSO-28 also showed higher F508del/4RK-CFTR function compared to that for VX-661. On the other hand, none of these compounds was able to rescue DD/AA-CFTR processing and function. In conclusion, these findings suggest that these four triazole compounds act as F508del-CFTR correctors. Furthermore, LSO-18 and LSO-24 seems to have a similar MoA to that of VX-445. On the other hand, LSO-39 and VX-661 are likely to share a similar MoA in rescuing F508del-CFTR. As LSO-39 and VX-661 did not show additivity in cells expressing F508del/R1070W-CFTR, these compounds are likely to be accommodated at the NBD1:ICL4 interface. Additionally, LSO-28 seems to rescue F508del-CFTR trafficking by a distinct mechanism of action.

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Characterization of F508del-CFTR rescue by corrector PTI-801

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Background: The most prevalent CF-causing mutation -- F508del -- impairs the folding of the CFTR protein, resulting in its endoplasmic reticulum retention and premature degradation. Despite some therapeutic advances that have been attained with the development and clinical approval of small molecules that rescue F508del-CFTR processing and function, the mechanism of action (MoA) of these compounds needs to be further elucidated.

Objectives: This study aims to shed light on the MoA of PTI-801, a recently described F508del-CFTR corrector.

Methods: The effects of PTI-801 were assessed by immunofluorescence microscopy, biochemical and functional assays in CFBE cell lines constitutively expressing F508del-CFTR. The MoA of PTI-801 was assessed by examining its additivity to correctors VX-661, VX-809, ABBV-2222 and FDL-169, low temperature, genetic revertants of F508del-CFTR (G550E, R1070W and 4RK) and the traffic-null variant DD/AA.

Results and discussion: The maximal rescue of F508del-CFTR processing and PM expression by PTI-801 was attained at the 3 μM dose and its efficiency was superior compared to those of VX-661, VX-809, ABBV-2222 and FDL-169. Co-administration of PTI-801 with VX-661, VX-809, ABBV-2222 or FDL-169 resulted in a greater rescue of F508del-CFTR processing, PM expression and function (upon acute stimulation with Fsk plus VX-770). Under low-temperature incubation, F508del-CFTR demonstrated additivity in processing and function with PTI-801 when chronically administered. However, PTI-801 was unable to restore channel gating when acutely administered on rescued F508del-CFTR. PTI-801 demonstrated additivity to the genetic revertants G550E, R1070W and 4RK, but was unable to rescue DD/AA trafficking. In summary, these preliminary findings suggest that PTI-801 acts as a pharmacological chaperone and may have different binding sites compared to other correctors, namely VX-661, VX-809, ABBV-2222 and FDL-169.

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Linking the compound database CandActCFTR and CFTR lifecycle map to predict possible active compound combinations

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During the last years different small-molecule therapeutics have been developed for clinical applications, which not only alleviate symptoms of CF-patients, but also amplify CFTR function directly. However, most of the therapeutics developed until now only target specific mutations or mutation classes, and are consequently not effective for all patients. The latest research efforts therefore focus on developing combination therapies to target multiple defects at once. For this purpose, high-throughput screens have been performed, where thousands of substances have been tested in different cell models. These result in a plethora of data and various candidate compounds, often with an unclear mode of action. In order to provide an overview over already tested compounds, we previously established the publicly available database CandActCFTR (https://candactcftr.ams.med.uni-goettingen.de/), where substances from currently more than 100 publications are listed and categorized according to their interaction with CFTR. It becomes apparent that for about 80 % of the compounds it is unknown whether they affect CFTR directly through a physical interaction, or indirectly through its interactome. In order to elucidate the mechanism of action for promising candidate substances and be able to predict possible synergistic effects of substance combinations, we used a systems biology approach to create a human- and machine-readable model of CFTR biogenesis and activity (https://cf-map.uni-goettingen.de). It is composed of a core model, manually curated from small-scale experiments in human cells, and a coarse model including interactors identified in large-scale efforts. Overall, the manually curated core model includes 170 different molecular entities and 156 reactions from 221 experimental publications. The high-throughput data layer encompasses 1384 unique proteins from four publications (Wang *et al.*, Pankow *et al.*, Matos *et al.* and Santos *et al.*). The overlap between the two data sources amounts to only 46 proteins. Additionally, as aid in the upkeep of the CFTR lifecycle model and to support manual literature curation in similar contexts, we are working on a text mining tool to curate molecular interactions relevant to a specific topic directly from publications. Here, the interactions are automatically parsed and categorized from the publications, displayed in a coarse model and can then be manually validated or rejected by domain experts. In order to link the compounds from the CandActCFTR database to the CFTR-Lifecycle map, we developed a small, easy-to-use tool, which utilizes published interaction data and is currently being expanded by interaction data from molecular docking efforts. By linking the compounds from the database to targets in the disease map, we can develop hypothesis on the mechanism of action for promising compounds and prospectively propose chemical scaffolds and compound families for testing. The model can ultimately be used to support the identification of potential targets inside the cell and evaluate which factors interact to produce as much functional CFTR as possible. Via this approach, different combinations of substances can then be proposed for laboratory testing.

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CFTR modulators and ganglioside GM1: new insight in the treatment of cystic fibrosis

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The new therapeutic strategies for patients with Cystic Fibrosis (CF) are aimed to act on the defect of the protein rescuing the CFTR function. The first treatment approved for patients carrying the most frequent mutation, the F508del, was based on the use of the corrector VX-809, and of the potentiator VX-770. Unfortunately, the efficacy of this treatment was time limited due to the accumulation of the VX-770 in the plasma membrane (PM) resulting in the instability of the rescued mutated-CFTR. In bronchial epithelial cells carrying F508del-CFTR, it has been demonstrated that the side effect of VX-770 is enhanced by the lack of an important lipid interactor of CFTR, such as the ganglioside GM1. Indeed, the exogenous administration of GM1 is able to increase the stability and function of F508del-CFTR rescued by the corrector and potentiator.

Nowadays a new formulation of CFTR modulators (Trikafta^{TM}) is approved and consists in the concomitant administration of two correctors VX-661 and VX-445, and of the potentiator VX-770. Despite this therapeutic approach ameliorates the clinical phenotype of the patients, in vitro experiments on CF bronchial epithelial cells point out that also in case of this formulation VX-770 induces a slight reduction in the F508del-CFTR rescued at the PM level by the correctors.

Considering the important role of the ganglioside GM1 in the stabilization of CFTR at the cell surface, our aim was to investigate its beneficial effect on the maturation of F508del-CFTR rescued by Trikafta^{TM} in order to optimize the effectiveness of novel F508del-CFTR modulators. Therefore, bronchial epithelial cells overexpressing the F508del-CFTR were treated with Trikafta^{TM}, with or without the exogenous administration of GM1, or its lyso-derivative form, the LIGA20. Importantly, exogenous administration of GM1 and LIGA20 seems to counteract the negative effects of VX-770 on rescued F508del-CFTR expression. However, these data are obtained evaluating the CFTR in a total cell lysate and not at the cell PM level where the protein exerts its function. For this reason, we have isolated the CFTR associated with the PM with respect to the intracellular counterpart exploiting the biotinylation of the proteins associated with the external leaflet of the PM. This condition allowed to isolate 90-100% of the biotinylated proteins without the contamination of intracellular components. We have observed that the F508del-CFTR corrected with Trikafta^{TM} is indeed associated with the PM, moreover, the co-treatment with this formulation and the GM1 showed even a more significant effect in the stabilization of CFTR at the cell surface.

Taken together, our data indicate that GM1 and its pharmacological derivative LIGA20 are important factors that participate in the stabilization of the CFTR at the PM. Based on this consideration, treatment with GM1 or its derivative could be considered as an innovative strategy to ameliorate the effectiveness of the modulators on the rescue of mutated CFTR.

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Analysis of CFTR folding using novel antibodies against TMDs

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CFTR folding is complex and hierarchical, beginning with insertion of the transmembrane segments into the endoplasmic reticulum membrane and co-translational folding of individual domains. Critical domain-domain interactions occur co- and post-translationally to form a physiologically stable structure. Despite this progress, mechanistic insight into the folding pathways of CFTR transmembrane domains and especially the latter stage of domain assembly is relatively unknown. Understanding how CFTR folds is important for mode of action studies of therapeutic compounds. We generated antibodies against the transmembrane domains of CFTR and in combination with radiolabel pulse-chase and limited proteolysis, established an assay that recapitulates de novo folding of CFTR in a temporal manner in cells. We found that NBD1 folds with fast kinetics compared to the other domains, supporting the notion that it acts as a 'hub' for other domains to assemble on. As CFTR folds, the intracellular loops in the TMDs and multiple sites in NBD2 become more packed and protected from protease cleavage. Application of the assay to understand how the DAD export motif within NBD1 contributes to transport of CFTR to the Golgi complex showed that mutations within this sequence are deficient in NBD1 folding and have impaired CFTR assembly. This strongly suggests that compromised domain assembly is the root source for inhibited forward transport of these CFTR mutants in the secretory pathway. The assay enhances current understanding on the folding of CFTR and how mutations disrupt CFTR traffic. It also provides a platform for investigating molecular mechanisms underlying the effect of (clinical) modulators on CFTR structure.

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CFTR activity is determined by the store-independent activation of SPCA2/STIM1/ORAI1 complex in secretory epithelial cells

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Introduction: Cystic fibrosis transmembrane conductance regulator (CFTR) regulates the composition of bodily fluids secreted by epithelial cells. For CFTR activation the cAMP/PKA signaling is crucial. However, several studies suggest that the intracellular Ca^{2+} signaling, which strongly depends on the store operated Ca^{2+} entry (SOCE) in epithelial cells, is also able to alter the activity of CFTR through an unknown mechanism.

Therefore, we aimed to clarify the molecular background of Ca^{2+} signaling-dependent CFTR regulation in primary, polarized epithelial cells.

Methods: Mouse and human pancreas, liver and airway organoid cultures and isolated pancreatic ductal fragments were used for RNA-seq, immunostaining and fluorescent Cl^-, Ca^{2+} and fluid secretion measurements. Protein-protein interactions were investigated by direct stochastic optical reconstruction microscopy (dSTORM) and cluster analysis while cAMP signaling was investigated with FRET.

Results: We demonstrated that the plasma membrane (PM) Ca^{2+} entry channel Orai1, which is activated during SOCE, mediates constitutively active extracellular Ca^{2+} entry in primary polarized epithelial cells (derived from the pancreas, liver and lung). This store independent activity is maintained by Secretory Pathway Ca^{2+}-ATPase 2 (SPCA2), which increases the Stim1-Orai1 interaction and Orai1 current in unstimulated cells. We found that Orai1 is co-expressed with CFTR in nanodomains of the apical PM and determines the activity of CFTR in epithelial cells from human and mice pancreas, liver and lung. Finally, we demonstrated that the regulation of CFTR by Orai1 involved Ca^{2+}/Calmodulin stimulated AC1, 3 and 8, which are located in the same protein nanodomain accompanied by CFTR and Orai1.

Conclusion: Our results suggest that the SPCA2 regulated, store-independent extracellular Ca^{2+} influx via Orai1 determines the activity CFTR in polarized epithelia, which is a novel form of regulation and have major physiological relevance.

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Alkalosis-induced hypoventilation in cystic fibrosis: the importance of efficient renal adaptation

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Objectives: The lungs and kidneys are pivotal organs in the regulation of body acid-base homeostasis. In cystic fibrosis, the impaired renal ability to excrete an excess amount of bicarbonate into the urine imposes an increased risk of metabolic alkalosis. This is caused by defective bicarbonate secretion in the β-intercalated cells of the collecting duct that requires both CFTR and pendrin for normal function. Metabolic alkalosis has been suggested to contribute to acute hypercapnic respiratory failure in patients with cystic fibrosis. In this study, we investigate the ventilatory consequences of impaired renal bicarbonate excretion during acute base-loading.

Methods: Using intermittently closed barometric respirometry, we studied the ventilatory consequences of an acute oral base-load in normal, CFTR knock-out and pendrin knock-out mice.

Results: In wild-type mice, oral base-loading induced a dose-dependent metabolic alkalosis, fast urinary removal of base and a moderate base-load did not perturb ventilation. In contrast, CFTR and pendrin KO mice, which are unable to rapidly excrete excess base into the urine, developed a marked and transient depression of ventilation lasting up to 4 hours when subjected to the same base-load.

Conclusion: Stimulation of CFTR and pendrin-dependent renal base elimination in response to an acute oral base-load is a necessary physiological function to avoid ventilatory depression. In cystic fibrosis, metabolic alkalosis may contribute to the commonly reduced lung function via suppression of ventilatory drive.

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Cystic fibrosis-related bone disease: CFTR class II mutations deregulate osteoclast formation and favor RANK+MCSFR+ circulating pre-osteoclasts

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Introduction: Cystic fibrosis-related bone disease (CFBD) is one of many comorbidities associated to CFTR mutations. CFBD leads to early decreased bone mineral density and increased fracture risk (Sermet-Gaudelus *et al.*, 2011). Bone resorption is under the control of osteoclast (cells able to resorb mineralized matrix) differentiated from circulating monocytes. We previously demonstrated an increased RANK+MCSFR+ circulating osteoclast precursors in G551D-CFTR patients (Velard *et al.*, 2018). In human CFTR-F508del primary human osteoclast culture, we showed a reduction in osteoclast number associated with a higher size and reduced resorption abilities (Jourdain *et al.*, 2021).

Purpose: Our aim is to identify putative impact of CFTR class II mutations on osteoclastogenesis.

Methods: Circulating pre-osteoclast monocytes were isolated from 16 CFTR class II mutations bearing patients (Reims Cystic Fibrosis Resource and Competence Centre - NCT04877223)(n=9 F508del homozygous, n=1 N1303K homozygous, n=3 F508del/class I mutation, n=3 F508del/class II mutation)(aged 19 to 51 years) and 13 healthy controls (EFS Grand Est - ALC/PIL/DIR/AJR/FO/606)(ages 22 to 39 years). PBMCs were labelled for membrane RANK and MCSFR receptors analysis on monocytes using flow cytometry. After 21 days of differentiation culture, osteoclasts were stained (Phalloïdine-AlexaFluor488®, Vinculine immunofluorescent staining, DAPI) to determine osteoclasts number per field, size and evaluate focal adhesion plate.

Results: We evidenced that class II mutations bearing patients exhibit increased double positive $RANK^+/MCSFR^+$ circulating monocytes compared to healthy donors (58 *vs* 28%, $p<0.05$). Osteoclasts exhibiting CFTR mutations (n=16) were 33% less numerous ($p<0.05$) and 104% larger ($p<0.05$) than healthy culture (n=13). No variation in osteoclasts shapes was demonstrated between healthy and CF cells, indicating a similar osteoclasts maturation. However, vinculin staining, evidencing the sealing zone in osteoclasts, was thinner and less co-located with actin ring in osteoclast bearing-class II CFTR mutation than healthy controls. None of the parameter studied was modified according to whether the patients bore a homozygous (n=9) or heterozygous (n=6) mutation ($p>0.05$ between CFTR homozygous and heterozygous patients).

Conclusion: In our study, we demonstrated an increased osteoclast differentiation potential in F508del-like patients by a rise of RANK+/MSCFR+ circulating osteoclasts precursors. Surprisingly, *in vitro*, we noticed a reduction in cell number and a higher size in culture for CF osteoclasts, without alteration of their shape. Also, F508del mutation could cause modification in cytoskeleton that decreased sealing zone formation and osteoclast function (Blangy *et al.*, 2020). These results could explain the lack of resorption activity observed in F508del-CFTR osteoclasts culture (Jourdain *et al.*, 2021), then causing dysregulation of bone homeostasis in CFBD-suffering patients.

Keywords: CFBD, F508del, Osteoclast, Monocytes

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CFTR impacts on bone cells differentiation from iPSCs: new insights in cystic fibrosis-related bone disease

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With the advances in medical care and increased survival of cystic fibrosis (CF) patients, co-morbidities affecting other organs than lungs have emerged, in particular CF-related bone disease (CFBD). The role of CFTR in CFBD has been studied in animal models and *in vitro* using human primary cells (Rosen, 2018; Jacquot, 2016). A delayed bone formation and maturation have been demonstrated in CF (Stalvey, 2013; Velard, 2014). However, knowledge gained from the above-mentioned models remain limited due to their relevance to human physiology or limited accessibility (i.e. bone biopsies).

The use of induced pluripotent stem cells (iPSCs) presents the potential to generate every cell type from one common accessible source and to study the developmental impact of CFTR on bone cells commitment.

Here, we show a successful method to generate both osteoblasts and osteoclasts from iPSCs coming from the same donor (healthy or F508del CF). Osteoblasts were obtained through embryoid bodies and mesenchymal stem cells (MSC) formation before the follow-up of the osteoblastic commitment over 21 days. For osteoclasts, a direct iPSC-derived monocyte precursors generation was achieved. At different time of differentiation, cells were fixed for immunostaining or lysed to collect mRNA and perform qRT-PCR. Secreted proteins were quantified by ELISA from culture supernatant.

Commitment toward osteoblasts succeeded for both line with an increase overtime of the osteoblastic markers (2.5-fold RUNX2 expression from MSC stage to day 14, 10-fold ALPL and COL1A1, and 1.8-fold BGLAP increased ($p<0.01$)), associated with a significant decrease of chondroblast and adipocyte transcription factors SOX9 and PPARG ($p<0.01$), and with the formation of calcification nodules on the latest time point. However, a delayed differentiation occurred in CF cells: RUNX2 and BGLAP were decreased by 30% and 50% respectively compared to healthy cells. This delay may result from differences in modulation of signaling pathways between CF and control, since in CF osteoblasts, BMP2 (pro-osteoblastic) was shown 10 times decreased, and at MSC stage, FGFR1 and SMAD2 (inhibitors of osteoblasts differentiation) were 35% overexpressed. As expected (Velard, 2014; Delion, 2016), the CF line displayed a reduced OPG production (60% decreased, $p<0.05$), perturbing the OPG/RANKL ratio which is pivotal for osteoclast differentiation and bone resorption.

Osteoclastogenesis was also achieved for both CF and control iPSC lines with the presence of multinucleated TRAP+ cells which phenotype characterization is on progress.

The achievement of differentiation into osteoblasts and osteoclasts from control and CF iPSCs proves the ability to use these as a biological source to model the impact of CFTR in CFBD.

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The cystic fibrosis urine bicarbonate challenge test

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Objectives: Decreased urine bicarbonate excretion ability is suggested to be a physiological result of renal CFTR malfunction in humans. The objective of this study was to apply an oral bicarbonate challenge in adult cystic fibrosis (CF) patients and investigate:

1. the association of CF disease characteristics with challenged urine bicarbonate excretion
2. Ivacaftor-Tezacaftor-Elexacaftor (Kaftrio®)-induced changes of challenged urine bicarbonate excretion

Methods: 50 adult CF patients were enrolled in this study. All completed a baseline bicarbonate challenge test and 40 completed a bicarbonate challenge test 6 months after initiating Kaftrio® therapy. At baseline, the association between urine bicarbonate excretion and genotype, lung function ($ppFEV_1$), pancreatic function, chronic pseudomonas infection, and sweat chloride was assessed. After 6 months, Kaftrio®-induced changes of challenged urine bicarbonate excretion were measured.

Results: At baseline, challenged urine bicarbonate excretion was associated with several CF disease characteristics. Bicarbonate excretion was higher in patients with residual function mutations and pancreatic-sufficient patients. Importantly, a higher bicarbonate excretion was associated with a higher $ppFEV_1$ and a lower risk of having chronic pseudomonas infection. Kaftrio® treatment increased bicarbonate excretion by 87%. The individual increase in bicarbonate excretion and lung function were not associated.

Conclusion: Individual urine test results correlate with typical key CF clinical disease parameters. This test detects a substantial treatment effect of Kaftrio® therapy resulting in a markedly improved ability to increase urine bicarbonate excretion. The urine test offers a new, simple, and safe functional assessment quantifying the biological consequences of reduced CFTR function and the extent of functional recovery after pharmacological treatment.

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VX-809 has multiple effects in normal mice

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Combinations of CFTR correctors such as Orkambi are currently in use to rescue CFTR trafficking mutants, particularly F508-del CFTR. Extensive preclinical testing of Orkambi has been conducted in patients homozygous for the F508-del mutation, in two randomized clinical trials called TRAFFICK (NCT01807923) and TRANSPORT (NCT01807949). Reported side effects include cough, labored breathing, headache, diarrhea, nausea, and increased creatinine phosphokinase, and in some patients, changes in liver enzymes. Another side effect is an increase in blood pressure (BP). For example, in a longitudinal study of CF patients taking Orkambi from April 1, 2016 to June 30, 2018, median systolic BP increased by 14 mmHg, and diastolic BP increased by 9 mmHg (Sergeev et, al., (2019) Safety and effectiveness of lumacaftor-ivacaftor in adults with cystic fibrosis: A single-center Canadian experience. *Canadian Journal of Respiratory, Critical Care, and Sleep Medicine*, 1-6). This side effect has the potential to produce chronic salt-dependent hypertension, particularly in those receiving long-term treatment with Orkambi. To examine the effects of VX-809 we treated normal mice daily with 20 mg/kg of VX-809 for 11 days. Consistent with what was found in patients, mice injected with VX-809 have altered liver enzymes. We also found reduced levels of heat shock protein 27 indicative of a diminished heat shock response. To determine the effects on wt-CFTR in kidney, we used confocal microscopy to examine immunostained tissue from normal mice treated with VX-809, focusing on medullary CFTR and ENaC, (the epithelial sodium channel). The data showed that treatment with VX-809 did not alter CFTR co-localization with the lateral membrane marker E-cadherin in normal mouse kidneys treated with VX-809. In contrast, CFTR co-localization with both the ER marker calnexin and the Golgi marker GM130 decreased significantly following treatment with VX-809; the effect was most notable for GM130. Surprisingly, co-localization with Na^+/K^+ ATPase, a basolateral membrane marker, increased 2-fold after VX-809 treatment. In medullary regions, VX-809 treatment did not alter α-ENaC's co-localization with either E-cadherin or calnexin. Moreover, α-ENaC did not co-localize with Na^+/K^+ ATPase, a marker of the basolateral membrane. The observation that ENaC did not co-localize with Na^+/K^+ ATPase, in contrast to the results for CFTR, was good evidence that VX-809 has a specific effect on CFTR, and not on ENaC. Thus, our results indicated that VX-809, by affecting the location of CFTR promotes an absorptive phenotype in the kidney and may be the cause of hypertension noted in patients taking Orkambi. We also showed that VX-809 treatment alters liver enzymes and reduces heat shock proteins, especially Hsp27.

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Integrative analysis of vascular impairment in models of cystic fibrosis

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Background: Cystic fibrosis (CF) is a multisystemic disease primordially affecting lungs but also associated with broad systemic complications. While CFTR is mainly studied in epithelial cells, it has an important (and overlooked) role in endothelial cells (ECs). Several evidences point out a vascular dysfunction in CF and link it to the development of pulmonary hypertension and CF-associated liver diseases (CFLD). However, the precise mechanism involving blood vessels in these life-threatening disorders is not fully understood.

Aims: We aimed to characterize, using meta-analysis, bulk and single cell RNA-sequencing (scRNA-seq), how CFTR-impairment impacts EC biology and could thus contribute to CF pathogenesis.

Material and methods: We used an unbiased transcriptomic approach in two complementary models of CFTR deficiency ($CFTR_{inh-172}$ & shRNAs) in primary human ECs. We extensively characterized and validated the changes in EC properties occurring upon CFTR dysfunction *in vitro*, *in vivo* & *ex vivo*. Additionally, we performed a meta-analysis to compare our results to CFTR-impaired epithelial models. Finally, we isolated ECs from human CF liver explants and performed scRNA-seq to capture endothelial heterogeneity and transcriptomic changes occurring at the single cell level.

Results: With this study we demonstrated that CF models of ECs display impaired proliferation, migration, mesenchymal state and autophagy. Remarkably, we also uncovered a profound pro-inflammatory state leading to increased leukocyte adhesion and extravasation. Finally, our scRNA-seq data revealed a distinct population of ECs, unique to samples from patients with CF, upregulating genes involved in coagulation and the complement cascade.

Conclusion: Our integrative data thus suggests that ECs are no innocent bystander in CF pathology, but rather may be central mediators of the exaggerated inflammatory phenotype. Besides, our work showed novel aspects of human liver ECs at single-cell level thereby supporting endothelial involvement in CFLD.

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Defective CFTR expression promotes epithelial cell damage in alcoholic pancreatitis and hepatitis by the impaired regulation of PMCA activity

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Background and aims: Alcoholic pancreatitis and hepatitis are frequent, potentially lethal diseases with limited treatment options. Our previous study reported that the expression of CFTR Cl^- channel is impaired by ethanol in pancreatic ductal cells leading to more severe alcohol-induced pancreatitis. In addition to determining epithelial ion secretion, CFTR has multiple interactions with other proteins, which may influence intracellular Ca^{2+} signaling. Thus, we aimed to investigate the impact of ethanol-mediated CFTR damage on intracellular Ca^{2+} homeostasis in pancreatic ductal epithelial cells and cholangiocytes.

Methods: Human and mouse pancreas and liver samples and *ex vivo* organoids were used to study ion secretion, intracellular signaling and protein expression and interaction. The effect of CFTR defect on PMCA4 activity was measured by microfluidimetrics. Protein-protein interaction was assessed by Direct Stochastic Optical Reconstruction Microscopy (dSTORM). The consequence of PMCA4 inhibition was analysed in a mouse model of alcohol-induced pancreatitis.

Results: The decreased CFTR expression impaired PMCA function and resulted in sustained intracellular Ca^{2+} elevation in ethanol-treated and mouse and human pancreatic organoids. Liver samples derived from alcoholic hepatitis patients and ethanol-treated mouse liver organoids showed decreased CFTR expression and function, and impaired PMCA4 activity. PMCA4 co-localizes and physically interacts with CFTR on the apical membrane of polarized epithelial cells, where CFTR-dependent calmodulin recruitment determines PMCA4 activity. The sustained intracellular Ca^{2+} elevation in the absence of CFTR inhibited mitochondrial function and was accompanied with increased apoptosis in pancreatic epithelial cells and PMCA4 inhibition increased the severity of alcohol-induced AP in mice.

Conclusion: Our results suggest that improving Ca^{2+} extrusion in epithelial cells may be a potential novel therapeutic approach to protect the exocrine pancreatic function in alcoholic pancreatitis and prevent the development of cholestasis in alcoholic hepatitis.

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Sterol profile alteration in CF patients: does CFTR protein matter?

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Patients with cystic fibrosis (CF) have reduced intestinal absorption of lipids and low plasma cholesterol, despite the enhanced endogenous synthesis. We evaluated the cholesterol metabolism by the analysis of plasma surrogate markers, i.e., lathosterol (hepatic synthesis marker), cholestanol and phytosterols (intestinal absorption markers) by a standardized gas chromatographic method. We also monitored the serum lipid profile and vitamin E levels. Hepatobiliary injury/function was evaluated by laboratory markers, such as aspartate aminotransferase (AST) and alanine transaminase (ALT) for hepatocytes injury, γ-glutamyltransferase (γ GT) and alkaline phosphatase (AP) for biliary injury, and bilirubin and albumin for liver function.

At baseline, we found that plasma cholesterol and vitamin E levels were significantly lower in the homozygous F508del patients than in patients compound heterozygous for F508del mutation and another severe mutation (hetero-deltaF508), although both type of groups were treated with the same dose of pancreatic enzyme replacement therapy (PERT) and vitamin E supplementation. Lumacaftor/ivacaftor treatment for 15 ± 6 months improved enterohepatic flux in homo-deltaF508 patients, but without an increase of serum cholesterol concentration that, on the contrary, decreased.

Unexpectedly, we observed a slightly decrease of serum cholesterol also in the Lumacaftor/ivacaftor untreated hetero-deltaF508 CF patients (Table 1), that, for this reason did not show a significant difference in the hepatobiliary injury/function compared to baseline values. However, the sterols profile, using the surrogate markers, may help the clinicians in the management of PERT and may contribute to monitor the effects of CFTR modulator therapy.

In conclusion, it remains intriguing to understand some aspects of cholesterol metabolism in CF patients. While a slight decrease in serum cholesterol is expected in the untreated group, it is not clear why treatment with modulators fails to improve serum cholesterol levels while improving the entero-hepatic flux in homo-deltaF508 patients; where do modulators fail?

Table 1 Follow-up of plasma sterols in hetero-deltaF508 CF patients

Sterols (mg/dL)	**Baseline**	**After 15 months**
Cholesterol	132 (42)	110 (24)
HDL-cholesterol (mg/dL)	*46 (14)*	*43 (10)*
LDL-cholesterol (mg/dL)	*58 (52-74)*	*66 (50-80)*
Phytosterols	*0.31 (0.19)*	*0.25 (0.21)*
Lathosterol	*0.46 (0.20)*	*0.32 (0.11)*
Cholestanol	0.23 (0.13)	0.22 (0.06)
Ratios (µg/mg)		
Phytosterols/cholesterol	2.28 (1.08)	3.05 (1.64)
Lathosterol/cholesterol	3.57 (1.30)	3.11 (1.29)
Cholestanol/cholesterol	1.74 (0.64)	1.97 (0.46)

Normal data are reported as mean (SD) and statistical differences were evaluated by paired Student t test. Non parametric data are reported as median (interquartile range) and statistical differences were evaluated by paired Wilcoxon test. n.s. not significant. * $p < 0.05$

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Identification of drugs activating CFTR-independent fluid secretion in nasal organoids based on a high-content screening assay

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Introduction: Alternative chloride channels are an interesting target to restore the epithelial fluid homeostasis in cystic fibrosis (CF) airway epithelia. As this approach bypasses cystic fibrosis transmembrane conductance regulator (CFTR), it might help a large proportion of people with CF, independent of their mutation type. A high-content screening assay with nasal organoids from individuals with CF was used to identify drugs that induced epithelial fluid secretion via alternative ion channels.

Methods: Nasal organoids from individuals with CF (n=4 donors) were cultured in 384-well plate format. A drug library containing 1400 FDA-approved drugs was screened on their ability to induce fluid secretion, based on organoid swelling. The primary screen was performed in 384-well plate format, with 2 compounds per well. Compounds which induced swelling were subsequently tested in a secondary screen in the more conventional 96 well-plate format in order to make a selection of hits. These hits were then tested in nasal cells from CFTR-null donors to confirm their CFTR-independency. To determine if the drugs activated the alternative chloride channel TMEM16A, gene knockouts were created using CRISPR-Cas9 gene editing in cells derived from individuals with CFTR-null mutations.

Results: Ninety compounds which induced fluid secretion in CF nasal organoids were identified in the primary screening assay. After the secondary screen, 13 hits were chosen based on the amount of swelling and their working mechanism. Most of the compounds also induced swelling in nasal organoids from CFTR-null donors, suggesting CFTR-independency. In order to test if the compounds activate TMEM16A, TMEM16A knockout nasal cells from CFTR-null mutations were created with a efficiency of 87-95%, which was determined by Sanger sequencing. Western blot showed reduced TMEM16A protein expression in the TMEM16A knockout cells and Ussing chamber experiments showed reduced UTP-induced current in the genetic knockout cells, indicating less TMEM16A activity.

Conclusion: We have identified and validated multiple FDA-approved drugs activating CFTR-independent swelling in nasal organoids. Using TMEM16A knockout nasal organoids, we will further determine the contribution of this specific alternative ion channel.

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ATP12A upregulation in airway epithelial cells by inflammatory stimuli

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In a previous study, we found that expression of ATP12A proton pump is increased in the bronchi of cystic fibrosis (CF) patients undergoing lung transplant (Scudieri et al., JCI Insight 2018). We asked whether ATP12A upregulation was due to the inflammation at an advanced stage of lung disease or a direct consequence of the CFTR basic defect. Therefore, we investigated ATP12A expression in nasal brushings of CF patients and control individuals. We found significantly higher ATP12A expression in the nasal mucosa of CF patients, even of very young age. In cultured sterile conditions, we found no difference in ATP12A expression between CF and non-CF airway epithelial cells, thus suggesting a role of inflammation in the upregulation of ATP12A in vivo. We treated CF and non-CF epithelia with a panel of pro-inflammatory cytokines that have been reported to be involved in CF. We found that the combination of IL-17 plus TNF-α was the most effective stimulus increasing ATP12A at the protein and functional level. We evaluated ATP12A function by measuring pH on the apical surface of bronchial epithelia under bicarbonate-free conditions. Non-CF cells under control conditions showed a relatively small but significant effect of ouabain and bafilomycin A1, which inhibit ATP12A and V-ATPase, respectively. The effect of the two agents was additive. The bafilomycin-sensitive component was absent in CF cells. Treatment of CF and non-CF cells with IL-17 plus TNF-α induced a marked acidification that was exclusively sensitive to ouabain. We analyzed the effect of IL-17 plus TNF-α at the transcriptome level using RNAseq. For comparison, we also investigated the individual effects of IL-17, TNF-α, and IL-4. This analysis revealed a profound change elicited by IL-17 plus TNF-α combination, including genes involved in transepithelial transport (e.g. SLC26A4, ATP12A), cytokine/chemokine signaling (CSF3, IL-19, CCL20, CXCL6, CXCL5, CXCL1), modulation of immune response and antimicrobial activity (IDO1, NOS2, DEFB4A, LTF, DEFB4B), and mucins (MUC5B). ATP12A upregulation at the mRNA levels was modest but significant. We performed also single cell RNA sequencing (scRNAseq) to further analyze the effects of IL-17 plus TNF-α treatment. The treatment caused the appearance of a cluster enriched with cells expressing ATP12A and SLC26A4. In conclusion, we found that IL-17 plus TNF-α treatment induces a complex program that involves profound changes in gene expression profile, probably to boost the ability of the airway epithelium to respond to inflammatory stimuli. ATP12A upregulation by pro-inflammatory condition may be detrimental, causing acidification of ASL that in CF cannot be rescued by CFTR-dependent bicarbonate secretion.

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SLC26A4 but not TMEM16A directly regulates ASL pH under inflamed conditions in nasal epithelia derived from donors with rare class I mutations

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Introduction: Airway surface liquid (ASL) pH is more acidic in CF due to faulty CFTR, which contributes to mucus stasis and bacterial infection. ASL pH is therefore a potential factor that could be targeted for future CF therapies. However, we lack a complete understanding of the complex mechanisms underlying ASL pH regulation. In addition to CFTR, possible targets, such as Na^+/H^+-exchanger, K^+H^+-ATPase, SLC26 ion transporters and TMEM16A, a calcium activated chloride channel, have all been suggested to regulate ASL pH, as well as the paracellular ion shunt. Our aim here was to investigate the potential role of the Cl^-/HCO_3^- exchanger, SLC26A4, and TMEM16A, in acid-base homeostasis in fully differentiated nasal CF airway cultures derived from donors with class I mutations, under both normal and inflammatory conditions.

Methods: SLC26A4 (26A4) and TMEM16A (T16A) knock-out (KO) cells were produced by CRISPR-Cas9 gene-editing of CF-null basal cells from 3 donors with class I mutations. The cells were then differentiated at an air-liquid interface (ALI) for 4 weeks. ASL pH was measured in real-time using a fluorescent plate-reader under thin film conditions (Saint-Criq et al., JOVE, 2019). To induce a pro-inflammatory phenotype, and increase the expression of 26A4 and T16A, cell cultures were treated with IL-4 [10ng/mL] for 48hrs.

Results: The baseline ASL pH in CF nasal epithelial cell cultures was 6.63±0.29 (n=27) and was unaffected by IL-4 treatment (6.56±0.48, n=27). However, in 26A4 KO epithelia, although baseline ASL pH was unaltered under normal conditions (6.64±0.30, n=12), it was markedly acidified (6.06±0.42, $p<0.0001$, n=12) in IL-4 treated cultures. In contrast, the baseline ASL pH for T16A KO epithelia, under both normal and IL-4 conditions, was significantly increased (6.82±0.26 pH, $p<0.05$ and, 6.88±0.53, $p<0.001$, respectively, n=15). The addition of the calcium agonist, carbachol (CCh) caused a small, but not significant, acidification of ASL pH under all the conditions tested. In contrast, the subsequent addition of the cAMP-agonist, forskolin (FSK), alkalinized ASL pH (ΔASLpH 0.27±0.37), a response that was significantly enhanced in IL-4 treated epithelia (ΔASLpH 1.01±0.44, $p<0.0001$). In T16A KO epithelia, with or without IL-4, the FSK-induced alkalinisation was unaffected. In 26A4 KO epithelia under normal conditions, although the FSK response was unaltered (ΔASLpH 0.18±0.21), it was significantly attenuated in 26A KO epithelia treated with IL-4 (ΔASLpH 0.44±0.24, $p<0.0001$).

Conclusion: Genetic KO of TMEM16A increased baseline ASL pH under all conditions but did not affect the response to calcium or cAMP agonists. This suggests that TMEM16A indirectly contributes to steady-state ASL pH homeostasis. Genetic KO showed that SLC26A4 plays a minor role in ASL pH homeostasis and response to agonists under non-inflammatory conditions. However, under IL-4 treatment, SLC26A4 KO significantly impacted ASL pH, leading to a more acidic pH, as well as a reduced response to the cAMP agonist. Overall, this study shows that TMEM16A indirectly contributes to ASL pH homeostasis, but highlights an important direct role of the non-CFTR transporter, SLC26A4, which suggests this transporter could be an important alternative target for ASL pH therapy in CF.

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Characterization of a new TMEM16A activity enhancer

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Background: Most pre-clinical drug discovery for Cystic Fibrosis (CF) aims to restore ion transport through mutation-specific CFTR rescue. However, ~15-20% of all individuals with CF worldwide will not benefit from these drugs, as they lack F508del in both alleles, as well as other common mutations which are targeted by those therapies. There is thus an unmet need for novel therapies that restore ion transport in all individuals with CF. One attractive strategy is to stimulate alternative Cl^- channels (ACCs) to compensate for the absence of functional CFTR, being TMEM16A/Anoctamin 1 (ANO1) a good candidate. Importantly, this approach applies to all individuals with CF, independently of their CFTR genotype.

Aim: To study the efficacy of a novel TMEM16A enhancer (FDA-01).

Methods: Hits identified in a FDA-approved drugs screen using human nasal organoids and swelling assay (Beekman lab, unpublished data) were rescreened here by the halide-sensitive (HS)-YFP assay in human CF bronchial epithelial (CFBE) cells (expressing TMEM16A endogenously but not CFTR) to determine their capacity to activate this ACC. The most promising hit, FDA-01, was selected to be studied in more detail also after TMEM16A knock-down. The FDA-01 effect on TMEM16A expression and intracellular localization was analyzed by Western blot (WB) and by a microscopy-based traffic assay, respectively [1] and further functionally validated by Ussing chamber measurements upon stimulation with ATP with and without the specific TMEM16A inhibitor, Ani9.

Results: FDA-01 was the most promising hit in the secondary HS-YFP screening, as it was shown to enhance TMEM16A activity under prolonged exposure (24h), with higher activating effect than E_{act}, a compound that stimulates Ca^{2+}-activated channels, namely TMEM16A [2]. In the presence of Ani9, FDA-01 had no effect, suggesting its specificity for TMEM16A. After TMEM16A knockdown, FDA-01 showed lower effect, further validating its TMEM16A specificity. Additionally, WB analysis showed that FDA-01 increases the total TMEM16A expression levels, partially explaining the observed increase in TMEM16A function. Ussing chamber experiments showed that FDA-01 has an effect in ATP responses, which was absent in the presence of Ani9, again suggesting the strong contribution of TMEM16A to the above ATP response.

Conclusion: This work highlights the high potential of drug repurposing for the development of a novel therapies for all individuals with CF, independently of their CFTR genotype.

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The RNA-binding protein HuR drives *Pseudomonas aeruginosa* adhesion to the CF airway epithelium by stabilizing Vav3 mRNA

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Pseudomonas aeruginosa (*Pa*) represents the leading cause of chronic airway infections in Cystic Fibrosis (CF). Bacterial adhesion to the host epithelial surface represents a decisive step for successful airway colonization. We have recently shown that the CF airway epithelium exhibits luminal platforms rich in fibronectin and β1 integrin, which promote the adhesion of *Pa* to the surface (Badaoui *et al., Cell Reports* 2020). This phenotype was induced by an increased expression of the guanine nucleotide exchange factor Vav3, which belong to the family of Rho GTPase activators. Here, we aimed to uncover the mechanisms that induce Vav3 overexpression in CF. We observed that primary human airway epithelial cells (HAECs) from CF donors showed an increased expression of HuR, a member of the Hu family of RNA-binding proteins that generally stabilizes target transcripts and promotes mRNA translation. Furthermore, the expression of tristetraprolin (TTP), a major binding competitor of HuR that promotes mRNA degradation, was decreased in CF HAECs suggesting a post-transcriptional imbalance towards higher mRNA stability. This post-transcriptional regulation imbalance was recapitulated in the Calu-3 airway epithelial cell line after *CFTR* knockdown by CRISPR-Cas9 (CFTR-KD). Moreover, ribonucleoprotein immunoprecipitation (RNP) revealed enriched interaction between HuR and Vav3 mRNA in CFTR-KD cells, which increased Vav3 mRNA stability as measured by Actinomycin D-chase experiments. Interestingly, blocking HuR-AREs interaction in CF primary HAECs and CFTR KD Calu-3 cells by CMLD-2, a competitive inhibitor of HuR, was able to prevent Vav3 overexpression. Consequently, CMLD-2 inhibited the formation of the fibronectin/β1 integrin apical complex. In addition, transepithelial electrical resistance measurement revealed that CMLD-2 treatment improved the epithelial integrity of the CFTR-KD epithelium. Finally, HuR inhibition by CMLD-2 prevented the adhesion of PAO1, a laboratory *Pa* strain, to the CFTR-KD epithelium. These results indicate that correction of the mRNA stability/degradation imbalance in CF may represent a potential therapeutic target by preventing the initiation of *Pa* infection.

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Identification of genes linking CFTR to airway epithelial regeneration

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Background: Upon infection, inflammation or damage of the airways, repair of the epithelial tissue is essential to restore the epithelial integrity and maintain lung function. Epithelial regeneration involves several coordinated processes, including cell adhesion, migration and proliferation to cover the wound and form a new monolayer before re-differentiating and reconstituting a polarized and intact epithelium. Dysfunctional CFTR has been reported to impair epithelial wound regeneration after injury [1] and also to delay wound closure in polarized cell lines and primaries [2]. In parallel, CFTR plasma membrane (PM) traffic is known to be regulated by factors only expressed in differentiated cells [3]. Despite that many studies relate CFTR traffic to epithelial wound healing, differentiation and regeneration [4], their mechanistic relationship to CFTR remains elusive.

Objective: To identify and characterize F508del-CFTR traffic rescuing factors that also restore the CF regeneration defect in human bronchial epithelial (CFBE) cells.

Methods: A novel high-throughput wound healing live-cell microscopy device and assay were developed and used to screen siRNAs targeting 220 genes which rescue F508del-CFTR traffic (unpublished data) to determine which of those genes also affect wound closure, migration and proliferation, in wt- and F508del-CFTR expressing cells. A wound healing model of human airway basal cells (BCi-NS1.1) cells with multipotent differentiation capacity [5] was cultured under differentiating air-liquid interface (ALI) conditions to further validate the hit genes in a fully-differentiated mucociliated epithelium.

Results: Through the primary wound healing screen we identified >30 hit genes that accelerate/delay wound closure in F508del- vs wt-CFTR expressing CFBE cells. Gene ontology of these genes revealed that they are mainly linked to the actin cytoskeleton. A hit validation screen revealed ~8 genes which when knocked-down (KD) accelerate wound closure in F508del-CFTR expressing CFBE cells, being currently under further characterization. Moreover, after successfully injuring fully-differentiated BCi-NS1.1 cells, and monitoring the wound closure, we observed an increase in wt-CFTR expression over time, hinting at CFTR relevant role in epithelial regeneration. Further studies are underway to characterize the wound healing screen hits regarding their impact on differentiation and regeneration.

Conclusion: We identified ~8 genes which rescue both F508del-CFTR PM traffic and the wound healing delay of CF cells. These data shed light on how these mechanisms are coupled in CF and identified potential drug targets in strategies aiming to rescue the CF regeneration defect.

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Trikafta mediated bicarbonate transport correction in F508del primary cell cultures

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Cystic fibrosis is an autosomal recessive disease caused by mutations in the *CFTR* gene. The latter encodes for the CFTR protein located at the apical membrane of epithelial cells. This transepithelial anion channel secretes chloride (Cl^-) and bicarbonate (HCO_3^-) ions into the airway surface liquid (ASL). F508del results in a defect of CFTR-mediated anion transport. It was recently shown that the combination of a potentiator (VX-770) and two correctors (VX-445/VX-661), TRI thereafter, corrects CFTR-mediated Cl^- secretion and induces ASL alkalinization under inflammatory conditions. In this work we investigate the CFTR-mediated HCO_3^- transport correction and its effect on pH, both under inflammatory and non-inflammatory conditions.

Bronchial and nasal epithelial cells were harvested from F508del homozygous patients and grown in an air/liquid interface. To study the *in vitro* effect of the triple combination [VX-445 (3µM), VX-661 (3µM), VX-770 (100nM)] and control (DMSO), primary cells were treated for 48h. Inflammation was induced by TNFα (10ng/mL) /IL17 (20ng/mL). Cl^- and HCO_3^- transport were then assessed by short circuit current (I_{sc}) in Ussing chamber by studying the variation of the response to CFTR inhibitors (Inh172, 10µM and GlyH101, 10µM) after addition of Forskoline (10µM). ASL pH was measured in a controlled atmosphere at 37°C, 5% CO2, using a microelectrode, in physiological conditions and using a Ringer without Cl^- to inhibit the pendrin Cl^-/HCO_3^- exchanger activity. Expression of different transporters was quantified by RTqPCR.

Triple combination increased HCO_{3-} ($p=0.001$) and Cl^- ($p<0.001$) ion transport in nasal (culture from 11 different patients) as well as bronchial epithelial cells (culture from 6 different patients, $p=0.0.3$). pH measurements showed TRI-mediated alkalinisation of ASL in physiological condition after stimulation of inflammation ($n= 6$, $p<0.05$). In condition of pendrin inhibition, TRI induced alkalinisation under basal ($n=6$, $p<0.05$) and inflammatory ($n=6$, $p<0.05$) conditions, unmasking CFTR HCO3- transport. CFTR and pendrin transcript level were significantly increased after stimulation of inflammation ($p<0.05$) but were not significantly modified by TRI.

CFTR-mediated Cl- and HCO3 transport is improved by TRI and participates in the alkalinisation of ASL pH. These results provide a better understanding of CFTR modulators effects on respiratory epithelium homeostasis.

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Evaluation of TMEM16A as a modifier of the cystic fibrosis lung phenotype utilizing CF patient-specific human induced pluripotent stem cells

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Previous studies have suggested that the calcium-activated chloride channel TMEM16A may serve as a potential pharmacological target to treat the CF lung phenotype. CFTR and TMEM16A are main contributors to chloride secretion within the airway epithelium. Interestingly, it was observed that TMEM16A expression is enhanced in CF as well as under mucus-hypersecretion associated conditions. However, the precise physiological function of TMEM16A remains largely unknown. It was hypothesized that, in CF either the stimulation of TMEM16A could improve mucociliary clearance, or that its inhibition could prevent mucus accumulation. However, so far no complex *in vitro* model of CF respiratory epithelial cells is available to investigate both conditions in more detail. Therefore, the project aims to utilize human induced pluripotent stem cell (hiPSC) derived respiratory epithelial cells with either TMEM16A knockout or overexpression to evaluate TMEM16A as a CF modifier by analyzing hiPSC-derived airway epithelial tissues.

The generation of homozygous TMEM16A knockout ($T16^{KO}$) and TMEM16A(*abc*) ($T16abc^{OX}$) overexpressing hiPSC lines was achieved utilizing hiPSCs from a healthy donor (WT), CF patient-specific (ΔF508 homozygous) (CF) and corresponding gene-corrected (Corr) CF-hiPSCs respectively. Analyses of all gene edited hiPSC lines verified their pluripotent properties and genomic stability after genome engineering. Utilizing a multistep protocol WT, CF, CF-$T16^{KO}$ and CF-$T16abc^{OX}$ hiPSCs have been successfully differentiated into airway epithelial tissues in Air-Liquid Interface (ALI) cultures showing expression of typical epithelial markers like TUBB4A, MUC5AC and TMEM16A. Initial studies are conducted on mature ALI cultures to analyze TMEM16A-dependent ion transport changes. Elucidating the physiological function of TMEM16A will be crucial to define the therapeutic intervention to target TMEM16A in CF therapy.

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Inhibition of Orai1 Ca^{2+} Channel has a protective role in the progression of chronic pancreatitis and rescues CFTR dysfunction in pancreatic ducts

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Introduction: Cystic fibrosis transmembrane conductance regulator (CFTR) has a key role in the maintenance of pancreatic ductal function carrying Cl^- and HCO_3^- ions across the apical membrane, beside this it has also been pathophysiology role. CFTR gene expression decreased, and the protein was mislocalized in pancreatic ductal cells during chronic pancreatitis (CP). Abolished CFTR expression causes a diminished fluid secretion, HCO_3^- secretion, decreased luminal pH, therefore leads to severe ductal dysfunction and inflammation. Physiologically CFTR co-localizes with Orai1, a store operated Ca^{2+} channel protein was found in the same macrodomain on the apical surface of ductal epithelial cells. STIM1-Orai1 interaction activates store operated Ca^{2+}entry, localizing plasma and endoplasmic reticulum membrane near CFTR, causing an elevation in local Ca^{2+} levels. It is known that sustained elevation of intracellular Ca^{2+} concentration induces injury to pancreatic tissue and the development of acute pancreatitis (AP). Orai1 is the most abundant Ca^{2+} channel in pancreas and its role in pathomechanism of AP is known, but its contribution to development of CP is not clear. CP is a progressive inflammatory disease characteristically accompanied by irreversible functional and structural damages of the pancreas without specific treatment. In the light of these facts, it is crucial to characterize whether the inhibition of Orai1 could restore the acinar and ductal functions in pancreatic tissue during CP.

Objective: The objective of our study to investigate the effect of Orai1 in the pathomechanism of CP in mice, focusing on the acinar and ductal functions regarding CFTR.

Methods: CP was induced by repetitive administration of cerulein for 2 weeks in wild type FVB/N mice. To test whether the inhibition of Orai1 may protect the pancreatic tissue during the repeated bouts of AP, selective Orai1 inhibitor (CM-C) was administered on the last 5 days as a post-treatment. The severity of CP was evaluated by analyzing histological parameters, *in vivo* pancreatic secretion, hydroxyproline (HyP) content and tissue amylase activity. Ductal function parameters were analyzed by $Cl^-$and HCO_3^- physiological measurements and CFTR immunostaining on isolated ducts. Inflammatory marker proteins and genes were characterized by immunohistochemistry and real time quantitative polymerase chain reaction.

Results: Pancreatic sections showed minimal intact parenchyma, acinar-ductal metaplasia and diffuse intestinal fibrosis in CP. Post-treatment with CM-C significantly reduced the severity of cerulein-induced CP, partially restoring the acinar functions, like *in vivo* pancreatic fluid secretion, amylase activity or HyP content. Inhibition of Orai1 prevented CP-induced inhibition of HCO_3^-secretion and restored the inhibitory effect of cerulein based on Cl^- measurements, suggesting the recovery of the ductal functions in isolated ducts. These results are supported by the observation that CM-C re-established the mislocalization of CFTR protein in isolated pancreatic ducts. Moreover, CM-C decreased the level of inflammatory marker mRNAs (Tnf, Tgfb1, Il1b) and immune cell marker proteins (CD3, CD8, CD19, F4/80, MPO) in CP.

Conclusions: We can conclude, that the selective inhibition of Orai1 reduced the severity of CP and restored the ductal function and physiological CFTR localization, which may open new therapeutic possibilities in the treatment of CP.

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iPSC-derived macrophages from cystic fibrosis patients for infection models and drug screening platforms

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Cystic fibrosis (CF) is an autosomal recessive disease driven by mutations in the CFTR gene, leading to increased lung infection susceptibility. The increasing number of infections caused by multidrug resistant (MDR) *Pseudomonas aeruginosa* are of particular concern for CF patients. Alveolar macrophages (AMs) form the first line of defense against airborne bacteria. However, recent evidence suggests AMs in CF to be hyperinflammatory and suboptimal phagocytes.

To study the impact of CFTR in macrophages and to evaluate novel treatment scenarios, a standardized *in vitro* macrophage model for CF would be highly valuable. Since direct isolation and use of primitive alveolar macrophages from CF patients are particularly challenging and hard to receive, alternative strategies should be considered. Here we present the first steps towards the development of an *in vitro* model using CF patient derived induced pluripotent stem cell (iPSC) and thereof derived primitive macrophages (iMac). In our innovative approach, iMac can be generated easily on demand, in a reproducible manner and in scalable quantity.

To establish our CF macrophage model, three human iPSC lines derived either from healthy wild type (WT) donor, diseased CF patient (F508del) and respective isogenic corrected CF (CorrCF; F508del heterozygous) were evaluated. All three iPSC lines were successfully differentiated into macrophages using our well-established macrophage differentiation protocol. Irrespective of the donor background, all generated iMac showed typical macrophage morphology and expressed macrophage-related surface markers such as CD14, CD45, CD86 and CD163. In contrast, CF-iMac showed a significantly impaired ability to phagozytose *P. aeruginosa* at multiplicity of infection (MOI) 1 and 10 when compared to WT-iMac. Interestingly, CorrCF-iMac showed an intermediate phagocytosis capacity which was clearly MOI dependent. Of note, while CorrCF-iMac could clear *P. aeruginosa* at low MOI, CorrCF-iMac showed impaired clearance capacity at higher MOI, which was very much similar to CF-iMac. Furthermore, CF-iMac showed a time-dependent delay to effectively initiate bacterial degradation, while WT-iMac efficiently eradicated more than 50% of its intracellular *P. aeruginosa* within the first hour of digestion. This result was further corroborated by electron microscope images of CF and WT-iMac within one hour *of P. aeruginosa* treatment. Here, internalized bacteria in CF-iMac tended to be visibly less degraded, which points towards a delayed anti-microbial activity of iMac in CF.

In summary, we are able to provide a new platform for the generation of primitive macrophages from CF patients. Using this system, we could demonstrate impairment in bacterial phagocytosis and killing of CF-iMac. We believe that iMac can be an essential tool in the development of a reliable *in vitro* infection model with potential to further research MDR bacteria clearance in CF as well as screen new drugs targeted towards the improvement of CF macrophage functionality.

P76

The downregulation of leukocytes MMP9 expression could be used as marker for monitoring and predicting the efficacy of therapies in cystic fibrosis

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It is well known that the clinical responses of cystic fibrosis (CF) patients to CFTR modulators vary by genotype, and sometimes even within the genotype. Thus, it is of great relevance to find biomarkers for monitoring therapies, particularly those predictive of an individual patient's response.

The significance of inflammation in CF and the finding that CFTR is expressed also in immune cells have raised interest in the role of these cells in this pathology. In particular, many studies have indicated that the restoration of CFTR function by modulators in leukocytes promotes also the normalization of inflammation and infection in CF patients. This correlation suggests that this cell type, being also easily and quickly isolated from patients, could be a useful cellular model to monitor the efficacy of CF therapies during clinical trials.

In this contest, we previously identified changes in specific proteomic profiles related to restored CFTR activity in CF leukocytes after *ex vivo* treatment with Ivacaftor. The results obtained highlighted the downregulation of proteins belonging to the leukocyte transendothelial migration, particularly the matrix metalloproteinase 9 (MMP9). Since the high expression of this protease potentially contributes to parenchyma lung destruction and dysfunction in CF, the downregulation of MMP9 could represent one of the possible positive effects of Ivacaftor in decreasing the disease progression.

The objective of our study aimed to confirm the involvement of MMP9, previously identified as downregulated following the rescue of CFTR activity, by an *in vivo* investigation.

We used leukocytes isolated from CF patients before and following both Ivacaftor or Trikafta therapy and we measured MMP9 levels by Immunoblotting. The results obtained were correlated with those evalueted by the CFTR activity GST-HS-YFP assay, before and at different times during the therapy.

This data showed that the therapies promoted a decrease in leukocytes MMP9 levels together with a recovery of CFTR activity in each sample analysed. Moreover, both parameters correlated with an improvement of some clinical parameters, such as FEV_1% and sweat tests.

At the light of our results we can conclude that both Ivacaftor and Trikafta therapy increased leukocytes CFTR activity and decreased MMP9 expression. Although additional studies are needed to confirm these data in a higher number of patients, downregulation of leukocytes MMP9 expression could be considered a promising biomarker suitable for application in a clinical laboratory setting, in order to accelerate a much-needed personalized medicine approach in CF.

P77

Increased pulmonary infiltration and attenuated phagocytosis defines perinatal dysfunction of innate immunity in cystic fibrosis

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In Cystic Fibrosis (CF) patients, cycles of infection and inflammation lead to fatal lung damage. While there is no doubt that diminished mucus transport is a major contributor to the impaired bacterial clearance, the relevance of the immune system for the early pulmonary pathophysiology is unclear. Initial steps of the disease cannot be followed in patients, simply because blood sampling in very young patients is restricted to rare occasions and systematic investigation of resident immune cells in the airway wall is almost impossible. Further, immune cells are highly sensitive to environmental influences, and their priming by cellular stress pathways or imprinting in the pro-inflammatory milieu of CF patients complicates the correlation of leukocyte deficits to primary CFTR dysfunction.

We made use of a pig model for CF to examine immune cells, the professional defenders against disease, before the onset of infection or inflammation. Within the first day of life, histological and proteomic examination as well as cytokine levels in BAL showed no sign of pneumonia or mucus accumulation in CF pigs. However, the isolation of cells from different tissues and their purification by density gradient revealed a significantly increased number of mononuclear cells in CF airways. By using flow cytometry, we identified mononuclear phagocytes as the accumulating leukocyte population. Furthermore, Ki-67 expression levels exhibited a significant enrichment of proliferative myeloid cells in CF lungs. Complementary immuno-histochemical staining for the macrophage (MΦ) marker CD68 localized MΦs in the interstitium or in attachment to the alveolar wall of neonatal CF lungs and specifically, digital image analysis revealed enrichment of $CD68^+$ cells in cranio-ventral compartments of apical and cardiac lobes, whereas in the caudal regions the diaphragmatic lobe did not show differences between CF and WT controls. In addition, stimulation of whole blood samples with opsonized *E. coli* indicated impaired phagocytic uptake and production of reactive oxygen species by CF phagocytes, which was coincided with a lower expression of the phagocytosis initiating type III Fc-gamma receptor, CD16 and the complement receptor 3, CD11b on monomyleoid cells from blood, spleen and lungs.

Increased infiltration into tissue of small airways and a reduced phagocytic potential indicate substantial changes in the myelo-monocytic lineage. Our results demonstrate that aberrations of the immune system in CF are congenital and recommend that specifc treatment of the immune system in patients should be considered at the earliest possible stage before secondary consequences of the destructive airway disease manifest.

P78

Wnt and TGFβ signaling in cystic fibrosis airway epithelium differentiation

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Cell proliferation and differentiation are important processes in the maturation of an epithelium during development and injury repair. These processes need to be tightly regulated to ensure an epithelium with a good structural integrity. Cystic Fibrosis (CF) is associated with a lack of structural integrity of the airway epithelium, suggesting a defect in differentiation, and ultimately results in infections and respiratory failure. We have recently shown that CF airway epithelia are associated with overexpression and apical localization of fibronectin, phenomena usually linked with defects in differentiation. However, the link between CFTR and differentiation defect is unclear. To uncover this, we compared primary cultures of CF (F508del mutation) human airway epithelial cells (HAECs) and a HAEC line, Calu-3, knocked-out for *CFTR* by CRISPR-Cas9 (*CFTR* KO) to control counterparts, grown at an air-liquid interface (ALI) as a trigger for differentiation through the inhibition of proliferation. RNA sequencing on HAECs and qPCR revealed that the Wnt and TGFβ signaling pathways were deregulated in CF airway epithelia, suggesting an imbalance between proliferation and differentiation. Indeed, Ki67 analysis revealed a significant decrease in cell proliferation after 3 days of ALI in the *CFTR* KO cells ($P < 0.0001$). Furthermore, as shown by immunoblotting and immunofluorescence experiments, the expression of the main signal transducer of the Wnt signaling pathway, β catenin, was significantly lower in *CFTR* KO cells at day 15 of ALI ($P < 0.05$). This decrease was caused by enhanced degradation of β catenin at D15 of ALI, ($P < 0.05$), as demonstrated by a cycloheximide chase assay. This mechanism is known to be induced by Wnt pathway inhibition. Indeed, DKK1, an inhibitor of the Wnt pathway, is over-secreted in *CFTR* KO cells. Moreover, an ectopic fibronectin deposition was observed in the *CFTR* KO cells during ALI, suggesting an impairment in differentiation potentially due to aberrant TGFβ signaling. In an attempt to correct these differences, *CFTR* KO monolayers were treated with CHIR99021 and SB431542, Wnt pathway activator and TGFβ pathway inhibitor respectively. Treatment with CHIR99021 was able to restore the expression of total and nuclear β catenin in *CFTR* KO cells. Interestingly, fibronectin expression was also decreased after treatment. On the other hand, treatment with SB431542 led to a decrease in the phosphorylation of Smad2, one of the main signal transducers of the TGFβ pathway, as well as a decrease in fibronectin expression in *CFTR* KO cells. Altogether, these results hint towards the interplay of Wnt and TGFβ pathways in the proliferation and differentiation of airway epithelial cells which are dysfunctional in the absence of CFTR. Further investigation is underway to provide more insight towards the initiation and progression of Cystic Fibrosis.

P79

Macrophages combatting *Pseudomonas aeruginosa* lung infection – a new treatment strategy for multidrug resistant bacterial infections in the CF lung

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Multidrug resistant pathogens are one of the main worldwide health concerns. This constitutes an extremely alarming problem especially when considering patients with lifelong pulmonary conditions such as cystic fibrosis (CF), which makes them particularly vulnerable to lung infections. Some strains of *Pseudomonas aeruginosa*, a nosocomial pathogen and common cause of severe lower respiratory tract infections in immunosuppressed or chronically ill patients, have been reported as resistant to nearly all available antibiotic treatments. As a key pathogen in CF, chronic *P. aeruginosa* infections are decisive for the course of the disease and directly related to progressive lung damage in CF lungs. It is therefore of main importance to establish a new therapeutic approach for those patients.

In preliminary studies, we transferred bone marrow from healthy animals into CF mice and were thus able to successfully demonstrate an improved immune response to airway infection with *P. aeruginosa*. To bypass the invasive step of bone marrow depletion as done before, we now want to apply macrophages directly to the lungs of CF mice, a so called pulmonary macrophage transplantation (PMT). Since alveolar macrophages represent the first line of defense against airborne bacteria, but are suboptimal phagocytes in CF patients a transplant of functional WT macrophages could be a way to treat and even prevent lung infections. Therefore, we here present our first steps towards the development of a novel cell based treatment of *P. aeruginosa* pulmonary infections in CF.

In order to corroborate our hypothesis, the following *in vivo* experiments were performed: Healthy wild type murine bone marrow derived macrophages (mBMDM), obtained by an established protocol, were intratracheally (i.t.) delivered to the lungs of CF mice. Twenty-four hours post transplantation, mice were challenged with i.t. administered *P. aeruginosa* and closely monitored during the following 24h. Along the infection progression, mice which received PMT recovered better (monitored by body temperature and multiparametric disease score) compared with the experimental group that only received the infection. We also observed a reduced hemorrhage in the broncho-alveolar lavage fluid of transplanted mice and a tendency towards the reduction of lung bacterial load.

In summary, the preliminary findings of this ongoing study are encouraging, but will need an expansion of data to confirm reliability of our results with regard to the potential future translation of this treatment to human patients.

P80

Liposomes efficiently activate innate immune system to counteract Pseudomonas aeruginosa infection in CF zebrafish model

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Multi-drug resistant (MDR) bacteria are insensitive to the antibiotics currently in use. This phenomenon, if not contained, will represent the main cause of death for humanity in 2050. The situation is even more worrying when considering patients with chronic bacterial infections, such as patients with Cystic Fibrosis (CF). The 80% of these patients is subjected to chronic lung infection mainly caused by *Pseudomonas aeruginosa* (*Pa*), that frequently develops antibiotic resistance. Besides the well-known role of *Pa* as extracellular bacterium, recent studies revealed that it can remain also intracellularly into host macrophages, a situation that leads to efficient escape mechanisms, especially in the CF context. Novel anti-infectious therapeutic approaches, based on the modulation of the host response (Host-directed therapy, HDT), have been proposed to counteract the emergence of antimicrobial resistance. An intriguing strategy is to stimulate host intracellular antimicrobial action through the use of liposomes carrying bioactive molecules. In a previous work we set-up a *Pa* infection model in CF zebrafish embryos to follow *in vivo* the progression and resolution of the infection and to assess the efficacy of pathogen-directed therapies (PDT) in terms of lethality, bacterial burden and pro-inflammatory response. In the present study, we are investigating in wild-type (WT) and CF zebrafish embryos the antibacterial effects of apoptotic body-like liposomes carrying bioactive lipids able to stimulate macrophage phagocytic activity. First, we tested the effect of liposomes in stimulating the host-immune response. When embryos were locally treated with liposomes and then infected with *Pa*, we observed an increased recruitment of macrophages toward the infection site. Also, systemic delivery of liposomes before *Pa* injection, led to an increased expression of anti-inflammatory cytokines, suggesting a more rapid resolution of the infection.

Indeed, we observed a decrease in the bacterial burden at both early and late infection stage, both with local and systemic *Pa* infection. Furthermore, we tested liposomes in a therapeutic setting, administering them after *Pa* infection. Also in this case, we observed a reduction of the bacterial load, in particular in WT embryos but less in CF embryos, according to their delay in the activation of the immune response. In conclusion, the combination between HDT and PDT strategies might represent a useful therapeutic approach to counteract the insurgence of MDR bacteria, limiting the antibiotic doses and time of administration.

P81

Monocyte integrin activation as a CFTR targeted drugs evaluation test in cystic fibrosis patients

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Background: Lung inflammation is a major cause of decline in respiratory function in CF patients. The accumulation of neutrophilic granulocytes (PMNs) in the inflamed lung depends on integrin activation. Previously we found that CFTR regulates the activation of integrins in monocytes. Integrin activation is defective in CF monocytes, leading to an unbalanced recruitment of phagocytes into the lung parenchyma, with exacerbated accumulation of PMNs. The discovery identifies CF as a new type of leukocyte adhesion defect disease (LAD-IV), indicating that a LAD background characterizes the pathogenesis of CF.

Hypothesis: Restoration of CFTR function by specific drugs restores LFA-1 activation in CF monocytes. Therefore, quantification of LFA-1 activation in monocytes constitutes a marker of CFTR activity and can be exploited to monitor the efficacy of CFTR correcting drugs.

Objective: To demonstrate that the measurement of LFA-1 activation in monocytes can be used to monitor the CF patient treatments with the CFTR targeted drugs ivacaftor, tezacaftor and elexacaftor.

Patients, materials and methods: Peripheral blood from CF patients selected for class II or III mutations before and after therapy. Monoclonal antibodies that detect low-intermediate and high affinity conformational epitopes of LFA-1. Activation of LFA-1 induced with chemotactic factors. Quantification of LFA-1 activation is measured by flow cytometry and adhesion assays.

Results: We analyzed monocytes of 38 CF patients treated with Ivacaftor+Tezacaftor+ Elexacaftor or Ivacaftor+Tezacaftor (Vertex Pharmaceuticals). The results show that in monocytes isolated from 25 out of 30 patients treated with Ivacaftor+Tezacaftor+ Elexacaftor and from 6 out of 8 patients treated with Ivacaftor+Tezacaftor, the therapy was able to correct the LFA-1 activation defect, measured as both affinity induction and adhesion. The affinity measure was more reproducible. Furthermore, a preliminary analysis in 15 patients of the change in respiratory capacity (FEV1) shows that its recovery is significantly greater ($p = 0.04$) in the group that presented LFA-1 affinity correction (6.5 ± 7, $n = 7$) compared to the group that did not show correction (2.3 ± 2, $n = 6$). Notably, the reduction of the concentration of chloride in sweat in the two groups, on the other hand, is entirely comparable. A much wider multiparameter statistical analysis is ongoing to confirm and increase the significance of these data.

Conclusion: The data show the correspondence between correction of the CFTR defect and correction of LFA-1 activation defect in CF patients and that the correction of the integrin defect appears to be associated with a greater recovery of FEV1 compared to the evaluation of the concentration of chloride in sweat. The data, therefore, support that the measurement of monocyte LFA-1 activation can be used as a test to monitor the effectiveness of CFTR targeted treatments.

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P83

Hydrogel-encapsulated niclosamide for topical treatment of inflammatory airway diseases

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Background: Previous work showed that the Ca^{2+}-activated Cl^- channel TMEM16A is upregulated in cystic fibrosis and asthma and accompanies goblet cell hyperplasia/metaplasia and mucus hypersecretion, being predominantly expressed in mucus-producing cells in the airway submucosal glands. Repurposing of the anthelminthic drug niclosamide -- a potent TMEM16A inhibitor -- was proposed as an effective treatment for inflammatory airway diseases. Niclosamide showed a potent inhibition of mucus production and mucus secretion in the airways, inhibition of the release of the CF-typical inflammatory mediator IL-8, and a pronounced induction of bronchorelaxation, shown both *in vivo* and *ex vivo*. While systemic application of niclosamide may lead to unwanted side effects, local application to the lungs may circumvent these problems, particularly when the drug is encapsulated into hydrogels.

Methods/results: In the present study, we achieved the encapsulation of niclosamide in polyethylene glycol hydrospheres of micrometer and nanometer size (Niclo-spheres). When applied to ovalbumin (OVA)-sensitized and challenged -- asthmatic -- mice via intratracheal instillation to the lungs, free niclosamide and Niclo-spheres strongly attenuated the overproduction of mucus, measured by mucins-specific alcian blue staining of paraffin-embedded lung tissue. All niclosamide formulations were also shown by immunohistochemistry to inhibit the secretion of the major proinflammatory mediator CLCA1 in the airways, and to improve mucociliary clearance in *ex vivo* asthmatic tracheas, roughly back to healthy control levels. Niclo-spheres were confirmed by whole-cell Patch-Clamp measurements to strongly inhibit TMEM16A, and by immunocytochemistry to inhibit mucus production, in CFBE and Calu-3 human airway epithelial cells, which is due to a pronounced inhibition of intracellular Ca^{2+} signals. Empty/unloaded particles were shown to have no effect in any parameter analysed.

Conclusions/future perspectives: The data suggests that poorly dissolvable drugs such as niclosamide can be deposited on the airway epithelium as hydrogel-encapsulated formulations for the resolution of inflammatory airway disease. This local application may present several advantages over systemic administration by limiting off-target effects and the need for repetitive dosing due to slow long-lasting release of the hydrogel-cargo. Conjugation of the hydrogel-particles with unique ligands designed to target airway cell type-specific surface receptors and prompt cell-specific internalization/uptake is the next step to achieve an enhanced specificity of niclosamide delivery to inflamed airways.

Keywords: Niclosamide, TMEM16A, cystic fibrosis, inflammatory airway disease

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P84

Extracellular vesicle lncRNA MALAT1 drives HDAC11-dependent chronic inflammation in cystic fibrosis airway neutrophils

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Objectives: We previously showed that polymorphonuclear neutrophils (PMNs) recruited to the airway lumen in people with CF undergo rapid transcriptional reprogramming, resulting in hyperexocytosis yet reduced bacterial killing. Together, this promotes chronic infections, feed-forward inflammation and structural tissue damage. These PMNs also secrete a large number of extracellular vesicles (EVs). Here, we hypothesized that EVs secreted by reprogrammed PMNs cause newly recruited PMNs to undergo similar pathological changes, thereby explaining the chronicity of CF airway inflammation.

Methods: EVs were characterized from CF sputum and conditioned media from airway-like neutrophil cultures derived from a lung transmigration model. EVs were first purified by differential centrifugation followed by fractionation on a 300 kDa MWCO and downstream analysis by RNA-seq, nanoflow cytometry and nanoparticle tracking analysis. To study recursive signaling, EVs from transmigrated CF-like and healthy neutrophils were applied to a naïve population of neutrophils recruited in a second round of transmigration. This secondary set of transmigrated neutrophils were characterized by RNA-seq and flow cytometry to assess activation and inflammatory poise. As a functional validation, secondary transmigrated neutrophils were incubated with *P. aeruginosa* (PA01) to assess bacterial killing capacity.

Results: Exposure of PMNs to EVs from CF sputum in an airway transmigration model induced hyperexocytosis and reduced bacterial killing, recapitulating in vivo findings. Removal of PMN-derived (CD66b+) EVs from CF sputum prevented hyperexocytosis and increased bacterial killing by recruited PMNs. Moreover, EVs from PMNs recruited to CF sputum in vitro induced hyperexocytosis and reduced bacterial killing by a second wave of recruited PMNs.

RNA-seq analysis identified lncRNA MALAT1 as differentially expressed in EVs from CF- or healthy-conditioned PMNs. Transfection of naïve PMNs with MALAT1 induced hyperexocytosis and reduced bacterial killing. The regulatory enzyme HDAC11 was rapidly upregulated in CF sputum exposed and MALAT-1 transfected PMNs. Transfection of naïve PMNs with HDAC11 resulted in hyperexocytosis, decreased bacteria killing and lead to the localization of MALAT1 to secreted EVs, while inhibiting HDAC11 blocked these processes. Finally, MALAT1 levels in CD66b+ EVs from adult CF patients correlated negatively with %FEV1 and increased during acute pulmonary exacerbations.

Conclusion: CF patient sputum contains a high concentration of EVs, particularly those originating from prior waves of inflammatory PMNs. PMNs recruited to the CF airway lumen are exposed to these EVs leading to MALAT1-dependent HDAC11 induction and subsequent packaging of MALAT1 into EVs. These PMNs exocytose primary granules and fail to clear invading pathogens. The released MALAT1+ CD66b+ EVs reprogram naïve PMNs to exhibit a similar phenotype resulting in feed forward reprogramming of PMNs and chronic airway disease. These findings identify MALAT1 and HDAC11 as potential targets for intervention to lessen inflammation and improve pathogen killing in CF airways and other diseases dominated by PMNs.

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P85

HiPSC-derived AECs as a novel platform to study the role of ionocytes in mucociliary clearance

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Cystic Fibrosis originates from biallelic mutations of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene. These result in increased mucus viscosity which reduces clearance in the airway epithelium and predisposes to bacterial infection and biofilm formation. The main cell type involved in this process are ciliated cells of the airway epithelium. These specialised cells orchestrate mucus movement upwards and outside the airways to keep them free of pathogens and external particles. Based on this essential function, ciliated cells have been a major focus for CF research. More recently, ionocytes have been identified as the cell type which expresses the highest levels of CFTR and they have been hypothesised to have a key role in CF. However, their exact function in the airway epithelium remains to be elucidated.

The study of ionocytes in the human airway and their role in disease remains challenging because of their rarity, the lack of availability of fresh tissue/brushings and the patient burden that their obtention implies. Furthermore, the comparison between patient and healthy donor samples has limitations due to genetic background-derived variability. For all these reasons, our group has decided to use of human induced pluripotent stem cells (hiPSCs) for disease modelling and for studying the airway epithelium. This model system allows the reprogramming of somatic cells from patients into pluripotent cells that can be differentiated into virtually any cell type, including AECs. However, production of ionocytes from hiPSCs remains to be demonstrated.

To address this question, we have developed a differentiation protocol to produce AECs from hiPSCs. This method includes a sorting step at day 16 to enrich for lung progenitors ($NKX2.1^{+}$), after which the cells can be expanded as 3D organoids before they are further matured. Importantly, the resulting cells can also be cryopreserved, allowing to re-start cultures from lung progenitors rather than having to differentiate hiPSCs every time, thereby increasing the practicality of the protocol. Maturation of the cells in air-liquid interface using an in-house chemically defined medium leads to the formation of a polarised pseudostratified epithelium that includes basal cells (p63, CK5), secretory cells (SCGB3A2, MUC5AC), ciliated cells (FOXJ1) with motile cilia and more importantly ionocytes (FOXI1), as shown by qPCR and immunocytochemistry analyses. To further investigate the role of ionocytes, we generated hiPSCs knock out for FOXI1, a transcription factor essential for their differentiation. $FOXI1^{KO}$ hiPSCs were differentiated into AECs and, when compared to their isogenic wildtype control, we observed a decrease in number of ciliated cells both at mRNA and protein level. In terms of functionality, the cells showed no significant difference in epithelial barrier properties (TEER) while ciliary function remains to be fully characterised.

Our approach constitutes a unique platform to study the importance of human ionocytes in cilia motility in health and in disease paving the way to more targeted treatments. Furthermore, the model has the potential to be useful for the study of other lung diseases and to bring us a step closer to regenerative therapy.

P86
Sputum handling for rheology

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Rheology as a tool to relate sputum physical properties to infection, inflammation or mucociliary clearance has been a hot topic for the last years. However, contrasting information from different studies is difficult as there is no stablished protocol on the way samples should, or mustn't be manipulated before rheometry.

Sputum samples go through different phases between their collection until the moment they are finally measured. In this cycle there are many conditions as storage temperature or accepted delay between the time of extraction and the measurement which must be controlled to obtain descriptive and comparable measurements.

The main objective of this study is to bring light into the handling of sputum samples to stablish a standardized and robust protocol before rheological measurements.

Therefore, the effect of freezing samples has been studied as well as the consequences of leaving them at room temperature over 24 hours. Moreover, sputum is a highly heterogeneous fluid making it complicated to obtain reproductible rheological measurements hence, the repercussions of homogenization have also been addressed. Finally, due to the health emergency caused by COVID19 the heating effect was studied as this was the technique to inactive potential virus in the samples.

Samples are collected from the Centre de Ressource et de Compétence de Mucoviscidose (CHU Grenoble). These expectorations are induced by drainage with the help of a physiotherapist and then kept at 4°C for less than one hour before testing.

- Heating consequences. Samples were divided into two aliquots: (1) raw aliquot and (2) heated aliquot at 56°C for 30 minutes in an orbital incubator at 30 rpm.
- Homogenization repercussions. Samples were separated into two aliquots: (1) raw aliquot and (2) vortexed aliquot for 30 seconds.
- Freezing effect. Samples were divided into three aliquots: (1) raw aliquot, (2) frozen aliquot by directly introducing it in a -80°C freezer and (3) frozen aliquot by snap freezing. Before measurements frozen samples were thawed quickly at 37°C.
- Aging repercussions are studied by rheometry in a controlled atmosphere through 24 hours.

On the one hand, this study has shown heating has a destructive effect leading to rheological prints characteristic in liquids more than gels. On the other hand, freezing and homogenization do not show significant outcomes: their variation in elasticity and viscosity is lower than the variation found for intra-sample reproductibility. However, snap freezing is preferred as it shows higher resemblance to the measurements for raw aliquots.

Therefore, we recommend storing samples, without heating, by snap freezing and homogenize them to ensure reproductibility.

P87

Antisense oligonucleotides as splicing modulators in intestinal and respiratory patient-derived model systems - novel insights into a potential therapy

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Rectal organoids provide a limitless resource for CF research and personalized therapy by study of the endogenous CFTR gene in its native context. Drug response in organoids has been shown to correlate to the response in cellular model systems such as HNEs and to clinical response as measured by lung function (FEV1) and sweat chloride levels. 10-15% of CF patients carry mutations affecting the correct splicing of the CFTR transcript. SpliSense is developing an antisense oligonucleotide (ASO)-based therapeutic approach to modulate aberrant CFTR splicing in patients carrying non-canonical splicing mutations and restore CFTR function. SpliSense has tested its ASOs in cellular systems as well as HNEs from patients carrying at least one 3849+10kB C->T allele (1), a mutation leading to the inclusion of a cryptic 84bp exon. The results show that free uptake of SPL84-23 leads to complete rescue of aberrant splicing and restoration of CFTR function.

In order to investigate the relevancy of organoids as a model system for testing ASO-based drugs, we developed a protocol for effective uptake of ASOs into intestinal organoids. We show that SPL84-23 modulates the splicing pattern in 3D organoids from several patients carrying the 3849 mutation as seen by a significant reduction of aberrant splicing, restoration of mature CFTR protein and significant response in the forskolin-induced swelling assay. We are currently investigating the functional effect of the ASO treatment in organoid-derived monolayers, by short-circuit current measurements. Our results show a significant effect of the ASO treatment on the splicing pattern and CFTR activity. This approach enables a direct comparison of CFTR functional rescue to HNEs from the same patient and by the same assay. Our results highlight the potential of organoids as a valuable model system for the development of ASOs as a novel therapeutic approach for CF patients carrying non-canonical splicing mutations.

1. Oren YS et al.*Journal of CF Vol.* 20,5 (2021): 865-875.

P88

Correction of the W1282X CFTR variant by gene editing with two techniques alternative to homology-directed repair

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CFTR modulators can treat ~90% of patients with cystic fibrosis, but this represents only about half of the known CF-causing variants described to date. CFTR class I mutations, which results in no protein production cannot be targeted by the currently available drugs. We have previously shown correction of the 2nd most common class I mutation W1282X using homology-directed repair (HDR) with Cas9 and Cas12a, resulting in full length protein expression and restoration of CFTR channel function. However, we also observed a high level of on-target indels which potentially limits this as a therapeutic approach[1]. Here, we report two alternatives approaches to correct this variant.

First, we used adenine base editing (ABE) in the HEK FLPin W1282X cell line and human nasal epithelial primary cells (HNE) homozygous for W1282X. NG-ABEmax and gRNA plasmids were either transfected with lipofectamine or electroporated in HEK or HNE cells respectively. Our results showed genomic correction of the W1282X mutation at a frequency of about 25%, but also a bystander effect at amino acid position 1283 which changed Arginine to Glycine (R1283G). The correction was linked with an increased level of corrected transcripts also carrying the bystander (~50% of total transcripts). Protein analyses of clones with both W1282 and G1283 showed expression of a full-length protein but with some impairment in the production of mature CFTR. Short circuit current (Isc) measurements on the corrected HNE cells showed restoration of protein function of about 15% of WT.

The second approach was to integrate a superexon spanning exon 23 to 27 into intron 22 of 16HBE14o- W1282X cell line using homology-independent targeted integration (HITI). Cells were transfected with Lipofectamine using Cas9/sgRNA and superexon$_{23\text{-}27}$ donor plasmids. We observed precise genomic integration of the superexon at a frequency of about 5%, and this resulted in 7% of CFTR transcripts being derived from the the superexon containing the WT sequence at position 1282. A clonal cell line containing the superexon in both alleles was derived and showed that about 70% of the transcripts contained the superexon with WT amino acid at 1282, while the remaining transcripts contained the W1282X variant. Western blot analyses of the clone showed restoration of a full-length mature protein. Measurement of Isc showed CFTR functional restoration of ~10% of WT. When treated with modulators VX-770, VX-661 and VX-445, the CFTR channel function was increased to ~30% of WT.

Further experiments will be required to see if it is possible to base edit W1282X without the bystander editing to enable functional restoration of CFTR activity, and/or to assess if the use of modulators could correct the negative effect of the bystander effect. However, the HITI of superexon$_{23\text{-}27}$ and its ability to restore CFTR at levels which are expected as beneficial to CF patients, merits further investigation, and has the added advantage that it should also correct other CF variants such as N1303K located in exon 24.

1. Santos, L. et al. Comparison of Cas9 and Cas12a CRISPR editing methods to correct the W1282X-CFTR mutation. *J Cyst Fibros* (2021).

P89

Development of in vitro transcribed mRNA therapeutics for cystic fibrosis

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Cystic fibrosis (CF) is a recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), affecting approximately 10,000 people in the UK. Absent/non-functional CFTR leads to thick, sticky mucus in the lung which results in chronic bacterial infection and inflammation. Gene replacement therapy with viral/non-viral vectors has been explored in the last 25 years but all failed to show significant clinical efficacy. *In vitro* transcribed (IVT) mRNA has emerged in the last few years as a new approach for protein replacement.

Our aim is to develop CFTR IVT mRNA therapy for CF to replace the missing wild type CFTR protein using receptor-targeted nanocomplex (RTN) formulations. We have developed RTN formulations that consist of liposomes epithelial receptor targeting peptides and the nucleic acid. Our approach was to first optimise RTN nanoparticles for transfection of primary cystic fibrosis bronchial epithelial (CFBE) cells, at submerged culture and air-liquid interface (ALI) cultures, as well as in mouse lung, using reporter IVT mRNAs. We then assessed delivery efficiency of CFTR mRNA in CFBE cells at submerged culture and ALI culture by immunostaining of protein. Functional testing for restoration of ion transport by Ussing chamber analysis is in progress and will be reported.

We first optimised the RTN formulations, comparing combinations of three different cationic liposomes and five peptides for their biophysical properties and transfection efficiency. We identified a novel formulation (size 130 nm, PDI 0.28, charge 38 mV) for mRNA delivery that achieved almost 100% cellular uptake efficiency and 90% transfection efficiency. There were no differences in transfection efficiency between primary normal human bronchial epithelial (NHBE) cells and CFBE cells. The same RTN formulation was able to deliver the mRNAs in ALI cultured cells and mouse lungs where luciferase expression in mouse lungs was ~200-fold higher than plasmid DNA encoded luciferase. CFTR mRNA was successfully delivered to NHBE and CFBE cells. In addition, we co-packaged the CFTR corrector VX-809 with CFTR mRNA into RTNs and found that this improved the expression of CFTR protein in CFBE cells in submerged culture by approximately 2 to 2.5-fold. CFTR protein translated from the IVT mRNA was detected by immunoblotting from 4-hour to at least 48-hour after the CFBE mRNA transfections.

In conclusion, CFTR IVT mRNA delivery is a promising novel therapeutic for cystic fibrosis. In addition, the flexibility of the RTN formulation allows co-delivery of CFTR mRNA with VX-809, which significantly improved CFTR expression.

P90

Anticodon engineered transfer RNAs efficiently suppress endogenous CFTR nonsense mutations

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Cystic Fibrosis (CF) is a genetic disease caused by mutations in cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The majority of the CF patient populations has benefited from the development of CFTR modulator drugs, which target defective CFTR proteins to restore the channel folding, trafficking and function. However, approximately 3% of CF patients with nonsense or premature termination codon (PTC) mutations are not eligible for CFTR modulator therapies. PTCs result in significant loss of CFTR transcripts through the nonsense-mediated decay (NMD) pathway and therefore nearly complete loss of CFTR protein, which eliminates the therapeutic target for modulators. Due to a critical need for the development of novel therapeutic approaches for CF patients with nonsense mutations, anticodon engineered transfer RNA (ACE-tRNA) therapy is currently being explored as a potential treatment option for these patient populations. With an engineered anticodon complementary to a PTC, ACE-tRNAs are designed to suppress PTCs by incorporating the correct amino acid and thereby generate full-length functional protein.

Recently, we generated and screened a library of ACE-tRNAs to target PTCs with high efficiency. Based on the initial screening results, we selected ACE-tRNAs with the best PTC suppression activity for CF-causing nonsense mutations, G542X, R1162X and W1282X. To determine the efficiency of ACE-tRNAs to rescue CFTR mRNA expression level and channel function, we performed cell-based assays in gene-edited human bronchial epithelial cell lines (16HBEge) with nonsense CF mutations. CFTR transcript level was significantly increased for 16HBEge-G542X, -R1162X and --W1282X cells following transient transfection with cDNAs encoding ACE-tRNAs. To correlate ACE-tRNA cassette numbers to PTC readthrough activity, we generated a 16HBEge-R1162X cell line that stably expresses arginine ACE-tRNAs using a piggyBac transposon technology. Excitingly, we determined that only ~16 copies of integrated ACE-tRNA per genome are sufficient to rescue significant levels of CFTR mRNA expression and channel function.

We next wanted to test the effect of NMD inhibitor and CFTR modulators in combination with ACE-tRNAs. As we expected, NMD inhibitor treatment had synergistic effects with ACE-tRNAs on rescuing both CFTR transcripts and functional protein levels. Further, because ACE-tRNAs seamlessly rescue full-length CFTR protein, there is now a target for CFTR modulators. Combination therapy with ACE-tRNAs and modulators in fact enhanced CFTR function. These findings suggest that ACE-tRNAs may be a promising therapeutic approach for nonsense-associated CF, especially in combination with readily available drugs.

P91

CFTR super exon splice site and polyA signal affect CFTR expression and function

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Gene editing and gene therapy are promising therapeutic approaches for cystic fibrosis (CF). Strategies to correct individual *CFTR* variants are not feasible for most CF-causing genetic variants as more than 1,700 genetic variants of the *CFTR* gene have been suggested to cause CF. Insertion of a partial cDNA (also known as a super exon) into the native *CFTR* genomic locus can restore the *CFTR* gene to a wildtype coding sequence for all mutations downstream of the insertion site while retaining the endogenous *CFTR* promoter and chromatin architecture. Several groups have successfully inserted *CFTR* super exons of various sizes, but much super exon biology remains unexplored. There are many variables for super exon insertion, such as insertion site, codon optimization, splice site, and polyadenylation signal sequence. We inserted three super exons with the same coding sequence into the same location of *CFTR* and found that both the super exon splice site and polyadenylation signal sequence can have significant consequences on super exon expression and function.

We inserted a super exon into intron 22 of the *CFTR* gene with the W1282X or N1303K CF-causing mutation. To separate super exon expression and function from the efficiency of super exon insertion in a population of cells, we isolated super exon-positive clonal cell lines. Super exon 1 consisted of the native exon 23 splice acceptor, native CFTR coding sequence for exons 23-27, and the BGH polyA signal. Surprisingly, this super exon resulted in poor (5-10% of WT) restoration of CFTR function. Analysis of *CFTR* mRNA isoforms revealed that many transcripts were splicing to the native exon 23 and bypassing the super exon. To address this issue, we created super exon 2, which has the same sequence as super exon 1, but with a stronger splice site. This super exon resulted in increased CFTR Function (~30% of WT) but was still not able to completely rescue CFTR expression and function. The third super exon had both a strong splice site as well as a different polyA signal sequence (SV40 polyA). This super exon resulted in robust CFTR functional rescue (50-100% of WT).

In summary, we have demonstrated successful targeted insertion of three *CFTR* super exons with the same coding but different flanking sequences, resulting in variable functional rescue of CFTR. These studies demonstrate that simple recapitulation of WT DNA coding sequence does not necessarily produce normal levels of WT mRNA transcript and complete functional rescue. Additionally, analysis of super exon function in a population of cells may mask inefficiencies in the super exon construct. Work is ongoing to evaluate these super exons in other CFTR cell types and in vivo, and to apply these improved super exon features (splice site and polyA signal sequence) to creating better, larger super exons that could help more people with CF.

P92

Optimization of nonsense suppressor tRNAs to lower the in vivo delivery hurdle

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Nonsense mutations or premature termination codons (PTCs) occur when a canonical triplet nucleotide codon is converted into one of three stop codons (TGA, TAG and TAA). These mutations make up 10-15% of all genetic lesions that cause disease including Cystic Fibrosis (CF). Nonsense mutations have been challenging targets for drug discovery and as a result there are currently no FDA-approved therapeutics. Current CF small molecule therapeutics target the aberrant CFTR channel, however as nonsense mutations prevent production of CFTR, about 3% of CF patients do not benefit from the available therapies. Instead, therapies for nonsense-associated CF require a shift in target to CFTR transcription, mRNA degradation, or translation of PTC-containing CFTR transcript.

We have recently demonstrated the capability of a panel of anticodon-edited tRNAs (ACE-tRNAs) to suppress common CF-causing PTCs in 16HBEGE cells harboring genomically encoded nonsense mutations. While ACE-tRNAs have been demonstrated to provide seamless rescue of full-length CFTR, delivery of ACE-tRNA DNA vectors to epithelial cells remains a significant hurdle. To lower this delivery hurdle, we set out to optimize the performance of our best performing "hit" ACE-tRNAs through alterations to the ACE-tRNA expression cassette and ACE-tRNA sequence. Increasing the potency of our ACE-tRNA per unit delivered theoretically allows us to deliver less ACE-tRNA to reach the same level of CFTR rescue.

To increase the potency of our ACE-tRNA hits we further engineered the sequences to promote transcription and maturation of the tRNA and improve interactions between the ACE-tRNA and the translational apparatus. ACE-tRNAs delivered as DNA will need to be expressed and processed and will benefit from optimization of expression and processing, while ACE-tRNAs delivered as DNA or RNA will benefit from improved intracellular stability or interactions with the translational apparatus. Here we report the results of screens to optimize the transcriptional and translational function of our best performing ArgTGA, LeuTGA, and GlyTGA ACE-tRNAs. We employed a high-throughput-cloning and -screening platform using a dual firefly and Nano-luciferase expression system to assay optimized ACE-tRNAs for PTC readthrough efficiency. We screened libraries containing over 1600 members to optimize the extragenic and intragenic sequences of ArgTGA, LeuTGA, and GlyTGA in 16HBE14o- cells. We were able to determine optimized elements that increase ACE-tRNA ArgTGA, LeuTGA, and GlyTGA PTC suppression efficiency between 2- and 4-fold, effectively lowering the amount of ACE-tRNA needed to deliver to the cell to rescue the same amount of CFTR. With these optimal transcription and translation elements we are well positioned for delivery of optimal ACE-tRNAs in vivo.

P93

Designing novel self-amplifying RNA and lipid nanoparticle for long-term restoration and enhancement of CFTR function

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Objective: Cystic fibrosis (CF) affects approximately 80,000 people worldwide but still lacks effective therapy for all mutation classes. Over the past years, several areas have been explored for potential therapy. Gene therapy based on DNA and mRNA showed potential regardless of the mutation class. mRNA based human cystic fibrosis transmembrane protein (hCFTR) gene supplementation therapy already proved beneficial and entered clinical trials, but a long-lasting supplementation therapy can be difficult to achieve. Self-amplifying RNA (sa-RNA) systems have proven to have high transient transgene expression, which together with an efficient delivery system can be beneficial. Here we present a novel sa-RNA for achieving long lasting hCFTR expression.

Method: The coding sequence (CDS) of hCFTR was codon optimized and cloned into a VEEV based sa-RNA backbone. *In vitro* expression of hCFTR was defined by RT-qPCR, Western Blotting (596 specific hCFTR antibody) and immunofluorescent staining (IF). Assays were performed 24 hours and 72 hours after transfection of CF bronchial epithelial (CFBE41o-) cells and lung carcinoma (A549) cells with our hCFTR saRNA. Immunofluorescent staining was performed by transfecting CFBE41o- cells and growing in air liquid interface till apical and basal side could be determined by TEER. Nucleus, cell membrane and hCFTR protein were stained and evaluated using fluorescence microscopy. *In vitro* functionality was evaluated using a yellow fluorescent protein (YFP)-based assay. Stably expressing halide-sensitive YFP CFBE41o- cells were transfected and evaluated for quenching of fluorescence signal after 24 and 48 hours.

Results: In both cell lines, RT-qPCR showed a 20,000 fold higher presence of the hCFTR mRNA. The functional glycosylated hCFTR protein (170 kDa band) was detected at different timepoints by Western Blot. Immunofluorescence showed significant migration of hCFTR protein to the apical side of CFBE41o- cells. Results of the YFP-based assay lead to a 7-fold higher functionality of the CFTR protein in transfected CFBE41o- cells.

Conclusion: Our study proved expression and functionality of hCFTR protein supplemented by sa-RNA based approach *in vitro*. A proof of concept study *in vivo* that studies the extent and duration of the CFTR correction is necessary and will be done using a CFTR knock-out mice model after finishing the ongoing optimization of an appropriate carrier.

P94

Correction of the CFTR 1717-1G>A splicing mutation through CRISPR based technology

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Almost 13% of mutations causing cystic fibrosis alter the correct splicing of the CFTR gene. Among these, the Class 1 1717-1G>A substitution is one of the most common mutation affecting approximately 1% of cystic fibrosis (CF) patients. This mutation consists in a G to A conversion in intron 11 which results in the inactivation of a conserved AG dinucleotide at the 3' end acceptor splice site leading to exon 12 skipping or recognition of an alternative cryptic splice site. Both altered splicing products generate a stop codon and consequent lack of CFTR protein synthesis which is associated with CF clinical development not treatable with any currently available pharmacological agent. Gene therapy, in particular CRISPR-Cas derived systems, is an emerging opportunity to permanently cure CF caused by any class type mutations. The recently developed adenine base-editing (ABE) and prime-editing (PE) technologies are capable of promoting precise genome editing in the absence of double-strand breaks (DSB) as opposed to the original CRISPR-Cas strategies thus preventing deleterious genotoxic effects. With this study we aim at developing a genome editing strategy to correct the 1717-1G>A mutation through the most recent CRISPR-Cas technology, DSB-free.

We have set up the correction strategy in 1717-1G>A HEK293 models that we have developed by using minigene constructs mimicking the splicing defect. We obtained up to 42% of correction using NG-ABE8e and NG-ABE8.20m base-editors through plasmid transfection. Nevertheless, both ABEs were associated with high levels of bystander edits, which resulted in the unwanted modifications near the mutated nucleotide with potential detrimental effects in CFTR protein synthesis. As alternative strategy we tested the most recent prime-editing approach which produced encouraging preliminary results with over 10% scarless correction and no detected indels.

Overall, we obtained results showing that the recent prime-editor CRISPR-Cas approach can be exploited to repair the 1717-1G>A providing precise modification over the bystander unwanted mutations introduced by the base-editors. Further optimization of the prime-editor strategies is ongoing to produce a more efficient correction of the 1717-1G>A splicing mutation.

P95

Transduction of Rhesus macaque lung after repeat dosing by AAV1 is enhanced by short-term prednisone treatment

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The major hurdle with gene therapy is development of neutralizing antibodies in response to repeat delivery that could potentially block expression of enough CFTR to be therapeutic. Given that turnover of airway cells may make gene transfer with recombinant adeno-associated virus (AAV)-based vectors transient, repeat dosing of AAV1-CFTR virus will ultimately be required. The goal is to assess whether repeat dosing of AAV1-CFTR administered to primates leads to widespread gene transfer and CFTR expression. To test this, we sprayed 2 doses of 10^{13} vector genomes (vg) of AAV1-Δ27-264 CFTR at 0 and 30 days, respectively, into the airways of 2 healthy male and 3 healthy female Rhesus monkeys, followed by a single dose of 10^{13} particles of AAV1-GFP at day 60. Monkeys were treated with methyl-prednisone succinate 2 mg/kg IM 24 hours before and after AAV1 administration. AAV1-Δ27-264 CFTR was shown to increase endogenous CFTR in Rhesus macaques via transcomplementation. Four treated monkeys and 1 control were sacrificed at day 90 and 1 treated monkey at 1 year. There were no adverse events related to the study, indicating that triple dosing with AAV1 vectors along with prednisone is safe for up to 1 year. Neutralizing antibody titers increased in all animals 30 days after the first dose. AAV1-Δ27-264 CFTR were detected at 10^{7} vg/μg or more of genomic DNA in all lung tissues of all 5 vector-treated animals. These data are remarkable because we detected 10 times as many vg/μg of AAV1-Δ27-264 and 40 times as many vg/μg of AAV1-GFP in the prednisone-treated animals than what we measured previously in the absence of prednisone. What is even more notable it that, despite being applied as a third dose, the vg/μg of AAV1-GFP was similar to that of AAV1-Δ27-264 CFTR. We had previously noted a 10-fold decrement in the vg/μg of AAV1-GFP compared to that of AAV1Δ27-264 CFTR in the monkeys not treated with prednisone. mRNA and protein expression for CFTR and GFP were detected. mRNA levels were also higher in the prednisone-treated animals than in virus-infected monkeys not treated with prednisone. AAV1-Δ27-264 CFTR and GFP were detected at 10^{6} vg/μg or more of genomic DNA in liver and 10^{5} vg/μg or more in the pancreas. Widespread immunostaining for CFTR and GFP was detected in lung surface epithelial and basal cells and in hepatic and pancreatic ducts. Expression of CFTR and GFP was detected at the same levels in the animal necropsied after 1 year. Repeat dosing of AAV1 is safe but increases neutralizing antibodies against capsid proteins. Detection of GFP protein expression after 2 doses of AAV1-CFTR suggests that, even though increases in neutralizing antibodies are evident, transduction by AAV1 based vectors still occurs. Short-term prednisone treatment boosts transduction, making repeated dosing more feasible. Finally, transduction of liver and pancreas may be an added benefit for multiorgan rescue of CFTR after lung delivery.

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P96

Determination of Tristetraprolin expression and regulation, a key anti-inflammatory protein, in CF

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Objectives: Regulatory elements like microRNA (miRNA) and RNA-Binding protein (RNA-BP) act independently or in synergic/antagonist manner by binding 3'UTR extremity of mRNA, inducing their degradation and/or inhibition of their translation. Tristetraprolin (TTP), an anti-inflammatory RNA-BP, is deregulated in CF cultures. TTP, when non-phosphorylated, destabilizes mRNA of pro-inflammatory cytokines, participating in resolution of inflammation. We defined how TTP expression is controlled in the aim to define a strategy for increasing the TTP proteins amount and for favoring its non-phosphorylated form, to control *in fine* the inflammatory response mechanisms.

Materials and methods: To evaluate the importance of the TTP-3'UTR regulatory elements and the impact of TTP overexpression on cytokines expression, reporter gene assays (luciferase) containing the 3'UTR regions of TTP, IL-8, IL-6 and TNFα, and quantification of mRNA and proteins amount have been conducted. Bronchial cells stably overexpressing TTP mRNA through its transcriptional activation by using the CRISPR activation (CRISPa) technology, were generated. The dynamic regulation of the TTP phosphorylation has also been assessed.

Results: To decorticate regulation of the TTP expression, we first studied the regulatory elements located in the TTP 3'UTR. We showed that the RNA-BP HuR and HuB had a positive effect on the TTP luciferase activity. On the opposite, two miRNAs miR-138 and miR-155 decreased TTP protein level by acting on its 3'UTR part (luciferase activity). This inhibitory effect is increased in inflammatory context with LPS (1mg/ml). Transfection of either miR-138 or miR-155 inhibitors, for miRNA knockdown, induced a restauration of TTP protein level. To validate the importance of these regulatory elements on TTP mRNA stability, we degenerated functional motifs on the TTP 3'UTR. Introduction of mutations on the binding sites for regulatory factors increased TTP expression. To confirm their binding on TTP-3'UTR extremity, we also designed modified oligonucleotides blockers (ONB) that mask binding sites for miRNAs and RNA-BP recruitment. Preliminary results showed an increase of mRNA TTP level, getting us new tools to overexpress TTP mRNA.

We next determined if overexpressing TTP mRNA is effective enough to decrease pro-inflammatory cytokines expression level. In presence of LPS, TTP overexpression led to a strong decrease in the tested cytokines expression at mRNA level by acting on their 3'UTR. The same results were found in TTP-CRISPRa bronchial cells. These findings confirm that TTP overexpression leads to a reduction in pro-inflammatory cytokines mRNA level in bronchial cells.

Finally, we showed that in CF cells the TTP phosphorylation state depends on the ERK/MK2 phosphorylation cascade. The use of an ERK pathway inhibitor drug induced a significant decrease in TTP phosphorylation, providing us proteins to target for controlling TTP phosphorylation status. We are now testing decoy peptides to prevent TTP phosphorylation, the inactive form of TTP, in a more specific manner than available drugs.

Conclusion: Identify destabilizing motifs of the TTP-3'UTR and define proteins essential for TTP phosphorylation, will led to conceive new molecular tools for inflammation resolution.

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P97

Base editing strategy to repair the *CFTR* 2789+5G>A splicing mutation

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The 2789+5G>A mutation, with a frequency of about 1% (CFTR2 database), is among the fifteen most frequent defects in *CFTR* causing cystic fibrosis (CF). This mutation causes an aberrant splicing which determines the production of a non-functional CFTR protein.

In this work, we applied CRISPR-Cas adenine base editors (ABE) to precisely correct this point mutation and restore the correct *CFTR* splicing pattern. To set the most effective base editing strategy we generated a minigene model mimicking the splicing defect in HEK293 cells. We obtained up to 40% correction of the genetic defect by inducing an A-to-G conversion in the mutated minigene model through lentiviral delivery of the ABEmax base editor. To further improve the efficacy of this approach we tested newly evolved base editors, ABE8e and ABE8.20m, which resulted in higher efficiency. Nevertheless, the most active base editors were associated with increased bystander activity which resulted in unwanted A-to-G transitions surrounding the mutation.

We tested our strategy in primary bronchial epithelial cells isolated from a compound heterozygous patient for the 2789+5G>A *CFTR* mutation. In this model we obtained 15% base correction by delivering the base editor as mRNA to limit unwanted A-to-G conversions at the target site (bystander activity) or throughout the genome (off-target activity).

The efficacy of this genome editing strategy was finally validated in intestinal organoids derived from CF patients (2789+5G>A/G542X). We obtained up to 10% A-to-G conversion which correlated with the restoration of correct splicing and functional recovery of CFTR measured by forskolin-induced swelling assay.

These results demonstrate that CRISPR-mediated base editing is an efficient method to restore the 2789+5G>A splicing defect and is potentially extendable to other CFTR mutations.

P100

Homology independent targeted integration at the AAVS1 safe harbour locus

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We aim to develop a genome editing strategy for cystic fibrosis (CF) using homology independent targeted integration (HITI) after targeted double-strand breaks by CRISPR-Cas9. HITI relies on non-homologous end joining (NHEJ), enabling editing of non-dividing lung epithelial cells, where homology directed repair (HDR) is not an option. HITI also enables targeted insertion of large pieces of cDNA, such as a CFTR-encoding superexon, into the genome, rescuing function regardless of CF genotype. We are targeting the safe harbor AAVS1 site for two reasons: firstly, we anticipate more robust expression from the endogenous PP1R12C gene promoter than possible at the CFTR locus with its tightly-regulated, relatively weak promoter; secondly, editing at a site distant from the CFTR locus reduces the risk of damaging any residual CFTR activity in the case of DSB formation without subsequent cDNA integration.

We first developed a primary cell model by lentiviral BMI-1 transduction of basal epithelial cells carrying a CFTR nonsense mutation (R709X). BMI-1 transduction has been shown to extend proliferative potential of primary cells. Using this cell model, we showed lack of CFTR activity by Ussing chamber analysis. For the HITI strategy, two donor constructs containing a promoterless GFP were introduced alongside AAVS1-targeting gRNA and Cas9. One plasmid contained only one gRNA cut site upstream of the GFP construct, resulting in a single linearized construct consisting of GFP and plasmid backbone. The second plasmid contained GFP flanked by two gRNA cut sites, resulting in a mixed population of excised donor and plasmid backbone. GFP expression was detected as a readout for successful genomic integration. The linearized donor plasmid was found to express GFP with nearly double the frequency as the excised donor plasmid. Genotypic integration was then verified via PCR analysis, confirming successful integration in the proper orientation.

High rates of GFP expression and genotypic integration suggest that HITI is a valid strategy for large construct integration at the AAVS1 safe harbour locus. In the future, we aim to integrate a CFTR superexon at this site. We will then expand corrected cells, differentiate using air-liquid interface culture, and measure CFTR expression and ion trafficking in both corrected and uncorrected cells. If successful, integration of a CFTR superexon could potentially serve as a "one size fits all" treatment for CF, regardless of patient genotype.

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Harnessing CRISPR-Cas9 technology to revert F508del-CFTR defect

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The most frequent mutation causing CF is the F508del mutation, an out-of-frame deletion of 3 nucleotides that lead to a silent codon change of isoleucine 507 and the loss of phenylalanine 508. The discovery and development of genome editing technologies, such as CRISPR-Cas nucleases, offer unprecedented strategies to reverse genetic mutations. Nevertheless, mutations like F508del, which consist of small deletions, remain difficult to correct through genome editing techniques.

A variety of reports, including mutation analysis in patients with a mild form of CF, suggest that secondary mutations in the F508del locus may neutralize the primary genetic defect, thus partially restoring the CFTR function. Therefore, the introduction of revertant mutations represents a potential strategy to counteract the F508del defect.

We initially evaluated the reversion level of known revertant mutations (as the R553Q, G550E, R555K and R1070W) by evaluating the amount of CFTR at the plasma membrane by flow-cytometry analysis. Various degree of recovery of plasma membrane CFTR was obtained, reaching WT-CFTR levels with the combination of two revertant mutations. CRISPR-Cas base editors were used to reproduce these revertant mutations in CF cell models. Cytosine or adenine deaminase enzymes fused to CRISPR-Cas9 allowed the editing of specific nucleotides without generating DNA double strand breaks. We observed that this strategy was partially efficient in reverting the F508del-CFTR defect, nevertheless to increase the correction efficiency we sought to identify novel revertant mutations.

To expand the pool of F508del-CFTR revertants, we created a library of F508del-CFTR mutants by introducing random mutations into the CFTR gene. Revertants population was initially selected by flow-cytometry analysis that allowed the identification of cells that express CFTR at the plasma membrane. Revertant mutations were identified by sequencing and will be further validated as a potential novel strategy to repair the F508del defect through genome editing.

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Repurposed tRNAs suppress nonsense mutations in CFTR and restore function

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Nonsense mutations convert sense codon to a termination codon, which is referred to as pre-mature termination codon (PTC). The newly emerged PTC activates mRNA surveillance pathways resulting in an abrupt termination of protein translation. No efficient treatment exists for cystic fibrosis patients with nonsense mutations. Multiple small molecule-based therapies failed in clinical trials as they are not selective to the PTC and cause pervasive readthrough ato natural termination codon; UAA, UGA and UAG. We developed a novel strategy to repurpose sense-codon decoding tRNAs into efficient PTC suppressors. The tRNA suppressor efficacy is optimized by modulating tRNA body sequences to individually fine-tune them to the chemical properties of the cognate amino acid. At clinically-relevant PTC in CFTR, the suppressor tRNAs reestablished expression and function in cell systems and patient-derived primary cultures of nasal epithelia in a manner relevant to CF clinical benefit. These results provide the framework for treating nonsense CFTR mutations with tRNAs tailored to each nonsense mutation.