2024

European Cystic Fibrosis Society

19th ECFS Basic Science Conference

Conference Programme & Abstract Book

Valletta, Malta

Chairpersons

Alexandre Hinzpeter, Patrick Harrison and Marie Egan

20 March – 23 March 2024

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CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

WELCOME FROM THE ECFS PRESIDENT AND THE PRESIDENT ELECT

It is a great pleasure to welcome you in Malta to the 19th European Cystic Fibrosis Conference dedicated to Basic Science.

This year we are delighted to welcome Dr. Alexandre Hinzpeter as the conference Chairperson who will be supported by Dr. Patrick harrison and Dr. Marie Egan as co-chairpersons.

Basic scientific discovery is critical to our understanding of CF. New therapies targeting the basic defect have been an extraordinary achievement coming from basic scientific research. But there are still many critical questions left unanswered and basic science in CF is needed more than ever to cure all patients with CF.

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 annual event in the ECFS programme.

Your active participation will contribute to a productive exchange of information and we hope productive collaborations.

A very warm welcome to an exciting conference.

Isabelle Fajac
ECFS President

Jane Davies
ECFS President Elect

WELCOME FROM THE CONFERENCE CHAIPERSONS

A very warm welcome to you to the 19th ECFS Basic Science Conference, located in Malta this year.

As you disembark, prepare to be captivated by the island's rich tapestry. Walk the fortified walls of Valletta, a UNESCO World Heritage Site, and feel the whispers of the Knights of St. John. Gaze upon the iconic Maltese Cross, a symbol of resilience and cultural pride. Let the turquoise waters of hidden coves and the golden hues of historical architecture surround you.

But your journey here transcends history. With this conference, you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment.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. Here you will have excellent opportunities to discuss your data and interact in a great atmosphere with the best of European and International experts in the field.

This year's programme features eight symposia with invited speakers and talks from submitted abstracts. There are also two keynote lectures and flash poster sessions. This conference will be a unique chance to discuss topical aspects of basic research in cystic fibrosis with international experts.

We welcome all scientists, not just from the field of CF research but also from a diverse range of related topics, to an exciting conference of high scientific quality.

Alexandre Hinzpeter
INSERM - Institut Necker Enfants Malades
France

Patrick Harrison
University College Cork
Ireland

Marie Egan
Yale University
United States

19th ECFS Basic Science Conference

20 – 23 March 2024, Valletta, Malta

Programme

Chairpersons: Alexandre Hinzpeter (Paris, FR), Patrick Harrison (Cork, IE) & Marie Egan (New Haven, US)

Wednesday, 20 March 2024 (Day 1)		
17:30-18:00	**Official Opening of the Conference by the Conference Chairpersons**	
18:00-19:00	**Opening Keynote Lecture:**	
	CFTR biogenesis and misfolding correction; the role of allosteric domain-domain coupling - Gergely Lukacs (CA)	
19:00-19:45	**Welcome Reception**	Level 6
19:45-21:30	*Dinner*	Level 2 - Floriani Hall

Thursday, 21 March 2024 (Day 2)		
07:30-08:45	*Breakfast*	Level 4 – Spice Island
08:45-10:30	**Symposium 1 – CFTR protein structure and function**	
	Chairs: Nicoletta Pedemonte (IT) / Gergely Lukacs (CA)	
08:45-09:10	CFTR structure, dynamics, and gating through the in silico microscope - Tamás Hegedűs (HU)	
09:10-09:35	Molecular mechanism of Cl^- conduction the open state of human CFTR - Régis Pomès (CA)	
09:35-10:00	CFTR NBD2 folding requires its assembly with TMDs rather than folded NBD1 - Ineke Braakman (NL)	
10:00-10:10	Abs. 02 - Exploring the activating effects of the inhibitors of type IV ABC transporters on CFTR gating – Paola Vergani (UK)	
10:10-10:20	Abs. 05 - Mechanism of action and binding site characterization of corrector *ARN23765* via Photo-Affinity Labeling (PAL) approach in live cells - Fabio Bertozzi (IT)	
10:20-10:30	Abs. 04 - Understanding the conformational landscape of NBD1 from CFTR: from normal function to cystic fibrosis - Arina Svoeglazova (BE)	
10:30-11:00	*Coffee break* & **Poster viewing**	
11:00-12:45	**Symposium 2 – CFTR genetics & CFTR expressing cells**	
	Chairs: Anna Cereseto (IT) / Batsheva Kerem (IL)	
11:00-11:25	Modulator responsiveness of 656 CFTR variants: from predicting patient treatability to structural insights - Martin Mense (US)	
11:25-11:50	Single-cell analyses of the human airways in health and respiratory diseases - Laure-Emmanuelle Zaragosi (FR)	
11:50-12:15	*CFTR* regulation in time and space - Ann Harris (US)	
12:15-12:25	Abs. 22 - Pre-clinical data demonstrates great promise for AAV gene therapy, one dose of AAV1 or 6-Δ27-264 CFTR successfully ameliorated clinical symptoms in G551D ferrets	

	after 5-weeks - Liudmila Cebotaru (US)	
12:25-12:35	Abs. 25 - Novel CFTR+ lung progenitor cells contribute to the dynamic developmental origins of fetal epithelial cell lineages - Amy Wong (CA)	
12:35-12:45	Abs. 20 - A modelling framework for epithelial airway fluid and ion transport with multiple cell types: implications for success or failure in gene therapies for cystic fibrosis - Omar Hamed (UK)	
12:45-14:30	*Lunch*	Level 5 – Admiral's Landing
14:30-15:30	**Flash Poster Session (even numbers)**	
	Chair: Neeraj Sharma (US)	
15:30-16:00	*Coffee break* & **Poster viewing**	
16:00-17:45	**Symposium 3 – Gene editing to correct any CFTR mutation**	
	Chairs: Stephen Hart (UK) / Ann Harris (US)	
16:00-16:25	Modulation of Double Strand Breaks repair to promote Cas9 and PEn dependent DNA insertions - Marcello Maresca (SE)	
16:25-16:50	Strategies to correct whole exons in CFTR by gene editing - Patrick Harrison (IE)	
16:50-17:15	Identification and evolution of novel CRISPR -Cas systems from the human microbiome - Anna Cereseto (IT)	
17:15-17:25	Abs. 28 - Rescuing G542X by Adenine Base Editing: A guide to restore function - Lucia Nicosia (IE)	
17:25-17:35	Abs. 33 - Site-specific gene targeting of chromosomal safe harbor and CFTR locus for correcting any CFTR mutation - Jim Hu (CA)	
17:35-17:45	Abs. 32 - CRISPR-ABE: a new strategy for the temporospatial control of editing to correct the W1282X mutation in the CFTR gene - Immacolata Zollo (PT)	
20:00-21:30	*Dinner*	Level 2 - Floriani Hall
21:30-23:00	**Evening Poster Session:** Posters with even numbers	
Friday, 22 March 2024 (Day 3)		
07:30-08:45	*Breakfast*	Level 4 – Spice Island
08:45-10:30	**Symposium 4 – Targeting stop codons and splicing defects**	
	Chairs: Jane Davies (UK) / Margarida Amaral (PT)	
08:45-09:10	Stabilization of RNA is critical to achieving functional level restoration of nonsense mutations by CFTR modulators - Neeraj Sharma (US)	
09:10-09:35	Reading through nonsense mutations with engineered tRNAs - Zoya Ignatova (DE)	
09:35-10:00	Targeting splicing mutations using oligonucleotides - Batsheva Kerem (IL)	
10:00-10:10	Abs. 43 - Identification of novel pharmacological inhibitors of nonsense-mediated RNA decay to rescue CFTR with premature termination codons - Arianna Venturini (IT)	
10:10-10:20	Abs. 42 - Upregulation of a nonsense mediated decay (NMD) insensitive CFTR mRNA isoform has therapeutic potential for the treatment of 3' CFTR PTC variants - Normand Allaire (US)	
10:20-10:30	Abs. 38 - Comparative study of readthrough molecules - Fabrice Lejeune (FR)	

10:30-11:00	*Coffee break* & **Poster viewing**	
11:00-12:45	**Symposium 5 – Gene(tic) therapies: are we ready for clinical research?** a patient organisation-initiated symposium	
	Chairs: Sylvia Hafkemeyer (DE) / Jeffrey Beekman (NL)	
11:00-11:25	Statements of people with CF regarding genetic therapies	
	A video on statements will be presented	
11:25-11:50	Enabling true 'informed' consent by empowering participants - Lorna Allen (UK)	
11:50-12:15	Delivery: What is feasible according to research and how does it fit to the expectations of people with CF – Stephen Hart (UK)	
12:15-12:40	Considerations to enhance patient access to genetic therapies - Stefano Zancan (IT)	
12:40-12:45	Final discussion and concluding remarks	
12:45-14:00	*Lunch*	Level 5 – Admiral's Landing
14:00-18:30	**Free Afternoon**	
18:30-19:30	**Flash Poster Session (odd numbers)** **Chair: Ineke Braakman (NL)**	
20:00 -21:30	*Dinner*	Level 2 – Floriani Hall
21:30-23:00	**Evening Poster Session:** Posters with odd numbers	
Saturday, 23 March 2024 (Day 4)		
07:30-08:45	*Breakfast*	Level 4 – Spice Island
08:45-10:30	**Symposium 6 – Targeting protein partners and alternative channels**	
	Chairs: Alexandre Hinzpeter (FR) / David Sheppard (UK)	
08:45-09:10	Mutant CFTR impacts Insulin-receptor signaling and regulates localization of tight junction proteins, disrupting the airway glucose barrier - Nael McCarty (US)	
09:10-09:35	Targeting ubiquitination to enhance modulator treatments - Nicoletta Pedemonte (IT)	
09:35-10:00	Targeting CFTR protein partners and alternative channels - Margarida Amaral (PT)	
10:00-10:10	Abs. 46 - Targeting ATP12A proton pump provides new therapeutic opportunities for cystic fibrosis - Giulia Gorrieri (IT)	
10:10-10:20	Abs. 58 - Enhancing apical loop currents in airway epithelia carrying CFTR non-sense mutations - Nathalie Baumlin (US)	
10:20-10:30	Abs. 47 - SLC26A9 modulators identified through High Throughput Screening (HTS) - Mohsen Esmaeili (CA)	
10:30-11:00	*Coffee break* & **Poster viewing**	

11:00-12:45	**Symposium 7 – Cell – bacteria relations: infection and inflammation in the era of modulators**	
	Chairs: Geneviève Héry-Arnaud (FR) / Dean Madden (US)	
11:00-11:25	Multi-lobe bronchoscopy reveals ETI's effects on regional lung infection and inflammation - Samantha Durfey (US)	
11:25-11:50	Effects of CFTR modulators on airway mucus, infection and inflammation in cystic fibrosis - Marcus Mall (DE)	
11:50-12:15	A Scottish exploration of the effects of Tez/Iva and Elex/Tez/Iva on systemic and pulmonary inflammation - from bench to bedside - Nicola Robinson (UK)	
12:15-12:25	Abs. 64 - GM1 ganglioside: new insight on its immunomodulatory capacity in CF - Dorina Dobi (IT)	
12:25-12:35	Abs. 65 - Exploring the relationship between *Pseudomonas aeruginosa* infection and SLC6A14 in cystic fibrosis - Manon Ruffin (FR)	
12:35-12:45	Abs. 72 - Differential effects of CFTR modulators on SARS-CoV-2 infectivity in cultured nasal, bronchial, and intestinal epithelia of people with cystic fibrosis - Loes den Hertog - Oosterhoff (NL)	
12:45-14:15	*Lunch*	Level 5 – Admiral's Landing
14:15-16:00	**Symposium 8 – Cell models, organoids in the enhanced life expectancy of patients**	
	Chairs: Marie Egan (US) / Patrick Harrison (IE)	
14:15-14:40	Functions of the pulmonary ionocyte in the proximal ferret airways - John Engelhardt (US)	
14:40-15:05	Intestinal organoids, CFTR function and tissue-specific disease - Jeffrey Beekman (NL)	
15:05-15:30	Role of enteroendocrine cell differentiation in the pathogenesis of CFRD - Daniel Zeve (US)	
15:30-15:40	Abs. 85 - Investigation of CFTR function and epithelial barrier properties at single cell resolution using multi-electrode array technology - Marjolein Ensinck (BE)	
15:40-15:50	Abs. 88 - Endometrium-derived organoids from cystic fibrosis patients to study the endometrial factor in the disease-associated fertility deficiency - Ellen De Pauw (BE)	
15:50-16:00	Abs. 79 - Elexacaftor/Tezacaftor/Ivacaftor treatment partially normalizes osteoclasts phenotype in cystic fibrosis-related bone disease - Johan Sergheraert (FR)	
16:00-16:30	*Coffee Break*	
16:30-17:30	**Closing Keynote lecture**	
	Bridging the Gap: scientific discovery to clinical benefit – Jane Davies (UK)	
20:00	*Dinner / Social Event*	

POSTER TITLES & AUTHORS

P1 Characterization of the tumor-suppressor character of CFTR in the context of colorectal cancer development in primary patient-derived intestinal organoids
Sacha Spelier, Isabelle van der Windt, Regina Hofland, Jeffrey Beekman

P2 Exploring the activating effects of the inhibitors of type IV ABC transporters on CFTR gating
Maria-Cristina Ardelean, Hannah J. Eldred, Paola Vergani

P3 The dual impact of INF2 on the regulation of CFTR
João F Ferreira, Carlos M Farinha

P4 Understanding the conformational landscape of NBD1 from CFTR: from normal function to cystic fibrosis
Arina Svoeglazova, Daniel Scholl, Maud Sigoillot, Rafael Colomer Martinez, Tom Kache, Marie Overtus, Chloé Martens, Jelle Hendrix, Cédric Govaerts

P5 Mechanism of action and binding site characterization of corrector *ARN23765* via Photo-Affinity Labeling (PAL) approach in live cells
Fabio Bertozzi, Elisa Romeo, Francesco Saccoliti, Angela Andonaia, Caterina Allegretta, Onofrio Laselva, Federico Falchi, Riccardo Occello, Nara Liessi, Andrea Armirotti, Stefania Girotto, Cristina Pastorino, Nicoletta Pedemonte, Tiziano Bandiera

P7 Nanobodies as therapeutics for cystic fibrosis
Marie Overtus, Tihomir Rubil, Floriane Debuisson, Anita Balázs, Benoît Chevalier, Alexandre Hinzpeter, Anne des Rieux, Marcus Mall, Cédric Govaerts

P8 Peptide CIGB-552 has a synergistic effect on CFTR-F508del combined with Elexacaftor/Tezacaftor/Ivacaftor
Benjamin Simonneau, Stéphanie Simon, Bénédicte Duriez, Fanny Degrugillier, Bruno Costes, Frédéric Becq, Maribel G. Vallespi, Pascale Fanen, Abdel Aissat

P9 Pi3Kγ modulation: A new frontier in maximizing F508del-CFTR stability for improved therapeutic outcomes
Alessandra Murabito, Marco Mergiotti, Valeria Capurro, Alessia Loffreda, Andrea Raimondi, Carlo Tacchetti, Nicoletta Pedemonte, Emilio Hirsch, Alessandra Ghigo

P10 Mapping CFTR-dependent and CFTR-independent luminal pH regulation along the crypt-villus axis of human small intestine
Ghazal Masarweh, Yanfen Jiang, Jessica Sarthi, Zachary Sellers

P11 Rescuing G85E, N1303K and F508del-CFTR by Elexacaftor or Bamocaftor combined to Tezacaftor/Ivacaftor in human airway epithelial cells
Sandra Mirval, Aline Santin, Thomas Carrez, Frédéric Becq

P13 Beneficial effect of orkambi in alcohol-induced acute pancreatitis
Viktoria Venglovecz, Anna Grassalkovich, Emese Tóth, Attila Ébert, József Maléth, Zoltán Rakonczay, Zsolt Galla, Péter Monostori, Péter Hegyi

P14 Gene therapy and modulator treatments for Cystic fibrosis: a combined therapeutic approach
Xavier Buin, Rosy Ghanem, Nathalie Benz, Mathieu Berchel, Pascal Trouvé, Tristan Montier

P15 A new approach to understand complex cases of cystic fibrosis and its related disorders
Clara Blotas, Mégane Collobert, Anaïs Le Nabec, Emmanuelle Masson, Claude Férec, Stéphanie Moisan

P17 DNA methylation markers predict pulmonary exacerbation and lung function degradation in Cystic Fibrosis
Albertina De Sario, Jorg Tost, Davide Caimmi, Manuela Pastore, Christelle Reynes, Florence Busato, Fanny Pineau, Mireille Claustres, Isabelle Vachier, Raphaël Chiron

P18 From epigenome-wide association studies (EWAS) To the functional analysis of differentially methylated regions in cystic fibrosis
Loréna Valdés, Jorg Tost, Isabelle Rivals, Milena Magalhães, Florence Busato, Laurent Mely, Sylvie Leroy, Marlène Murris, Mireille Claustres, Davide Caimmi, Isabelle Vachier, Raphaël Chiron, Albertina De Sario

P19 Macrophages as a tool to study innate immunity and to develop a cell-based immunotherapy for cystic fibrosis
Claudio Rodríguez-González, Sylvia Merkert, Silke Hedtfeld, Stephanie Tamm, Ulrich Martin, Anna-Maria Dittrich, Nico Lachmann, Burkhard Tümmler, Antje Munder

P20 A modelling framework for epithelial airway fluid and ion transport with multiple cell types: implications for success or failure in gene therapies for cystic fibrosis
Omar Hamed, Guy W.J. Moss, Vivek Dua

P21 Detection of CFTR mRNA and protein in immune cells via quantitative real-time PCR and Western blot
Alexander Schnell, Stephanie Tamm, Silke Hedtfeld, Claudio Rodriguez Gonzalez, André Hoerning, Nico Lachmann, Frauke Stanke, Antje Munder, Anna-Maria Dittrich

P22 Pre-clinical data demonstrates great promise for AAV gene therapy, one dose of AAV1 or 6-Δ27-264 CFTR successfully ameliorated clinical symptoms in G551D ferrets after 5-weeks.
Cristian Ciobanu, William Guggino, Liudmila Cebotaru

P23 Pharmacological improvement of CFTR function rescues airway epithelial homeostasis and host defense in children with cystic fibrosis
Jennifer Loske, Mirjam Völler, Sören Lukassen, Mirjam Stahl, Loreen Thürmann, Anke Seegebarth, Jobst Röhmel, Sebastian Wisniewski, Marey Messingschlager, Stephan Lorenz, Sven Klages, Roland Eils, Irina Lehmann, Marcus A. Mall, Saskia Trump, Simon Y. Graeber

P24 Beyond Kaftrio: mechanistic insights to maximize N1303K-CFTR rescue
Iwona Pranke, Valeria Capurro, Benoit Chevalier, Emanuela Pesce, Natacha Rémus, Isabelle Sermet-Gaudelus, Alexandre Hinzpeter, Nicoletta Pedemonte

P25 Novel CFTR+ lung progenitor cells contribute to the dynamic developmental origins of fetal epithelial cell lineages
Amy Wong, Henry Quach, Spencer Farrell, Kayshani Kanagarajah, Michael Wu, Xiaoqiao Xu, Prajkta Kallurkar, Andrei Turinsky, Christine Bear, Felix Ratjen, Sidhartha Goyal, Theo Moraes

P26 Characterization of the mechanism of action of approved and novel CFTR modulating drugs on G85E-CFTR protein activity and maturation
Mariateresa Lena, Valeria Tomati, Valeria Capurro, Emanuela Pesce, Cristina Pastorino, Marco Di Luca, Federico Cresta, Federico Zara, Luis J.V. Galietta, Renata Bocciardi, Carlo Castellani, Nicoletta Pedemonte

P27 Extension of the Homology-Directed Repair (HDR) Editing window to repair all CF-causing mutations in Exon 12
Emma Collins, Joss Murray, Joana Alves, Patrick Harrison

P28 Rescuing G542X by Adenine Base Editing: A guide to restore function
Lucia Nicosia, Roberta Valeria La Torre, Iwona Pranke, Joss Murray, Lisa Lonetti, Kader Cavusoglu-Doran, Ciaran Lee, Paola Melotti, Isabelle Sermet, Claudio Sorio, Martina Scallan, Patrick Harrison

P29 Correction of all Exon 12 variants using a template-jumping prime editing strategy
Joss Murray, Patrick Harrison

P30 Characterization of CRISPR-Cas cell-derived vesicles for genome editing applications in cystic fibrosis
Elena Gurrieri, Giulia Maule, Marta Stancampiano, Ambra Sarracino, Antonio Casini, Anna Cereseto

P31 Functional correction of the CFTR 1717-1G>A splicing mutation using an adenine base editor with minimal bystander editing
Alessandro Umbach, Annalisa Santini, Daniela Guidone, Mattijs Bulcaen, Sam Thierie, Giulia Maule, Matteo Ciciani, Marianne Carlon, Luis Galietta, Anna Cereseto

P32 CRISPR-ABE: a new strategy for the temporospatial control of editing to correct the W1282X mutation in the CFTR gene
Immacolata Zollo, Lúcia Santos, Joana Alves, Patrick T. Harrison, Carlos M. Farinha

P33 Site-specific gene targeting of chromosomal safe harbor and CFTR locus for correcting any CFTR mutation
Jim Hu, Ziyan R. Chen, Ranmal Bandara, Zhichang P. Zhou, Rongqi Duan, Amy Wong, Christine Bear, Hartmut Grasemann

P34 Journey to the Centre of the Airway: A VP22 cell-penetrating Base Editor as a novel tool for tackling lung basal cells' encasement
Joana Alves, Lisa Lonetti, Lúcia Santos, Carlos M. Farinha, Patrick T. Harrison

P35 Base-editors delivery through fusogenic vesicles
Marta Stancampiano, Giulia Maule, Elena Gurrieri, Ambra Sarracino, Antonio Casini, Anna Cereseto

P36 Optimization of Gene insertion strategies for restoration of CFTR expression in airway epithelium
Cristina Barilla', Shingo Suzuki, Andras Rab, Eric Sorscher, Brian R Davis

P37 Functional analysis of gene-edited CF variant G542X.
Issie Rose, Lucia Nicosia, Miriam Greenwood, Kader Doran-Cavusoglu, Patrick Harrison, Stephen Hart, Deborah Baines

P38 Comparative study of readthrough molecules
Fabrice Lejeune, Catherine Leroy, Sacha Spelier, Jeffrey Beekman

P39 Optimization of blocking oligonucleotides as therapies to correct consequences of deep intronic mutations
Chloé Felgerolle, Karine Deletang, Magali Taulan-Cadars

P41 Enhanced rescue of CFTR nonsense mutations under inflammatory stimuli
Anna Borrelli, Arianna Venturini, Fabiana Ciciriello, Luis J.V. Galietta

P42 Upregulation of a Nonsense Mediated Decay (NMD) insensitive CFTR mRNA isoform has therapeutic potential for the treatment of 3' CFTR PTC variants
Normand Allaire, Jae Seok Yoon, Mathew Armstrong, Mercy Chado, Andrey Sivachenko, Priyanka Bhatt, Kevin Coote, Hermann Bihler, Calvin Cotton, Martin Mense

P43 Identification of novel pharmacological inhibitors of nonsense-mediated RNA decay to rescue CFTR with premature termination codons
Arianna Venturini, Anna Borrelli, Fabiana Ciciriello, Isabelle Sermet-Gaudelus, Luis Juan Vicente Galietta

P44 Exploring novel therapeutic targets: Unraveling signatures linked to differentially expressed genes and proteins in G542X-CFTR
Andreia F A Henriques, Lúcia Santos, Carlos M Farinha

P46 Targeting ATP12A proton pump provides new therapeutic opportunities for cystic fibrosis
Giulia Gorrieri, Floriana Guida, Ilaria Musante, Rosaria Casciaro, Fabiana Ciciriello, Federico Zara, Paolo Scudieri

P47 SLC26A9 modulators identified through High Throughput Screening (HTS)
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P48 Identification of novel epistatic modifiers that influence CFTR folding trajectory through ribosomal interaction
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P49 Hypoxia reduces TRPA1 activity in cystic fibrosis bronchial epithelial cells
Khilian Pascarel, Isabelle Fixe, Jenny Colas, Christine Barrault, Frederic Becq, Clarisse Vandebrouck

P50 Insight into the mechanism of action of VX770 to develop a new CFTR-independent therapeutic proposal
Michela Rubin, Ilaria Artusi, Valentina Bosello-Travain, Monica Rossetto, Maria Luisa Di Paolo, Anna Pianazzola, Angela Menna, Giorgio Cozza

P51 Proteomic analysis of cultured bronchial epithelium secretome reveals the presence of ion transport proteins
Daniela Guidone, Luis J. V. Galietta

P52 Low density lipoproteins and F508del CFTR: the controversial role of the cholesterol in cystic fibrosis
Anna Tamanini, Nicoletta Loberto, Laura Mauri, Rosaria Bassi, Dorina Dobi, Christian Boni, Elena Baldisseri, Diletta Onorato, Federica Quiri, Nicoletta Pedemonte, Valentino Bezzerri, Debora Olioso, Massimo Aureli

P53 Role of TRPV4 in innate defense mechanisms of the airway epithelium
Michele Genovese, Arianna Venturini, Daniela Guidone, Anna Borrelli, Luis J. V. Galietta

P54 Highway to cell: Cell-penetrating peptide to internalize the CFTR-stabilizing iCAL36 peptide
Prisca Boisguerin, Emilie Josse, Quentin Seisel, Magali Taulan-Cadars

P55 From CFTR to a CF signalling network: a systems biology approach to study Cystic Fibrosis
Matthieu Najm, Victor Laigle, Loredana Martignetti, Matthieu Cornet, Mairead Kelly-Aubert, Isabelle Sermet, Laurence Calzone, Véronique Stoven

P56 A Novel Peptide Targeting the A-kinase anchoring function of PI3Kγ that increase cAMP and potentiate CFTR
Angela Della Sala, Laura Tasca, Cosmin Stefan Butnarasu, Giulia Prono, Valentina Sala, Enrico Millo, Leonardo Terranova, Stefano Aliberti, Alberto Massarotti, Sonja Visentin, Emilio Hirsch, Alessandra Ghigo

P57 Inhibition of Orai1-Mediated Calcium Signaling Ameliorates Lung Disease in G551D Cystic Fibrosis Ferrets
Robert Tarran, M. Flori Sassano, Sasha Goriounova, Jason Iskarpatyoti, Alan Schmalstig, Dylan Glawe, Shahab Fakhari, John Engelhardt, Doug Bartels, Saira Ahmad

P58 Enhancing apical loop currents in airway epithelia carrying CFTR non-sense mutations
Nathalie Baumlin, Neerupma Silswal, John S. Dennis, Michael D. Kim, Matthias Salathe

P59 The Orai1 antagonist, ELD607, reduces chronic neutrophilic inflammation in a βENaC mouse model
Flori Sassano, Saira Ahmad, Matthew Biggart, Nikki Worthington, Troy Rogers, Barbara Grubb, Robert Tarran

P60 Septin 7-dependent defense mechanisms against *Pseudomonas aeruginosa* are impaired in cystic fibrosis bronchial epithelial cells
Sylvain Brax, Clémence Gaudin, Claire Calmel, Harriet Corvol, Manon Ruffin, Loic Guillot

P61 Targeting platelet activation with pro-resolving lipid mediators: an innovative strategy to dampen lung inflammation in cystic fibrosis
Simona D'Orazio, Matteo Mucci, Giulia Ferri, Giada Messina, Maria Tredicine, Alessia Lamolinara, Manuela Iezzi, Pietro Ripani, Mark Looney, Antonio Recchiuti, Domenico Mattoscio

P62 Tackling phage resistance to increase the robustness of phage therapy for curing *Pseudomonas aeruginosa* infections in patients with cystic fibrosis
Francesca Forti, Allegra Laricchia, Marco Cafora, Jimena Nieto Noblecia, Lisa Cariani, Anna Pistocchi, Federica Briani

P63 *Ex vivo* pig lung as a new CF model for the study of *Pseudomonas aeruginosa* infection and phage therapy application
Marco Cafora, Francesca Forti, Freya Harrison, Federica Briani, Anna Pistocchi

P64 GM1 ganglioside: new insight on its immunomodulatory capacity in CF
Dorina Dobi, Nicoletta Loberto, Laura Mauri, Rosaria Bassi, Christian Boni, Elena Baldisseri, Diletta Onorato, Federica Quiri, Valentino Bezzerri, Debora Olioso, Anna Tamanini, Nicoletta Pedemonte, Valeria Tomati, Giulio Cabrini, Alessandro Rimessi, Alice Rossi, Alessandra Bragonzi, Massimo Aureli

P65 Exploring the relationship between *Pseudomonas aeruginosa* infection and SLC6A14 in cystic fibrosis
Julia Mercier, Claire Calmel, Manon Fleury, Sylvain Brax, Erika Sutanto, Anthony Kicic, Jean Michel Sallenave, Harriet Corvol, Loïc Guillot, Manon Ruffin

P66 Investigating the therapeutic potential of phages as antibacterials and immunomodulators in a zebrafish model of cystic fibrosis
Anna Pistocchi, Marco Cafora, Francesca Forti, Nicoletta Loberto, Laura Belleri, Rosaria Bassi, Dorina Dobi, Massimo Aureli, Federica Briani

P67 Unveiling the effect of ETI therapy on *Pseudomonas aeruginosa* persistence and adaptation through RNA expression profiling
Morgana Letizia, Lauren E. Whiteley, Frances L. Diggle, Edward F. McKone, Pradeep Singh, Marvin Whiteley

P68 Identification of molecular determinants that govern morphotype-specific physiology in Mycobacterium abscessus
Brittany Ross, Emma Evans, Marvin Whiteley

P70 Impact of triple therapy on mucus rheology and bacteriology in cystic fibrosis
Lydia Esteban Enjuto, Jeremy Patarin, Max Maurin, Hugues Bodiguel

P71 Conformational regulation of transcription in a *Pseudomonas aeruginosa* epoxide-based virulence circuit
Susu He, Noor Taher, Kelli Hvorecny, Michael Ragusa, Christopher Bahl, Alison Hickman, Fred Dyda, Dean Madden

P72 Differential effects of CFTR modulators on SARS-CoV-2 infectivity in cultured nasal, bronchial, and intestinal epithelia of people with cystic fibrosis
Loes den Hertog - Oosterhoff, Gimano Amatngalim, Shannon Smits, Dorien de Jong, Suzanne Kroes, Heleen Sonneveld, Jori Symons, Wilco Nijenhuis, Lukas Kapitein, Robert-Jan Lebbink, Monique Nijhuis, Jeffrey Beekman

P73 Mutliomic approach to identify possible mechanisms of action of HEMTs and to propose new therapeutic targets
Mairead Kelly-Aubert, Emmanuelle Bardin, Matthieu Cornet, Véronique Stoven, Isabelle Sermet-Gaudelus

P74 In vitro characterisation of drug candidates against *Mycobacterium abscessus*
Lea Mann, Markus Lang, Adrian Richter

P75 *N*α-aroyl-*N*-aryl-phenylalanine amides are active against a broad panel of non-tuberculous mycobacteria
Markus Lang, Uday S. Ganapathy, Thomas Dick, Adrian Richter

P76 Development of a novel CF lung disease model based on CF patient-specific human induced pluripotent stem cells
Mark-Christian Jaboreck, Sylvia Merkert, Laurien Czichon, Janina Zöllner, Nicole Cleve, Jan Hegermann, Mia Mielenz, Silke Hedtfeld, Frauke Stanke, Tihomir Rubil, Anita Balázs, Marcus Mall, Ruth Olmer, Ulrich Martin

P77 Characterization A559T-CFTR variant in patient-derived intestinal organoids and nasal cells in response to CFTR modulators correlated with in vivo clinical response to ETI therapy.
Paola Melotti, Karina Kleinfelder, Anca Manuela Hristodor, Marina Bertini, Claudio Sorio

P78 A platform for biomarkers evaluation, pathophysiology studies, and therapeutic development based on patient-derived cells collected by nasal brushing
Giulia Gorrieri, Floriana Guida, Ilaria Musante, Rosaria Casciaro, Fabiana Ciciriello, Federico Zara, Paolo Scudieri

P79 Elexacaftor/Tezacaftor/Ivacaftor treatment partially normalizes osteoclasts phenotype in cystic fibrosis-related bone disease
Johan Sergheraert, Christine Guillaume, Claire Dumortier, Julien Braux, Muriel Griffon, Bruno Ravoninjatovo, Pierre-Régis Burgel, Clémence Martin, Loïc Pierrard, Sophie C Gangloff, Marie-Laure Jourdain, Frédéric Velard

P80 Automation workflow for forskolin induced swelling assay in cystic fibrosis: advancing personalized medicine through high-throughput screening with primary intestinal organoids
Roos-Anne Samsom, Bram Bosch, Heleen Sonneveld-van Kooten, Jeffrey Beekman, Sam van Beuningen

P81 Theratyping of R347P, R347H, T465N and L227R CFTR variants in patient-derived rectal organoids correlates with clinical response to CFTR modulators in matched CF patients.
Karina Kleinfelder, Alessia Carpene, Paola Melotti, Elena Spinelli, V Fainiardi, Giovanna Pisi, Vito Terlizzi, Claudio Sorio

P82 Label free prediction of primary airway epithelial cell staining
Bram Bosch, Shannon Smits, Gimano Amatngalim, Jeffrey Beekman, Sam van Beuningen

P83 Amplifier PTI-428 enhances the effect of VX-661/VX-445/VX-770 in patient-derived airway organoids
Suzanne Kroes, Gimano Amatngalim, Shannon Smits, Sara Hageraats, Heleen van Kooten - Sonneveld, Jeffrey Beekman

P84 An iPSC-derived bronchial epithelial model to study nonsense mutations in Cystic Fibrosis
Stecy Mienanzambi, Benjamin Simonneau, Pascale Fanen, Bénédicte Duriez

P85 Investigation of CFTR function and epithelial barrier properties at single cell resolution using multi-electrode array technology
Marjolein M. Ensinck, Alessandra Venz, Anabela S. Ramalho, Saeedeh Ebrahimi Takalloo, François Vermeulen, Dries Braeken, Marianne S. Carlon

P86 Optimization of secretory cell dependent CFTR function measurements in 3D airway organoids derived from submerged differentiated nasal epithelia
Shannon Smits, Loes den Hertog-Oosterhoff, Henriette Dreyer, Jeffrey Beekman, Gimano Amatngalim

P87 *In vitro* and *in vivo* efficacy of elexacaftor/tezacaftor/ivacaftor in people with cystic fibrosis carrying rare CFTR variants
Lucie Borek Dohalska, Eva Furstova, Stepanka Novotna, Pavel Drevinek, Tereza Dousova

P88 Endometrium-derived organoids from cystic fibrosis patients to study the endometrial factor in the disease-associated fertility deficiency
Ellen De Pauw, Marjolein M. Ensinck, Beau Gommers, Stefan Timmerman, Lieven Dupont, Diether Lambrechts, Marianne S. Carlon, Hugo Vankelecom

P89 Challenges in interpreting discordant in-vitro responses to modulator combination Elexacaftor, Tezacaftor and Ivacaftor in different tissue models derived from an individual harbouring ultra-rare genotype: H609R/2184insA
Tarini Gunawardena, Wan Ip, Claire Bartlett, Kai Du, Sowmya Thanikachalam, Neil Sweezey, Theo Moraes, Christine Bear, Tanja Gonska

AWARD WINNERS

ECFS Young Fellows Travel Award

Marjolein Ensinck (BE)
Chloé Felgerolle (FR)
Daniela Guidone (IT)
Omar Hamed (UK)
Joss Murray (IE)
Johan Sergheraert (FR)
Sacha Spelier (NL)
Immacolata Zollo (PT)

Student Helper Award

Maria-Cristina Ardelean (UK)
Ellen De Pauw (BE)
Lucia Nicosia (IE)
Arina Svoeglazova (BE)

Free Registration Young Researchers

Supported by the Italian Cystic Fibrosis Research Foundation
Anna Borrelli
Angela Della Sala
Dorina Dobi
Simona D'Orazio
Alessandra Murabito
Arianna Venturini
Supported by the Canadian CF Foundation
Mohsen Esmaeili
Tarini Gunawardena

20 March — 18:00–19:00

Opening Keynote Lecture

CFTR biogenesis and misfolding correction; the role of allosteric domain-domain coupling

Naoto Soya[1], Haijin Xu[1], Ariel Roldan[1], Zhengrong Yang[2], Haoxin Ye[1], Fan Jiang[2], Aiswarya Premchandar[1], Guido Veit[1], John Kappes[2], Tamas Hegedus[3], Gergely L. Lukacs[1]

[1]Department of Physiology and Biochemistry, McGill University, Montréal, QC, Canada,, [2]Heersink School of Medicine, University of Alabama School of Medicine, Birmingham, AL, USA, [3]Department of Biophysics and Radiation Biology, Semmelweis University, 1085 Budapest, Hungary

The rapid cotranslational folding of small single-domain proteins has been well established. In contrast, the folding kinetics of large multi-domain proteins with complex topology (e.g. members of the ATP-binding cassette (ABC) transporter family, including CFTR), are usually impeded by accumulation of co- and post-translational folding intermediates both in vitro and in vivo. This phenomenon may explain that ~50% of newly synthesized wild-type (WT) CFTR nascent chains are targeted for degradation by the endoplasmic (ER) quality control during their post-translational maturation. In line, single-molecule and population studies of soluble multi-domain proteins suggest that inter-domain interactions are indispensable for their posttranslational conformational biogenesis.

ABCC transporters typically consists of two nucleotide binding domains (NBD1,2) and transmembrane domains (TMD1,2). While the allosteric regulation of TMDs conformational states by the NBDs dimerization-dissociation cycle of native transporters is well established, the contribution of domain-domain interactions in ABC-transporters folding, misfolding, and conformational rescue by pharmacological chaperones remains incompletely understood.

Results will be presented to provide an overview of molecular/cellular mechanism of the complex domain-folding and -misfolding of ABCC-transporters during their biogenesis at the ER, as well as the mutant rescue mechanism by the VX-809/VX-609 and VX-445 CFTR folding correctors that confer life-changing clinical improvements for number of CF patients. Using targeted mutational- and/or pharmacophore-induced perturbations, our working model integrates results that were obtained on three ABCC-transporters (CFTR(ABCC7), MRP1(ABCC1), and ABCC6) at three complementary levels. i) At the cellular level, the transporters' biosynthetic processing, expression, and metabolic stability, as well as their domains' conformational stability, are monitored. ii) At the isolated NBD1 level, the domains' thermal stability and backbone NHs conformational dynamics are assessed. Changes of purified CFTR variants conformational dynamics are determined both at the backbone NHs dynamics and fast atomic motions level, by using hydrogen-deuterium exchange and mass spectrometry (HDX-MS), and molecular dynamic (MD) simulations, respectively.

Mutational uncoupling or stabilization of the NBD1-TMDs interfaces can compromise or facilitate the CFTR(ABCC7)-, MRP1(ABCC1)-, and ABCC6-transporters post-translational coupled domain-folding in the ER globally and at individual domain level without energetic or kinetic destabilization of the NBD1. Furthermore, we provide evidence indicating that association of VX-809 and/or VX-445 folding correctors to the recently identified TMD1 and TMD2 drug binding sites, respectively, allosterically or orthosterically rescue the kinetically trapped post-translational folding intermediates of the P67L and F508del mutants by rewiring the inter-domain allosteric-networks.

We propose that dynamic allosteric domain-domain communications not only regulate the function of CFTR and other ABCC-transporters but are indispensable to tune the folding landscape of their post-translational intermediates. These allosteric networks can be compromised by genetic mutations, and reinstated by correctors, offering a framework for mechanistic understanding of CFTR and other ABCC-transporters (mis)folding and improving the efficiency of folding correction.

I thank the past members of my laboratory and collaborators for their unwavering support and dedication along the challenging path of CF research, as well as the funding provided by CIHR, CF Foundation, CF Canada, and CFI.

21 March — 08:45–10:30

Symposium 01- CFTR protein structure and function

S1.1 CFTR structure, dynamics, and gating through the in silico microscope

Tamás Hegedűs

Department of Biophysics and Radiation Biology, Semmelweis University and HUN-REN Biophysical Virology Research Group, Budapest, Hungary

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein impact its folding, domain stability, and inter-domain assembly. Recent advancements in clinical therapeutics have seen the successful application of several drugs targeting common CFTR mutations. However, gaps remain in our understanding of their efficacy and mechanisms of action, which are critical for the development of new drugs. Specifically, detailed atomic insights into the open channel pore are essential for designing potentiators.

Over the past seven years, significant progress has been made in structural modeling of CFTR. All human CFTR cryo-EM structures, derived from Yue Chen's laboratory, capture both inactive (inside-open, channel-closed) and active conformations (inside-closed), including structures with bound drugs and ΔF-CFTR coordinates. While these 3D models have undeniably enhanced our understanding of CFTR's structural properties, they also present limitations due to their static nature, offering only snapshots of the protein. This limitation hinders the comprehension of mutation effects on dynamics and function, as well as the understanding of channel gating mechanisms. In addition, structures obtained under activation conditions (e.g., in the presence of ATP, phosphorylation, and potentiators) fail to show an open ion pathway. This might be attributed to a kink in the TM8 helix, absent in avian CFTR structures, which display TM7 and TM8 helices detached from their usual location.

To study the effects of mutations and drug binding on CFTR structure and dynamics, we have employed various in silico methods. In addition to experimental structures, we used homology modeling to generate CFTR models possessing a TM8 helix without any kink. Furthermore, we applied AlphaFold for structure prediction based solely on sequence alignment, resulting in a kink-free TM8 structure, albeit with low-confidence predictions in the extracellular halves of TM7 and TM8 (as indicated by low pLDDT scores).

Using these models, some including bound drugs, we conducted molecular dynamics simulations to analyze CFTR dynamics with both kinked and straight helices, examining their pore-opening capabilities. Subsequently, we explored the changes in drug-protein interactions and how drug binding affects CFTR dynamics. Our in silico approaches and computational tools enhance our comprehension of the structure, function, and drug interactions of CFTR, thereby facilitating the refinement of pharmacotherapies.

S1.2 Molecular mechanism of Cl- conduction in the open state of human CFTR

Régis Pomès

Molecular Medicine, Hospital for Sick Children, Toronto, Ontario, Canada Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

By providing atomic-level information on a wide range of time scales extending from the femtosecond to the microsecond and beyond, molecular dynamics simulations can help bridge the gap between structure and function of biological ion channels. I will present recent and ongoing simulation studies of ion permeation and gating in the Cl- channel cystic fibrosis transmembrane regulator (CFTR). A molecular model of the open state of human CFTR was derived from extensive simulations of a nearly-open cryo-EM structure in an explicit lipid bilayer, providing insight into the structural and physico-chemical basis of channel gating and ion permeation. Open-state conformations of the channel were reached by spontaneous relaxation of pore-forming helices to a regular pentameric arrangement of TM1, 6, 8, 11 and 12 in which the hydrophobic bottleneck of the pore forms a continuous, hydrated path to permeating Cl- ions. Simulations performed under a wide range of applied voltage leads to predicted Cl^- conductance in quantitative agreement with published electrophysiological data. The open-state structure is further validated by agreement with experimentally demonstrated open state features. The analysis of the ion permeation mechanism reveals the role of specific pore residues in the solvation and mobility of Cl- ions. This work opens the way to detailed analyses of the structural and functional consequences of single-point mutations implicated in cystic fibrosis.

S1.3 CFTR NBD2 folding requires its assembly with TMDs rather than folded NBD1

Ineke Braakman, Hanneke Hoelen, Tamara Hillenaar, Jisu Im, Peter van der Sluijs

Cellular Protein Chemistry, Faculty of Science, Utrecht University, The Netherlands

Programme note: the details are not authorised for inclusion

P2

S1.4 Exploring the activating effects of the inhibitors of type IV ABC transporters on CFTR gating

Maria-Cristina Ardelean, Hannah J. Eldred, Paola Vergani

University College London, Neuroscience, Physiology and Pharmacology, London, GB

CFTR, a Type IV ABC transporter, uniquely functions as an anion channel. Cystic fibrosis (CF)-causing gating mutations in CFTR reduce open probability (Po). Potentiators are a class of compounds that increase CFTR Po and present a therapeutic strategy for patients with gating mutations. VX-770 (ivacaftor; Vertex Pharmaceuticals) is the only approved potentiator. Cryo-EM studies revealed bound VX-770 at the interface between the hinge-like discontinuity in transmembrane helix 8 (TM8) and the lipid bilayer[1]. To gain mechanistic insight, we explored how compounds initially developed for other Type IV ABC transporters affect CFTR activity and how they interact with VX-770.

The Genetech-developed G-compounds inhibit MsbA, a homodimeric bacterial ABC transporter[2]. Among these, G907 binds at two homologous sites on MsbA, one corresponding to the portal between TM4 and TM6, on the CFTR anion permeation pathway, not far from the VX-770 binding site. Unexpectedly, we found that G-compounds activated CFTR[3].

P-gp, another Type IV ABC transporter, plays a crucial role in the efflux of xenobiotics. Elacridar and zosuquidar are potent third-generation P-gp inhibitors. They bind in the substrate-binding pocket on P-gp, corresponding to CFTR's inner vestibule, which is also relatively close to the VX-770 binding site on CFTR[4].

To investigate the effects of these molecules on CFTR, we employed a high-content assay which evaluates ion-channel activity at the cellular level in HEK-293 cells[5]: CFTR, tagged with a halide-sensitive yellow fluorescent protein (YFP, H148Q/I152L) and mCherry, a red cytosolic fluorescent marker for image segmentation, are co-expressed from a bicistronic plasmid. Quenching of YFP-CFTR fluorescence following extracellular iodide addition reports on CFTR activity.

Elacridar and zosuquidar modestly enhanced the activity of partially phosphorylated CFTR. Furthermore, less-than-additive effects were observed in combination with VX-770 (table 1), suggesting direct CFTR targeting. The mismatch between the inhibition of other ABC transporters and activation of CFTR might reflect an effect on the dynamics of the extracellular end of TM8 in CFTR, which recent results suggest might be modulated by both potentiator VX-770 and inhibitor CFTRinh-172[6,7]. Future studies are planned to assess the effects of VX-770 on G907/elacridar/zosuquidar binding. A better understanding of how these molecules interact on CFTR could help clarify gating and pharmacological mechanisms.

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Table 1: AAC_9 (area above YFP-quenching curve, 9 s after iodide addition, RFU*s)

acute treatment	***mean***	***(95 % CI)***	***N***
0.5 µM forskolin	3.72	(3.42, 4.02)	12
0.5 µM forskolin+10 µM elacridar	4.74	(4.31, 5.16)	12
0.5 µM forskolin+10 µM zosuquidar	4.81	(4.41, 5.22)	12
0.5 µM forskolin+3 µM VX-770	5.57	(5.25, 5.89)	16
0.5 µM forskolin+3 µM VX-770+10 µM elacridar	5.9	(5.40, 6.39)	8
0.5 µM forskolin+3 µM VX-770+10 µM zosuquidar	5.95	(5.42, 6.47)	8

P5

S1.5 Mechanism of action and binding site characterization of corrector *ARN23765* via Photo-Affinity Labeling (PAL) approach in live cells

Fabio Bertozzi[1], Elisa Romeo[2], Francesco Saccoliti[1], Angela Andonaia[1], Caterina Allegretta[3], Onofrio Laselva[3], Federico Falchi[4], Riccardo Occello[4], Nara Liessi[5], Andrea Armirotti[5], Stefania Girotto[2], Cristina Pastorino[6], Nicoletta Pedemonte[6], Tiziano Bandiera[1]

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Cystic Fibrosis (CF) is a genetic disease characterized by an impairment in the synthesis or function of CF transmembrane conductance regulator (CFTR) anion channel, caused by mutations in CFTR gene. Small-molecule compounds addressing the basic defect of the disease have been identified and are referred to as CFTR modulators.[1] Among these, the F508del-CFTR corrector *ARN23765,* discovered by our group, showed high potency in rescuing the function of mutant CFTR in primary human bronchial epithelial cells derived from CF patients homozygous for the F508del mutation.[2] Despite the validated pharmacological effects, *ARN23765* mechanism of action and binding site had not yet been conclusively defined. CFTR correctors could act either directly by binding to CFTR or by interacting with the machinery responsible for protein synthesis, trafficking and maturation.[3,4] To the best of our knowledge, no data are so far available disclosing the interaction of modulators with CFTR (either wt- or F508del-) in a native cellular environment.

Along with the identification of the primary biological target(s), our project aimed to uncover the mechanism of action and the binding site of *ARN23765* to its target protein in living cells. Photo-Affinity Labeling (PAL)[5] approach was pursued as a suited strategy to perform such investigation. *ARN23765*-derived photo-affinity probes (PAPs) were designed and synthesized by introducing both a small photo-reactive moiety to capture bio-molecules in its close proximity, and a reporter/purification tag allowing adducts identification in biochemical studies.[6]

Taking advantage of the PAL methodology, we demonstrated the in situ interaction of *ARN23765*-derived PAPs with CFTR in wt- and F508del-CFTR overexpressing CFBE41o- cells. Additionally, functional studies using CFTR domains identified the interface between MSD1-NBD1 as the protein region involved in *ARN23765*-induced correction. The putative binding site of *ARN23765* on CFTR was elucidated by a combination of computational (molecular docking and molecular dynamics) analyses and site-directed mutagenesis studies, which identified the primary amino acid residues involved in the effective interaction of the corrector with CFTR.

To the best of our knowledge, our study is the first to disclose the interaction of a corrector probe to wt- and mutant F508del-CFTR in an intact cellular setting. Furthermore, these results contribute to elucidate the molecular bases of CFTR rescuing induced by *ARN23765*, showing its direct binding to CFTR and the stabilization of the interface between MSD1-NBD1 protein region through the interaction with specific amino acids.

This work was supported by the Italian Cystic Fibrosis Research Foundation (FFC Ricerca) as part of the "FFC#4/2020 and FFC#2/2022" projects.

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P4

S1.6 Understanding the conformational landscape of NBD1 from CFTR: from normal function to cystic fibrosis

Arina Svoeglazova[1], Daniel Scholl[1], Maud Sigoillot[1], Rafael Colomer Martinez[1], Tom Kache[2], Marie Overtus[1], Chloé Martens[1], Jelle Hendrix[2], Cédric Govaerts[1]

[1]Université Libre de Bruxelles, Faculty of Sciences, Chemistry department, Brussels, BE, [2]Hasselt University, Advanced Optical Microscopy Centre and Biomedical Research Institute, Hasselt, BE

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel regulating fluid transport in epithelial tissues. The most common mutation is the deletion of phenylalanine at the 508 position (F508del) in the nucleotide-binding domain 1 (NBD1), which leads to channel degradation and ion transport lack.

We developed a collection of nanobodies targeting human NBD1 (Sigoillot, M. et al, 2019) and isolated nanobody G11a, that stabilizes an alternative conformation of NBD1, topologically different from the canonical NBD1 fold. By single-molecule FRET (smFRET) microscopy, canonical and alternative conformational equilibrium was regulated by ATP presence. The F508del mutation presence in the domain favored the alternative state (Scholl, D. et al, 2021). To probe the functional and physiological role of the different conformation, we have developed a point mutant of NBD1 (W401A), strongly promoting the alternative state. We are now investigating the mutation effect on the protein structure and function.

We also identified nanobody T1a, a high-affinity NBD1 binder which, remarkably, induces NBD1 destabilization in a “F508del mutation” manner. The F508del mutation-like effects of T1a can provide a unique tool to investigate the CF-causing mutation molecular basis.

On the one hand, we want to trace how F508del mutation leads to NBD1 destabilization using T1a nanobody. On the other hand, we aim to examine the NBD1 alternative state natural. In our research we use biochemical (ELISA), physical (smFRET, thermal shift assay), and structural (X-ray crystallography, hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) methods. The alternative fold could be a functional state of the domain, but in the destabilizing mutation presence it becomes the weakest NBD1 state and leads to its misfolding.

21 March — 11:00–12:45

Symposium 02 - CFTR genetics & CFTR expressing cells

S2.1 Modulator responsiveness of 656 CFTR variants: from predicting patient treatability to structural insights

Hermann Bihler[1], Andrey Sivachenko[1], Linda Millen[2], Priyanka Bhatt[1], Amita Thakerar Patel[3], Justin Chin[1], Violaine Bailey[1], Isaac Musisi[1], André LaPan[1], Normand E. Allaire[1], Joshua Conte[1], Noah R. Simon[4], Amalia S. Magaret[4], Karen S. Raraigh[5], Garry R. Cutting[5], William R. Skach[6], Robert J. Bridges[3], Phil J. Thomas[2], Martin Mense[1]

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The US Food and Drug Administration has approved the Vertex medicine Trikafta® (marketed as Kaftrio® in Europe) for the treatment of cystic fibrosis (CF) when the disease is caused by at least one allele of the 178 CFTR variants for treatment listed on the FDA drug label. The majority of the variants were approved based only on in vitro data and the clinical safety record of the treatment. Recognizing that there are hundreds more CFTR variants that have not been assessed by Vertex for their responsiveness to Trikafta®, a study was undertaken to identify CFTR variants that result in increased chloride (Cl-) transport function by the CFTR protein after treatment with the CFTR-modulator combination elexacaftor/ tezacaftor/ivacaftor (ELX/TEZ/IVA), the active ingredients of Trikafta®. The data may benefit people with CF (pwCF) who are not currently eligible for modulator therapies, and they also provide insights into structure-activity relationships, i.e., the mechanisms of functioning and interactions in the CFTR protein.

Methods: Plasmid DNA encoding 655 CFTR variants and wild-type (WT) CFTR were transfected into Fisher Rat Thyroid cells that do not natively express CFTR. After 24 hours of incubation with control or TEZ and ELX, and acute addition of IVA, CFTR function was assessed using the transepithelial current clamp conductance assay. Each variant's forskolin/cAMP-induced baseline Cl- transport activity, responsiveness to IVA alone, and responsiveness to the TEZ/ELX/IVA combination were measured in three different laboratories. Western blots were conducted to evaluate CFTR protein maturation and complement the functional data.

Results and Conclusions: 253 variants not currently approved for CFTR modulator therapy showed low baseline activity (<10% of normal CFTR Cl- transport activity). For 152 of these variants, treatment with ELX/TEZ/IVA improved the Cl- transport activity by ≥10% of normal CFTR function, which is suggestive of clinical benefit has been the threshold for variant label inclusion by the FDA. ELX/TEZ/IVA increased CFTR function by ≥10 percentage points for an additional 140 unapproved variants with ≥10% but <50% of normal CFTR function at baseline. These findings significantly expand the number of rare CFTR variants for which ELX/TEZ/IVA treatment should result in clinical benefit.

The data also reveal that the majority of residues associated with putative gating and conductance variants are located at the outer mouth of the CFTR pore or on the inside of the NBD domains, as well as lining the anion-conducting pore of the protein. A smaller group of variants resulting in changes at residues 314, 347, 352, 924 and 997 display much reduced sensitivity to the CFTR inhibitor Inh-172. Mapping these residues onto the 3D structure of CFTR illustrates that they are closely coordinated.

S2.2 Single-cell analyses of the human airways in health and respiratory diseases

Laure-Emmanuelle Zaragosi

Institut de Pharmacologie Cellulaire et Moléculaire, CNRS, Inserm, Université Côte d'Azur

The defence function of the lung airway epithelium results from homeostasis of a complex cellular ecosystem which can be altered in chronic respiratory diseases. We have used single-cell RNA profiling to investigate the precise composition of the distinct cell types and differential gene expression at different levels of the lung airways.

Our initial single-cell atlas of the human healthy airways was established in young healthy adults and was then integrated in a larger Human Lung Cell Atlas (HLCA) which combines 49 datasets of the human lung into a single atlas spanning over 2.4 million cells from 486 individuals. The HLCA captures the cellular diversity of the human lung by describing the characteristics of 62 distinct cell types including rare cell types.

Smaller variations between normal and pathological samples were more directly assessed by comparing patients with healthy age-matched volunteers. A first example was for patients suffering from early stages of Chronic Obstructive Pulmonary Disease (COPD) for which a new dataset of 119 samples was assembled after collecting cells by brushings or biopsies from the nose to the 6th division of the airways. This first COPD airway cell atlas better characterized rare cell types and identified early traits of the disease, illustrated by modifications in cell composition and gene expression.

We have applied a similar approach to evaluate the modifications of the nasal epithelium of cystic fibrosis patients at the onset of a Trikafta therapy. Samplings were performed before and after treatments, and single-cell RNAseq was used to quantify the extend of restoration of the nasal mucosa in terms of cell composition and gene expression.

The benefits of integrating healthy and disease single-cell atlas in larger resources leads to a more robust characterization and better recovery of cell types and states. The global HLCA defines a general computational strategy transferrable to the analysis of other pathological samples.

S2.3 *CFTR* regulation in time and space

Ann Harris

Case Western Reserve University

Mechanisms underlying the regulation of the *CFTR* gene in secretory cells in the airway and intestinal epithelium are broadly understood. The locus exists in its own topologically associating domain (TAD), within which cell-type selective *cis*-regulatory elements (CREs) are recruited to the gene promoter to drive gene expression. Most CREs are transcription factor-dependent enhancers that work cooperatively in the nucleus. Less is known about *CFTR* regulation in relatively inaccessible epithelial cells in the pancreatic duct, the bile duct and the efferent ducts of the epididymis, all cell types where *CFTR* transcript levels are high. Furthermore, *CFTR* regulation in rare cell types such as the pulmonary ionocyte/CFTR high cells in the lung, and equivalent clear cells in the caput epididymis, has not been elucidated.

Recent data identifying CREs and regulatory mechanisms in these poorly studied epithelial cell types will be presented, together with aspects of temporal regulation of *CFTR* that may be relevant to novel therapeutics.

P22

S2.4 Pre-clinical data demonstrates great promise for AAV gene therapy, one dose of AAV1 or 6-Δ27-264 CFTR successfully ameliorated clinical symptoms in G551D ferrets after 5-weeks.

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Developing a gene therapy for CF is still a critical need especially for patients who either are not approved for corrector therapy or do not tolerate it. One way to show definitively that gene therapy can be therapeutic is to rescue defective CFTR function in a relevant animal model that recapitulates, as closely as possible, human disease. To accomplish this, we used the ferret model bearing the G551D mutation which responds to the potentiator, VX-770, similar to humans (1). The goal was to assess whether dosing of AAV1/6-Δ27-264-CFTR vectors administered to G551D ferrets in the absence of proteasome inhibitors leads to widespread gene transfer, expression and phenotypic rescue. The G551D ferrets were removed from VX-770 treatment 7 days before AAV treatment. 1 dose of 10^{11} vg of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR was sprayed into the airways and injected intravenously into eight, 3-month-old male and female ferrets. 2-G551D and 2-wild-type ferrets were kept as controls. Animals were necropsied at 35 days post-instillation.

AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR were detectable at ≥10^6 vg/ug-genomic-DNA in trachea, bronchi, lung, ileum, liver and pancreas. mRNA expression was detected at ≥10^6 copies/ug-genomic-DNA in trachea, bronchus, lung, pancreas and ileum and ≥10^5 copies/ug-genomic-DNA in liver. Widespread CFTR protein expression was seen in the tissues of all the treated animals above levels measured in untreated wild-type and G511D ferrets. CFTR protein expression above untreated animal levels following a single dose of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR suggests that transduction by AAV1 or 6 based vectors occurs in airways, liver ileum and pancreas of AAV treated G551D ferrets. The gross morphology of the treated ferrets resembled that of the normal ferrets compared to the untreated G551D ferrets. Minor amounts of mucous was present compared to untreated animals.

The results are important in that we were able to transduce trachea and lung despite the barriers associated with airway delivery that include thick airway mucous, AAV degradation and potential inhibitory factors in the sputum. Transduction of the G551D ferrets in the organs affected in CF provide promise for rescue of multiorgan disease in CF. Funded by NIDDK.

1. Sun X, Yi Y, Yan Z, Rosen BH, Liang B, Winter MC, et al. In utero and postnatal VX-770 administration rescues multiorgan disease in a ferret model of cystic fibrosis. Sci Transl Med. 2019;11(485):eaau7531.

P25

S2.5 Novel CFTR+ lung progenitor cells contribute to the dynamic developmental origins of fetal epithelial cell lineages

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Human fetal lung development remains largely unexplored. The scarcity of embryonic and prenatal lung tissues and the lack of appropriate human models have impeded efforts to study human-specific processes underlying fetal lung development. Consequently, the etiology of congenital lung diseases caused by cessation of lung development or the origins of progressive lung diseases that manifests early in neonatal life such as cystic fibrosis (CF), is poorly understood. Here, we sequenced >150,000 single cells using 3' 10X Genomics from 19 healthy human fetal lungs from gestational weeks 10-19. We outline a time course atlas of cells broadly categorized as epithelial, stromal, immune, endothelial and Schwann cell populations that make up the developing human fetal lung and identified a total of 58 unique cell types. Interestingly estimating RNA velocity reveal the existence of several fetal uncommitted progenitor cells that express abundant levels of CFTR and gives rise to ciliated, pulmonary neuroendocrine cell and basal/club cell lineages. RNA velocity-based inferences of cell lineage trajectories showed complex developmental dynamics emerging from a progenitor cell that express SCGB3A2+SFTPB+CFTR+ (Triple Positive, TP). This may be explained by temporal changes in cell-cell signalling and altering the microenvironment of signals driving specific cell fate during differentiation. We focused on Wnt signalling to the TP cells and show multiple cell-cell communications with these cells via Wnt/receptor interactions. Specifically, temporal changes in WNT2 ligand-receptor interactions suggest a role of Wnt2 in regulating TP cell fate. Previous studies have found a role of CFTR in regulating canonical Wnt/beta catenin pathway in intestinal stem cell niches and kidney development. We then used siRNA targeting *CFTR* in fetal lung explants and found changes in fetal epithelial differentiation and airway formation, with a concomitant increase in beta catenin. Our work reveals novel developmental trajectories of some epithelial subtypes and reveal how CFTR may regulate airway epithelial morphogenesis. Uncovering the role of CFTR in human lung development will expand our understanding of how dis-regulation of CFTR impact congenital disorders and affect disease pathogenesis.

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S2.6 A modelling framework for epithelial airway fluid and ion transport with multiple cell types: implications for success or failure in gene therapies for cystic fibrosis

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Epithelial ion transport regulates the depth of airway surface liquid (ASL), a thin fluid layer (0.1-10 μm) lining the airway epithelium, enabling the vital mucociliary clearance of mucus-trapped pathogens in the upper airways. ASL homeostasis is regulated by a complex collaboration of ion channels, transporters, and tight junction proteins. In cystic fibrosis (CF), where this system is disrupted, the ASL becomes dehydrated creating sticky mucus that is difficult to clear, resulting in chronic lung infections.

Existing mathematical models of ASL regulation typically treat the system as a single, idealised cell containing all the important elements of fluid/ion transport. However, airway epithelia comprise patchworks of distinct cell types, each with specialised functions. To explore the implications of this for ASL homeostasis, we developed a multicellular quantitative framework to understand the bioelectric properties of healthy and CF epithelia and, in the latter case, the implications of cell diversity for gene therapy.

Our mathematical framework for modelling multicellular epithelia has its basis in the equivalent electrical circuit and direct modelling of ion fluxes for Na^+, Cl^- and K^+. The solution of the model provides numerical values of cellular/ASL ion concentrations, membrane potentials and cellular/ASL heights comparable to those reported in the literature. The modelling framework is quite flexible, such that cells can be made distinct by the presence/proportion of channels expressed on their surfaces. Lateral flow of ions between neighbouring cells was also incorporated into the model.

We began our examination of multicellular modelling by considering an extreme case where all essential secretory channels/transporters were included in one cell type whilst absorptive machinery was exclusively located within a second, neighbouring cell type. We found that even with exaggerated differences, cells could stably maintain internal ionic compositions and membrane potentials distinct from their neighbours.

Next, using a less extreme scenario, we examined ASL homeostasis in a two-cell model of healthy airway epithelium. This highlighted a key role for the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and basolateral Cl^- channels (ClC_{ba}). The presence of ClC_{ba} in the absence of NKCC resulted in a cell absorbing Cl^- from the ASL while a neighbouring cell with NKCC present and ClC_{ba} absent secretes Cl^-. Both cell types were modelled expressing apical CFTR, thus suggesting a cell type-dependent role in both Cl^- absorption and secretion.

Taking this model further, we simulated CF epithelia and subsequent gene therapy in multiple situations. First, we considered the case where perfect localisation of CFTR to the apical membrane occurs for both secretory and absorptive cell types. This improved ASL hydration, increasing ASL depth from 3 μm to ~6.0 μm, near the height of extended cilia. Next, we considered a scenario where CFTR overexpression led to mis-sorting of the protein via equal localisation to apical and basolateral membranes. This resulted in very limited hydration, increasing ASL depth to ~4.4 μm, and would not form a successful therapy.

We conclude that such models can help to elucidate the roles of different cell types in ASL regulation and inform the design and development of successful gene therapies.

21 March — 16:00–17:45

Symposium 03 - Gene editing to correct any CFTR mutation

S3.1 Modulation of Double Strand Breaks repair to promote Cas9 and PEn dependent DNA insertions

Marcello Maresca

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The CRISPR-Cas9 system is a powerful tool for genome engineering, but low efficiency of targeted gene integration is a significant challenge for therapeutic applications, especially in non-dividing cells. We developed new approaches to enhance the efficiency of targeted integrations in mammalian cells. This includes 2HDR a method using a mix of small molecule inhibitors to facilitate gene insertion in dividing cells, and PEn/2iPEn employing an RT/DNA polymerase-driven strategy to promote templated insertions via NHEJ-dependent and NHEJ-independent pathway. The use of the specified DNA repair inhibitors, combined with our newly developed nuclease, PsCas9, reduces the potential for unwanted on-target and off-target effects.

S3.2 Strategies to correct whole exons in CFTR by gene editing

Patrick T. Harrison

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One of the limiting factors in the clinical development of CRISPR gene editing is the relatively short editing range. For example, base editing can correct many different CF-causing mutations, but each one will require development of a separate guide RNA, and ultimately separate clinical testing and regulatory approval. Conventional prime editing has a longer range of ~30 bp and could potentially correct small clusters of mutations but not an entire exon. And whilst homology-directed repair can be used to correct mutations across a small window with a single donor and guideRNA combination, it's not yet possible to correct multiple mutations with using a superexon integration strategy which could ultimately disrupt regulatory elements if used clinically with a systemic delivery approach.

Here, I describe two different approaches we are using in our lab to correct all the variants in exon 12 of CFTR, including splice variants at the -1 and +1 position. An update on other techniques suitable for this approach will also be reviewed.

S3.3 Identification and evolution of novel CRISPR -Cas systems from the human microbiome

Anna Cereseto, Nicola Segata, Matteo Ciciani, Elisabetta Visentin, Ilaria Bonuzzi, Lorenzo Lucchetta, Giulia Vittoria Ruta

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CRISPR technologies are transforming the bio-medicine field by providing new therapeutic concepts for the treatment of diseases through genetic repairs and deployment of disease protecting factors. Nonetheless, the currently available CRISPR nucleases and derived technologies do not address the hurdles related to genome modification in gene therapy applications. Challenges are imposed by specific properties of CRISPR tools which includes high molecular weight limiting their compatibility with most commonly delivery vectors including lipid nanoparticles, target sequence constraints, immunogenicity and heterogeneous efficiency and precision throughout the genome. We recently focused on the development of new technologies by retrieving CRISPR systems from a large databank of the human microbiome and through a directed evolution approach to enhance the activity of the prokaryotic enzymes to eukaryotic environment. This work led us to the discovery of new CRISPR systems and the enhancement of Cas nucleases with compelling features for gene therapy applications.

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S3.4 Rescuing G542X by Adenine Base Editing: A guide to restore function

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G542X is the 2nd most common CF-causing mutation. A premature termination codon (TGA) and the consequent nonsense mediated decay prevent CFTR protein synthesis and, thus, CFTR-mediated chloride transport. Here we outline a strategy to rescue this variant at the DNA level by gene editing and we describe the effects of modulators to boost CFTR activity following editing. Our approach involves Adenine Base Editing (ABE), a CRISPR-based system that allows the transition of A-T base pairs into G-C base pairs in a safe and efficient manner. Whilst ABE cannot precisely repair G542X (as this would require A-T to C-G editing), it can be used to convert G542X into G542R, an alternative CFTR variant that has previously been shown to retain approximately 30% of WT activity [1]. In our approach, a G542X-specific single-guide RNA (sgRNA) directs a highly catalytic base editor, NG-ABE8e [2], to edit the A on the opposite strand of the TGA codon into a G, converting the G542X stop codon to CGA, which encodes Arginine (G542R).

Editing of G542X into G542R by ABE was tested in patient-derived intestinal organoids, where the designed sgRNA and the selected Adenine Base Editor were delivered as ribonucleoproteins (RNPs) encapsulated in engineered Virus-Like Particles (eVLPs [3]), that deliver their cargo without replicating or integrating. Restoration of CFTR function was assessed by Forskolin-Induced Swelling (FIS) assay and/or Short Circuit Current (I_{sc}) assay, on multiple donors and in two different labs. Levels of DNA editing were detected by high-throughput sequencing.

Following transduction of intestinal organoids homozygous for G542X, FIS (after 7 days) and Isc assays (after 21 days) both showed restoration of CFTR function. FIS assay data analysis reported a significant swelling of treated organoids, up to 22% of the total (non-sorted) population. The percentage of swelling organoids was also eVLPs-concentration dependent and correlated with DNA editing levels. I_{sc} detected CFTR-mediated chloride transport, up to 10% of WT levels, and showed a clear response to Elexacaftor/Tezacaftor/Ivacaftor (ETI) treatment, which appeared to further increase G542R-CFTR activity. Amplicon-sequencing confirmed that CFTR functional rescue is specific to edited organoids only.

Our focus is now to optimise editing levels through the refinement of sgRNA design and eVLPs production. Our ultimate goal is to test this strategy *in vivo*, utilising suitable CF model systems of the disease, to evaluate its impact on the CF phenotype, and determine if there is any variation in editing efficiency in specific cell types.

[1] Xue et al. Identification of the amino acids inserted during suppression of CFTR nonsense mutations and determination of their functional consequences. Hum Mol Genet. 2017

[2] Richter et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol. 2020

[3] Banskota et al. Engineered virus-like particles for efficient in vivo delivery of therapeutic proteins. Cell. 2022

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S3.5 Site-specific gene targeting of chromosomal safe harbor and CFTR locus for correcting any CFTR mutation

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Objective: The development of CFTR modulator drugs was a major milestone in translating cystic fibrosis (CF) discoveries into treatment that benefits most CF patients. However, some people with CF do not respond to these new drugs, and around the world others with CF cannot afford them. Our long term goal is to develop a gene therapy that can benefit all patients. We plan to use a helper-dependent adenoviral (HD-Ad) vector to deliver a CRISPR-Cas9 system and a gene expression cassette as donor DNA to achieve permanent CFTR expression in lung epithelial cells. One of the unique advantages of our system is that following gene integration, there is no residual Cas9 expression, which eliminates the potential risk of the host immune system attacking the cells with gene integration.

Methods: We used pig cells and selected the GGTA1 (genomic safe harbor) and CFTR loci for testing our strategy. HD-Ad vectors do not have any adenoviral genes and have a large capacity for delivery. We pack gene editing cassettes for Cas9 and a guide RNA as well as a donor gene expression cassette (for reporter *LacZ* or human *CFTR* expression) in a single vector. Following the delivery of vectors into cultured epithelial cells, site-specificity of gene integration is determined by junction PCR and DNA sequencing. The reporter gene expression is analyzed by X-gal staining or quantification using chemiluminescent assays, while the CFTR expression is determined by Western blotting and the CFTR function is analyzed by FLiPR assays.

Results: We have previously shown success in targeting the pig GGTA1 locus. In this study, we focus on the pig CFTR locus. We demonstrated high efficiency in Cas9-mediated DNA cleavage by ddPCR and showed precise integration of *LacZ* or human *CFTR* expression cassettes into the pig CFTR locus by junction PCR and Sanger sequencing. We detected transgene mRNA and protein expression when the reporter or CFTR expression cassette was integrated. The efficiency of the *LacZ* integration into the CFTR locus is close to 10%, similar to that for the GGTA1 locus. We are in the process of performing FLiPR assays to assess the CFTR function.

Conclusions: These results validated the potential of our *CFTR* gene targeting strategy in therapeutic developments.We showed that Cas9 expression was eliminated following transgene integration, which is important for *in vivo* applications since Cas9 is a foreign protein and elimination of its expression would avoid immune responses to gene-corrected cells. We are working on strategies to further enhance the efficiency of gene correction in order to translate our strategy into *in vivo* applications.

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S3.6 CRISPR-ABE: a new strategy for the temporospatial control of editing to correct the W1282X mutation in the CFTR gene

Immacolata Zollo[1], Lúcia Santos[1], Joana Alves[1,2], Patrick T. Harrison[2], Carlos M. Farinha[1]

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In recent years, new CRISPR editing tools have increasingly attracted the interest of the scientific community due to their double strand break-free editing capabilities that can significantly reduce Indel formation. In particular, Adenine Base Editors (ABE) have been successfully used to correct nonsense mutations in the *CFTR* gene. This approach uses a Cas9 nickase (nCas9) fused to a TadA enzyme that can convert any adenine (A) in the editing window into guanine (G). Consequently, this system may have a non-specific activity on the nearby adenines, known as bystander effects, that hinders its potential use.

One way to reduce unwanted edits on off-target sites is to minimize the duration of the nuclease activity by splitting Cas9 into two halves[1]. Using a similar strategy, we developed a Split-ABE version, consisting of two fusion proteins – NLS-FKBP12-Cas9(C)-NLS and TadA-NES-Cas9(N)-FRB – and showed this can dimerize and edit in presence of rapamycin. We have previously reported that this ability to temporally control base editing, also limits the bystander editing adjacent to the W1282X target site[2].

Encouraged by these results, here we describe the development of this system to include spatial control of the editing. Our approach is based on the integration of cell-specific promoters upstream of the Split-ABE cassette into a lentiviral vector (pLVX) through sequential cloning. This will allow expression of the Split-ABE in specific cell types in the airway epithelium.

We started by modifying the backbone of pLVX to replace the Puromycin resistance gene with the Hygromycin one and to eliminate restriction sites that would hamper later cloning steps. Then the Split-ABE cassette was isolated through digestion from the construct described above and ligated with the novel pLVX-Hygro. Lastly, the following promoters will be inserted upstream the Split-ABE cassette: KRT5, FOXJ1, FOXI1, MUC5AC and CC16 to control the expression of the CRISPR-Split-ABE system in the basal cells, ciliated cells, ionocytes, goblet cells and club cells, respectively.

The five final plasmids will be used to generate lentiviral particles in order to transduce a variant of the BCi-NS1 cells carrying the nonsense mutation W1282X. This cell line is an appropriate cellular model to evaluate the temporospatial control of editing due to its ability to differentiate into all the cell types of the airway epithelium.

In summary, here we present a strategy for the temporospatial control of ABE to correct the W1282X mutation, the sixth most common Cystic Fibrosis (CF) - causing variant, through a Split-ABE version. Our main long-term goal is to understand which cell types and how many of them need to be edited to rescue enough function of CFTR that can translate into clinical benefit for individuals with CF.

1. Zetsche B. et al. 2015. Nat Biotechnol. 33:139-42.

2. Santos L et al., 2022. 46th ECFS conference, Vienna, WS17.02.

Acknowledgements: Work supported by grant HARRIS21G0 from CFF and centre grants UIDP/04046/2020 and UIDB/04046/2020 (doi: 10.54499/UIDB/04046/2020) from FCT, Portugal (to BioISI).

22 March — 08:45–10:30

Symposium 04 - Targeting stop codons and splicing defects

S4.1 Stabilization of RNA is critical to achieving functional level restoration of nonsense mutations by CFTR modulators

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Trikafta, a CFTR modulator treatment that specifically corrects the dysfunctional protein, has significantly increased the quality of life and median life expectancy for those suffering from Cystic Fibrosis (CF). Unfortunately, those harboring nonsense variants cannot reap the benefits of Trikafta, as the mRNA decay caused by the nonsense variant leads to the production of a minimal, or no protein at all. Thus, it is vital to identify additional combinations that can counteract the consequences of nonsense variants in order to make treatment options accessible to all people with CF.

We utilized two model systems: a) Human nasal epithelial (HNE) cells, and b) Flp-In stable cells. In total, 12 people with CF, 20 carrier parents, one unrelated carrier, and 8 healthy controls were recruited. Each person with CF had at least one copy of nonsense variant. Cells were treated with combinations of the following - ASOs to NMD-critical transcripts, small molecule inhibitors of NMD, readthrough compounds, and CFTR modulator (Trikafta). CFTR mRNA expression, protein processing and function were evaluated.

CFBE cells bearing W1282X CFTR EMG treated with a readthrough compound or Trikafta alone resulted in a minimal recovery of CFTR function. However, upon treatment with combination of NMD-ASO, readthrough, and Trikafta, we observed a remarkable recovery of CFTR function, e.g., treatment with SMG6-ASO, ELOX-02 and Trikafta, ΔIsc= 32.5±13.2 μA/cm2, corresponding to ~20% of wildtype CFTR function in this system. In NEs harboring W1282X, the same combination yielded a significant increase in CFTR function (2.4±0.1μA/cm2), corresponding to ~18% of control. ASO in combination with readthrough compound and CFTR corrector were able to make full-length glycosylated CFTR protein. Importantly, qRT-PCR results for CFBEs and HNEs support that CFTR RNA was increased 10-fold under these treatment conditions and bulk-RNA sequencing confirmed that the ASOs drastically reduced NMD-causing target genes. ASOs among NMD inhibitors and ELOX-02 among readthrough compounds were least toxic and resulted in higher recovery of CFTR expression and function. R1162X did not respond to this combination.

Altogether, our findings suggest that therapies for CF resulting from nonsense mutations will benefit from taking into account RNA stability and drug toxicity. It is clear that recovery of function from some of these variants, including W1282X, benefits greatly from the addition of Trikafta following successful stabilization of RNA.

S4.2 Reading through nonsense mutations with engineered tRNAs

Zoya Ignatova

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Engineering tRNAs to readthrough nonsense mutation-associated premature termination of mRNA translation can restore protein synthesis and function. However, tRNA-based gene therapy has not yielded an optimal combination of clinical efficacy and safety and there is presently no treatment for individuals with nonsense mutations. We developed a mutation-tailored strategy to refactor native tRNAs into efficient suppressor tRNA (sup-tRNAs) that are suitable for both administrations, as lipid nanoparticle (LNP) or as adeno-associated viral (AAV) formulations. I will present our recent advances in the development of tRNA therapeutics with high activity and safety in cell and animal models and discuss different formulation approaches for single or chronic treatment modalities.

S4.3 Targeting splicing mutations using oligonucleotides

Efrat Ozeri-Galai[1], Lital Friedman[1], Asaf Cohen[1], Yifat S Oren[1], Eitan Kerem[2], Gili Hart[1], Batsheva Kerem[1,3]

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SpliSense is developing antisense oligonucleotides (ASOs) as inhaled drugs for treatment of Cystic Fibrosis (CF) patients carrying specific rare mutations that are not responding to the current available CFTR modulators. Among our targeted mutations is the non-canonical splicing mutations 3849+10kb C->T (3849) mutation, that generates both aberrant and correct splicing of the CFTR gene.

SPL84, an ASO drug specifically targeting the 3849 pre-mRNA, has demonstrated complete rescue of CFTR activity in HBE and HNE cells from patients homozygous and heterozygous for the 3849 +10kb C-to-T mutation upon basal exposure (1). As SPL84 is an inhaled drug it reaches the lung epithelial cells via the apical side. SPL84 was further shown to penetrate rapidly through the mucus layer into the epithelial cells. Moreover, it was shown to efficiently penetrate into the epithelial cell nuclei, where it acts to modulate CFTR splicing. Importantly, as confirmed by the CFFT lab, apical treatment of SPL84 in cells from patients homo or heterozygous for the 3849-mutation led to correction of the splicing defect and to rescue of CFTR activity. Moreover, SPL84's effect was superior compared to TRIKAFTA in 3849 HBE cells heterozygotes for the 3849 and F508 del mutations.

The demonstrated efficient penetration of SPL84 from the apical side through the mucus layer into cell nuclei, resulting in the rescue of CFTR activity, models the inhaled drug's exposure in patients' lungs and and highlight the potential of SPL84 to provide a significant clinical benefit for patients carrying the 3849 mutation. Aiming to develop SPL84 as a drug, we further conducted preclinical toxicology studies which showed that SPL84 is well tolerated in mice and monkeys when administrated via inhalation.

Recently, we completed a Phase 1 study (SPL84-CL-001-P), a randomized, double-blind, placebo-controlled, single ascending dose (SAD) study evaluating the safety, tolerability, and pharmacokinetics (PK) of SPL84 administered by inhalation in healthy volunteers (HV). The ASO dosing in all subjects was completed, with no overall safety issues observed. There were no significant SPL84-related adverse events, and no significant effect on vital signs, clinical laboratory values, ECG, physical examination, or pulmonary function. Systemic exposure of SPL84 was low, as expected for an inhaled product, and tended to be dose dependent.

Altogether, the efficient penetration of SPL84 through the mucus layer from the apical side into the cell nuclei, leading to the rescue of CFTR activity, is a promising indication for its therapeutic potential. A first in human single dose study demonstrated safety in HVs supports progression to treatment. The safety profile observed in the first in human single dose study in HVs supports the progression of SPL84 to the treatment of CF patients with decreased lung function carrying the 3849 +10 Kb C->T mutation with SPL84 in a study planned to be initiated later this year.

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S4.4 Identification of novel pharmacological inhibitors of nonsense-mediated RNA decay to rescue CFTR with premature termination codons

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Background: Many patients with cystic fibrosis (CF) currently benefit from the pharmacological therapy with Kaftrio®. However, there is still a significant percentage of CFTR mutations that are insensitive to CFTR modulators, like premature termination codons (PTCs). The rescue of CFTR with PTCs faces two limiting steps: 1) the nonsense-mediated RNA decay (NMD) mechanism that, by degrading mRNAs carrying PTCs, strongly reduces CFTR transcript levels; 2) PTCs cause the arrest of protein synthesis, with the production of a truncated and nonfunctional CFTR protein. The combination of NMD inhibitors with readthrough agents, which overcome the translation arrest, can maximize rescue of CFTR with PTCs. NMD inhibitors can also be effective in the absence of a readthrough agent for PTCs localized at the carboxy-terminus of the CFTR sequence such as W1282X. Our aim is to find novel compounds able to modulate NMD mechanism without interfering with readthrough, by screening a chemical library with a CFTR functional assay.

Methods: The 16HBE14o^{-} cell line with W1282X (obtained from CFF) was stably transduced with the halide-sensitive yellow fluorescent protein (HS-YFP). These cells are used to screen a chemical library containing more than 9,000 approved drugs, investigational drugs, and compounds with known biological activity (mechanistic probes). The most active compounds are then studied with secondary biochemical and functional assays to clarify their mechanism of action and to assess their effects on other PTCs. The most promising compounds will be also tested with short-circuit current recordings on airway epithelial cells derived from people with CF, alone or in combination with readthrough agents and CFTR modulators.

Results: Until now, we have screened ~7,500 compounds, and different positive hits have been identified. Among these, we found a molecule (NMDi-01) that induces 4 times as much CFTR activity compared to negative control. We tested NMDi-01 at multiple concentrations to generate a dose-response relationship and assess its potency (EC_{50} = 0.65 μM). We conducted additional experiments to establish its mechanism of action. NMDi-01 was ineffective on F508del-CFTR, suggesting that it acts as a real NMD inhibitor and not as a general CFTR gene transcription enhancer or amplifier. When we evaluated NMDi-01 effect on W1282X-CFTR at transcriptional and protein level, we found that this compound causes a nearly 15-fold increase in CFTR transcript levels and promote W1282X-CFTR function by markedly enhancing the protein expression. These effects are compatible with strong NMD inhibition. In parallel, other hits from the screening are being characterized. Most active compounds belong essentially to two main groups: proteasome inhibitors and kinase inhibitors. These compounds work in the submicromolar or low micromolar range. For kinase inhibitors, experiments of phosphoproteomic analysis are currently ongoing to identify the site of action and the specificity of compounds.

Conclusions: We expect to discover NMD inhibitors and novel biological targets and processes associated with NMD that could increase CFTR rescue in patients with PTCs.

Acknowledgements: This project is supported by CFF (GALIET22I0) and the European Cystic Fibrosis Society / CF Europe (post-doctoral research fellowship to Arianna Venturini).

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S4.5 Upregulation of a Nonsense Mediated Decay (NMD) insensitive CFTR mRNA isoform has therapeutic potential for the treatment of 3' CFTR PTC variants

Normand Allaire, Jae Seok Yoon, Mathew Armstrong, Mercy Chado, Andrey Sivachenko, Priyanka Bhatt, Kevin Coote, Hermann Bihler, Calvin Cotton, Martin Mense

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Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR) protein. Of the 2000+ documented *CFTR* gene variants at least 719 are established CF-causing variants with an additional 49 variants of varying clinical consequence (https://cftr2.org/). Based on genotype, ~90% of PwCF can now benefit from highly effective modulator therapy (HEMT) directed at the underlying cause of their disease; however, the remaining 10% have *CFTR* variants including nonsense mutations that are not approved for current modulator treatments.

Nonsense or Premature Termination Codon (PTC) variants are challenging to address therapeutically, with currently no available therapies. PTC mutations generally elicit Nonsense-Mediated mRNA Decay (NMD) resulting in 80-90% reduction of full-length mRNA abundance. The remaining CFTR mRNA may give rise to lowly expressed truncated protein which is often non-functional and/or a small amount of full-length protein by PTC readthrough. The reduction of mRNA via NMD is a hallmark of the PTC disease pathology and a major hurdle for any PTC therapy.

Previously we showed that a naturally occurring, lowly expressed truncated CFTR mRNA transcript (e22 trunc) resulting from utilization of an alternative polyadenylation (APA) site in intron 22 is resistant to NMD and yields expression of a truncated CFTR protein that has partial function when treated with modulators. In immortalized 16HBEge cells expressing CFTR W1282X, this truncated mRNA can be upregulated via ASO blockade of exon 22/23 splicing to near wild type levels of full length CFTR, resulting in rescue of ~ 25% WT function (50 AUC/min [uA/cm^2] when combined with CFTR modulator treatment.

As therapeutic proof of concept, we have now translated the approach of ASO-mediated blockade of exon 22/23 splicing to the gold standard for therapeutic development, primary CF hBE cells, for this study hBEs of the CFTR W1282X genotype. We have optimized steric blocking splice donor (SD) and splice acceptor (SA) ASO dose levels and exposure times for air-liquid-interface (ALI) cultures to yield e22 trunc mRNA levels approaching those of WT FL CFTR mRNA. The expressed truncated protein product is trafficked to the plasma membrane and in functional studies in presence of a CFTR potentiator we observed Cl^- transport of ~25% WT function (9.32 AUC/min [mA/cm^2]), which is well within the therapeutically relevant range. This work provides evidence for a novel and promising approach for the treatment of PwCF harboring W1282X and possibly other PTC variants near the 3' end of CFTR.

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S4.6 Comparative study of readthrough molecules

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Around 10% of mutations causing cystic fibrosis are nonsense mutations. These class I mutations are among the most impactful and are not addressed by CFTR modulators. It is possible to restore the expression of a gene carrying a nonsense mutation by using molecules capable of activating readthrough of the premature termination codon. Several molecules having such activity have been reported for decades. These molecules were identified by different labs and tested on very different cellular models, not allowing a direct comparison between the readthrough molecules. It is therefore very difficult to rank them according to their effectiveness. We carried out a comparative study to evaluate the effectiveness of around ten readthrough molecules (including, ataluren, ELX-02, DAP or TLN468) on identical cellular models and on the three stop codons (cell lines expressing firefly luciferase mRNA carrying a nonsense mutation and organoids derived from cystic fibrosis patient cells carrying a nonsense mutation in CFTR). We were thus able to determine the effective concentrations of each of the molecules tested, the corrected stop codon preferences and the contribution of nonsense-mediated mRNA decay to the readthrough efficiency. Our results show that efficiency can be sensitive to the model used. However, we observe 3 groups of molecules: the first comprising the molecules which do not show activity under our experimental conditions, a second group in which a readthrough activity is measured homogeneously between the molecules and a third group containing a few molecules who surpass others. Following the results of the clinical trials of ataluren and ELX-02 which concluded a lack of effectiveness of these two molecules, this type of comparative study could possibly be predictive of success in the clinical trial phase.

22 March — 11:00–12:45

Symposium 05 - Gene(tic) therapies: are we ready for clinical research?

S5.1 Enabling true 'informed' consent by empowering participants

Lorna Allen

Cystic Fibrosis Trust (UK)

How can we ensure that potential participants are fully informed in order to give true 'informed' consent? The complex nature of genetic therapies and the possible impact on future choices or options demands a longer, more detailed and supported decision-making process for participants. Who is expected to take part in these trials and why, and do the inclusion/exclusion criteria support that profile?

Genetic therapies introduce another layer of consideration beyond the immediate burden of trial participation and the impact of anticipated outcome -- they require some consideration of the development pipeline, the timescales, industry uncertainties and commercial sensitivities and how these factors may relate to an individual's health status and trajectory. How can we provide appropriate educational resources for patients and their specialist clinical teams whilst also maintaining realistic expectations for the CF community and balancing the participation demands on a minority sector?

S5.2 Delivery: What is feasible according to research and how does it fit to the expectations of people with CF?

Stephen L. Hart, Ruhina Maeshima

Genetics and Genomic Medicine Department, UCL Great Ormond Street Institute of Child Health, London, UK

In recent years, highly effective triple modulators have been approved that can benefit 90% of people with CF, but for the remaining 10% or more, who, for different reasons cannot benefit from modulators, there is a lack of alternative effective therapies. For these people, genetic therapies have long been proposed as the most likely source of hope, yet to date no effective genetic therapies have emerged for CF despite exciting advancements in recent years in other diseases. For example, AAV viral therapies have been developed for several genetic diseases including retinal degeneration, haemophilia and neuromuscular diseases. Newer technologies, such as mRNA delivered by LNPs, have shown their potential in vaccines for SARS-CoV-2 while CRISPR gene editing therapy is now approved for sickle cell disease and advancing into trials for other diseases. There is much research into the use of these highly promising technologies for CF, while our understanding of CF continues to advance, such as the recent discovery that secretory cells and ionocytes are the highest expressers of CFTR so the major target for correction. However, the main limiting factor in implementing these approaches for CF, remains the challenge of their delivery to the lung epithelium.

Gene replacement therapy, mRNA transcript therapy, and some gene editing strategies, offer the potential for a one-size-fits all treatment, regardless of the CFTR variants involved. CFTR mRNA therapy, offers high levels of expression, relative the plasmid DNA, with no risk of genomic integration and its associated risks of oncogenicity. Delivery of mRNA requires a nanocarrier and Lipid NanoParticles (LNPs) are the most promising type of formulation. The efficacy of this approach depends on the ability of inhaled, nebulised, mRNA nanocarrier to overcome the mucociliary barriers to transfect the epithelium. Targeted transfection of the correct cells in the epithelium, secretory cells and ionocytes, may provide a further challenge. Alternatively, systemic delivery avoids the mucociliary barrier and perhaps better access to basal cells, but presents challenges of biodistribution and penetration of the endothelium to reach the epithelium.

Airway delivery provides the most immediate access for CFTR mRNA to the lung epithelium and so we are currently focusing on that approach, but it is essential for its efficacy that nanoparticles are able to penetrate mucus. Air liquid interface cultures (ALI) of CF epithelial cells provide a physiologically relevant model to evaluate therapies for molecular and functional correction. We have developed a peptide-targeted lipid nanoparticle for siRNA, mRNA and Cas9 ribonucleoprotein (RNP) delivery, and I will discuss their improvements in mucus penetration and delivery evaluated in ALI cultures and in vivo, as well as developments in delivery in the wider field.

S5.3 Considerations to enhance patient access to genetic therapies

Stefano Zancan

Fondazione Telethon ETS

For a patient having access to a therapy means to have a concrete possibility to receive it. This is mainly possible by participating into a clinical trial or when the therapy is on the market. This presentation describes few examples on how Fondazione Telethon ETS, a no-profit organization committed to find a cure for rare disease, tries to facilitate patient access to therapy indirectly through innovative approach to accelerate drug development and reducing associated cost, and directly by supporting patients participating in clinical trials and lately by maintaining a commercial therapy on the market.

23 March — 08:45–10:30

Symposium 06 - Targeting protein partners and alternative channels

S6.1 Mutant CFTR impacts Insulin-receptor signaling and regulates localization of tight junction proteins, disrupting the airway glucose barrier

Nael A. McCarty[1,3], Analía J. Vazquez Cegla[1,3], Kymry T. Jones[1,3], Guiying Cui[1,3], Michael Koval[2,3]

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In Cystic Fibrosis (CF), the loss of CFTR function leads to the well-known triad of infection, inflammation, and obstruction, resulting in progressive lung function decline. As pwCF develop CF-related diabetes (CFRD), lung function decline is accelerated. Little is known about how the development of systemic hyperglycemia worsens airway function in CF.

Our published work from the McCarty and Koval labs detailed the discovery of fundamental defects in the handling of glucose in the CF airway, including impacts on both the function of tight junction proteins in limiting flux of glucose into the airway and the function of insulin-dependent glucose transporters to remove airway glucose from the airway surface liquid (ASL), two arms that together generate an "airway glucose barrier." Furthermore, our results show that non-CF airway epithelial cells responded to insulin by enhancing glucose barrier function, while cells expressing mutant CFTR (CF cells) responded to insulin by weakening barrier function.

New data suggest that while insulin-mediated signaling in non-CF cells occurs through the "metabolic" pathway involving Akt, supportive of tight junction integrity and glucose uptake, insulin-mediated signaling in CF cells occurs through the "mitogenic" pathway involving MEK/ERK, which is detrimental to barrier integrity. These differences in insulin signaling may arise from alterations in expression of insulin receptor subtypes. We show that one outcome of mutant CFTR expression and exposure to hyperglycemic conditions is an inability to correctly localize multiple tight junction proteins, including claudin-4 and ZO-1, to the cell periphery, and that these processes are variably sensitive to insulin and treatment with ETI. Furthermore, mutant CFTR impacts the trafficking of GLUT4 protein to the plasma membrane. Combined, these effects lead to inability to regulate ASL glucose abundance, which has likely multiple negative impacts on pulmonary physiology.

S6.2 Targeting ubiquitination to enhance modulator treatments.

Emanuela Pesce[1], Valeria Tomati[1], Valeria Capurro[1], Cristina Pastorino[1], Mariateresa Lena[2], Nicoletta Pedemonte[1]

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In Cystic Fibrosis (CF) several mutations, including the deletion of phenylalanine 508 (F508del), in the CF transmembrane conductance regulator (CTFR) leads to defective folding, that is detected by quality control systems of the cell and results in subsequent premature degradation of the mutant protein that mainly occurs through the proteasomal machinery. In other cases, such as for the N1303K-CFTR, its degradation involves the ER-associated-autophagy pathway. CFTR folding defects can be targeted with pharmacological agents named correctors, however, the extent of rescue is variable. While for the F508del-CFTR variant, the maximal rescue obtained with corrector combinations is close to 60-70% of normal CFTR function, for other variants the rescue is much lower or even absent. Thus, the search for novel drugs showing different and additive mechanisms (with respect to correctors) has recently increased. Among them, drugs that modulate CFTR proteostasis environment are particularly attractive to enhance therapy effectiveness further. One of the most promising CF drug targets is the ubiquitin ligase RNF5, which promotes F508del-CFTR degradation. After the discovery of the first inhibitor of RNF5, the extensive exploration of the structure-activity relationships of the chemical class led to an optimized analogue, with improved corrector activity good tolerability and no toxic side effects. The E1 ubiquitin-activating enzyme (UBA1) is another possible target. Indeed, its inhibition by TAK-243, a small-molecule in clinical trials for other diseases, boosts the rescue of F508del-CFTR induced by correctors. Interestingly, this new combinatory approach leads to improved CFTR rescue also in the case of rare CF-causing mutations, including N1303K. These results suggest that the efficacy of correctors combination can be further improved by concomitantly targeting the misfolding detection machinery at the beginning of the ubiquitination cascade. This will open new therapeutic options for low/non-responding rare misfolded CFTR mutants.

This work was supported by Cystic Fibrosis Foundation grant PEDEMO20G0 and by Fondazione per la Ricerca sulla Fibrosi Cistica grants FFC #9/2019 and FFC #10/2021 to NP. VT acknowledges grant GR-2018--12367126 by Ministero della Salute. Work in NP lab is also supported by the Italian Ministry of Health through Cinque per mille and Ricerca Corrente.

S6.3 Targeting CFTR protein partners and alternative channels

Margarida D. Amaral, Cláudia Rodrigues, Violeta Railean, Ines Pankonien

BioISI – Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Lisboa Portugal

Besides transporting anions across epithelia, CFTR has been implicated in other functions. Indeed, it is emerging in the literature that CFTR associates to the actin cytoskeleton, epithelial cell junctions and extracellular matrix (ECM) proteins which substantiates its role in cell junction formation, actin cytoskeleton organization and in the formation and maintenance of epithelial apical basolateral polarity.

Several key interactors contribute to CFTR tethering to the actin cytoskeleton, notably the Na+/H+ exchanger regulatory factor isoform 1 (NHERF1). We and others have shown that stimulating this interaction directly or indirectly (via Rac1 signalling or calpain inhibition) favours wt- and F508del-CFTR anchoring at the plasma membrane [1].

Given this close interaction to the actin cytoskeleton, it is not surprising that CFTR plays a role in cell junction formation and vice-versa. Indeed, we and others have shown that in the absence of functional CFTR, cells exhibit decreased epithelial tightness, as measured by lower transepithelial electrical resistance, and mislocalized / disorganized epithelial proteins, with concomitant upregulation of mesenchymal markers [2]. In parallel, our data also show that the dedifferentiation process of epithelial-mesenchymal-transition (EMT) also occurs in CF cells and native tissues, even in the absence of TGF-□1 mediated inflammation. We have started to explore the pathways and networks linking dysfunctional CFTR to EMT, having identified several central node proteins, including transcriptional factors KLF4, Twist1 and Yap1 (reviewed in [3]).

By mapping these networks to those of CFTR interactors [4] and those associated with other non-CFTR channels and transporters, like TMEM16A and SLC26A9 [5], we aim to identify key players that can be targeted to compensate for the absence of CFTR-mediated ion transport in CF but that also rescue CFTR vital role in maintaining the structure and function of epithelial polarized cells at the level of the cytoskeleton and cell junctions.

[1] Matos et al, J Biol Chem 2019; Matos et al (2018) Sci Rep 2018; Loureiro et al, Sci Signal 2015; Matos et al, ACS Chem Biol 2013.

[2] Quaresma et al, Cell Death Dis 2020; Quaresma et al, Life Sci Alliance 2022; Sousa et al, Int J Mol Sci 2020; Sousa et al, Cells 2020.

[3] Pankonien et al, Int J Mol Sci 2022.

[4] Lim et al, Mol Syst Biol 2022

[5] Pinto et al, J Mol Biol 2022; Pinto et al, J Exp Pharmacol 2021; Pinto et al, Int J Mol Sci 2022

Work supported by UIDB/04046/2020 (DOI: 10.54499/UIDB/04046/2020) and UIDP/04046/2020 (DOI: 10.54499/UIDP/04046/2020) Centre grants from FCT, Portugal (to BioISI).

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S6.4 Targeting ATP12A proton pump provides new therapeutic opportunities for cystic fibrosis

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In cystic fibrosis (CF), the loss of function of CFTR causes multiple airway defects, as dehydration and acidification of the ASL, and mucus accumulation, which impair mucociliary transport and favor the chronic infection and inflammation of the lungs [PMID: 34090606]. Correction of CF abnormalities may be obtained by restoring the function of CFTR with mutation-specific treatments or through airway physiology-directed therapies. Indeed, it has been speculated that beneficial effects could be obtained by modulating other channels and transporters in order to increase CFTR-independent anion secretion (TMEM16A, SLC26A9, SLC26A4) or inhibit acidification (ATP12A). Despite the increasing interest in these targets, their location in the airways and their real contribution to epithelial homeostasis is poorly known and sometimes controversial. Moreover, specific modulators are still lacking.

In this study, we aimed to define the precise expression and role in the airways of TMEM16A, SLC26A9, SLC26A4, and ATP12A and to find drug candidates for the most promising targets.

We used *ex-vivo* human samples and advanced *in-vitro* models to assess the targets' expression by spatial biology. RNAscope *in situ* hybridization in healthy lung sections revealed a higher expression of ATP12A and SLC26A4, with a proximal--distal gradient, while TMEM16A appeared with a very low expression in human bronchi and was undetectable in more distal regions. SLC26A9 was absent in most epithelial and glandular cells of the airways, except for rare epithelial cells (< 1%) showing high expression. By mRNA-protein co-detection we identified these cells as pulmonary neuroendocrine cells (PNECs). Then, we investigated alternative targets expression in asthmatic and CF airways by RNAscope on asthmatic lung sections and by immunofluorescence on nasal brushings collected from CF and non-CF individuals. The expression of all targets, except SLC26A9, was found increased under these chronic inflammatory conditions.

To investigate the alternative targets' role in the airways, based on the findings obtained in native tissues showing different expression patterns along the respiratory tract, we used *in vitro* models resembling large and small airways epithelia, including non-CF and CF nasal epithelia, and epithelia generated from immortalized bronchial and bronchiolar basal cells (BCi-NS1.1 and SABCi-NS1.1). In all models, TMEM16A, SLC26A4, and ATP12A were induced by inflammatory conditions (IL-4 or IL-17 + TNFα), independently of the CF genotype, whereas SLC26A9 was poorly detected. Then, a series of microscopy-based assays highlighted a prevalent role of ATP12A in controlling ASL properties, with its inhibition producing effects considered beneficial for CF: less acidic pH, increased thickness and decreased viscosity. Accordingly, we initiated the search for drug-like modulators by developing a novel *in vitro* model with functional expression of ATP12A suitable for high-throughput screening purposes. The screening of a library of antisense oligonucleotides (ASOs) targeting human ATP12A produced several hits able to decrease ATP12A expression and proton secretion.

In conclusion, our study evidenced ATP12A as a promising target for CF. Importantly, we found antisense-drug candidates able to suppress ATP12A expression, a potential CFTR-independent approach to normalize ASL properties in CF airways.

This study was supported by Vertex CF Research Innovation Award and Italian Cystic Fibrosis Foundation

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S6.5 Enhancing apical loop currents in airway epithelia carrying CFTR non-sense mutations

Nathalie Baumlin, Neerupma Silswal, John S. Dennis, Michael D. Kim, Matthias Salathe

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Objectives: Highly effective modulators for mutant cystic fibrosis transmembrane conductance regulator (CFTR) have made a significant difference for close to 90% of people with cystic fibrosis (pwCF). However, ~10% of pwCF with two minimal function mutations or mutations not amenable to modulators have currently limited options. Ion channels alternative to CFTR could compensate and thus serve as therapeutic targets in CF airway disease. For this, the apical loop current seems critical as apical potassium exit enhances chloride conductance and fluid availability on the airway surface. TMEM16A potentiators are being developed. In addition, there is a new recognition that CFTR modulators could act as large conductance, calcium-activated potassium (BK) channel potentiators. Our hypothesis is that simultaneous BK and TMEM16A potentiation may have beneficial effects on parameters of mucociliary function in pwCF with minimal function CFTR mutations.

Methods: Normal human bronchial epithelial (NHBE) and cystic fibrosis bronchial epithelial (CFBE) cells were cultured at the air-liquid interface and allowed to differentiate for at least 4 weeks. All assays were performed 24h after treatments. Potentiation of TMEM16A was achieved using Rg3 (15 μM), a major component of red ginseng aqueous extract, and potentiation of BK using elexacaftor (VX-445; 5 μM). Nesolicaftor (PTI-428, 10 μM) acting via poly(rC)-binding protein 1 (PCBP1) was used to stabilize LRRC26 (gamma subunit of BK) and TMEM16A mRNAs in the presence of inflammation, simulated with recombinant TGF-β1 (5 ng/mL). Short-circuit currents of TMEM16A and BK were recorded in Ussing chambers. Ciliary beat frequency (CBF) was measured using SAVA pre- and post-treatments. Airway-surface liquid (ASL) volumes were estimated by meniscus scanning pre- and post-treatments.

Results: Elexacaftor potentiated BK currents in NHBE and CFBE cells with minimal function CFTR mutations and partially rescued TGF-β1-induced BK dysfunction. Rg3 potentiated TMEM16A currents in CFBE cells and partially restored TGF-β1-induced TMEM16A dysfunction. Elexacaftor and Rg3 in CFBE cells had a marginally improved effect on TMEM16A compared to Rg3 alone. In NHBE cells, nesolicaftor enhanced BK and TMEM16A conductance, and increased ASL volumes and CBF. TGF-β1-induced inflammation decreased BK activity, ASL volumes and CBF. These effects were rescued by nesolicaftor.

Conclusions: Our data show that enhancing apical loop currents even in inflammatory environments by potentiation of alternative ion channels in pwCF with minimal function CFTR mutations is feasible. These “triple” therapies to address nonsense mutations with molecules already tested in human beings could see quick turnarounds into clinical trials.

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S6.6 SLC26A9 modulators identified through High Throughput Screening (HTS)

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Background: Enhancing non CFTR-mediated anion secretion has been proposed as a mutation-agnostic therapeutic approach for the treatment of cystic fibrosis (CF) and could be effective for minimal function genotypes without approved CFTR modulators. Human population genetic evidence identified SLC26A9 as a modifier of CF pancreatic disease and lung function. SLC26A9 is an epithelial chloride transporter that is expressed mainly in airway and gastric epithelia and assumed to contribute epithelial surface hydration. Here we aim to identify small molecules activating SLC26A9.

Methods: To identify SLC26A9 modulators, we carried out a high-throughput screening (HTS) campaign of >100,000 chemically diverse compounds using a fluorescent cell-based assay which measures membrane potential (MP) to monitor ion transport in GripTite 293 MSR cell line generated with inducible expression of human SLC26A9. After the primary screen, we implemented a computational hit-to-lead design for the prioritized compounds. We employed SiteMap, a computational tool, to identify potential drug-binding pockets on SLC26A9. To enhance the reliability of these findings, we integrated homology-based deep learning methods (DiffDock), which help validate the proposed binding sites. Following this, we utilize molecular docking and similarity-based scoring techniques to navigate the vast realm of molecular possibilities in the Enamine REAL Space. This approach enables the selection of top candidate molecules that show promising potential as SLC26A9 agonists. To analyze lead candidates, the parent compounds along with their derivatives have been examined for their potential effect on SLC26A9 using the fluorescent MP assay. cAMP-Glo Max Assay was used to assess intracellular cAMP levels.

Results: The HTS was robust with a mean plate Z' value of 0.65, mean signal to baseline (S/B) of 2.65 and the overall rate for identification of activators was 0.015%. Fifteen potential compounds have been prioritized as SLC26A9 agonists based on enhanced MP responses, significant dose-response curves and drug-like properties. These compounds did not induce MP response in counter screens using cells without SLC26A9 expression and did not stimulate cellular cAMP levels in CFTR-expressing 16HBE14o- and Capan-1 cells suggesting no indirect CFTR activation. Most of the 15 compounds reproduced their agonistic effect in H2122 lung and CFPAC-1 pancreatic cell lines with endogenous expression of SLC26A9. Specificity of four of the most potent agonists was verified using RNAi knockdown experiments against SLC26A9. Moreover, MP assay results in 16HBE14o- cells indicated that most of the 15 compounds at their EC50 concentrations have no impact on FSK-induced CFTR response. Analyzing derivatives of prioritized compounds indicated that 6 derivatives from 6 different parental compounds enhanced SLC26A9 response. The EC50 determination of the selected derivatives indicated high potency compounds.

Conclusion: The identified compounds will provide useful research tools to elucidate the roles of SLC26A9 as a potential target for CF therapy. Further optimization of SLC26A9 agonists for potency and pharmacological properties are in progress to select compounds for Ussing measurements in human airway epithelial cell cultures and mucociliary clearance assays.

23 March — 11:00–12:45

Symposium 07 - Cell – bacteria relations: infection and inflammation in the era of modulators

S7.1 Multi-lobe bronchoscopy reveals ETI's effects on regional lung infection and inflammation

SL Durfey [1], SG Kapnadak[2], MC Radey1[1], M Teresi[3], T Gambol[2], HS Hayden[1], A Waalkes[4], MM Willmering[5,6], JD Godwin[2], L Boyken[7], M Stroik[8], A Vo, SB Singh[3], AF Feder[9], SJ Salipante[4], C Steele[10], JC Woods[5,6], D Stoltz[8,11], T Pena[3,11], JP Clancy[12], ML Aitken[2], PK Singh[1]

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Background: Elexacaftor/tezacaftor/ivacaftor (ETI) improves lung function, reduces sputum pathogen density, and dampens pro-inflammatory mechanisms in CF cells. Despite these remarkable benefits, most people infected with Pseudomonas aeruginosa (Pa) remain infected after treatment. The mechanisms explaining persistent infection and the relationship between lung infection and inflammation after ETI are unknown. We tested two hypotheses: (1) Pre-treatment lung damage would associate with infection persistence after ETI. (2) Despite ETI's effects in abrogating pro-inflammatory mechanisms, lung regions with persistent Pa infection would remain inflamed, and the extent of regional inflammation would correlate with Pa density.

Methods: We used bronchoscopy to sample the airways of five lung segments per subject. To reduce between-region contamination, a new disposable bronchoscope was used for each segment and washings were collected after wedging. The same lung regions in each subject were sampled again after 18 months of ETI, and Pa density, CT-based lung damage, neutrophil elastase, and a multiplex panel of cytokines were measured.

Results: Before ETI, nearly all (43/45) lung regions were Pa-positive across the nine adult subjects studied. After ETI, one third of lung regions became culture negative for Pa (18/45 regions were Pa-negative post-ETI).

We first tested our hypothesis that pre-existing lung damage would be associated with persistence. Pa persistence after ETI was associated with higher lung damage, based on Brody CT scores ($p = 0.03$). We then examined the Brody subscores to identify which types of lung damage were associated with Pa persistence. We found that pre-treatment airway thickness and air trapping were higher in regions with persistent Pa ($p = 0.04$ and $p = 0.01$, respectively), while bronchiectasis and mucus plugging did not differ. We confirmed these results using quantitative computer algorithm methods.

We then investigated the relationship between persistent Pa and inflammation after ETI. We observed a strong association between neutrophil elastase (NE) levels and Pa culture density before and after ETI ($p<0.0001$). The coefficient of determination (R2 value) between these variables was higher post-ETI than pre-ETI (R2=0.76 vs 0.31). Furthermore, NE became undetectable in regions clearing Pa, while average NE in regions remaining infected was 2.0 ug/mL ($p < 0.001$). We then determined whether the increased association with Pa was specific to NE. We found that the correlations between Pa density and individual cytokines increased after ETI for 53 of 96 measured cytokines and decreased for 3 cytokines. Cytokines that increased in association with Pa after treatment include IL-1β, IL-8, TNFα, and IL-17A.

Conclusions: We examined the relationship between regional lung damage and regional infection clearance post-ETI. We found that Pa persisted in regions with greater degrees of lung damage, specifically airway thickening and air trapping.

We also examined the relationship between regional lung pathogen density and regional inflammation. Our findings support the hypothesis that regional inflammation in patients on ETI correlates with regional Pa density. Indeed, correlations between these parameters increased after ETI, and NE was generally only detectable in Pa-positive segments. These findings suggest that modulators' primary inflammation-dampening effects may not alleviate inflammation in persistently infected lung regions.

S7.2 Effects of CFTR modulators on airway mucus, infection and inflammation in cystic fibrosis

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CFTR dysfunction leads to abnormal viscoelastic properties of airway mucus, impaired mucociliary clearance and mucus plugging, which sets the stage for chronic polymicrobial infection and neutrophilic inflammation that are key drivers of progression of structural lung damage in patients with cystic fibrosis (CF). The triple combination CFTR modulator therapy elexacaftor/tezacaftor/ivacaftor (ETI) showed unprecedented improvements in lung function and other clinical outcomes in clinical trials and post- approval observational studies in patients with CF with at least one copy of the common *F508del* allele. We recently demonstrated that ETI improves F508del-CFTR function in the airways of patients with one or two *F508del* alleles to ~40 to 50% of normal CFTR activity, however, data on the effects of this level of restoration of CFTR function on key downstream pathologies in the CF airways, i.e. increased viscoelasticity of airway mucus, chronic inflammation and infection remain limited.

In the context of Modulate-CF (NCT04732910), a prospective observational study of real-world effects of ETI conducted in Germany, we therefore determine the longitudinal effects of ETI on rheology, the microbiome, inflammation markers and the proteome in sputum samples from CF patients with one or two *F508del* alleles in a post-approval setting. For this substudy, we recently performed the first interim analysis of data obtained from longitudinal sputum samples taken before and 1, 3 and 12 months after initiation of ETI in a cohort of 79 CF patients aged 12 years and older throughout the first 12 months of therapy. Data from CF patients were compared to sputum samples from 10 healthy controls that were enrolled in this study to be able to determine residual disease activity in CF after initiation of ETI therapy. We found that ETI improved the elastic modulus (G') and the viscous modulus (G") of CF sputum at 3 and 12 months after initiation of therapy. Further, ETI decreased the relative abundance of *Pseudomonas aeruginosa* in CF sputum at 3 months and increased the microbiome α-diversity at all timepoints. ETI also reduced markers of neutrophilic inflammation such as IL-8 at 3 months and free neutrophil elastase (NE) activity at all timepoints, and shifted the CF sputum proteome towards healthy. However, none of the sputum outcome measures in CF patients treated with ETI reached levels close to those to those obtained in sputum from healthy individuals.

This study is currently ongoing and will be extended to younger age groups as well as longer treatment periods. The results of our first interim analysis show that ETI leads to substantial improvements in sputum viscoelastic properties, chronic airway infection and inflammation as well as abnormalities in airway proteome signatures in patients with CF 12 years and older with at least one *F508del* allele that are sustained throughout the first 12 months of therapy. Our data on residual disease activity under this highly effective CFTR modulator therapy also indicate that additional therapeutic strategies may be needed to control airway infection and inflammation in adolescent and adult CF patients with chronic lung disease

S7.3 A Scottish Exploration of Tez/Iva and Elex/Tez/Iva on systemic and pulmonary inflammation- From bench to bedside

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Background:

Uncontrolled and pro-longed inflammation is a major cause of lung damage in people with CF (pwCF). Clinical trials have demonstrated that the highly effective CFTR modulator combination Elexacaftor-Tezacaftor-Ivacaftor (Elex/Tez/Iva) improves lung function and quality of life, but the impact of this treatment on inflammatory pathways is unknown. We conducted a prospective observational study in pwCF. Research samples were collected during the initial year of treatment and analysed for inflammatory biomarkers of CF disease. A small subset also underwent CT scans.

Aim:

To determine the impact of Elex/Tez/Iva triple combination CFTR modulator therapy on systemic and pulmonary inflammation in people with CF.

Methods:

Eligible adults with CF were selected by genotype and recruited when commencing Tez/Iva or Elex/Tez/Iva therapy. Participants returned for review at 1 month, 3 months and 1 year after commencing therapy and research blood samples were collected at these time points. 10 pwCF were recruited for CT chest scans before commencing treatment with Elex/Tez/Iva and following one year of treatment. The inflammatory biomarkers calprotectin and C-reactive protein (CRP) as well as cytokines IL-6, IL-8 and IL-10 and MMP-10 were measured in patient serum by ELISA. Proteomic analysis of 10 modulator naïve people with CF were also analysed for inflammation and organ damage proteins at baseline and 1 year.

Results:

There was a four-fold reduction in C-reactive protein in the initial 3 months after commencing treatment (n=73, $p=0.0021$). IL-6 was significantly reduced (n=49, $p=0.0023$) and correlated with CRP reduction, however, there was no significant change seen in circulating levels of IL-8 or IL-10. Both IL-6 and CRP remain significantly reduced at 1 year. MMP-10 was significantly reduced after 3 months treatment (n=48, $p<0.0001$) and remained significantly reduced at 1 year ($p<0.0009$). Calprotectin, a biomarker of exacerbation risk, was significantly reduced (n=38, $p=0.0028$) after 3 months of Elex/Tez/Iva in patients who had not been on a previous modulator. pwCF who were previously treated with Tez/Iva and switched to Elex/Tez/Iva at the start of the study had lower levels of calprotectin at baseline. Calprotectin remained significantly reduced at 1 year Elex/Tez/Iva treatment (n=40 $p<0.0009$). Proteomic analysis at 1 year showed a significant reduction in IL-6, IL-20 and MMP-10 (n=10) in those who were modulator naive.

Subjective improvements were seen in all 10 CT scans following 1 year of Elex/Tez/Iva therapy. Qualitative reports described a reduction in mucus plugging and bronchial wall thickening in all scans. Significant reductions were seen in the Global index of airway wall thickening ($p<0.0001$), PRAGMA disease scores ($p=0.04$), and PRAGMA airway wall thickening ($p=0.003$). A reduction in PRAGMA bronchiectasis score was seen in 7/10 pwCF following 1 year of treatment, despite only 4/10 being documented by radiologists. A reduction in thoracic lymphadenopathy was also observed.

Conclusion:

Significant improvements are seen in markers of systemic and pulmonary markers of inflammation following Elex/Tez/Iva treatment. These appear to be sustained over the first year of therapy. Resolution of inflammation in CF may therefore contribute to the clinical improvements seen in pwCF commencing Elex/Tez/Iva.

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S7.4 GM1 ganglioside: new insight on its immunomodulatory capacity in CF

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The availability of new modulators for the rescue of the mutated CFTR opens a new scenario for the treatment of patients with Cystic Fibrosis (CF). In the previous years, we discovered a new interactor of CFTR, consisting in the ganglioside GM1, which is fundamental for the stability of the channel at the plasma membrane (PM). In bronchial epithelial cells, the absence of CFTR at the cell surface is associated with a decrease in the content of GM1. Interestingly, restoring the PM content of this ganglioside by its administration increases the stability of the mutated channel rescued by CFTR-modulators, such as Orkambi or Kaftrio formulations. To note, GM1 ganglioside is an essential modulator of innate and adaptive immune response, and it was reported to directly regulate the clearance of *P. aeruginosa* from the respiratory tract, which is downregulated in CF bronchial cells.

Based on this evidence, we investigated the role of this ganglioside in the host-pathogen interactions in CF.

First of all, xenophagic clearance activity was measured in both CF and non-CF bronchial cells exposed to GM1 in order to test whether the administration of GM1 ameliorates the clearance of *P. aeruginosa*. Reduced xenophagic clearance of invading pathogen was detected in CF cells compared to non-CF cells, as higher number of colony-forming units (CFU)/ml of invading bacteria was observed in CF bronchial cells. However, at 48-hour pre-treatment, GM1 administration rescued the bacterial clearance capacity of CF cells reducing the number of colony-forming units (CFU)/ml of invading bacteria. Through a bacterial invasion assay, we excluded that the reduction of intracellular bacteria in CF cells exposed to GM1 for 48 hours depended by a lowering bacterial invasion capacity.

Afterwards, the safety of GM1 administration via aerosol was assessed in *in vivo* models performing experiments on WT mice (C57Bl/6) treated with different doses (1, 5 and 20 mg/kg/day) of GM1 in saline solution. Mice were treated with the ganglioside 24 hours before, as well as immediately after the injection of PBS used to induce stress that mimics acute infection. Over time, for maximum 24 hours, the safety of this treatment was monitored by keeping under observation the well-being, weight, and temperature of mice. Although some reduction, concerning both the body weight and the temperature, was observed between mice treated with GM1 and vehicles, these differences were not statistically significant. Therefore, in light of these results in these conditions GM1 at all doses tested does not appear to induce toxicity.

Concerning the importance of GM1 in the stability of CFTR at the PM and its immunomodulatory role, the results of this study could serve for the development of new therapeutic strategies to improve the efficacy of the treatments of CF patients, and their immunomodulatory capacities.

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S7.5 Exploring the relationship between *Pseudomonas aeruginosa* infection and SLC6A14 in cystic fibrosis

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Introduction: Large-scale genetic studies on people with cystic fibrosis (pwCF) have highlighted the pivotal role of the *SLC6A14* (solute carrier family 6 member 14) gene in modulating lung function and the early onset of *Pseudomonas aeruginosa* infection. We know that SLC6A14 encodes an amino acid transporter highly expressed in bronchial epithelial cells and plays a crucial role in amino acid concentration within cell cytoplasm. However, its precise functional involvement in CF lung physiopathology, especially in the context of *P. aeruginosa* infection, remains poorly defined.

Objective: This study aims to elucidate the mechanisms by which SLC6A14 influences lung function in the pathological context of CF, both in aseptic and *P. aeruginosa* conditions.

Methods: We used Calu-3 knockdown for CFTR (Calu-3 CFTR-KD) and primary airway epithelial cells from healthy donors or pwCF. SLC6A14 mRNA and protein expression were analyzed through real-time quantitative PCR, western blot, and immunofluorescence. SLC6A14 amino acid transport was assessed by using Arginine Monohydrochloride L-[2,3,4-^{3}H] uptake. *P. aeruginosa* infection was mimicked using purified virulence factors such as lipopolysaccharide (LPS), LasB elastase (LasB), or flagellin (Fla), along with live PAO1wt or PAO1ΔlasB strains. Additionally, transcriptomic and proteomic analyses were performed on fully differentiated bronchial epithelial cells from pwCF treated with the SLC6A14 inhibitor alpha-methyltryptophan (α-MT).

Results: In CF airway epithelial cells (*ex vivo*), *SLC6A14* mRNA levels were increased while protein levels were drastically decreased compared to those of cells from healthy donors. Exposure to virulence factors from *P. aeruginosa*, such as LPS and Fla, increased both SLC6A14 mRNA and protein levels. However, live *P. aeruginosa* infection led to an increase in *SLC6A14* mRNA levels and a decrease in SLC6A14 protein levels. We then demonstrated that this decrease is attributable to LasB elastase. Transcriptomic and proteomic analyses of primary fully differentiated CF bronchial epithelial cells revealed that several transcripts and proteins were differentially expressed in α-MT-treated cells compared to control cells with enriched gene sets related to wound healing, cell junction organization, cell-cell adhesion, macroautophagy, *S. aureus* infection and viral infection pathways.

Conclusion: Our study allows to determine that, during the course of CF, the protein expression levels of SLC6A14 may be influenced by infection thus affecting the activity of SLC6A14. Within the bronchial epithelium, a reduced expression and/or activity of SLC6A14 might impact pathways essential for preserving epithelial barrier integrity. Exploring interconnections between SLC6A14 and the diverse proteins implicated in these pathways may unveil novel therapeutic targets aimed at enhancing the host response to infection in the CF context.

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S7.6 Differential effects of CFTR modulators on SARS-CoV-2 infectivity in cultured nasal, bronchial, and intestinal epithelia of people with cystic fibrosis

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Introduction: People with cystic fibrosis (pwCF) are in general more susceptible to respiratory infections due to CFTR dysfunction in airway epithelial cells, however during the COVID-19 pandemic, pwCF were not deemed more susceptible to SARS-CoV-2. *In vitro* studies have shown impaired SARS-CoV-2 infectivity in CFTR deficient bronchial cells compared to wild type-controls, but differences in infection across different tissues are not known. To further understand the effect of CFTR dysfunction on SARS-CoV-2 infectivity, we conducted SARS-CoV-2 infection studies in cultured airway and intestinal epithelial cells from pwCF, in the absence or presence of CFTR modulators.

Methods: Nasal brushings (n=10), bronchial (n=10), and intestinal (n=5) biopsies were obtained from pwCF, including individuals with a F508del homozygous and heterozygous genotype. After expansion, both nasal and bronchial airway epithelial cells were differentiated in air-liquid interface cultures on transwell inserts. The intestinal epithelium was expanded as organoids, and afterwards cultured as submerged 2D monolayers on transwell inserts. Airway and intestinal epithelial cells were stimulated with vehicle or Elexacaftor, Tezacaftor and Ivacaftor (ETI) and infected at the apical side with strain /NL/2020SARS-CoV-2 (MOI 1). Infectivity was assessed by qPCR using primers targeting the envelope (E) gene at 7 days post infection.

Results: SARS-CoV-2 infected airway and intestinal epithelial cells of pwCF displayed differences in viral load, with the lowest viral load in bronchial epithelial cells, and the highest viral load in intestinal epithelial cells. Treatment with ETI did not result in a significant difference in viral load compared to vehicle treatment in nasal epithelial cells. However, upon ETI treatment we observed a significant increased viral load in bronchial epithelial cells, and a reduced viral load in intestinal epithelial cells compared to vehicle.

Conclusion: Our results revealed differences in SARS-CoV-2 infectivity between different epithelial tissues from pwCF, and contrasting effects of ETI CFTR modulator therapy. In line with previous studies, CFTR dysfunction in bronchial epithelia decreased SARS-CoV-2 infectivity, and furthermore was reversed by CFTR repairing drugs.

23 March — 14:15–16:00

Symposium 08 - Cell models, organoids in the enhanced life expectancy of patients

S8.1 Functions of the pulmonary ionocyte in the proximal ferret airways

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Speciation leads to adaptive changes in organ cellular physiology and creates challenges for studying rare cell type functions that diverge between humans and mice. Rare CFTR-rich pulmonary ionocytes exist throughout the cartilaginous airways of humans, but limited presence and divergent biology in the proximal trachea of mice has prevented the use of traditional transgenic models to elucidate ionocyte functions in the airway. Here we describe the creation and use of conditional genetic ferret models to dissect pulmonary ionocyte biology and function by enabling ionocyte lineage tracing (*FOXI1*-Cre^{ERT2}::ROSA-TG), ionocyte ablation (*FOXI1*-KO), and ionocyte-specific deletion of *CFTR* (*FOXI1*-Cre^{ERT2}::*CFTR* $^{L/L}$). By comparing these models to cystic fibrosis (CF) ferrets, we demonstrate that ionocytes control airway surface liquid (ASL) absorption, secretion, pH, and mucus viscosity-leading to reduced ASL volume and impaired mucociliary clearance in CF, *FOXI1*-KO, and *FOXI1*-Cre^{ERT2}::*CFTR* L/L ferrets. These processes are regulated by CFTR-dependent ionocyte transport of Cl^- and HCO_3^-. Single-cell transcriptomics and *in vivo* lineage tracing revealed three subtypes of pulmonary ionocytes and a *FOXI1*-lineage common rare cell progenitor for ionocytes, tuft, and neuroendocrine cells during airway development. Thus, rare pulmonary ionocytes perform critical CFTR-dependent functions in the proximal airway that are hallmark features of CF airway disease. These studies provide a road map for using conditional genetics in the first non-rodent mammal to address gene function, cell biology, and disease processes that have greater evolutionary conservation between humans and ferrets.

S8.2 Intestinal organoids, CFTR function, and tissue-specific disease

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Human intestinal organoids (hIO) are multicellular structures that grow in 3D in vitro from adult stem cells from human instestinal tissue biopsies. These patient-derived cells can be important to understand how genetic components of disease translate into (individual) clinical disease expression, but also to explore novel mechanisms of disease. We and others have shown that fluid secretion phenotypes in hIO can be used to quantify CFTR function. In this presentation, I will discuss how different assay conditions can be used to quantify CFTR function across different magnitudes. HIO fluid secretion phenotypes can inform on high levels of CFTR function that discriminate between non-CF and CF, but also at lower levels of CFTR function to study disease expression within the CF population. Optimization strategies for these assays will be discussed, and the impact of such measurements (+/- CFTR modulators) for disease expression across different tissues will be discussed. Additionally, we are exploring how hIO may identify CFTR-dependent mechanisms that could contribute to the strongly increased prevalence of colorectal cancer that is observed in aging people of CF. I will share some unpublished, preliminary data showing CFTR-dependent functional consequences beyond fluid secretion in pairs of isogenic CFTR-deficient hIO.

S8.3 The role of enteroendocrine cell differentiation in the pathogenesis of CFRD

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Due to improvements in CF care, multiple comorbidities have become increasingly prevalent in the CF community, including CF related diabetes (CFRD). CFRD now affects about 20% of adolescents and up to 50% of adults with CF, and is associated with a decrease in pulmonary function, poor weight gain, and increased mortality. The pathogenesis of CFRD is multifactorial and involves defects in insulin secretion and increased insulin resistance. Focusing on insulin secretion, it has been proposed that reduced beta cell mass and beta cell dysfunction both play a key role. However, recent studies have shown that the incretin effect is also impaired in patients with CFRD. The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and the glucagon-like peptide 1 (GLP-1), are secreted from enteroendocrine (EE) cells of the intestine in response to oral nutrient intake, with GIP predominately secreted from the duodenum and GLP-1 predominately secreted from the ileum and rectum. Upon release, the incretin hormones act on beta cells (among other targets) to produce and secrete insulin. Serum levels of both GIP and GLP-1 are decreased in patients with CFRD compared to healthy controls, suggesting that the diminished incretin effect may represent a major mechanism underlying CFRD. Interestingly, a recent study has shown that in both patients with CF and in a mouse model of CF, the number of EE cells is decreased compared to controls. Therefore, it is possible that the reduction in GIP and GLP-1 levels observed in patients with CFRD is due to decreased EE cell number (and possibly EE cell lineage development from intestinal stem cells (ISCs)) and not simply from decreased hormone production.

Reduced EE cell number has also been identified in patients with obesity and type 2 diabetes mellitus (T2DM), two diseases that, like CFRD, show defects in insulin secretion. Loss of EE cells in each of these conditions suggests dysfunction at the level of ISC differentiation into EE cells. In fact, deletion of the cystic fibrosis transmembrane conductance regulator (CFTR) gene has been shown to alter ISC function. Specifically, loss of CFTR leads to increased ISC proliferation and altered differentiation. Whether CFTR is essential for EE cell differentiation has not been evaluated. Further, specific CFTR modulators, including the combination of elexacaftor, tezacaftor, and ivacaftor (ETI), have been shown to improve insulin secretion without improving insulin sensitivity, suggesting a possible direct effect on EE cell differentiation, but the mechanisms responsible for this remain unknown. Our previous and preliminary data show that human ISCs can be robustly differentiated into the EE lineage using ex vivo organoid cultures, and, using single cell RNA sequencing, we have shown that CFTR is expressed in human ISCs.

Because of the above, we have derived human intestinal organoids from patients with CF and CFRD to explore the role of CFTR in EE cell differentiation, as well as the ability of ETI treatment to alter ISC function. Through these studies, we hope to better understand the pathogenesis of CFRD and the role incretin hormones play as possible therapies.

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S8.4 Investigation of CFTR function and epithelial barrier properties at single cell resolution using multi-electrode array technology

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Objectives: CFTR is involved in many processes throughout different organs. To date however, most CFTR modulator studies have focused on their effect on CFTR mediated ion transport and hydration in the airway epithelium. Nevertheless, the extrapulmonary effects of CFTR modulator therapy were recently identified as one of the top ten research priorities by the CF community, acknowledging in particular the major burden of gastro-intestinal symptoms for people with CF (PwCF) (Rowbotham et al, 2023). Moreover, although it is assumed that CFTR modulator treatment reaches and acts similarly in all CFTR expressing cells within an epithelium, current assays lack the required spatial resolution to study potential differences in modulator rescue of CFTR function between cells. We therefore set out to investigate the effect of CFTR modulator treatments on CFTR ion channel function and barrier integrity in individual intestinal epithelial cells using the UZ Leuven CF organoid biobank and imec's high density multi-electrode array chip.

Methods: CFTR ion channel function and local barrier integrity were studied using electrical impedance recordings on multi-electrode array chips with >16.000 subcellularly sized (8µm) electrodes. For initial studies, we used the intestinal colon cancer cell line CaCo2 which expresses CFTR endogenously. Cells were grown for 7-14 days on collagen I-coated chips, after which baseline impedance (barrier integrity) was recorded at 1 kHz. Next, cells were treated for 20 minutes with CFTR activator forskolin (10µM) or a combination of forskolin and CFTR inhibitor Inh-172 (50µM) and impedance was recorded once more. CFTR function was quantified as the percentage change between both recordings. Barrier integrity was also evaluated separately through immunocytochemistry for tight junction protein ZO-1 and by measuring apparent permeability with FITC-dextran 3-5kDa (FD4).

Results: As a proof of concept, we used CaCo2 cells to measure CFTR function with high spatial resolution by impedance recordings. We observed a CFTR-specific impedance drop of ~20% upon CFTR stimulation by forskolin in CaCo2 cells (vs. ~3% in mock treated controls; p=0.0003), which was completely inhibited by co-incubation with Inh-172 (~1% drop; p=0.0047; vs. mock: p=0.96). To confirm that this was due to CFTR channel function, we showed that tight junctions were not affected by these treatments as epithelial permeability of FD4 was unaffected and ZO-1 localization intact. We are currently investigating CFTR function and local barrier integrity in organoid-derived monolayers from non-CF controls and PwCF, with and without CFTR modulator treatment.

Future perspectives: We here present the first exciting data on the use of high-density electrical impedance recordings in CaCo2 cells that allow to study simultaneously barrier integrity and CFTR channel function at single cell resolution. We are currently using this assay to evaluate the effect of CFTR modulator treatment on PwCF-derived primary intestinal monolayers at the single cell level. Next, we aim to correlate this single cell CFTR rescue with CFTR protein expression. We envision that this novel model will contribute to unravelling the full effects of CFTR modulator treatment, in the gastro-intestinal tract and beyond.

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S8.5 Endometrium-derived organoids from cystic fibrosis patients to study the endometrial factor in the disease-associated fertility deficiency

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Introduction/objectives: Due to their increased life expectancy in this highly effective modulator therapy (HEMT) era, people with CF (PwCF) become increasingly interested in starting a family. However, female CF patients suffer from deficient fertility, often facing problems to become pregnant. Underlying reasons remain understudied. In particular, it is largely unknown whether dysfunction of the endometrium, the womb's inner lining and key tissue for embryo implantation and development, is involved. Pregnancy rates have increased since the HEMT, but the direct impact of these CF medicines on the endometrium is unknown. These gaps are mainly due to lack of appropriate research models. Therefore, we developed endometrium-recapitulating organoid models from CF patients and started to decode the role of the endometrium in CF-associated fertility deficiency and the impact of CFTR modulators on endometrial (dys-)function.

Methods: Endometrial organoids (EMO) were developed from CF patient-derived endometrial biopsies. These CF EMO were molecularly and functionally compared to healthy endometrium-derived EMO to start uncovering aberrations that underlie CF fertility deficiency. First, the EMO were characterized using RT-qPCR, and histochemical (e.g. periodic acid-Schiff) and immunofluorescence stainings. The HS-YFP quenching assay was used to probe CFTR function in the EMO and their response to CFTR modulators and CFTR-inh172. EMO were exposed to defined hormonal (estrogen (E2) and progesterone (P4)) regimens to recapitulate the different menstrual cycle phases *in vitro*. Bulk RNA-sequencing (seq) of EMO was performed before and after hormonal treatment to elucidate differences in endometrial responsiveness between the CF and healthy condition. Interesting genes identified were validated by RT-qPCR.

Results: We successfully developed organoids from CF patient endometrium. Before, we have shown that EMO from healthy endometrium can reliably reproduce the menstrual cycle phases under defined E2/P4 exposure. Here, we found cycle phase-dependent *CFTR* and (opposite) *ENAC* expression levels in EMO, similar to *in vivo*. Moreover, CFTR functionality (in healthy EMO) could be validated by HS-YFP quenching and CFTR-inh172 inhibition. Next, the CFTR defect as well as response to CFTR modulators (*i.e.* ETI) was demonstrated in CF EMO. In addition, CF EMO showed a thicker mucus layer as compared to healthy EMO. Currently, we are deciphering whether and how menstrual cycle phases, including the embryo receptivity stage, are different between CF and healthy endometrium, as avatared by their EMO. Intriguingly, CF EMO showed a heightened response to E2 (*i.e.* hormonal treatment to recapitulate the proliferative phase) with higher proliferative activity and visually more dying organoids, which can be explained by decreased E2-inactivating capacity in the CF condition. In addition, we found a lower expression response of key receptivity markers in the CF EMO upon hormonal recapitulation of the embryo receptivity stage. Additional mining of the RNA-seq data will now provide further insights in endometrial aberrations in PwCF.

Discussion: We established a new organoid model from CF patient-derived endometrium which provides a valuable novel research tool to shed light on the endometrium-centred causes in CF-associated fertility deficiency and to explore the impact of CFTR modulators and other therapies on the endometrium.

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S8.6 Elexacaftor/Tezacaftor/Ivacaftor treatment partially normalizes osteoclasts phenotype in cystic fibrosis-related bone disease

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Personnalized therapies and innovative treatment enhance life expectancy in patients bearing CF mutations. Cystic fibrosis-related bone disease (CFBD) is a frequent comorbidity in CF patients. CFBD is characterized by low bone density and increased risk of fracture, even during childhood. However, mechanisms explaining the impact of defective CFTR function on bone homeostasis remain not fully elucidated. Preliminary data demonstrated that loss of CFTR function led to impaired osteoblastogenesis as well as a rise in the number of circulating pre-osteoclastic $RANK^+MCSFR^+$ monocytes. **We aimed to** study the impact of CFTR class II mutation channel loss of activity and its correction by modulator treatment on osteoclast (OC) phenotype. **Circulating** pre-OC monocytes were isolated from 33 patients bearing CFTR Class II mutations (NCT04877223) and 23 matched healthy controls (age and gender) (ALC/PIL/DIR/AJR/FO/606). Membrane RANK and MCSFR receptors were labelled for flow cytometry study. Sera levels of pro-inflammatory markers (IL-6, IL-8 and TNF-α), bone remodeling markers (CTX, PINP) and S1P were evaluated by ELISA. After 14 days, OC were plated on dentin slices for 7 days to evaluate resorption activity. After 21 days of differentiation, OC were stained (Phalloïdin-AlexaFluor488®, Vinculin-immunofluorescent staining, DAPI) to determine osteoclasts number, size and evaluate focal adhesion complexes. S1P supernatants levels were assessed by ELISA. Pre-OC monocytes were treated with prolonged exposure to Elexacaftor, Tezacaftor and Ivacaftor (ETI 3µM/3µM/1µM). **We evidenced** an increased proportion of $RANK^+$ $MCSFR^+$ cells in CF patients' blood (2.3-fold; $p<0.05$) compared to controls. Interestingly, we observed an increased of bone remodeling markers: CTX (1.,12 fold; $p<0.05$) and PINP (1.18-fold; $p<0.05$), and S1P (1.42-fold; $p<0.05$) concentrations from CF sera compared to control, independently from pro inflammatory markers. OC exhibiting CFTR mutations were less numerous but larger than non-CF ones (1.5-fold; $p<0.05$). ETI treatment did not restore number of OC but significantly decreased (-70%; $p<0.05$) their size towards non-CF ones. S1P levels in CF OC supernatants were higher compared to controls (+180%; $p<0.05$), after treatment even if levels were lower (-39%; $p<0.05$) they remained higher than in non-CF (+72%; $p<0.05$). Resorption activity that was strongly reduced in CF cultures (40-fold; $p<0.05$), was enhanced in treated culture compared to untreated (12-fold; $p<0.05$) without reaching non-CF levels. Vinculin staining was thinner and less co-localized with actin ring in OC bearing-CFTR Class II mutation than in control. ETI treatment partially restored cytoskeleton organization. **Our results** tend to demonstrate defective OC differentiation and resorption process in OC bearing class II CFTR mutations, which are partially restored by CFTR modulator treatment. This could explain disruption in bone homeostasis observed in CF patients.

23 March — 16:30–17:30

Closing Keynote Lecture

S9.1 Bridging the Gap: scientific discovery to clinical benefit

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The 'Valley of Death'- that place we've all heard of but none of us want to be stranded in. Where huge number of drugs lose their footing and never make it out alive. The pre-clinical/ clinical divide that separates winners from losers. Those making it across the valley, then face the mountain range of later-phase, pivotal trials to enable clinical access.

CF has celebrated some major wins in recent years, most notably of course with CFTR modulators, which are transforming the lives of many. However, this has led to the emergence of a yawning chasm between this majority of the population, and others, who largely due to bad luck in the genetic lottery, are unable to benefit. Focus has switched to this subgroup in recent years, with growing discovery/ development programmes in genetic therapies and other, mutation-targeted approaches. In parallel, essential research continues in complication-directed therapies (antimicrobials, anti-inflammatories), which will remain a need for the growing proportion of people living with CF into later adulthood.

In order for this welcome research to reach clinical potential, we should focus early as a multidisciplinary field on identifying and mitigating likely challenges to translation. I will use some historical examples of drugs being developed for CF which have failed such translational challenges, and others meeting with success. In what I hope will be a thought-provoking discussion format, we will consider both operational and scientific 'red flags' and seek proactively to identify more subtle 'amber flags'. Examples of good practice, eg. early-stage patient involvement & engagement, patient-centred study design, population scoping through Patient Registries and development of target product profiles (TPPs) will be discussed. The roles of trial networks and standardized outcome measures, pioneered by ECFS CTN and CFF TDN will be highlighted.

There will never be a 'one-size-fits-all' solution to the challenges we face in maximizing the clinical potential of scientific discovery. But there are likely ways that together, we can bridge the gaps and reduce the risk to valley-crossing pioneers.

P1

Characterization of the tumor-suppressor character of CFTR in the context of colorectal cancer development in primary patient-derived intestinal organoids

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The last decade of CF research is characterized by a massive paradigm shift in terms of the development of highly effective modulator treatments (HEMTs), that effectively treat various CFTR mutations. Whilst this is clearly major progress, the aging CF population faces new difficulties. Recent studies show that a third of all adult CF patients suffer from a range of comorbidities such as a large increased risk of developing several epithelial cancers, including colorectal cancer (CRC). The prevalence of CRC in the non-CF population between 40-49 years old is 2-5%, whereas in CF patients the CRC prevalence is around 23%. Understanding how dysregulated CFTR could result in the increased chance of developing CRC is pivotal in order to prevent the build-up of polyps, potential outgrowth of polyps and as such advancing care and quality of life for pwCF.

In line with this increased prevalence of CRC in pwCF, various animal and cell line models point out the connection between CFTR-loss of function (LoF) and dysregulation of intestinal stem cells. However, results are contradictory due to the complexity of both CFTR-LoF as well as CRC development. Consequences of CFTR-LoF can be linked to cell-autonomous effects as well as indirect effects, such as the pro-inflammatory state of the gastro-intestinal system. Understanding the extent and the exact underlying molecular mechanism of the link between CFTR-LoF and CRC is however absolutely essential to ultimately prevent polyp formation and/or progression in pwCF.

To address this, we exploited patient-derived intestinal organoid (PDIO) cultures isolated from rectal biopsies with a wide range of genotypes. We furthermore create CRISPR-engineered organoid isogenic organoid lines to compare wildtype CFTR with CFTR-LoF within the same genetic background. This resource offers a unique opportunity to characterize cell-autonomous consequences of CFTR-LoF. First pilot experiments indicate indeed phenotypical differences in terms of proliferation speed and single cell outgrowth, where CFTR-LoF is associated with an increase in proliferative capacity. We are furthermore characterizing proliferation on a more molecular level, amongst others by means of proliferation-specific markers (e.g. EdU) and cell cycle analysis. We expect to have those results at the time of the conference.

We furthermore started with identification of underlying alteration of molecular pathways associated with CRC development in CFTR-LoF PDIOs. We are characterizing differences in activity of molecular pathways upon CFTR-LoF that associate particularly with CRC development, e.g. WNT, TGF-B, EGFR, and P53 signaling. Furthermore we are currently characterizing differences in gene expression profiles in an unbiased manner by means of bulk and single cell RNA sequencing. The bulk RNA sequencing already gives interesting leads, such as the decreased expression of WNT pathway inhibitor DKK1 upon CFTR-LoF, which could associate with enhanced proliferative capacities.

Overall, we believe in this new era of CF-research it is pivotal to understand how cell-autonomous effects of CFTR-LoF connect to the increased risk of CRC and aim to contribute to this important field of study.

P2

Exploring the activating effects of the inhibitors of type IV ABC transporters on CFTR gating

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CFTR, a Type IV ABC transporter, uniquely functions as an anion channel. Cystic fibrosis (CF)-causing gating mutations in CFTR reduce open probability (Po). Potentiators are a class of compounds that increase CFTR Po and present a therapeutic strategy for patients with gating mutations. VX-770 (ivacaftor; Vertex Pharmaceuticals) is the only approved potentiator. Cryo-EM studies revealed bound VX-770 at the interface between the hinge-like discontinuity in transmembrane helix 8 (TM8) and the lipid bilayer[1]. To gain mechanistic insight, we explored how compounds initially developed for other Type IV ABC transporters affect CFTR activity and how they interact with VX-770.

The Genetech-developed G-compounds inhibit MsbA, a homodimeric bacterial ABC transporter[2]. Among these, G907 binds at two homologous sites on MsbA, one corresponding to the portal between TM4 and TM6, on the CFTR anion permeation pathway, not far from the VX-770 binding site. Unexpectedly, we found that G-compounds activated CFTR[3].

P-gp, another Type IV ABC transporter, plays a crucial role in the efflux of xenobiotics. Elacridar and zosuquidar are potent third-generation P-gp inhibitors. They bind in the substrate-binding pocket on P-gp, corresponding to CFTR's inner vestibule, which is also relatively close to the VX-770 binding site on CFTR[4].

To investigate the effects of these molecules on CFTR, we employed a high-content assay which evaluates ion-channel activity at the cellular level in HEK-293 cells[5]: CFTR, tagged with a halide-sensitive yellow fluorescent protein (YFP, H148Q/I152L) and mCherry, a red cytosolic fluorescent marker for image segmentation, are co-expressed from a bicistronic plasmid. Quenching of YFP-CFTR fluorescence following extracellular iodide addition reports on CFTR activity.

Elacridar and zosuquidar modestly enhanced the activity of partially phosphorylated CFTR. Furthermore, less-than-additive effects were observed in combination with VX-770 (table 1), suggesting direct CFTR targeting. The mismatch between the inhibition of other ABC transporters and activation of CFTR might reflect an effect on the dynamics of the extracellular end of TM8 in CFTR, which recent results suggest might be modulated by both potentiator VX-770 and inhibitor CFTRinh-172[6,7]. Future studies are planned to assess the effects of VX-770 on G907/elacridar/zosuquidar binding. A better understanding of how these molecules interact on CFTR could help clarify gating and pharmacological mechanisms.

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Table 1: AAC_9 (area above YFP-quenching curve, 9 s after iodide addition, RFU*s)

acute treatment	***mean***	***(95 % CI)***	***N***
0.5 µM forskolin	3.72	(3.42, 4.02)	12
0.5 µM forskolin+10 µM elacridar	4.74	(4.31, 5.16)	12
0.5 µM forskolin+10 µM zosuquidar	4.81	(4.41, 5.22)	12
0.5 µM forskolin+3 µM VX-770	5.57	(5.25, 5.89)	16
0.5 µM forskolin+3 µM VX-770+10 µM elacridar	5.9	(5.40, 6.39)	8
0.5 µM forskolin+3 µM VX-770+10 µM zosuquidar	5.95	(5.42, 6.47)	8

P3

The dual impact of INF2 on the regulation of CFTR

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Background: Old and new data has made increasingly clear that CFTR is at a crossing between the cytoskeleton and signaling pathways, especially cAMP signaling. Regulation of both CFTR trafficking (particularly, plasma membrane (PM) stability) and channel activation is a complex process involving protein partners functioning as molecular switches. Fine tuning of this regulation requires integrity of correct cytoskeletal organization, as the cytoskeleton is responsible for the scaffolding that stabilizes CFTR at the PM and brings several interacting proteins to CFTR's proximity, among which cAMP sensors, such as PKA and EPAC1, have a prominent role [1]. Regulation of CFTR also occurs with recruitment of several actin cytoskeleton dynamics regulators to CFTR's proximity, namely INF2. INF2 is an unusual member of the formin family because of its unique ability to accelerate actin filament depolymerization, adding to the nucleation and elongation activities common to all formins and also its unique ability to associate reversibly with the cytosolic leaflet of the endoplasmic reticulum (ER). INF2 has been reported as a negative regulator of CFTR PM stability [2].

Aims: The main goal of this work was to characterize the role of INF2 in the regulation of CFTR both at the PM and the endoplasmic reticulum (ER).

Methods: We used CFBE cells expressing wt-, F508del- or N1303K-CFTR and analyzed them using Western blot and cycloheximide chase assay.

Results: INF2 knock-down (KD) promotes an increase in mature wt-CFTR levels, even after treatment with the corrector combination VX-661 + VX-445. For F508del-CFTR, INF2 KD improves rescue by VX-661 + VX-445 independently of EPAC1 activation. In a cycloheximide-chase assay, INF2 KD decreased rescued F508del-CFTR turnover, with an increase in protein half-life, suggesting that the stabilizing effect is caused by a decrease in degradation. INF2 KD also promotes a stabilizing effect on immature CFTR levels, for wt-, F508del-, and N1303K-CFTR. These results indicate that INF2 has a dual role in the regulation of CFTR, both at the PM and ER. The next steps in this study will be to assess the influence of INF2 expression levels and CFTR expression system (endogenous vs heterologous expression) on the stabilizing effect, in different models (and also from different tissues), to potentially highlight INF2 as a novel target for modulation to develop combinatorial therapies for CF.

Conclusions: Our results shed light into how an actin cytoskeleton dynamics regulator regulates CFTR, exploring the crosstalk between cAMP signaling and the cytoskeleton to modulate CFTR levels and possible rescue.

Acknowledgements: Work was supported by FFC Ricerca grant FFC#2/2023 and FCT/MCTES, Portugal - grants UIDB/04046/2020 and UIDP/04046/2020 (https://doi.org/10.54499/UIDB/04046/2020) to BioISI and PhD fellowship 2021.06174.BD to JFF.

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P4

Understanding the conformational landscape of NBD1 from CFTR: from normal function to cystic fibrosis

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel regulating fluid transport in epithelial tissues. The most common mutation is the deletion of phenylalanine at the 508 position (F508del) in the nucleotide-binding domain 1 (NBD1), which leads to channel degradation and ion transport lack.

We developed a collection of nanobodies targeting human NBD1 (Sigoillot, M. et al, 2019) and isolated nanobody G11a, that stabilizes an alternative conformation of NBD1, topologically different from the canonical NBD1 fold. By single-molecule FRET (smFRET) microscopy, canonical and alternative conformational equilibrium was regulated by ATP presence. The F508del mutation presence in the domain favored the alternative state (Scholl, D. et al, 2021). To probe the functional and physiological role of the different conformation, we have developed a point mutant of NBD1 (W401A), strongly promoting the alternative state. We are now investigating the mutation effect on the protein structure and function.

We also identified nanobody T1a, a high-affinity NBD1 binder which, remarkably, induces NBD1 destabilization in a “F508del mutation” manner. The F508del mutation-like effects of T1a can provide a unique tool to investigate the CF-causing mutation molecular basis.

On the one hand, we want to trace how F508del mutation leads to NBD1 destabilization using T1a nanobody. On the other hand, we aim to examine the NBD1 alternative state natural. In our research we use biochemical (ELISA), physical (smFRET, thermal shift assay), and structural (X-ray crystallography, hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) methods. The alternative fold could be a functional state of the domain, but in the destabilizing mutation presence it becomes the weakest NBD1 state and leads to its misfolding.

P5

Mechanism of action and binding site characterization of corrector *ARN23765* via Photo-Affinity Labeling (PAL) approach in live cells

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Cystic Fibrosis (CF) is a genetic disease characterized by an impairment in the synthesis or function of CF transmembrane conductance regulator (CFTR) anion channel, caused by mutations in CFTR gene. Small-molecule compounds addressing the basic defect of the disease have been identified and are referred to as CFTR modulators.[1] Among these, the F508del-CFTR corrector *ARN23765,* discovered by our group, showed high potency in rescuing the function of mutant CFTR in primary human bronchial epithelial cells derived from CF patients homozygous for the F508del mutation.[2] Despite the validated pharmacological effects, *ARN23765* mechanism of action and binding site had not yet been conclusively defined. CFTR correctors could act either directly by binding to CFTR or by interacting with the machinery responsible for protein synthesis, trafficking and maturation.[3,4] To the best of our knowledge, no data are so far available disclosing the interaction of modulators with CFTR (either wt- or F508del-) in a native cellular environment.

Along with the identification of the primary biological target(s), our project aimed to uncover the mechanism of action and the binding site of *ARN23765* to its target protein in living cells. Photo-Affinity Labeling (PAL)[5] approach was pursued as a suited strategy to perform such investigation. *ARN23765*-derived photo-affinity probes (PAPs) were designed and synthesized by introducing both a small photo-reactive moiety to capture bio-molecules in its close proximity, and a reporter/purification tag allowing adducts identification in biochemical studies.[6]

Taking advantage of the PAL methodology, we demonstrated the in situ interaction of *ARN23765*-derived PAPs with CFTR in wt- and F508del-CFTR overexpressing CFBE41o- cells. Additionally, functional studies using CFTR domains identified the interface between MSD1-NBD1 as the protein region involved in *ARN23765*-induced correction. The putative binding site of *ARN23765* on CFTR was elucidated by a combination of computational (molecular docking and molecular dynamics) analyses and site-directed mutagenesis studies, which identified the primary amino acid residues involved in the effective interaction of the corrector with CFTR.

To the best of our knowledge, our study is the first to disclose the interaction of a corrector probe to wt- and mutant F508del-CFTR in an intact cellular setting. Furthermore, these results contribute to elucidate the molecular bases of CFTR rescuing induced by *ARN23765*, showing its direct binding to CFTR and the stabilization of the interface between MSD1-NBD1 protein region through the interaction with specific amino acids.

This work was supported by the Italian Cystic Fibrosis Research Foundation (FFC Ricerca) as part of the "FFC#4/2020 and FFC#2/2022" projects.

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P7

Nanobodies as therapeutics for cystic fibrosis

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For patients bearing the F508del mutation, cystic fibrosis is due to a thermal destabilization of the CFTR chloride channel which, in turn, does not reach the plasma membrane to regulate fluid balance across the epithelia. Although they provide significant clinical improvement, approved correctors do not thermally stabilize the protein nor lead to recovery of wild-type like function, indicating that improvements are needed. In this context, we developed nanobodies that specifically bind NBD1 and thermally stabilize CFTR. When transfected, these nanobodies are able to restore the maturation and the function of mutant CFTR in strong synergy with approved correctors (Trikafta). In the perspective of therapeutics, we propose to use lipid nanoparticules (LNPs) containing nanobody-encoding mRNA as means to deliver stabilizing nanobodies to the cell interior. We have tested two different types of LNPs to specifically target the lungs. The first LNPs are designed to resist to the nebulization in order to directly reach the airways. The second LNPs were developed to be injected in the bloodstream and target the lungs thanks to a selective organ targeting (SORT) lipid. We show that both mRNA formulations enable efficient expression of stabilizing nanobodies leading to improved maturation and cell-surface expression of F508del-CFTR. This effect is highly synergistic with that of approved correctors (Trikafta). Moreover, this treatment leads to an increase of chloride conductance in CFBE cells as shown by Ussing chamber assay. Our work opens a novel strategy to improve the current therapies to treat cystic fibrosis.

P8

Peptide CIGB-552 has a synergistic effect on CFTR-F508del combined with Elexacaftor/Tezacaftor/Ivacaftor

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Objectives: Cystic fibrosis is an autosomal recessive disease in which mutations in the CFTR gene lead to a reduced life expectancy in carriers, partly due to the rapid loss of respiratory functions. CFTR-F508del is the most frequent mutation, leading to a mislocalized and non-functional CFTR protein. The tri-therapy Elexacaftor/Tezacaftor/Ivacaftor is now given to patients carrying CFTR-F508del mutation. However, the plasma membrane stability of CFTR-F508del protein is also compromised, and the combination of correctors and potentiators does not address this instabilityAdditional strategies are still in progress to reinforce mutated CFTR maturation/function/stability, and CFTR interactome taking part in its biogenesis could be a source of strategies. Among CFTR interactors, we have previously identified COMMD1 as a potential therapeutic target, which overexpression favors the plasma membrane expression of CFTR. However, overexpressing COMMD1 directly in patients is still a challenge. In 2013, a cell-penetrating peptide named CIGB-552 was discovered as a COMMD1 protein stabilizer. We evaluate the therapeutic potential of CIGB-552 in cystic fibrosis context.

Methods:

1. FITC Tagged version of CIGB-552 was used to evaluate its uptake on cell models cultured submerged or in air-liquid interface.
2. HS-eYFP assay was performed for measuring CFTR quenching ratio.
3. Short-circuit current was recorded to evaluate specific chloride flux through CFTR channel.
4. Western blot was used to evaluate CFTR maturation and COMMD1 expression.

Results and conclusion: We demonstrate that CIGB-552 is non-toxic and preferentially enters CFTR-F508del expressing cells without modifying COMMD1 expression or localization in our cystic fibrosis cell models. CIGB-552 is not a potentiator nor a corrector but acts synergistically with Elexacaftor/Tezacaftor/Ivacaftor in improving chloride efflux and increasing transepithelial potential difference of CFTR-F508del cells. However, addition of CIGB-552 with Elexacaftor/Tezacaftor/Ivacaftor does not modify C-band intensity of CFTR-F508del. The mechanism implied by CIGB-552 with the COMMD1 protein in this positive effect are still undeciphered. But recent studies show COMMD1 as a key protein in plasma membrane protein recycling involving the complex COMMANDER. We hypothesized that CIGB-552 could favor the recycling role of COMMD1 in sending back proteins to the plasma membrane from endosomes. CIGB-552 is currently in phase I clinical trial for solid-tumor cancer, meaning CIGB-552 could be quickly and safely repurposed to treat cystic fibrosis in combination with Vx-445/661/770.

P9

Pi3Kγ modulation: A new frontier in maximizing F508del-CFTR stability for improved therapeutic outcomes

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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 plasma membrane (PM) anion channel expressed in epithelial cells. For patients carrying the F508del-CFTR variant, in 2020 EMA approved the combination therapy ETI that has shown high effectiveness in clinics. Even if it restores defective channel folding and gating, ETI rescues F508del-CFTR function by only 50% compared to wild-type, underlying the need to find add-on treatments to maximize its efficacy. In this regard, CFTR "stabilizers", aka molecules that secure the channel at the PM and decrease its degradation rate, represent a promising therapeutic strategy since they can target the stability defect of F508del-CFTR not addressed by ETI.

Hypothesis and Objectives: We previously showed that a PI3Kγ mimetic peptide targeting the PKA-anchoring function of PI3Kγ (PI3Kγ MP; n° WO2016103176) can increase the PM amount of F508del-CFTR without acting as a CFTR corrector, making us hypothesize that the compound can function as a CFTR stabilizer. For these reasons, the project aims to investigate whether PI3Kγ MP could represent an alternative strategy to maximize the efficacy of ETI by stabilizing the F508del-CFTR at PM.

Methods: Cell surface protein biotinylation and western blotting were used to evaluate CFTR PM density in HEK293T and CFBE41o- cells upon PI3Kγ MP treatment. CFTR activity was instead examined by performing Ussing chamber measurements in F508del primary bronchial epithelial cells.

Results: Through experiments on F508del-CFBEo- cells, we observed that the PI3Kγ MP, when used in conjunction with ETI, significantly increases the presence of CFTR at the cell surface. This suggests that upon peptide treatment the corrected CFTR is less susceptible to endocytosis and/or recycled at the membrane. By performing a cycloheximide chase assay, we found that the quantity of F508del-CFTR that has not been degraded after 6 hours of CHX treatment is double when the PI3Kγ MP is added to the triple combination therapy, thus indicating that the peptide prolongs the half-life of the corrected F508del-CFTR. Most importantly, in F508del bronchial epithelia treated with ETI and the PI3Kγ MP, total CFTR activity upon maximal activation was significantly higher compared to ETI alone, indicating that CFTR stabilization can improve ETI efficiency in primary cells.

Conclusions: These findings highlight the therapeutic potential of the peptide as an add-on therapy with ETI for CF patients responsive to these CFTR modulators.

P10

Mapping CFTR-dependent and CFTR-independent luminal pH regulation along the crypt-villus axis of human small intestine

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Background: In CF, dysfunctional and/or absent CFTR causes reduced intestinal chloride and bicarbonate secretion, resulting in decreased small intestinal luminal pH. This pH imbalance may contribute to epithelial injury and nutrient malabsorption. It remains unclear what cell populations are responsible for CFTR-dependent and CFTR-independent pH regulation. Our aim was to combine transcriptomic and functional analysis of chloride, bicarbonate, and proton transport in healthy and CF duodenum to identify populations of intestinal cells responsible for luminal pH regulation.

Methods: Undifferentiated and differentiated enteroids (Wnt/R-Spondin removal for 1-5 days) were cultured as submerged monolayers for RNA sequencing (n=6; 3 healthy/2 passages each) and combined short-circuit current (I_{sc})/pH-stat (5 healthy, 1 F508del/F508del CF). Simultaneous I_{sc}/pH-stat measurements allow detection of electrogenic (e.g., CFTR) and electroneutral (e.g., NHE3) acid-base transport. $CFTR_{inh}$-172 (20mM, apical), forskolin (10mM, basolateral), and elexacaftor/tezacaftor (3mM x48 hours)/ivacaftor (0.3mM) (ETI) were used to measure CFTR function. S3226 (10mM, apical) was used to inhibit NHE3.

Results: Undifferentiated and Day1-diff enteroids had increased mRNA expression of *LGR5, PCNA, OLFM4, MYC, CD44,* indicating a crypt phenotype. Day3 and Day5 enteroids had increased mRNA expression of *ALPI, APOA4, SI, NT5E, APOC3*, indicating a villus phenotype. *CFTR* and *SLC9A3* (NHE3) mRNA was highest in crypt enteroids. I_{sc} was highest in crypt enteroids, whereas bicarbonate secretion was highest in Day5-diff villus enteroids (n=8-24, $P<0.05$). CFTRinh-172 inhibited baseline I_{sc} in crypt (n=4-5, $P<0.05$) and Day3-diff villus enteroids (n=5, $P<0.05$), but not Day5-diff villus enteroids (n=4, $P>0.05$). In contrast, $CFTR_{inh}$-172 did not inhibit bicarbonate secretion in crypt or villus enteroids (n=4-6, $P>0.05$). S3226 alone increased baseline bicarbonate secretion in crypt and villus enteroids (n=5-13, $P<0.05$), but did not alter the lack of CFTRinh-172 effect on bicarbonate secretion (n=6, $P>0.05$). CF enteroids (n=2-4) generally showed lower baseline I_{sc} and bicarbonate secretion than healthy controls in crypt and villus enteroids. ETI only increased baseline I_{sc} in crypt-like enteroids ($P<0.05$). Forskolin, without S3226, increased Isc in crypt and villus enteroids (n=6-13, P0.<0.05), but only increased bicarbonate secretion in Day5- diff villus enteroids. With S3226, forskolin no longer stimulated a significant increase in bicarbonate secretion in Day5-diff enteroids (n=8-11, $P>0.05$). In CF enteroids, forskolin (with S3226) did not increase I_{sc} or bicarbonate secretion in crypt or villus enteroids (n=2-3, $P>0.05$). ETI only increased forskolin-stimulated I_{sc} in crypt enteroids (n=5, $P<0.05$), with no effect on forskolin-stimulated bicarbonate secretion in crypt or villus enteroids (n=3-4, $P<0.05$).

Conclusions: CFTR-mediated chloride secretion predominantly occurs in crypt enterocytes and may be improved with ETI. In contrast, CFTR-dependent bicarbonate secretion may be low across crypt and villus regions and may not be significantly improved with ETI. Forskolin's ability to increase bicarbonate ion availability and luminal pH appears to be primarily due to inhibition of NHE3-mediated proton secretion in villus enterocytes. Ongoing functional mapping of CFTR-dependent and CFTR-independent chloride and acid-base transport may identify therapeutic strategies to improve gastrointestinal symptoms in CF.

P11

Rescuing G85E, N1303K and F508del-CFTR by Elexacaftor or Bamocaftor combined to Tezacaftor/Ivacaftor in human airway epithelial cells

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Background and purpose: New life-changing pharmacological drugs are approved for more and more people with Cystic Fibrosis (pwCF). However, not all the defects of class 2 CF variants are corrected, such as thermal instability as an example. Here, we compared the effect of Elexacaftor (VX445)/Tezacaftor (VX661)/Ivacaftor (VX770) (hereafter noted ETI) to that of Bamocaftor (VX659)/Tezacaftor/Ivacaftor (hereafter noted BTI) on the three class 2 CFTR variants F508del, G85E and N1303K and investigated CFTR correction of function, maturation and thermal instability at 37°C.

Experimental approach: We combined electrophysiology and biochemical methods such as Ussing chamber (recording of the transepithelial short circuit current, noted Isc), whole-cell patch clamp recordings and western blot. Our cell models are the CF bronchial epithelial CFBE41o cell lines WT-, F508del-, G85E- and N1303K-CFTR CFBE and primary human airway epithelial cells obtained from F508del homozygous patients. Cells were treated at 37°C either 24h (F508del) or 48h (G85E and N1303K) with ETI and BTI. The concentrations used are: 1, 3 or 10 µM for the correctors VX445 and VX659; 3 µM for the corrector VX661 and 1 µM for the potentiator VX770. CFTR dependent Isc was stimulated by 1 (F508del) or 10 µM (G85E and N1303K) of fsk and inhibited by 10 µM of $CFTR_{inh172}$.

Key results: The correction of function and maturation of F508del-, G85E- and N1303K-CFTR by BTI is improved compared to the effect of ETI and depends on the duration and concentration of treatment. After 2h, at physiological temperature, about half of the F508del-CFTR dependent transepithelial current (Isc) corrected by ETI or BTI is still present, which is not the case in their absence. Whatever the condition, the amplitudes of Fsk-dependent Isc and $CFTR_{inh172}$-dependent Isc are significantly increased when BTI is used compared to ETI for F508del-, G85E- and N1303K-CFTR.

Conclusions and implications: The level of correction of function and maturation of F508del-, G85E- and N1303K-CFTR by BTI is significantly increased compared to ETI. Although not fully eliminated, the thermal instability of F508del-CFTR is also less pronounced after BTI compared to ETI, suggesting a better and longer correction level with BTI.

Supported by Vaincre la mucoviscidose

P13

Beneficial effect of orkambi in alcohol-induced acute pancreatitis

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Background and aims: Long-term alcohol use is one of the causes of acute pancreatitis (AP). Our workgroup has shown that ethanol (EtOH) decreases the expression and activity of the cystic fibrosis transmembrane conductance regulator (CFTR), which plays a key role in alcohol-induced AP development. It is well known, that Orkambi can correct impaired CFTR function in cystic fibrosis (CF) patients. Thus our **aim** in this study was to investigate whether Orkambi can mitigate alcohol-induced AP.

Materials and methods: Guinea pig pancreatic ducts were pre-treated with different concentrations of EtOH (30, 50 and 100 mM) for 12 hours alone or in combination with Ivacaftor (VX770) and/or Lumacaftor (VX-809), and CFTR expression and activity were evaluated by immunostaining and by patch clamp technique, respectively. Alcoholic AP was induced in Orkambi-treated guinea pigs, and standard laboratory and histological parameters were measured.

Results: Ivacaftor and Lumacaftor alone or in combination dose-dependently restored the apical expression and activity of CFTR after EtOH treatment *in vitro*. Oral administration of Orkambi reduced the severity of alcohol-induced AP and restored impaired CFTR activity and expression.

Conclusion: Orkambi is able to restore the CFTR defect caused by EtOH and decreases the severity of alcohol-induced pancreatitis. This is the first *in vivo* pre-clinical evidence of Orkambi efficacy in the treatment of alcohol-induced AP.

The research was funded by the Hungarian Ministry of Innovation and Technology, National Research, Development and Innovation Fund (TKP2021-EGA-23 to PH), the Translational Neuroscience National Laboratory programme (RRF-2.3.1-21-2022-00011 to PH), the National Research, Development and Innovation Office (SNN134497 to VV, K135874 to ZR and K131996 to PH) and the Translational

P14

Gene therapy and modulator treatments for Cystic fibrosis: a combined therapeutic approach

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Nowadays, the pharmacological approach based on modulators is one of the main strategies to treat people with Cystic Fibrosis (pwCF). Indeed, these molecules have considerably improved the pwCF lung capacity and quality of life. Trikafta® (combination of CFTR correctors and potentiator) for instance, enables a gain of 10% of FEV_1. However, about 30% of pwCF in France still suffer from pulmonary complications because they do not take these treatments. In addition, various and numerous side effects are related to their use such as infection and depression. Other molecules acting on *CFTR* mRNA are currently under investigations. One of them, nesolicaftor (PTI-428) is a CFTR amplifier that stabilizes *CFTR* mRNA and, therefore, allows an increase of the amount of CFTR protein in either non-CF or p.Phe508del cells. Although clinical trials did not demonstrate any significant result on pwCF pulmonary function, CFTR protein level in patient's nasal mucosa was higher meaning that this molecule could represent an interest for pwCF. Another strategy based on non-viral gene therapy still represents a potential alternative approach owing to its mutation independence nature and its high level of innocuity. However, last clinical trial showed that lipid-based gene therapy alone is yet not enough efficient to significantly change the clinical state of patients.

We studied the possible impact of the CFTR amplifier nesolicaftor, on transgene expression following lipid-based nanoparticles. Our hypothesis was based on the assumption that nesolicaftor could act on h*CFTR* mRNA expressed from plasmid and therefore increase the amount of CFTR protein. Our goal is therefore to look if modulators and gene therapy combined could lead to a benefit impact and therefore compensate for their respective weaknesses and provide a benefit impact on pwCF treatment.

CFBE41o- cells (homozygote p.Phe508del) were transfected using an optimized CpG free plasmid encoding hCFTR (pGM169) complexed with a lipid-based nanoparticle, BSV163/DOPE and/or treated with different concentrations of nesolicaftor (10µM or 30µM). Twenty-four hours later, cell viability was assayed and mRNA was extracted. For RT-qPCR, specific primers were designed to distinguish endogenous CFTR mRNA from the transgene mRNA. Furthermore, CFBE41o- KO cells (knocked-out for h*CFTR*) were treated with nesolicaftor 30µM and transfected as described before. Forty-eight hours later, protein expression was assayed by immunoblotting.

After 24h of treatment on CFBE41o- cells, nesolicaftor (30µM) increased both endogenous and transgene CFTR mRNA by 5,8 and 2,6 times, respectively. Additionally, the association of nesolicaftor and gene delivery was not toxic (80% cell viability). After 48h of treatment on CFBE41o- KO, exogenous CFTR protein amount was increased by 36%.

These in vitro results demonstrated that nesolicaftor can act on endogenous and exogenous CFTR mRNA and as a consequence, on CFTR protein amount. This encourages us to perform additional experiments to further study the impact of the combined approach on exogenous CFTR protein.

P15

A new approach to understand complex cases of cystic fibrosis and its related disorders

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Cystic fibrosis (CF) is the most common severe hereditary genetic disease in the Caucasian population, which affects almost 1 over 2500 births. More than 2100 variations have been reported for the causing gene, CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). However, there are still 1-2% of incomplete genotype and above all a misunderstanding of clinical heterogeneity observed in people with CF. Another misleading case is the presence of related disorders (CFTR-RD) as congenital bilateral absence of vas deferens (CBAVD) and pancreatitis. The pathogenesis leading to CF or CFTR-RD is not clear. To answer that, we put effort on understanding the cis-regulation of the CFTR gene. Dysfunctions in cis-regulation are described as involved in numerous pathologies, known as “enhanceropathies”. By mapping CFTR putative cis-regulatory elements (CREs), we aim to gain more insights into its tissue-specific pattern of gene expression.

We use 4C (Circular Chromosome Conformation Capture) in intestinal, pancreatic and epididymis cells to map CRE-promoter interactions. Subsequently, to identify candidate CREs (cCREs), we integrated epigenomic data as H3K27ac (enhancer), H3K27me3 (silencer) and CTCF (CCCTC-binding factor) marks. To functionally validate identified cCREs, we perform in vitro reporter gene assay. Targeted high throughput sequencing is then applied to different patients' cohort to detect cis-regulatory variants.

Many CRE-promoter interactions have been identified, allowing us to define cis-regulation models for each tissue of interest. We highlight some similarities but also few tissue-specific interactions. Most regulatory regions have an enhancer effect. The first unique silencer of the CFTR gene localizes at +507,6 kb from the last codon has been identified. It is the first reported CRE outside the topologically associating domain of the CFTR locus. Once we have a better understanding of the cis-regulation at the CFTR locus, we sequence each cCRE and detect several regulatory variants. A dozen of SNP (single nucleotide polymorphism) have a significant different frequency compared to the French population. To validate their impact, functional tests are performed.

We provide new elements which could help, in the future, to better understand the physiopathology of CFTR diseases. To go further, we want to define the transcription factors involved in the regulation of the CFTR gene. In addition, we need to use CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology to have an endogenous tool that can lead us to confirm the gene regulation within these three models.

Thus, this work allows us to better understand the three-dimensional organization of the CFTR locus in order to improve the management of patients.

P17

DNA methylation markers predict pulmonary exacerbation and lung function degradation in Cystic Fibrosis

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Although tremendous progress has been done in cystic fibrosis treatment, robust biomarkers are still required to monitor the disease and guide treatments. Herein, we asked whether DNA methylation levels at specific CpG sites are predictive markers. Using MethylEpic array hybridization, we profiled 64 sputum samples from the MethylBiomark cohort and assessed the correlation between DNA methylation levels and clinical traits of interest in cystic fibrosis. We combined Welch t-test and logistic regressions to assess categorical variables and general linear models for continous variables. Sex and age were used as covariables. The MethylBiomark cohort consists of 50 patients with cystic fibrosis who were enrolled in the CF care center of Montpellier, France. They were aged 12 to 30 year and carried two severe (class I, II) *CFTR* variants in *trans*. Sputum samples were collected at four time points, during a 18-month follow-up. The clinical course of cystic fibrosis is characterized by episodes of pulmonary exacerbation, which are associated with progressive loss of lung function and increased mortality. We found that DNA methylation levels at 23 CpG sites predicted patients with pulmonary exacerbation. The top2-markers were reassessed in independent sputum samples from the same cohort, using pyrosequencing (Pyromark, Qiagen). They map to the *ANKRD53* gene, encoding a cytoskeletal potein that may contribute to inflammation, and to *GRIK2* which is expressed in smooth muscle cells in the bronchus and is altered in chronic obstructive pulmonary disease. Currently, pulmonary exacerbation are defined using clinical criteria that vary by institution. If clinically relevant threshold are established for methylation levels, these new epigenetic markers could be helpful to homogeneize pulmonary exacerbation definition and patient management across institutions. We also found that DNA methylation levels at specific CpG sites correlated either with lung function ($FEV1_{pp}$) or with longitudinal measures of lung function ($FEV1_{pp}$ variations). Overall, DNA methylation markers in sputum samples predicted pulmonary exacerbation, disease severity and lung function decline in patients with cystic fibrosis. These new markers may improve the follow-up of patients by refining patient classification and helping initiate the appropriate treatment in time.

P18

From epigenome-wide association studies (EWAS) To the functional analysis of differentially methylated regions in cystic fibrosis

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The general aim of this study is to identify genes and gene pathways responsible for the high degree of variability in disease severity, comorbidities and survival in cystic fibrosis. Twin studies showed that lung disease severity in CF patients depends almost equally on genetic and environmental factors. To address the role of non-inherited factors in cystic fibrosis, we carry out epigenome-wide associated studies (EWAS) in a cohort of CF patients and healthy controls, and functional analyses in selected differentially methylated genes.

The MethylCF cohort (51 CF patients F508del/F508del, 24 controls) was enrolled in four CF centers in the South of France (Montpellier, Hyères, Nice et Toulouse). DNA methylation was analysed with the MethylEpic array (genome-wide) and by pyrosequencing PyroMarkQ24 (selected CpG sites). Transcription levels were measured by RNAseq and qPCR. Functional analyses are carried out in human primary bronchial cells (Epithelix) and cell lines (Beas-2B, THP-1). The predicted *cis*-regulatory sequences (enhancers and promoters) are analysed with *in vitro* luciferase reporter assays and in LPS-treated cells to visualize endogenous regions. DNA methylation, RNA and protein expression are measured by pyrosequencing, qPCR and western blotting, respectively.

Herein, we show that DNA methylation was greatly altered in blood samples from CF patients and gain/loss of methylation correlated with clinical traits (disease, FEV1, BMI, diabetes). Of interest, the JAK/STAT pathway was particularly affected. Five top-ranked differentially methylated CpG sites were replicated by pyrosequencing. Following a bioinformatic analysis, we showed that methylation changes were overrepresented in predicted enhancers while also present in gene promoters. Currently, *in vitro* functional studies in human primary cells and cell lines are carried out to assess whether gain/loss of DNA methylation affect gene transcription. We generated preliminary results in differentially methylated regions associated with genes involved in cytokines regulation (*SOCS3*, *LYN*), apoptosis (*FADD*, *LYN*), immune response (*ATP11A, LYN*), calcium homeostasis (*ASPH*) and diabetes (*RGS1*). Specifically, 2 CpG sites at *SOCS3* (*Suppressor Of Cytokine Signaling 3*) were less methylated in blood samples from CF patients than controls. Methylation loss negatively correlated with lung function, and positively correlated with an increase of gene expression in blood samples. *SOCS3* was induced in LPS-treated human monocytes (THP1), but no methylation change was observed after a single treatment (acute exposure). Work is in progress to assess whether multiple LPS doses (chronic exposure) do change DNA methylation.

Overall, EWAS disclose new genes that are associated with clinical traits of interest in cystic fibrosis, however, functional analyses are needed to understand the molecular mechanisms responsible for these associations.

P19

Macrophages as a tool to study innate immunity and to develop a cell-based immunotherapy for cystic fibrosis

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People with cystic fibrosis (PwCF) have an increased susceptibility to lung infections. For a long time, it was thought that the absence or dysfunction of the CFTR ion channel in the airway epithelial cells, which leads to a build-up of mucus in the lungs, is instrumental in creating an ideal niche for opportunistic pathogens such as *Pseudomonas aeruginosa* to colonize the CF lungs. However, it has become known that alveolar macrophages, which form the first line of defence against airborne bacteria, are also impaired in CF.

Since there are no established cell lines for CF macrophages, we used induced pluripotent stem cells (iPSC) from CF patients and differentiated these into macrophage-like cells (iMacs). These iMacs can be generated in a standardized and scalable manner. iMacs from a CF patient homozygous for the p.Phe508del mutation were compared with those from a healthy donor (WT). Both, CF and WT iMacs showed morphology and the expression of surface markers typical for mature macrophages. Strikingly, CF iMacs showed a lower ability to phagocytose bacteria when cultured with *P. aeruginosa* for 6 hours compared to WT iMacs. Similarly, CF iMacs showed a delayed ability to efficiently kill intracellular bacteria compared to WT iMacs. Additionally, we found a higher lysosomal pH in CF iMacs and, upon *P. aeruginosa* infection, a potential mitochondrial fragmentation and a more hyperinflammatory phenotype compared to WT iMac.

In parallel to our investigations with human macrophages, we transferred murine WT macrophages derived from hematopoietic bone marrow stem cells, into the lungs of a CF mouse model before (preventive approach) or after (therapeutic approach) an intratracheal lung infection with *P. aeruginosa*. Twenty-four hours after infection, lungs were harvested, homogenized and inflammatory parameters and lung bacterial numbers were compared to control animals without pulmonary macrophage transfer (PMT). In both treatment strategies, PMT reduced the number of colony forming units in the lungs of mice, but only the preventive approach was effective in significantly limiting lung damage caused by the infection, as shown by lower hemoglobin levels in the broncho-alveolar lavage fluids and histopathological scoring.

In summary, our results are encouraging with regard to a preventive treatment strategy in which innate immune cells could be used in CF patients' lungs. Our studies on iPSC-derived macrophages show that CFTR plays an important role in these immune cells, making the iMacs an ideal model for the study of innate immunity in the CF lung and potentially leading to the development of a cell-based therapy.

P20

A modelling framework for epithelial airway fluid and ion transport with multiple cell types: implications for success or failure in gene therapies for cystic fibrosis

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Epithelial ion transport regulates the depth of airway surface liquid (ASL), a thin fluid layer (0.1-10 μm) lining the airway epithelium, enabling the vital mucociliary clearance of mucus-trapped pathogens in the upper airways. ASL homeostasis is regulated by a complex collaboration of ion channels, transporters, and tight junction proteins. In cystic fibrosis (CF), where this system is disrupted, the ASL becomes dehydrated creating sticky mucus that is difficult to clear, resulting in chronic lung infections.

Existing mathematical models of ASL regulation typically treat the system as a single, idealised cell containing all the important elements of fluid/ion transport. However, airway epithelia comprise patchworks of distinct cell types, each with specialised functions. To explore the implications of this for ASL homeostasis, we developed a multicellular quantitative framework to understand the bioelectric properties of healthy and CF epithelia and, in the latter case, the implications of cell diversity for gene therapy.

Our mathematical framework for modelling multicellular epithelia has its basis in the equivalent electrical circuit and direct modelling of ion fluxes for Na^+, Cl^- and K^+. The solution of the model provides numerical values of cellular/ASL ion concentrations, membrane potentials and cellular/ASL heights comparable to those reported in the literature. The modelling framework is quite flexible, such that cells can be made distinct by the presence/proportion of channels expressed on their surfaces. Lateral flow of ions between neighbouring cells was also incorporated into the model.

We began our examination of multicellular modelling by considering an extreme case where all essential secretory channels/transporters were included in one cell type whilst absorptive machinery was exclusively located within a second, neighbouring cell type. We found that even with exaggerated differences, cells could stably maintain internal ionic compositions and membrane potentials distinct from their neighbours.

Next, using a less extreme scenario, we examined ASL homeostasis in a two-cell model of healthy airway epithelium. This highlighted a key role for the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and basolateral Cl^- channels (ClC_{ba}). The presence of ClC_{ba} in the absence of NKCC resulted in a cell absorbing Cl^- from the ASL while a neighbouring cell with NKCC present and ClC_{ba} absent secretes Cl^-. Both cell types were modelled expressing apical CFTR, thus suggesting a cell type-dependent role in both Cl^- absorption and secretion.

Taking this model further, we simulated CF epithelia and subsequent gene therapy in multiple situations. First, we considered the case where perfect localisation of CFTR to the apical membrane occurs for both secretory and absorptive cell types. This improved ASL hydration, increasing ASL depth from 3 μm to ~6.0 μm, near the height of extended cilia. Next, we considered a scenario where CFTR overexpression led to mis-sorting of the protein via equal localisation to apical and basolateral membranes. This resulted in very limited hydration, increasing ASL depth to ~4.4 μm, and would not form a successful therapy.

We conclude that such models can help to elucidate the roles of different cell types in ASL regulation and inform the design and development of successful gene therapies.

P21

Detection of CFTR mRNA and protein in immune cells via quantitative real-time PCR and Western blot

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Background and Aims: The *Cystic Fibrosis Conductance Transmembrane Regulator* gene encodes for the CFTR ion channel, which is responsible for the transport of chloride and bicarbonate across the plasma membrane. Mutations in the gene result in impaired ion transport, subsequently leading to perturbed secretion in all exocrine glands and thereby the multi organ disease cystic fibrosis (CF). In recent years several studies report on CFTR expression also in immune cells as demonstrated by immunofluorescence, flow cytometry and immuno blotting. However, these data are mainly restricted to single cell populations and show a large variation depending on the used methodology. Here, we investigated *CFTR* transcription and protein expression by standardized protocols in a comprehensive panel of immune cells.

Methods: We applied a high-resolution Western blot protocol using a combination of highly specific monoclonal CFTR antibodies that has been optimized for the detection of CFTR in epithelial cells to healthy primary immune cell subpopulations sorted by flow cytometry and used immortalized cell lines as controls. Specificity of CFTR protein detection was controlled by peptide competition and enzymatic Peptide-N-Glycosidase-F (PNGase) digest. *CFTR* transcripts were analyzed by quantitative real-time PCR and normalized to the level of epithelial T84 cells as a reference.

Results:*CFTR* mRNA expression could be shown for primary $CD4^+$ T cells, NK cells as well as differentiated THP-1 and Jurkat T cells. In contrast, we failed to detect *CFTR* transcripts for $CD14^+$ monocytes and undifferentiated THP-1 cells as well as B cells and $CD8^+$ T cells. Prominent immunoreactive bands were detectable by immunoblotting with the combination of four CFTR antibodies, targeting different epitopes of the CFTR protein. However, in biosamples from non-epithelial origin, these CFTR-like protein bands could be unmasked as false positive by peptide competition or PNGase digest, meaning that observed mRNA transcripts were not necessarily be translated into CFTR protein which could be detected via immunoblotting.

Discussion: Our results confirm that mRNA expression in immune cells is many times lower than in cells of epithelial origin. The immunoreactive signals in immune cells turned out to be false positive and may be provoked by the presence of a high-affinity protein with similar epitope. Non-specific binding (e.g. Fab-interaction with glycosyl branches) might also contribute to false-positive signals. Our findings highlight the necessity of accurate controls such as CFTR-negative cells as well as peptide competition and glycolytic digest in order to identify genuine CFTR protein by immuno blotting. Our data furthermore suggest that CFTR protein expression data from techniques such as histology, for which the absence of a molecular weight or other independent control prevents unmasking of false-positive immunoreactive signals, has to be interpreted carefully as well.

P22

Pre-clinical data demonstrates great promise for AAV gene therapy, one dose of AAV1 or 6-Δ27-264 CFTR successfully ameliorated clinical symptoms in G551D ferrets after 5-weeks.

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Developing a gene therapy for CF is still a critical need especially for patients who either are not approved for corrector therapy or do not tolerate it. One way to show definitively that gene therapy can be therapeutic is to rescue defective CFTR function in a relevant animal model that recapitulates, as closely as possible, human disease. To accomplish this, we used the ferret model bearing the G551D mutation which responds to the potentiator, VX-770, similar to humans (1). The goal was to assess whether dosing of AAV1/6-Δ27-264-CFTR vectors administered to G551D ferrets in the absence of proteasome inhibitors leads to widespread gene transfer, expression and phenotypic rescue. The G551D ferrets were removed from VX-770 treatment 7 days before AAV treatment. 1 dose of 10^{11} vg of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR was sprayed into the airways and injected intravenously into eight, 3-month-old male and female ferrets. 2-G551D and 2-wild-type ferrets were kept as controls. Animals were necropsied at 35 days post-instillation.

AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR were detectable at ≥10^6 vg/ug-genomic-DNA in trachea, bronchi, lung, ileum, liver and pancreas. mRNA expression was detected at ≥10^6 copies/ug-genomic-DNA in trachea, bronchus, lung, pancreas and ileum and ≥10^5 copies/ug-genomic-DNA in liver. Widespread CFTR protein expression was seen in the tissues of all the treated animals above levels measured in untreated wild-type and G511D ferrets. CFTR protein expression above untreated animal levels following a single dose of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR suggests that transduction by AAV1 or 6 based vectors occurs in airways, liver ileum and pancreas of AAV treated G551D ferrets. The gross morphology of the treated ferrets resembled that of the normal ferrets compared to the untreated G551D ferrets. Minor amounts of mucous was present compared to untreated animals.

The results are important in that we were able to transduce trachea and lung despite the barriers associated with airway delivery that include thick airway mucous, AAV degradation and potential inhibitory factors in the sputum. Transduction of the G551D ferrets in the organs affected in CF provide promise for rescue of multiorgan disease in CF. Funded by NIDDK.

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P23

Pharmacological improvement of CFTR function rescues airway epithelial homeostasis and host defense in children with cystic fibrosis

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Rationale: Pharmacological improvement of CFTR function with elexacaftor/tezacaftor/ivacaftor (ETI) provides unprecedented improvements in lung function and other clinical outcomes to patients with cystic fibrosis (CF). However, ETI effects on impaired mucosal homeostasis and host defense at the molecular and cellular level in the airways of CF patients remain unknown.

Objectives: To investigate effects of ETI on the transcriptome of nasal epithelial and immune cells from children with CF at the single cell level.

Methods: Nasal swabs from 13 children with CF and at least one *F508del* allele aged 6 to 11 years were collected at baseline and three months after initiation of ETI, subjected to scRNA-seq and compared to swabs from 12 age-matched healthy children.

Measurements and main results: Proportions of *CFTR*-positivecells were decreased in epithelial basal, club and goblet cells, but not in ionocytes from children with CF at baseline and were restored on ETI therapy to near healthy levels. Single cell transcriptomics revealed an impaired interferon signalling and reduced expression of MHC I and II encoding genes in epithelial cells of CF children at baseline, which was partially restored by ETI. Additionally, ETI therapy markedly reduced the inflammatory phenotype of immune cells, particularly of neutrophils and macrophages.

Conclusions: Pharmacological improvement of CFTR function improves innate mucosal immunity and reduces immune cell inflammatory responses in the upper airways of children with CF at the single cell level, highlighting the potential to restore epithelial homeostasis and host defense in CF airways by early initiation of ETI therapy.

P24

Beyond Kaftrio: mechanistic insights to maximize N1303K-CFTR rescue

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Introduction: N1303K is the fourth most frequent Cystic Fibrosis (CF) causing mutation. We investigated the mechanism underlying N1303K-CFTR rescue by ELX/TEZ/IVA (ETI).

Methods: We studied CFTR expression and maturation with ETI combination by Western Blot and nanoluciferase HiBiT complementation assay. CFTR activity was tested in differentiated Human Nasal Epithelial Cells (HNEC) by Short Circuit Current in Ussing chamber and in CFBE41o- cells by YFP-Based Assay. We evaluated N1303K-CFTR maturation and activity under control condition or after incubation with different CFTR modulator combinations in the various cell models.

Results: ELX and TEZ combination increased N1303K-CFTR expression level, but not its maturation in transiently transfected HEK-293 cells, 16HBE cell lines and HNECs. Interestingly, in control condition, a small amount of N1303K-CFTR was detected at the cell surface. Upon treatment with ETI, N1303K-CFTR exhibited a significant increase in the total amount whithout increasing the proportion of channels at the cell surface in contrast to F508del-CFTR which exhibited a significant 3-fold increase of the cell surface ratio. Upon inhibition of protein synthesis by cycloheximide, in control conditions, N1303K-CFTR decreased over time less than F508del-CFTR. ELX/TEZ treatment slowed down the turn-over rate of both N1303K-CFTR and F508del-CFTR. Autophagy inhibition did not enhance N1303K-CFTR rescue by ELX/TEZ. N1303K-CFTR activity was variable in in HNECs. It was maximized by the combination of ETI with apigenin (API) in 16HBE cell lines and in HNECs. API addition to ETI improved Forced Expiratory Volume in 1 second by 10% in a N1303K homozygous patient.

Conclusion: N1303K causes an atypical maturation defect associated with a variable response to ETI. ETI efficacy is enhanced by API in the respiratory epithelium.

P25

Novel CFTR+ lung progenitor cells contribute to the dynamic developmental origins of fetal epithelial cell lineages

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Human fetal lung development remains largely unexplored. The scarcity of embryonic and prenatal lung tissues and the lack of appropriate human models have impeded efforts to study human-specific processes underlying fetal lung development. Consequently, the etiology of congenital lung diseases caused by cessation of lung development or the origins of progressive lung diseases that manifests early in neonatal life such as cystic fibrosis (CF), is poorly understood. Here, we sequenced >150,000 single cells using 3' 10X Genomics from 19 healthy human fetal lungs from gestational weeks 10-19. We outline a time course atlas of cells broadly categorized as epithelial, stromal, immune, endothelial and Schwann cell populations that make up the developing human fetal lung and identified a total of 58 unique cell types. Interestingly estimating RNA velocity reveal the existence of several fetal uncommitted progenitor cells that express abundant levels of CFTR and gives rise to ciliated, pulmonary neuroendocrine cell and basal/club cell lineages. RNA velocity-based inferences of cell lineage trajectories showed complex developmental dynamics emerging from a progenitor cell that express SCGB3A2+SFTPB+CFTR+ (Triple Positive, TP). This may be explained by temporal changes in cell-cell signalling and altering the microenvironment of signals driving specific cell fate during differentiation. We focused on Wnt signalling to the TP cells and show multiple cell-cell communications with these cells via Wnt/receptor interactions. Specifically, temporal changes in WNT2 ligand-receptor interactions suggest a role of Wnt2 in regulating TP cell fate. Previous studies have found a role of CFTR in regulating canonical Wnt/beta catenin pathway in intestinal stem cell niches and kidney development. We then used siRNA targeting *CFTR* in fetal lung explants and found changes in fetal epithelial differentiation and airway formation, with a concomitant increase in beta catenin. Our work reveals novel developmental trajectories of some epithelial subtypes and reveal how CFTR may regulate airway epithelial morphogenesis. Uncovering the role of CFTR in human lung development will expand our understanding of how dis-regulation of CFTR impact congenital disorders and affect disease pathogenesis.

P26

Characterization of the mechanism of action of approved and novel CFTR modulating drugs on G85E-CFTR protein activity and maturation

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Background: Cystic Fibrosis (CF) is a genetic disease due to a mutation that causes loss of function of the CFTR anion channel. The G85E is a class II variant that causes a severe folding and trafficking defect. According to the CFTR2 database, globally the G85E has an allelic frequency of 0.4%. In Italy this frequency increases, reaching 1.07%. At present, in Europe the G85E variant is still considered orphan of therapies, since it is not included among those for which CFTR-modulating drugs have been approved. However, G85E is included in the list of 177 variants for which the triple combination Elexacaftor/Tezacaftor/Ivacaftor (ETI) has been approved by FDA in the USA.

Aims: We conducted a functional and biochemical characterization of the G85E-CFTR variant through heterologous expression systems and ex-vivo models based on the use of patient-derived human nasal epithelial cells (HNEC) in order to investigate its responsiveness to approved drugs and novel preclinical compounds.

Methods: HNEC were obtained by nasal brushing of CF patients, processed and differentiated on Snapwell supports in air-liquid interface. Once differentiated they were used to carry out electrophysiological and biochemical analyses to evaluate the response to the different modulators. In parallel, we also exploited the YFP functional assay and analysis of protein stability on heterologous expression systems.

Results: Our study demonstrated that, as expected, treatment of primary airway cells with ETI causes a significant (but modest) rescue of CFTR function, that reaches 15-25% of the activity measured in epithelia derived from non-CF subjects. We also demonstrated a detrimental effect of chronic treatment with Ivacaftor on the rescue of the G85E variant, that may further limit clinical efficacy of this therapy. Among the compounds under preclinical development, we identified a novel combination of modulators that can rescue G85E-CFTR up to 25-35% of the CFTR activity displayed by non-CF epithelia with no evidence of negative effects upon chronic potentiator treatment.

Conclusions: Our studies suggest that ETI causes a very partial rescue of G85E-CFTR, suggesting that optimized modulators might be needed for this variant. Interestingly, our analyses highlighted the existence of novel combination of modulators endowed with higher efficacy and leading to increased rescue of G85E-CFTR maturation and activity.

Acknowledgements: 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).

P27

Extension of the Homology-Directed Repair (HDR) Editing window to repair all CF-causing mutations in Exon 12

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Precision gene editing using CRISPR is a promising therapeutic approach to treat genetic disorders, such as cystic fibrosis (CF). Double-stranded breaks induced by Cas9/gRNA can be precisely repaired by homology-directed repair (HDR) using DNA donors as templates. However, HDR strategies are limited by their inability to efficiently mediate repair beyond a very short distance from the target site. In genetic disorders caused by multiple mutations, such as CF, this limitation restricts the clinical application and therapeutic range of potential therapies.

Exon 12 of the *CFTR* gene contains 18 different CF-causing variants. While single CF-causing mutations have been corrected using HDR, extension of HDR to repair additional mutations has proved challenging. This is reported to be hindered by collapse of the D-loop formed between the DNA donor template and target DNA. We hypothesised that the high sequence homology typically shared between the donor and target DNA triggers early D-loop collapse[1]. We propose incorporation of silent mutations into the donor template will reduce sequence homology to a level that lets the D-loop remain open and continue HDR-driven repair. Here, we aim to test this hypothesis and develop a single donor template and gRNA with the capacity to efficiently repair all CF-causing mutations across exon 12.

The gRNA was targeted to intron 12 and HDR templates (HDRTs) with reduced target homology were designed using wild-type exon 12 as a template to which several base substitutions were introduced. Synonymous codons were used when making base changes, such that the HDRT DNA sequence differed to that of wild-type exon 12, yet the protein coding sequence remained the same. In parallel, a HDRT with a single base change in the PAM site was made to model conventional HDR. Three mutant cell lines were generated to test the HDRTs, with mutations located 5' (c.1585-1G>A), in the middle (G542X) or 3' of exon 12 (c.1679+1G>A). Thus, each cell line acted as a molecular ruler in which the extent of HDR-mediated repair could be measured. Cell lines were created using the HEK293-Flp-In system and expression-minigenes (EMGs) containing full-length *CFTR* cDNA[2]. *CFTR* EMGs were modified to include abridged introns 11 and 12 flanking exon 12 and an individual *CFTR* mutation.

Amplicon sequencing of HDRT- and Cas9/gRNA-transfected cells revealed HDR efficiency was similar across all three mutant cell lines when transfected with a HDRT containing multiple base changes (7.5% c.1585-1G>A, 10.3% G542X, 10.3% c.1679+1G>A). In contrast, using the conventional HDR model, editing was highest in c.1679+1G>A mutant cells (8.0%) and declined the further the mutation was located from the gRNA target site (4.7% G542X, 1.3% c.1585-1G>A). This preliminary data suggests the HDR editing window can be extended when a donor template with reduced target homology is used. This strategy holds promise as a therapeutic approach to repair all CF-causing mutations in exon 12.

[1]Byrne et al. (2015) *Nucleic Acids Research.* PMID:**25414332**

[2]Sharma et al. (2014) *Human Mutation.* PMID:**25066652**

We thank Dr Sharma and Dr Cutting for the HEK293-Flp-In cells and EMG-CFTR-cDNA plasmid. This work is supported by Science Foundation Ireland (20-FFP-P-8661).

P28

Rescuing G542X by Adenine Base Editing: A guide to restore function

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G542X is the 2nd most common CF-causing mutation. A premature termination codon (TGA) and the consequent nonsense mediated decay prevent CFTR protein synthesis and, thus, CFTR-mediated chloride transport. Here we outline a strategy to rescue this variant at the DNA level by gene editing and we describe the effects of modulators to boost CFTR activity following editing. Our approach involves Adenine Base Editing (ABE), a CRISPR-based system that allows the transition of A-T base pairs into G-C base pairs in a safe and efficient manner. Whilst ABE cannot precisely repair G542X (as this would require A-T to C-G editing), it can be used to convert G542X into G542R, an alternative CFTR variant that has previously been shown to retain approximately 30% of WT activity [1]. In our approach, a G542X-specific single-guide RNA (sgRNA) directs a highly catalytic base editor, NG-ABE8e [2], to edit the A on the opposite strand of the TGA codon into a G, converting the G542X stop codon to CGA, which encodes Arginine (G542R).

Editing of G542X into G542R by ABE was tested in patient-derived intestinal organoids, where the designed sgRNA and the selected Adenine Base Editor were delivered as ribonucleoproteins (RNPs) encapsulated in engineered Virus-Like Particles (eVLPs [3]), that deliver their cargo without replicating or integrating. Restoration of CFTR function was assessed by Forskolin-Induced Swelling (FIS) assay and/or Short Circuit Current (I_{sc}) assay, on multiple donors and in two different labs. Levels of DNA editing were detected by high-throughput sequencing.

Following transduction of intestinal organoids homozygous for G542X, FIS (after 7 days) and Isc assays (after 21 days) both showed restoration of CFTR function. FIS assay data analysis reported a significant swelling of treated organoids, up to 22% of the total (non-sorted) population. The percentage of swelling organoids was also eVLPs-concentration dependent and correlated with DNA editing levels. I_{sc} detected CFTR-mediated chloride transport, up to 10% of WT levels, and showed a clear response to Elexacaftor/Tezacaftor/Ivacaftor (ETI) treatment, which appeared to further increase G542R-CFTR activity. Amplicon-sequencing confirmed that CFTR functional rescue is specific to edited organoids only.

Our focus is now to optimise editing levels through the refinement of sgRNA design and eVLPs production. Our ultimate goal is to test this strategy *in vivo*, utilising suitable CF model systems of the disease, to evaluate its impact on the CF phenotype, and determine if there is any variation in editing efficiency in specific cell types.

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[2] Richter et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol. 2020

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P29

Correction of all Exon 12 variants using a template-jumping prime editing strategy

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Exon 12 of *CFTR* hosts a range of different classes of cystic fibrosis-causing mutations. While exon 12 is one of the shortest exons (1.5% of the *CFTR* mRNA), it harbours 7.1% of all *CFTR* mutations (CFTR2). G542X alone contributes to 2.5% of all CFTR mutations, however the remaining exon 12 mutations occur with much lower frequencies. Due to the low frequency of each of these mutations, correcting all exon 12 mutations with a single gene editing strategy is an attractive approach. Recently, a prime editing variation called template-jumping prime editing (TJ-PE) was developed with the potential to replace longer sequences, typically 100-1000 bp, of a target genomic locus (Zheng et al., 2023). In this system, a TJ-PE guide RNA (TJ-pegRNA) directs a prime editor (PE2) to the desired protospacer sequence located upstream of the first mutation in target region, creating a single-stranded nick in the genomic DNA. This generates a free 3'-flap which anneals to the primer binding sequence of the TJ-pegRNA, followed by reverse transcription of the reverse transcriptase template (RTT) by PE2. This nascent DNA sequence is integrated into the genome aided by a downstream sequence which anneals to a downstream 3'-flap generated by a nicking guide RNA on the opposite strand. A TJ-pegRNA with an RTT encoding exon 12 and a downstream sequence complementary to intron 12 should replace exon 12, therefore correcting all exon 12 mutations with a single pegRNA/nicking gRNA combination.

This concept was tested with a TJ-pegRNA aiming to delete 90 bp and insert 200 bp into a control locus in HEK293T cells. Using PEmax we measured 28% insertion efficiency, and sequencing confirmed that the RTT was inserted precisely between the two target protospacer sequences. Next, we designed TJ-pegRNAs to replace *CFTR* exon 12. We first designed gRNAs targeting intron 11 on the top strand and intron 12 on the bottom strand. These gRNAs were paired and transfected, followed by measuring synergistic efficiency in deleting exon 12. The intron 11 gRNA was used to design the spacer and primer binding site 1 sequence of the exon 12 replacement TJ-pegRNA, and the intron 12 gRNA was used as the second nicking gRNA for TJ-PE. The RTT of the exon 12 replacement TJ-pegRNA was subsequently designed, identical to the sequence length between the two nicks as well as retaining the sequence identity for wild-type exon 12. A second TJ-pegRNA was also designed with a shorter RTT, being the exon 12 sequence as well as 100 bp of both intronic sequences. We hypothesise that the shorter RTT sequence will improve editing efficiency with this strategy. The resulting TJ-pegRNAs could correct any exon 12 mutation in the endogenous CFTR gene, as well as the aid the development of *in vitro* cell models with specific exon 12 mutations.

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Acknowledgements

This work is supported by DRUMM22G0-COLLAB from the CFF Foundation.

P30

Characterization of CRISPR-Cas cell-derived vesicles for genome editing applications in cystic fibrosis

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Recent advancements in genome editing technologies resulted in the development of correction approaches for several Cystic Fibrosis Transmembrane Regulator (*CFTR*) mutations, proven efficient in Cystic Fibrosis (CF) patient-derived cell models. However, one major obstacle remains the *in vivo* delivery to airway cells.

We previously published a traceless CRISPR-Cas delivery tool, VEsiCas (Montagna et al., Mol Ther Nucleic Acids 2018), which we now are modifying for effective and precise delivery to the lung epithelium aiming at the advancement of gene therapy in CF. These cell-derived vesicles carrying the adenine base editor (BE) ABE8e-SpCas9, named BE-vesicles, showed efficient targeting of cultured Huh7 and CFBE41o- cell lines and base editing in multiple loci including *CFTR*.

Characterization of the BE-vesicles represents a crucial step to standardize the production to improve the delivery and genome editing efficacy of the cargo base-editor.

For BE-vesicles isolation, culture media of transfected cells was subjected to sequential centrifugation steps with final ultracentrifugation, and different isolation protocols based on ultracentrifugation were tested and compared.

Nanoparticle tracking analysis (NTA) was performed to determine particle concentration and size distribution. NTA experiments showed a heterogeneous particle size distribution, with the most represented populations between 100 and 150 nm. Different timing of cell transfection and BE-vesicles release have been tested, with 12 and 48 hours, respectively, as the optimal combination for the higher number of released particles, with no significant differences in mean and mode diameters.

Immunoblotting experiments on transfected cells and derived BE-vesicles revealed the presence of the expected specific markers typically enriched in extracellular vesicles. Consistently, cellular markers remained undetected, thus confirming the quality of the vesicles. Moreover, we obtained preliminary results on Cas9 quantification in BE-vesicles by ELISA assay of detergent-lysed vesicles. The analysis of non-denatured particles to evaluate the incorporation of Cas9 by imaging flow cytometry and the characterization with sgRNA quantification by qPCR of the BE-vesicles is currently ongoing.

The development of BE-vesicles tailored to the lung epithelium will be key to deploy genome editing technologies for the treatment of *CFTR* mutations causing CF.

P31

Functional correction of the CFTR 1717-1G>A splicing mutation using an adenine base editor with minimal bystander editing

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The 1717-1G>A is a prevalent *CFTR* Class 1 mutation, affecting around 1% of individuals with cystic fibrosis according to the CFTR2 database. This specific mutation disrupts a conserved AG dinucleotide at the 3' end acceptor splice site, leading to the skipping of exon 12 or the recognition of a newly formed cryptic splice site. Both alternative splicing products introduce a premature stop codon, preventing the correct synthesis of the CFTR protein.

This study aims to develop cutting-edge CRISPR/Cas-based genome engineering to correct the 1717-1G>A mutation, leveraging adenine base editing technologies.

To establish the CRISPR/Cas strategy, a cellular model of the 1717-1G>A mutation was generated using HEK293 cells. After searching for the best editing tool for the specific mutation we selected a strategy based on adenine base editing (ABE) technology, SpRY-ABE9, composed of an engineered PAM variant of SpCas9 and one of the latest versions of ABE. Through plasmid transfection, we obtained over 30% A>G correction using SpRY-ABE9, with minimal bystander editing and similar degree of CFTR recovery indicated by proper protein localization at the cell membrane.

To maximize the efficacy of the editing strategy through RNA delivery, we extensively optimized the sgRNA scaffold and mRNA capping encoding SpRY-ABE9. RNA delivery of the SpRY-ABE9 in 1717-1G>A patient-derived bronchial epithelial cells and intestinal organoids showed mutation correction exceeding 10% on the target adenine, corresponding to efficient restoration of CFTR function.

In summary, our data indicate that the recently developed ABE9 represents a promising strategy, balancing editing efficiency and minimizing bystander editing. This approach holds potential for restoring CFTR function, showing a significant advancement in addressing the 1717-1G>A mutation, which currently lacks pharmaceutical treatment options.

P32

CRISPR-ABE: a new strategy for the temporospatial control of editing to correct the W1282X mutation in the CFTR gene

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In recent years, new CRISPR editing tools have increasingly attracted the interest of the scientific community due to their double strand break-free editing capabilities that can significantly reduce Indel formation. In particular, Adenine Base Editors (ABE) have been successfully used to correct nonsense mutations in the *CFTR* gene. This approach uses a Cas9 nickase (nCas9) fused to a TadA enzyme that can convert any adenine (A) in the editing window into guanine (G). Consequently, this system may have a non-specific activity on the nearby adenines, known as bystander effects, that hinders its potential use.

One way to reduce unwanted edits on off-target sites is to minimize the duration of the nuclease activity by splitting Cas9 into two halves[1]. Using a similar strategy, we developed a Split-ABE version, consisting of two fusion proteins – NLS-FKBP12-Cas9(C)-NLS and TadA-NES-Cas9(N)-FRB – and showed this can dimerize and edit in presence of rapamycin. We have previously reported that this ability to temporally control base editing, also limits the bystander editing adjacent to the W1282X target site[2].

Encouraged by these results, here we describe the development of this system to include spatial control of the editing. Our approach is based on the integration of cell-specific promoters upstream of the Split-ABE cassette into a lentiviral vector (pLVX) through sequential cloning. This will allow expression of the Split-ABE in specific cell types in the airway epithelium.

We started by modifying the backbone of pLVX to replace the Puromycin resistance gene with the Hygromycin one and to eliminate restriction sites that would hamper later cloning steps. Then the Split-ABE cassette was isolated through digestion from the construct described above and ligated with the novel pLVX-Hygro. Lastly, the following promoters will be inserted upstream the Split-ABE cassette: KRT5, FOXJ1, FOXI1, MUC5AC and CC16 to control the expression of the CRISPR-Split-ABE system in the basal cells, ciliated cells, ionocytes, goblet cells and club cells, respectively.

The five final plasmids will be used to generate lentiviral particles in order to transduce a variant of the BCi-NS1 cells carrying the nonsense mutation W1282X. This cell line is an appropriate cellular model to evaluate the temporospatial control of editing due to its ability to differentiate into all the cell types of the airway epithelium.

In summary, here we present a strategy for the temporospatial control of ABE to correct the W1282X mutation, the sixth most common Cystic Fibrosis (CF) - causing variant, through a Split-ABE version. Our main long-term goal is to understand which cell types and how many of them need to be edited to rescue enough function of CFTR that can translate into clinical benefit for individuals with CF.

1. Zetsche B. et al. 2015. Nat Biotechnol. 33:139-42.

2. Santos L et al., 2022. 46th ECFS conference, Vienna, WS17.02.

Acknowledgements: Work supported by grant HARRIS21G0 from CFF and centre grants UIDP/04046/2020 and UIDB/04046/2020 (doi: 10.54499/UIDB/04046/2020) from FCT, Portugal (to BioISI).

P33

Site-specific gene targeting of chromosomal safe harbor and CFTR locus for correcting any CFTR mutation

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Objective: The development of CFTR modulator drugs was a major milestone in translating cystic fibrosis (CF) discoveries into treatment that benefits most CF patients. However, some people with CF do not respond to these new drugs, and around the world others with CF cannot afford them. Our long term goal is to develop a gene therapy that can benefit all patients. We plan to use a helper-dependent adenoviral (HD-Ad) vector to deliver a CRISPR-Cas9 system and a gene expression cassette as donor DNA to achieve permanent CFTR expression in lung epithelial cells. One of the unique advantages of our system is that following gene integration, there is no residual Cas9 expression, which eliminates the potential risk of the host immune system attacking the cells with gene integration.

Methods: We used pig cells and selected the GGTA1 (genomic safe harbor) and CFTR loci for testing our strategy. HD-Ad vectors do not have any adenoviral genes and have a large capacity for delivery. We pack gene editing cassettes for Cas9 and a guide RNA as well as a donor gene expression cassette (for reporter *LacZ* or human *CFTR* expression) in a single vector. Following the delivery of vectors into cultured epithelial cells, site-specificity of gene integration is determined by junction PCR and DNA sequencing. The reporter gene expression is analyzed by X-gal staining or quantification using chemiluminescent assays, while the CFTR expression is determined by Western blotting and the CFTR function is analyzed by FLiPR assays.

Results: We have previously shown success in targeting the pig GGTA1 locus. In this study, we focus on the pig CFTR locus. We demonstrated high efficiency in Cas9-mediated DNA cleavage by ddPCR and showed precise integration of *LacZ* or human *CFTR* expression cassettes into the pig CFTR locus by junction PCR and Sanger sequencing. We detected transgene mRNA and protein expression when the reporter or CFTR expression cassette was integrated. The efficiency of the *LacZ* integration into the CFTR locus is close to 10%, similar to that for the GGTA1 locus. We are in the process of performing FLiPR assays to assess the CFTR function.

Conclusions: These results validated the potential of our *CFTR* gene targeting strategy in therapeutic developments.We showed that Cas9 expression was eliminated following transgene integration, which is important for *in vivo* applications since Cas9 is a foreign protein and elimination of its expression would avoid immune responses to gene-corrected cells. We are working on strategies to further enhance the efficiency of gene correction in order to translate our strategy into *in vivo* applications.

P34

Journey to the Centre of the Airway: A VP22 cell-penetrating Base Editor as a novel tool for tackling lung basal cells' encasement

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Gene editing of the progenitor lung basal cells (BC) may have the potential to permanently correct any CF-causing mutation in the *CFTR* gene. Whilst luminal epithelial cells (LEC) can be readily transfected with DNA or RNA molecules encoding gene-editing constructs, the complex architecture of the lung pseudostratified epithelium (LPE) potentially causes an impediment to the direct targeting of BC. Considering this, we decided to determine if it is possible to deliver a gene-editing cargo to the LEC that could then transfer the gene-editing ribonucleoprotein (RNP) complex to the nuclei of BC cells below.

Inspired by previous studies[1] using HSV-1 viral protein 22 (VP22) cell-penetrating peptide, we developed VP22-GFP fusion proteins and a novel adenine base editor (ABE), VP22-ABE9-SpRY. We hypothesize that VP22-ABE RNPs may retain the VP22 intrinsic nuclear localization and intercellular trafficking capacity, thus being capable of travelling through the LPE and successfully delivering gene-editing complexes to BC.

Expression vectors encoding for fusion proteins VP22-GFP and VP22-ABE9-SpRY were designed using gBlocks encoding for a VP22 sequence and cloning them under control of a CMV promotor in GFP and ABE9-SpRY expression vectors, respectively. To initially characterise the pVP22-GFP vector, HEK293T cells were transfected and high levels of GFP expression were detected by flow cytometry 48 hours post-transfection. Moreover, a distinct nuclear localisation pattern of the protein was observed through fluorescence microscopy relative to a pGFP control vector that lacked VP22. To assess the base editing capacity of VP22-ABE9-SpRY, we initially targeted the *HEK2* gene in HEK293T cells and co-transfected the cells with VP22-ABE9-SpRY or ABE9-SpRY and the HEK2 gRNA. A-to-G editing efficiency of the A5 position was assessed 72h post-transfection with both BEs achieving similar levels of ~70%.

To test the cell-penetrating capacity of the VP22 vectors in a LPE model, we used an immortalized human airway basal cell line, termed BCi-NS1.1, which due to its multipotent differentiation capacity can be fully-differentiated for 28-days under air-liquid interface (ALI) culture conditions into different cell types of the LPE. Additionally, we developed a BCi-NS1.1 cell line with the *CFTR* W1282X variant, that when under the observation of 28-days ALI cultures allows for the identification of ciliated cells, however, the beating seems to be impaired relative to WT cultures, most likely due to increased mucus production.

Using these models, we will assess the A-to-G editing efficiency of VP22-ABE9-SpRY *vs.* ABE9-SpRY using different gRNAs in models of fully differentiated ALI cultures of BCi-NS1.1 WT and W1282X, that mimic the healthy and CF LPE. Briefly, 72h post-transfection ALI cultures will be detached and dissociated from the transwell inserts to allow for sorting of BC ($NGFR^{+ve}$) using flow cytometry. With this approach, we will compare editing levels using VP22-ABE9-SpRY *vs.* classical ABEs between the entire pool of cells and the BC ($NGFR^{+ve}$) cells, so as to determine the shuttle capacity of VP22 RNPs complexes into BC.

[1]Elliott & O'Hare, (1999) Gene Therapy PMID:10341888

We thank Dr. Umbach and Prof. Cereseto for pCMV-SpRY-ABE9. This work was supported by FCT (UI/BD/153376/2022) and the CF Foundation (HARRISP21G0)

P35
Base-editors delivery through fusogenic vesicles

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CRISPR-Cas9 editing tools have provided powerful and effective strategies to correct *CFTR* mutations in cellular models. However, future perspectives of *in vivo* applications are limited by the lack of efficient and safe delivery methods to target lungs, the most affected organ in Cystic Fibrosis (CF) patients. Promising delivery methods consist in viral derived vectors having efficient mechanisms of cellular entry and with tuneable tissue tropisms.

Aiming at developing a new and effective delivery system for genome editing agents to the airway epithelia for the treatment of CF, we recently exploited VEsiCas (VSV-G enveloped vesicles) generated in our lab (Montagna *et al*, Mol Ther Nucleic Acids, 2018). These vesicles have been demonstrated to efficiently deliver CRISPR-Cas9 as ribonucleoprotein complexes (RNPs) limiting the off-target editing and unwanted alteration in the genome due to transient CRISPR-Cas activity.

Here we adapted VEsiCas for the delivery of Adenine Base Editors (ABE) which have been demonstrated to efficiently reverse CF mutations in the absence of DNA double strand breaks (DSB). The incorporation of ABE was evaluated by measuring editing activity on different cell lines (CFBE41o-, Huh7, CaCo2, HEK293) and loci (PCSK9, TRAC, B2M, CFTR). We observed an efficient editing activity among different sgRNAs tested, thus suggesting an advantageous target flexibility of the VEsiCas delivery system.

Finally, in order to optimize VEsiCas tropism towards the lung tissue we are focusing in substituting the VSV-G with viral envelops with higher target compatibility with airway epithelial cells, namely base editor vesicles (BE-vesicles). To explore various viral envelops we initially tested pseudotyped lentiviral vectors by measuring GFP lentiviral transgene delivery in CF immortalized cell lines (CFBE41o-). Selectedcandidate envelopes, are currently under evaluation.

The development of BE-vesicles with high loading and fusogenic potentials for airway epithelial cells is an important groundwork for gene therapy advancement to treat mutations causing CF particularly those that are not sensitive to CFTR modulators developed for the clinic.

P36

Optimization of Gene insertion strategies for restoration of CFTR expression in airway epithelium

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Objective: Our objective is to develop a universal gene editing strategy capable of curing individuals with Cystic Fibrosis (CF). CF can be caused by a diverse set of mutations distributed across the 250 thousand base pairs of the *CFTR* gene. Because of the large number of mutations already reported, there is a strong interest in directly targeting either a partial or full-length *CFTR* cDNA into the endogenous *CFTR* locus, rather than developing mutation-specific approaches. This strategy has the advantage of correcting or compensating for all *CFTR* mutations downstream of the integration site. If this can be achieved while retaining the native *CFTR* chromatin structure and regulatory sequences, it also has the possibility of restoring appropriate cell-type specific expression. We have previously demonstrated the ability to efficiently target integration of AAV-delivered partial *CFTR cDNAs* into *CFTR* introns 7 or 8 of CF airway basal cells with restoration of CFTR expression and function in the derived airway epithelium (Suzuki et al. Mol Ther 2020). To correct for all or nearly all *CFTR* mutations, it likely would be necessary to target integration of the partial *CFTR* cDNA sequences into the most upstream regions of *CFTR* sequences. In this regard, we are seeking to develop a single donor vector capable of efficient *CFTR exons 2-27 (CFTR2-27)* integration into intron 1 (employing a synthetic splice acceptor [SA]) or exon/intron 2 (utilizing the native exon 2 SA) enabling appropriately regulated CFTR expression.

Methods: We have recently performed a comparative evaluation of targeted integration into a variety of sites distributed across intron 1, exon 2 and intron 2 with the goal of identifying target sites that can be efficiently targeted -- and transgene constructs that give rise to robust expression. We incorporated two reporter cassettes in the donor construct, *pgk-mScarlet* for selection of targeted cells and a chimeric *CFTR-luciferase* to read out levels of expression. This enabled us to perform a comparative assessment of levels of transgene expression in fully differentiated airway epithelia derived exclusively from targeted cells.

Results: Our study has revealed clear differences in expression level as a function of integration site further modulated by the choice of poly adenylation (polyA) sequences. For those integration sites and polyA sequences exhibiting highest luciferase expression, we have also been evaluating delivery and expression of the desired *CFTR2-27* transgene. Preliminary results suggest that even modest targeting efficiency at select sites can restore *CFTR* activity to approximately 10% of wild-type. Since some of the transgene constructs exceed AAV packaging limits, we have been evaluating alternative strategies to deliver *CFTR2-27*.

Conclusions: Although this project is being performed in the context of AAV-mediated delivery and homologous recombination-mediated integration, we anticipate that this examination will be highly informative irrespective of the delivery vector used or the precise method of integration. Furthermore, we are interested in evaluating how these findings in ex vivo can be translated into direct in vivo targeting of CF airway epithelium.

Funded by Cystic Fibrosis Foundation (DAVIS19XX0)

P37

Functional analysis of gene-edited CF variant G542X.

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The Cystic Fibrosis (CF) causing variant G542X (**G**GA>**T**GA) results in premature termination of translation of the cystic fibrosis transmembrane regulator (CFTR) protein, and nonsense-mediated decay of the CFTR mRNA resulting in almost complete loss of functional CFTR protein expression. This leads to defective anion transport and the development of CF disease pathology. Currently available CF modulator therapies cannot be used to treat this variant, but an Adenine Base Editor (ABE) and guide RNA combination developed and optimized by P. Harrison, L. Nicosia and K. Doran-Cavusoglu can convert the G542X stop codon to G542R, a variant which retains about 30% of WT activity and is amenable to modulator therapy. The plasmids encoding this ABE and guide RNA were encapsulated in lipid-based nanocomplexes developed by S. Hart and M. Greenwood, which have demonstrated reduced immunogenicity and high uptake in epithelial cells, were delivered to human bronchial epithelial cells (HBECs) harboring the G542X CFTR variant. Ion transport was measured as short circuit current (Isc) across resistive epithelial monolayers. Cultures were equilibrated standard using standard physiological salt solution before addition of pharmacological drugs, forskolin, 10 μM (bilateral) to activate CFTR; CFTRInh-172, 10 μM (apical) to inhibit CFTR; and ouabain, 100 μM (basolateral) to inhibit Na+K+ATPase activity. 14 days post transfection with nanoparticles, Sanger sequencing showed 17% of alleles had been edited to render the variant treatable with modulators. Isc measurements showed that even as little as 17% of editing (heterologous mixture with unedited G542X cells) partly restores CFTR activity to 60% that of normal levels when in combination with modulators (n = 6). This is demonstrated in the comparatively greater CFTRInh-172 inhibitable current compared to G542X samples (p=0.015). This data provides proof-of-concept for partial restoration of anion transport by gene editing of G542X.

P38
Comparative study of readthrough molecules

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Around 10% of mutations causing cystic fibrosis are nonsense mutations. These class I mutations are among the most impactful and are not addressed by CFTR modulators. It is possible to restore the expression of a gene carrying a nonsense mutation by using molecules capable of activating readthrough of the premature termination codon. Several molecules having such activity have been reported for decades. These molecules were identified by different labs and tested on very different cellular models, not allowing a direct comparison between the readthrough molecules. It is therefore very difficult to rank them according to their effectiveness. We carried out a comparative study to evaluate the effectiveness of around ten readthrough molecules (including, ataluren, ELX-02, DAP or TLN468) on identical cellular models and on the three stop codons (cell lines expressing firefly luciferase mRNA carrying a nonsense mutation and organoids derived from cystic fibrosis patient cells carrying a nonsense mutation in CFTR). We were thus able to determine the effective concentrations of each of the molecules tested, the corrected stop codon preferences and the contribution of nonsense-mediated mRNA decay to the readthrough efficiency. Our results show that efficiency can be sensitive to the model used. However, we observe 3 groups of molecules: the first comprising the molecules which do not show activity under our experimental conditions, a second group in which a readthrough activity is measured homogeneously between the molecules and a third group containing a few molecules who surpass others. Following the results of the clinical trials of ataluren and ELX-02 which concluded a lack of effectiveness of these two molecules, this type of comparative study could possibly be predictive of success in the clinical trial phase.

P39

Optimization of blocking oligonucleotides as therapies to correct consequences of deep intronic mutations

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Introduction: Since years, several CFTR therapeutic strategies have been approved and the number of *CFTR* mutations targeted by therapeutic agents is increasing. Nevertheless, approximately 10%-20% of patients remain not eligible to CFTR modulators, particularly patients with splicing mutations (~12% of the reported *CFTR* mutations). We specifically focused on deep intronic mutations that induce aberrant splicing patterns, mostly the insertion of an intronic region in the mRNA sequence (cryptic exon, CE), generally leading to creation of a premature termination codon. We used an approach of modified oligonucleotides (Target Site Blockers, TSB), to specifically target and block deep intronic mutations effect through their binding on aberrant splicing sites of pre-mRNA. We have designed TSBs for 10 deep intronic mutations and we assessed their effectiveness on minigene plasmids by RNA analysis. Now, we developed a luciferase minigene assay to optimize TSB efficacy for correctly regulating pre-mRNA splicing in bronchial cells.

Materials and methods: We previously constructed minigene plasmids containing intronic sequences (400-600pb) encompassing either the normal or the mutated sequence for various intronic mutations. Chemically modified TSBs (*Locked Nucleic Acid*, LNA) were specifically designed for each deep intronic mutation included in this study, and treatments at different concentrations (from 20nM to 1uM) were performed on cells. Various cellular models have been used: Beas 2b transfected with minigene plasmids and primary cultures from CF patients' nasal cells cultured in monolayers or in epithelium (ALI, Air Liquid Interface). Efficiency of AONs to prevent inclusion of CE was assessed by analyzing RNAs and proteins extracted 24h and 48h post-transfection (by RT-PCR, fragments analysis or western blot).

Results: Minigene assays showed that oligonucleotides were efficient to block inclusion of CE for c.1680-883A>G, c.1680-886A>G, c.3469-1304C>G, c.3874-4522A>G and c.1585-9412A>G mutations, with an increase by 20-30% of normal mRNA rate, up to 70% for c.1680-883A>G and c.1680-886A>G mutations at low concentration (50nM), in bronchial cell lines and in primary nasal cultures. These promising results lead us to address the issue of the rapid TSB optimization including the choice of chemical modifications added on ribose constitutive of TSB and of the agents of delivery, to efficiency generate wider proportion of WT mRNA. To help us in screening all these TSB, we have elaborated new minigenes containing *Luciferase* gene sequence interrupted by mutated intronic sequences. Thus Luciferase activity would be detected only in normal splicing conditions and might be screened in 96-wells plate, reducing cost and turnaround time in lab. We next designed TSB with different chemical modifications already published and/or used in clinical trials (*Locked Nucleic Acid*, LNA ; *O-Methoxyethyl*, OME ; *O-Methyl*, OM...). We also aim to vary proportions of modified nucleotides as well as their positions in TSB sequence and to redefine the optimal TSB concentration and the internalization method.

Conclusion: Our findings demonstrated the efficacy of TSB-based strategy to restore normal *CFTR* mRNA and we propose optimizing TSB design. TSB-based strategy is an appropriate manner to offer new therapy purposes for CF patients harboring deep intronic mutations.

P41

Enhanced rescue of CFTR nonsense mutations under inflammatory stimuli

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In cystic fibrosis (CF), premature termination codons (PTCs) hamper the correct CFTR protein translation, producing severely truncated forms of the CFTR chloride channel insensitive to present CFTR modulators (correctors, potentiators). A possible strategy to targets PTC is the pharmacological read-through (RT) achieved by compounds that act on the stalled ribosome at the PTC site, allowing continuation of the protein synthesis. Such correction is limited by the nonsense mediated mRNA decay (NMD), that decreases the amount of mutated-mRNA susceptible of pharmacological RT. Recently, small molecules acting as eRF3 degraders, such as CC-90009, have been reported as effective on PTCs (1,2). As we previously reported (3), PTCs differently respond to rescue maneuvers, with the G542X as the most sensitive PTC to RT agents, and the W1282X as the most responding mutation to NMD inhibition. We evaluated CC-90009 as a RT agent and/or NMD inhibitor. In 16HBE14o- cells, we found little effect of CC-90009, by itself or in combination with other compounds, in rescuing W1282X-CFTR. In contrast, CC-90009 was effective on G542X-CFTR. Using short-circuit current (Isc) recordings on 16HBE14o- cells expressing G542X-CFTR, we found a significant CFTR rescue by CC-90009 in combination with a RT agent. We confirmed these results on primary human bronchial epithelial cells (HBECs) from a G542X/G542X patient. Isc recordings showed that the triple combination of CC-90009, VX-809 as a corrector and G418 as RT agent, elicited a CFTR function that was nearly 40% of that measured in non-CF HBECs. Our data suggest that CC-90009 likely behaves not as RT agent itself, but rather cooperating with RT agents in a synergistic way. Intriguingly, the same drug combination elicited negligible activity in G542X/G542X nasal epithelial cells (HNECs) derived from the same patient.

It has been reported that the inflammatory cytokines TNFα and IL-17A enhance the efficacy of CFTR modulators in rescuing F508del-CFTR in HBECs (4). We asked if inflammation could also influence correction of PTCs. We carried out Isc recordings on G542X/G542X HBECs treated for 72 h with IL4 or TNFα/IL-17. Cells were also treated with or without a triple compound combination (CC-90009, VX-809 and RT agent) in the last 24 h before Isc experiments. We found that cytokine-treated epithelia showed an enhanced response to the triple compound combination, with the best condition (IL-4 plus triple combo) leading to a nearly 90% of normal CFTR function. We are currently investigating the molecular basis of this behaviour, both at the transcriptional and protein levels. Moreover, ongoing analysis by Fluorescence Recovery After Photobleaching (FRAP) experiments will be informative on the effects of these treatments on airway surface liquid (ASL) properties.

This work is supported by Cystic Fibrosis Foundation (GALIET22I0) and by an ECFS/CFE postdoctoral fellowship (to Arianna Venturini).

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P42

Upregulation of a Nonsense Mediated Decay (NMD) insensitive CFTR mRNA isoform has therapeutic potential for the treatment of 3' CFTR PTC variants

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Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR) protein. Of the 2000+ documented *CFTR* gene variants at least 719 are established CF-causing variants with an additional 49 variants of varying clinical consequence (https://cftr2.org/). Based on genotype, ~90% of PwCF can now benefit from highly effective modulator therapy (HEMT) directed at the underlying cause of their disease; however, the remaining 10% have *CFTR* variants including nonsense mutations that are not approved for current modulator treatments.

Nonsense or Premature Termination Codon (PTC) variants are challenging to address therapeutically, with currently no available therapies. PTC mutations generally elicit Nonsense-Mediated mRNA Decay (NMD) resulting in 80-90% reduction of full-length mRNA abundance. The remaining CFTR mRNA may give rise to lowly expressed truncated protein which is often non-functional and/or a small amount of full-length protein by PTC readthrough. The reduction of mRNA via NMD is a hallmark of the PTC disease pathology and a major hurdle for any PTC therapy.

Previously we showed that a naturally occurring, lowly expressed truncated CFTR mRNA transcript (e22 trunc) resulting from utilization of an alternative polyadenylation (APA) site in intron 22 is resistant to NMD and yields expression of a truncated CFTR protein that has partial function when treated with modulators. In immortalized 16HBEge cells expressing CFTR W1282X, this truncated mRNA can be upregulated via ASO blockade of exon 22/23 splicing to near wild type levels of full length CFTR, resulting in rescue of ~ 25% WT function (50 AUC/min [uA/cm^2] when combined with CFTR modulator treatment.

As therapeutic proof of concept, we have now translated the approach of ASO-mediated blockade of exon 22/23 splicing to the gold standard for therapeutic development, primary CF hBE cells, for this study hBEs of the CFTR W1282X genotype. We have optimized steric blocking splice donor (SD) and splice acceptor (SA) ASO dose levels and exposure times for air-liquid-interface (ALI) cultures to yield e22 trunc mRNA levels approaching those of WT FL CFTR mRNA. The expressed truncated protein product is trafficked to the plasma membrane and in functional studies in presence of a CFTR potentiator we observed Cl^- transport of ~25% WT function (9.32 AUC/min [mA/cm^2]), which is well within the therapeutically relevant range. This work provides evidence for a novel and promising approach for the treatment of PwCF harboring W1282X and possibly other PTC variants near the 3' end of CFTR.

P43

Identification of novel pharmacological inhibitors of nonsense-mediated RNA decay to rescue CFTR with premature termination codons

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Background: Many patients with cystic fibrosis (CF) currently benefit from the pharmacological therapy with Kaftrio®. However, there is still a significant percentage of CFTR mutations that are insensitive to CFTR modulators, like premature termination codons (PTCs). The rescue of CFTR with PTCs faces two limiting steps: 1) the nonsense-mediated RNA decay (NMD) mechanism that, by degrading mRNAs carrying PTCs, strongly reduces CFTR transcript levels; 2) PTCs cause the arrest of protein synthesis, with the production of a truncated and nonfunctional CFTR protein. The combination of NMD inhibitors with readthrough agents, which overcome the translation arrest, can maximize rescue of CFTR with PTCs. NMD inhibitors can also be effective in the absence of a readthrough agent for PTCs localized at the carboxy-terminus of the CFTR sequence such as W1282X. Our aim is to find novel compounds able to modulate NMD mechanism without interfering with readthrough, by screening a chemical library with a CFTR functional assay.

Methods: The 16HBE14o^{-} cell line with W1282X (obtained from CFF) was stably transduced with the halide-sensitive yellow fluorescent protein (HS-YFP). These cells are used to screen a chemical library containing more than 9,000 approved drugs, investigational drugs, and compounds with known biological activity (mechanistic probes). The most active compounds are then studied with secondary biochemical and functional assays to clarify their mechanism of action and to assess their effects on other PTCs. The most promising compounds will be also tested with short-circuit current recordings on airway epithelial cells derived from people with CF, alone or in combination with readthrough agents and CFTR modulators.

Results: Until now, we have screened ~7,500 compounds, and different positive hits have been identified. Among these, we found a molecule (NMDi-01) that induces 4 times as much CFTR activity compared to negative control. We tested NMDi-01 at multiple concentrations to generate a dose-response relationship and assess its potency (EC_{50} = 0.65 μM). We conducted additional experiments to establish its mechanism of action. NMDi-01 was ineffective on F508del-CFTR, suggesting that it acts as a real NMD inhibitor and not as a general CFTR gene transcription enhancer or amplifier. When we evaluated NMDi-01 effect on W1282X-CFTR at transcriptional and protein level, we found that this compound causes a nearly 15-fold increase in CFTR transcript levels and promote W1282X-CFTR function by markedly enhancing the protein expression. These effects are compatible with strong NMD inhibition. In parallel, other hits from the screening are being characterized. Most active compounds belong essentially to two main groups: proteasome inhibitors and kinase inhibitors. These compounds work in the submicromolar or low micromolar range. For kinase inhibitors, experiments of phosphoproteomic analysis are currently ongoing to identify the site of action and the specificity of compounds.

Conclusions: We expect to discover NMD inhibitors and novel biological targets and processes associated with NMD that could increase CFTR rescue in patients with PTCs.

Acknowledgements: This project is supported by CFF (GALIET22I0) and the European Cystic Fibrosis Society / CF Europe (post-doctoral research fellowship to Arianna Venturini).

P44

Exploring novel therapeutic targets: Unraveling signatures linked to differentially expressed genes and proteins in G542X-CFTR

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Background: The *CFTR* gene encompasses over 2,100 documented genetic variants, grouped into classes (Class I-VII) based on their impact on the CFTR protein. Among these, F508del, a Class II variant, stands out as the most prevalent mutation in persons with cystic fibrosis (pwCF). While modulators are available for the treatment of F508del (and several other variants), approximately one in ten pwCF still lacks a specific treatment, including those with nonsense mutations.

Nonsense mutations (Class I) profoundly disrupt protein production, with 177 such mutations identified. These mutations introduce premature stop codons (PTCs), leading to the activation of nonsense-mediated decay (NMD), a cellular process that degrades mRNA containing PTCs. Notably, the G542X mutation (c.1624G>T) is the most prevalent among nonsense mutations, further underlining the need for targeted therapeutic interventions [1].

Managing CF goes beyond the prevalent F508del mutation. This underscores the critical need for customized strategies, especially when dealing with the array of diverse mutations found in affected individuals. Understanding the intricacies of CFTR mutations is imperative for the progress of personalized treatment modalities. Such insights play a pivotal role in advancing therapies that can effectively meet the requirements of pwCF.

Our aim here is to understand the network of differentially expressed transcripts and proteins in cells bearing nonsense mutations that might contribute to NMD of CFTR transcript and the absence of protein in these cells.

Methods: We used transcriptomics and proteomics datasets from 16HBE14o- gene edited cell lines with WT and 6 different CF-causing variants [2] and focused on G542X mutation specific signatures. For validation, we used quantitative RT-PCR and targeted proteomics.

Results: We identified 201 differentially expressed transcripts and 35 differentially expressed proteins unique for G542X. Gene Ontology Enrichment analysis reveals that GO Biological process terms such as “regulation of transcription” and “regulation of RNA metabolic process” are most representative for the transcripts whereas “Protein Biosynthesis” and “RNA-binding” are enriched for proteins – suggesting the association of these datasets with processes affecting the expression of CFTR mRNA in cells bearing G542X-CFTR.

Ongoing validation of the identified transcripts revealed 4 upregulated, and 3 downregulated genes. We are currently assessing the effect of knocking down the 4 upregulated genes in the overall CFTR mRNA and protein levels to ultimately provide new targets for pharmacological modulation. The most promising targets will also be used in combination with other strategies to identify synergistic and additive effects that can be explored in pwCF that currently have no treatment options available.

Conclusions: Our study will allow us to test various strategies to correct nonsense mutations and uncover a combined approach where our identified targets lead to NMD inhibition and PTC readthrough to rescue CFTR in these individuals.

Acknowledgements: Work supported by Emily’s Entourage and center grants to BioISI UIDB/04046/2020 and UIDP/04046/2020 (https://doi.org/10.54499/UIDB/04046/2020) from Foundation for Science and Technology, Portugal.

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P46

Targeting ATP12A proton pump provides new therapeutic opportunities for cystic fibrosis

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In cystic fibrosis (CF), the loss of function of CFTR causes multiple airway defects, as dehydration and acidification of the ASL, and mucus accumulation, which impair mucociliary transport and favor the chronic infection and inflammation of the lungs [PMID: 34090606]. Correction of CF abnormalities may be obtained by restoring the function of CFTR with mutation-specific treatments or through airway physiology-directed therapies. Indeed, it has been speculated that beneficial effects could be obtained by modulating other channels and transporters in order to increase CFTR-independent anion secretion (TMEM16A, SLC26A9, SLC26A4) or inhibit acidification (ATP12A). Despite the increasing interest in these targets, their location in the airways and their real contribution to epithelial homeostasis is poorly known and sometimes controversial. Moreover, specific modulators are still lacking.

In this study, we aimed to define the precise expression and role in the airways of TMEM16A, SLC26A9, SLC26A4, and ATP12A and to find drug candidates for the most promising targets.

We used *ex-vivo* human samples and advanced *in-vitro* models to assess the targets' expression by spatial biology. RNAscope *in situ* hybridization in healthy lung sections revealed a higher expression of ATP12A and SLC26A4, with a proximal--distal gradient, while TMEM16A appeared with a very low expression in human bronchi and was undetectable in more distal regions. SLC26A9 was absent in most epithelial and glandular cells of the airways, except for rare epithelial cells (< 1%) showing high expression. By mRNA-protein co-detection we identified these cells as pulmonary neuroendocrine cells (PNECs). Then, we investigated alternative targets expression in asthmatic and CF airways by RNAscope on asthmatic lung sections and by immunofluorescence on nasal brushings collected from CF and non-CF individuals. The expression of all targets, except SLC26A9, was found increased under these chronic inflammatory conditions.

To investigate the alternative targets' role in the airways, based on the findings obtained in native tissues showing different expression patterns along the respiratory tract, we used *in vitro* models resembling large and small airways epithelia, including non-CF and CF nasal epithelia, and epithelia generated from immortalized bronchial and bronchiolar basal cells (BCi-NS1.1 and SABCi-NS1.1). In all models, TMEM16A, SLC26A4, and ATP12A were induced by inflammatory conditions (IL-4 or IL-17 + TNFα), independently of the CF genotype, whereas SLC26A9 was poorly detected. Then, a series of microscopy-based assays highlighted a prevalent role of ATP12A in controlling ASL properties, with its inhibition producing effects considered beneficial for CF: less acidic pH, increased thickness and decreased viscosity. Accordingly, we initiated the search for drug-like modulators by developing a novel *in vitro* model with functional expression of ATP12A suitable for high-throughput screening purposes. The screening of a library of antisense oligonucleotides (ASOs) targeting human ATP12A produced several hits able to decrease ATP12A expression and proton secretion.

In conclusion, our study evidenced ATP12A as a promising target for CF. Importantly, we found antisense-drug candidates able to suppress ATP12A expression, a potential CFTR-independent approach to normalize ASL properties in CF airways.

This study was supported by Vertex CF Research Innovation Award and Italian Cystic Fibrosis Foundation

P47

SLC26A9 modulators identified through High Throughput Screening (HTS)

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Background: Enhancing non CFTR-mediated anion secretion has been proposed as a mutation-agnostic therapeutic approach for the treatment of cystic fibrosis (CF) and could be effective for minimal function genotypes without approved CFTR modulators. Human population genetic evidence identified SLC26A9 as a modifier of CF pancreatic disease and lung function. SLC26A9 is an epithelial chloride transporter that is expressed mainly in airway and gastric epithelia and assumed to contribute epithelial surface hydration. Here we aim to identify small molecules activating SLC26A9.

Methods: To identify SLC26A9 modulators, we carried out a high-throughput screening (HTS) campaign of >100,000 chemically diverse compounds using a fluorescent cell-based assay which measures membrane potential (MP) to monitor ion transport in GripTite 293 MSR cell line generated with inducible expression of human SLC26A9. After the primary screen, we implemented a computational hit-to-lead design for the prioritized compounds. We employed SiteMap, a computational tool, to identify potential drug-binding pockets on SLC26A9. To enhance the reliability of these findings, we integrated homology-based deep learning methods (DiffDock), which help validate the proposed binding sites. Following this, we utilize molecular docking and similarity-based scoring techniques to navigate the vast realm of molecular possibilities in the Enamine REAL Space. This approach enables the selection of top candidate molecules that show promising potential as SLC26A9 agonists. To analyze lead candidates, the parent compounds along with their derivatives have been examined for their potential effect on SLC26A9 using the fluorescent MP assay. cAMP-Glo Max Assay was used to assess intracellular cAMP levels.

Results: The HTS was robust with a mean plate Z' value of 0.65, mean signal to baseline (S/B) of 2.65 and the overall rate for identification of activators was 0.015%. Fifteen potential compounds have been prioritized as SLC26A9 agonists based on enhanced MP responses, significant dose-response curves and drug-like properties. These compounds did not induce MP response in counter screens using cells without SLC26A9 expression and did not stimulate cellular cAMP levels in CFTR-expressing 16HBE14o- and Capan-1 cells suggesting no indirect CFTR activation. Most of the 15 compounds reproduced their agonistic effect in H2122 lung and CFPAC-1 pancreatic cell lines with endogenous expression of SLC26A9. Specificity of four of the most potent agonists was verified using RNAi knockdown experiments against SLC26A9. Moreover, MP assay results in 16HBE14o- cells indicated that most of the 15 compounds at their EC50 concentrations have no impact on FSK-induced CFTR response. Analyzing derivatives of prioritized compounds indicated that 6 derivatives from 6 different parental compounds enhanced SLC26A9 response. The EC50 determination of the selected derivatives indicated high potency compounds.

Conclusion: The identified compounds will provide useful research tools to elucidate the roles of SLC26A9 as a potential target for CF therapy. Further optimization of SLC26A9 agonists for potency and pharmacological properties are in progress to select compounds for Ussing measurements in human airway epithelial cell cultures and mucociliary clearance assays.

P48

Identification of novel epistatic modifiers that influence CFTR folding trajectory through ribosomal interaction

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CF drug discovery efforts have resulted in generation of elexacaftor-tezacaftor-ivacaftor (ETI) and other corrector/potentiator therapies clinically approved for F508del and ~170 additional CFTR variants. Many patients receiving CFTR modulators have experienced significant improvement in health outcomes, but a substantial portion of the global CF population does not benefit from these small molecules. It is imperative to continue developing new therapeutic options that address underlying genetic defects not only for patients with the most common form of CF, but also for individuals carrying rare CFTR variants.

Our group has utilized an innovative approach to identify novel genetic modifiers of ABC-C protein processing by modeling different subclasses of CFTR variants in a *Saccharomyces cerevisiae* phenomic system. Mutations analogous to those observed in people with CF are introduced into the CFTR homologue, yeast oligomycin resistance-1 (YOR1), which faithfully reproduce pathogenic defects associated with corresponding CFTR variants encoded in human cells. For example, the F508del-CFTR equivalent (yor1-F670del) confers protein instability, misfolding, endoplasmic reticulum (ER) retention, and diminished function. Synthetic genetic arrays are performed with these constructs individually mated to ~5,000 knockout/knockdown single mutants from the yeast deletion strain library (YDSL), with enhanced oligomycin resistance serving as an indicator of restored ABC-C protein activity. In the case of yor1-F670del, improved processing has been observed following depletion of specific ribosomal proteins such as RPL12/uL11. Using siRNA, we have also shown reducing mammalian RPL12 levels by ~50% robustly augments functional expression of F508del-CFTR in polarized monolayers of Fischer rat thyroid (FRT) cells, CF bronchial epithelia, and primary human airway epithelia.

In the present study, we conducted a second *S. cerevisiae* screen to discover cellular factors that further modulate the RPL12-silencing effect on F508del biogenesis. A yor1-F670del/rpl12a double mutant was crossed with YDSL single gene knockout/knockdown strains for genome-wide assessment of RPL12 epistasis in the context of yor1-F670del functional rescue. Phenomic data revealed numerous RPL12 interactors involved in pathways such as ER-associated protein folding and 80S ribosome assembly, among others. Evolutionary conservation of genetic interactions was evaluated by partial (~50%) siRNA-based inhibition of human homologues expressed in FRT epithelia. Cells stably transduced with F508del-CFTR cDNA and a horseradish peroxidase tag in the fourth extracellular loop were employed to quantify restoration of mutant CFTR trafficking to the plasma membrane. Cellular toxicity was tested using a commercial kit (Promega CellTiter-Fluor Assay). Silencing RPL12 together with certain other gene products – most notably RPL21 or RPS11 – conferred additive (~3-fold) or synergistic (~5-fold) rescue of F508del cell surface localization. Cell viability was reduced ~10-50% by most gene knockdowns or treatment with CFTR modulators (positive controls). Ongoing work will examine whether dual suppression of RPL12 ± epistatic amplifiers, alone or in combination with ETI, leads to enhancement of F508del-CFTR maturational efficiency and/or transepithelial ion transport.

Our findings describe new genetic modifiers of ABC-C protein folding trajectory that work by epistatic interactions with the ribosome. Importantly, these cellular factors may serve as therapeutic targets to enhance correction of both prevalent and rare CFTR processing variants. This work was supported by the U.S. CFF and NIH.

P49

Hypoxia reduces TRPA1 activity in cystic fibrosis bronchial epithelial cells

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Previous studies have demonstrated that calcium homeostasis is abnormal in human airways epithelial cells affected by the F508del-CFTR mutation [1,2]. Intracellular calcium is in fact modulated by various entities that are deregulated in cystic fibrosis (CF): Transient Receptor Potential (TRP) channels [3], mitochondria [4], endoplasmic reticulum [2]. However, the hypoxia state of cells in CF has never been included as a regulating factor of calcium transport. As we are newly interested in the effect of the modulation oxygen level on ion channels in the pathophysiological context of CF, we decided to focus on TRPA1. We chose this channel which is part of the TRP superfamily, for multiples reasons: i) multiples TRPs entities have interactions with CFTR [5] ii) it is sensitive to oxygen variations intrinsically [6] and iii) it has been demonstrated as involved in inflammatory mechanisms in CF [7].

Our study aims to better characterize the effects of hypoxia on TRPA1 channel in the pathological context of cystic fibrosis.

We used CF bronchial epithelial cell lines (CFBE /WT and /F508del), grown and expended in normoxia (21% O_2) and then switched to hypoxia (1% O_2), in a HypoxyLab(tm) workstation, for 2 to 24 hours. Cells were treated by dimethyl sulfoxide (DMSO) or ETI (Elexacaftor/Tezacaftor/Ivacaftor) for 24 hours. The accumulation and quantification of the TRPA1 protein was studied using western-blot. TRPA1 activity was measured by recording the variations of intracellular calcium using the Fluo 4-AM probe following stimulation by a specific agonist for this channel: Allyl IsoThioCyanate (AITC; 30µM -- 100µM). Immunostainings were performed with anti-TRPA1 (1/400) and ZO-1 (1/400) primary antibodies, in order to visualize the effect of hypoxia on TRPA1 localization.

The global quantity of TRPA1 in CFBE /WT, /F508del and /F508del corrected by ETI, is not altered after within the 24h range in 1% O_2 atmosphere. However, the activity of the channel is reduced in hypoxia in all cell types and independently of the ETI correction. Surprisingly, in normoxia the CFTR treatment seems to affect the TRPA1 activity. Finally, our preliminary results seem to indicate that hypoxia could modify the localisation of TRPA1 in CFBE.

In the future, our project will focus on two different problematics: i) how ETI modulate TRPA1 activity and which of its component is involved in this modulation and ii) do shorter hypoxia times (<24 hours) induce activity and localisation changes attributed to TRPA1.

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P50

Insight into the mechanism of action of VX770 to develop a new CFTR-independent therapeutic proposal

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Background: Cystic Fibrosis (CF) is a genetic disorder, caused by mutations of CFTR gene [1]. Current therapy (Trikafta®) works on a limited number of mutations, hence the need of new therapeutic proposals in CF. The prevalent CFTR variant, F508del, induces the misfolding and accumulation of CFTR in the ER with an oxidative imbalance related to an overproduction of reactive oxygen species (ROS). Specifically, high ROS are associated with depletion of glutathione (GSH), whose synthesis is regulated by NF-E2-related factor 2 (Nrf2), and which fosters the oxidative damage of polyunsaturated fatty acids (PUFA) via lipid peroxidation (LPO) [2]. LPO can lead to the onset of ferroptosis, an iron-mediated form of cell death.

Objectives: The hypothesis is that restoring proteostasis might help in the recovery of mutated CFTR. Therefore, this project aims at investigating the impact of VX compounds on oxidative stress and subsequently exploiting the identified mechanism of action to define new non-CFTR-directed therapeutic strategies.

Methods: CFBE41o- parental were used as CF cell models. Upon 24, 48 or 72h-treatments with VX compounds, LPO levels were detected using C11-Bodipy. Resazurin-based cell viability assay was used to assess protective role of VX compounds against pharmacologically induced ferroptosis in CFBE41o- and HT1080 cell lines. Liposome-based oxygen consumption measurement and cyclic voltammetry were used to determine a direct interaction between compounds and lipid radicals. Moreover, GSH quantitative determination was performed with Tietze assay, while Nrf2 pathway was characterized with western blot analysis.

Results: The CFTR potentiator VX770 surprisingly diminishes lipid peroxides to physiological level in CFBE41o- parental cells and exerts a protective role against pharmacologically induced ferroptosis. The latter result was reproduced in the CFTR lacking cell line HT1080, thus proving the independency of the anti ferroptotic mechanism from the chloride channel. Late and mild Nrf2 activation suggests that this pathway is not the primary mechanism through which VX770 protects cells against LPO and ferroptosis. Conversely, electrochemical techniques reveal that VX770 behaves like a radical trapping antioxidant.

Conclusions: The collected data unveil a CFTR-independent mechanism of action of VX770 that directly modulates oxidative factors within CF models. These results pave the way to a new therapeutic approach based on the idea that, similarly to VX770, radical scavengers could be employed as standalone CFTR potentiators or in combination with CFTR correctors to treat a wide range of mutations.

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Funding: Italian Cystic Fibrosis Foundation (FFC#4/2021)

P51

Proteomic analysis of cultured bronchial epithelium secretome reveals the presence of ion transport proteins

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The airway epithelium represents a defense barrier against pathogens. Defective bacterial eradication with recurrent infection and inflammation is a common hallmark of many chronic respiratory diseases, including cystic fibrosis (CF). In our previous study (Guidone et al., JCI Insight, 2022) we found that treatment of human bronchial epithelium in vitro with IL-17A plus TNF-a has a high impact on cell transcriptome, with upregulation of genes involved in antimicrobial response, neutrophil recruitment and transepithelial ion transport. The overall result of ion transport changes is a marked hyperviscosity of the airway surface, that is reversed by b-adrenergic stimulation. The switch from the hyperviscous to fluid state is dependent on CFTR since it is not observed in CF epithelia. In vivo, this inability may sustain a vicious cycle of inflammation and mucociliary clearance impairment. Given the high impact of IL-17A/TNF-a treatment on epithelial surface properties, we decided to characterize the secretome of bronchial epithelial cells with/without cytokines. Mass spectrometry analysis of fluid collected from the apical surface revealed the presence of many defense molecules and chemoattractants for immune cells (IL-19, CSF3, CXCL17, CCL20). Surprisingly, we also found a specific set of membrane proteins including ATP12A, SLC26A4, DUOX2, SLC5A1, but not CFTR or ENaC. By FACS, we found evidence of extracellular vesicles (EVs) in the supernatant of bronchial epithelial cells, with increased abundance upon treatment with IL-17A/TNF-a. We hypothesize that the presence of EVs may be implicated in communication of regulatory stimuli to target epithelial and immune cells and/or in direct bactericidal activity. However, the role of ion transport proteins in such EVs needs to be clarified.

ATP12A activity, which is upregulated by different pro-inflammatory stimuli, particularly IL-17A plus TNF-a (Guidone et al., JCI Insight 2022), is considered detrimental in CF. However, there are still many gaps in our knowledge regarding ATP12A regulation, trafficking, and activity. To address these gaps, we investigated ATP12A interactome by immunoprecipitation and mass-spectrometry analysis (IP/MS) in bronchial epithelial cells treated with/without IL-17A plus TNF-a. In both conditions, as one of the top interactors, we found ATP1B1, the ATP12A b-subunit. Intriguingly, only in cells with cytokine treatment, we found as interactors SLC26A4, CFTR, SCNN1B and SCNN1G, suggesting the formation of an ion transport complex. We are currently investigating how the IP/MS data reflect the types of interactions of ATP12A in EVs.

Work supported by Fondazione Italiana per la Ricerca sulla Fibrosi Cistica (grant FFC#9/2022).

P52

Low density lipoproteins and F508del CFTR: the controversial role of the cholesterol in cystic fibrosis

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In recent times, there has been a tremendous increase in knowledge regarding the various factors contributing to the clinical phenotype of individuals affected by cystic fibrosis (CF). Specifically, numerous pieces of evidence point to alterations in the lipid compositions of bronchial epithelial cells with mutations impacting the trafficking of CFTR at the plasma membrane level. Our research has demonstrated that human primary bronchial epithelial cells, differentiated in vitro from individuals with CF carrying the biallelic F508del mutation, exhibit an increased content of ceramide and globotriaosylceramide at the apical membrane. Conversely, there is a decrease in the levels of glucosylceramide and gangliosides, particularly GM1. This reduction in GM1 levels partly counteracts the rescue effects of Kaftrio, as GM1 is a crucial lipid that interacts with CFTR, promoting its stability and half-life at the apical surface.

Controversial data exist regarding the cholesterol content, another lipid that, along with GM1, contributes to the organization of specific membrane domains. Our novel experimental approaches have allowed us to determine that CF bronchial epithelial cells carrying the F508del mutation exhibit reduced cholesterol levels. This reduction induces increased mobility of CFTR at the plasma membrane, negatively impacting the channel's stability. Furthermore, increasing cholesterol content at the cell surface enhances CFTR activity.

Additionally, we have discovered that the exogenous administration of low-density lipoproteins (LDL), a physiological process distributing cholesterol throughout the body's cells, is an adjuvant to Kaftrio treatment in rescuing CFTR with the F508del mutation. Due to the significant properties of this lipid, in the near future, we plan to conduct experiments aimed at investigating the potential beneficial effects of LDL in combination with Kaftrio for all mutations affecting the folding and stability of the channel at the plasma membrane—a crucial aspect in the fight against CF

P53

Role of TRPV4 in innate defense mechanisms of the airway epithelium

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Background: TRPV4 channel mediates calcium influx through the plasma membrane of many different cell types. In airway epithelia, TRPV4 may be involved in the transduction of mechanical stimuli (i.e. perturbation of ciliary beating by enhanced viscosity) and the response to bacterial components such as LPS (Andrade et al., J Cell Biol 2005; Alpizar et al., Nat Commun 2017). We previously observed that activation of TRPV4 by pharmacological agents results in a compartmentalized calcium influx that leads to CFTR but not TMEM16A activation (Genovese et al., J Physiol 2019). Instead, we found that TMEM16A is activated by extracellular UTP through purinergic mobilization of intracellular calcium. This may depend on expression of TRPV4 and TMEM16A in separate cell types.

Aims: Our aim was to investigate the localization of TRPV4 in airway epithelial cells, its response to bacterial components, and its ability to induce H_2O_2 production via calcium-dependent dual oxidases (DUOX1/2) as a bactericidal mechanism.

Methods: TRPV4 site of expression was assessed by immunofluorescence with antibodies against TRPV4, cilia, and CFTR in human nasal epithelial cells freshly collected by nasal brushings of both cystic fibrosis (CF) and non-CF individuals. The effect of LPS on TRPV4 function (calcium influx) was evaluated on both non-CF bronchial epithelia and FRT cells (which have endogenous expression of TRPV4). The link between TRPV4-mediated calcium influx and H_2O_2 production were assessed using the fluorescent CM-H2 DCFDA probe in bronchial epithelia treated with or without IL-17A/TNF-α or IL-4.

Results: TRPV4 was detected in the apical membrane of ciliated cells. CFTR expression in such cells was undetectable whereas it was found in non-ciliated cells.

Regarding the response to bacterial components, we found no effect of LPS, derived from Pseudomonas aeruginosa or Escherichia coli, on TRPV4. In contrast, the addition of the TRPV4 opener GSK 1016790A, added as control, induced the expected cytosoliccalcium elevation.

Finally, we investigated the link between TRPV4 and H_2O_2. Under basal conditions, untreated epithelia showed undetectable H_2O_2 production. There was instead a significant basal H_2O_2 production in epithelia treated with IL-17/TNF-α or IL-4. GSK 1016790A-dependent stimulation of TRPV4 significantly enhanced H_2O_2 generation in untreated epithelia and this effect was further enhanced in epithelia treated with IL-4.

Conclusions: The calcium-dependent influx mediated by TRPV4 does not have a global effect on the epithelium but may be connected to specific antimicrobial epithelial mechanisms. In particular, TRPV4 stimulation causes H_2O_2 production, thus remarking the role played by TRPV4 in anti-bacterial defense.

Acknowledgements: Our work was supported by Fondazione Italiana per la Ricerca sulla Fibrosi Cistica (grant FFC#9/2022).

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Highway to cell: Cell-penetrating peptide to internalize the CFTR-stabilizing iCAL36 peptide

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Cystic fibrosis (CF) is the most common fatal genetic disorder in populations of European descent. CF is due to loss-of-function mutations in CFTR (*cystic fibrosis transmembrane conductance regulator*)[(a)], an epithelial ion channel strongly involved in fluid and ion homeostasis. Its activity being required for airway mucociliary clearance, CF patients suffer from airway obstruction and chronic infection.

One of the three known functional defects associated with the most prevalent mutation, p.Phe508del-CFTR, concerns the stability of this chloride channel at the apical membrane of endothelial cells. Previously, our group has reported the development of CAL inhibiting peptides (iCAL) which rescue CFTR activity (11% increase in chloride efflux)[(b),(c)]. However, optimization of the peptide sequence in terms of metabolic stability and cellular delivery is still necessary to obtain more specific inhibitors.

In the present study, optimization of the peptidyl inhibitor was performed by coupling five different cell-penetrating peptides (CPP)[(d)]. Screening these CPP-iCAL36 peptides under different conditions was performed to undestand their cellular internalization properties and to select TatRI as the optimal CPP for iCAL36 delivery. More importantly, using this TatRI-iCAL36 peptide, we were able to reveal for the first time an additive increase in the CFTR amount in the presence of VX-445/VX-809 compared to VX-445/VX-809 treatment alone[(e)]. Finally, we present first results of iCAL36 peptide sequence optimization.

This finding is a significant contribution to the development of CFTR-stabilizing peptides in addition to currently used treatments (small-molecule correctors or potentiators) for CF patients.

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P55

From CFTR to a CF signalling network: a systems biology approach to study Cystic Fibrosis

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Although Cystic Fibrosis (CF) is a monogenic disease, its overall physio-pathology cannot be solely explained by the loss of the CFTR chloride channel function. Indeed, CFTR belongs to a yet not fully deciphered network of proteins (CF network hereafter) participating in various signalling pathways. Our assumption is that the absence of a functional CFTR at the membrane causes perturbations of its direct protein partners, further propagating functional dysregulations in the CF network. Based on 10 publicly available transcriptomic datasets from CF and control epithelial respiratory cells, we used systems biology technologies to build a network that recapitulates CF signalling dysregulations. Based on Gene Sets Enrichment Analysis (GSEA) (1), we showed that 16 pathways from the KEGG database were over- or under-activated in at least 3 studies. These pathways shared many proteins in common, which allowed to connect them into a network, defining our CF network. Overall, the network comprises 330 proteins and 529 interactions and contains rich, but complex information.

Topological analysis of the network allowed identification of:

1. A few source nodes, i.e. proteins known to be direct interactors of CFTR that may initiate the propagation of dysregulations. This result is in favor of the above hypothesis. These source nodes include CSNK2A1, EZR, PLCB1/3, PRKACA, SRC, SYK and TRADD.

2. A list of 35 output nodes (mainly transcription factors) that trigger a panel of deleterious CF cellular phenotypes. These phenotypes are consistent with CF pathology and include inflammation, perturbation in innate immune response, perturbation of cytoskeleton, or perturbation in apoptosis-related processes, which shows that the approach did capture relevant biological information about CF. Indeed, the output nodes include NFKB1/2, RELA/B, FOS and JUN that are well-known to be involved in inflammation and cell proliferation, various caspases, and several IRFs transcription factors involved in innate immunity response.

3. Nodes with high betweenness centrality, i.e. hub node proteins, through which a high proportion of the flow of dysregulations propagates to ultimately reach output nodes.

Source nodes and nodes with high betweenness centrality appear as candidate targets to reduce the propagation of dysregulations within the network, and consequently mitigate deleterious CF phenotypes. Interestingly, specific inhibitors and even marketed drugs are available for some of these proteins that may be of interest for further experimental validation in cellular CF models. Such validation experiments should involve biological readouts related to the network cellular phenotypes such as inflammation or wound repair. If confirmed, this would offer new therapeutic approaches for CF, particularly for patients who are not eligible to CFTR modulators.

Although systems biology technologies have been essentially developed for complex diseases like cancer, our study demonstrates their interest to better understand biological features that characterize CF, and to propose new hypotheses that can be further tested experimentally. Finally, they offer original approaches to identify therapies that are complementary to the restoration of the defective gene.

1. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Subramanian et al, PNAS, 102(43), 2005.

P56

A Novel Peptide Targeting the A-kinase anchoring function of PI3Kγ that increase cAMP and potentiate CFTR

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Objectives: The second messenger 3′,5′-cyclic adenosine monophosphate represents the primary trigger for the cystic fibrosis transmembrane conductance regulator (CFTR) activation, the ion channel whose mutations lead to the rare genetic disease cystic fibrosis (CF). Previously, we demonstrated that, in the lungs, phosphoinositide 3-kinase γ (PI3Kγ) acts as a A-kinase anchoring protein which negatively regulates cAMP by favoring the activation of cAMP-degrading enzymes. Here, we hypothesize that targeting PI3Kγ scaffold activity enhances cAMP in epithelial cells, leading to CFTR modulation.

Methods: A library of 23 cell-penetrating peptides was examined for their ability to disrupt the PI3Kγ/PKA complex. To understand how they interacted with PKA, we used PEP-FOLD3.561 to predict their structure and, subsequently, HADDOCK52 to pinpoint the details of interaction with PKA regulatory subunit-α, the known binding surface for AKAPs. Among them, a non-natural peptide named KITH-ADS26 (Patent pending n. 102023000025458) was selected for further analysis as it significantly raised cAMP levels in bronchial epithelial cells. Next, we assessed whether the peptide could be employed to reinstate the activity of the cAMP-dependent chloride channel CFTR, to this aim, CFBE F508del CFTR cells expressing the halide-sensitive yellow fluorescent protein (HS-YFP), which allows evaluation of CFTR activity in the cell membrane by calculating the fluorescence quenching rate resulting from iodide influx were used.

Results: We found that KITH-ADS26 is a selective PI3Kγ/PKA-RIIα peptide disruptor with a tight binding affinity for PKA, having a dissociation constant (K_D) in the nanomolar range, as assessed by steady-state fluorescence and stopped flow techniques. Moreover, KITH-ADS26 triggered cAMP elevation both *in vitro* in bronchial epithelial and *in vivo* in cells in the airway tract of mice upon intratracheal administration. Finally, cAMP elevation enhanced the efficacy of the standard-of-care in rescuing the function of the most prevalent CFTR mutant (F508del-CFTR).

Conclusions: Altogether, these data support the use of KITH-ADS26 for pharmacological manipulation of PI3Kγ-dependent cAMP signaling.

P57

Inhibition of Orai1-Mediated Calcium Signaling Ameliorates Lung Disease in G551D Cystic Fibrosis Ferrets

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Cystic fibrosis is an inherited genetic disease caused by dysregulation of the CFTR anion channel. Clinical manifestations of this disease include airway dehydration, mucus stasis and unrelenting cycles of infection and inflammation. Lung infection and inflammation continue to be a problem for CF patients despite the development of highly effective modulator therapies. Further, ~10% of CF patients are ineligible for these therapies and have severe CF lung disease. We have recently developed ELD607, a specific Orai1 inhibitor[1]. In HEK293T cells, ELD607 was CFTR mutation agnostic and inhibited Orai1 equally well in the absence of CFTR, with WT CFTR, or with W1282X. G551D, DF508 or R117H CFTR (all n=6). Inhaled ELD607 serves to rebalance the lung's immune system. Indeed, we previously found that ELD607 lowered lung neutrophils, raised alveolar macrophages, prevented lung damage and reduced bacterial CFUs in the lungs of mice infected with both *P. aeruginosa* and *S. aureus*[1]. Here, we tested the hypothesis that ELD607 would reduce lung inflammation in $CFTR^{G551D/G551D}$ ferrets that had developed significant CF lung disease. $CFTR^{G551D/G551D}$ ferrets were reared on a CFTR potentiator and lung disease developed over several months following discontinuation of drug. ELD607 was dosed either intratracheally or intravenously at 1 mg/kg daily over 3 days. Tissues were harvested on the third day. ELD607-naïve G551D and wild-type ferrets were also studied as controls. Both intravenous and intratracheal dosing of ELD607 were well-tolerated and blood chemistry remained within the normal range [albumin, alkaline phosphatase, creatine, alanine amino acid transferase, bilirubin, Ca^{2+}, K^+, Na^+ were all normal]. The naïve G551D ferrets had CF disease and exhibited raised BAL neutrophils and reduced BAL alveolar macrophages compared to wild-type ferrets. ELD607 reduced BAL neutrophils and increased alveolar macrophages after either intratracheal or intravenous delivery. ELD607 reduced BAL neutrophils [CF naïve, 74.7±9.0; CF IT ELD607 9.5±0.75; CF IV ELD607 31.5±3.5] and increased BAL macrophages [CF naïve, 34.1±3.6%; CF IT ELD607 89±1.4; CF IV ELD607 65.5±3.3]. Taken together, these data indicate that ELD607 safely reduced lung neutrophilia in CF G551D ferrets. This is the first report of ELD607 efficacy in a non-rodent disease model. Histology/proteomics and chronic studies with ELD607 in G551D ferrets are ongoing. However, our interim data support the overarching hypothesis that ELD607 acts as an immunomodulator that can normalize neutrophilic inflammation to reduce CF lung disease.

Funding Sources: This research was supported by the CFF and the NIH/NHLBI.

Reference: [1]Ahmad S. et al. 2023. AJRCCM. doi: 10.1164/rccm.202308-1393OC.

P58

Enhancing apical loop currents in airway epithelia carrying CFTR non-sense mutations

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Objectives: Highly effective modulators for mutant cystic fibrosis transmembrane conductance regulator (CFTR) have made a significant difference for close to 90% of people with cystic fibrosis (pwCF). However, ~10% of pwCF with two minimal function mutations or mutations not amenable to modulators have currently limited options. Ion channels alternative to CFTR could compensate and thus serve as therapeutic targets in CF airway disease. For this, the apical loop current seems critical as apical potassium exit enhances chloride conductance and fluid availability on the airway surface. TMEM16A potentiators are being developed. In addition, there is a new recognition that CFTR modulators could act as large conductance, calcium-activated potassium (BK) channel potentiators. Our hypothesis is that simultaneous BK and TMEM16A potentiation may have beneficial effects on parameters of mucociliary function in pwCF with minimal function CFTR mutations.

Methods: Normal human bronchial epithelial (NHBE) and cystic fibrosis bronchial epithelial (CFBE) cells were cultured at the air-liquid interface and allowed to differentiate for at least 4 weeks. All assays were performed 24h after treatments. Potentiation of TMEM16A was achieved using Rg3 (15 μM), a major component of red ginseng aqueous extract, and potentiation of BK using elexacaftor (VX-445; 5 μM). Nesolicaftor (PTI-428, 10 μM) acting via poly(rC)-binding protein 1 (PCBP1) was used to stabilize LRRC26 (gamma subunit of BK) and TMEM16A mRNAs in the presence of inflammation, simulated with recombinant TGF-β1 (5 ng/mL). Short-circuit currents of TMEM16A and BK were recorded in Ussing chambers. Ciliary beat frequency (CBF) was measured using SAVA pre- and post-treatments. Airway-surface liquid (ASL) volumes were estimated by meniscus scanning pre- and post-treatments.

Results: Elexacaftor potentiated BK currents in NHBE and CFBE cells with minimal function CFTR mutations and partially rescued TGF-β1-induced BK dysfunction. Rg3 potentiated TMEM16A currents in CFBE cells and partially restored TGF-β1-induced TMEM16A dysfunction. Elexacaftor and Rg3 in CFBE cells had a marginally improved effect on TMEM16A compared to Rg3 alone. In NHBE cells, nesolicaftor enhanced BK and TMEM16A conductance, and increased ASL volumes and CBF. TGF-β1-induced inflammation decreased BK activity, ASL volumes and CBF. These effects were rescued by nesolicaftor.

Conclusions: Our data show that enhancing apical loop currents even in inflammatory environments by potentiation of alternative ion channels in pwCF with minimal function CFTR mutations is feasible. These “triple” therapies to address nonsense mutations with molecules already tested in human beings could see quick turnarounds into clinical trials.

P59

The Orai1 antagonist, ELD607, reduces chronic neutrophilic inflammation in a βENaC mouse model

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Rationale: In patients with cystic fibrosis (CF), chronic bacterial infection, inflammation and frequent exacerbations are caused by defective CFTR via dehydration and mucus buildup in the airways. Bronchiectasis and respiratory failure are the result of injury and remodeling due to repeated cycles of inflammation. Hence, management of airway inflammation represents a vital aspect of CF treatment. The only currently available, approved anti-inflammatory therapy to treat these patients is ibuprofen. CFTR-/- transgenic mice lack functional CFTR and do not develop CF-like lung disease. Mice overexpressing the beta subunit of the epithelial Na+ channel (βENaC mice) spontaneously develop spontaneous neutrophilic inflammation and mucus dehydration, and exhibit significant mortality, posing as a robust murine model for chronic CF lung disease. Orai1 is a plasma membrane Ca2+ channel that regulates inflammation by controlling gene expression and cytokine secretion. Its activity is increased in CF airways. We hypothesized that Orai1 inhibition would reduce inflammation in the βENaC mice. ELD607, a fully optimized Orai1 antagonist, has been developed to evaluate whether it could inhibit Orai1 locally in the lungs to reduce pulmonary inflammatory responses in mice with chronic inflammation via inhalation.

Methods: Neonate βENaC mice and their WT littermates were exposed daily (intranasally) to ELD607 (0.5 mg/kg) or vehicle for 10 days and examined for survival. Bronchoalveolar lavages (BAL) and whole lung tissues were collected 24 h after the last ELD607 dose, on day 11 to perform Ussing chamber studies and lung histology.

Results: βENaC neonates treated with ELD607 for 10 days exhibited reduced neutrophilia (90%) and lung macrophage levels were restored to the normal range (n=9/group). Pro-inflammatory biomarkers of neutrophilia and lung injury (neutrophil elastase, lactate dehydrogenase, and total protein in BAL) were significantly reduced in ELD607-treated mice compared to naïve. After 10 days, 100% of WT mice survived, regardless of treatment, while ELD607-exposed βENaC mice significantly increased survival from 45% to 95% (n=7-10/group). ELD607 treatment significantly reduced goblet cell metaplasia by ~10-fold and mucus obstruction by 75% (n=10-12/group). However, chronic ELD607 dosing had no effect on either isoprenaline or amiloride-sensitive currents (markers of CFTR and ENaC activity respectively) in either WT or βENaC mice (n=3 per group).

Conclusions: Chronic ELD607 inhalation significantly reduced pulmonary inflammation, goblet cell metaplasia, mucus obstruction and lung damage; leading to increased survival, despite not affecting βENaC nor CFTR activity. These data indicate that inhibition of Orai1-mediated inflammation can significantly ameliorate CF-like lung disease without affecting the primary defection (i.e. increased Na+ absorption). In conclusion, ELD607 is a novel, inhaled immunomodulator that may be suitable for treating CF lung disease.

P60

Septin 7-dependent defense mechanisms against *Pseudomonas aeruginosa* are impaired in cystic fibrosis bronchial epithelial cells

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In people with cystic fibrosis (pwCF), dysfunction of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein results in increased mucus viscosity. The resulting alteration in mucociliary clearance generates an environment conducive to the persistent colonization of the airways by pathogens, notably *Pseudomonas aeruginosa* (*Pa*). The presence of *Pa* is associated with a deterioration of lung function in pwCF. It is therefore crucial to identify the specific cellular components involved in the bronchial response to *Pa* infection, paving the way for the development of innovative therapeutic approaches.

In this study, we aimed to understand the role of septin (SEPT) 7 in the process of *Pa* intracellular infection in bronchial epithelial cells. SEPT7 belongs to the septin cytoskeleton which is involved in epithelial barrier maintenance and anti-infective response processes. Among the 13 SEPT proteins, SEPT7 is particularly important. It is involved in increasing the cell membrane rigidity, preventing from bacterial internalization. It is also involved in trapping intracellular bacteria in cage-like structures, leading to their degradation. However, its role in bronchial *Pa* infections in CF remains unknown.

We used CF and non-CF (healthy donors) primary bronchial epithelial cells, and bronchial epithelial cell lines, 16HBE with wild-type *CFTR* (16HBE-WT) or with F508del-*CFTR* variant (16HBE-F508del) and BEAS-2B. In addition to submerged cultures, primary differentiated bronchial epithelial cells grown at the air-liquid interface were used. Fluorescent (GFP) PAK and PAO1 strains of *Pa* were used. An interfering RNA (siRNA) specific for SEPT7 was used to inhibit SEPT7 expression. The study of SEPT7 expression was carried out using qPCR, western blot, immunofluorescence and immunohistochemistry. Internalization experiments of *Pa* were performed using tobramycin to remove extracellular bacteria.

We observed no difference in the expression levels (mRNA and protein) or localization of SEPT7 between CF and non-CF bronchial epithelial cells. SEPT7 immunostaining on lung biopsies from healthy or CF donors also showed no difference in expression. Cellular infection with *Pa* did not modulate SEPT7 expression either. We also showed that SEPT7 is able to form cage-like structures around intracellular *Pa* in all our CF and non-CF cell models.

In non-CF bronchial epithelial cells (BEAS-2B and 16HBE-WT), inhibition of SEPT7 led to an increase in the number of intracellular *Pa* and a decrease of interleukin 6 (IL-6) production, indicating that SEPT7 is involved in the control of infection and subsequent inflammation. However, in CF bronchial epithelial cells (16HBE-F508del and primary cells) these effects were not found, suggesting an alteration in these SEPT7-dependent mechanisms. Furthermore, the use of Elexacaftor-Tezacaftor-Ivacaftor combination did not correct this alteration, suggesting that CFTR function is not directly involved in these processes.

Our results showed that SEPT7 plays an essential role in the control of *Pa* infection of bronchial epithelial cells which is altered in the CF context. Further work will aim to determine the extent to which this alteration could promote persistent infection of CF bronchial epithelial cells.

P61

Targeting platelet activation with pro-resolving lipid mediators: an innovative strategy to dampen lung inflammation in cystic fibrosis

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Despite the advent of highly effective modulators (HEM), chronic lung inflammation remains the primary cause of morbidity and death in cystic fibrosis (CF). We previously reported that platelets (PLT) are key contributors of CF pathological inflammation, as PLT show a hyperactive phenotype that support lung inflammation while dampening beneficial pro-resolution mechanisms. Importantly, our more recent evidence highlights that the PLT activation is not reverted by HEM, indicating that PLT hyperreactivity remains a vexing problem in CF that calls the exploration of new therapeutic strategies.

Thus, innovative approaches to diminish PLT activation could be useful to counter regulate CF inflammation, bacterial infection, and lung damage in CF. To this end, here we tested the potential of resolvins (RvD), key proresolving lipid mediators (SPMs) that drive the resolution phase of inflammation, in targeting PLT hyperactivation and restoring an effective pro-resolution program. To address this, 2 specific aims are pursued:

1. Identifying specific anti-PLT RvD ex vivo

2. Testing ex vivo and in vivo bio-actions of the selected RvD

To this end, purified PLT from CF patients were used to evaluate PLT activation after exposure to RvD. Then, the identified anti-PLT RvD were tested ex vivo and in vivo in CFTR KO mice acutely infected with planktonic *P.aeruginosa*. Bacterial CFU, differential leukocyte count in bronchoalveolar lavage fluids (BAL), lipidomic by mass spectrometry and histological (IHC) analyses of lungs were carried as readout of infection, inflammation, and resolution.

Among the tested RvD, RvD3 significantly reduced Thromboxane B2 (TxB2) release, a canonical marker of PLT activation, when PLT were challenged with pro-inflammatory stimuli. In infected CFTR KO mice, RvD3 specifically reduced TxB2 production by PLT since its effect were abolished in CFTR KO PLT depleted mice, thus mirroring ex vivo results. In line with this, CD62P-stained sections by IHC revealed a decreased PLT activation.

Importantly, in acute pneumonia RvD3 reduced neutrophils (PMN) infiltration in BAL and total bacterial count in a TxB2-dependent manner, therefore suggesting a PLT-dependent shift in inflammation towards resolution. Consistent with this, using purified CF-derived blood cells ex vivo, RvD3 stimulated clearance of *P. aeruginosa* by PMN only in presence of PLT. Strikingly, in vivo RvD3 restored the altered balance of pro-inflammatory versus pro-resolving lipid mediators by lowering levels of pro-inflammatory lipids (TxB2, PGE2, PGD2, LTB4) as compared to SPM levels (LXA4, LXB4, RvE2, RvE4) in lungs of infected mice.

Together, these results define the potency and efficacy of RvD3 as antiplatelet strategy to reduce the inflammation-based pathology in CF. In particular, by dampening PLT activation, RvD3 promotes a quick resolution of lung inflammation characterized by a reduced PMN infiltration, bacterial load and lowered levels of pro-inflammatory mediators. Hence, RvD3 could be exploited as innovative PLT-based approach to improve the lung inflammatory status of people with CF.

P62

Tackling phage resistance to increase the robustness of phage therapy for curing *Pseudomonas aeruginosa* infections in patients with cystic fibrosis

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Patients with Cystic Fibrosis (CF) are highly susceptible to lung infections caused by different bacteria, among which *Pseudomonas aeruginosa* (Pa). Phage therapy, namely exploiting phages (bacteria-specific viruses) to kill infecting bacteria, represents a promising strategy for curing bacterial infections refractory to antibiotics in these patients. We assembled a collection of phages able to kill Pa clinical strains isolated from patients with CF and developed a four-phage cocktail (CK4) able to treat Pa infections in animal models among which a *cftr*-loss-of-function zebrafish. However, the success of phage therapy may be endangered by the emergence of mutant bacteria resistant to phages. Addressing this challenge constitutes the focal point of our research. In a previous work, we found that CK4-resistant mutants are easily isolated from cultures of the susceptible PAO1 strain grown in either a standard laboratory medium or an artificial sputum medium mimicking the composition of the airway fluid of people with CF. In both cases, CK4 resistant mutants are defective in lipopolysaccharide (LPS) biosynthesis, suggesting that all CK4 components exploit the LPS as receptor for adsorption. Indeed, all phages that we have collected so far from environmental samples rely on either LPS or type IV pilus (T4P) as receptors. Heterogeneity of LPS and lack of pili may explain the widespread phage-resistance shown by Pa clinical strains isolated from CF patients. This makes of pivotal importance the identification and/or construction of phages using different receptors that may make the therapy more robust towards resistance. We have found and are currently characterizing six new natural phages, some of which grow on multi-phage resistant or even pan-resistant strains isolated form people with CF. Overall, our data show that natural Pa phages not relying on LPS or T4P for adsorption are relatively rare. Moreover, we have implemented a mutagenesis approach to derive phages with altered host range building upon already characterized phages potentially applicable to therapy. The preliminary results of this approach will be presented. Finally, we are testing whether the treatment with Trikafta may select bacteria with altered phage-susceptibility profile. The results we have obtained so far show no correlation between the treatment with Trikafta and phage resistance degree.

P63

Ex vivo pig lung as a new CF model for the study of *Pseudomonas aeruginosa* infection and phage therapy application

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Chronic bacterial infections affect individuals whose normal immune defenses are compromised, including those with cystic fibrosis (CF). *Pseudomonas aeruginosa* (*Pa*) colonization of the abnormally thick mucus in the CF respiratory tract leads to intractable biofilm infections of the lower airways. Despite repeated antibiotic administration, episodes of acute pulmonary exacerbation occur, eventually leading to death from respiratory failure. To counteract this type of antibiotic-refractory infection, phage therapy, the use of exogenous bacteriophages (or phages, viruses able to infect specifically bacteria), has regained increasing interest. In a previous study, we isolated and characterized new phages able to kill *Pa* laboratory and clinical strains *in vitro* and to counteract *Pa* acute infections in different animal models, among which a CF zebrafish embryo model. However, these models do not reproduce all the aspects of the CF airway, in particular concerning the biofilm formation. At present, biofilm studies are mainly based on assays developed on abiotic surfaces. There is the need to develop models that can recapitulate the *in vivo* environment, therefore reproducing the metabolic state of the pathogen. With the present work, we are setting up the *ex vivo* pig lung (EVPL) CF model, a established system that mimics the physicochemical environment of the human CF airways allowing for growing *in vivo* biofilm-like. In particular, the incubation of swine bronchial and bronchiolar tissue with an artificial sputum medium mimicking the composition of the airway fluid of people with CF, allows to recapitulate a chronic CF pulmonary infection. We are setting up the EVPL CF model to study infections from different strains of *Pa* (i.e. collection of laboratory strains as PAO1 or PA14 and strains derived from individuals with CF treated with modulators) and to validate the antimicrobial potential of new or already characterized phages of our collection, alone or in combination with antibiotics. The preliminary results of this approach will be presented. Notably, the EVPL model is established and used by various research groups, providing a solid foundation for findings. The present study aims to demonstrate the potency of a new CF model to test the efficacy of phage therapy against *Pa* biofilm, in order to address an unsolved issue of phage therapy. The topic is of absolute importance for individuals with CF as *Pa* is difficult to eradicate due to its high mutational adaptation rate. Moreover, the introduction of a cheap and easy-of-use CF model, together with studies in CF human cells, could speed-up the translational potential of the introduction of phages into clinics.

P64

GM1 ganglioside: new insight on its immunomodulatory capacity in CF

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The availability of new modulators for the rescue of the mutated CFTR opens a new scenario for the treatment of patients with Cystic Fibrosis (CF). In the previous years, we discovered a new interactor of CFTR, consisting in the ganglioside GM1, which is fundamental for the stability of the channel at the plasma membrane (PM). In bronchial epithelial cells, the absence of CFTR at the cell surface is associated with a decrease in the content of GM1. Interestingly, restoring the PM content of this ganglioside by its administration increases the stability of the mutated channel rescued by CFTR-modulators, such as Orkambi or Kaftrio formulations. To note, GM1 ganglioside is an essential modulator of innate and adaptive immune response, and it was reported to directly regulate the clearance of *P. aeruginosa* from the respiratory tract, which is downregulated in CF bronchial cells.

Based on this evidence, we investigated the role of this ganglioside in the host-pathogen interactions in CF.

First of all, xenophagic clearance activity was measured in both CF and non-CF bronchial cells exposed to GM1 in order to test whether the administration of GM1 ameliorates the clearance of *P. aeruginosa*. Reduced xenophagic clearance of invading pathogen was detected in CF cells compared to non-CF cells, as higher number of colony-forming units (CFU)/ml of invading bacteria was observed in CF bronchial cells. However, at 48-hour pre-treatment, GM1 administration rescued the bacterial clearance capacity of CF cells reducing the number of colony-forming units (CFU)/ml of invading bacteria. Through a bacterial invasion assay, we excluded that the reduction of intracellular bacteria in CF cells exposed to GM1 for 48 hours depended by a lowering bacterial invasion capacity.

Afterwards, the safety of GM1 administration via aerosol was assessed in *in vivo* models performing experiments on WT mice (C57Bl/6) treated with different doses (1, 5 and 20 mg/kg/day) of GM1 in saline solution. Mice were treated with the ganglioside 24 hours before, as well as immediately after the injection of PBS used to induce stress that mimics acute infection. Over time, for maximum 24 hours, the safety of this treatment was monitored by keeping under observation the well-being, weight, and temperature of mice. Although some reduction, concerning both the body weight and the temperature, was observed between mice treated with GM1 and vehicles, these differences were not statistically significant. Therefore, in light of these results in these conditions GM1 at all doses tested does not appear to induce toxicity.

Concerning the importance of GM1 in the stability of CFTR at the PM and its immunomodulatory role, the results of this study could serve for the development of new therapeutic strategies to improve the efficacy of the treatments of CF patients, and their immunomodulatory capacities.

P65

Exploring the relationship between *Pseudomonas aeruginosa* infection and SLC6A14 in cystic fibrosis

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Introduction: Large-scale genetic studies on people with cystic fibrosis (pwCF) have highlighted the pivotal role of the *SLC6A14* (solute carrier family 6 member 14) gene in modulating lung function and the early onset of *Pseudomonas aeruginosa* infection. We know that SLC6A14 encodes an amino acid transporter highly expressed in bronchial epithelial cells and plays a crucial role in amino acid concentration within cell cytoplasm. However, its precise functional involvement in CF lung physiopathology, especially in the context of *P. aeruginosa* infection, remains poorly defined.

Objective: This study aims to elucidate the mechanisms by which SLC6A14 influences lung function in the pathological context of CF, both in aseptic and *P. aeruginosa* conditions.

Methods: We used Calu-3 knockdown for CFTR (Calu-3 CFTR-KD) and primary airway epithelial cells from healthy donors or pwCF. SLC6A14 mRNA and protein expression were analyzed through real-time quantitative PCR, western blot, and immunofluorescence. SLC6A14 amino acid transport was assessed by using Arginine Monohydrochloride L-[2,3,4-^{3}H] uptake. *P. aeruginosa* infection was mimicked using purified virulence factors such as lipopolysaccharide (LPS), LasB elastase (LasB), or flagellin (Fla), along with live PAO1wt or PAO1ΔlasB strains. Additionally, transcriptomic and proteomic analyses were performed on fully differentiated bronchial epithelial cells from pwCF treated with the SLC6A14 inhibitor alpha-methyltryptophan (α-MT).

Results: In CF airway epithelial cells (*ex vivo*), *SLC6A14* mRNA levels were increased while protein levels were drastically decreased compared to those of cells from healthy donors. Exposure to virulence factors from *P. aeruginosa*, such as LPS and Fla, increased both SLC6A14 mRNA and protein levels. However, live *P. aeruginosa* infection led to an increase in *SLC6A14* mRNA levels and a decrease in SLC6A14 protein levels. We then demonstrated that this decrease is attributable to LasB elastase. Transcriptomic and proteomic analyses of primary fully differentiated CF bronchial epithelial cells revealed that several transcripts and proteins were differentially expressed in α-MT-treated cells compared to control cells with enriched gene sets related to wound healing, cell junction organization, cell-cell adhesion, macroautophagy, *S. aureus* infection and viral infection pathways.

Conclusion: Our study allows to determine that, during the course of CF, the protein expression levels of SLC6A14 may be influenced by infection thus affecting the activity of SLC6A14. Within the bronchial epithelium, a reduced expression and/or activity of SLC6A14 might impact pathways essential for preserving epithelial barrier integrity. Exploring interconnections between SLC6A14 and the diverse proteins implicated in these pathways may unveil novel therapeutic targets aimed at enhancing the host response to infection in the CF context.

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Investigating the therapeutic potential of phages as antibacterials and immunomodulators in a zebrafish model of cystic fibrosis

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The alarming diffusion of bacteria resistant to the antibiotics currently in use and the delay in the pipeline for the discovery of new efficient drugs is a serious problem for bacterial infections. The situation is even worrying in pathological conditions, such as in individuals with cystic fibrosis (CF). CF individuals are frequently affected by severe chronic infection, in particular Pseudomonas aeruginosa (Pa), and recently, clinical trials to treat them with an inhaled phage cocktail have been started. Phage therapy is considered generally safe but some aspects deserve to be further studied, especially considering phage interactions with the host immune system. We have started investigating: i) what happens when phages get in touch with eukaryotic cells in wild-type (WT) and CF models; ii) how phages elicit the modulation of the host immune system; iii) which phage component(s) activate anti-inflammatory cascade. We addressed the fate of phages administered to WT or CF human cell lines specific for airway epithelium (CFBE or CuFi-1 F508del, respectively) or innate immune system (THP-1). Moreover, we dissected the molecular mechanisms through which phages modulate the host immune system in WT and CF zebrafish embryos, taking advantage of transgenic lines that allow the direct visualization of innate immune cells (i.e., neutrophils, macrophages), that are conserved from zebrafish to human. Finally, we are isolating and purifying proteins, the most promising among the phages composing our four-phage cocktail, to test which exerts the anti-inflammatory effects previously demonstrated. The discovery of the mechanisms involved in phage/host immune system interaction in normal and pathological conditions will be relevant for the CF community. This work aims to clarify unsolved issues of phage therapy that cannot be addressed in patients, with the final goal of making it a reliable and safe therapeutic option.

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Unveiling the effect of ETI therapy on *Pseudomonas aeruginosa* persistence and adaptation through RNA expression profiling

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The CFTR modulator therapy Elexakaftor/Tezacaftor/Ivacaftor (ETI) has revolutionized the treatment landscape for 90% of people with cystic fibrosis (pwCF), substantially improving CF symptoms. Despite the significant progress made in CF treatment, we currently have a limited understanding of the impact of ETI on other aspects of the disease, such as pathogenic burden and airway infection. *Pseudomonas aeruginosa* is a predominant pathogen in pwCF. *P. aeruginosa* rapidly adapts to the CF lung environment causing chronic infections that are highly resistant to host defenses and antimicrobial treatments. Ongoing studies, based on bacterial culturing and genomic sequencing, suggest that *P. aeruginosa* prevalence generally declines during the initial months of ETI but rebounds and remains at relatively stable levels thereafter. Despite the significance of these results, there is an urgent need for investigations aimed at understanding the physiological changes and transcriptional response that occur in *P. aeruginosa* after the initiation of ETI therapy to help shape clinical care as CF infections evolve. To address this gap, we performed longitudinal transcriptomic studies on *P. aeruginosa* in CF sputum samples from a cohort of 10 participants. Samples were collected before and at 2-days, 1-week, 1-year, and 2-years following the initiation of ETI therapy. Initial analyses reveal that post-ETI, *P. aeruginosa* maintains a human-infection gene expression signature previously identified in the Whiteley lab using machine learning approaches. In addition, *P. aeruginosa* transcriptomes at 2-days and 1-week post-ETI exhibited small numbers of differentially-regulated genes compared to pre-ETI transcriptomes, likely due to the high variability in the *P. aeruginosa* transcriptomes between the study participants post-ETI. Ongoing investigations aim to examine transcriptional changes at later timepoints and define an ETI-specific *P. aeruginosa* transcriptional signature as *P. aeruginosa* adapts to the post-ETI CF lung environment.

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Identification of molecular determinants that govern morphotype-specific physiology in Mycobacterium abscessus

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Bacterial colony morphology is typically stable and heritable, yet certain species exhibit morphologic change that confers a survival advantage when confronted with an environmental shift. *Mycobacterium abscessus* (MAB) is an emerging pathogen among people with Cystic Fibrosis (CF). Initial infection is thought to be caused by the smooth colony morphotype of MAB which causes asymptomatic or symptomatic infection. Over time, MAB can adapt to a rough colony morphology via point mutations in the biosynthetic machinery of the glycopeptidolipid. This rough morphotype exacerbates inflammation, leading to lung function decline and disease progression. To gain a deeper understanding of the physiologic differences of MAB in its two distinct forms, we generated transposon insertion mutant libraries and sequenced them to quantitatively assess gene essentiality. Analysis under nutrient-rich conditions revealed 13 unique essential genes in the smooth morphotype and 53 in the rough morphotype. Notably, we identified *pknA* as a gene uniquely required for the survival of the rough morphotype and verified this through CRISPRi experiments using rough CF clinical isolates. PknA encodes a serine-threonine protein kinase that govern various aspects of bacterial physiology, including cell division, translation, lipid metabolism, and central metabolism. We also used our transposon libraries to identify genes important for survival of both morphotypes to antimicrobials, including nitric oxide. We identified distinct sets of genes required to overcome NO exposure. Both morphotypes require *EmbC* which is known to mediated survival to various stresses, but smooth MAB uniquely requires the dipeptide importer *dpp* and adenosine salvaging while rough requires a putative polyketide cyclase. These results may explain the differential fitness of MAB morphotypes in response to NO exposure. Together, our findings show that MAB smooth and rough morphotypes contain distinct genetic determinants for growth and tolerance to antimicrobials, which are likely critical for CF lung infections.

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Impact of triple therapy on mucus rheology and bacteriology in cystic fibrosis

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Rationale: The abnormal viscoelasticity of cystic fibrosis (CF) mucus significantly impacts the microenvironment within the airways, influencing microbial colonization and persistence. This interplay between mucus rheology and bacteriology is a key determinant in the chronic respiratory infections commonly observed in CF patients. The introduction of the triple therapy with elexacaftor/tezacaftor/ivacaftor (ETI), has revolutionised the treatment landscape for individuals with CF. This treatment helps to restore the normal activity of the CFTR protein on the cell surface, improving chloride transport and potentially reducing the viscosity of mucus in the airways. Understanding the changes in mucus rheology and bacteriology before and after the administration of ETI therapy is pivotal for evaluating the therapeutic efficacy of this innovative medication.

Methods: We collected sputum samples from 7 CF adults before and after receiving ETI treatment from the “Centre de Ressource et de Compétence de Mucoviscidose" (CRCM) at Grenoble Alpes University Hospital. The study was approved by French research ethics committee (case number 20.09.08.61213). Two expectorations were collected at each visit through autogenic drainage with the assistance of a physiotherapist. Samples for rheometry were stored at -80°C after saliva removal and bacterial count after incubation in TSA plates was carried out with the second tube. Rheometry was measured for each sample at low and high deformation obtaining the viscoelastic modulus (G*) and the critical stress () which is a measure of the mucus strength.

Results: The expectorated volume decreased by 41 ± 17% following ETI treatment, except for one patient who exhibited a twofold increase. Post-ETI treatment, the incidence of patients infected with *pseudomonas aeruginosa*, *staphylococcus aureus* and *burkholderia cepacia* halved. Conversely, while no samples showed infection with *haemophilus influenzae* or *achromobacter xylosoxidans* before treatment, after treatment, three patients presented *haemophilus influenzae* and one *achromobacter xylosoxidans*.

Furthermore, sputum rheometry revealed a reduction of G* and by 59 ± 13% and 57 ± 15%, respectively, in samples collected within the first 10 months of treatment initiation. In contrast, two out of the three samples collected more than 10 months after treatment initiation showed no significant alterations in their rheology, while the third one exhibited a substantial increase in G* and . This increase was attributed to an exacerbated patient with heightened bacterial colonization and a low FEV1%.

Conclusions: In summary, our study emphasizes the significant positive impact of elexacaftor/tezacaftor/ivacaftor triple therapy on mucus rheology and bacteriology in cystic fibrosis (CF) patients. The reduction in expectorated volume, decreased incidence of specific infections, and improved mucus rheology within the initial 10 months of treatment highlight the transformative effects of ETI. While most samples beyond this period demonstrated sustained positive responses, exceptions underscore the importance of individualized considerations in CF treatment. These findings shed light on the evolving understanding of the complex relationship between mucus mechanical behaviour and bacterial dynamics in the context of CF treatment with ETI therapy.

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Conformational regulation of transcription in a *Pseudomonas aeruginosa* epoxide-based virulence circuit

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Even in the era of highly effective modulator therapies, the opportunistic pathogen *Pseudomonas aeruginosa* (Pa) infects the airways of patients with cystic fibrosis (CF) patient airways. Pa produces a virulence factor Cif that is associated with worse patient outcomes. Cif is an epoxide hydrolase that reduces cell-surface abundance of the cystic fibrosis transmembrane conductance regulator (CFTR) and sabotages pro-resolving signals that regulate inflammatory responses. The expression of Cif is regulated by a divergently transcribed TetR family transcriptional repressor CifR.

CifR represents the first reported bacterial transcriptional regulator that is sensitive to certain epoxide activators, meaning that *cif* expression can be derepressed in the presence of some substrates. However, neither the interaction of CifR with cognate operator sequences nor its mechanism of activation has been investigated at a molecular level. Using biochemical and structural approaches, we have uncovered the molecular mechanisms controlling this complex virulence operon. We present here the first crystallographic structures of CifR alone and in complex with operator DNA, resolved in a single crystal lattice.

Significant conformational changes are observed between these two structures. They suggest how CifR regulates the expression of the virulence gene *cif*. Interactions between the N-terminal extension of CifR and the DNA minor groove of the operator play a significant role in the operator recognition of CifR. In contrast, there is a surprising lack of direct base recognition in the major groove. We also determined that cysteine residue Cys107 is critical for epoxide sensing and DNA release, most likely through a covalent mechanism. These results offer new insights into the stereochemical regulation of an epoxide-based virulence circuit in a critically important clinical pathogen.

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Differential effects of CFTR modulators on SARS-CoV-2 infectivity in cultured nasal, bronchial, and intestinal epithelia of people with cystic fibrosis

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Introduction: People with cystic fibrosis (pwCF) are in general more susceptible to respiratory infections due to CFTR dysfunction in airway epithelial cells, however during the COVID-19 pandemic, pwCF were not deemed more susceptible to SARS-CoV-2. *In vitro* studies have shown impaired SARS-CoV-2 infectivity in CFTR deficient bronchial cells compared to wild type-controls, but differences in infection across different tissues are not known. To further understand the effect of CFTR dysfunction on SARS-CoV-2 infectivity, we conducted SARS-CoV-2 infection studies in cultured airway and intestinal epithelial cells from pwCF, in the absence or presence of CFTR modulators.

Methods: Nasal brushings (n=10), bronchial (n=10), and intestinal (n=5) biopsies were obtained from pwCF, including individuals with a F508del homozygous and heterozygous genotype. After expansion, both nasal and bronchial airway epithelial cells were differentiated in air-liquid interface cultures on transwell inserts. The intestinal epithelium was expanded as organoids, and afterwards cultured as submerged 2D monolayers on transwell inserts. Airway and intestinal epithelial cells were stimulated with vehicle or Elexacaftor, Tezacaftor and Ivacaftor (ETI) and infected at the apical side with strain /NL/2020SARS-CoV-2 (MOI 1). Infectivity was assessed by qPCR using primers targeting the envelope (E) gene at 7 days post infection.

Results: SARS-CoV-2 infected airway and intestinal epithelial cells of pwCF displayed differences in viral load, with the lowest viral load in bronchial epithelial cells, and the highest viral load in intestinal epithelial cells. Treatment with ETI did not result in a significant difference in viral load compared to vehicle treatment in nasal epithelial cells. However, upon ETI treatment we observed a significant increased viral load in bronchial epithelial cells, and a reduced viral load in intestinal epithelial cells compared to vehicle.

Conclusion: Our results revealed differences in SARS-CoV-2 infectivity between different epithelial tissues from pwCF, and contrasting effects of ETI CFTR modulator therapy. In line with previous studies, CFTR dysfunction in bronchial epithelia decreased SARS-CoV-2 infectivity, and furthermore was reversed by CFTR repairing drugs.

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Mutliomic approach to identify possible mechanisms of action of HEMTs and to propose new therapeutic targets

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New highly effective CFTR modulator therapies (HEMT) have changed the prognosis and quality of life of CF patients, and functional studies have ascertained their role in restoring CFTR-dependent Cl^- secretion. Other CFTR-related pathways could be improved by these treatments but to date their exact mechanism of action remains incompletely understood. We therefore undertook a multiomic study to decipher which pathways could be involved in HEMT's mechanisms of action.

We collected nasal epithelial cells from eight F508del homozygous patients and seven WT age- and sex-matched controls. Primary cultures were amplified and differentiated at air-liquid interface. Cultures were then treated with VX-445/VX-661/VX-770 or DMSO for 48h before collecting samples for transcriptomics, proteomics, and untargeted metabolomics.

In F508del-CFTR cultures, the effect of VX-445/VX-661/VX-770 was seen at all levels with differentially expressed genes, proteins, and metabolites. Moreover, when performing enrichment analysis, several pathways overlapped and were found to be differentially regulated at all three levels. This unraveled a possible role of decreased KRAS and hypoxia signaling and increased anti-oxidant and interferon response signaling. Pathway analysis also suggested a metabolic shift with a modification of lipid metabolism.

The convergence of transcriptomics, proteomics and metabolomics underlines the robustness of such multiomic approaches and could help identify new HEMT molecular or cellular targets.

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In vitro characterisation of drug candidates against *Mycobacterium abscessus*

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Non-tuberculous mycobacteria (NTM) are environmental bacteria and opportunistic pathogens that pose a particular threat to patients with cystic fibrosis or a weakened immune system [1]. Due to its resistance to numerous classes of antibiotics, *Mycobacterium abscessus* causes infections that are very difficult to treat [2]. We have therefore focused our drug discovery efforts on agents that are effective against *Mycobacterium abscessus*. In this context, we are investigating synthetic RNA polymerase inhibitors [3] with bactericidal activity against the resistant pathogen. We optimised a synthetic procedure for the drug class [3], several derivatives have been synthesised and also characterised within the working group [4].

We developed a method to simplify the determination of the minimum bactericidal inhibitory concentration (MBC). For this purpose we are using a fluorescent dye to stain the bacteria so that microscopic, image-based evaluation can be used. In this model, both individual antibiotics and combinations can be analysed for their bactericidal efficacy.

By working with the fluorescent dye 3-HC-2 Tre, a fluorescent lable coupled to trehalose, which is actively and selectively taken up by mycobacteria, we are also contributing to the development of diagnostic methods [5] [6]. With our work on the development of active substances against *Mycobacterium abscessus*, we make a contribution to improving the treatment and diagnosis of cystic fibrosis patients infected with mycobacteria.

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*N*α-aroyl-*N*-aryl-phenylalanine amides are active against a broad panel of non-tuberculous mycobacteria

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Infections with non-tuberculous mycobacteria (NTM) pose a particular risk for cystic fibrosis patients. The prevalence of pulmonary NTM infections in CF patients is increasing worldwide, with the *Mycobacterium abscessus* complex and the *Mycobacterium avium* complex being the most clinically significant. Nevertheless, CF patients are also infected with other NTM species, some of which are characterized by various resistance and susceptibility profiles. The robustness of these bacteria against the human immune system, as well as their intrinsic antibiotic resistance to common anti-infectives, makes treatment problematic and fraught with complications (Johansen et al., 2020). The management of the diseases is further complicated by the complex and expensive identification of the pathogens, making new anti-infectives with a broad activity spectrum against NTM extremely important.

We are investigating AAPs (Nα-aroyl-N-aryl-phenylalanine amides) as promising drug candidates that show high efficacy *in vitro* against certain mycobacteria (Low et al., 2017; Mann et al., 2022). Just like rifampicin, the substance class targets the inhibition of bacterial RNA polymerase, with different binding sites making cross-resistance unlikely (Lin et al., 2017). Based on the hit substance MMV688845 from the Pathogen Box® substance library (Medicines for Malaria Ventures, MMV (Richter et al., 2018)), a structurally diverse selection of AAPs has now been tested against a wide panel of different NTM species to verify their potential as broad-spectrum anti-NTM drugs. The panel that was tested for AAP susceptibility includes all *M. abscessus* complex subspecies as well as a set of clinical isolates, the human pathogenic type strains of the *M. avium* complex and variety of other NTM. The tested AAPs displayed generally active against the *M. abscessus* complex and the *M. avium* complex with MIC_{90} values in the low micromolar range comparable to those of clarithromycin. Apart from that *M. xenopi* and *M. simiae* showed less susceptibility to AAPs.

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Development of a novel CF lung disease model based on CF patient-specific human induced pluripotent stem cells

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Adequate modelling of CF lung disease is challenging aspect of CF research and currently relies on the utilisation of model systems like animal models, primary respiratory cells and immortalised cell lines. However, these model systems show significant drawbacks as they do not sufficiently recapitulate all aspects of human pathophysiology and/or are limited by accessibility of patient-specific cells. Therefore, induced pluripotent stem cells (hiPSCs) are emerging as a novel model system of CF lung disease and hold the potential to overcome previous drawbacks. hiPSCs possess a virtually unlimited proliferation capacity and can be generated from healthy (WT) and diseased donors by reprogramming of somatic cells. Moreover, differentiation of hiPSCs enables to generate and study a multitude of CF-affected cell lineages, like respiratory epithelial cells. In addition, genome engineering of hiPSCs is already well established to achieve seamless gene corrections, gene knockouts or integration of transgenes on a clonal basis. In our study, we developed a novel CF lung disease model based on CF patient-specific hiPSCs that carry the CFTR ΔF508 mutation and demonstrated its utility as a tool in CF research. A multistep differentiation protocol was applied to WT and CF hiPSCs to generate respiratory epithelial cells in air-liquid-interface cultures (iALI cultures) that contained a structured epithelium comprised of ciliated, goblet and basal cells. Molecular and functional analyses verified the airway specification of iALI cultures and revealed high similarities compared to primary derived respiratory epithelial cells (pALI cultures). Moreover, our analyses confirmed the manifestation of a CF-like disease phenotype in CF iALI cultures that was characterised by: (I) an impaired CFTR protein expression, (II) reduced transepithelial chloride conductance, (III) reduced ciliary beating and (IV) an altered mucous layer ultrastructure shown by electron microscopy. Furthermore, the application of CFTR modulator drugs (ETI: elexacaftor-tezacaftor-ivacaftor) enabled to modulate and partially rescue the disease phenotype of CF iALI cultures.

Our hiPSC-based CF disease model will represent a valuable tool to model CF lung disease and will serve as a platform to better understand CF pathophysiology, to test drug candidates and to develop novel therapies of CF.

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Characterization A559T-CFTR variant in patient-derived intestinal organoids and nasal cells in response to CFTR modulators correlated with in vivo clinical response to ETI therapy.

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Currently, the triple-drug combination, elexacaftor (E)/tezacaftor (T)/ivacaftor (I) (ETI, Trikafta®) has been demonstrated to have high efficacy and safety for CF patients harboring mutations that are eligible for this treatment. The inclusion of new CFTR variants in this list is possible following the in vitro demonstration of responsiveness to CFTR modulators. Here, we characterized a rare CFTR variant A559T and evaluated its functional restoration after in vitro treatment in patient-derived cells, and we correlated the ex-vivo data with the clinical effect of off-label ETI therapy in the same subject. Previous work done in human immortalized bronchial epithelium registered the absence of response of A559T-CFTR to any FDA-approved modulator. Our recently published data showed that A559T-CFTR presents a minimal function. The acute addition of I, following CFTR activation by forskolin, had no significant increment of baseline level of anion transport in both colonoids and nasal cells, confirming recent data published by other authors. However, the combined treatment, ET, significantly increases the chloride secretion in A559T colonoids and human nasal epithelium cells (hNEC), reaching approximately 10% of WT-CFTR function. These results were confirmed by forskolin-induced swelling assay and by western blotting in rectal organoids. We now describe the clinical response of this subject by reporting the results of 7 months of treatment with off-label ETI. We recorded an increment in lung function (FEV1%) from 46% to 52% of the predicted value. The volume and viscosity of the sputum were decreased. Resistance to physical effort was investigated by 6 minutes walking test (6MWT) showing improvements with variations following exercise before and after treatment for 3 months with ETI: SpO_2 enhanced from 86% to 91% with minimum SpO_2 values from 84% to 90%; respiratory rate decreased from 20 to 12 and from 28 to 20 apm, before and after exercise respectively. We detected a weight gain of 4.9 kg with BMI from 20.20 to 22.00 after 7 months of treatment). During ETI therapy, IV and oral antibiotics were not prescribed, hospitalization was not required, and there were no reasons anymore to consider inclusion in the list of lung transplantation. The study reveals the predictivity power of theratyping performed rectal or nasal derived cells. Both models show a significant response to ETI that has been confirmed in vivo for this case. providing a strong rationale for treating patients carrying this variant with ETI: the discrepancy with data obtained in immortalized cell line models suggests that primary cells should be utilized whenever possible to validate the results obtained in heterologous systems.

Acknowledgments: This research was funded by the Italian Cystic Fibrosis Research Foundation (FFC) #9/2020 (Delegazione FFC di Pesaro, Rivarolo Canavese e Gruppo sostegno FFC di Fidenza) to P.M.; Lega Italiana Fibrosi Cistica onlus (C.S. and P.M.); and American CFF, grant number Assael08A0 (P.M.).

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A platform for biomarkers evaluation, pathophysiology studies, and therapeutic development based on patient-derived cells collected by nasal brushing

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Therapeutic challenges and cases of inconclusive diagnosis (CRMS/CFSPID individuals) persist despite significant advancements in the knowledge and treatment of cystic fibrosis (CF). Giving the growing evidence of CFTR's role in tissues other than the airways, and the goal of a more personalized medicine, it is imperative to seek novel disease biomarkers, experimental models, and new therapeutic targets. An accessible patient-specific source of respiratory epithelium is the nasal cavity, where the *regio respiratoria* is located at the lower and middle nasal turbinates [PMID: 31853193; PMID: 31082105]. Primary nasal epithelial cells (hNEC) collected by nasal brushing have become a standard in recent years, but their potential is still underestimated.

In this study, we assessed the potential of hNECs for *ex vivo* studies to find novel biomarkers and *in vitro* applications involving advanced models for pathophysiology studies and therapeutic development.

hNECs were collected from healthy and CF individuals by a brushing for nostril. Immediate fixation coupled to immunodetection or mRNA extraction for transcriptomics was performed for *ex vivo* studies, while expansion of the basal cells was executed for *in vitro* applications.

Freshly-fixed samples were used to evaluate a novel potential biomarker for CF, the *ionocytes' CFTR content* (ICC). We assessed the genotype-phenotype-ICC relationships in a cohort of non-CF, CF, and CRMS/CFSPID subjects. Moreover, we enrolled CF patients starting TRIKAFTA. In accordance with genotype-phenotype, we found crescent ICC levels in the following groups: CF patients with severe mutations < CF patients with mild mutation/CRMS/CFSPID < CF patients treated with TRIKAFTA < non-CF individuals. Importantly, by comparing the ICC with other clinical parameters, we found a significant negative correlation with the sweat chloride levels.

For the *in vitro* studies, we retrieved roughly 500'000 basal cells from each brushing, and we expanded them to tens of millions until passages three-five. The basal cells were then nitrogen-stored, differentiated in 2D-ALI models, or reprogrammed into induced pluripotent stem cells (iPSCs). By comparing transcriptomic data from *ex vivo* and *in vitro* samples, we found similar signatures between native and *in vitro* differentiated airway epithelia, indicating that the basal cells retained their differentiation abilities after the expansion phase. Then, we applied microscopy-based assays to investigate the airway surface liquid (ASL) properties of 2D-ALI models kept under control or inflammatory conditions. Evaluation of ASL pH, thickness, and viscosity evidenced inflammatory-specific effects, as the acidification and hyperviscosity caused by IL-17 + TNFα, and highlighted the differential contribution of various channels and transporters, among which ATP12A proton pump emerged as a major player.

We also explored hNECs suitability for the generation of iPSCs. We obtained and fully characterized iPSC clones from non-CF and CF hNECs with similar efficiency to other classical sources (fibroblasts or PBMC) and initiated their differentiation into progenitor cells of the three germ layers as a base for multiorgan-on-chip systems.

In conclusion, in our study we demonstrated the potential of hNECs for the development of patients-specific platforms allowing *ex vivo* and *in vitro* studies, including evaluating novel biomarkers, exploring physio-pathological mechanisms, and developing advanced and personalized multi-organ models.

P79

Elexacaftor/Tezacaftor/Ivacaftor treatment partially normalizes osteoclasts phenotype in cystic fibrosis-related bone disease

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Personnalized therapies and innovative treatment enhance life expectancy in patients bearing CF mutations. Cystic fibrosis-related bone disease (CFBD) is a frequent comorbidity in CF patients. CFBD is characterized by low bone density and increased risk of fracture, even during childhood. However, mechanisms explaining the impact of defective CFTR function on bone homeostasis remain not fully elucidated. Preliminary data demonstrated that loss of CFTR function led to impaired osteoblastogenesis as well as a rise in the number of circulating pre-osteoclastic $RANK^+MCSFR^+$ monocytes. **We aimed to** study the impact of CFTR class II mutation channel loss of activity and its correction by modulator treatment on osteoclast (OC) phenotype. **Circulating** pre-OC monocytes were isolated from 33 patients bearing CFTR Class II mutations (NCT04877223) and 23 matched healthy controls (age and gender) (ALC/PIL/DIR/AJR/FO/606). Membrane RANK and MCSFR receptors were labelled for flow cytometry study. Sera levels of pro-inflammatory markers (IL-6, IL-8 and TNF-α), bone remodeling markers (CTX, PINP) and S1P were evaluated by ELISA. After 14 days, OC were plated on dentin slices for 7 days to evaluate resorption activity. After 21 days of differentiation, OC were stained (Phalloïdin-AlexaFluor488®, Vinculin-immunofluorescent staining, DAPI) to determine osteoclasts number, size and evaluate focal adhesion complexes. S1P supernatants levels were assessed by ELISA. Pre-OC monocytes were treated with prolonged exposure to Elexacaftor, Tezacaftor and Ivacaftor (ETI 3µM/3µM/1µM). **We evidenced** an increased proportion of $RANK^+$ $MCSFR^+$ cells in CF patients' blood (2.3-fold; $p<0.05$) compared to controls. Interestingly, we observed an increased of bone remodeling markers: CTX (1.,12 fold; $p<0.05$) and PINP (1.18-fold; $p<0.05$), and S1P (1.42-fold; $p<0.05$) concentrations from CF sera compared to control, independently from pro inflammatory markers. OC exhibiting CFTR mutations were less numerous but larger than non-CF ones (1.5-fold; $p<0.05$). ETI treatment did not restore number of OC but significantly decreased (-70%; $p<0.05$) their size towards non-CF ones. S1P levels in CF OC supernatants were higher compared to controls (+180%; $p<0.05$), after treatment even if levels were lower (-39%; $p<0.05$) they remained higher than in non-CF (+72%; $p<0.05$). Resorption activity that was strongly reduced in CF cultures (40-fold; $p<0.05$), was enhanced in treated culture compared to untreated (12-fold; $p<0.05$) without reaching non-CF levels. Vinculin staining was thinner and less co-localized with actin ring in OC bearing-CFTR Class II mutation than in control. ETI treatment partially restored cytoskeleton organization. **Our results** tend to demonstrate defective OC differentiation and resorption process in OC bearing class II CFTR mutations, which are partially restored by CFTR modulator treatment. This could explain disruption in bone homeostasis observed in CF patients.

P80

Automation workflow for forskolin induced swelling assay in cystic fibrosis: advancing personalized medicine through high-throughput screening with primary intestinal organoids

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Since a decade the forskolin induced swelling assay (FIS assay) is being used to test medication for cystic fibrosis (CF), a disease caused by mutations in the CFTR gene. By conducting the assay on primary intestinal organoids obtained from CF patients, personalized medicine can be facilitated through this method. This assay is performed by many different people throughout the time, which can cause person to person variance, but also day to day variance by one person. An automated workflow can limit these variances and allow for high-throughput screening on a daily basis. With the use of different machinery, we designed an automation workflow system. This includes an incubator, microscope, various liquid handlers, and a robotic arm to move plates from place to place. With the liquid handler (OT2) from Opentrons it is possible to pipette small Matrigel droplets with patient-derived organoids in a consistent manner in a standard 96-culture well plate. After the Matrigel droplet formation in the incubator, medium can be added using a reagent dispenser (MultifloFX), small compounds and forskolin concentrations are automatically dispensed using small droplets (I.DOT) for high accuracy. The KX-2 robotic arm can move the plate with droplets between all places within the automation setup. A high-end automated microscope (Zeiss CD7) can image the organoids over time for further analysis. Lastly, using Artificial Intelligence (AI) we standardized and automated the analysis of the FIS assay based on simple bright field imaging. This whole process can run completely autonomous in a very stable manner using specialized orchestration software (SRUN-Overlord, PAA).

This pipeline allowed us to create a high-throughput analysis for cystic fibrosis but will also provide opportunities for future assays and other culture techniques within this system. The advantage of this system is that it can be upgraded with different machinery if needed to be able to perform more complex assays in the future. Finally, we strongly believe that this assay standardization and automation is critical for providing trustworthy increased assay throughput.

P81

Theratyping of R347P, R347H, T465N and L227R CFTR variants in patient-derived rectal organoids correlates with clinical response to CFTR modulators in matched CF patients.

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CF is a life-limiting disease that is manageable by the recent use of mutation-targeted CFTR modulators. Response to therapy on an individual level has been proposed to be predicted in vitro through the evaluation of the response of stem cell-based organoids to CFTR modulators. Here, we develop patient-derived intestinal organoids from CF subjects carrying one of the following CFTR missense variants: T465N, L227R, R347H and R374P. Of note, T465N is a rare and still poorly characterized variant found in a CF patient presenting Q39X/T465N genotype that still awaits CFTR therapy approval. We then evaluated their functional impact on CFTR protein function and their restoration at molecular (western blot) and functional levels (FIS assay and/or electrophysiology technique) in response to various CFTR modulators: lumacaftor (L)-ivacaftor (I), tezacaftor (T) -I, and elexacaftor (E) T-I. Moreover, clinical data of the CF patients bearing R374P in homozygous or in compound heterozygous as well as for the subjects harboring R347H, L227R and T465N variants were collected before and after CFTR modulator therapy. The forskolin-induced swelling assay (FIS) showed that the T465N, R347H and R347P variants respond to treatments with TI and ETI, while L227R only showed a negligible increase in swelling after LI or ETI treatment. The transepithelial current measurement done in colonoids registered a significant enhanced CFTR dependent anions secretion upon TI or ETI treatment for T465N, R347H and R347P variants reaching up to 19% of WT CFTR activity. On the other hand, the increase of L227R-CFTR activity upon CFTR modulators was marginal, representing less than 5% of WT CFTR functional activity compared to a non-CF sample. We also detected a strong intensification of the expression of the fully glycosylated CFTR protein after ET treatment for the variants R347P, R347H and T465N-CFTR. The protein expression level of L227R was only minimally increased by the treatments. In vivo treatment with LI for the subject L227R/L227R had no significant effect, only permitted to record a reduced sweat chloride concentration (from 104 mmol/L to 75 mmol/L) while BMI and FEV1 remained essentially unchanged. Trikafta administration on the patient carrying the R347P/R347P genotype was associated with a significant improvement of his clinical parameters with 19% increase in FEV1 and sweat chloride reduction from 74 to 39 (mmol/L). In conclusion, functional and protein expression assays in colonoids correctly predicted clinical benefits or lack of the same after treatment, confirming the ability of intestinal organoids to predict in vivo drug efficacy.

Acknowledgments: This research was funded by the Italian Cystic Fibrosis Research Foundation (FFC) #9/2020 (Delegazione FFC di Pesaro, Rivarolo Canavese e Gruppo sostegno FFC di Fidenza) to P.M.; Lega Italiana Fibrosi Cistica onlus (C.S. and P.M.); and American CFF, grant number Assael08A0 (P.M.).

P82

Label free prediction of primary airway epithelial cell staining

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Fluorescence microscopy is a powerful technique for identifying cells or cellular structures. Researchers use fluorescence microscopy to analyse the topology, location and distribution of cells or cell structures and is both highly specific and highly sensitive. A large challenge however is that fluorescence microscopy is usually performed on fixed cells and has a maximum of 4-5 immunofluorescent markers. With the use of Artificial Intelligence (AI), we attempt to predict fluorescent staining on non-fixated and living cell cultures based on bright field microsopy. Automatic staining predictions in airway epithelium will allow for repeated measurements in a single well over long periods of time. This can improve current protocols in human disease models for respiratory diseases such as cystic fibrosis (CF). Primary human airway cells are widely used to study various airway diseases, so we use airway basal stem cells obtained from patients with respiratory diseases such as cystic fibrosis, Primary ciliary dyskinesia and healthy patients.

Here we developed convolutional neural networks (CNN) models to predict staining in conventional air-liquid interface cultures and submerged airway cultures. CNNs are a class of neural networks specifically designed for image analysis. CNNs implement one or more kernel based analysis steps which allow models to learn both high and low level visual features. By stacking model predictions a theoretical unlimited amount of markers can be predicted on a single bright field image. The models are trained and tested on cilia (SiR-tubulin) and nuclei (DAPI) staining of airway epithelial cell cultures derived from airway basal stem cells.

We show that the developed AI models are able to predict the nuclei staining in airway cultures to a very high accuracy. Furthermore, we are now able to predict cilia staining and thereby ciliated cells purely on the morphological features of these cells using AI and bright field microscopy. After further optimization of these models they can be used to study airway cell cultures in more detail in a live cell setting. This will for example allow for the study of airway cell differentiation in CFTR loss-of-function experiments.

Lastly, this research is CF-overarching and can be implemented for fundamental studies on a broad range of airway diseases.

P83

Amplifier PTI-428 enhances the effect of VX-661/VX-445/VX-770 in patient-derived airway organoids

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Background: CFTR-modulator therapy has proven to change the disease course of many people with CF (pwCF) who are eligible for treatment. Consequently, the identification of reliable biomarkers that can predict efficacy of modulating therapy becomes crucial in discovering effective drugs for all pwCF. Over the past years different patient-derived organoid (PDOs) models have emerged to assess the effect of CFTR-modulator therapy. Forskolin-induced swelling (FIS) of PDO can be used to quantify CFTR protein function. Currently, FIS of intestinal organoids is most validated and has proven effective in predicting clinical drug response. The potential of applying FIS to predict drug efficacy in other types of PDOs is more premature. However, other types of PDOs might be more sensitive to pick up CFTR modulator effect which cannot be detected in intestinal organoids due to their different cellular background. FIS in intestinal PDOs is completely CFTR dependent where airway organoid models are known to have multiple ion channels responsible for swelling.

In this project, sensitivity in CFTR modulator repairing effects were compared between paired patient-derived intestinal and airway organoids. Performing FIS to test drug efficacy in both intestinal and airway organoids may help more patients towards eligibility of new therapy.

Methods: FIS upon CFTR modulator incubation was compared in 6 homozygous F508del paired patient-derived intestinal, nasal and bronchial organoids to compare sensitivity. CFTR modulator monotherapy and combinations tested were VX-770, PTI-808, VX-445, VX-661/VX-445/VX-770, PTI-801/PTI-808, PTI-428, VX-770/PTI-428, PTI-808/PTI-428, VX-445/PTI-428, VX-661/VX-445/VX-770/PTI-428 and PTI-801/PTI-808/PTI-428. CFTR repairing effects will also be validated on mRNA level.

Results: We observed that amplifier PTI-428 increased the efficacy of VX-661/VX-445/VX-770 in paired homozygous F508del nasal and bronchial but not in intestinal PDOs.

Conclusions: Effect of the amplifier PTI-428, on top of VX-661/VX-445/VX-770, could only be detected in patient-derived airway organoids and not in intestinal organoids. Previous research has shown that PTI-428 increases CFTR biosynthesis via PCBP1-mediated regulation of CFTR mRNA. We hypothesize that CFTR mRNA levels are saturated in intestinal organoids but not in airway organoids. This contrast in saturation levels suggests the potential for PTI-428 to have an effect specifically in the airways and not in the intestine. More research is needed to further understand these differences. To conclude, both intestinal and airway organoids are complementary models to study the specific drug effects of individual tissues.

P84

An iPSC-derived bronchial epithelial model to study nonsense mutations in Cystic Fibrosis

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Objectives: Cystic fibrosis is a rare autosomal recessive disease with more than 2,000 *CFTR* gene mutations identified in patients, most patients (80%) carrying at least the F508del mutation on one allele. The *CFTR* gene encodes an ion channel located at the apical surface of numerous epithelia, including the respiratory and pancreatic epithelia. Among the various organs affected, the progressive destruction of the lungs significantly impacts the patient's quality of life and life expectancy. Since almost four years, a disease-modifying therapy, the Kaftrio®, has been offered to patients with the F508del mutation. However, given the large number and diversity of *CFTR* gene mutations, many patients remain without an efficient treatment. Among the mutations that do not respond to Kaftrio®, are nonsense and splicing mutations leading to the absence of CFTR protein.

The team, as part of a French consortium supported by the association Vaincre la Mucoviscidose, is starting a collaborative research program (Di-T-CAP, ClassFirstTheraRead) aimed at identifying and validating CFTR modulators for class nonsense and splicing mutations. Reliable and robust validation of such molecules requires improved *in vitro* human models as close as possible to physiological conditions. Our objective in the consortium is to establish airway epithelia derived from induced pluripotent stem cells (iPSC) and grown at air-liquid interface (ALI). Mutated epithelia will be generated with a CRISPR/Cas-9 approach from a control iPSC line.

Methods:

1. Differentiation of the PCi-CAU2 iPSC WT line into airway epithelium grown in ALI,
2. Characterization of the structure, composition, and integrity of the epithelium through immunostaining identifying epithelial cell types (ciliated cells, basal cells, secretory cells, club cells, and ionocytes), and measurement of transepithelial electrical resistance parameters (TEER)

Results/Conclusions: Differentiating iPSCs from the PCi-CAU2 cell line into bronchial epithelium proceeded successfully through several steps in appropriate culture media. After 80% of iPSCs were differentiated into definitive endoderm cells ($CKIT^+/CXCR4^+$), 9.6% of these cells differentiated into lung progenitors ($CD47^{high}/CD26^{low}$). Further differentiation yielded 23.6% bronchial basal cells ($NGFR^+/EpCAM^+$). The culture of these cells under ALI conditions led to the development of a pseudostratified bronchial epithelium, which was further characterized by histochemistry. The TEER of this epithelium is comparable to that of an epithelium derived from human nasal epithelial cells. Immunofluorescence staining experiments revealed the presence of basal ($KRT5^+$), ciliated ($acetyltubulin^+$), secretory ($MUC5AC^+$), and club ($SCGB1A1^+$) cells. The Ionocytes still need to be identified since we observed marginal CFTR-positive expressing cells.Our results demonstrate that the iPSC PCi-CAU2 line can differentiate into a bronchial epithelium with morphology characteristics and TEER measures comparable to those of an HNEC-derived epithelium. The iPSC lines carrying the CFTR nonsense mutations are currently being constructed in collaboration with the iPSC-CRISPR platform at I-Stem. Bronchial epithelium derived from the differentiation of this mutated cell line will be used to test the efficacy of molecules modulating those mutations and restoring the CFTR function.

P85

Investigation of CFTR function and epithelial barrier properties at single cell resolution using multi-electrode array technology

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Objectives: CFTR is involved in many processes throughout different organs. To date however, most CFTR modulator studies have focused on their effect on CFTR mediated ion transport and hydration in the airway epithelium. Nevertheless, the extrapulmonary effects of CFTR modulator therapy were recently identified as one of the top ten research priorities by the CF community, acknowledging in particular the major burden of gastro-intestinal symptoms for people with CF (PwCF) (Rowbotham et al, 2023). Moreover, although it is assumed that CFTR modulator treatment reaches and acts similarly in all CFTR expressing cells within an epithelium, current assays lack the required spatial resolution to study potential differences in modulator rescue of CFTR function between cells. We therefore set out to investigate the effect of CFTR modulator treatments on CFTR ion channel function and barrier integrity in individual intestinal epithelial cells using the UZ Leuven CF organoid biobank and imec's high density multi-electrode array chip.

Methods: CFTR ion channel function and local barrier integrity were studied using electrical impedance recordings on multi-electrode array chips with >16.000 subcellularly sized (8µm) electrodes. For initial studies, we used the intestinal colon cancer cell line CaCo2 which expresses CFTR endogenously. Cells were grown for 7-14 days on collagen I-coated chips, after which baseline impedance (barrier integrity) was recorded at 1 kHz. Next, cells were treated for 20 minutes with CFTR activator forskolin (10µM) or a combination of forskolin and CFTR inhibitor Inh-172 (50µM) and impedance was recorded once more. CFTR function was quantified as the percentage change between both recordings. Barrier integrity was also evaluated separately through immunocytochemistry for tight junction protein ZO-1 and by measuring apparent permeability with FITC-dextran 3-5kDa (FD4).

Results: As a proof of concept, we used CaCo2 cells to measure CFTR function with high spatial resolution by impedance recordings. We observed a CFTR-specific impedance drop of ~20% upon CFTR stimulation by forskolin in CaCo2 cells (vs. ~3% in mock treated controls; p=0.0003), which was completely inhibited by co-incubation with Inh-172 (~1% drop; p=0.0047; vs. mock: p=0.96). To confirm that this was due to CFTR channel function, we showed that tight junctions were not affected by these treatments as epithelial permeability of FD4 was unaffected and ZO-1 localization intact. We are currently investigating CFTR function and local barrier integrity in organoid-derived monolayers from non-CF controls and PwCF, with and without CFTR modulator treatment.

Future perspectives: We here present the first exciting data on the use of high-density electrical impedance recordings in CaCo2 cells that allow to study simultaneously barrier integrity and CFTR channel function at single cell resolution. We are currently using this assay to evaluate the effect of CFTR modulator treatment on PwCF-derived primary intestinal monolayers at the single cell level. Next, we aim to correlate this single cell CFTR rescue with CFTR protein expression. We envision that this novel model will contribute to unravelling the full effects of CFTR modulator treatment, in the gastro-intestinal tract and beyond.

P86

Optimization of secretory cell dependent CFTR function measurements in 3D airway organoids derived from submerged differentiated nasal epithelia

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Introduction: Secretory cells represent a major CFTR expressing cell type of the airway epithelium that is affected in cystic fibrosis (CF). In order to further study CFTR function in secretory epithelial cells in 3D airway organoids, we aimed to optimize a previously established culture protocol in which organoids are generated from nasal-brushing derived airway epithelial cells differentiated on conventional submerged cell culture plates.

Methods: Nasal brushings derived airway basal cells were obtained from a healthy control (HC) and CF subject. Airway basal cells were expanded in submerged conditions, and subsequently confluent monolayers were differentiated in submerged conditions. The differentiation medium was supplemented with bone morphogenetic protein 4 (BMP-4), a growth factor that induces secretory cell differentiation. After 7, 10, and 14 days of differentiation, airway organoids were generated by fragmenting the differentiated cell layer and embedding the fragments into basement membrane extract droplets. Generated 3D airway organoids were subsequently used to study CFTR function in a forskolin-induced swelling (FIS) assay. To increase CFTR function in secretory epithelial cells, organoids were pre-incubated for five days with interleukin-1β/interleukin-17/TNFα. To determine CFTR repair, CF airway organoids were pre-treated with vehicle, Ivacaftor or Elexacaftor/Tezacaftor/Ivacaftor (ETI).

Results: Submerged differentiation of airway epithelial cells with BMP-4 resulted in higher abundance of secretory cells compared to the control conditions. 7 days of differentiation was sufficient to observe a significant increase in modulator response in organoids. The response was further increased after 10 and 14 days of differentiation. HC organoids pre-treated with the cytokine combination of interleukin-1β/interleukin-17/TNFα showed enhanced fluid transport after addition of forskolin. Moreover, cytokine treated CF organoids displayed elevated responses to Ivacaftor and ETI in FIS assays.

Conclusion: The addition of BMP-4 in our differentiation medium led to faster maturation of secretory airway epithelial cells. Moreover, pre-incubation of secretory cell enriched airway organoids with interleukin-1β/interleukin-17/TNFα increased CFTR function and modulator responses in FIS assays, possibly resembling enhanced CFTR function during airway inflammation in CF. This optimized culture model can increase the scalability of *in vitro* studies while creating a biologically relevant model to further study CFTR function in secretory airway epithelial cells in CF.

P87

In vitro and *in vivo* efficacy of elexacaftor/tezacaftor/ivacaftor in people with cystic fibrosis carrying rare CFTR variants

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Objectives: CFTR modulator (CFTRm) therapy with elexacaftor/tezacaftor/ivacaftor (ETI) has been approved for people with CF (pwCF) carrying at least one copy of the F508del. In the United States, 178 rare *CFTR* gene variants responsive in FRT cells have the ETI label extension. Other *in vitro* studies, such as patient-derived intestinal organoids (IOs), have also been anecdotally used to predict the *in vivo* response to CFTRm in pwCF with rare CFTR gene variants, leading to the initiation of ETI therapy.

Methods: The *in vitro* effect of ETI (expressed as AUC at 0.128 μM concentration of forskolin) was measured using FIS assay in IOs from nine pwCF carrying rare non-F508del genotypes (D1152H/N1303K; I336K/1717-1G>A; G551D/R553X; 2789+5G>A/3659delC; W865X/S945L; N1303K/3849+10kbC>T; 621+1G>T/3849+10kbC>T; 3849+10kbC>T/CFTRdele2,3). The effect of ETI *in vivo* could be assessed 4 weeks (change of ppFEV1 and BMI) and 6 months (sweat chloride concentration, SCC) after initiation of the therapy in five of these pwCF who received treatment under managed access program.

Results: FIS assay showed increased swelling of all IOs treated with ETI. Clinical data showed improvement of mean ppFEV1 value by 11.0 percentage points ($n=5$, $p \leq 0.05$)) compared to the mean value before treatment, BMI remained stable throughout the follow up. We observed a significant reduction in SCC by 40.0 mmol/l ($n=3$, $p \leq 0.01$). Two PwCF were switched to ETI from ivacaftor (IVA), both FIS in IOs and clinical data showed superiority of ETI over IVA.

Conclusion: *In vitro* measurements in IOs could serve as a prediction tool to extend the approval of ETI and facilitate personalized medicine in PwCF carrying rare *CFTR* variants.

Supported by Ministry of Health of the Czech Republic, grant nr. NU23-07-00079

P88

Endometrium-derived organoids from cystic fibrosis patients to study the endometrial factor in the disease-associated fertility deficiency

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Introduction/objectives: Due to their increased life expectancy in this highly effective modulator therapy (HEMT) era, people with CF (PwCF) become increasingly interested in starting a family. However, female CF patients suffer from deficient fertility, often facing problems to become pregnant. Underlying reasons remain understudied. In particular, it is largely unknown whether dysfunction of the endometrium, the womb's inner lining and key tissue for embryo implantation and development, is involved. Pregnancy rates have increased since the HEMT, but the direct impact of these CF medicines on the endometrium is unknown. These gaps are mainly due to lack of appropriate research models. Therefore, we developed endometrium-recapitulating organoid models from CF patients and started to decode the role of the endometrium in CF-associated fertility deficiency and the impact of CFTR modulators on endometrial (dys-)function.

Methods: Endometrial organoids (EMO) were developed from CF patient-derived endometrial biopsies. These CF EMO were molecularly and functionally compared to healthy endometrium-derived EMO to start uncovering aberrations that underlie CF fertility deficiency. First, the EMO were characterized using RT-qPCR, and histochemical (e.g. periodic acid-Schiff) and immunofluorescence stainings. The HS-YFP quenching assay was used to probe CFTR function in the EMO and their response to CFTR modulators and CFTR-inh172. EMO were exposed to defined hormonal (estrogen (E2) and progesterone (P4)) regimens to recapitulate the different menstrual cycle phases *in vitro*. Bulk RNA-sequencing (seq) of EMO was performed before and after hormonal treatment to elucidate differences in endometrial responsiveness between the CF and healthy condition. Interesting genes identified were validated by RT-qPCR.

Results: We successfully developed organoids from CF patient endometrium. Before, we have shown that EMO from healthy endometrium can reliably reproduce the menstrual cycle phases under defined E2/P4 exposure. Here, we found cycle phase-dependent *CFTR* and (opposite) *ENAC* expression levels in EMO, similar to *in vivo*. Moreover, CFTR functionality (in healthy EMO) could be validated by HS-YFP quenching and CFTR-inh172 inhibition. Next, the CFTR defect as well as response to CFTR modulators (*i.e.* ETI) was demonstrated in CF EMO. In addition, CF EMO showed a thicker mucus layer as compared to healthy EMO. Currently, we are deciphering whether and how menstrual cycle phases, including the embryo receptivity stage, are different between CF and healthy endometrium, as avatared by their EMO. Intriguingly, CF EMO showed a heightened response to E2 (*i.e.* hormonal treatment to recapitulate the proliferative phase) with higher proliferative activity and visually more dying organoids, which can be explained by decreased E2-inactivating capacity in the CF condition. In addition, we found a lower expression response of key receptivity markers in the CF EMO upon hormonal recapitulation of the embryo receptivity stage. Additional mining of the RNA-seq data will now provide further insights in endometrial aberrations in PwCF.

Discussion: We established a new organoid model from CF patient-derived endometrium which provides a valuable novel research tool to shed light on the endometrium-centred causes in CF-associated fertility deficiency and to explore the impact of CFTR modulators and other therapies on the endometrium.

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Challenges in interpreting discordant in-vitro responses to modulator combination Elexacaftor, Tezacaftor and Ivacaftor in different tissue models derived from an individual harbouring ultra-rare genotype: H609R/2184insA

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Introduction: Assessing CFTR function under *in-vitro* conditions have provided valuable insights on drug responses for patients with CF (pwCF). Human nasal epithelial cells (HNECs) and intestinal organoids (HIOs) are such pre-clinical drug testing platforms that can be helpful in studying the drug responses of highly effective modulator therapies (HEMTs), specifically for those patients with rare CF causing mutations. H609R is a rare CFTR missense mutation, prevalent in the Ecuadorian population (19%). Previous studies on H609R-HEK-293 cells have shown 50% wildtype rescue of CFTR by the fluorescence-based membrane potential (FMP) assay when treated with Elexacaftor (E), Tezacaftor (T) and Ivacaftor (I) (Laselva et al., J. Pers. Med. 2021). In order to evaluate the consequences of the H609R mutation in relevant tissue context we report *in-vitro* responses observed in HNECs and HIOs of a 5-year-old H609R/2184insA patient with explicit consent.

Methods: Nasal epithelial cells (HNECs) and rectal biopsies were collected from the donor as a part of the CF Individualized Therapy (CFIT) Study (Eckford et al., J. Cyst. Fibros. 2019). The HNECs were cultured until passage 3 and cells were differentiated under air liquid interface (ALI) conditions for 14 days. HNECs were treated with Elexacaftor and Tezacaftor for 48hr and subjected to Ussing Chamber studies and FMP studies to assess the CFTR function. 3D HIOs were transformed to 2D HIOs and treated with ETI and the CFTR functional response was studied by Ussing chamber studies.

Results: Immuno-fluorescence staining by ZO-1 in HNECs showed poor differentiation of cultures. The Ussing chamber measurements following ETI treatment was ΔIeq -0.75± 0.5 mA/cm^2 which is lesser than a 10% forskolin+ Ivacaftor mean observed for non-CF HNECs. However, the FMP measurements showed that HNECs treated with ET lead to an Ivacaftor % potentiation of 32.81±1.05 ΔF/F0 which was higher than a 50% non-CF mean (19.1 ΔF/F0). In contrast, Ussing chamber measurements in HIOs measured a forskolin current of -15.6±2.2 mA/cm^2 post ETI treatment which is more than the 10% of the forskolin response observed for HIOs in non-CF individuals.

Conclusions: The current study presents a case where theratyping studies of an ultra-rare mutation in two different primary tissue models derived from the same patient led to two different interpretations. Future studies will focus on defining the tissue specific properties that enabled robust rescue by Trikafta(tm) (ETI) in colonic tissue and poorer responses in nasal epithelial cultures.