2019

European Cystic Fibrosis Society

16th ECFS Basic Science Conference

Conference Programme & Abstract Book

Dubrovnik, Croatia

Chairpersons

Isabelle Callebaut, Carlos Farinha and Martin Mense

27 March – 30 March 2019

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CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

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italian cystic fibrosis research foundation

WELCOME FROM THE ECFS PRESIDENT

Dear Friends and Colleagues,

It is a great pleasure to welcome you in Croatia to the 16th European Cystic Fibrosis Conference entirely dedicated to Basic Science.

This year we are delighted to welcome Prof. Callebaut as the conference Chairperson who will be supported by Prof. Farinha and Dr. Mense as co-chairpersons.

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

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

WELCOME FROM THE CONFERENCE CHAIRPERSONS

A very warm welcome to you to the 16th ECFS Basic Science Conference located, this year, in Croatia.

It's easy to see why Dubrovnik is known as the, "Pearl of the Adriatic" as soon as one arrives. By taking a walk around the historic centre, outstanding views can be seen from the famous city walls encircling the city. Built to protect Dubrovnik from invaders in days gone by, history seeps out from the stones, allowing tourists to step back in time and discover the long, rich history. In the past, Dubrovnik was known for fruitful merchandising and maritime trading. Nowadays it is one of the most popular tourist destinations in the Adriatic region.

A main feature of the Basic Science Conference is the large presence of young participants, including PhD students and post-docs, who are instrumental to the success of this annual event with their passion and participation in lively discussions.

During the week there will be ample opportunity to share and explore your data with others and interact in this beautiful setting with the best of European and International experts within this field.

This year's programme features 8 symposia with international experts and invited talks from submitted abstracts. As for the last editions we will also have two keynote lectures, flash poster sessions and a session from the ECFS Basic Science Working Group. On Saturday a flash paper session will be organised in parallel with a Journal of Cystic Fibrosis workshop on how to get published. We will end the programme with the closing keynote lecture. 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.

Isabelle Callebaut
Sorbonne University, Paris
France

Carlos Farinha
University of Lisboa
Portugal

Martin Mense
CFFT Lab
United States

2019 ECFS Conference

New Frontiers in Basic Science of Cystic Fibrosis

27-30 March 2019, Dubrovnik, Croatia

Programme

Chairpersons:

Isabelle Callebaut (Paris, FR), Carlos Farinha (Lisbon, PT), Martin Mense (Lexington, US)

Wednesday, 27 March 2019 (Day 1)	
13:30-17:00	**Pre-Conference Seminar**
	CFTR: new insights on structure and function, and implications for modulation
	Chairs: Bertrand Kleizen (NL) - David Sheppard (UK)
13:30-13:35	Introduction - Bertrand Kleizen (NL)
13:35-14:10	The allosteric effects of the F508del mutation in human CFTR elucidated by cryo-EM enabled by protein engineering - John Hunt (US)
14:10-14:45	Conformational landscape of CFTR from experimental and simulation data - Isabelle Callebaut (FR)
14:45 – 15:15	*Coffee break*
15:15-15:50	On the molecular mechanism of action for CFTR potentiators - Tzyh-Chang Hwang (US)
15:50-16:25	CFTR therapeutics: current evidence, gaps in knowledge, and future directions - Isabelle Sermet-Gaudelus (FR)
16:25-16:55	Discussion
16:55-17:00	Conclusion - David Sheppard (UK)
17:30-18:00	**Official Opening of the Meeting by the Conference Chairpersons**
18:00-19:00	**Opening Keynote Lecture**
	The genetics and genomics of CF – Garry Cutting (US)
19:00-19:45	**Welcome Reception**
19:45-21:30	*Dinner*

Thursday, 28 March 2019 (Day 2)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Genetics, genomics, and transcriptomics**
	Chairs: Garry Cutting (US) – Alexandre Hinzpeter (FR)
08:45-09:10	Enhancers, structural elements and 3D organization: key features in regulation of *CFTR* gene expression - Ann Harris (US)
09:10-09:35	Update on cystic fibrosis modifier genes - Harriet Corvol (FR)
09:35-10:00	Restoration of the CFTR function by antisense oligonucleotide splicing modulation - Batsheva Kerem (NL)
10:00-10:10	Abstract 01 - Identification of *CFTR cis*-regulatory variants - Mégane Collobert (FR)
10:10-10:20	Abstract 02 - Comparison of three genome editing techniques to correct the common W1282X mutation responsible for cystic fibrosis - Karen Mention (IE)
10:20-10:30	Abstract 06 - Recruitment to cystic fibrosis airway fluid licenses transcription and subsequent acquisition of the pathological GRIM fate by human neutrophils - Rabindra Tirouvanziam (US)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 2 – Rare mutations and complex alleles**
	Chairs: Isabelle Sermet-Gaudelus (FR) – Nicoletta Pedemonte (IT)
11:00-11:25	*CFTR* complex alleles - Alexandre Hinzpeter (FR)
11:25-11:50	Make sense out of nonsense with antisense - Shuling Guo (US)
11:50-12:15	A shortcut to bringing more patients with rare genotypes into the tent? - Phil Thomas (US)
12:15-12:25	Abstract 10 - Characterization of the rare S955P-CFTR mutation and its response to modulators in cellular models and patient-derived materials - Sofia Ramalho (PT)
12:25-12:35	Abstract 09 - The cystic fibrosis mutations L927P and I336K modulate CFTR pore dynamics during channel gating, but are rescued by ivacaftor and lumacaftor - Majid K. Al Salmani (UK)
12:35-12:45	Abstract 08 - Not all stop codons are created equal, neither do they always stop: morals from single-channel studies of E60X- and G542X-CFTR - Jiunn-Tyng Yeh (US)
12:45-14:30	*Lunch*
14:30-15:30	**Flash Poster Session (even numbers)**
	Chair: Bertrand Kleizen (NL)
15:30-16:00	*Coffee break* & **Poster viewing**
16:00-17:45	**Symposium 3 – Epithelial channelome: (i) Folding and structure**
	Chairs: Isabelle Callebaut (FR) – Ineke Braakman (NL)
16:00-16:25	Mechanism of ion conduction and gating in the calcium-activated chloride channel TMEM16A - Raimund Dutzler (CH)
16:25-16:50	A minimal helical hairpin motif provides molecular-level insights into misfolding and pharmacological rescue of CFTR - Michael Schlierf (DE)
16:50-17:15	NBD1 cotranslational folding intermediates as therapeutic targets - Bill Skach (US)

17:15-17:25	Abstract 13 - The regulatory insertion enables novel conformations of NBD1 of CFTR - Daniel Scholl (BE)
17:25-17:35	Abstract 18 - CFTR conformational landscape explored by computational approaches - Ahmad Elbahnsi (FR)
17:35-17:45	Abstract 15 - Identifying the molecular targets for CFTR potentiators GLPG1837 and VX-770 - Han-I Yeh (US)
17:45-18:00	*Break*
18:00-19:45	**ECFS Basic Science Working Group session**
	Activating alternative chloride channels to treat CF: friends or foes?
	Chairs: Margarida Amaral (PT) - Jeff Beekman (NL)
18:00-18:05	Introduction
18:05-18:50	Round table 1: Can we regulate TMEM16A independently of Ca^{2+}? And TMEM16A /SLC26A9 independently of CFTR? Moderators: Jeff Beekman & Karl Kunzelmann
18:05-18:10	Facts about ANO1 - Karl Kunzelmann (DE)
18:10-18:13	Abstract 32 - EACT increases intracellular calcium levels by a TMEM16A-independent mechanism - Henry Danahay (UK)
18:13-18:16	Abstract 111 - Niclosamide repurposed for the treatment of inflammatory airway disease – Roberta Benedetto (DE)
18:16-18:19	Abstract 34 - Identification of novel ANO1/TMEM16A regulators as alternative therapeutic targets for cystic fibrosis - Madalena Pinto (PT)
18:19-18:50	Overall Discussion
18:50-19:00	Short Break
19:00-19:45	Round table 2: TMEM16A and mucus Moderators: Margarida Amaral & Luis Galietta
19:00-19:03	Abstract 30 - TMEM16A channel function does not influence goblet cell numbers in the human airway epithelium - Henry Danahay (UK)
19:03-19:06	Abstract 28 - Cell Proliferation Upregulates ANO1 in Mucus Cell Hyperplasia – Filipa Simões (PT)
19:06-19:09	Abstract 105 - The attached stratified mucus in obstructive airway disease is detached by calcium removal - Dalia Fakih (SE)
19:09-19:12	Abstract 31 - Physiological role and therapeutic importance of TMEM16A chloride channel in the airway epithelium - Arianna Venturini (IT)
19:12-19-45	Overall Discussion
19:45-21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Even numbers

Friday, 29 March 2019 (Day 3)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4 – Epithelial channelome: (ii) Cell physiology and ion transport**
	Chairs: Margarida Amaral (PT) – Phil Thomas (US)
08:45-09:10	Exploiting species differences to investigate CFTR - David Sheppard (UK))
09:10-09:35	Anionophores: Small-molecule CFTR surrogates as mutation agnostic CF drug candidates - Roberto Quesada (ES)
09:35-10:00	ATP12A – pig models - Michael Welsh (US)
10:00-10:10	Abstract 20 - Impact of CFTR function on airway mucus properties - Martial Delion (BE)
10:10-10:20	Abstract 21 - Early onset of airway mucus obstruction associated with increased mortality in neonatal *Slc26a9* deficient mice - Pamela Millar-Büchner (DE)
10:20-10:30	Abstract 24 - Dual role of pendrin as a bicarbonate secretion pathway and CFTR modulator in well-differentiated human nasal and bronchial epithelial cells - John W. Hanrahan (CA)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 5 - CFTR processing, trafficking and interactions**
	Chairs: Carlos Farinha (PT) - Luis Galietta (IT)
11:00-11:25	A Proteomic Variant Approach (ProVarA) for personalized medicine of inherited and somatic disease – Darren Hutt (US)
11:25-11:50	Novel CFTR regulators identified by means of a functional genomics approach and their possible mechanisms of action - Nicoletta Pedemonte (IT)
11:50-12:15	Functional genomics of F508del-CFTR: Illuminating traffic mechanisms for innovative therapeutic strategies - Margarida Amaral (PT)
12:15-12:25	Abstract 47 - CFTR processing mutations cause distinct trafficking and functional defects - Marjolein Ensinck (BE)
12:25-12:35	Abstract 49 - Role of the proteasome in the biosynthetic arrest of SLC26A9 by F508del-CFTR - Yukiko Sato (CA)
12:35-12:45	Abstract 48 - Monitoring the phospho-occupancy of CFTR in respiratory epithelia using mass spectrometry - Aiswarya Premchandar (CA)
12:45-14:00	*Lunch*
14:00-18:30	**Free Afternoon**
18:30-19:30	**Flash Poster Session (odd numbers)**
	Chair: Bertrand Kleizen (NL)
19:30 -21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Odd numbers

Saturday, 30 March 2019 (Day 4)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 6 – Inflammation and host-pathogen interactions**
	Chairs: Marcus Mall (DE) – Gunnar Hansson (SE)
08:45-09:10	Implication of host antimicrobial peptides in the modulation of bacterial-bacterial interactions in CF airways - Lhousseine Touqui (FR)
09:10-09:35	Viral-bacterial co-infections in cystic fibrosis – Jennifer Bomberger (US)
09:35-10:00	Biofilm formation in cystic fibrosis - Tom Coenye (BE)
10:00-10:10	Abstract 67 - Effects of short-term lumacaftor-ivacaftor therapy on lung microbiome in Phe508del homozygous patients with cystic fibrosis - Sébastien Boutin (DE)
10:10-10:20	Abstract 56 - Metabolic reprogramming of cystic fibrosis macrophages by the IRE1a-XBP1 pathway leads to an exaggerated inflammatory response - Samuel Lara-Reyna (UK)
10:20-10:30	Abstract 55 - SPLUNC1 peptidomimetics inhibit Orai1 to reduce pulmonary inflammation - Saira Ahmad (US)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 7 – Model systems**
	Chairs: Martin Mense (US) – Jeff Beekman (NL)
11:00-11:25	A single cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte - Aron Jaffe (US)
11:25-11:50	Mechanisms of airway epithelium repair in cystic fibrosis - Marc Chanson (CH)
11:50-12:15	Understanding altered airway mucins sialyation and decreased mucociliary transport in cystic fibrosis pig airways using single cell gene expression – airway cells - Pascal Barbry (FR)
12:15-12:25	Abstract 82 - Development of a steady-state lumen area screening assay to measure CFTR function in organoids of cystic fibrosis patients - Marne Hagemeijer (NL)
12:25-12:35	Abstract 76 - Human induced pluripotent stem cell derived p63 expressing epithelial cells provide a personalized model for CF lung epithelium - Bob Scholte (NL)
12:35-12:45	Abstract 75 - Primary human nasal epithelial cell air/liquid interface cultures: an *in vitro* model system for assessing CFTR function - Calvin Cotton (US)
12:45-14:15	*Lunch*

14:15-16:10	**Symposium 8 – Therapeutical approaches**	
	Chairs: Martina Gentzsch (US) – David Sheppard (UK)	
14:15-14:40	Novel developments in genetic therapies for cystic fibrosis - Stephen Hart (UK)	
14:40-15:05	Update on CF gene therapy – Chris Boyd (UK)	
15:05-15:30	Co-potentiators as a novel therapeutic paradigm for CFTR mutations that are not responsive to available modulators - Peter Haggie (US)	
15:30-15:40	Abstract 87 - Genetic repair of CFTR function in cystic fibrosis organoids using CRISPR/Cas9 adenine base editing - Eyleen de Poel (NL)	
15:40-15:50	Abstract 102 - The pharmacology of novel TMEM16A potentiator compounds - Martin Gosling (UK)	
15:50-16:00	Abstract 33 - Identification of pharmacological modulators of the TMEM16A chloride channel by high-throughput screening - Michele Genovese (IT)	
16:00-16:10	Abstract 90 - CFTR super exon partially corrects W1282X-CFTR - Hillary Valley (US)	
16:10-16:45	*Coffee Break*	
16:45-17:45	**Workshop: How to get published – a perspective from JCF**	**Breakout Room**
	Chairs: Cliff Taggart (UK) – David Spencer (UK)	
	Cliff Taggart, JCF Editor and David Spencer, JCF publisher, will give an overview of the Journal of Cystic Fibrosis including how to publish basic science findings and what the editors and reviewers look for when reviewing manuscripts submitted to the Journal.	
16:45-17:45	**Flash Paper Session**	
	Chair: Marc Chanson (CH)	
16:45 – 16:58	Esomeprazole increases airway surface liquid pH in primary cystic fibrosis epithelial cells - Vinciane Saint-Criq (UK)	
16:58 – 17:11	*Staphylococcus aureus* impacts *Pseudomonas aeruginosa* chronic respiratory disease in murine models - Cristina Cigana (IT)	
17:11 – 17:25	Targeting of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein with a technetium-99m imaging probe - Filipa Mendes (PT)	
17:25 – 17:35	Abstract 85 - Lack of cystic fibrosis transmembrane conductance regulator causes low cortical bone thickness and high cortical porosity in newborn pigs - Frédéric Velard (FR)	
17:35 – 17:45	Abstract 86 - Pancreatic ductal fluid and bicarbonate secretion of the ferret and pig models of cystic fibrosis (CF) - Emese Tóth (HU)	
17:45-18:00	*Break*	
18:00-19:00	**Closing Keynote lecture**	
	Organoids for cystic fibrosis research - Jeffrey Beekman (NL)	
20:30	*Dinner / Social Event*	

POSTER TITLES & AUTHORS

P1 Identification of *CFTR cis*-regulatory variants
Mégane Collobert, Karen Rouault, Carine L'Hostis, Marie-Pierre Audrézet, Claude Férec, Stéphanie Moisan

P2 Comparison of three genome editing techniques to correct the common W1282X mutation responsible for cystic fibrosis
Karen Mention, Kader Cavusoglu-Doran, Lucia Santos, David Sanz, Martina Scallan, Patrick Harrison

P3 Epistatic effect of complex alleles in cystic fibrosis phenotype – the protein translation view
Robert Rauscher, Giovana B. Bampi, Kathryn E. Oliver, Marta G. Ferrer, Disha Joshi, David Mark, Sivagurunathan Sutharsan, Manfred Ballmann, Eric J. Sorscher, Zoya Ignatova

P4 Variation Spatial Profiling (VSP): the evolving genome driving cystic fibrosis
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P5 Increased SLC6A14 expression shows more severe cystic fibrosis phenotypes with tissue- and sex-specific contributions
Mohsen Esmaeili, Gengming He, Naim Panjwani, Fan Wang, Benjamin Dekoven, Katherine Keenan, Julie Avolio, Fan Lin, Lei Sun, Johanna M Rommens, Lisa J Strug

P6 Recruitment to cystic fibrosis airway fluid licenses transcription and subsequent acquisition of the pathological GRIM fate by human neutrophils
Camilla Margaroli, Dalia Arafat-Gulick, Fangxu Sun, Haydn Kissick, Ronghu Wu, Greg Gibson, Rabindra Tirouvanziam

P7 Implication of miR-199a-3p in bronchic inflammation in cystic fibrosis patients
Pauline Bardin, Emmeline Marchal-Duval, Florence Sonneville, Sabine Blouquit-Laye, Harriet Corvol, Olivier Tabary

P8 Not all stop codons are created equal, neither do they always stop: morals from single-channel studies of E60X- and G542X-CFTR
Jiunn-Tyng Yeh, Tzyh-Chang Hwang

P9 The cystic fibrosis mutations L927P and I336K modulate CFTR pore dynamics during channel gating, but are rescued by ivacaftor and lumacaftor
Majid K. Al Salmani, Sangwoo T. Han, Samuel J. Bose, Garry R. Cutting, David N. Sheppard

P10 Characterization of the rare S955P-CFTR mutation and its response to modulators in cellular models and patient-derived materials
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P11 Functional impact of the rare CFTR variants W57G/A234D and CFTR modulator theratyping in a CF patient
Paola Melotti, Francesca Rescigno, Alessia Farinazzo, Valeria Esposito, Karina Kleinfelder, Marina Bertini, Emily Pintani, Gloria Tridello, Luca Rodella, Angelo Cerofolini, Francesco Lombardo, Filippo Catalano, Francesco Tomba, Hugo deJonge, Carlo Castellani, Claudio Sorio

P12 Repurposing drugs for cystic fibrosis subjects with rare *CFTR* mutations using intestinal organoids: An update on the Rainbow project
Eyleen de Poel, Jesse E. Brunsveld, Annelot M. Vonk, Hugo Oppelaar, Sabine Heida-Michel, Margot Geerdink, Gitte Berkers, Peter van Mourik, Sylvia Boj, Robert G.J. Vries, Kors van der Ent, Marne C. Hagemijer, Jeffrey M. Beekman

P13 The regulatory insertion enables novel conformations of NBD1 of CFTR
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P14 Unraveling new conformations of CFTR and their possible role in cystic fibrosis
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P15 Identifying the molecular targets for CFTR potentiators GLPG1837 and VX-770
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P16 VX-770 potentiates CFTR function by interacting with MSD2
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P18 CFTR conformational landscape explored by computational approaches
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P19 *In silico* search for the chloride pathway in CFTR
Bianka Farkas, Hedvig Tordai, Rita Padányi, János Gera, Gábor Paragi, Tamas Hegedűs

P20 Impact of CFTR function on airway mucus properties
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P21 Early onset of airway mucus obstruction associated with increased mortality in neonatal *Slc26a9* deficient mice
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Alessia Farinazzo, Francesca Rescigno, Valeria Esposito, Anna Baruzzi, Stefano Aliberti, Francesco Blasi, Vito Terlizzi, Giovanni Taccetti, Luca Rodella, Angelo Cerofolini, Francesco Lombardo, Filippo Catalano, Francesco Tomba, Sonia Volpi, Karina Kleinfelder, Hugo de Jonge, Claudio Sorio, Paola Melotti

P24 Dual role of pendrin as a bicarbonate secretion pathway and CFTR modulator in well-differentiated human nasal and bronchial epithelial cells
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P25 *CFTR* is functionally expressed in a subpopulation of endocrine cells of the mammalian islet of Langerhans and modulates insulin secretion
Mauricio Di Fulvio, Marika Bogdani, Lisa Kelly, Myrian Velasco, Marcia Hiriart, Lydia Aguilar-Bryan

P26 CFTR correctors restore the alcohol-induced expression defect in pancreatic ductal cells
Anna Grassalkovich, József Maléth, Tamara Madácsy, Petra Pallagi, Zoltán Rakonczay Jr., Péter Hegyi, Viktória Venglovecz

P27 Interactions between the exocrine and endocrine pancreas under pathological conditions and the effect of CFTR function
Attila Ébert, Glória Stefán, Emese Tóth, Dawood Khan, Péter Hegyi, Viktória Venglovecz

P28 Cell proliferation upregulates ANO1 in mucus cell hyperplasia
Filipa Simões, Margarida Quaresma, Iris AL Silva, Luka Clarke, Karl Kunzelmann, Margarida D. Amaral

P29 Dysfunction of Anoctamin 1 (Ano1) in F508del mouse pancreatic b-cell
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P30 TMEM16A channel function does not influence goblet cell numbers in the human airway epithelium
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P31 Physiological role and therapeutic importance of TMEM16A chloride channel in the airway epithelium
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P32 E_{ACT} increases intracellular calcium levels by a TMEM16A-independent mechanism
Sarah Lilley, Henry Danahay, Holly Charlton, Kathryn Adley, Roy Fox, Martin Gosling

P33 Identification of pharmacological modulators of the TMEM16A chloride channel by high-throughput screening
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P34 Identification of novel ANO1/TMEM16A regulators as alternative therapeutic targets for cystic fibrosis
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P35 Partial Epithelial to Mesenchymal Transition (EMT) in CF: CF as an epithelial differentiation disorder
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P37 Evaluation of different growth conditions on acid-base homeostasis in non-CF and CF airway epithelial cells
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P41 PLCβ3 is involved in forskolin-dependent CFTR activation
Chloé Grébert, Sandra Mirval, Frédéric Becq, Clarisse Vandebrouck

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P48 Monitoring the phospho-occupancy of CFTR in respiratory epithelia using mass spectrometry
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P50 TGF-β1 recruits LMTK2 to inhibit CFTR-mediated chloride transport in human bronchial epithelial cells
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P51 Investigating the drug-drug plasma protein binding and cytochrome interactions of ivacaftor
Elena Schneider-Futschik, Daniel Hoyer, Tony Velkov

P52 HspB5, a phospho-regulated corrector of F508del-CFTR
Fanny Degrugillier, Benjamin Simonneau, Virginie Escabasse, Pascale Fanen, Stéphanie Simon

P53 Keratin 8-dependent regulation of CFTR and alpha-1-antitrypsin targeting
Iwona Pranke, Aiswarya Premchandar, Nesrine Baatallah, Grazyna Faure, Danielle Tondelier, Sara Bitam, Imran Haq, James Irving, Pieter Hiemstra, Alexandre Hinzpeter, Olivier Namy, Michal Dadlez, Isabelle Sermet-Gaudelus, David Lomas, Aleksander Edelman

P54 Hit optimization for the development of novel ubiquitin-ligase RNF5 inhibitors
Irene Brusa, Federico Falchi, Elvira Sondo, Nicoletta Pedemonte, Marinella Roberti, Andrea Cavalli

P55 SPLUNC1 peptidomimetics inhibit Orai1 to reduce pulmonary inflammation
Saira Ahmad, Maria Sassano, Tongde Wu, Robert Tarran

P56 Metabolic reprogramming of cystic fibrosis macrophages by the IRE1a-XBP1 pathway leads to an exaggerated inflammatory response
Samuel Lara-Reyna, Thomas Scambler, Jonathan Holbrook, Heledd Jarosz-Griffiths, Chi Wong, Sinisa Savic, Daniel Peckham, Michael McDermott

P58 The PDZ containing domain protein, cal, links CFTR to the regulation of inflammation
Liudmila Cebotaru, William Guggino

P59 The cellular prion protein (PrP^C) modulates CFTR-associated inflammation
Johanna Cormenier, Amal Kouadri, Laurence Macari, Nadia Alfaidy, Mohamed Benharouga

P60 Cathepsin G activity reporters detect chronic airway inflammation by microscopy and flow cytometry
Matteo Guerra, Dario Frey, Matthias Hagner, Susanne Dittrich, Michelle Paulsen, Marcus Mall, Carsten Schultz

P61 Involvement of the RhoGTPase activator Vav3 in the CF airway epithelium remodelling
Mehdi Badaoui, Alice Zoso, Marc Bacchetta, Bernhard Wehrle-Haller, Marc Chanson

P62 Oxidative stress-associated lipid biomarkers in bronchoalveolar lavage correlate with CF lung disease severity and progression
Hamed Horati, Camilla Margaroli, Mieke Veltman, Matthew B. Kilgore, Joshua D. Chandler, Harm A.W.M. Tiddens, Rabindra Tirouvanziam, Hettie M. Janssens, Bob J. Scholte

P63 Oxidative stress and abnormal bioactive lipid metabolism in CF bronchial epithelial cells in air-liquid interface culture, partial correction by Glutathione, Fenretinide and Orkambi
Mieke Veltman, Marta Stolarczyk, Nick Klymiuk, Andrea Bähr, Juhi Shah, Dusan Garic, Juan Bautista De Sanctis, John Hanrahan, Danuta Radzioch, Bob J. Scholte

P64 CFTR-F508del mutation affects differentiation and functional activity of bone-resorbing osteoclasts
Jacky Jacquot, Marie-Laure Jourdain, Christine Guillaume, Julien Braux, Dominique Hubert, Frédéric Velard

P65 The normal and CF airway peptidomes regulate cell growth
Robert Tarran, Maria Sassano, Xie Ling, Bryan Zorn, Nathan Simpson, Jurnorain Gani, Matthew Wolfgang, Xian Chen, Kai Hilpert, Deborah Baines

P66 The role of IL-17s/IL-17Rs immune axis in airway defense and immunopathology during *Pseudomonas aeruginosa* infection
Giulia Rizzo, Alessandro Migliara, Barbara Sipione, Fabio Saliu, Alessandra Bragonzi, Cristina Cigana, Angelo Lombardo, Nicola Lorè

P67 Effects of short-term Lumacaftor-Ivacaftor therapy on lung microbiome in Phe508del homozygous patients with cystic fibrosis
Sébastien Boutin, Simon Gräber, Mark Wielpütz, Cornelia Joachim, Sabine Wege, Olaf Sommerburg, Mirjam Stahl, Alexander Dalpke, Marcus Mall

P69 Identification of specific microRNAs in CF bronchial epithelial cells infected by *Pseudomonas aeruginosa*
Viviane Balloy, Rachel Legendre, Odile Sismeiro, Hugo Varet, Jean-Yves Coppée, Harriet Corvol, Christian Herr, Michel Chignard, Loïc Guillot

P70 Distinguishing active bacterial populations associated with clinical exacerbations in cystic fibrosis patients by culture and DNA and RNA based molecular profiling
Gayatri Nair, Michael Surette, Laura Rossi, Andreas Freitag

P71 Novel compounds to enhance the eradication and reduce the virulence of respiratory infections
Lucy Sykes, Hajer Taleb, Danielle Williams, James Preece, Paul Rice, Daniel Neef, Graham Dixon

P72 Respiratory epithelial cells can remember infection
Jeanne Bigot, Juliette Guitard, Loïc Guillot, Harriet Corvol, Michel Chignard, Christophe Hennequin, Viviane Balloy

P73 ASL contribution to airway epithelial protection against *Pseudomonas aeruginosa*
Juliette Simonin, Alexandre Luscher, Mehdi Badaoui, Aderonke Sofoluwe, Marc Bacchetta, Davide Losa, Thilo Köhler, Christian van Delden, Marc Chanson

P74 Set of preclinical mouse models of respiratory infection to evaluate antibiotic efficacy
Cristina Cigana, Serena Ranucci, Alice Rossi, Ida De Fino, Mededе Melessike, Alessandra Bragonzi

P75 Primary human nasal epithelial cell air/liquid interface cultures: an *in vitro* model system for assessing CFTR function
Calvin Cotton, Masih Rezaee, James Chmiel, Kimberly McBennett, Erica Roesch

P76 Human induced pluripotent stem cell derived p63 expressing epithelial cells provide a personalized model for CF lung epithelium
Bob J. Scholte, Ruth Olmer, Sandra Baus, Sylvia Merkert, Mieke Veltman, Hettie Janssens, Ulrich Martin

P77 High throughput screening for modulators of p.Phe508del CFTR activity applying an organotypic functional assay based on genetically engineered CF disease-specific induced pluripotent stem cells (iPSCs)
Sylvia Merkert, Madline Schubert, Janina Zöllner, Lena Engels, Mieke Veltman, Silke Radetzki, Luis Galietta, Jens Peter von Kries, Bob Scholte, Ruth Olmer, Ulrich Martin

P78 The CFIT Program iPSC resource can generate CF-relevant tissues for studies of drug responses
Paul Eckford, Jiaxin Jiang, Sunny Xi, Zoltán Bozóky, Amy Wong, Janet Rossant, Felix Ratjen, Christine Bear

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John Mahoney, Megan Simpkinson, Alexander Stuffer, Aleksander Szymaniak, Phil Thomas, Martin Mense

P80 Gene and base edited cell models of cystic fibrosis-causing mutations
Lúcia Santos, David J. Sanz, Kader Cavusoglu-Doran, Karen Mention, Elena Rojas, Carlos M. Farinha, Patrick T. Harrison

P81 Establishment and reproducible screening of a large-scale CF patient derived colon organoid biobank
Jasper Mullenders, Marvin Statia, Annelot Vonk, Begoña Aguilera, Jeffrey Beekman, Kors Van der Ent, Rob Vries, Sylvia Boj

P82 Development of a steady-state lumen area screening assay to measure CFTR function in organoids of cystic fibrosis patients
Marne Hagemeijer, Sylvia Suen, Jesse Brunsveld, Paul van Haaren, Hettie Janssens, Karin de Winter-de Groot, Kors van der Ent, Jeffrey Beekman

P83 High reproducibility of forskolin-induced swelling of organoids across three academic laboratories
Annelotte M. Vonk, Anabela Santo Ramalho, Iris A.L. Silva, Hugo M. Botelho, Peter van Mourik, Sabine Michel, Rutger Bor, Begona Aguilera, Jasper Mullenders, Sylvia F. Boj, Robert Vries, Kris de Boeck, Margarida D. Amaral, Cornelis K. van der Ent, Jeffrey M. Beekman

P84 Development of a humanized CF mouse model
Angélique Mottais, Younès Achouri, Mathilde Beka, Audrey Reynaerts, Martial Delion, Teresinha Leal

P85 Lack of cystic fibrosis transmembrane conductance regulator causes low cortical bone thickness and high cortical porosity in newborn pigs
Frédéric Velard, Julien Braux, Marie-Laure Jourdain, Ignacio Caballero-Posadas, Nathalie Winter, Mustapha Si-Tahar, Nikolai Klymiuk, Andrea Baehr, Sophie C. Gangloff, Jacky Jacquot

P86 Pancreatic ductal fluid and bicarbonate secretion of the ferret and pig models of cystic fibrosis (CF)
Emese Tóth, Pavana Rotti G, Viktória Venglovecz, Petra Pallagi, Zoltán Jr. Rakonczay, József Maléth, Aliye Uc, John Engelhardt, Péter Hegyi

P87 Genetic repair of CFTR function in cystic fibrosis organoids using CRISPR/Cas9 adenine base editing
Eyleen de Poel, Maarten H. Geurts, Sylvia W.F. Suen, Jeffrey M. Beekman, Hans Clevers

P88 CFTR correction in primary airway epithelial cells with CRISPR/Cas9 as a proof of principle *ex vivo* gene therapy for Cystic Fibrosis
Afroditi Avgerinou, Marisa Ofrim, Maximillian Woodall, Amy Walker, Chris O' Callaghan, Deborah Baines, Stephen Hart, Paola Bonfanti

P90 CFTR super exon partially corrects W1282X-CFTR
Hillary Valley, Rebecca Seymour, Yi Cheng, Katherine Bukis, Kevin Coote, Hermann Bihler, Philip Thomas, Jerome Mahiou, Martin Mense

P91 CFTR protein detection in organoids from healthy and CF patients with nonsense mutations support using the organoid model to test ELX-02 mediated CFTR readthrough restoration
Shira Landskroner-Eiger, Chagai Rot, Matthew Goddeeris, Iris Alroy, Jasper Mullenders, Neal Sharpe

P92 Exploring splicing modulation approaches using antisense oligonucleotides in cystic fibrosis organoids
Juliet Lefferts, Kors van der Ent, Jeffrey Beekman, Marne Hagemeijer

P93 Restoration of CFTR function by antisense oligonucleotide splicing modulation in respiratory and intestinal primary model systems
Batsheva Kerem, Yifat S. Oren, Ofra Avizur, Efrat Ozeri Galai, Michal Irony Tur-Sinai, Noemie Stanleigh, Venkateshwar Mutyam, Steven M. Rowe, Anita Golec, Aurélie Hatton, Iwona Pranke, Isabelle Sermet-Gaudelus

P94 The effect of CF sputum on CFTR mediated Cl^- transport and ASL height. Is gene editing sufficient to restore function in the CF environment?
Maximillian Woodall, Robert Tarran, Deborah Baines, The Cystic Fibrosis Trust Gene Editing Strategic Research Centre

P95 Effective transduction of Rhesus macaque lung and human enteroids with AAV1-CFTR
Murali Yanda, William Guggino, Liudmila Cebotaru

P96 Prolonged incubation with CFTR modulators and forskolin induced swelling on organoids with N1303K mutation
Anabela S. Ramalho, Eva Fürstová, Lieven Dupont, Marijke Proesmans, Francois Vermeulen, Kris De Boeck

P97 Two novel corrector classes with the potential to be developed for new combination therapies
John Preston Miller, Soheil Aghamohammadzadeh, Daniel Parks, Jack Ryan, Jiefei Geng, Sucharitha Parthasarathy, Adriana Villella, Min Wu, Benito Munoz

P98 Rescue of F508del-CFTR by Corrector RDR01752
Miquéias Lopes-Pacheco, Iris Silva, Elvira Sondo, Nicoletta Pedemonte, Margarida Amaral

P99 A novel amplifier-enhanced high throughput screen based on CFTR functional activity identifies read through modulators of G542X-CFTR
Danijela Dukovski, Adriana Villella, Soheil Aghamohammadzadeh, Po-Shun Lee, Benito Munoz, John P. Miller

P100 Ganglioside GM1 improves the plasma membrane stabilization of F508del-CFTR rescued by the use of CFTR modulators
Nicoletta Loberto, Giulia Mancini, Carlotta Trabucchi, Maria Cristina Dechecchi, Giuseppe Lippi, Laura Mauri, Debora Olioso, Sandro Sonnino, Nicoletta Pedemonte, Valeria Tomati, Giulio Cabrini, Anna Tamanini, Massimo Aureli

P101 Rescue F508del CFTR with nanobodies
Marie Overtus, Maud Sigoillot, Daniel Scholl, Marianne Carlon, Toon Laeremans, Els Pardon, John R. Riordan, Jan Steyaert, Cédric Govaerts

P102 The pharmacology of novel TMEM16A potentiator compounds
Henry Danahay, Sarah Lilley, Holly Charlton, Roy Fox, Brian Button, Martin Gosling

P103 ATP12A as an alternative therapeutic target in cystic fibrosis lung disease
Paolo Scudieri, Ilaria Musante, Arianna Venturini, Emanuela Caci, Luis J.V. Galietta

P104 Novel therapeutic approaches based on small molecule transmembrane anion transporters to improve the mucociliary properties of cystic fibrosis airway epithelia
Ambra Gianotti, Valeria Capurro, Livia Delpiano, Marcin Mielczarek, Israel Carreira-Barral, Maria Garcia-Valverde, Roberto Quesada, Alessandra Ludovico, Debora Baroni, Oscar Moran, Emanuela Caci

P105 The attached stratified mucus in obstructive airway disease is detached by calcium removal
Dalia Fakih, Joan Antoni Fernandez Blanco, Anna Ermund, Gunnar C. Hansson

P106 Inhaled therapy with a cell-permeable PI3Kgamma mimetic peptide to limit bronchoconstriction and lung inflammation in cystic fibrosis
Alessandra Murabito, Valentina Sala, Cosmin Stefan Butnarasu, Kai Ren, Francesca Ungaro, Sonja Visentin, Emilio Hirsch, Alessandra Ghigo

P107 Exploiting a PI3Kγ mimetic peptide as a CFTR modulator in cystic fibrosis
Alessandra Murabito, Kai Ren, Flora Pirozzi, Nancy L. Quinney, Paola Melotti, Claudio Sorio, Andrea Raimondi, Carlo Tacchetti, Carlo Laudanna, Martina Gentzsch, Emilio Hirsch, Alessandra Ghigo

P108 Characterization of biological activity of RNF5 inhibitors as potential drugs for cystic fibrosis basic defect
Emanuela Pesce, Elvira Sondo, Emanuela Caci, Valeria Tomati, Andrea Armirotti, Andrea Cavalli, Nicoletta Pedemonte

P109 LMTK2 inhibits PP1c to regulate the TGF-β1 signaling pathway in cystic fibrosis
Daniel F. Cruz, Maja Köhn, Carlos M. Farinha, Agnieszka Swiatecka-Urban

P110 Inhibition of autoinflammation in cystic fibrosis using small molecule therapy
Heledd H. Jarosz-Griffiths, Thomas Scambler, Samuel Lara Reyna, Jonothan Holbrook, Chi Wong, Shelly Pathak, Fabio Martinon, Sinisa Savic, Michael McDermott F, Daniel Peckham

P111 Niclosamide repurposed for the treatment of inflammatory airway disease
Inês Cabrita, Roberta Benedetto, Rainer Schreiber, Karl Kunzelmann

P112 Open randomised study on docosahexaenoic acid, 5-methyltetrahydrofolate and vitamin B12 supplementation in cystic fibrosis pediatric patients: focus on fatty acids, inflammation and blood cells membrane
Paola Melotti, Gloria Tridello, Sonia Volpi, Marianna Passiu, Ilaria Meneghelli, Sira Cordioli, Claudio Sorio, Gabriella Bergamini, Elisa Calcaterra, Mariaserena Boraso, Mario Salmona, Luisa Diomede, Patrizia Risè, Baroukh Maurice Assael, Ciro D'Orazio

P114 Intranasal *Lactobacilli* administration: a new way to prevent *Pseudomonas aeruginosa* respiratory tract infections
Rosyne Lagrafeuille, Marie-Sarah Fangous, Charles-Antoine Guilloux, Stéphanie Gouriou, Philippe Gosset, Sophie Vallet, Geneviève Héry-Arnaud, Rozenn Le Berre

P115 Is cystic fibrosis related bone disease reversible? New data on CFTR potentiation and osteoclast precursor monocytes
Christine Guillaume, Marie-Laure Jourdain, Dina Abdallah, Nicola Ronan, Yvonne McCarthy, Evelyn Flanagan, Barry Plant, Frédéric Velard, Jacky Jacquot

P116 Novel molecular imaging tools for cystic fibrosis
Vera Ferreira, João Correia, João Gonçalves, Carlos Farinha, Filipa Mendes

P117 Sputum rheology: a robust biophysical marker for the monitoring of cystic fibrosis patients
Jérémy Patarin, Étienne Ghiringhelli, Matthieu Robert de Saint Vincent, Claire Cracowski, Boubou Camara, Sébastien Quétant, Cécile Bosc, Christophe Pison, Jean-Louis Quésada, Jean-Luc Cracowski

AWARD WINNERS

ECFS Young Fellows Travel Award

Saira Ahmad (US)
Afroditi Avgerinou (UK)
Sébastien Boutin (DE)
Jesse Brunsveld (NL)
Mégane Collobert (FR)
Eyleen de Poel (NL)
Martial Delion (BE)
Emese Tóth (HU)

Student Helper Award

Marjolein Ensinck (BE)
Samuel Lara-Reyna (UK)
Karen Mention (IE)
Margarida Quaresma (PT)

Free Registration Young Researchers

Supported by the Italian CF Research Foundation

Irene Brusa
Michele Genovese
Onofrio Laselva
Alessandra Murabito
Emanuela Pesce
Paolo Scudieri
Arianna Venturini

Vaincre La Mucoviscidose Travel Award

Pauline Bardin
Jeanne Bigot
Johanna Cormenier
Fanny Degrugillier
Ahmad Elbahnsi
Rosyne Lagrafeuille

Mukoviszidose eV Travel Award

Giovana B. Bampi
Roberta Benedetto
Pamela Millar Büchner
Madalena Pinto

27 March — 18:00–19:00

Opening Keynote Lecture

The genetics and genomics of CF

Garry Cutting

Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, MD USA

Cystic fibrosis is caused by dysfunction of a single gene. As such, all manifestations can be attributed directly or indirectly to altered function of CFTR. Variants in the *CFTR* gene determine the degree to which CFTR function is affected that, in turn, determines the severity of disease at the level of the cell, organ and individual. Thus, capturing all of the DNA variation in human CFTR and understanding the effect of each variant upon CFTR function will inform our efforts to dissect mechanism and develop effective treatments. To this end, CF researchers have been cataloguing variants in CFTR in the CF Mutation Database since the identification of the CFTR gene. Over 2000 variants have been reported primarily in individuals with CF but also in persons with clinical features that overlap with CF. Differentiating which variants cause CF and by what mechanism became essential with the development of clinically-successful CFTR targeted drugs. The Clinical and Functional Translation of CFTR (CFTR2) project was implemented to address this challenge. Data collected from ~89,000 individuals with CF revealed that ~520 variants are carried by 3 or more individuals worldwide and another ~1100 variants are very rare, occurring in only one or two individuals worldwide. The CFTR2 team has completed annotation of 410 of the 520 variants using clinical, functional and population criteria. Responses to ivacaftor and lumacaftor were robustly correlated with residual function for almost all of the 410 variants that produce CFTR protein. To refine our understanding of the relationship between CFTR function and CF disease severity, the CFTR2 team assigned function to 226 CFTR genotypes that revealed logarithmic relationship with sweat [Cl^-], pancreatic status and lung function. As predicted by the log relationship, modest increases in CFTR function were associated with clinically relevant improvements in cross-sectional FEV1% predicted over a broad range of ages. Plotting of clinical trial results against the benchmarks established from the CFTR2 data revealed that sweat gland and lung dysfunction were reversible to a degree observed in individuals with a lifetime of higher CFTR function. While CFTR variants are an important determinant of CF disease variability, alterations elsewhere in the genome play a role, especially for complications such as neonatal intestinal obstruction (aka meconium ileus), CF-related diabetes and, to a lesser extent, body mass index, lung infection and airway dysfunction. Association analysis conducted by the International CF Modifier Consortium has been particularly successful in locating common DNA variants in non-coding regions of the genome that influence severity of individual and multiple traits. Many of the modifier loci contain genes that encode proteins of biologic relevance to CF or have plausible roles in organ level disease. Whole genome sequencing performed on ~5200 individuals in the U.S. is currently being analyzed to explore loci identified by association analysis and to find rare DNA variants that modify CF. In summary, genetics and genomics provide a framework to understand the mechanisms underlying disease variation that can be used to inform molecular-based treatment of CF.

28 March — 08:45–10:30

Genetics, genomics and transcriptomics

S1.1 Enhancers, structural elements and 3D organization: key features in regulation of *CFTR* gene expression.

Monali NandyMazumdar, Shiyi Yin, Jenny Kerschner, Alekh Paranjapye, Shih-Hsing Leir, Ann Harris

Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, OH-44106, USA

Programme note: the details are not authorised for inclusion

S1.2 Update on cystic fibrosis modifier genes

Harriet Corvol

Sorbonne University, St Antoine Research Center and Pediatric CF center, Trousseau Hospital, Paris France

Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disease in Caucasians caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel expressed in epithelial cells throughout the body. Over 2,000 mutations in the *CFTR* gene have been described, the most frequent being F508del. The disease affects many organs, as the pancreas, the liver, the intestine, and most critically the lungs.

Although CF is recognized as a single gene disorder, considerable phenotypical diversity exists among patients with the same *CFTR* mutations. Besides environmental factors, it has been shown that additional genetic modifiers contribute to this variability: organ dysfunctions due to the altered CFTR protein may for example be enhanced by genotypic variation in the genes participating in some key metabolic pathways. Modifier gene studies aim at identifying these genes, to improve knowledge of CF pathophysiology, as well as to identify new therapeutic targets.

Early on, gene modifiers in CF were looked for using candidate gene approaches with mixed results, altogether due to small sample size and poor choice of targets. Setting up large cohorts of CF patients was the necessary step to increase power, and consortiums were created in North-America and France (International CF Gene Modifier consortium). Whole genome analyzes (GWAS) and candidate gene studies performed by this consortium identified several genetic loci associated with the variability of numerous CF phenotypes, including lung disease, meconium ileus, liver disease, CF related diabetes, immunotrypsinogen and sweat chloride measurements, as well as host defense. Recently, modifier genes were also shown to be associated with the interindividually variable airway response to targeted therapies such as ivacaftor.

Identification of these modifiers is a challenge that should offer not only a way to distinguish those patients at risk of developing a more severe disease and to accordingly adapt the care, but also to better understand the physiopathological mechanisms of CF and enable the development of new drugs towards a personalized medicine.

S1.3 Restoration of the CFTR function by antisense oligonucleotide splicing modulation

Batsheva Kerem

Department of Genetics, The Hebrew University, Jerusalem, Israel

CFTR mutations are divided to different classes according to their effect on the protein function. We are studying mutations affecting the correct splicing of the CFTR transcripts. The project that I will present is focused on a common CFTR splicing mutation, the 3849+10kb C-to-T, which leads to inclusion of an 84 bp cryptic exon between exons 22-23 in the mature__mRNA. This cryptic exon contains an in-frame stop codon that leads to degradation of a significant fraction of the mRNA by the NMD pathway as well as to the production of prematurely truncated nonfunctional proteins.

We aim to develop an antisense oligonucleotides (ASOs)-based therapy to modulate the level of correctly spliced CFTR transcripts. Using an in house developed algorithm we designed ASOs, targeted to prevent the recognition of the cryptic exon. Screening of these ASOs led to the identification of several lead ASOs that significantly decrease the level of aberrantly spliced CFTR__mRNA and increase the level of correctly spliced CFTR mRNA. Importantly, in human primary nasal epithelial cells (HNEs) these ASOs led to a significant increase in the level of correct splicing leading to a complete restoration of the CFTR function. Altogether, our results highlight the potential of our lead ASO as a therapeutic approach for CF patients carrying splicing mutations.

P1

S1.4 Identification of *CFTR cis*-regulatory variants

Mégane Collobert[1], Karen Rouault[1,2], Carine L'Hostis[1,3], Marie-Pierre Audrézet[1,2], Claude Férec[1,2], Stéphanie Moisan[1,2]

[1]Univ Brest, Inserm, EFS, UMR 1078, GGB, Brest, France, [2]Laboratoire de Génétique Moléculaire et d'Histocompatibilité, CHRU de Brest, Brest, France, [3]Association Gaétan Saleün, Brest, France

The *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR*) gene was identified in 1989. Although, more than 2000 mutations have been discovered, some patients with cystic fibrosis or *CFTR*-related disorders (*CFTR*-RD) have incomplete genotypes or present extreme phenotypes. Regulatory elements described in the promoter, cannot alone explain the complex tissue-specific regulation of this gene. In recent years, the development of chromatin conformation study techniques has identified several long-range regulatory elements as involved in this control expression (Moisan et al., 2016). The objective of this project is to study the involvement of '*cis*-ruption', that is the dysfunction of a *cis* -regulatory element, in cystic fibrosis (CF) and CFTR-RD (*Congenital Bilateral Absence of Vas Deferens,* CBAVD).

17 *cis*-regulatory regions of *CFTR* gene were sequenced by a hight-throughput approach (Fluidigm) in these 112 patients. The frequency of identified variants in our patient's groups was compared European population, with GnomAD database.

In a homogeneous group of 25 CBAVD patients carrying only one F508del mutation, 8 variants display a frequency significantly different in this group compared to European population. In particularly, one variant is 40 times more frequent in CBAVD patients carrying a F508del mutation than in European population. This variant is located in *cis* -regulatory region of intron 21, and especially, in an important transcription factor binding site, EP300. EP300 is essential for gene's transcription. Enhancer tests are realised to measure the effect of the intron 21 region on the activity of *CFTR* promoter in intestinal (Caco-2) and airway (16HBE14o-) cells. The intron 21 region alone has not effect on the activity of *CFTR* gene promoter in intestinal and airway cells. However, by combining the enhancer of intron 21 and the enhancer of intron 11 (strong enhancer described in intestinal cells), a strong cooperative effect is observed on the *CFTR* promoter activity in intestinal cells. These two enhancers have common transcription factor binding sites. In particular, binding sites of the EP300, TCF12, REST and CEBPB (UCSC). However, the EP300 regulatory element interacts with TCF12 and CEBPB. This could explain the cooperative effect of these *cis*-regulatory regions of intron 11 and 21 on the *CFTR* promoter activity. Actually, a sequencing of a control cohort (167 fathers carrying one F508del mutation) is underway.

Enhancer tests with the insertion of the variant of interest in the combination of intron 11 and 21 enhancers are in progress to determine the impact of the variant on the *CFTR* promoter activity. Also, we will study the binding of transcription factor by *Chromatin Immunoprecipitation*, as well as the chromatin organization by *Chromosome Conformation Capture Carbon Copy*.

P2

S1.5 Comparison of three genome editing techniques to correct the common W1282X mutation responsible for cystic fibrosis

Karen Mention[1], Kader Cavusoglu-Doran[1], Lucia Santos[1,2], David Sanz[1], Martina Scallan[3], Patrick Harrison[1]

[1]University College Cork, Physiology, Cork City Center, Ireland, [2]BioISI - Biosystems & Integrative Sciences Institute, Lisbon, Portugal, [3]University College Cork, Microbiology, Cork City Center, Ireland

Ninety percent of the CF-causing mutations cannot be treated with clinically available drugs. W1282X is one of the most common non-druggable variants with a prevalence of 1.2% (Cftr.org). This class I mutation is caused by the change of a G to an A in the 3846 position of the cDNA, and creates a premature TGA stop codon, making cells unable to synthesize a full-length protein, resulting in virtually no CFTR protein available at the cell surface. Whilst many groups have reported the correction of F508del mutation by genome editing technology, there are no published reports on the correction of the W1282X mutation. In this study, we present a comparison of three different techniques to correct the W1282X mutation in HBE W1282X mutant cell lines (1).We compared Homologous directed repair (HDR) approach using two different CRISPR proteins, SpCas9 and AsCas12a (Cpf1) in a RiboNucleoProtein (RNP)/guideRNA complex format. The donor template was a single strand oligonucleotide of the non-target sequence, containing the corrected mutation flanked by asymmetric homology arms. In both cases, we observed ~20% precise editing by HDR, but we also found a significant level of indels caused by NHEJ repair. Given the high level of NHEJ relative to HDR, and the recent observation that double-stranded breaks can lead to unwanted chromosomal rearrangements (2), we have also tested Cas9-adenine base-editing (ABE) to correct this mutation. ABE can convert an A:T to G:C base pair with high efficiency without the formation of a DSB (3). We have recently confirmed that this enzyme shows high levels of precise editing, with very low levels of indels. However, a current limitation of this technique is that the target residue must lie in a window of 4nt to 7nt region on the same DNA strand as the 5'-NGG-3' protospacer adjacent motif (PAM). There is only one PAM sequence close to the W1282 mutation site, but the targeted A would be outside the base-editing window, and we have been unable to detect ABE using the wild-type Cas9-ABE7.10. However, the mutation is potentially amenable to editing with the recently described xCas9-ABE, which has less constraints on the PAM sequence (4). If it is possible to correct W1282X efficiently with xCas9-ABE, this may be the most suitable for preclinical development. Indeed, a recent study using HDR demonstrates that off-target sites editing using Cas9-ABE is 20-fold lower than for Cas9/gRNA (5).

This project has been possible thanks to research grants from the CF Trust and the CF Foundation.

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2. Kosicki, M., K. Tomberg, and A. Bradley. Nat Biotechnol, 2018. **36**(8): p. 765-771.

3. Gaudelli, N.M., et al. Nature, 2017. **551**(7681): p. 464-471.

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5. Liang, P., et al. Nat Commun, 2019. **10**(1): p. 67.

P6

S1.6 Recruitment to cystic fibrosis airway fluid licenses transcription and subsequent acquisition of the pathological GRIM fate by human neutrophils

Camilla Margaroli[1], Dalia Arafat-Gulick[2], Fangxu Sun[2], Haydn Kissick[3], Ronghu Wu[2], Greg Gibson[2], Rabindra Tirouvanziam[1]

[1]Emory University School of Medicine, Pediatrics, Atlanta, United States, [2]The Georgia Institute of Technology, School of Biology, Atlanta, United States, [3]Emory University School of Medicine, Urology, Atlanta, United States

Rationale: Blood neutrophils acquire a pathological fate upon recruitment to CF airway fluid, leading to reduced bacterial killing despite active granule release, immunomodulatory, and metabolic activities (aka, “GRIM” fate). Our group identified this pathological fate in adults [Tirouvanziam et al. PNAS, 2008], and recently in infants [Margaroli et al., AJRCCM, 2018] with CF. Because of their visibly hypercondensed chromatin, blood neutrophils are thought as transcriptionally silent. In contrast with this conventional view, we hypothesized that the profound changes in neutrophils leading to the GRIM fate, following recruitment to the CF airways, were dependent upon transcriptional licensing.

Methods: We used an in vitro model that recapitulates neutrophil recruitment into CF airway fluid and GRIM fate acquisition [Forrest et al., JLB, 2018], to mass-produce GRIM neutrophils and conduct parallel transcriptomic profiling by RNASeq and proteomic profiling by mass spectrometry.

Results: Recruitment into CF airway fluid leads to rapid transcriptional licensing in neutrophils, with increase in total RNA content and major shifts in mRNA and proteins. In particular, computational analysis revealed the expression of immunomodulatory activities reminiscent of myeloid-derived suppressor cells, and metabolic properties consistent with enhanced survival of the GRIM neutrophils. Interestingly, granule enzymes were not resynthesized by GRIM neutrophils. As expected, recruitment of blood neutrophils toward the chemoattractant control leukotriene B4 did not lead to acquisition of the GRIM fate, but induced transcripts related to phagolysosomal acidification, and led to efficient bacterial killing. Finally, blockade of transcription by the RNA polymerase II inhibitor alpha-amanitin early after recruitment of neutrophils into CF airway fluid blocked GRIM phenotype acquisition and restored bacterial killing capacity.

Conclusions: Neutrophils are transcriptionally licensed upon recruitment to CF airway fluid. This transcriptional licensing in turn causes functional changes leading to the GRIM fate, including active granule release and inhibition of bacterial killing. RNA-targeted therapy can be envisioned to modulate the fate and function of neutrophils and potentially ameliorate inflammation and control of infection in children and adults with CF in whom GRIM neutrophils are a dominant presence in the airways.

Support: CF Foundation (TIROUV17G0, MCCART15R0), NIH (R01 HL126603).

28 March — 11:00–12:45

Rare mutations and complex alleles

S2.1 *CFTR* complex alleles

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CFTR complex alleles are characterized by the presence of more than one mutation or sequence variation. These can affect multiple biological steps, such as mRNA production and processing, protein folding, function or stability. *In vivo* and *in vitro* assays enable to assign specific defects to each mutation. A growing number of data indicate that some mutations are associated with multiple defects, e.g. affecting both mRNA splicing and channel function. In the context of complex alleles, defects associated to each mutation can be additive. Conversely, some *cis* mutations can reduce the defects associated to an otherwise more severe mutation. This was shown for the c.[-102T>A;S549R] complex allele where enhanced expression levels associated with -102T>A attenuated the usually severe clinical phenotype observed with S549R. Nonetheless, the additivity of defects affecting different processes appears hard to predict rendering genetic counseling difficult without a complete knowledge of the biological impact of these variants.

Additionally, as only a subset of the most frequent mutations is usually explored for diagnosis, complex alleles are often overseen. A better knowledge of each patient's genotype will be obtained with the introduction of Next Generation Sequencing. This already enabled to identify variants in alleles carrying F508del that could affect expression levels and in fine disease severity.

On another hand, a better understanding of defects associated to complex alleles will be key to propose specific therapeutics adapted to each patient's genotype. Therapeutic approaches have been recently developed to alleviate specific defects, such as molecules stabilizing CFTR (correctors) and molecules enhancing CFTR activity (potentiators). *Cis* mutations can affect treatment efficacy, as suggested by some F508del patients which are non-responsive to Orkambi treatment and carry exonic *cis* mutations affecting either mRNA splicing or channel folding. The effect on treatment outcome of variants affecting expression levels still needs to be explored. If positive, enhancing CFTR F508del expression levels would enhance treatment efficacy. This could be achieved by increasing transcriptional activity, stabilizing *CFTR* transcripts or enhancing exon 10 inclusion. While still not introduced in clinics, these approaches should be beneficial for all treatments targeting CFTR restoration.

In conclusion, CFTR complex alleles could be more frequent than previously thought giving rise to a more divers landscape of disease causing alleles. Predicting the final effect of mutations affecting different cellular processes on disease severity or treatment efficacy is still challenging. Nonetheless, *cis* mutations appear to affect F508del corrective therapies in a subset of patients. This could also be the case for other mutations, highlighting the importance to validate treatment efficacy in the context of complex alleles and the need to identify pertinent co-therapies to enhance treatment efficacy.

S2.2 Make sense out of nonsense with antisense

Melissa M. Keenan[1], Lulu Huang[1], Nikole Jordan[2], Eric Wong[2], Jerome Mahiou[2], Feng Liang[2], Hermann Bihler[2], Martin Mense[2], Shuling Guo[1], Brett P. Monia[1]

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Antisense technology is a validated platform for drug development. Antisense oligonucleotides (ASOs) treatments can downregulate their target mRNAs through an RNase H mechanism; alternatively, through steric blocking mechanisms, ASOs can modulate splicing or upregulate target mRNAs leading to increased protein production. Nonsense mutations generate premature termination codons (PTCs) that produce truncated proteins and subject mRNA transcripts to rapid degradation through the nonsense mediated decay (NMD) pathway. Approximately 10% of cystic fibrosis (CF) patients have at least one nonsense mutated CFTR allele, for which no targeted therapies are yet available.

We have developed ASOs targeting multiple players in the NMD pathway and evaluated these ASOs in cellular models of human CFTR nonsense mutations. In a mini gene system in HEK293 cells expressing CFTR W1282X mutation, SMG1-ASO mediated NMD inhibition upregulates the RNA, protein and surface-localized protein expression of the truncated W1282X gene product. Additionally, these ASOs increase the CFTR chloride channel function in CRISPR-Cas9-edited human bronchial epithelial cells homozygous for the W1282X mutation. This functional improvement is further enhanced when SMG1-ASO is combined with readthrough compound or CFTR modulator. Our data demonstrate that antisense therapy that modulate the NMD pathway has the potential to generate a functional or partially functional CFTR protein, thereby providing benefit to CF patients.

S2.3 A shortcut to bringing more patients with rare genotypes into the tent?

L. Millen[1], I. Musisi[2], A. Thakerar Patel[3], K. Raraigh[4], P. Oyem[2], N. Allaire[2], D. Benjamin[2], H. Bihler[2], R. Bridges[3], G. Cutting[4], M. Mense[2], A. Sivachenko[2], P. Thomas[1,2]

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A transient FRT transfection system, amenable to rapid assessment of CFTR function and folding, has been leveraged to evaluate the liability of hundreds of rare, disease-associated variants and their responsiveness to approved small molecule therapeutics. Transepithelial choride movement and CFTR processing were measured at multiple sites by blinded observers. This presentation will serve to update the progress on this project and to present some examples of new knowledge that can be gleaned from these large data sets.

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S2.4 Characterization of the rare S955P-CFTR mutation and its response to modulators in cellular models and patient-derived materials

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Background: More than 2,000 variants have been described to date in the *CFTR* gene, most of them presumed to be CF-causing. Among such large number of variants, many of them are very rare – with over 1,000 existing in less than five patients worldwide. For some of these mutations, since the functional defect has not been characterized yet, the prediction of disease outcome is difficult. Thus, there is an unmet need to functionally characterize these mutations at the molecular and cellular level in patient-derived materials when available, and in relevant cellular models for better disease diagnosis/prognosis. Moreover, the specific defect of each mutation is essential for mutation-specific therapy. S955P-CFTR (c.2863T>C) is a novel mutation located in exon 17 of *CFTR* gene which has not been previously characterized and was recently found in heterozygosity with F508del in an 8-week old Czech boy. The patient had IRT elevation (118 ng/ml) and repeatedly higher levels of sweat chloride (50.5; 55.3 mmol/l).

Aim: Here we aim to assess the molecular/functional defect of S955P-CFTR, both in patient-derived materials and in a cellular model, and to determine its responsiveness to available CFTR modulators.

Methods: CFTR function was measured in rectal biopsies from a CF patient bearing S955P by Ussing chamber and in intestinal organoids by the forskolin-induced swelling (FIS) assay. Additionally, assessment of S955P-CFTR protein expression and maturation was performed by Western blot, in a novel CFBE-based cell line stably expressing the S955P CFTR mutant (produced by *in vitro* mutagenesis), as well as in intestinal organoids from the above CF patient. The CFBE cell line was also used to measure CFTR activity at baseline and after treatment with modulators (potentiator genistein alone or with correctors VX-809 and VX-661) by Ussing chamber measurements.

Results and Discussion: Data from rectal biopsies and the intestinal organoids from a CF patient bearing S955P/F508del showed relatively high levels of residual CFTR activity, probably due to the S955P allele. The FIS assay of the intestinal organoids after treatment with CFTR modulators showed an significant increase in CFTR activity in the presence of VX809/VX770 and VX661/VX770, probably due to the F508del allele. Data from CFBE cells show that both mature and immature forms of S955P-CFTR are present, suggesting that this mutant has normal processing. Ussing chamber assays suggest that S955P-CFTR has residual function that is however not increased by treatment with CFTR modulators (VX-809 and VX-661). Altogether our results suggest that S955P is a class IV mutation and highlight the relevance of characterizing rare CFTR mutants using combined data from cellular models and patient samples, towards a personalized therapy approach.

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S2.5 The cystic fibrosis mutations L927P and I336K modulate CFTR pore dynamics during channel gating, but are rescued by ivacaftor and lumacaftor

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Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7), a unique member of the ATP-binding cassette (ABC) transporter superfamily. Unlike other ABC transporters, which function as active transporters, CFTR acts as an ATP-gated Cl^- ion channel that allows passive diffusion of substrate anions across the plasma membrane. This specialised role resulted from the evolution of the membrane-spanning domains (MSD1 and MSD2), transforming CFTR from an alternating access pathway with two gates to a pore with a single gate essential for fast and energetically-efficient anion transport. The CFTR pore is lined by transmembrane segment 1 (M1), M6, M8 and M12. Here, we investigate the action of two CF-associated mutations, L927P and I336K, which introduce structural changes to M8 and M6, respectively, at the narrowest part of the CFTR pore. To observe CFTR activity, we performed single-channel patch-clamp recording on excised inside-out membrane patches from Chinese hamster ovary (CHO) cells that were incubated at 27 ºC for ≥ 2 days to promote the plasma membrane expression of mutant CFTR. Voltage was --50 mV in the presence of a Cl^- concentration gradient ($[Cl^-]_{int}$, 147 mM; $[Cl^-]_{ext}$, 10 mM). The intracellular concentration of ATP and PKA were 1 mM and 75 nM, respectively, and temperature was 37 ºC. Both mutations formed PKA-activated CFTR Cl^- channels gated by intracellular ATP. However, their unitary current amplitudes were reduced by ~40 % ($n \geq 4$) when compared with currents recorded from membrane patches containing wild-type CFTR. Moreover, open probability values of both channels were >83 % ($n \geq 4$) lower than that of wild-type CFTR. The bursting pattern characteristic of wild-type CFTR channel gating was severely disrupted by L927P. By contrast, I336K-CFTR had two modes of channel gating which we termed mode-1 and -2. Mode-1 lacked the bursting pattern observed in wild-type CFTR, while mode-2 retained it. I336K-CFTR predominantly dwelt in mode-1 and only infrequently resided in mode-2. Ivacaftor enhanced the activity of both L927P- and I336K-CFTR in excised inside-out membrane patches, while treatment with both lumacaftor and ivacaftor enhanced transepithelial Cl^- currents across I336K-CFTR-expressing bronchial epithelia. The location of L927 and I336 within the transmembrane segments suggests that CF mutations at these residues might influence conformational changes within the pore during the gating cycle. We hypothesize that the proline introduced in L927P-CFTR possibly extends the discontinuity of M8 and reduces its structural flexibility during pore rearrangements following ATP binding. By contrast, I336K possibly locks the pore in a low conduction constricted state by reducing its flexibility. The switching of I336K-CFTR between two modes of channel gating raises the interesting possibility that I336K-CFTR activity might be enhanced by stabilising mode-2 channel gating.

Supported by NIH and CFFT; MKA-S was the recipient of a scholarship from the Sultanate of Oman.

P8

S2.6 Not all stop codons are created equal, neither do they always stop: morals from single-channel studies of E60X- and G542X-CFTR

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Cystic fibrosis (CF) is caused by loss-of-function mutations in the *CFTR* gene encoding a phosphorylation-activated but ATP-gated chloride channel. Among six classes of pathogenic mutations, Class I mutations, including premature termination codon (PTC) mutations, result in little or no protein expression. Although PTCs dramatically reduce the expression of CFTR, they can be suppressed endogenously or by reagents such as aminoglycoside antibiotics (e.g., G418). During this "read-through" process, a range of near-cognate tRNAs is used for translation, leading to various missense mutations at the position of PTC.

In this study, we investigated the characteristics of E60X and G542X, a PTC mutation at the N-terminus of CFTR and one in the first nucleotide binding domain (NBD1), respectively. Western blot experiments showed distinct differences between the read-through proteins of E60X and G542X. Significant amounts of non-glycosylated, band A E60X-CFTR proteins were detected, indicating full-length proteins were produced, but they failed to undergo glycosylation, as the core-glycosylated band B and complex-glycosylated band C were barely detectable. In contrast, we hardly saw any band for G542X-CFTR. These differences may be due to varied susceptibility of specific PTCs to read-through.

Although Western blotting showed no clear evidence for surface expression of E60X or G542X, whole-cell patch-clamp experiments recorded forskolin-dependent currents with a current density of 5.9±1.6 pA/pF (n=7) for E60X. Furthermore, application of the gating modulator genistein increased whole-cell currents by ~3-fold, indicating the presence of partially functional proteins. Moreover, pre-treating the cells with Lumacaftor, an FDA-approved CFTR corrector, or G418, a read-through reagent, increased whole-cell current density by ~5-fold. In inside-out patches from cells pre-treated with Lumacaftor, the application of PKA and ATP activated E60X-CFTR currents with a single-channel amplitude of 0.34±0.01 pA (n=5) that is similar to WT, but with a lowered open probability (P_o) of 0.21±0.03 (n=6), which can be increased to 0.7±0.07 (n=4) by N^6-phenylethyl-2'-deoxyATP, a high-affinity ATP-analogue. These results suggest that the read-through products of E60X-CFTR possess not only an intact anion permeation pathway, but the nucleotide binding domains where ATP or its analogue binds. Interestingly, the P_o of E60X-CFTR pre-treated with G418 was 0.5±0.07 (n=4), indicating a change in the read-through products. Comparing to the single-channel behaviors of E60Y/Q/S, the proposed read-through products for E60X, the P_o of Lumacaftor-treated E60X is more consistent with E60Y/S, but with E60Q for G418-treated channels.

In contrast, G542X-CFTR pre-treated with Lumacaftor or G418 presented a P_o of .2, which is different from those of G542R/C/W, the proposed read-through products (~0.7 for G542R, ~0.5 for G542C, and no detectable activity for G542W). These observations underscore the notion that the function of read-through products varies with stop codons and/or the position of the stop codon. Importantly, regardless of these variabilities, applying the CFTR potentiator GLPG1837 greatly increases the P_o of all the read-through products, hence suggesting a possible treatment strategy of combining CFTR correctors (e.g., Lumacaftor), read-through reagents (e.g., G418) and CFTR potentiators (e.g., GLPG1837). In summary, our studies unveil biophysical and pharmacological properties of the read-through products of E60X- and G542X-CFTR at a single-channel level. ~0

28 March — 16:00–17:45

Epithelial channelome : (i) Folding and structure

S3.1 Mechanism of ion conduction and gating in the calcium-activated chloride channel TMEM16A

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The calcium-activated chloride channel TMEM16A is expressed at the apical side of airway epithelia and its activation was thus proposed as potential therapeutic mechanism for the treatment of cystic fibrosis. The protein mediates anion-selective currents upon an increase of the intracellular Ca^{2+}concentration. TMEM16A is part of a protein family that comprises channels for ions and lipids with a single conserved molecular architecture. The protein is a dimer with each subunit containing an ion conduction pore. Both pores in the dimeric protein function as independent entities with respect to ion conduction and gating. Ca^{2+} binds to a site located within the transmembrane domain in vicinity of the pore in the non-conducting conformation. Ca^{2+} binding renders the electrostatics attractive for anions and it provides interactions for the conformational rearrangement of an α-helix, which lines part of the ion conduction path. Consequently, the movement of a transmembrane helix that closes the pore in the absence of ligand, towards a position that comes into physical contact with Ca^{2+}, directly couples ligand binding to pore opening.

S3.2 A minimal helical hairpin motif provides molecular-level insights into misfolding and pharmacological rescue of CFTR

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Our meagre understanding of cystic fibrosis transmembrane conductance regulator (CFTR) misfolding and its reversal by small-molecule correctors hampers the development of mechanism-based therapies of cystic fibrosis (CF). Here, we exploit a helical hairpin construct---the simplest proxy of membrane-protein tertiary contacts---containing CFTR's transmembrane helices 3/4 and its corresponding CF-phenotypic mutant V232D to gain molecular-level insights into CFTR misfolding and drug rescue by the pharmacological corrector Lumacaftor (VX-809). Using a single-molecule approach to study hairpin conformations in lipid bilayers, we find that the wild-type hairpin is well folded, while the V232D mutant assumes an open conformation in bilayer thicknesses mimicking the endoplasmic reticulum. We further find that the E217G hairpin exhibits an altered adaptive packing behavior stemming from an additional GXXXG helix--helix interaction motif created in the mutant hairpin. This suggests that the misfolding and functional defect caused by the E217G mutation arises from an impaired conformational adaptability of TM helical segments in CFTR. Addition of Lumacaftor reverses the aberrant opening of the V232D hairpin to restore a compact state as in the wild type. Interestingly, it also further stabilizes the wildtype hairpin. The observed membrane escape of the V232D hairpin and its reversal by Lumacaftor complement cell-based analyses of the full-length protein, thereby providing *in vivo*/*in vitro* correlates of misfolding and drug-action mechanisms of CFTR. The tendency to stabilize hairpin insertion suggests that Lumacaftor has a general mode of action likely connected to its membrane-destabilizing properties, through which it efficiently improves maturation of various CFTR mutants.

S3.3 NBD1 cotranslational folding intermediates as therapeutic targets

Hideki Shishido, Jae Seok Yoon, William R. Skach

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Most cases of cystic fibrosis (CF) are caused by loss of CFTR expression due to genetic mutations that decrease protein folding efficiency. However, little is known about how CF mutations affect the de-novo folding landscape as CFTR is synthesized in the cell. Here we use a FRET-based system wherein translationally incorporated donor and acceptor fluorophores enable one to examine conformational changes of nascent NBD1 translation intermediates. Our previous results revealed that NBD1 utilizes an orchestrated folding pathway in which the N-terminal, a-helical, and b-sheet core subdomains undergo sequential and controlled folding events that reflect gain and subsequent loss of conformational entropy as the nascent polypeptide emerges from the ribosome exit tunnel (Khushoo et al, Mol. Cell. 2011, and Kim et al, Science. 2015). During this process, ribosome attachment significantly delays folding of the a-subdomain, which in turn is required to ensure folding competence of the b-sheet core. In addition, synonymous codon substitutions that increased the a-subdomain translation rate, decreased NBD1 folding efficiency in vitro and promoted aggregation of full length CFTR in cells. We refer to this process as "translational tuning" to indicate that the ribosome synchronizes translation rate and folding kinetics via selective codon usage, while stabilizing and destabilizing nascent polypeptide structure to optimize folding outcome. We now show that CF-causing mutations not only disrupt trafficking of full-length protein, but also cotranslationally perturb the NBD1 folding pathway. Two mutations, A455E and L558S, located within different subdomains of NBD1 showed similar effects, disrupting folding of both α-subdomain and the β-sheet core. This folding defect was observed only transiently during a brief window of synthesis, consistent with a perturbation in the timing of sequential subdomain compactions. Surprisingly, the ribosome also played an important in cotranslational misfolding: first by destabilizing the misfolded state, and subsequently by facilitating eventual compaction of the entire domain. Finally, suppressor mutations (S492P and I539T) that partially stabilize F508del CFTR, eliminated the observed cotranslational folding defect induced by A455E (but not L558S) and partially restored trafficking of full-length A455E CFTR. These results identify cotranslational folding intermediates as potential pharmacological targets, and suggest that drugs which restore the cotranslational folding pathway may reflect a therapeutic strategy for treating CF and other misfolding diseases. (Funded by CFF)

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S3.4 The regulatory insertion enables novel conformations of NBD1 of CFTR

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Introduction: The role of conformational dynamics of NBD1 of CFTR in CF pathogenicity is well established. For example, the presence of an intrinsically disordered segment called the regulatory insertion (RI) is connected to the deleterious effect of delF508, as its removal has been shown to restore mutant CFTR maturation and function *in vitro*. The RI is unique to CFTR, its function is unknown and it destabilizes the protein significantly.

Approach: We have used nanobodies as crystallization chaperones for NBD1. Nanobodies are antigen binding fragments derived from llama antibodies. One of these nanobodies that we crystallized in complex with NBD1 interacts directly with the regulatory insertion. In this crystal structure NBD1 adopts a conformation that is significantly different from the classsical state observed in all crystal structures available in the protein data bank as the RI domain is now adopting a defined conformation and the N-terminus is not structured.

We used Double Electron-Electron Resonance (DEER) in conjunction with single molecule Förster Resonance Energy Transfer (smFRET) to characterize the conformational dynamics of NBD1 and test whether or not this new conformation is present in solution and in absence of the nanobody.

Results and conclusion: An equilibrium exists between the classically observed state and the alternate state we are reporting here.

At low levels of ATP (submillimolar) the classical state of NBD1 is less populated than the newly discovered state. Only high levels of ATP (mM) combined with deletion of the RI result in NBD1 adopting exclusively the classical state.

These results show that NBD1 adopts a complex conformational landscape due to the presence of the RI and that it can be modulated by ATP.

We hypothesize that deletion of the RI rescues ΔF508-CFTR by restricting the conformational landscape (ie. promoting the classical state). As a corollary, preventing transition to the alternate conformation of the RI may provide novel therapeutic approaches to overcome the effect of destabilizing mutations in NBD1 such as F508del.

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S3.5 CFTR conformational landscape explored by computational approaches

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CFTR protein represents an important target for drug discovery, with challenging issues related to the development of modulators specific for each class of mutations. Today, it is well recognized that the pleiotropic defects induced by some mutations affecting the CFTR protein, such as F508del, can be rescued by combination of pharmacotherapies addressing single defects. Combinations of modulators, correctors but also potentiators, targeting different sites of the CFTR protein, are thus actively investigated, with special emphasis on modulators that act in a synergic way and on rare mutations.

In this context, 3D structure provides critical information to understand the molecular basis of defects induced by mutations and potential ligand-binding sites that can be occupied by modulators. Knowledge on CFTR 3D structure was gained during the last years by comparative modelling, followed by cryo-EM, giving access to different conformations of the channel that can be explored in an integrative way. These however give only information on a few, discrete conformational states and do not inform about the plasticity of potential ligand-binding sites and the effect of ligand binding on the whole protein.

Tooking advantage of these structural data, we carried out standard molecular dynamics (MD) and enhanced dynamics (metadynamics) in explicit solvent using wild-type and mutated CFTR channel embedded in a lipid membrane. These simulations provided a larger exploration of CFTR conformational landscape and revealed some crucial features about CFTR functions and dynamics. These discriminating characteristics, involving specific salt-bridges, NBDs/ICLs interfaces and channel cavities, were used to generate a precise cartography of CFTR states classifying any channel conformation(s) and allowing thus to better characterize mechanisms underlying CFTR mutations but also modulations.

By combining experimental and theoretical methods, we identified some potential binding sites for modulators. We thus conducted several MD simulations of CFTR in presence of different modulators, correctors and potentiators (in-house and others). These simulations allowed us to support the binding site predictions, provide interactions schemes for each ligand, demonstrate the plasticity of some binding sites to accommodate the ligands and understand for some of them the molecular basis of their action.

These studies open new perspectives for the rational, structure-based design of specific modulators.

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S3.6 Identifying the molecular targets for CFTR potentiators GLPG1837 and VX-770

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In the past three years, major breakthroughs in solving the atomic structures of CFTR, which provide exquisite chemical features of this ion channel protein, make structure-based drug design a real possibility. However, to date the binding sites for CFTR modulators remain largely unknown. In this study, we first constructed a homology model of human CFTR based on the cryo-EM structure of the phosphorylated and ATP-bound zebrafish ortholog and then utilized in silico molecular docking to identify potential binding sites for GLPG1837, a CFTR potentiator that has been proposed to share a common binding site with the FDA-approved drug VX770 (Ivacaftor). Among the identified four binding sites, the one with the highest score (site I) includes three amino acids (D924, N1138, and S1141) located at the interface between CFTR's two transmembrane domains. Using the patch-clamp technique to measure CFTR currents at different concentrations of GLPG1837, we observed side-chain specific changes in the apparent affinity for GLPG1837 at all three positions. Specifically, glutamate, asparagine and alanine substitutions at position 924 cause a graded right-ward shift of the dose-response curves. On the other hand, the apparent affinity of GLPG1837 is increased either when N1138 is replaced with hydrophobic amino acids, including N1138F, N1138Y, and N1138L, or when S1141 is substituted by arginine (S1141R) and lysine (S1141K). Consistently, current relaxation time constant upon removal of VX770 is shortened by D924N but prolonged by N1138L and S1141K, supporting the notion that these three amino acids in site I also contribute to binding of VX-770. In contrast, alanine substitution of the residues in the other three lower-scored sites (site II, III, and IV) fails to change the affinity for GLPG1837. Lately, the structure of human CFTR with dimerized NBDs is solved by cryo-EM, allowing us to perform docking simulation on human CFTR and compare the results with those based on a homology model. Despite a similar ranking score for site I, a new binding site (site II_N) located at the interface between transmembrane segment 4, 5, and 8 is revealed. Among the five aromatic amino acids (F229, F236, Y304, F312 and F931) that compose site II_N, Y304F and F312A decrease the apparent affinity for GLPG1837 whereas F236A poses minimal effect. While more experiments are in progress to define the potential role of site II_N in the binding of potentiators, our data so far support site I as a likely binding site for both GLPG1837 and VX770.

28 March — 18:00–19:45

ECFS Basic Science Working Group Symposium

Activating Alternative Chloride Channels to Treat CF: Friends or Foes?

Chairs: Margarida Amaral (PT) - Jeff Beekman (NL)

18:00-18:05 Introduction

18:05-18:50 **Round table 1: Can we regulate TMEM16A independently of Ca2+? And TMEM16A /SLC26A9 independently of CFTR?**

Moderators: Jeff Beekman & Karl Kunzelmann

18:05-18:10 Facts about ANO1 - Karl Kunzelmann (DE)

18:10-18:13 Abstract 32 - EACT increases intracellular calcium levels by a TMEM16A-independent mechanism - Henry Danahay (UK)

18:13-18:16 Abstract 111 - Niclosamide repurposed for the treatment of inflammatory airway disease - Roberta Benedetto (DE)

18:16-18:19 Abstract 34 - Identification of novel ANO1/TMEM16A regulators as alternative therapeutic targets for cystic fibrosis - Madalena Pinto (PT)

18:19-18:50 Overall Discussion

18:50-19:00 Short Break

19:00-19:45 **Round table 2: TMEM16A and mucus**

Moderators: Margarida Amaral & Luis Galietta

19:00-19:03 Abstract 30 - TMEM16A channel function does not influence goblet cell numbers in the human airway epithelium - Henry Danahay (UK)

19:03-19:06 Abstract 28 - Cell Proliferation Upregulates ANO1 in Mucus Cell Hyperplasia - Filipa Simões (PT)

19:06-19:09 Abstract 105 - The attached stratified mucus in obstructive airway disease is detached by calcium removal - Dalia Fakih (SE)

19:09-19:12 Abstract 31 - Physiological role and therapeutic importance of TMEM16A chloride channel in the airway epithelium - Arianna Venturini (IT)

19:12-19-45 Overall Discussion

29 March — 08:45–10:30

Epithelial channelome : (ii) Cell physiology and ion transport

S4.1 Exploiting species differences to investigate CFTR

David N. Sheppard

University of Bristol, School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences Building, University Walk, Bristol BS8 1TD, UK

Programme note: the details are not authorised for inclusion

S4.2 Anionophores: Small-molecule CFTR surrogates as mutation agnostic CF drug candidates

Maria Garcia-Valverde [1], Israel Carreira-Barral[1], Marcin Mielczarek[1], Ambra Gianotti[2], Valeria Capurro[2], Livia Delpiano[2], Alessandra Ludovico[3], Debora Baroni[3], Oscar Moran[3], Emanuela Caci[2], Roberto Quesada[1]

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Cystic fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. As a result, the production and/or function of the CFTR protein is compromised. CTFR is a transmembrane channel protein expressed in epithelial tissues, which facilitates the transport of chloride and bicarbonate. Impairment of this function results in pathophysiological manifestations including high salt content in the sweat, pancreatic enzyme insufficiency and obstructive lung disease. The last few years have witnessed most important advances in the understanding of the pharmacological correction of CFTR expression and function. The approval of drugs developed using this strategy such as Kalydeco™, Orkambi™ and Symdeko™ developed by Vertex Pharmaceuticals and the strong drug development pipeline within the pharma industry bode well for providing therapeutic solutions to the majority of CF patients. Despite these successes, the urgent need of other therapeutic persists. The performance of the current drugs dramatically depends on the type of mutation carried by the patient and it is still far from optimal for numerous patients. Moreover CF patients harboring mutations resulting in no CFTR production such as class I mutations are not expected to benefit from this strategy. Therefore there is a significant interest in developing mutation agnostic therapies.

Following our interest in developing small molecules capable of facilitating the permeation of small anions such as chloride and bicarbonate across lipid membranes, anionophores, we envisaged the possibility of using them a minimalistic CFTR function surrogates. This concept represents a potential universal remedy for all CF patients. We have screened a large collection of candidates and characterized their activity as anion transporters in both liposomes and in isolated cultured cells. Treatment of CF patient-derived differentiated bronchial epithelia cultures with some of these compounds reduce fluid reabsorption and improve the rheological properties of the secreted mucus. Functional correction of these two key parameters in CF pathophysiology represents a promising proof of concept for the development CF drug candidates using this strategy.

S4.3 Submucosal glands produce mucus strands that initiate mucociliary transport

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Lungs are continually assaulted by bacteria, viruses, and toxic particles. To protect lungs from these challenges and to maintain near-sterility, mammals have evolved multiple airway defenses. One important component of airway defense is mucociliary transport (MCT), in which mucus binds bacteria and particulates and cilia propel the mucus out of the lung. In humans and other large mammals, the cartilaginous airways contain submucosal glands (SMGs) that produce mucus. We developed an *in vivo* CT-based imaging assay of MCT by tracking microdisks in spontaneously breathing, non-intubated, newborn pigs. Results from *in vivo* and *ex vivo* studies revealed that mucus emerges from SMG ducts onto the airway surface in the form of rope-like strands containing a MUC5B core. MUC5AC from goblet cells forms small threads and sheets that wind around the strands. As the strands elongate, the force from cilia beating breaks them, and they sweep over the surface. To test the function of mucus strands in non-CF airways, we disrupted them by applying reducing agents *tris*-(2-carboxyethyl)phosphine and dithiothreitol. They decreased the fraction of moving spheres and delayed initiation of movement for spheres that did move both *in vivo* and *ex vivo*. These findings indicate that submucosal glands produce mucus in the form of strands and that the strands initiate movement of large particles, facilitating their removal from airways. In contrast, in CF SMGs, loss of CFTR-mediated Cl^- and HCO_3^- secretion increases mucus elasticity reducing its breaking, and as a result the strands sometimes remain anchored to SMG ducts, disrupting MCT and impairing host defenses.

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S4.4 Impact of CFTR function on airway mucus properties

Martial Delion[1], Boya Wang[2], Sophia Samir[2], Lolita Radet[2], Rodney Gilmore[2], David B. Hill[2], Martina Gentzsch[2], Richard, C Boucher[2], Camille Ehre[2]

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Introduction: Cystic fibrosis (CF) is characterized by the buildup of thick, viscous mucus in several organs, particularly the lungs. Gel-forming mucins (mainly MUC5B and MUC5AC in the lungs) are high-molecular-weight glycoproteins responsible for the viscoelastic properties of mucus and play a key role in the CF pathogenesis. In CF airways, abnormal mucus properties drive mucus stasis and airway obstruction but the pathophysiologic link with CFTR malfunction is unclear. Mutations within CFTR reduce transepithelial Cl^- and $HCO3^-$ secretion, which leads to ionic imbalance and volume depletion. Airway dehydration causes mucin hyperconcentration, increasing the interactions between mucin monomers (i.e., mucin entanglement). CFTR dysfunction is also associated with oxidative stress, which may increase disulfide bond formation (i.e., mucin crosslinking). Additionally, deficient bicarbonate secretion affects $Ca2^+$ chelation, which may compromise mucin unfolding/expansion (i.e., mucin compaction). Understanding the dominant biochemical change(s) (i.e., mucin entanglement, covalent crosslinking, electrostatic compaction) caused by CFTR malfunction is critical to identify new therapeutic targets aimed to "reverse" mucus abnormalities in CF.

Methods: Primary and immortalized human bronchial epithelial (HBE) cells were obtained from CF patients carrying either the G551D or F508del mutation. Human nasal epithelial (HNE) cells were collected from normal and CF subjects. Modulation of CFTR activity was ensured by either correction by small molecules (Vx770- Vx809) or pharmacological inhibition with the specific CFTR inhibitor $CFTR_{Inh172}$. We used these cell systems to study the impact of CFTR activity on mucin properties using biochemical assays (Western blotting, HPLC, IHC), biophysical measurements (macro/microrheology) and scanning electron microscopy (SEM). Change in spontaneous mucociliary transport (MCT) was also assessed using fluorescent microbeads tracking.

Results: Chronic treatment with CFTR modulators in our primary and immortalized cell cultures resulted in enhanced CFTR-mediated short-circuit currents. In G551D cells, Ivacaftor treatment significantly decreased total mucin concentration by ~30%, increased mucus pH slightly, and increased ciliary beat amplitude by ~30%, suggesting a physical change in the mucin network surrounding the cilia. G551D- CFTR rescue decreased MUC5B but not MUC5AC crosslinking as shown by electrophoretic mobility shift on mucin agarose gel. In F508del-HBE cells, the duration and velocity of mucociliary transport (MCT) was significantly reduced compared to non-CF cells but when treated with a combination of Ivacaftor and Lumacaftor, F508del-HBE cells partially restored MCT. In addition, changes in CFTR function strongly affected mucin secretion in freshly harvested nasal cells. Similarly, CFTR rescue significantly reduced MUC5B and MUC5AC secretion in CF nasal cells. Inversely, CFTR inhibition resulted in mucin hyperconcentration (1.5-fold change) in non-CF nasal cells, which correlated with a change in MUC5B/MUC5AC ratio and a tighter mucus network by SEM.

Conclusion: Functional CFTR rescue affected mucin interactions by decreasing mucin entanglement and crosslinking. In contrast, CFTR inhibition increased mucin concentration and molecular weight/size. CFTR dysfunction alters MUC5B/MUC5AC ratio and mucin network organization, which both likely affect the biophysical properties of airway mucus. Hence, treatments directly targeting mucus (e.g. mucolytics reducing disulfide bonds) could be a promising therapeutic strategy, independent of inflammation or patient genotype.

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S4.5 Early onset of airway mucus obstruction associated with increased mortality in neonatal *Slc26a9* deficient mice

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Background: Recent evidence suggests that the epithelial Cl^- channel SLC26A9 is associated with disease severity in patients with cystic fibrosis (CF) (Strug LJ. et al., 2016) and other muco-obstructive lung diseases such asthma (Anagnostopoulou P. et al., 2012) and bronchiectasis (Bakouh N. et al., 2013).These studies support a role of SLC26A9 as a modifier of muco-obstructive lung diseases. However, the in vivo role of SLC26A9 in lung development and disease remains unclear.

Aim: The aim of this study was to investigate the *in vivo* role of the SLC26A9 Cl^- channel during early postnatal adaptation.

Methods: We compared the pulmonary phenotype of wild-type (WT) and *Slc26a9*- deficient (*Slc26a9*$^{-/-}$) mice on a C57BL/6 background including survival, histology, micro computed tomography (µCT) imaging, wet/dry lung weight and oxygen saturation measurements. On a functional level, transepithelial potential differences (PD) of cultured tracheal explants of WT and *Slc26a9*$^{-/-}$ mice was assessed.

Results: All genotypes were represented at birth according to Mendelian ratios. Within the first 30 minutes of life, *Slc26a9*$^{-/-}$ mice showed signs of severe respiratory distress, reduced oxygen saturation ($p< 0.01$) and spontaneous mortality of 48%. To determine if developmental defects were responsible for the death of the *Slc26a9*$^{-/-}$ mice, we performed histological analysis on vital organs at the embryonic day 17 (E17), but did not detected differences in *Slc26a9*$^{-/-}$ compared to WT. Further, both genotypes presented a similar rate of lung liquid clearance at birth ($p=0.56$). To evaluate the contribution of the SLC26A9 Cl^- channel to the airway epithelial ion transport in the neonatal lung, PD was measured in cultured tracheal explants and found to be lower in *Slc26a9*$^{-/-}$ (-18 ± 6 mV) compared to WT mice (-41 ± 6 mV; $p< 0.05$). Histological analyses immediately after birth revealed severe mucus obstruction through the whole tracheobronchial tree of *Slc26a9*$^{-/-}$ mice compared to WT mice ($p< 0.01$). These findings were confirmed by µCT showing early onset airway mucus obstruction ($p< 0.05$) associated with atelectasis compared to WT mice.

Conclusions: In summary, the *Slc26a9*$^{-/-}$ mice shows an early airway muco-obstructive phenotype associated with decreased epithelial ion transport *in vivo*. Taken together, our data support that SLC26A9 mediated Cl^-/fluid secretion plays a critical role in airway mucus clearance in the neonatal lung and that this process may be essential for normal postnatal adaptation to the air-breathing conditions.

Supported by the BMBF (82DZL00401 and 82DZL004A1)

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S4.6 Dual role of pendrin as a bicarbonate secretion pathway and CFTR modulator in well-differentiated human nasal and bronchial epithelial cells

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Introduction: Bicarbonate facilitates mucin unpacking and bacterial killing on the airway surface however its transport mechanisms are not well understood. The activation of CFTR channel by phosphorylation stimulates conductive Cl^- and HCO_3^- efflux and this is defective in CF. The anion exchanger pendrin (SLC26A4) mediates electroneutral HCO_3^- efflux and is upregulated by proinflammatory cytokines. The relative contributions of these pathways remain uncertain and may depend on physiological state.

Aim: To examine pendrin and CFTR expression in the airways and their roles in HCO_3^- secretion across human nasal and bronchial epithelia cells.

Methods: Fresh tissue and well differentiated bronchial and nasal epithelial cells that had been cultured at the air-liquid interface for 1 month were used. Intracellular pH was measured using BCECF-AM during apical or basolateral exposure to low-Cl^- solution containing HCO_3^- to monitor anion exchange. Cells were transduced using multiple adenoviral shRNAs targeting pendrin mRNA transcripts. CFTR-dependent short-circuit current (Isc) was measured in Ussing chambers after adding amiloride. Airway surface liquid (ASL) height was monitored using confocal microscopy. Whole-cell Cl^- current was measured in BHK cells stably expressing CFTR with or without transient co-expression of EGFP-pendrin.

Results: In native bronchial tissue, both CFTR and pendrin were most abundant at the apical pole of ciliated surface cells, with little CFTR or pendrin detected in submucosal glands. IL-4 dramatically increased pendrin mRNA levels and apical pendrin immunostaining in well-differentiated primary nasal and bronchial cells, confirming previous studies by others. Exposure to low-Cl^- apical solution caused intracellular alkalinization (ΔpH_i) that was enhanced 4-fold in cells that had been pretreated with IL-4. ΔpH_i was not altered by DIDS or $CFTR_{inh}$-172 but was reduced in pendrin knockdown cells. Forskolin increased ΔpH_i and this stimulation was prevented by $CFTR_{inh}$-172, implicating CFTR. Surprisingly, forskolin only increased ΔpH_i when pendrin expression had been upregulated by IL-4. This dependence of ΔpH_i on pendrin suggests there is minimal electrical coupling between Cl^- and HCO_3^- fluxes and that CFTR activation increases anion exchange-mediated HCO_3^- influx. Conversely, increasing pendrin with IL-4 or by heterologous expression increased forskolin-stimulated, $CFTR_{inh}$-172-inhibitable current ~2-fold in both epithelial and non-epithelial cells, respectively.

Conclusions: Pendrin mediates most HCO_3^- secretion across airway surface epithelium during inflammation and enhances CFTR-mediated electrogenic Cl^- secretion.

Support: Cystic Fibrosis Canada, Canadian Institutes of Health Research, Canada Foundation for Innovation

29 March — 11:00–12:45

CFTR processing, trafficking and interactions

S5.1 A Proteomic Variant Approach (ProVarA) for personalized medicine of inherited and somatic disease

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The advent of precision medicine for genetic diseases has been hampered by the large number of variants that cause familial and somatic disease, a complexity that is further confounded by the impact of genetic modifiers. To begin to understand differences in onset, progression and therapeutic response that exist among disease-causing variants, we present the proteomic variant approach (ProVarA), a proteomic method that integrates mass spectrometry with genomic tools to dissect the etiology of disease. To illustrate its value, we examined the impact of variation in cystic fibrosis (CF), where 2025 disease-associated mutations in the CF transmembrane conductance regulator (CFTR) gene have been annotated and where individual genotypes exhibit phenotypic heterogeneity and response to therapeutic intervention. A comparative analysis of variant-specific proteomics allows us to identify a number of protein interactions contributing to the basic defects associated with F508del- and G551D-CFTR, 2 of the most common disease-associated variants in the patient population. We demonstrate that a number of these causal interactions are significantly altered in response to treatment with Vx809 and Vx770, small molecule therapeutics that respectively target the F508del and G551D variants. ProVarA represents the first comparative proteomic analysis among multiple disease-causing mutations, thereby providing a methodological approach that provides a significant advancement to existing proteomic efforts in understanding the impact of variation in CF disease. We posit that the implementation of ProVarA for any familial or somatic mutation will provide a substantial increase in the knowledge base needed to implement a precision medicine-based approach for clinical management of disease.

S5.2 Novel CFTR regulators identified by means of a functional genomics approach and their possible mechanisms of action

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Deletion of phenylalanine 508 (F508del) in the CFTR chloride channel causes a processing defect of the mutant protein resulting in its retention in the ER with subsequent degradation, and a gating defect due to reduced channel activity. CFTR F508del displays also a decreased plasma membrane residency time as a result of high internalization rate coupled with defective recycling. Since CFTR quality control / trafficking is a complex process with multiple checkpoints, and an effective drug therapy for CF will rely on the combination of different correcting manoeuvres to drive the proper folding of CFTR domains and to improve mutant processing i.e. pharmacological chaperons could be combined with proteostasis regulators.

Several proteins have been already identified as proteostasis regulators, acting throughout the CFTR F508del maturation pathway: 1) ER folding and assembly [the ERAD pathway Derlin-1, p97/VCP, RNF5/RMA1, chaperones systems HSP90, HSP70, small-HSP and the SUMOylation pathway]; 2) peripheral control [Dab2, CAL, NHERF-1, COMMD1]; 3) kinases cascades. These proteins have a potential therapeutic role for CF: from this point of view, particularly interesting is the E3 ubiquitin ligase RNF5.

By using a functional genomics approach based on siRNA screening, we identified 37 proteins whose silencing results in a significant rescue of F508del-CFTR activity. The identified targets include proteins associated to F508del-CFTR degradation (like UBA52 and UBXN6) and sumoylation (i.e. UBE2I, UBA2), transcription factors (i.e. MLLT6, CHD4, TRIM24) and also proteins with unknown function like FAU.

Now our work is focused on: 1. the characterization, prioritization and dissection of the mechanism(s) of action by which downregulation of selected proteins leads to mutant CFTR rescue; 2. the evaluation of global changes induced by target downregulation on cell morphology and functions. To this aim, the effects of silencing/overexpression of specific targets have been evaluated on rescue of mutant CFTR processing and concurrent possible pleiotropic effects by means of biochemical and electrophysiological techniques and by high-content imaging and analysis.

The results indicates that, while some of the targets appears particularly promising and specific for CFTR (like FAU), other can be associated to deleterious effects due to dysregulation of specific cell pathways and/or to undesired effects on ion transepithelial transport.

This work was supported by the Italian Cystic Fibrosis Foundation (Fondazione per la Ricerca sulla Fibrosi Cistica) and by the Italian Ministry of Health (Ricerca Corrente and 5permille)

S5.3 Functional genomics of F508del-CFTR: Illuminating traffic mechanisms for innovative therapeutic strategies

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Although it is known for long that F508del, the most common CF-causing mutation (~85% of patients) leads to protein misfolding and recognition by the endoplasmic reticulum quality control (ERQC) that targets it for proteasomal degradation, we are still missing the mechanisms and pathways involved in this process.

We have used a functional genomics approach for a global characterization of F508del-CFTR traffic defect. This consisted in the development of a high-throughput microscopy assay in human bronchial epithelial cells to identify factors that rescue traffic of F508del-CFTR to the cell surface and applied it to screen a library of siRNAs targeting the druggable genome (~9,000 genes). Functional classification of hits according to ERQC checkpoints, overlap with previous CF transcriptomics and proteomic studies provide hit relevance in CF and non-overlap with general secretome help defining specificity of F508del-CFTR traffic regulators.

Overall, our results point to a complex involvement of several cellular functions in the regulation of the CF physiopathology, and define novel traffic pathways and molecular targets that can be potentially manipulated by new drugs in a synergistic way for the greater benefit of individuals with CF. Moreover, these regulators can also help defining the mechanism of action of existing CFTR corrector drugs.

Work supported by UID/MULTI/04046/2013 centre grant from FCT, Portugal (to BioISI)

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S5.4 CFTR processing mutations cause distinct trafficking and functional defects

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Introduction: Approved and investigational CFTR correctors and potentiators have the possibility to treat up to 90% of CF patients. Still, 10% of patients remain in need of specific therapies. This group comprises patients with two minimal function alleles, including certain severe protein processing mutations, e.g. N1303K and G85E. We established a flow cytometry assay to quantify single cell CFTR plasma membrane (PM) density. After validating the assay using the well-characterized processing mutation F508del, we next aimed at studying mutations with a known or suggested processing defect, i.e. G85E, A455E and N1303K and their responses to corrector VX-809 and potentiator VX-770.

Methods: We generated HEK293T cell lines stably expressing mutant CFTR with a 3HA-tag in the 4th extracellular loop (Sharma et al, 2004) under control of the CMV promoter after puromycin selection. G551D and WT served as controls for normal trafficking. We assessed CFTR maturation by western blot (WB), CFTR expression by immunocytochemistry (ICC) and quantified PM density by flow cytometry (% gated * median fluorescence intensity, MFI). CFTR function was assessed by halide sensitive YFP quenching assay ($1-F/F_0$). In parallel, CFTR maturation and function were studied in primary intestinal organoids overexpressing the respective CFTR mutants. CFTR function was assessed by forskolin induced swelling.

Results: On WB, G85E, A455E, F508del and N1303K showed a similar defect in CFTR maturation, namely an absent or strongly reduced band C. However, quantitative analysis of PM density by flow cytometry revealed distinct differences between mutants, i.e. absence of PM CFTR for G85E (0.2% PM expression relative to WT), low levels of PM CFTR for F508del (2.4%) and A455E (4.3%) and medium levels for N1303K (12.3%). As a reference, gating mutant G551D resulted in 109.9% PM CFTR. VX-809 increased PM density by 6-fold for F508del, 2.7-fold for A455E and 1.7-fold for N1303K, but failed to correct G85E. CFTR function in the YFP assay was low to absent at baseline for all processing mutants (1-8%), compared to 42% for WT. Combined VX-809+VX-770 increased CFTR function by 8.3-fold for F508del over DMSO, 2.8-fold for A455E and 2.3-fold for N1303K. For G85E no functional rescue was observed. In transduced organoids, WB confirmed the maturation defect as in HEK293T cell lines (no band C for the processing mutants). Both endogenous and overexpressed G85E and N1303K failed to functionally respond to VX-809+VX-770, in contrast to F508del, resulting in ~70% organoid swelling. A455E analysis is ongoing.

Conclusion: The flow cytometry based PM density assay provides a first rapid and quantitative method using stable cell lines to identify trafficking defects of the many poorly studied rare *CFTR* mutations. Although mutants G85E, A455E, F508del and N1303K at first sight presented with a similar processing defect on WB, the flow assay identified distinct trafficking defects. Currently we are characterizing the rare mutations E60K and E92K. The findings obtained in cell lines will be fully compared to data in primary cell models to ensure maximal translational potential.

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S5.5 Role of the proteasome in the biosynthetic arrest of SLC26A9 by F508del-CFTR

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Introduction: Currently available drugs that correct F508del-CFTR misfolding provide only modest clinical benefit for most CF patients, therefore alternative anion channels and other potentially druggable targets are being explored. SLC26A9 is a constitutively active anion channel expressed in human airways that modifies the severity of CF airway disease in patients with the G551D-CFTR mutation. The functional expression of complex glycosylated SLC26A9 is reduced in cells that express F508del-CFTR mediated in part by a PDZ- and CAL-dependent mechanism. SLC26A transporters also interact with the regulatory (R) domain of WT-CFTR through its Sulfate Transporter AntiSigma factor antagonist (STAS) domain, however the role of this interaction in SLC26A9 biosynthetic arrest is uncertain. Indeed, it is not known if the interaction with immature F508del-CFTR in the endoplasmic reticulum (ER) contributes to the retention and proteasomal degradation of SLC26A9.

Aim: To understand the interaction between SLC26A9 and CFTR and its impact on SLC26A9 expression.

Methods: BHK cells overexpressing wild-type (WT) or F508del-CFTR and parental BHK cells lacking CFTR were transiently transfected with SLC26A9 cDNA. SLC26A9 protein was quantified in lysates by immunoblotting and at the plasma membrane by cell surface biotinylation. SLC26A9 levels were assessed in well-differentiated CF and non-CF primary human bronchial epithelial cells (pHBEs) by immunofluorescence staining and confocal microscopy.

Results: Total and plasma membrane SLC26A9 expression were both lower in BHK cells when co-expressed with F508del-CFTR than when expressed alone or with WT-CFTR. Similar results were obtained when well-differentiated CF and non-CF primary human bronchial epithelial cells (pHBEs) were studied by confocal imaging. Since F508del-CFTR misfolding leads to its retention in the ER and subsequent proteasomal degradation, we examined the effects of proteasome inhibitors on SLC26A9 degradation. Inhibiting the proteasome increased SLC26A9 immunofluorescence in F508del-CFTR homozygous pHBE cells but not in non-CF pHBEs, suggesting there is enhanced proteasomal degradation of SLC26A9 with F508del-CFTR in pHBEs. This difference in proteasome inhibitor sensitivity was not observed in non-epithelial cells overexpressing SLC26A9 with F508del-CFTR or WT-CFTR. Apical SLC26A9 levels increased in pHBEs and in non-epithelial cells when F508del-CFTR was partially corrected using low temperature or VX-809, and this rescue was mimicked by co-transfecting cells with WT-CFTR. The rate of SLC26A9 degradation was measured when expressed alone or with WT-CFTR or F508del-CFTR. In the presence of the protein synthesis inhibitor cycloheximide, degradation of immature SLC26A9 was enhanced in F508del-expressing cellssuggesting there is CFTR-dependent, proteasome-mediated degradation of SLC26A9 at the ER.

Conclusions: These results suggest that ER retention of F508del-CFTR and SLC26A9 leads to premature degradation of both proteins and SLC26A9 biosynthetic arrest is due in part to an interaction between them. Disrupting this interaction will make SLC26A9 an exciting therapeutic target for most CF patients.

Support: Studentships from CF Canada, and Fonds de recherche du Québec – Santé to YS, and grants from the Canada Foundation for Innovation to DYT and CF Canada to JWH.

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S5.6 Monitoring the phospho-occupancy of CFTR in respiratory epithelia using mass spectrometry

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Background: CFTR channel activity is regulated primarily by cAMP-dependent, protein kinase (PKA)-dependent phosphorylation of the regulatory domain (RD) and ATP-dependent dimerization of the nucleotide-binding domains (NBDs). It is established that the intrinsically unstructured RD phosphorylation is a prerequisite for pore opening upon ATP-induced dimerization of the NBDs. To prevent CFTR activation by PKA, fifteen consensus PKA phosphorylation sites had to be mutated in the RD and NBD1 (15SA), suggesting that the non-phosphorylated RD displacement is cooperatively regulated by multiple phosphosites. However, we still lack a quantitative method to monitor the dynamics of CFTR phosphosites occupancy in order to directly investigate its role as a functional determinant of channel gating in its native environment. To this end, we implemented a novel mass spectrometry-based (MS) workflow to quantify the phospho-occupancy of most of the PKA consensus sites in WT-CFTR in transduced human bronchial epithelia (CFBE).

Methods: We engineered a CFTR construct with His_6-biotin-His_6 (HBH) tag at the N-terminus and a 3HA-tag on the fourth extracellular loop (HBH-CFTR). The plasma membrane expression and the short circuit current measurements (I_{sc}) of the HBH-CFTR were similar to that of the CFTR-3HA variant. We performed a label-free, targeted MS data acquisition on the affinity-enriched CFTR by combining electron-transfer dissociation (ETD) and higher-energy collision dissociation (HCD), referred to as EThcD fragmentation.

Results: We measured the constitutive phospho-occupancy of ten CFTR PKA consensus sites (S422, S660, S670, S686, S700, S712, S737, S753, S768, and S795) in both resting and stimulated conditions in CFBE. Most sites registered at least 5% elevated phosphorylation levels upon forskolin stimulation, consistent with the model that phosphorylation of multiple sites synergistically contributes to the channel activation. Interestingly, S686, which was essential in CFTR activation based on previous mutational analysis, was not amenable to forskolin-induced phosphorylation. We also compared the phosphorylation levels between adenylyl cyclase (AC) stimulation and inhibition by forskolin and SQ22536, respectively. S660, S670, S700, S712, S737, S753 and S795 showed a significant increase in their phosphorylation level by PKA activation. Having established the phosphorylation levels in the basal and stimulated CFTR, we tested the acute activation mechanism of CFTR upon cigarette smoke condensate (CSC) exposure. The phospho-occupancy of the PKA sites, with the exception of two sites, was similar to that of the forskolin-stimulation, suggesting the involvement of PKA activation upon CSC exposure. Ongoing experiments compare the effect β2-adrenergic receptor, vasoactive intestinal peptide receptor and AC activation on the of phospho-occupancy of CFTR.

Conclusion: Our EThcD-MS technique helps to monitor the phospho-occupancy of multiple phosphosites simultaneously under resting and stimulated physiological and pathological conditions. This technique can further be implemented to study the phospho-occupancy of WT and mutant CFTRs in primary human bronchial epithelia.

Supported by: Cystic Fibrosis Canada Fellowship (AP), CIHR, CFF Therapeutics Inc.

30 March — 08:45–10:30

Inflammation and host-pathogen interactions

S6.1 Implication of host antimicrobial peptides in the modulation of bacterial-bacterial interactions in CF airways.

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Airways infections is a major problem that determines the life expectancy of patients with cystic fibrosis (CF). These infections vary significantly with the age of patients and *Staphylococcus aureus* (SA) is the most commonly isolated bacterium from young CF patients. With the increase of age of patients, *Pseudomonas aeruginosa* (PA) becomes predominant and in the adult patients it represents the major bacteria in the lung. However, the mechanisms responsible for this age-related infection switch from SA to PA remained unclear. We hypothesize that molecules, such as antimicrobial peptides (AMPs), produced by the host or by PA play a role in the progressive and selective elimination of SA from CF airways. We first identified the type-IIA-secreted phospholipase A2 (sPLA2-IIA), a host enzyme with high bactericidal activity, as a major factor involved in the killing of SA in CF airways. We showed that this enzyme exhibits high killing capacity toward Gram-positive bacteria including SA, with almost no effects on the Gram-negative one. The levels of sPLA2-IIA increase in the expectorations of CF patients in an age-dependent manner and is mainly expressed by their bronchial epithelial cells (BECs). Expression of sPLA2-IIA is markedly induced by PA in these cells and in lungs in a guinea-pig model of pulmonary infection by PA. Both laboratory strains of PA and isolates from CF patients induced sPLA2-IIA expression. Induction of sPLA2-IIA expression in BECs is mediated by the injection by PA of the toxin ExoS via the type-III secretion system (T3SS) and involves the transcription factor KLF-2. In mouse and guinea-pig models of lung co-infection by PA and SA, sPLA2-IIA production promotes SA killing in airways. Transgenic mice over-expressing sPLA2-IIA constitutively exhibit very low levels of SA in their airways and, most generally, an airway microbiota in which the Gram-positive bacteria are virtually absent. In parallel, our studies showed that BECs from CF patients produce constitutively the AMP, cathelicidin LL-37 that was also detected in epithelia of lung explants from CF patients. We showed that LL-37 kill preferentially PA compared to SA *in vitro.* This killing was observed with both laboratory and clinical strains of PA isolated from CF patients. Interestingly, infection by PA, but not with SA strains, down-regulated the LL-37 expression by BECs of CF patients. This inhibition is mediated via the injection of the toxin ExoS within BECs, but the signaling pathways involved in this down-regulation is still unknown. In BECs in which the expression of LL-37 was silenced by siRNA the multiplication of PA was higher than that in BECs with normal LL-37 expression. This suggested that down-regulation of LL-37 expression by PA may favor the multiplication of this bacterium within BECs. Thus, induction of sPLA2-IIA expression promotes the elimination of SA in CF airways and its replacement by PA, a process facilitated by the down-regulation of LL-37 production in airways cells. Overall, our studies suggest that a bacterium can eradicate another bacterium by manipulating the host immunity, and that AMPs produced by the host contribute to the modulation of airways microbiota evolution.

S6.2 Viral-bacterial co-infections in cystic fibrosis

Jennifer Bomberger

University of Pittsburgh

Viral-bacterial co-infections are becoming increasingly recognized as a cause of morbidity and mortality in chronic lung disease, including cystic fibrosis (CF). Polymicrobial infections frequently occur in the CF respiratory tract, but the underlying mechanisms of how viral-bacterial interactions mediate pathogenesis remain poorly understood. In CF patients, clinical observations link the acquisition of chronic *Pseudomonas aeruginosa* infection with seasonal respiratory virus infections (notably, respiratory syncytial virus (RSV), rhinovirus and influenza A). Recently, we demonstrated that respiratory viral infection, and the subsequent innate immune antiviral interferon (IFN) response, promoted biofilm growth of *P. aeruginosa*, mediated through the disruption of nutritional immunity. We are investigating the underlying innate immune pathways involved in promoting *P. aeruginosa* biofilm growth, with a focus on immunometabolism shifts in the respiratory epithelium. We have identified interferon-regulated genes that promote *P. aeruginosa* biofilm biogenesis by altering the metabolic state of the epithelium, as well as IRGs that induce competitive interactions of *P. aeruginosa* with other microbes (including *Staphylococcus aureus*) in polybacterial biofilm communities. Clinical studies are also underway to examine nutritional immunity defects during viral co-infections that support *P. aeruginosa* dominance in the CF respiratory tract and transition to a chronic infection. Our research aims to improve our understanding of the mechanisms by which respiratory viral infections alter host immunity to support chronic bacterial infections, with the long-term goal of identifying new therapeutic targets to prevent chronic *P. aeruginosa* infections in the CF respiratory tract.

S6.3 Biofilm formation in cystic fibrosis

Tom Coenye

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Biofilms are communities of microbial cells that are embedded in an at least partially self-produced matrix. *In vivo* biofilms can occur as surface-attached communities (e.g. on implanted devices) but also as aggregates of bacteria embedded in host material (e.g. sputum in the lungs of CF patients, wound tissue). Biofilm-associated micro-organisms are phenotypically very different from planktonic cells and one of the hallmarks of biofilm infections is their recalcitrance to conventional antimicrobial therapy. Many organisms encountered in respiratory tract infections in CF are capable of forming biofilms and it is clear that the biofilm phenotype plays a role in the difficulties associated with treating these infections. In this presentation I will present an overview of the mechanisms involved in biofilm-associated tolerance to antibiotics, with a focus on the role of metabolic adaptation. In addition, I will highlight the importance of using appropriate model systems to study biofilm biology in general and biofilm tolerance in particular. Finally, I will present a brief discussion on the value of standardized susceptibility testing for microbial biofilms in clinical practice.

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S6.4 Effects of short-term Lumacaftor-Ivacaftor therapy on lung microbiome in Phe508del homozygous patients with cystic fibrosis

Sébastien Boutin[1,2], Simon Gräber[3], Mark Wielpütz[4], Cornelia Joachim[5], Sabine Wege[6], Olaf Sommerburg[7], Mirjam Stahl[8], Alexander Dalpke[9], Marcus Mall[3]

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The interplay between cystic fibrosis (CF) airways microbiota and the disease severity and CFTR malfunction were demonstrated by several studies. The discovery of CFTR correcotr and potentiator was a breakthrough discovery but the influence of those therapy on the microbiome of the lung is still not fully explored. The aim of our study was to analysed the short-term effects of lumacaftor-ivacaftor on the lung microbiome in Phe508del homozygous patients

15 patients were included in this study sputum was use to sampled the lung microbiota at baseline and 8 to 16 weeks after initiation of therapy with the approved dose of lumacaftor 400 mg in combination with ivacaftor 250 mg every 12 h. Samples were aliquoted (200 µL) and treated with PMA to avoids subsequent PCR amplification of extracellular DNA. DNA extractions were performed using the QIAamp Mini Kit. Microbiome was explored by amplifying the the V4 region of the 16S rRNA gene (515F and 806R) and PCR amplicons were sequenced in a Illumina Miseq sequencing system with 250 cycles. In parallel, the number of 16S copies was quantified by quantitative PCR (qPCR). Sequences were analyzed with the R package dada2. Raw sequences were filtered and trimmed for good quality and merge as contigs. Ribosomal sequence variants (RSV) were assigned to taxonomy using the Silva database (version 132). RSV assigned to eukaryotes, archae and chloroplast were removed from the analysis.

Pseudomonas aeruginosa was present in five patients at baseline and no change was seen through therapy with lumacaftor-ivacaftor. Other common genera found in the cohort were Staphylococcus, Neisseria, Veillonella and Prevotella, but no significant changes in single generas were observed under therapy. Nevertheless, treatment with lumacaftor-ivacaftor significantly increased the alpha-diversity of the microbiome of Phe508del homozygous patients. This effect was mostly due to a significant increase in the richness after lumacaftor-ivacaftor therapy. Dominance as the relative abundance of the most dominant RSV and the overall bacterial burden showed tendencies of reduction under therapy.

Those results indicates that the combination of lumacaftor and ivacaftor in Phe508del homozygous patients influence the microbiome of the lungs. This influencerelate in an increase of the α-diversity not due to the decrease of the most abundant RSV (dominance) but mostly to an improved colonization by the rare biosphere. This rare biosphere is mostly constituted of anaerobes like Prevotella, Veillonella and aerobic commensals like Neisseria. This would indicate that the CFTR restoration allow less stringent selection pressure on the migrants from the upper airways by a modification of the ecological niche. It was previously shown in several studies that a more diverse microbiome in the lower airways was correlated with a better lung function and milder disease. The absence of decrease in the pathogen load would indicate that CFTR modulation alone do not allow eradication of the infection however the increase in the diversity would indicate that an early CFTR modulation might prevent or delay the first infection by conserving a diverse microbiota in the lower airways offering potential competitors against the gram-negative pathogens.

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S6.5 Metabolic reprogramming of cystic fibrosis macrophages by the IRE1a-XBP1 pathway leads to an exaggerated inflammatory response

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Introduction: Cystic fibrosis (CF) is a monogenic recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR). CFTR mutations cause dysregulation of membrane channel function with intracellular accumulation of misfolded proteins, particularly in DF508 mutations, leading to Endoplasmic Reticulum (ER) stress and activation of the IRE1a-XBP1 pathway. Activation of IRE1a leads to splicing of XBP1 (XBP1s), a transcription factor linked to production of proinflammatory cytokines and an important regulator of cellular metabolism. XBP1s is elevated in several CF immune cells with further implications in inflammation. Classically activated M1 macrophages are important initiators of the inflammatory response, and these proinflammatory cells exhibit high levels of glycolysis. There is evidence indicating that the IRE1a-XBP1 pathway regulates M1-M2 macrophage polarisation and controls mitochondrial activity and energy consumption in immune cells. We hypothesised that the exaggerated inflammatory response seen in CF, with high levels of XBP1s, can be associated with a metabolic dysfunction in M1 macrophages.

Aim: To investigate ER stress in specific immune cells from CF patients, the metabolic state of these cells and also to determine whether this is associated with a proinflammatory phenotype.

Methods: Peripheral blood mononuclear cells (PBMCs), isolated neutrophils, monocytes, lymphocytes, and human blood monocyte-derived macrophages (BMDM) from CF patients and healthy control (HC) volunteers were used to evaluate ER stress, using quantitative real-time PCR (qPCR). BMDM were differentiated for 7 days, in the presence of either GM-CSF or M-CSF, and then activated on the 6^{th} day with LPS and IFNg for M1 macrophages, or IL-4 and IL-13 for M2 macrophages, respectively. M1-M2 macrophage ratios were measured using flowcytometry. IL-6 and TNF levels were measured by qPCR and ELISA. The metabolic profiles of monocytes and M1 macrophages were analysed in real time by quantifying the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in the Seahorse XFe96 analyser. XBP1s was blocked using 4m8c and MKC-3946 inhibitors. Significant values ($P< 0.05$).

Results: Gene expression analysis revealed a significant increase in *BiP, IRE1a, XBP1s, ATF4, GADD34*, *IL-6* and *TNF* in PBMCs from CF patients. Further analyses revealed that only neutrophils and monocytes showed a significant increase of ER stress markers, more pronounced in monocytes. *BiP, IRE1a, ATF4,* and *CHOP expression* were significantly increased in neutrophils, while *IRE1a, ATF4, PERK, ERDJ4, CHOP, GADD34*, and *TNF* were all significantly increased in monocytes. The proportion of CF M2 macrophages was significantly lower; although, the proportion of CF M1 macrophages was unaffected, *IL-6* and *TNF* production were significantly higher, with significantly higher levels of *BiP, XBP1s, ERDJ4, TNF and IL-6.* Monocytes and M1 macrophages showed significantly higher levels of OCR and ECAR with an increased glycolytic reserve. Finally, inhibition of XBP1s, before macrophage polarisation, reduced the high levels of OCR and ECAR lowering the production of IL-6 and TNF in CF M1 macrophages.

Conclusions: These findings suggest that CF M1 macrophages undergo systematic metabolic reprogramming to cope with their increased energy demands. This hypermetabolic state is associated with high levels of XBP1s production, driving a proinflammatory phenotype in CF M1 macrophages.

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S6.6 SPLUNC1 peptidomimetics inhibit Orai1 to reduce pulmonary inflammation

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CF patients undergo cycles of chronic bacterial infection and inflammation that lead to persistent neutrophilia and bronchiectasis. Indeed, lung disease is the major cause of morbidity and mortality in CF patients. Therefore, one of the cornerstones of a comprehensive treatment plan is the use of anti-inflammatory drugs. However, beyond ibuprofen, there are currently no effective anti-inflammatory drugs to treat CF patients. Orai1 is a plasma membrane Ca^{2+} channel that contributes to the development of chronic inflammation by changing gene expression and stimulating cytokine secretion. The short palate lung and nasal epithelial clone 1 (SPLUNC1) is a highly abundant, multi-functional, secreted protein that plays a critical role in maintaining lung health. SPLUNC1 binds to and negatively regulates Orai1, thus inhibiting store operated Ca^{2+} entry[1]. Furthermore, SPLUNC1 knockout mice exhibit a hyperinflammatory phenotype[2]. While SPLUNC1 is present in airway secretions from healthy individuals, it is diminished in immunocompromised patients, such as those with CF[1,3]. We have identified the region of SPLUNC1 that binds to Orai1 and created a peptidomimetic of this region, called α6. Here, we have explored its ability to regulate Orai1. To determine if the α6 peptide could inhibit Orai1, HEK293T cells and CF primary human bronchial epithelial cells (HBECs) were cultured and thapsigargin-induced Ca^{2+} release ± SPLUNC1 or ± α6 was measured. The peptide inhibited Ca^{2+} influx in a similar fashion as SPLUNC1, with an IC_{50} of 920.1 nM. This inhibitory effect was replicated in multiple pulmonary cell types including neutrophils and T-cells. Since secretion of the chemoattractant IL-8 is Ca^{2+} dependent, α6 was then tested to determine whether it could prevent IL-8 secretion in CF HBECs. Supernatant of mucopurulent material (SMM) +/- α6 was added mucosally and serosal IL-8 secretion was measured. SMM significantly increased IL-8 secretion, however α6 reduced IL-8 secretion to baseline levels. Additionally, α6 retained efficacy in the proteolytic SMM environment, suggesting that α6 is anti-inflammatory and not immunosuppressive. In conclusion, the α6 peptide significantly inhibits Orai1 and reduces pulmonary inflammation, indicating that it may serve as a novel, inhaled anti-inflammatory peptide.

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2. Thaikoottathil, J.V., et al. *Am J Respir Cell Mol Biol* **47**, 253-260 (2012)
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30 March — 11:00–12:45

Model systems

S7.1 A single cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte

Lindsey W. Plasschaert [1,2], Rapolas Žilionis[3,4], Rayman Choo-Wing[1,2], Virginia Savova[3,5], Judith Knehr [6], Guglielmo Roma[6], Allon M. Klein [3], Aron B. Jaffe[1,2]

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The functions performed by epithelial tissues are determined by the types, abundance, and distribution of differentiated cells generated by stem/progenitor cells. The ability to restore tissue function requires an understanding of how physiological tasks are distributed among cell types, and how cell states vary between homeostasis and disease settings. This is well exemplified in the conducting airway epithelium where basal stem cells give rise to specialized lumenal cells that perform mucociliary clearance. While much is known about the mucous-producing goblet cells and ciliated cells, less is known about the specification and functions of rare cell types in the airway, which include pulmonary neuroendocrine cells (PNECs) and brush cells. We recently performed single-cell RNA-sequencing on thousands of human bronchial epithelial cells and mouse tracheal epithelial cells to generate an atlas all of the cell types in the conducting airway. Our analysis uncovered a novel, rare cell type, which we refer to as the 'pulmonary ionocyte' due to its enrichment of ion transporters including *CFTR*, the gene mutated in Cystic Fibrosis. Using a combination of immunofluorescence, signaling pathway modulation, and electrophysiology, we found that Notch signaling is required and *FOXI1* expression is sufficient to drive pulmonary ionocyte formation, and that ionocytes are a major source of CFTR activity in the conducting airway epithelium.

S7.2 Mechanisms of airway epithelium repair in cystic fibrosis

Marc Chanson[1], Alice Zoso[1], Marc Bacchetta[1], Sylvain Lemeille[2], Aderonke Sofoluwe[1]

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Pathological remodeling of the airway epithelium is commonly observed in CF. Thus, tissue repair is critical to restore integrity and maintenance of the epithelium barrier function. Epithelial repair is a multi-step process initiated by progenitor cell migration into the injured area, proliferation and re-differentiation to all cell types that polarize to reconstitute a normal airway epithelium. There is evidence that CFTR plays a role in airway epithelial cell (AEC) proliferation and migration, but the consequences of CFTR dysfunction on CF AEC repair are unknown.

I will summarize current knowledge on the mechanisms regulating AEC regeneration and repair gained from mouse models. To better understand dynamics of the re-differentiation process in the CF pathology, we performed RNA-Sequencing during the initial stages of a wound repair model in non-CF (8 donors) and CF (7 patients homozygous for F508del CFTR mutation) human AEC in primary cultures at the air-liquid interface. For transcriptomic analysis, we considered the differentially expressed genes ($p< 0.01$) with a ± 2 fold change threshold and a False Discovery Rate of 5% correction. Up- and down-regulated genes were extracted and used for Gene Ontology, Gene Set Enrichment and Leading Edge analyses. I will describe the outcome of the transcriptomic analysis, which revealed differences in important biological processes during wound repair of CF AECs. The most represented pathways include "cytoskeletal remodeling" and "cell cycle" categories with highlighted pathways like "cell adhesion", "extracellular matrix remodeling" and "signal transduction". Experiments were also carried out in the presence of flagellin (1µg/ml flagellin for 24h) to mimic *Pseudomonas aeruginosa* infection. Upon flagellin stimulation, comparisons between CF and non-CF samples indicated pathways related to "signal transduction" and "innate and adaptive immune response". The results suggest that CFTR dysfunction is associated with pathological regeneration and repair of the CF airway epithelium.

S7.3 Understanding altered airway mucins sialyation and decreased mucociliary transport in cystic fibrosis pig airways using single cell gene expression

Pascal Barbry

Vaincre la Mucoviscidose & Association Grégory Lemarchal.

Programme note: the details are not authorised for inclusion

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S7.4 Development of a steady-state lumen area screening assay to measure CFTR function in organoids of cystic fibrosis patients

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We have developed sensitive functional assays that utilize intestinal organoids of cystic fibrosis (CF) patients to measure (residual) CF transmembrane conductance regulator (CFTR) function and response to CFTR modulating therapies. CFTR function (restoration) using this preclinical model system can be determined by quantitation of luminal fluid secretion via measurement of (i) forskolin-induced swelling (FIS) or (ii) the forskolin-independent steady-state lumen area (SLA) of CF organoids. Importantly, the dynamic range of both assays varies in that FIS is more responsive for CFTR function measurements at low CFTR function, whereas at higher CFTR activity SLA is more responsive. The SLA assay has a reduced throughput when compared to the FIS assay, as organoid lumen segmentation has to be performed manually, whereas FIS can be quantified automatically.

Here, we developed our conventional SLA assay into a medium-to-high throughput screening format by using a fluorescent live cell imaging dye that labeled the lumen of organoids specifically, allowing for automatic lumen segmentation and calculation of the percentage of the lumen area of the total organoid area (SLA). We generated an optimized and efficient labeling protocol in intestinal organoid cultures and demonstrated that CFTR function, as measured by well-characterized FIS responses in reference organoids, was not negatively affected by the labeling procedure. CFTR function quantitation in organoids by this novel approach with varying CFTR activity (ranging from low to wild-type CFTR activity) was benchmarked to previously published (conventional) SLA measurements and corresponded well to this reference data set. In contrast to the conventional assay our new approach was able to measure lumens in CF organoids. Finally, to perform medium-to-high throughput SLA measurements we developed a CellProfiler and ImageJ analysis pipeline which allows for automatic image segmentation and removal and manual correction of incorrectly segmented objects. As a proof-of-concept we measured residual CFTR function in a small patient cohort of individuals with the R117H-CFTR mutation on at least one allele using our novel SLA assay and data analysis pipeline.

In conclusion, we successfully developed our conventional SLA assay into an automated medium-to-high throughput screening assay, which expands the toolbox of existing CFTR function measurements and will be especially useful to study CFTR function (restoration) in CF organoids with high (residual) CFTR activity by existing but also future (next-generation) CFTR modulators.

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S7.5 Human induced pluripotent stem cell derived p63 expressing epithelial cells provide a personalized model for CF lung epithelium

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Introduction: hiPSC can be used to develop organotypic cell culture models to study the pathophysiology of rare and common variants of Cystic Fibrosis, and the efficacy of experimental therapeutics. In contrast to committed stem/progenitor cells, hiPSC can be clonally modified by gene editing using TALEN and CRISPR/CAS9, and differentiated to multiple epithelial lineages. Moreover, this allows the generation of CFTR corrected lines from CF patients, which provides a valid wild type control in comparative analysis, and future potential for cell replacement therapy (Merkert S et al: Stem Cell Res. 2017, 23:95-97). Developing robust differentiation protocols that allow large scale air-liquid interface (ALI) culture of hiPSC derived airway cells is still challenging.

Approach: We have generated F508del CFTR, corrected, and N1303K CFTR hiPSC, a folding mutant prominent in the Mediterranean population, for which no effective correctors are available. Fluorescent markers monitoring epithelial differentiation: NKX2.1 (NKX2.1eGFP), the airway basal cell marker p63 (p63mVenusnuc NeoR), and CFTR (CFTR-dTomato) were introduced by TALENs in the respective gene loci. In F508del CFTR lines used for HT screening a YFP halide sensitive probe was introduced.

Methods: hiPSC were subjected to a 20 day differentiation and selection protocol, which reproducibly yielded a population of NKX2.1-GFP/p63-Venusnuc neoR expressing cells (~85%). After expansion of p63+ hiPSC in media used to differentiate primary airway basal cells in ALI culture (BEGM, Pneumacult), cells were transferred to standard ECM coated membrane inserts (Costar). At different intervals, cells were analyzed by QPCR, electrophysiology, confocal and electron microscopy.

Results: Depending on the ECM coating, p63+ iPSC on inserts reached variable TEER values within five days before transfer to ALI (500-2500 Ohm/cm2.). QPCR analysis of ALI cultured p63+ hiPSC revealed expression of general airway markers (NKX2.1, SOX2, FOXA2, SOX9, NGFR, CK5), ciliated cells (FOXJ1, CFTR, SCNN1A/ENACa) and secretory cell markers (CCSP, MUC5AC). Microscopy showed clusters of high cuboid epithelial cells expressing lateral (ECAD) and tight junctions (ZO-1), airway markers (KRT5, KRT8, KRT14, FOXJ1, P63). Multi-ciliated (TUBIV+) and secretory cells (CCSP, MUC5AC) were observed at high frequency, confirmed by electron microscopy.

Conclusions: We report efficient generation of hiPSC derived basal airway progenitor cells using a selectable p63 marker, which can be used to generate airway epithelia at air-liquid interface. hiPSC p63+ derived F508del airway cells will be used to validate novel F508CFTR activating compounds from a high throughput screen on iPSC derived CFTR expressing intestinal cells. In F508del CFTR mutant mice, and human primary bronchial epithelial cells in air liquid interface culture we observe oxidative stress and abberant bioactive lipid metabolism, resulting in enhanced pro-inflammatory and pro-fibrotic signaling. hiPSC derived airway cells can be used to study this in an isogenic model not affected by previous pathology, that can be modified by gene editing.

Support: ERARE INSTINCT, NIH R01HL126603, Dutch CF foundation NCFS (HIT-CF1).

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S7.6 Primary human nasal epithelial cell air/liquid interface cultures: an *in vitro* model system for assessing CFTR function

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Traditional methods for primary culture of human airway epithelial cells rarely allowed more than 1-2 passages before significant loss of epithelial function. Recently developed approaches including irradiated fibroblast feeder layers and inclusion of rho-associated protein kinase inhibitor enable >6 passages (10^5 to 10^6 fold expansion of cell number) with good retention of epithelial function. The "gold standard" for in vitro testing of CFTR correctors and potentiators is well-differentiated primary human bronchial epithelial cells obtained from lungs transplanted from CF patients. The number of different patient samples obtained in this manner is small and the range of CFTR mutations available for study is limited. The goal of this work is to establish methods for collection, culture, and expansion of primary human nasal epithelial cells, derived from non-CF volunteers and CF subjects to examine of inter-subject variation in CFTR expression, function, and response to CFTR modulator drugs. Nasal brushings were obtained from >20 healthy, non-CF volunteers and >100 CF subjects. Nasal cells were collected a second time (>2 months later) from a subset of the subjects. Samples were expanded (to passage 2) and cryopreserved. For analysis of CFTR function and expression, cells from multiple passages were seeded onto filter supports for establishment of air/liquid interface culture. The epithelial monolayers were transferred to Ussing chambers 15-60 days after seeding for measurements of CFTR-dependent Cl secretion. Viable epithelial cell cultures were established from nearly all samples and cultures from CF and non-CF subjects exhibited large amiloride-sensitive sodium current. Forskokin-stimulated and I^{172}-inhibited current was readily observed in all non-CF monolayers, but was minimal in CF monolayers. CFTR mRNA expression across non-CF subjects varied by ~10-fold and was correlated to CFTR Cl current. Cultures derived from CF subjects exhibited a broad range of responses to CFTR modulators among different CFTR genotypes and within subjects homozygous for F508del CFTR. The response to VX-809/VX-770 varied ~5X between least and most responsive cultures from F508del/F508del cells. There is good group-wise correlation between in vitro responses in cultured cells and the FEV1 response obtained from clinical trials data. We are currently working to assess the correlation between individual patient clinical response to Kalydeco, Orkambi, and Symdeko and the in vitro nasal epithelial cell culture responses to these modulators. From studies of primary human nasal epithelial cell cultures we have also identified two rare mutations (L145H and L138ins) that respond to VX-809 and VX-661. These studies support the use of primary human nasal epithelial cell cultures for testing CFTR modulators.

(Supported by NIH and the Cystic Fibrosis Foundation)

30 March — 14:15–16:10

Therapeutical approaches

S8.1 Novel developments in genetic therapies for cystic fibrosis

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Despite the recent development of small molecule therapeutics for cystic fibrosis (CF), there remain individuals with CFTR mutations for whom no treatments are available. Gene therapy offers an alternative approach and has been investigated in numerous trials by gene replacement approaches using viral or non-viral vectors although no clinically effective treatment has emerged so far. Most of the problems appear to be related to efficiency and duration of transgene delivery and expression. While new viral or non-viral vectors may improve efficacy of approaches to gene replacement therapy, a wide range of alternative nucleic acids therapies are under development, including gene editing, gene silencing and mRNA therapeutics, which may be more effective than "traditional" gene therapy approaches.

RNA-based therapeutics, siRNA and mRNA, are active in the cytoplasm of the epithelial cells, avoiding the need to cross the nuclear envelope which is a major barrier to DNA-based genetic therapies. RNA-based therapies do however require repeated delivery at regular intervals so non-immunogenic carriers are necessary. Gene editing approaches have the advantage that transient expression of Cas9 in the epithelial cells in the presence of guide RNA and template, donor DNA, can lead to gene correction with for long term efficacy for the life time of the correct cell, with CFTR expression regulated by endogenous transcription factors.

For all genetic therapy strategies, nucleic acid delivery remains a key challenge to their development. New viral vectors remain of interest but our research, focuses on delivery by non-viral nanoparticles which are particularly promising for RNA-based therapies. Many different nanoparticle formulations have been described but our nanoparticle formulation comprises a mixture of cationic, receptor-targeting peptides, lipids and nucleic acids, which self-assemble on mixing at precise ratios into nanoparticles of about 100 nm. The peptide contains an oligolysine domain for electrostatic self--assembly with nucleic acids and an epithelial receptor-binding ligand for targeted transfection of airway epithelial cells. The lipid helps to mediate endosomal escape, enhancing lung epithelial transfection but also can be used to modulate the surface properties of the nanoparticle, for example, to better penetrate mucus.

These lipid/peptide formulations were originally developed for delivery of pDNA and minicircle DNA, in vitro and in vivo, but have since been adapted successfully for delivery of siRNA, mRNA and CRISPR/Cas9 ribonucleoprotein (RNP) complexes, opening up a wide range of therapeutic opportunities for CF. We are developing these different nucleic acid therapies in transfections of air liquid interface cultures of primary human CF epithelial cells for functional analysis of therapeutic effects, and in normal mice for assessing in vivo delivery. I will report on our progress in developing these nanoparticles for delivery of RNA-based therapies for CF, delivering siRNA to silence the epithelial sodium channel (αENaC), delivery of CRISPR/Cas9 formulations for therapeutic gene editing of CF mutations, and mRNA delivery to replace the nucleic acid template for CFTR protein production.

S8.2 Update on CF gene therapy

Chris Boyd [1,2]

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Although a large number of cystic fibrosis (CF) gene therapy trials have been carried out over the last two decades, the question of whether CFTR gene transfer to the lungs can be made efficient enough to ameliorate CF lung disease remains an open one. The UK CF Gene Therapy Consortium has undertaken a programme of both non-viral and lentivirus-mediated gene therapy. Efficacy of the non-viral formulation pGM169/GL67A has been assessed in a double-blind, placebo-controlled multi-dose Phase IIb trial in which subjects received 12 doses of pGM169/GL67A or placebo at monthly intervals. The trial met its FEV_1-based primary endpoint and showed, for the first time, that non-viral gene transfer can stabilise CF lung disease. However, the response was comparatively modest and further improvements in gene transfer efficiency are required. To achieve this we have developed a novel lentiviral vector (rSIV.F/HN) which provides efficient (log orders better than pGM169/GL67A) and long-lasting expression (~ 2 years) in mouse airways after a single topical dose. We have recently partnered with Boehringer Ingelheim and Oxford BioMedica to progress towards a first-in-man lentivirus trial in CF.

S8.3 Co-potentiators as a novel therapeutic paradigm for CFTR mutations that are not responsive to available modulators

Peter M. Haggie, Puay-Wah Phuan , A.S. Verkman

University of California, San Francisco

Modulator therapies - such as KALYDECO and SYMDEKO - that rescue defective gating and / or cellular processing of G551D-, F508del-, and ~30 additional CFTR mutations have become a central paradigm in CF drug development. Clinical application of triple drug combinations incorporating dual corrector / potentiator combinations for heterozygous F508del- CF subjects, with the second allele encoding a residual function or minimal function CFTR mutation, is anticipated in the near future. Despite these tremendous advances, there remains a major unmet need for the development of therapeutic approaches for ~10 % of the CF community that will not be responsive to available therapies and / or late-stage clinical development candidates. We introduced the concept of potentiator combination (co-potentiator) therapy to rescue defective gating of the truncated protein product generated by the W1282X mutation in CFTR (J. Biol. Chem. 2017, 292:771-785). These studies demonstrated that a novel phenylsulfonamide-pyrrolopyridine potentiator (ASP-11) discovered by high-throughput screening, when used in combination with VX-770, fully normalized the gating of the W1282X truncated protein to wild type levels. The potentiator combination was ~8-fold more effective than either potentiator used individually, indicating a synergistic response that may be due to compound actions at distinct sites to modulate CFTR conformation. The co-potentiator paradigm was extended to the N1303K mutation, which is the 4th most common CF-causing mutation worldwide, with high prevalence (~5--20 %) in Northern Africa and Europe. In an FRT cell line expressing halide sensitive YFP and N1303K-CFTR, a panel of approved drugs and previously reported CFTR folding modulators (including butyrate, C3, C18, but not VX-661) produced N1303K-CFTR functional responses when assayed with forskolin and VX-770; however, relative responses of potentiators (including VX-770) was limited and novel potentiators identified by compound screening were not better than VX-770. To investigate whether co-potentiators may work in synergy to rescue defective N1303K-CFTR channel, we tested the previously identified ASP-11 with VX-770. Similar to W1282X-CFTR, co-potentiators (VX-770 / ASP-11) elevated N1303K-CFTR channel by ~8-fold relative to either compound used alone (J. Cystic Fibrosis. 2018, 17:595-606). Significantly, application of VX-770 / ASP-11 to nasal epithelial cells isolated from a homozygous N1303K CF subject revealed functional rescue that would be therapeutically relevant. In initial medicinal chemistry studies, synthetic routes were developed to derivatize the ASP scaffold and compounds with low micromolar efficacy were identified. Assessment of the efficacy of the co-potentiator paradigm for additional CFTR mutations that are not amenable to available modulators is currently on going. In addition, high throughput screening to identify novel co-potentiator scaffolds has been conducted. These studies support the development of potentiator combinations for CFTR mutants, such as N1303K, that are not adequately treated by single potentiators. A combination of two potentiators, with or without a corrector, may be efficacious for multiple CF-causing CFTR mutants with no available therapy.

Supported by National Institute of Health, the Cystic Fibrosis Foundation, Emily's Entourage and CFRI

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S8.4 Genetic repair of CFTR function in cystic fibrosis organoids using CRISPR/Cas9 adenine base editing

Eyleen de Poel[1,2], Maarten H. Geurts[3], Sylvia W.F. Suen[1,2], Jeffrey M. Beekman[1,2], Hans Clevers[3]

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The monogenetic and autosomal recessive characteristics of cystic fibrosis (CF) allows for the use of gene editing techniques to restore defective CFTR function at its root. This is particularly interesting for patients with severe mutations that cannot be rescued by current small molecule therapies.

Previously, we showed that the CRISPR/Cas9 system could be used to genetically restore CFTR function in F508del/F508del organoids. However, conventional CRISPR/Cas9-mediated homology directed repair (HDR) has several disadvantages, such as undesirable off-target effects and the generation of indels or translocations at the target site. The recently developed novel CRISPR/Cas9 base editing technology circumvents these issues as this technique induces a permanent conversion of one base pair to another at a locus without generating double-stranded DNA breaks and could therefore potentially be a great tool to repair severe (class I) *CFTR* mutations.

Here, we used for the first time the newest adenine base editor to irreversibly convert specific A-T to C-G in patient-derived organoids harboring the R758X/R758X CFTR genotype. We also used an xCas9-adenine base editor to restore CFTR function in F508del/R553X organoids, thereby demonstrating the possibility for heterozygous repair by base editors. Furthermore, we showed for the first time that xCas9-adenine base editor can be used to repair a disease-causing mutation on an orthogonal (non NGG) PAM (NGT). In addition to the conventional adenine base editors, a recently published optimized version was also used to test the hypothesis if increasing expression of the base editor due to expression and nuclear localization optimization enhances gene editing efficiency. Transfection of organoids with the base editors was performed by electroporation, according to previously published protocols, with the exception that organoids were grown on Wnt surrogate instead of Wnt-conditioned medium 1 week prior to and 4 weeks after electroporation. Functional rescue of CFTR was assessed using the forskolin-induced swelling assay in these cultures. Multiple gene-corrected organoids per genotype were generated and identified by an increase in organoid swelling upon forskolin stimulation for one hour. These individual organoids were picked and passaged multiple times until a clonal organoid culture was developed. We demonstrated by Western blotting the production of full length CFTR protein and confirmed by Sanger sequencing the adenine base editing event in the CFTR gene in these clones. To assess potential off-target base editing, whole genome sequencing will be performed in the near future.

Altogether, we demonstrated proof of concept for gene correction by the newest adenine base editors in intestinal organoids derived from CF patients showing the potential of correcting the CFTR defect at the root of the disease in primary human CF cells.

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S8.5 The pharmacology of novel TMEM16A potentiator compounds

Henry Danahay[1], Sarah Lilley[2], Holly Charlton[2], Roy Fox[2], Brian Button[3], Martin Gosling[1]

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TMEM16A was recently identified as a calcium-activated chloride conductance and a key orchestrator of anion secretion in the human airway epithelium (Caputo et al 2008; Schroeder et al 2008; Yang et al 2008). It is now clinically established that promoting anion secretion in the airway leads to enhanced mucus clearance and reduced exacerbation frequency in CF patients and as such TMEM16A represents an important target for the next generation of mucokinetics. Importantly, positive regulators of TMEM16A function will be expected to be of benefit in all CF patients, irrespective of their CFTR mutational status.

Using 4 parallel screening approaches, we identified several chemically diverse, low molecular weight compounds that potentiated TMEM16A function. These hit compounds were validated for TMEM16A function using a patch-clamp assay under conditions where $[Ca^{2+}]_i$ was tightly buffered at an EC_{20} for TMEM16A channel activity. This enabled hits that activated TMEM16A by non-specifically elevating $[Ca^{2+}]_i$ to be rapidly filtered out from the hit list.

The efficacy of *bona fide* TMEM16A potentiators translated through to function in ion transport studies in CF-HBE. Pre-treatment of CF-HBE with TMEM16A potentiators for between 5 min to 96h resulted in an enhancement of Ca^{2+}-mediated anion-secretory responses that were sensitive to the TMEM16A blocker, Ani9. Measurements of $[Ca^{2+}]_i$ confirmed that TMEM16A potentiators had no effect on calcium mobilization, consistent with a direct effect on the channel.

A Series 1 TMEM16A potentiator, ETX001, increased the secretion of airway surface liquid (ASL) in CF-HBE. The ETX001-driven increase in ASL height was further enhanced in cells that had been pre-treated with IL-13 to boost TMEM16A expression. A close structural analogue of ETX001, ETX002, that is inactive on TMEM16A, did not increase ASL height.

Together, these data support the concept that potentiators of the alternative airway chloride conductance, TMEM16A, can restore anion conductance and fluid secretion in both primary CF cells. Enterprise Therapeutics are advancing TMEM16A potentiators into clinical development.

Caputo et al (2008) Science 322(5901):590-594

Schroeder et al (2008) Cell 134(6):1019-1029

Yang et al (2008) Nature 455(7217):1210-1215

P33

S8.6 Identification of pharmacological modulators of the TMEM16A chloride channel by high-throughput screening

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TMEM16A (anoctamin-1) is a calcium-activated chloride channel expressed in airway epithelial cells, particularly under conditions of mucus hypersecretion. Activation of TMEM16A by calcium agonists results in enhanced chloride and bicarbonate secretion. The physiological role of TMEM16A, which is upregulated under conditions that favor mucus hypersecretion, and its involvement in cystic fibrosis (CF) are still unclear. Our aim is to identify novel pharmacological modulators of TMEM16A function. Using the halide-sensitive yellow fluorescent protein (HS-YFP) functional assay, we screened a maximally-diverse chemical library (11,300 compounds). For this purpose, we used FRT cells co-expressing TMEM16A (abc isoform) and HS-YFP. Cells were stimulated with a low concentration of UTP to induce partial activation of TMEM16A. TMEM16A potentiators and inhibitors are detected as compounds that accelerate or slow down the rate of HS-YFP quenching, respectively. Controls during the screening included the Ani9 inhibitor (Seo et al., PLoS One 11:e0155771, 2016) and a TMEM16A potentiator that we identified in a previous study. After screening the whole library in duplicate, we found 24 compounds that increase TMEM16A activity above a threshold equal to 50% of the positive control. These hits are evaluated in secondary assays based on: i) null FRT cells, to rule out compounds acting on other channels/transporters; ii) CFPAC-1 cells (which have endogenous TMEM16A expression), to confirm activity in a second cell type; iii) stimulation of TMEM16A with ionomycin, to bypass purinergic receptors; iv) evaluation of intracellular calcium mobilization with a fluorescent probe, to detect compounds with an indirect mechanism of action. Compounds with the best characteristics will be tested in CF human bronchial epithelial cells (short-circuit current experiments) to assess the effect on calcium-dependent chloride secretion. The screening also identified possible inhibitors of TMEM16A. Novel pharmacological modulators of TMEM16A (potentiators and inhibitors) could be useful as tools of research and as possible therapeutic agents to improve mucociliary function in CF and other respiratory diseases.

This work is supported by CFF (grant GALIET17G0).

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S8.7 CFTR super exon partially corrects W1282X-CFTR

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Gene editing and gene therapy are promising therapeutic approaches for cystic fibrosis (CF). Strategies to correct individual CFTR variants are not feasible for most CF-causing genetic variants as more than 1,700 genetic variants of the CFTR gene have been suggested to cause CF. Insertion of a partial cDNA (also known as a super exon) into the native CFTR genomic locus can restore the CFTR gene to a wt coding sequence for all mutations downstream of the insertion site while retaining the endogenous CFTR promoter. However, the functional consequences of super exon insertion into CFTR are largely unexplored. Large fragment targeted insertion through gene editing, such as the introduction of a super exon, remains a technical challenge because it conventionally requires the low efficiency homology directed repair (HDR) pathway. As an alternative, homology independent targeted integration (HITI) is a recently developed technique for large fragment targeted insertion that bypasses the HDR pathway.

To explore the viability of the approach and functional consequences of partial cDNA insertion into CFTR, we employed both conventional HDR and HITI approaches for insertion of a small super exon into CFTR in gene edited 16HBE14o- cells expressing W1282X CFTR (cell line CFF-16HBEge CFTR W1282X). Previously, exonic sequences have been targeted for CFTR super exon insertion; however, we targeted intron 22 with CRISPR/Cas9 to avoid unproductive editing events (indels) in the CFTR-coding sequence. The inserted large fragment contains the ~250 distal nucleotides of intron 22 (to restore the natural splice donor of exon 23), a super exon coding for the native CFTR exons 23-27, and a bovine growth hormone (BGH) polyA sequence. Both the conventional HDR and HITI approaches yielded several clonal cell lines containing the desired genomic super exon sequence, which is predicted to yield a functional CFTR. As expected, the native genomic CFTR sequence distal to the intron 22 insertion site, including the W1282X mutation, was retained downstream of the inserted super exon and BGH polyA sequence.

Cells expressing the CFTR super exon allele no longer expressed truncated CFTR protein characteristic of the W1282X variant. Instead, Western blots revealed full length CFTR protein. Consistent with the Western analysis, we observed partially rescued CFTR function (~5-10% of WT function) in electrophysiological assays. Unexpectedly, the CFTR mRNA expressed from the super exon allele was subject to nonsense-mediated mRNA decay (NMD), very similar to the native W1282X-CFTR mRNA, as demonstrated by reduced mRNA levels that could be increased 5-8 fold with the NMD-inhibitor SMG1i.

In summary, we have demonstrated successful large fragment targeted insertion of a CFTR super exon through two editing strategies, resulting in partial functional rescue of CFTR. However, these studies also highlight the need for additional characterization to fully elucidate the functional consequences of super exons as a gene editing strategy for CF. Work is ongoing to further assess the impact and processing of super exon alleles, e.g., for different CFTR mutations and other insertion sites.

30 March — 16:45–17:45

How to get published – a perspective from JCF

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Moderators: Cliff Taggart (UK) - David Spencer (UK)

Cliff Taggart, JCF Editor and David Spencer, JCF publisher, will give an overview of the Journal of Cystic Fibrosis including how to publish basic science findings and what the editors and reviewers look for when reviewing manuscripts submitted to the Journal.

FP1.1 Esomeprazole increases airway surface liquid pH in primary cystic fibrosis epithelial cells

Livia Delpiano[1], Joseph J. Thomas[1], Annabel R. Yates[1], Sarah J. Rice[2], Michael A. Gray[1], Vinciane Saint-Criq[1]

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Respiratory failure, driven by airways mucus obstruction, chronic inflammation and bacterial infections, is the main cause of mortality and morbidity in people with cystic fibrosis (CF) due to defects in the Cl- and HCO−3 transport activity of the CF Transmembrane conductance Regulator (CFTR). Most recent pre-clinical and clinical studies have focused on restoring CFTR function by enhancing its trafficking or transport activity and show promising results. However, there are a significant number of patients that will not benefit from these CFTR-targeted therapies and it is therefore important to identify new non-CFTR targets that will restore lung function, by-passing CFTR dysfunction. The H+/K+-ATPase, ATP12A, has recently been identified as a potential novel target for CF therapies, since its acute inhibition by ouabain was shown to help restore mucus viscosity, mucociliary transport, and antimicrobial activity using in vitro CF airway models, and this effect was linked to an increase in the pH of the airway surface liquid (ASL). Here, we have evaluated the potential therapeutic use of ouabain by investigating the effect of chronically treating fully differentiated CF primary human airway epithelial cells (hAECs) with ouabain, under thin film conditions, resembling the in vivo situation. Our results show that although chronic treatment increased ASL pH, this correlated with a deleterious effect on epithelial integrity as assessed by LDH release, transepithelial electrical resistance, fluorescein flux, and ion transport. Since ATP12A shares approximately 65% identity with the gastric H+/K+-ATPase (ATP4A), we investigated the potential of using clinically approved ATP4A proton pump inhibitors (PPIs) for their ability to restore ASL pH in CF hAECs. We show that, despite not expressing ATP4A transcripts, acute exposure to the PPI esomeprezole, produced changes in intracellular pH that were consistent with the inhibition of H+ secretion, but this response was independent of ATP12A. More importantly, chronic exposure of CF hAECs to esomeprazole alkalinized the ASL without disrupting the epithelial barrier integrity, but this increase in ASL pH was consistent with a decrease in mRNA expression of ATP12A. We conclude that PPIs may offer a new approach to restore ASL pH in CF airways, which is independent of CFTR.

Full Reference of the Paper:

Esomeprazole Increases Airway Surface Liquid pH in Primary Cystic Fibrosis Epithelial Cells. Delpiano L, Thomas JJ, Yates AR, Rice SJ, Gray MA, Saint-Criq V. Front Pharmacol. 2018 Dec 11;9:1462. doi: 10.3389/fphar.2018.01462. eCollection 2018. PMID: 30618754

FP1.2 *Staphylococcus aureus* impacts *Pseudomonas aeruginosa* chronic respiratory disease in murine models.

C. Cigana[1], I. Bianconi[1], R. Baldan[2], M. De Simone[1], C. Riva[1], B. Sipione[1], G. Rossi[3], D.M. Cirillo[2], A. Bragonzi[1]

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Background: Staphylococcus aureus and Pseudomonas aeruginosa are key bacterial pathogens of the respiratory tract in patients with cystic fibrosis (CF). Although P. aeruginosa chronic bronchial infection is associated with a poorer prognosis, the consequences of S. aureus colonization on CF outcomes are controversial.

Methods: In this paper, murine models of infection resembling traits of the CF human airways disease have been revisited using an infection schedule that mimics the sequence of events of pulmonary disease in CF patients. First, mice were infected with S. aureus, embedded in agar beads; this was followed by P. aeruginosa infection and analysis of bacterial load, leukocyte infiltration, and lung tissue damage.

Results: We reveal that (1) S. aureus promotes severe lesions including abscess formation, (2) S. aureus increases the risk of subsequent chronic P. aeruginosa respiratory infection, and (3) once the chronic infection has been established, P. aeruginosa influences most of the inflammatory responses independent of S. aureus.

Conclusions: Our findings established the significance of S. aureus colonization per se and the impact on the subsequent P. aeruginosa infection. This would point towards a thorough assessment for the need of treatment against S. aureus.

Full Reference of the Paper:

Cigana C, Bianconi I, Baldan R, De Simone M, Riva C, Sipione B, Rossi G, Cirillo DM, Bragonzi A. Staphylococcus aureus Impacts Pseudomonas aeruginosa Chronic Respiratory Disease in Murine Models. J Infect Dis. 2018 Mar 5;217(6):933-942. doi: 10.1093/infdis/jix621. PubMed PMID: 29216403.

FP1.3 Targeting of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein with a technetium-99m imaging probe

Vera F. C. Ferreira[1], Bruno L. Oliveira[1,2], João D. Santos[3], João D. G. Correia[1], Carlos M. Farinha[3], Filipa Mendes[1]

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Cystic fibrosis (CF) is caused by mutations in the gene that encodes the CF transmembrane conductance regulator (CFTR) protein. The most common mutation, F508del, leads to almost total absence of CFTR at the plasma membrane, a defect potentially corrected via drug-based therapies. Herein, we report the first proof-of-principle study of a noninvasive imaging probe able to detect CFTR at the plasma membrane. We radiolabeled the CFTR inhibitor, CFTRinh-172a, with technetium-99m via a pyrazolyl-diamine chelating unit, yielding a novel 99mTc(CO)3 complex. A non-radioactive surrogate showed that the structural modifications introduced in the inhibitor did not affect its activity. The radioactive complex was able to detect

plasma membrane CFTR, shown by its significantly higher uptake in wild-type versus mutated cells. Furthermore, assessment of F508del CFTR pharmacological correction in human cells using the radioactive complex revealed differences in corrector versus control uptake, recapitulating the biochemical correction observed for the protein.

Full Reference of the Paper:

ChemMedChem. 2018 Jul 18;13(14):1469-1478. doi: 10.1002/cmdc.201800187. Epub 2018 Jun 12.

P85

FP1.4 Lack of cystic fibrosis transmembrane conductance regulator causes low cortical bone thickness and high cortical porosity in newborn pigs

Frédéric Velard[1], Julien Braux[1], Marie-Laure Jourdain[1], Ignacio Caballero-Posadas[2], Nathalie Winter[2], Mustapha Si-Tahar[3], Nikolai Klymiuk[4], Andrea Baehr[4], Sophie C. Gangloff[1], Jacky Jacquot[1]

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Background: People with cystic fibrosis exhibit growth defects and brittle bones. This observation has been attributed, in part, to malnutrition and chronic pulmonary inflammation. We tested the hypothesis that disruption of the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene directly affects bone microarchitecture and integrity by studying bone of newborn $Cftr^{-/-}$ pigs.

Methods: We examined femoral cortical and trabecular bones of $Cftr^{-/-}$ pigs less than 24h after birth using micro computed tomography (m CT, Skyscan 1076, Bruker). Scans were performed with the following settings: tube voltage, 80 kV; tube current, 0.125 mA; and voxel size, 17 x 17 x 17 mm (x, y, z). Three-dimensional images were rebuilt and analysed using the NRecon GPU version and CTAn (Bruker) software programs, respectively. The cortical bone porosity and structure were defined using a 3.4 mm wide region centered on the middle of the femur. A total of 37 newborn $Cftr^{-/-}$ piglets (24 males and 13 females) and 18 newborn $Cftr^{+/+}$ piglets (8 males and 10 females) was subjected to mCT scan.

Results: Compared to newborn $Cftr^{+/+}$ pig controls, $Cftr^{-/-}$ femoral bone exhibited significantly lower total volume (TV), bone volume (BV) and bone volume density (BV/TV) but only in females. However, the $Cftr^{-/-}$ bone mineral density (BMD) in trabecular and cortical tissues was significantly reduced in both sexes, compared to $Cftr^{+/+}$ piglets. Interestingly, focusing at the porosity of cortical bone in $Cftr^{-/-}$ pigs as a determinant of bone fragility associated with high fracture risk, we observed higher closed porosity with a marked increase of closed pore surface in cortical bone of $Cftr^{-/-}$ pigs (+18,7% for males and +48% for females). These results suggest a lower bone remodelling, lower interconnectivity within the vascular network, and increased bone fragility in $Cftr^{-/-}$ animals. No significant difference was observed in the open cortical porosity, whatever the gender.

Conclusion: Altogether, these data highlight the critical regulatory role of CFTR in bone development and maintenance, and suggest that some bone defects in people with cystic fibrosis are likely primary.

This work was, in part, supported by the Association French Vaincre la Mucoviscidose.

P86

FP1.5 Pancreatic ductal fluid and bicarbonate secretion of the ferret and pig models of cystic fibrosis (CF)

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Cystic fibrosis (CF) is a lethal genetic disease affecting several organs, including the pancreas. There are several animal models are available to study the CF related pancreatic tissue damage although they have limitations (e.g. the mouse model). Recently a cystic fibrosis transmembrane regulator (CFTR) knockout ferret and pig model have been generated.

Our aim was to investigate the fluid and bicarbonate secretion in the CF and wild type (WT) ferret and pig pancreatic ducts.

Intra/interlobular pancreatic ducts were isolated from newborn CF and WT ferret and pig pancreata. Expression of CFTR (cystic fibrosis transmembrane regulator) was detected by immunohistochemistry.Resting pH, buffer capacity and Cl^-/HCO_3^-exchange activity were evaluated by microfluorometry. Intracellular Cl^- efflux was measured by the Cl^- indicator MQAE ((*N*-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide). To inhibit the CFTR activity we used the CFTR inhibitior c2992-172 (Sigma) in 10 µM concentration in Cl^- free HCO_3^- solution. CFTR-dependent chloride efflux was calculated as the difference in alterations of Cl^- free HCO_3^- solution fluorescence in the absence and presence of CFTR selective inhibitor CFTRinh-172. The rate of chloride efflux induced by HCO_3^- after substitution of chloride by nitrate in the perfusion medium was measured by the change in fluorescence of MQAE. Fluid secretion of ducts were examined by videomicroscopy.

Resting intracellular pH of pancreatic ductal epithelial cells was 7.17±0.08 in ferrets. Functionally active sodium/hydrogen exchangers and sodium/bicarbonate cotransporters were detected in WT pancreatic ducts in ferrets. Intracellular Cl^- efflux measurements revealed a strong Cl^- efflux in pancreatic ducts isolated from WT ferrets ($\Delta F/F_0$=1.5±0.03) and pigs ($\Delta F/F_0$=1.6±0.04). Notably, bicarbonate secretion was significantly decreased in CF ferret ducts compared to WT ($p< 0.04$) Fluid secretion measurements revealed a significant increase in fluid secretion to HCO_3^- and 5µM forskolin and 100 µM IBMX in both WT pig and WT ferret ducts. In CF ferret and pig ducts increase of the fluid secretion were not detected during the stimulation period with 5µM forskolin and 100 µM IBMX, while in WT ferret and pig ducts stimulation of the fluid secretion occurred.

Absence of the CFTR leads to decreased or completely abolished pancreatic ductal fluid secretion both in ferrets and pigs. Pancreatic ductal bicarbonate secretion is also significantly decreased in ferrets.

30 March — 18:00–19:00

Closing Key Note Lecture

Organoids for cystic fibrosis research

Jeffrey Beekman

Regenerative Medicine Utrecht, and Pediatric Pulmonology, University Medical Center Utrecht, The Netherlands

In vitro patient-derived cell cultures play a critical role to understand CF disease and treatment thereof. Recent culture technologies facilitate the in vitro expansion of human somatic or adult stem cells, and their differentiation into 3D structures called organoids. These organoids retain characteristics of the in vivo tissue and provide new opportunities for drug development and personalized medicine. This presentation will focus on the development of CFTR-dependent phenotypic screening assays in intestinal organoids, and the relations between in vitro observations and in vivo disease and CFTR modulator response. Furthermore, I will summarize published and ongoing studies on new 3D model systems from various organ systems and approaches for improved pharmacological and genetic repair of CFTR or fluid secretion. The impact from these studies are being discussed in the context of a rapidly changing therapeutic landscape of CF.

P1

Identification of *CFTR cis*-regulatory variants

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The *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR*) gene was identified in 1989. Although, more than 2000 mutations have been discovered, some patients with cystic fibrosis or *CFTR*-related disorders (*CFTR*-RD) have incomplete genotypes or present extreme phenotypes. Regulatory elements described in the promoter, cannot alone explain the complex tissue-specific regulation of this gene. In recent years, the development of chromatin conformation study techniques has identified several long-range regulatory elements as involved in this control expression (Moisan et al., 2016). The objective of this project is to study the involvement of '*cis*-ruption', that is the dysfunction of a *cis* -regulatory element, in cystic fibrosis (CF) and CFTR-RD (*Congenital Bilateral Absence of Vas Deferens,* CBAVD).

17 *cis*-regulatory regions of *CFTR* gene were sequenced by a hight-throughput approach (Fluidigm) in these 112 patients. The frequency of identified variants in our patient's groups was compared European population, with GnomAD database.

In a homogeneous group of 25 CBAVD patients carrying only one F508del mutation, 8 variants display a frequency significantly different in this group compared to European population. In particularly, one variant is 40 times more frequent in CBAVD patients carrying a F508del mutation than in European population. This variant is located in *cis* -regulatory region of intron 21, and especially, in an important transcription factor binding site, EP300. EP300 is essential for gene's transcription. Enhancer tests are realised to measure the effect of the intron 21 region on the activity of *CFTR* promoter in intestinal (Caco-2) and airway (16HBE14o-) cells. The intron 21 region alone has not effect on the activity of *CFTR* gene promoter in intestinal and airway cells. However, by combining the enhancer of intron 21 and the enhancer of intron 11 (strong enhancer described in intestinal cells), a strong cooperative effect is observed on the *CFTR* promoter activity in intestinal cells. These two enhancers have common transcription factor binding sites. In particular, binding sites of the EP300, TCF12, REST and CEBPB (UCSC). However, the EP300 regulatory element interacts with TCF12 and CEBPB. This could explain the cooperative effect of these *cis*-regulatory regions of intron 11 and 21 on the *CFTR* promoter activity. Actually, a sequencing of a control cohort (167 fathers carrying one F508del mutation) is underway.

Enhancer tests with the insertion of the variant of interest in the combination of intron 11 and 21 enhancers are in progress to determine the impact of the variant on the *CFTR* promoter activity. Also, we will study the binding of transcription factor by *Chromatin Immunoprecipitation*, as well as the chromatin organization by *Chromosome Conformation Capture Carbon Copy*.

P2

Comparison of three genome editing techniques to correct the common W1282X mutation responsible for cystic fibrosis

Karen Mention[1], Kader Cavusoglu-Doran[1], Lucia Santos[1,2], David Sanz[1], Martina Scallan[3], Patrick Harrison[1]

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Ninety percent of the CF-causing mutations cannot be treated with clinically available drugs. W1282X is one of the most common non-druggable variants with a prevalence of 1.2% (Cftr.org). This class I mutation is caused by the change of a G to an A in the 3846 position of the cDNA, and creates a premature TGA stop codon, making cells unable to synthesize a full-length protein, resulting in virtually no CFTR protein available at the cell surface. Whilst many groups have reported the correction of F508del mutation by genome editing technology, there are no published reports on the correction of the W1282X mutation. In this study, we present a comparison of three different techniques to correct the W1282X mutation in HBE W1282X mutant cell lines (1).We compared Homologous directed repair (HDR) approach using two different CRISPR proteins, SpCas9 and AsCas12a (Cpf1) in a RiboNucleoProtein (RNP)/guideRNA complex format. The donor template was a single strand oligonucleotide of the non-target sequence, containing the corrected mutation flanked by asymmetric homology arms. In both cases, we observed ~20% precise editing by HDR, but we also found a significant level of indels caused by NHEJ repair. Given the high level of NHEJ relative to HDR, and the recent observation that double-stranded breaks can lead to unwanted chromosomal rearrangements (2), we have also tested Cas9-adenine base-editing (ABE) to correct this mutation. ABE can convert an A:T to G:C base pair with high efficiency without the formation of a DSB (3). We have recently confirmed that this enzyme shows high levels of precise editing, with very low levels of indels. However, a current limitation of this technique is that the target residue must lie in a window of 4nt to 7nt region on the same DNA strand as the 5'-NGG-3' protospacer adjacent motif (PAM). There is only one PAM sequence close to the W1282 mutation site, but the targeted A would be outside the base-editing window, and we have been unable to detect ABE using the wild-type Cas9-ABE7.10. However, the mutation is potentially amenable to editing with the recently described xCas9-ABE, which has less constraints on the PAM sequence (4). If it is possible to correct W1282X efficiently with xCas9-ABE, this may be the most suitable for preclinical development. Indeed, a recent study using HDR demonstrates that off-target sites editing using Cas9-ABE is 20-fold lower than for Cas9/gRNA (5).

This project has been possible thanks to research grants from the CF Trust and the CF Foundation.

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P3

Epistatic effect of complex alleles in cystic fibrosis phenotype – the protein translation view

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Marked phenotypic heterogeneity exists among patients with cystic fibrosis, despite identical CFTR genotype. This suggests that beyond primary disease-causing mutations, other factors – such as inter- or intragenic variation – must play a role during clinical course and/or individual response to small molecule therapeutics. Using unbiased, genome-wide approaches, we identified a common synonymous single nucleotide polymorphism (sSNP) in CFTR, T2562G, and determined its effect on cellular phenotype. Although the T2562G sSNP by itself does not result in CF pathophysiology, our biochemical studies show that this polymorphism alters CFTR folding, channel activity, and pharmacologic rescue. When T2562G is present *in cis* with other disease-associated variants, the mutation markedly influences CFTR by slowing translation kinetics. T2562G epistasis is heavily dependent on the position of the CF-causing mutation in full-length CFTR, but also on the type of disease-associated codon substitution. These results indicate an entirely new contributor to both molecular phenotype and modulator drug responsiveness in cystic fibrosis. Moreover, such epistatic interactions may have greater impact on CF disease severity and efficacy of pharmacologic intervention than previously considered.

P4

Variation Spatial Profiling (VSP): the evolving genome driving cystic fibrosis

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Cystic fibrosis (CF) is a familial autosomal recessive disease, caused by a hierarchical combination of 1) genotypic diversity in the CF transmembrane conductance regulator (CFTR), 2) diversity in response to its many unique cellular and subcellular environments, and 3) diversity in response to CF patient lifestyle, age and clinical management. A search for a universal platform to understand and manage misfolding disease led to the development and implementation of **V**ariation **S**patial **P**rofiling (VSP) (Wang and Balch, Cell Reports, 2018, Bridging Genomics to Phenomics at Atomic Resolution Using VSP, **24**: 2013) (doi: **10.1016/j.celrep.2018.07.059**). VSP is a Gaussian Process (GP)-based machine learning (ML) language base that applies the new biological principle of **S**patial **C**o**V**ariance (SCV) as a unifying platform to globally define the role of patient specific genotypes, their phenotype(s) and their responses to therapeutics. VSP generates a genomic platform that helps us to understand sequence-to-function-to-structure relationships spanning the entire CFTR fold. Given the complexity of CF disease leading to variable clinical presentation and therapeutic responses even among identical genotypes, we have applied VSP/SCV to address how CF pathophysiology can be rebalanced to mitigate variation specific misfolding challenges. VSP demonstrates that current therapeutics differentially alter the range and plasticity of specific clusters of CFTR residues to adjust SCV tolerance of the fold to initiate trafficking and or restore loss-of-function at the surface as a tissue dependent response. The VSP platform provides insight into unanticipated SCV relationships found in the CF population that can be used to develop a precision approach for clinical management of the patient.

P5

Increased SLC6A14 expression shows more severe cystic fibrosis phenotypes with tissue- and sex-specific contributions

Mohsen Esmaeili[1], Gengming He[1], Naim Panjwani[1], Fan Wang[1], Benjamin Dekoven[1], Katherine Keenan[1], Julie Avolio[1], Fan Lin[1], Lei Sun[2,3], Johanna M Rommens[1,4], Lisa J Strug[1,2]

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Individuals with cystic fibrosis (CF) have variable disease severity across their affected organs that is contributed to by genetic background beyond the causal *CFTR* mutations. Analysis by the International CF Gene Modifier Consortium indicate that CF lung disease and meconium ileus susceptibility are influenced by adjacent loci, separated by 200kb, on chromosome X between *SLC6A14*, an amino acid transporter, and *AGTR2*, the type 2 angiotensin receptor. We sought to understand how the associated SNPs from both lung disease and meconium ileus susceptibility (designated as the 'far' and 'near' upstream *SLC6A14* regions, respectively) contribute to CF phenotypes using ex vivo transcriptome, public data and in vitro transcriptional reporter studies. None of the associated SNPs occur in coding regions.

First we used RNA sequencing analyses of human primary bronchiole and nasal cells to investigate whether nasal cells provide a suitable airway model to study modifier genes in CF. In 13-paired samples we observed strong correlation of gene expression patterns between the two models, particularly for apical membrane component genes that were considered relevant epithelial factors (93/116 showed FDR< 0.1, including *CFTR*). Notably, *SLC6A14* pairwise expression patterns with apical constituents across the two models were highly correlated ($p=2.4x10^{-5}$); *AGTR2* showed no expression in either cell type. Having established the nasal cells as an airway model for genetic modification studies in CF, we demonstrated that the lung function-associated SNPs in the far upstream region aligned with nasal cell *SLC6A14* transcript levels from 70 Canadian CF participants, supporting that increased expression was detrimental to lung function, with similar effect in males and females.

Although we were restricted to adult stage data, we integrated the meconium ileus associated SNPs from the near upstream region of *SLC6A14* with expression quantitative trait loci (eQTL) of gastrointestinal organ tissues from the Genotype Tissue Expression (GTEx) consortium. Interestingly, the data support that the pancreas ($p=1.12x10^{-10}$), in contrast to the intestine, may be the source of the meconium ileus susceptibility, and provided evidence of a sex difference with male bias. As this near upstream region encompasses cis promoter elements including a steroid receptor and other common transcription factor binding sites, we used transient transfection and luciferase gene reporter assays with a 2.4 kb segment containing the transcription start site of *SLC6A14* and the strongest associated SNPs for meconium susceptibility in the CFPAC-1 cell line. Increased gene expression is observed with the risk haplotypes consistent with the direction of expression effects observed in the GTEx data. It was further evident that the steroid receptor site annotated in the promoter segment was functional in CFPAC-1 cells upon addition of receptor and steroid hormone. Analyses of other lines, including those from lung, are continuing.

Integration of GWAS and cross-tissue gene expression data implicate gene regulation of *SLC6A14* as being responsible for disease modification, with increased expression being detrimental to CF phenotypes. Underlying issues of sex influence and tissue source (and likely development stage) contribute critical elements that need further delineation.

P6

Recruitment to cystic fibrosis airway fluid licenses transcription and subsequent acquisition of the pathological GRIM fate by human neutrophils

Camilla Margaroli[1], Dalia Arafat-Gulick[2], Fangxu Sun[2], Haydn Kissick[3], Ronghu Wu[2], Greg Gibson[2], Rabindra Tirouvanziam[1]

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Rationale: Blood neutrophils acquire a pathological fate upon recruitment to CF airway fluid, leading to reduced bacterial killing despite active granule release, immunomodulatory, and metabolic activities (aka, “GRIM” fate). Our group identified this pathological fate in adults [Tirouvanziam et al. PNAS, 2008], and recently in infants [Margaroli et al., AJRCCM, 2018] with CF. Because of their visibly hypercondensed chromatin, blood neutrophils are thought as transcriptionally silent. In contrast with this conventional view, we hypothesized that the profound changes in neutrophils leading to the GRIM fate, following recruitment to the CF airways, were dependent upon transcriptional licensing.

Methods: We used an in vitro model that recapitulates neutrophil recruitment into CF airway fluid and GRIM fate acquisition [Forrest et al., JLB, 2018], to mass-produce GRIM neutrophils and conduct parallel transcriptomic profiling by RNASeq and proteomic profiling by mass spectrometry.

Results: Recruitment into CF airway fluid leads to rapid transcriptional licensing in neutrophils, with increase in total RNA content and major shifts in mRNA and proteins. In particular, computational analysis revealed the expression of immunomodulatory activities reminiscent of myeloid-derived suppressor cells, and metabolic properties consistent with enhanced survival of the GRIM neutrophils. Interestingly, granule enzymes were not resynthesized by GRIM neutrophils. As expected, recruitment of blood neutrophils toward the chemoattractant control leukotriene B4 did not lead to acquisition of the GRIM fate, but induced transcripts related to phagolysosomal acidification, and led to efficient bacterial killing. Finally, blockade of transcription by the RNA polymerase II inhibitor alpha-amanitin early after recruitment of neutrophils into CF airway fluid blocked GRIM phenotype acquisition and restored bacterial killing capacity.

Conclusions: Neutrophils are transcriptionally licensed upon recruitment to CF airway fluid. This transcriptional licensing in turn causes functional changes leading to the GRIM fate, including active granule release and inhibition of bacterial killing. RNA-targeted therapy can be envisioned to modulate the fate and function of neutrophils and potentially ameliorate inflammation and control of infection in children and adults with CF in whom GRIM neutrophils are a dominant presence in the airways.

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P7

Implication of miR-199a-3p in bronchic inflammation in cystic fibrosis patients

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INTRODUCTION: In patients with cystic fibrosis (CF), at the pulmonary level, the ionic imbalance caused by CFTR chloride channel dysfunction leading to a thickening mucus in the airways. The resulting hyperviscosity promotes bacterial colonization and the establishment of infection/inflammation cycles which in the long term degrade the pulmonary epithelium and consequently reduce the respiratory function of CF patients. From a molecular point of view, it has been shown that the NF-κB signaling pathway is hyperactivated in this context, however its origin is not yet known. In other pathologies, it has been shown that this pathway can be regulated by non-coding RNA as microRNA (miR).

OBJECTIVE: To study the involvement of miR in pulmonary inflammation observed in CF patients.

METHODS: Primary CF and non-CF human bronchial epithelial cells cultured in air-liquid interface were used to perform a RNAseq and a small RNAseq. The functional study that followed was performed on bronchial epithelial cells line from CF patients (CFBE41o-). These cells were transfected with miR-199a-3p mimic in order to increase its expression and stimulated by pro-inflammatory cytokines, calcium modulators and by Pseudomonas aeruginosa (Pa).

RESULTS: Using RNAseq, we showed that 50% of deregulated mRNA are linked to the NF-κB signaling pathway in CF patients. By a small RNAseq on the same cells, an analysis of all microRNA, we showed a decrease in miR-199a-3p expression in CF patients. The expression of miR-199a-3p is decreased and inversely correlated with an increase of IKKβ and IL-8 expression in bronchial explants from CF patients compared to non-CF patients, thus confirming the small RNAseq and RNAseq data. MiR-199a-3p modulates the expression of IKKβ through a direct interaction at its 3´-UTR in CFBE. Increase miR-199a-3p expression leading to a decrease in protein expression of IKKβ, NF-κB activity, and IL-8 secretion. However, as the origin of miR-199a-3p deregulation is unknown, we focused on the pro-inflammatory, calcium and infectious context. Stimulation by pro-inflammatory cytokines (IL-1β +/- TNF-α) showed modulation of miR-199a-3p expression. However, the use of calcium modulators such as thapsigargin and bapta, respectively, highlighted a decreasing trend and a significant increase in miR-199a-3p expression. The study of the impact of pro-infectious stimulus such as Pa by the PAK strain or by lipopolysaccharide (LPS) and/or flagellin, two virulence factors expressed by Pa is currently in progress.

CONCLUSION: MiR-199a-3p has a negative regulatory role in the NF-κB signaling pathway and that its poorly expression in CF patients contributes to a chronic pulmonary inflammation. From a fundamental point of view, the origin of miR-199a-3p expression deregulation remains to be elucidated.

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P8

Not all stop codons are created equal, neither do they always stop: morals from single-channel studies of E60X- and G542X-CFTR

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Cystic fibrosis (CF) is caused by loss-of-function mutations in the *CFTR* gene encoding a phosphorylation-activated but ATP-gated chloride channel. Among six classes of pathogenic mutations, Class I mutations, including premature termination codon (PTC) mutations, result in little or no protein expression. Although PTCs dramatically reduce the expression of CFTR, they can be suppressed endogenously or by reagents such as aminoglycoside antibiotics (e.g., G418). During this "read-through" process, a range of near-cognate tRNAs is used for translation, leading to various missense mutations at the position of PTC.

In this study, we investigated the characteristics of E60X and G542X, a PTC mutation at the N-terminus of CFTR and one in the first nucleotide binding domain (NBD1), respectively. Western blot experiments showed distinct differences between the read-through proteins of E60X and G542X. Significant amounts of non-glycosylated, band A E60X-CFTR proteins were detected, indicating full-length proteins were produced, but they failed to undergo glycosylation, as the core-glycosylated band B and complex-glycosylated band C were barely detectable. In contrast, we hardly saw any band for G542X-CFTR. These differences may be due to varied susceptibility of specific PTCs to read-through.

Although Western blotting showed no clear evidence for surface expression of E60X or G542X, whole-cell patch-clamp experiments recorded forskolin-dependent currents with a current density of 5.9±1.6 pA/pF (n=7) for E60X. Furthermore, application of the gating modulator genistein increased whole-cell currents by ~3-fold, indicating the presence of partially functional proteins. Moreover, pre-treating the cells with Lumacaftor, an FDA-approved CFTR corrector, or G418, a read-through reagent, increased whole-cell current density by ~5-fold. In inside-out patches from cells pre-treated with Lumacaftor, the application of PKA and ATP activated E60X-CFTR currents with a single-channel amplitude of 0.34±0.01 pA (n=5) that is similar to WT, but with a lowered open probability (P_o) of 0.21±0.03 (n=6), which can be increased to 0.7±0.07 (n=4) by N^6-phenylethyl-2'-deoxyATP, a high-affinity ATP-analogue. These results suggest that the read-through products of E60X-CFTR possess not only an intact anion permeation pathway, but the nucleotide binding domains where ATP or its analogue binds. Interestingly, the P_o of E60X-CFTR pre-treated with G418 was 0.5±0.07 (n=4), indicating a change in the read-through products. Comparing to the single-channel behaviors of E60Y/Q/S, the proposed read-through products for E60X, the P_o of Lumacaftor-treated E60X is more consistent with E60Y/S, but with E60Q for G418-treated channels.

In contrast, G542X-CFTR pre-treated with Lumacaftor or G418 presented a P_o of .2, which is different from those of G542R/C/W, the proposed read-through products (~0.7 for G542R, ~0.5 for G542C, and no detectable activity for G542W). These observations underscore the notion that the function of read-through products varies with stop codons and/or the position of the stop codon. Importantly, regardless of these variabilities, applying the CFTR potentiator GLPG1837 greatly increases the P_o of all the read-through products, hence suggesting a possible treatment strategy of combining CFTR correctors (e.g., Lumacaftor), read-through reagents (e.g., G418) and CFTR potentiators (e.g., GLPG1837). In summary, our studies unveil biophysical and pharmacological properties of the read-through products of E60X- and G542X-CFTR at a single-channel level. ~0

P9

The cystic fibrosis mutations L927P and I336K modulate CFTR pore dynamics during channel gating, but are rescued by ivacaftor and lumacaftor

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Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7), a unique member of the ATP-binding cassette (ABC) transporter superfamily. Unlike other ABC transporters, which function as active transporters, CFTR acts as an ATP-gated Cl^- ion channel that allows passive diffusion of substrate anions across the plasma membrane. This specialised role resulted from the evolution of the membrane-spanning domains (MSD1 and MSD2), transforming CFTR from an alternating access pathway with two gates to a pore with a single gate essential for fast and energetically-efficient anion transport. The CFTR pore is lined by transmembrane segment 1 (M1), M6, M8 and M12. Here, we investigate the action of two CF-associated mutations, L927P and I336K, which introduce structural changes to M8 and M6, respectively, at the narrowest part of the CFTR pore. To observe CFTR activity, we performed single-channel patch-clamp recording on excised inside-out membrane patches from Chinese hamster ovary (CHO) cells that were incubated at 27 ºC for ≥ 2 days to promote the plasma membrane expression of mutant CFTR. Voltage was --50 mV in the presence of a Cl^- concentration gradient ($[Cl^-]_{int}$, 147 mM; $[Cl^-]_{ext}$, 10 mM). The intracellular concentration of ATP and PKA were 1 mM and 75 nM, respectively, and temperature was 37 ºC. Both mutations formed PKA-activated CFTR Cl^- channels gated by intracellular ATP. However, their unitary current amplitudes were reduced by ~40 % ($n \geq 4$) when compared with currents recorded from membrane patches containing wild-type CFTR. Moreover, open probability values of both channels were >83 % ($n \geq 4$) lower than that of wild-type CFTR. The bursting pattern characteristic of wild-type CFTR channel gating was severely disrupted by L927P. By contrast, I336K-CFTR had two modes of channel gating which we termed mode-1 and -2. Mode-1 lacked the bursting pattern observed in wild-type CFTR, while mode-2 retained it. I336K-CFTR predominantly dwelt in mode-1 and only infrequently resided in mode-2. Ivacaftor enhanced the activity of both L927P- and I336K-CFTR in excised inside-out membrane patches, while treatment with both lumacaftor and ivacaftor enhanced transepithelial Cl^- currents across I336K-CFTR-expressing bronchial epithelia. The location of L927 and I336 within the transmembrane segments suggests that CF mutations at these residues might influence conformational changes within the pore during the gating cycle. We hypothesize that the proline introduced in L927P-CFTR possibly extends the discontinuity of M8 and reduces its structural flexibility during pore rearrangements following ATP binding. By contrast, I336K possibly locks the pore in a low conduction constricted state by reducing its flexibility. The switching of I336K-CFTR between two modes of channel gating raises the interesting possibility that I336K-CFTR activity might be enhanced by stabilising mode-2 channel gating.

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P10

Characterization of the rare S955P-CFTR mutation and its response to modulators in cellular models and patient-derived materials

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Background: More than 2,000 variants have been described to date in the *CFTR* gene, most of them presumed to be CF-causing. Among such large number of variants, many of them are very rare – with over 1,000 existing in less than five patients worldwide. For some of these mutations, since the functional defect has not been characterized yet, the prediction of disease outcome is difficult. Thus, there is an unmet need to functionally characterize these mutations at the molecular and cellular level in patient-derived materials when available, and in relevant cellular models for better disease diagnosis/prognosis. Moreover, the specific defect of each mutation is essential for mutation-specific therapy. S955P-CFTR (c.2863T>C) is a novel mutation located in exon 17 of *CFTR* gene which has not been previously characterized and was recently found in heterozygosity with F508del in an 8-week old Czech boy. The patient had IRT elevation (118 ng/ml) and repeatedly higher levels of sweat chloride (50.5; 55.3 mmol/l).

Aim: Here we aim to assess the molecular/functional defect of S955P-CFTR, both in patient-derived materials and in a cellular model, and to determine its responsiveness to available CFTR modulators.

Methods: CFTR function was measured in rectal biopsies from a CF patient bearing S955P by Ussing chamber and in intestinal organoids by the forskolin-induced swelling (FIS) assay. Additionally, assessment of S955P-CFTR protein expression and maturation was performed by Western blot, in a novel CFBE-based cell line stably expressing the S955P CFTR mutant (produced by *in vitro* mutagenesis), as well as in intestinal organoids from the above CF patient. The CFBE cell line was also used to measure CFTR activity at baseline and after treatment with modulators (potentiator genistein alone or with correctors VX-809 and VX-661) by Ussing chamber measurements.

Results and Discussion: Data from rectal biopsies and the intestinal organoids from a CF patient bearing S955P/F508del showed relatively high levels of residual CFTR activity, probably due to the S955P allele. The FIS assay of the intestinal organoids after treatment with CFTR modulators showed an significant increase in CFTR activity in the presence of VX809/VX770 and VX661/VX770, probably due to the F508del allele. Data from CFBE cells show that both mature and immature forms of S955P-CFTR are present, suggesting that this mutant has normal processing. Ussing chamber assays suggest that S955P-CFTR has residual function that is however not increased by treatment with CFTR modulators (VX-809 and VX-661). Altogether our results suggest that S955P is a class IV mutation and highlight the relevance of characterizing rare CFTR mutants using combined data from cellular models and patient samples, towards a personalized therapy approach.

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P11

Functional impact of the rare CFTR variants W57G/A234D and CFTR modulator theratyping in a CF patient

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Background: Over 2000 different variants in the CFTR gene have been described: not all of them cause CF. Clinical and laboratory-based information about the 374 most common CFTR variants are available in the CFTR2 database (www.cftr2.org) describing 312 CF-causing variants, 36 of varying clinical consequence, 13 non CF-causing and 13 of unknown significance. This study focused on a CF patient carrying the very rare (n=10 patients in CFTR2 database) CF causing missense CFTR variant W57G (c.169T>G), *in trans*with the A234D (c.701C>A) missense variant that is not described in the above-mentioned database. The complex allele A234D-I1027T has been previously reported (https://www.cysticfibrosisjournal.com/article/S1569-1993(06)80002-0/pdf)to be probably a neutral variant if isolated but might significantly impair CFTR function when associated with a CFTR mutation. Measurements of CFTR function *in-vivo*and *ex-vivo*have been developed for evaluation of the functional impact of rare variants as well as for investigating the effects of CFTR modulators on rare / of varying clinical consequence genotypes.

Ivacaftor is a CFTR potentiator approved by Food and Drug for treating 33 CFTR variants in CF patients with no evidences about its effects on the W57G/A234D CFTR genotype. CFTR modulator theratyping has been proposed to identify rare CFTR variants that are responsive to approved or in development drugs (Clancy et al. JCF 2018).

Methods: This study focuses on a 36 years old female CF patient followed-up since 2014 at CF Center of Verona with CFTR genotype W57G/A234D, abnormal sweat Cl (112 mmol/L), pancreatic sufficiency, lung infection (Pseudomonas aeruginosa), impaired lung function (FEV1: 65% of predicted value), bilateral bronchiectasis, kidney stones, recurrent sinusitis. Nasal Potential Difference and Intestinal Currents Measurements) according to ECFS SOPs were both abnormal supporting CF diagnosis. Imaged Beta-Adrenergic Sweat Rate Test (IBASR) test was performed as previously described (Bergamini et al. JCF 2017). Intestinal organoids were obtained from rectal biopsy after intestinal crypts isolation. The rescue of CFTR function was evaluated by FIS assay according to the Utrecht Medical Center Protocol with simultaneous treatment with forskolin (ranging 0.02- 5uM) and ivacaftor (3μM). Organoids were directly acquired by confocal live cell microscopy (Leica, TCS-SP5) from baseline to 60min using ImageJ software.

Results: CFTR dependent sweating was undetectable in two separate tests in this patient, consistently with CF diagnosis. The simultaneous treatment with ivacaftor and forskolin showed by FIS assay a significant 1.8±0.04,n=86 fold of increase ($p < 0.0001$ vs untreated) after 60 min but no significant swelling in the absence of ivacaftor.

Conclusions: Our results support the functional impact of the rare/of varying clinical consequence CFTR genotype W57G/A234D by standardized sweat Cl, NPD and ICM measurements as well as by experimental *in-vivo*and *in-vitro*assays in different CFTR expressing tissues. Successful targeting of this CFTR genotype in intestinal organoids by ivacaftor suggest it as therapeutic candidate for this patient, based on CFTR modulator theratyping. IBASR, NPD, ICM, Cl sweat tests are all available for testing the effects of ivacaftor in this patient should it will be available for her therapy.

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P12

Repurposing drugs for cystic fibrosis subjects with rare *CFTR* mutations using intestinal organoids: An update on the Rainbow project

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In the RAINBOW project we are screening >1,400 FDA-approved drug compounds, including ivacaftor, lumacaftor and ORKAMBI®, for their potential to modify fluid secretion in intestinal organoids generated from more than 150 Dutch CF patients carrying *CFTR* mutations with a prevalence of < 0.5% in the Dutch CF population. As fluid secretion in intestinal organoids is strictly CFTR-dependent, we anticipated that the majority of hit compounds modify fluid secretion, either directly or indirectly via CFTR.

We have performed a primary screen in 85 organoid cultures with various rare genotypes using our high-content 384-well forskolin-induced swelling assay (HCS-FIS). The screening of >60.000 conditions was feasible, and data from >50 screened plates indicated an average Z'- factor >0,5 indicating an excellent screening performance of our high-throughput screening platform. Identified primary hits were validated using the conventional FIS assay in 96-well plates. Out of 52 confirmed hits that modified epithelial fluid secretion, we selected the 12 most promising hits based on

(i) oral route of administration,

(ii) availability on the Dutch market and

(iii) likely long-term safety characteristics.

The 12 most promising hit compounds are currently being tested individually and as add-on therapy to ORKAMBI® in all organoids from the RAINBOW cohort.

We previously published results of this project on international CF conferences. Here we provide an updated list of data derived from 120 patient organoids. Overall, the data supports that fluid secretion assays in intestinal organoids facilitate relative large-scale individual drug screening efforts, potentially leading to the repurposing of CF and non-CF therapies as individual modulators of CFTR. Future experiments are needed to further validate hits and to establish in vivo safety and efficacy.

P13

The regulatory insertion enables novel conformations of NBD1 of CFTR

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Introduction: The role of conformational dynamics of NBD1 of CFTR in CF pathogenicity is well established. For example, the presence of an intrinsically disordered segment called the regulatory insertion (RI) is connected to the deleterious effect of delF508, as its removal has been shown to restore mutant CFTR maturation and function *in vitro*. The RI is unique to CFTR, its function is unknown and it destabilizes the protein significantly.

Approach: We have used nanobodies as crystallization chaperones for NBD1. Nanobodies are antigen binding fragments derived from llama antibodies. One of these nanobodies that we crystallized in complex with NBD1 interacts directly with the regulatory insertion. In this crystal structure NBD1 adopts a conformation that is significantly different from the classsical state observed in all crystal structures available in the protein data bank as the RI domain is now adopting a defined conformation and the N-terminus is not structured.

We used Double Electron-Electron Resonance (DEER) in conjunction with single molecule Förster Resonance Energy Transfer (smFRET) to characterize the conformational dynamics of NBD1 and test whether or not this new conformation is present in solution and in absence of the nanobody.

Results and conclusion: An equilibrium exists between the classically observed state and the alternate state we are reporting here.

At low levels of ATP (submillimolar) the classical state of NBD1 is less populated than the newly discovered state. Only high levels of ATP (mM) combined with deletion of the RI result in NBD1 adopting exclusively the classical state.

These results show that NBD1 adopts a complex conformational landscape due to the presence of the RI and that it can be modulated by ATP.

We hypothesize that deletion of the RI rescues ΔF508-CFTR by restricting the conformational landscape (ie. promoting the classical state). As a corollary, preventing transition to the alternate conformation of the RI may provide novel therapeutic approaches to overcome the effect of destabilizing mutations in NBD1 such as F508del.

P14

Unraveling new conformations of CFTR and their possible role in cystic fibrosis

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In most patients suffering from cystic fibrosis, the causing mutation is the removal of F508 in the NBD1 domain of CFTR (DelF508), a change that lead to structural destabilization of the domain and subsequently of the channel. The host laboratory has recently identified a new conformational state of NBD1 in which major structural rearrangements can be observed. Among these, the structuration of a destabilising intrinsically disordered region called the regulatory insertion (RI). Since this segment is involved in the destabilization of the domain and is required for this conformation we formulate the hypothesis that this new NBD1 conformer might have physio-pathological implications.

In order to probe the functional consequences of this conformer we need to promote either of the observed conformation in a cellular context and test their ability to modulate expression, maturation and channel activity.. Using the crystal structures as guides we will engineer modifications in the isolated NBD1 domain by introducing point mutations, by deleting some regions or by locking some segments with disulfide bridges. The modulation of the equilibrium between the different states of the protein can be then observed with single molecule fluorescence, crystallography or binding assays methods. The most promising mutations will be transposed to the full-length protein in order to establish the functional consequence of the conformational change in the full length wild-type and in the DelF508 mutant.

The first results are encouraging as the mutations promoted one or the other conformation. We were able to lock a double cysteine mutant in the classical conformer as revealed by binding assays. A construct of the domain depleted of the N-terminus was crystalized under the unusual alternative conformation. Finally, the introduction of a point mutation successfully shifted the equilibrium towards the classical conformation.

Thus we now have our first molecular tools to lock either of conformations to further study their implications in a cellular context with the full-length protein. Moreover, the effects of those conformations on the deletion of the F508 will be investigated.

P15

Identifying the molecular targets for CFTR potentiators GLPG1837 and VX-770

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In the past three years, major breakthroughs in solving the atomic structures of CFTR, which provide exquisite chemical features of this ion channel protein, make structure-based drug design a real possibility. However, to date the binding sites for CFTR modulators remain largely unknown. In this study, we first constructed a homology model of human CFTR based on the cryo-EM structure of the phosphorylated and ATP-bound zebrafish ortholog and then utilized in silico molecular docking to identify potential binding sites for GLPG1837, a CFTR potentiator that has been proposed to share a common binding site with the FDA-approved drug VX770 (Ivacaftor). Among the identified four binding sites, the one with the highest score (site I) includes three amino acids (D924, N1138, and S1141) located at the interface between CFTR's two transmembrane domains. Using the patch-clamp technique to measure CFTR currents at different concentrations of GLPG1837, we observed side-chain specific changes in the apparent affinity for GLPG1837 at all three positions. Specifically, glutamate, asparagine and alanine substitutions at position 924 cause a graded right-ward shift of the dose-response curves. On the other hand, the apparent affinity of GLPG1837 is increased either when N1138 is replaced with hydrophobic amino acids, including N1138F, N1138Y, and N1138L, or when S1141 is substituted by arginine (S1141R) and lysine (S1141K). Consistently, current relaxation time constant upon removal of VX770 is shortened by D924N but prolonged by N1138L and S1141K, supporting the notion that these three amino acids in site I also contribute to binding of VX-770. In contrast, alanine substitution of the residues in the other three lower-scored sites (site II, III, and IV) fails to change the affinity for GLPG1837. Lately, the structure of human CFTR with dimerized NBDs is solved by cryo-EM, allowing us to perform docking simulation on human CFTR and compare the results with those based on a homology model. Despite a similar ranking score for site I, a new binding site (site II_N) located at the interface between transmembrane segment 4, 5, and 8 is revealed. Among the five aromatic amino acids (F229, F236, Y304, F312 and F931) that compose site II_N, Y304F and F312A decrease the apparent affinity for GLPG1837 whereas F236A poses minimal effect. While more experiments are in progress to define the potential role of site II_N in the binding of potentiators, our data so far support site I as a likely binding site for both GLPG1837 and VX770.

P16

VX-770 potentiates CFTR function by interacting with MSD2

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CFTR protein consists of two membrane-spanning domains (MSD1, MSD2) with four intracellular loops (ICL1-4), two nucleotide-binding domains (NBD1, NBD2) and a regulatory (R) domain (Riordan et al., 1989; Gadsby et al., 2006). Importantly, of the 2000 disease-causing CFTR mutations, the most common, the deletion of phenylalanine at position 508 (F508del). F508del (Class II mutation) causes intradomain instability as well as poor interdomain assembly of the full-length protein, particularly mediated through the NBD1/ICL4 interface, while G551D (Class III) causes a channel 'gating' defect mediated through the NBD1/NBD2 interface.

Accordingly, two small molecule-based therapies, ORKAMBI™ and KALYDECO™, have been developed for patients bearing F508del and G551D (and other gating mutations), respectively. KALYDECO™ is comprised of ivacaftor (VX-770), a small molecule potentiator which increases the open probability of G551D-CFTR channels, while ORKAMBI™ is comprised of both VX-770 and lumacaftor (VX-809), a corrector molecule (Van Goor et al., 2009; Van Goor et al. 2011). The clinical efficacy of ORKAMBI, however, is relatively modest (Boyle et al. 2014) and better correctors are needed.It has also been demonstrated in vitro that VX-770 exerts a deleterious effect on the stability of the F508del mutant protein after correction by VX-809 (Cholon et al. 2014, Veit et al 2014).

Identification of the binding sites of VX-770 and VX-809 on CFTR protein may facilitate development of new modulators which are able to repair additional functional and folding defects (e.g. other domains and interfaces in F508del and defects in non-F508del and non-G551D mutations). While it has been identified that VX-809 directly modulates CFTR by stabilizing MSD1 (Ren et al. 2013, Loo et al. 2013, Laselva et al 2018), the binding site of VX-770 is currently unknown.

We studied the binding site of VX-770 using a photo-probe of VX-770 (Hamilton et al. 2018) where a biotin tag has been introduced onto the molecule. First we validated that this VX-770-biotin probe retains CFTR channel potentiator activity in HEK-293 cells using the fluorometric imaging plate reader (FLIPR) assay. We then demonstrated that the VX-770-Biotin probe covalently binds to WT-CFTR (reacted by photolysis) and competes with both VX-770 and an active analog of the molecule (SE-03) but not with an inactive VX-770 analog, (SE-02). Using a series of CFTR truncation fragments, we demonstrated that the VX-770-Biotin probe strongly interacts with MSD2 but not MSD1. However, this probe interacts with NBD1-R domain to a small extent. Therefore, we have demonstrated that chronic treatment of VX-770 exerts a deleterious effect on the stability of CFTR fragments containing MSD2 domains.

These findings support the concept that VX-770 enhances CFTR channel activity by interacting with MSD2 and may stabilizes the intra-molecular interaction at the interface between MSD2 and ICL4 (NBD1).

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P18

CFTR conformational landscape explored by computational approaches

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CFTR protein represents an important target for drug discovery, with challenging issues related to the development of modulators specific for each class of mutations. Today, it is well recognized that the pleiotropic defects induced by some mutations affecting the CFTR protein, such as F508del, can be rescued by combination of pharmacotherapies addressing single defects. Combinations of modulators, correctors but also potentiators, targeting different sites of the CFTR protein, are thus actively investigated, with special emphasis on modulators that act in a synergic way and on rare mutations.

In this context, 3D structure provides critical information to understand the molecular basis of defects induced by mutations and potential ligand-binding sites that can be occupied by modulators. Knowledge on CFTR 3D structure was gained during the last years by comparative modelling, followed by cryo-EM, giving access to different conformations of the channel that can be explored in an integrative way. These however give only information on a few, discrete conformational states and do not inform about the plasticity of potential ligand-binding sites and the effect of ligand binding on the whole protein.

Tooking advantage of these structural data, we carried out standard molecular dynamics (MD) and enhanced dynamics (metadynamics) in explicit solvent using wild-type and mutated CFTR channel embedded in a lipid membrane. These simulations provided a larger exploration of CFTR conformational landscape and revealed some crucial features about CFTR functions and dynamics. These discriminating characteristics, involving specific salt-bridges, NBDs/ICLs interfaces and channel cavities, were used to generate a precise cartography of CFTR states classifying any channel conformation(s) and allowing thus to better characterize mechanisms underlying CFTR mutations but also modulations.

By combining experimental and theoretical methods, we identified some potential binding sites for modulators. We thus conducted several MD simulations of CFTR in presence of different modulators, correctors and potentiators (in-house and others). These simulations allowed us to support the binding site predictions, provide interactions schemes for each ligand, demonstrate the plasticity of some binding sites to accommodate the ligands and understand for some of them the molecular basis of their action.

These studies open new perspectives for the rational, structure-based design of specific modulators.

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P19

In silico search for the chloride pathway in CFTR

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Cystic fibrosis (CF), a lethal monogenic disease, is caused by pathogenic variants of the CFTR chloride channel. Most of the CF mutations, including the most frequent ΔF508 deletion, affect protein folding, stability and lead to diminished apical anion conductance of epithelial cells. The recently published cryo-EM structures of full-length human and zebra fish CFTR provide a good model to gain insight into the structure-function relationship of CFTR variants. Considering that none of the static structures, although they were determined in the phosphorylated and ATP-bound active state, shows an open pathway for chloride permeation, we aimed to study the dynamics of the ATP-bound zebra fish CFTR structure (PDBID:5W81) using *in silico* methods. First, we performed extensive molecular dynamics (MD) simulations to generate a conformational ensemble of the protein. Then we used channel detecting algorithms to identify conformations with an opened channel between the intracellular and extracellular sides. The simulations indicate a main intracellular entry at TM4/6 and a secondary one at TM10/12 in accordance with electrophysiological experiments. A bottleneck region involves numerous amino acids from TM1, TM6, and TM12. The majority of the channel lining residues has been shown to be part of the chloride permeation pathway by experimental studies. We can observe in our trajectories that because of the break in TM8 the chloride permeation pathway is exposed to the membrane bilayer, thus hydrophobic lipid tails also take part in forming the channel wall. Since chloride ions entered the pathway in our equilibrium simulations, but did not traverse the bottleneck region, we performed metadynamic simulations to accelerate the sampling and to calculate the free energy surface of chloride passing. We found that this narrow permeation path exhibits only a minor energetic barrier, thus most likely the short timescale (100 ns) of our simulations limited the chloride passage. In summary, our *in silico* study describes a potential chloride channel pathway based on a recent cryo-EM structure in detail.Our results help to understand the gating of the CFTR chloride channel, thus to advance strategies to rescue dysfunctional mutants.

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P20
Impact of CFTR function on airway mucus properties

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Introduction: Cystic fibrosis (CF) is characterized by the buildup of thick, viscous mucus in several organs, particularly the lungs. Gel-forming mucins (mainly MUC5B and MUC5AC in the lungs) are high-molecular-weight glycoproteins responsible for the viscoelastic properties of mucus and play a key role in the CF pathogenesis. In CF airways, abnormal mucus properties drive mucus stasis and airway obstruction but the pathophysiologic link with CFTR malfunction is unclear. Mutations within CFTR reduce transepithelial Cl^- and $HCO3^-$ secretion, which leads to ionic imbalance and volume depletion. Airway dehydration causes mucin hyperconcentration, increasing the interactions between mucin monomers (i.e., mucin entanglement). CFTR dysfunction is also associated with oxidative stress, which may increase disulfide bond formation (i.e., mucin crosslinking). Additionally, deficient bicarbonate secretion affects $Ca2^+$ chelation, which may compromise mucin unfolding/expansion (i.e., mucin compaction). Understanding the dominant biochemical change(s) (i.e., mucin entanglement, covalent crosslinking, electrostatic compaction) caused by CFTR malfunction is critical to identify new therapeutic targets aimed to "reverse" mucus abnormalities in CF.

Methods: Primary and immortalized human bronchial epithelial (HBE) cells were obtained from CF patients carrying either the G551D or F508del mutation. Human nasal epithelial (HNE) cells were collected from normal and CF subjects. Modulation of CFTR activity was ensured by either correction by small molecules (Vx770- Vx809) or pharmacological inhibition with the specific CFTR inhibitor $CFTR_{Inh172}$. We used these cell systems to study the impact of CFTR activity on mucin properties using biochemical assays (Western blotting, HPLC, IHC), biophysical measurements (macro/microrheology) and scanning electron microscopy (SEM). Change in spontaneous mucociliary transport (MCT) was also assessed using fluorescent microbeads tracking.

Results: Chronic treatment with CFTR modulators in our primary and immortalized cell cultures resulted in enhanced CFTR-mediated short-circuit currents. In G551D cells, Ivacaftor treatment significantly decreased total mucin concentration by ~30%, increased mucus pH slightly, and increased ciliary beat amplitude by ~30%, suggesting a physical change in the mucin network surrounding the cilia. G551D- CFTR rescue decreased MUC5B but not MUC5AC crosslinking as shown by electrophoretic mobility shift on mucin agarose gel. In F508del-HBE cells, the duration and velocity of mucociliary transport (MCT) was significantly reduced compared to non-CF cells but when treated with a combination of Ivacaftor and Lumacaftor, F508del-HBE cells partially restored MCT. In addition, changes in CFTR function strongly affected mucin secretion in freshly harvested nasal cells. Similarly, CFTR rescue significantly reduced MUC5B and MUC5AC secretion in CF nasal cells. Inversely, CFTR inhibition resulted in mucin hyperconcentration (1.5-fold change) in non-CF nasal cells, which correlated with a change in MUC5B/MUC5AC ratio and a tighter mucus network by SEM.

Conclusion: Functional CFTR rescue affected mucin interactions by decreasing mucin entanglement and crosslinking. In contrast, CFTR inhibition increased mucin concentration and molecular weight/size. CFTR dysfunction alters MUC5B/MUC5AC ratio and mucin network organization, which both likely affect the biophysical properties of airway mucus. Hence, treatments directly targeting mucus (e.g. mucolytics reducing disulfide bonds) could be a promising therapeutic strategy, independent of inflammation or patient genotype.

P21

Early onset of airway mucus obstruction associated with increased mortality in neonatal *Slc26a9* deficient mice

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Background: Recent evidence suggests that the epithelial Cl^- channel SLC26A9 is associated with disease severity in patients with cystic fibrosis (CF) (Strug LJ. et al., 2016) and other muco-obstructive lung diseases such asthma (Anagnostopoulou P. et al., 2012) and bronchiectasis (Bakouh N. et al., 2013).These studies support a role of SLC26A9 as a modifier of muco-obstructive lung diseases. However, the in vivo role of SLC26A9 in lung development and disease remains unclear.

Aim: The aim of this study was to investigate the *in vivo* role of the SLC26A9 Cl^- channel during early postnatal adaptation.

Methods: We compared the pulmonary phenotype of wild-type (WT) and *Slc26a9*- deficient (*Slc26a9*$^{-/-}$) mice on a C57BL/6 background including survival, histology, micro computed tomography (µCT) imaging, wet/dry lung weight and oxygen saturation measurements. On a functional level, transepithelial potential differences (PD) of cultured tracheal explants of WT and *Slc26a9*$^{-/-}$ mice was assessed.

Results: All genotypes were represented at birth according to Mendelian ratios. Within the first 30 minutes of life, *Slc26a9*$^{-/-}$ mice showed signs of severe respiratory distress, reduced oxygen saturation ($p< 0.01$) and spontaneous mortality of 48%. To determine if developmental defects were responsible for the death of the *Slc26a9*$^{-/-}$ mice, we performed histological analysis on vital organs at the embryonic day 17 (E17), but did not detected differences in *Slc26a9*$^{-/-}$ compared to WT. Further, both genotypes presented a similar rate of lung liquid clearance at birth ($p=0.56$). To evaluate the contribution of the SLC26A9 Cl^- channel to the airway epithelial ion transport in the neonatal lung, PD was measured in cultured tracheal explants and found to be lower in *Slc26a9*$^{-/-}$ (-18 ± 6 mV) compared to WT mice (-41 ± 6 mV; $p< 0.05$). Histological analyses immediately after birth revealed severe mucus obstruction through the whole tracheobronchial tree of *Slc26a9*$^{-/-}$ mice compared to WT mice ($p< 0.01$). These findings were confirmed by µCT showing early onset airway mucus obstruction ($p< 0.05$) associated with atelectasis compared to WT mice.

Conclusions: In summary, the *Slc26a9*$^{-/-}$ mice shows an early airway muco-obstructive phenotype associated with decreased epithelial ion transport *in vivo*. Taken together, our data support that SLC26A9 mediated Cl^-/fluid secretion plays a critical role in airway mucus clearance in the neonatal lung and that this process may be essential for normal postnatal adaptation to the air-breathing conditions.

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P23

Selective bicarbonate transport defects in human rectal biopsies with CFTR gene variants

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Background: Among physiological assays currently used to study CFTR function and activity, ICM (Intestinal Current Measurements) is a powerful*ex-vivo* assay that allows the fine-diagnosis of CF, the evaluation of CFTR modulators efficacy, and the separate measurements of electrogenic chloride vs. bicarbonate secretion in patients with CFTR variants that differentially affect the permselectivity of the CFTR channel for these anions. Variants that preferentially affect bicarbonate transport are expected to be associated with pancreatic disorders (pancreatic insufficiency; pancreatitis) rather than intestinal or lung disease, considering the key role of bicarbonate in pancreatic ductal function. In this study we have exploited ICM to detect CFTR variants resulting in bicarbonate rather than chloride transport defects in the intestine.

Methods: ICM on rectal biopsies from 8 non-CF subjects, 8 CF patients, and 8 patients carriers of CFTR variants with inconclusive diagnosis (UNK) were performed in recirculating Ussing chambers systemaccording to the procedure of European Cystic Fibrosis Society ICM Standard Operating Procedure adapted by using a chloride-free or bicarbonate-free buffer in order to test ions transport mediated by CFTR channel. The transepithelial Isc (Short Circuit Current) registered across the tissue samples is a direct measure of ions movement across the epithelium after stimulation with CFTR-linked agonists (forskolin, carbachol) added to the serosal side of the rectal biopsies.

Results: Normal tracings for both anions were observed in non-CF controls (247±128 for Cl-, 177±137 μA/cm^{2}for HCO_3^-, n=8), while in CF (n=2) and UNK (n=6) CFTR genotypes the CFTR agonists differentially affected bicarbonate transport in presence of similar chloride transport levels. Isc for Cl^- was 130±37 vs 136±51 μA/cm^2 (n = 8, ns) in the two groups, whereas HCO_3^-associated currents were 38±10 (in patients with genotypes W1282X/R117H/IVS8 T7/T7; c.91C>T/R31C; T5/T7-TG11/12; G576A/R668C; I807M) vs 171±131 μA/cm^2(in patients with CFTR genotypes F508del/V938G; R74W+/-; 1717-1G>A+/-) (n= 8, p=0,03). Phenotypes of these patients include bronchiectasis and/or lung infection, rhinosinusitis congenital bilateral absence of vas deferens and only in one case pancreatitis (F508del/V938G).

Conclusions: Alteration of bicarbonate transport might be associated with selected CFTR variants, suggesting a different mechanism of regulation of the transport of chloride and bicarbonate anions in human rectal biopsies. Clinical phenotypes of milder forms of CF than classical ones include not only pancreatic damage but also lung disease. However, to find out whether a selective bicarbonate transport defect can be associated to a specific clinical phenotype and severity of disease, the collection of a larger series of cases is required. Such CFTR function defects could be detected specifically by physiological bioassays such as ICM that are able to evaluate also the functional response of CFTR gene variants to CFTR channel modulators.

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P24

Dual role of pendrin as a bicarbonate secretion pathway and CFTR modulator in well-differentiated human nasal and bronchial epithelial cells

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Introduction: Bicarbonate facilitates mucin unpacking and bacterial killing on the airway surface however its transport mechanisms are not well understood. The activation of CFTR channel by phosphorylation stimulates conductive Cl^- and HCO_3^- efflux and this is defective in CF. The anion exchanger pendrin (SLC26A4) mediates electroneutral HCO_3^- efflux and is upregulated by proinflammatory cytokines. The relative contributions of these pathways remain uncertain and may depend on physiological state.

Aim: To examine pendrin and CFTR expression in the airways and their roles in HCO_3^- secretion across human nasal and bronchial epithelia cells.

Methods: Fresh tissue and well differentiated bronchial and nasal epithelial cells that had been cultured at the air-liquid interface for 1 month were used. Intracellular pH was measured using BCECF-AM during apical or basolateral exposure to low-Cl^- solution containing HCO_3^- to monitor anion exchange. Cells were transduced using multiple adenoviral shRNAs targeting pendrin mRNA transcripts. CFTR-dependent short-circuit current (Isc) was measured in Ussing chambers after adding amiloride. Airway surface liquid (ASL) height was monitored using confocal microscopy. Whole-cell Cl^- current was measured in BHK cells stably expressing CFTR with or without transient co-expression of EGFP-pendrin.

Results: In native bronchial tissue, both CFTR and pendrin were most abundant at the apical pole of ciliated surface cells, with little CFTR or pendrin detected in submucosal glands. IL-4 dramatically increased pendrin mRNA levels and apical pendrin immunostaining in well-differentiated primary nasal and bronchial cells, confirming previous studies by others. Exposure to low-Cl^- apical solution caused intracellular alkalinization (ΔpH_i) that was enhanced 4-fold in cells that had been pretreated with IL-4. ΔpH_i was not altered by DIDS or $CFTR_{inh}$-172 but was reduced in pendrin knockdown cells. Forskolin increased ΔpH_i and this stimulation was prevented by $CFTR_{inh}$-172, implicating CFTR. Surprisingly, forskolin only increased ΔpH_i when pendrin expression had been upregulated by IL-4. This dependence of ΔpH_i on pendrin suggests there is minimal electrical coupling between Cl^- and HCO_3^- fluxes and that CFTR activation increases anion exchange-mediated HCO_3^- influx. Conversely, increasing pendrin with IL-4 or by heterologous expression increased forskolin-stimulated, $CFTR_{inh}$-172-inhibitable current ~2-fold in both epithelial and non-epithelial cells, respectively.

Conclusions: Pendrin mediates most HCO_3^- secretion across airway surface epithelium during inflammation and enhances CFTR-mediated electrogenic Cl^- secretion.

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P25

CFTR is functionally expressed in a subpopulation of endocrine cells of the mammalian islet of Langerhans and modulates insulin secretion

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Approximately 35-50% of patients with cystic fibrosis (CF) develop a new and unique form of age- dependent diabetes, CF-related diabetes (CFRD), characterized by a combination of features from type 1 and type 2 diabetes mellitus in a milieu of chronic infection, pancreatic insufficiency, and malabsorption. The resulting hyperglycemia, which endures upon progressive insulin-secreting β-cell dysfunction greatly increases morbidity and mortality. The consensus mechanism for insulin secretion encompasses β-cell plasma membrane depolarization and Ca^{2+}influx via voltage-gated Ca^{2+}channels in response to glucose. This initial glucose-dependent β-cell plasma membrane depolarization is due to a hyperpolarizing K^+current mainly controlled by K_{ATP}-channels and a largely ignored depolarizing anionic (Cl^-) conductance. Although several Cl^-channels including *CFTR*are known to participate in β-cell electrical activity and insulin secretion, further characterization has languished amidst controversy surrounding the perceived relevance of Cl^-channels, mainly *CFTR,*in the islet as well as their uncertain expression and function in insulin-secreting β-cells. The aim of this study was to systematically demonstrate *CFTR*expression within the context of a Cl^-channel machinery that includes, *ANO1*, a Ca^{2+}-activated Cl^-channel and all subunits of the volume-regulated anion channel (*LRRC8A-E,*VRAC). To accomplish this, we used a combined molecular, electrophysiological, immunological (19 antibodies) and functional approach. Our initial results reveal heterogeneous Cl^-channel expression in all endocrine cells of the pancreatic islet;*CFTR*is present in ~30% of β-cells and in some, but not all, α/δ/γ- or PP-cells whereas *ANO1*is found throughout all endocrine cells of the islet. Notably, all subunits of VRAC are expressed at different levels within different endocrine cells of the islet. Further, we demonstrate that *CFTR*modulates the secretory response in normal human and mouse islets suggesting that this channel plays a role in amplifying the secretory response to the K_{ATP}triggering signal.

P26

CFTR correctors restore the alcohol-induced expression defect in pancreatic ductal cells

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Introduction: Heavy alcohol intake is one of the most common causes of acute pancreatitis (AP). Our group previously showed that ethanol causes severe functional defect and impaired expression of the cystic fibrosis transmembrane conductance regulator (CFTR), which increases the severity of acute alcohol-induced pancreatitis. New compounds such as VX-770 (Ivacaftor) and VX-809 (Lumacaftor) are available to correct the impaired CFTR function and expression in cystic fibrosis patients with specific mutations, which might be utilized in the treatment of alcohol-induced AP.

Aim: Our aim was to test the effect of VX-809 and VX-770 on the CFTR expression during ethanol exposure.

Materials and methods: CFTR expression was evaluated by immunofluorescent staining in Capan-1 cells and isolated guinea pig pancreatic ducts. Images were captured by confocal microscopy.

Results: Exposure of Capan-1 cells and guinea pig pancreatic ductal cells to 100mM ethanol for 12 hours significantly decreased the plasma membrane expression of CFTR. In parallel the cytoplasmic CFTR expression was increased. The application of 10μM VX-809 beside the alcohol significantly improved the plasma membrane expression of CFTR in Capan-1 and pancreatic ductal cells. The plasma membrane expression also increased by 10μM VX-770 or the combination of VX-809 and VX-770 in pancreatic ductal cells during ethanol exposure.

Conclusion: These preliminary findings suggest that VX-809 and VX-770 might be able to restore the CFTR expression defect caused by alcohol. Further extended in vitro and in vivo studies need to clarify the effect of the 2 drugs on alcohol-induced pancreatic injury.

P27

Interactions between the exocrine and endocrine pancreas under pathological conditions and the effect of CFTR function

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Background: The exocrine and endocrine pancreas work in close interaction with each other in which the CFTR Cl^- channel plays an essential role by regulating ductal secretory processes and as shown by some studies, β-cell insulin secretion. The role of the channel under certain pathological conditions is not completely known, therefore our **aim** in this study was to investigate the exocrine and endocrine functions in diabetes- or pancreatitis-induced wild-type (WT) and CFTR knock out (KO) mice.

Methods: Intra-interlobular pancreatic ductal fragments were isolated from WT and CFTR KO mice by enzymatic digestion. Pancreatic ductal fluid and HCO_3^- secretion was measured by *in vivo* fluid secretion measurements and fluorescence microscopy. Islet functions were investigated by serum insulin and glucagon measurements. Pancreas tissue sections were prepared and immunhistologically stained against insulin, glucagon and CFTR to observe islet and ductal morphology. Pancreatitis was induced by intraperitoneal injection of cerulein and disease severity was assessed by evaluation of histological sections and serum amylase measurements. Diabetes was induced by ip. administration of streptozotocin and disease development was confirmed by glucose tolerance test.

Results: Pancreatic ductal fluid and HCO_3^- secretion significantly increased in diabetic and decreased in pancreatitis-induced mice. Serum levels of insulin decreased in diabetic animals, whereas the serum levels of glucagon was unaltered. The absence of CFTR decreased ductal fluid and HCO_3^- secretion and resulted lower serum insulin levels. Immunhistological staining of insulin and glucagon in pancreas tissue sections revealed a morphological change in CFTR KO islets with higher content of α-cells that localize towards the center.

Conclusion: Our results suggest that CFTR Cl^- channel plays a key role in ductal HCO_3^- secretion and has a direct or indirect role in islet function and structure.

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P28

Cell proliferation upregulates ANO1 in mucus cell hyperplasia

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Background: The human airway epithelium consists in a pseudostratified multicellular layer formed by different cell types expressing a wide variety of ion channels. One of these is the calcium-activated chloride channel (CaCC) Anoctamin 1 (ANO1), which was found to be upregulated by IL-4, a Th2 cytokine that induces mucus cell hyperplasia. Interestingly, ANO1 is also over-expressed in several cancers and reported as a cell proliferation and migration biomarker, thus being a plausible player in proliferation and metastasis.

Aim: Our aim is to study how epithelial differentiation influences ion transport and mucus production during differentiation and inflammation.

Methods: For this purpose, we used the human airway Basal Cell Immortalized Non-Smoker (BCi-NS1.1) line that differentiates into all epithelial cell types present in the airways when cultured under Air-Liquid Interface (ALI) for 30 days.

Results and Discussion: We assessed the levels of ANO1 and MUC5AC (a major airway mucus component) during differentiation and found an inverse correlation: while ANO1 is almost absent in differentiated cells, MUC5AC is abundantly expressed. Moreover, IL-4 induced ANO1 upregulation with a concomitant increase in cell proliferation (assessed by Ki-67). However, the ANO1 induction was inhibited by the proliferation blocker mitomycin C. Consistently, promotion of differentiation into mucus-producing cells with the Notch1 ligand DLL4 did not induce any change in ANO1 expression levels, despite the significant increase in MUC5AC levels. These results suggest that upregulation of this channel is specific to mucus cell hyperplasia (i.e., proliferation), but not metaplasia. Moreover, MUC5AC induction occurs independently of ANO1 expression.

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P29

Dysfunction of Anoctamin 1 (Ano1) in F508del mouse pancreatic b-cell

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Cystic Fibrosis (CF) is complicated by a form of diabetes (CFRD) mainly characterized by a gradual decline in insulin secretion. Although not apparent at birth, its prevalence increases with age, complicating CF in about 50% of the patients > 35 years, worsening their condition and increasing mortality. Yet little is known on the underlying mechanisms and the functional defects of CFRD within the β-cell. Interestingly, the Anoctamin 1 (Ano1) channel, a calcium-activated chloride channel, has been shown to contribute to insulin secretion and its activity appears to be linked to CFTR function.

First Ano1 is expressed by mouse pancreatic b-cells as observed in rat and human b-cells. By immunohistochemistry we localized Ano1 in pancreatic islets and by patch clamp analysis we detected a Ca^{2+}-activated anion channel. Increase in extracellular glucose is a pacemaker inducing cyclic oscillations in b-cell membrane potential. After an initial depolarization, due to closure of the K_{ATP} channels, alternating phases of depolarization (active) and repolarization (silent) phases can be observed partially explained by the gating of Ano1. During the active phases, spikes then repeated bursts of action potentials (AP) occur witnessing that the threshold for opening voltage-dependent Ca^{2+} channels has been reached, allowing Ca^{2+} entry and triggering insulin release.

By patch clamp, we studied the fluctuations of the b-cell membrane potential induced by glucose, 11.1 mM. Their pattern was similar in young F508del mice (< 1 year) vs WT mice, except a hyperpolarized b cell membrane resting potential. On the contrary this pattern was altered in aged F508del mice (>1 year) when compared to matched WT mice. In these F508del mice, the resting membrane potential was hyperpolarized (-81 vs -74 mV in WT; p=0.0002) and the number of AP/30 min was drastically reduced (811 vs 1394 in WT; p=0.01) with the bursting second phase peak amplitude partially repolarized (-29 vs -15 mV in WT; p=0.009). Moreover the duration of the silent phase separating the first spiking active phase and the second bursting continuous phase was increased (715 vs 275 sec in WT; p=0.025). The Ano1 potentiator bromophenyltetrazolbenzamide (Fact) was used to further study Ano1 contribution, first in WT mice. When Fact was added simultaneously with the increased glucose concentration, no silent phase was anymore observed and the number of AP/30 min was increased from 1394 to 3475 (p=0.0002). When Fact was added after the increase in glucose concentration, the AP events/30 min increased from 1728 to 3240 (p=0.01) while the AP peak was further depolarized (-16 vs -21 mV; p=0.007). Thus in F508del as in WT b-cell, Fact increased AP number and at least partially corrected the decreased electrical activity of F508del b-cells.

Insulin secretion by isolated islets is clearly increased by Fact in WT mice. Its effect in aged F508del mice was therefore examined here.

In conclusion, b-cell from F508del mice exhibit membrane hyperpolarization and as the mice aged, dysfunctional Ano1 gating appears leading to reduced number of AP/30 min. Fact appears to correct Ano1 dysfunction in F508del mice and thus is of potential interest in CFRD.

P30

TMEM16A channel function does not influence goblet cell numbers in the human airway epithelium

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A role for the calcium activated chloride channel, TMEM16A, in the regulation of airway goblet cell populations has been recently reported (Kondo et al., 2017; Lin et al., 2015; Qin et al., 2016). The published data to support a role for TMEM16A in driving the formation of goblet cells, are largely based on gene silencing and the use of non-specific TMEM16A blockers (e.g. niflumic acid). It is unclear whether this proposed function is therefore dependent on the ion channel activity of TMEM16A or through an alternative aspect of the proteins function. Using a potent TMEM16A channel blocker (Ani9) and a recently identified TMEM16A potentiator compound (ETX003; Enterprise Therapeutics; proprietary), we have evaluated whether channel function can regulate goblet cell numbers in primary cultures of human bronchial epithelial (HBE) cells.

Primary HBE (3 donor codes; non-CF) were cultured for 2 weeks at air-liquid interface (ALI) on permeable supports and formed a well-differentiated mucociliary epithelium. On ALI day 15, cells were treated with either: 1) vehicle, 2) IL-13 (10 ng/mL) or 3) ETX003 (1 μM) with each group in either the absence or presence of the TMEM16A blocker, Ani9 (10 μM). ETX003 is a TMEM16A potentiator with an EC_{50} of 150-300 nM for the potentiation of chloride secretion in patch clamp (FRT-TMEM16A) and ion transport (HBE). Ani9 fully blocks TMEM16A function in both patch clamp and HBE ion transport studies. Cells were cultured under these conditions for 96h before fixation and staining with antibodies directed against MUC5AC (goblet cells) and acetylated α-tubulin (ciliated cells). Goblet and ciliated cell numbers were quantified using an automated image acquisition (Zeiss Axiovert) and analysis system (Image J).

IL-13 induced a significant increase in the density of goblet cells (7-25-fold depending on donor code) based on the increased staining for MUC5AC, that was unaffected by co-administration of Ani9. ETX003 also failed to influence goblet cell numbers and Ani9 was likewise without effect. Finally, the co-administration of ETX003 with IL-13 also failed to modify goblet cell numbers.

Together, these data do not support a role for the ion channel function of TMEM16A in the regulation of goblet cell numbers in primary HBE.

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P31

Physiological role and therapeutic importance of TMEM16A chloride channel in the airway epithelium

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Loss of function of CFTR in the airways is the cause of multiple defects resulting in impairment of mucociliary clearance and innate defense mechanisms. Correction of cystic fibrosis (CF) abnormalities may be obtained by restoring the function of CFTR with mutation-specific treatments or by modulating other proteins. In this respect, airway epithelia express a second type of anion channel, the TMEM16A protein (also known as anoctamin-1), which is a calcium-activated chloride channel. TMEM16A is localized on the surface epithelium and in sub-mucosal glands, where it is abundantly expressed in mucous cells, particularly under inflammatory conditions. The stimulation of this alternative anion channel could be a way to circumvent the CFTR defect, especially for those cystic fibrosis patients expressing undruggable CFTR mutants. However, it is also possible that inhibition, rather than activation, is needed to decrease mucus in CF airways (Benedetto et al., FASEB J 2018).

We found that modification of TMEM16A carboxy-terminus results in channels that are active even at low intracellular calcium concentations (Scudieri et al., SciRep 2016).

Our strategy is to express this genetically-activated version of TMEM16A (GA-TMEM16A) in CF airway epithelial cells to understand if this results in correction of CF abnormalities. We use lentiviral vectors to deliver GA-TMEM16A in well-differentiated CF bronchial epithelial cells. As a control, we also have a genetically-inactivated version of TMEM16A (GI-TMEM16A). After the assessment of the efficiency of infection and expression of the different versions of TMEM16A protein, we are proceeding with the evaluation of function and the consequences on ASL properties. By short-circuit current recordings we found that the activity of GA-TMEM16A is quite persistent after stimulation with low concentration of calcium-elevating agents in contrast to the wild-type TMEM16A protein whose activity is typically transient. As expected, GI-TMEM16A had no function if compared to endogenous TMEM16A. Regarding ASL properties, we observed an increased ASL thickness in epithelia expressing GA-TMEM16A compared to WT-TMEM16A and control CF epithelia. Studies are in progress to evaluate the effect of TMEM16A enhanced function in mucus production and release.

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E_{ACT} increases intracellular calcium levels by a TMEM16A-independent mechanism

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The N-aroylaminothiazole E_{ACT} was first described by Namkung et al. (2011) as an activator of the calcium-activated chloride channel, TMEM16A. Subsequently, E_{ACT} has been used as a tool compound by investigators to describe a wide variety of putative physiological functions of TMEM16A. The aim of the present study was to compare the pharmacology of E_{ACT} with alternative potentiators of TMEM16A that have been recently discovered by high-throughput screening.

Consistent with the original reported pharmacology, E_{ACT} increased anion secretory responses in models of epithelial ion transport that could be attenuated with the TMEM16A blocker, Ani9. Similarly, novel TMEM16A potentiators from 3 structurally distinct chemical series also increased the Ani9-sensitive anion secretion in these ion transport models.

To understand the mechanism of activation of these anion secretory currents, the effects of E_{ACT} and the novel TMEM16A potentiators on levels of intracellular calcium ($[Ca^{2+}]_i$) were evaluated. The acute addition of E_{ACT} to primary CF-HBE induced a concentration-dependent increase in $[Ca^{2+}]_i$. Pre-treatment of cells with Ani9 had no effect on the E_{ACT}-induced rise in $[Ca^{2+}]_i$. In contrast, the novel TMEM16A potentiators had no effect on $[Ca^{2+}]_i$.

The observation that E_{ACT} could increase $[Ca^{2+}]_i$ questioned the reported pharmacological mechanism of TMEM16A activation by this molecule ie. via a direct interaction with the channel. To address whether E_{ACT} could directly activate TMEM16A in the absence of an elevation of $[Ca^{2+}]_i$, patch-clamp studies were performed under conditions of buffered $[Ca^{2+}]_i$. Under these conditions, with $[Ca^{2+}]_i$ tightly clamped, E_{ACT} showed no evidence of any activity on TMEM16A. In contrast, the novel TMEM16A potentiators all showed a potent increase in channel function.

Together, these data do not support the description of E_{ACT} as a direct TMEM16A modulator but are consistent with its activation of TMEM16A being indirect, the result of an as yet undefined mechanism leading to an elevation of $[Ca^{2+}]_i$. Furthermore the recent proposal that TMEM16A positively regulates $[Ca^{2+}]_i$ (Cabrita et al., 2017) is not consistent with the lack of effect of either the TMEM16A blocker Ani9 or the novel potent and selective TMEM16A potentiators on $[Ca^{2+}]_i$. Our data suggest that literature reports of TMEM16A function that have relied on the use of E_{ACT} as a pharmacological tool should be interpreted with caution.

Namkung et al (2011) FASEB J 25(11):4048-4062

P33

Identification of pharmacological modulators of the TMEM16A chloride channel by high-throughput screening

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TMEM16A (anoctamin-1) is a calcium-activated chloride channel expressed in airway epithelial cells, particularly under conditions of mucus hypersecretion. Activation of TMEM16A by calcium agonists results in enhanced chloride and bicarbonate secretion. The physiological role of TMEM16A, which is upregulated under conditions that favor mucus hypersecretion, and its involvement in cystic fibrosis (CF) are still unclear. Our aim is to identify novel pharmacological modulators of TMEM16A function. Using the halide-sensitive yellow fluorescent protein (HS-YFP) functional assay, we screened a maximally-diverse chemical library (11,300 compounds). For this purpose, we used FRT cells co-expressing TMEM16A (abc isoform) and HS-YFP. Cells were stimulated with a low concentration of UTP to induce partial activation of TMEM16A. TMEM16A potentiators and inhibitors are detected as compounds that accelerate or slow down the rate of HS-YFP quenching, respectively. Controls during the screening included the Ani9 inhibitor (Seo et al., PLoS One 11:e0155771, 2016) and a TMEM16A potentiator that we identified in a previous study. After screening the whole library in duplicate, we found 24 compounds that increase TMEM16A activity above a threshold equal to 50% of the positive control. These hits are evaluated in secondary assays based on: i) null FRT cells, to rule out compounds acting on other channels/transporters; ii) CFPAC-1 cells (which have endogenous TMEM16A expression), to confirm activity in a second cell type; iii) stimulation of TMEM16A with ionomycin, to bypass purinergic receptors; iv) evaluation of intracellular calcium mobilization with a fluorescent probe, to detect compounds with an indirect mechanism of action. Compounds with the best characteristics will be tested in CF human bronchial epithelial cells (short-circuit current experiments) to assess the effect on calcium-dependent chloride secretion. The screening also identified possible inhibitors of TMEM16A. Novel pharmacological modulators of TMEM16A (potentiators and inhibitors) could be useful as tools of research and as possible therapeutic agents to improve mucociliary function in CF and other respiratory diseases.

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Identification of novel ANO1/TMEM16A regulators as alternative therapeutic targets for cystic fibrosis

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Background: A promising therapeutic strategy for Cystic Fibrosis (CF) explores the modulation of alternative (non-CFTR) apical membrane Cl^- channels. Due to its role as a Ca^{2+}-activated Cl^- channel, its close relationship with CFTR, and its expression in the airways, TMEM16A/Anoctamin 1 (ANO1) has been suggested as a possible drug target for CF.

Aim: To identify regulators of ANO1 as potential drug targets for novel “mutation-agnostic” therapies for CF.

Methods: We have generated CF Bronchial Epithelial (CFBE) cells expressing double-tagged ANO1 with an enhanced green fluorescent protein (eGFP) fused to the C-terminus and a triple hemagglutinin (3xHA) tag at an extracellular loop, under an inducible promoter (Tet-ON). These cells were used in a microscopy-based traffic screen to identify ANO1 regulators. The hits obtained with the ANO1 traffic screen were validated using the halide sensitive (HS)-YFP assay in HEK 293 cells expressing exogenous ANO1. The effect of the resulting main hits on ANO1 function was then assessed by whole-cell patch-clamp. The mechanism of action (MoA) of the identified ANO1 regulators is currently being studied using biochemical and physiological assays.

Results and Discussion: A high-throughput siRNA screen for ANO1 traffic using a library of siRNAs targeting 691 genes previously identified as CFTR regulators or CFTR-interacting proteins (Amaral lab, unpublished data) revealed 179 genes regulating ANO1 PM traffic. These hits are involved in pathways including regulation of transcription, G-protein coupled receptor (GPCR) signalling, phosphorylation, inflammatory response, and proliferation. A secondary screen using the HS-YFP assay identified 30 hits that modulate the ATP-induced YFP-quenching in HEK-ANO1-HS-YFP cells. Out of the 30 hits, 8 were further selected for functional validation through whole-cell patch-clamp, based on their biological functions. Two GPCRs were discovered as the strongest ANO1 regulators, and their MoA is currently being explored. These results will possibly reveal alternative drug targets to compensate for the absence of CFTR function from which all people with CF could benefit.

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Partial Epithelial to Mesenchymal Transition (EMT) in CF: CF as an epithelial differentiation disorder

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Background: Although CF is mostly a channelopathy being CFTR mostly studied for its role as an anion channel, this plasma membrane (PM) protein has also been implicated in cellular processes apparently unrelated to ion transport, namely epithelial cell differentiation and, when dysfunctional, cancer. CFTR-defective cells/epithelia have indeed impaired differentiation, according to a growing amount of evidence which show in CF:

1) compromised development (e.g. tracheal malformations and CBAVD);

2) potentiation of tissue fibrosis;

3) abnormal wound healing;

4) defects in the tight junctions (TJs) and barrier function; and

5) increased epithelial cell proliferation.

Consistently, CFTR itself has been described as a tumour suppressor gene in several cancers. Transcriptomic data identified that a dedifferentiation/epithelial to mesenchymal transition (EMT) signature is characteristic of the CF epithelium. EMT is a complex process whereby fully differentiated (polarized) epithelial cells change into a mesenchymal phenotype, giving rise to apolar fibroblastoid cells. EMT was recently found to be active in several chronic lung diseases, like COPD and IPF, which share overall features with CF. In these diseases, EMT was not an "all-or-none" process, with cells displaying hybrid epithelial/mesenchymal states, in a process termed “partial EMT”.

Aim: To demonstrate the occurrence of (partial) EMT in CF epithelia, and to elucidate the fundamental role of CFTR in the maintenance of epithelial differentiation.

Methods: We used three types of materials:

1) native lung/bronchial tissue from CF patients and non-CF controls;

2) primary cultures of human bronchial epithelial cells (HBEs); and

3) human CFBE cell line stably expressing wt or F508del-CFTR.

Cryocuts of human CF and non-CF airway tissue were characterized by immunofluorescence (IF) regarding expression and localization of epithelial (e.g. E-cadherin, ZO1, CK18, claudin1); mesenchymal (e.g. N-cadherin, vimentin); EMT (SNAIL, TWIST); and proliferation (KI67) markers. Polarized CFBE and HBEs were tested using a similar panel of markers by both IF and Western blot (WB). Measurements of TEER and wound healing assays were also performed on these cellular models to measure differentiation and proliferation.

Results and Discussion: IF revealed a mislocalization of epithelial markers in CF vs control tissues/cells, revealing a general disorganization of epithelial structures, as well as an increase in EMT and proliferation markers in CF materials. The increase in mesenchymal protein levels was confirmed by WB in both F508del-CFTR CFBE and HBEs from CF patients vs controls. These CF cells also displayed significantly reduced TEER values, consistent with impaired barrier function/TJ defects, and an impaired migration but/and increased proliferation in wound healing assays. Altogether, these data indicate that CF cells are shifted to a less differentiated/more proliferative state. Our results confirm that dysfunctional CFTR has a direct impact on epithelial differentiation and, moreover, partial EMT occurs in CF bronchial epithelial cells.

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Targeting proton secretion in CF airway epithelial cells to help restore airway homeostasis

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Background: Targeting the acidic airway surface liquid (ASL) pH in Cystic Fibrosis (CF) could potentially help restore bacterial killing, ASL hydration and mucociliary clearance. Both CFTR and the non-gastric H^+/K^+-ATPase ATP12A have been implicated in ASL pH regulation. However other transporters and ion channels might also be involved. We have recently shown that chronic treatment with the clinically approved ATP4A Proton Pump Inhibitor (PPI), esomeprazole (Eso), was able to significantly increase ASL pH in CF airway cells without impairing epithelial integrity[1]. CRISPR-Cas9 experiments showed that the Eso-induced ASL alkalinisation was ATP12A-dependent. Surprisingly, acute exposure to this PPI induced an intracellular acidification that was ATP12A-independent. Our current work now focuses on (i) identifying the H^+ transporter targeted by Eso and (ii) testing other PPIs as well as potassium-competitive acid blockers (P-CABs) on ASL pH homeostasis.

Methods: ASL pH of primary human airway epithelial cells (hAECs) was measured using a mixture of dextran-coupled pHrodo, (0.1mg/ml) and AlexaFluor488 (0.1 mg/ml), diluted in a modified Krebs solution in the presence of either vehicle control (DMSO) or the K^+-competitive inhibitor of H^+/K^+-ATPases SCH-28080 (SCH, 10 µM), or the NHE inhibitor EIPA (3 µM). The next day, ASL pH was recorded in a temperature and CO_2 equilibrated plate-reader. Moreover, the effect of the inhibition of H^+ channels and other H^+ transporters on pH regulation was also assessed by intracellular pH (pH_i) experiments, using cells loaded with the pH-sensitive dye BCECF-AM.

Results: In non-CF hAECs, overnight apical treatment with EIPA did not change resting ASL pH (CTL: 6.76±0.25, EIPA: 6.84±0.14, n=5, p=0.39). In a similar way, SCH had no effect on ASL pH (CTL: 6.86±0.27, EIPA: 7.05±0.13, n=5, p=0.19). Moreover, both inhibitors had no effect on the forskolin-induced alkalinisation of the ASL. In CF hAECs, acute exposure to K^+-free solution (inhibiting H^+/K^+-ATPase), $ZnCl_2$ (100 µM, blocking the voltage gated H^+ channel HVCN1) and Eso (300 µM) induced an acidification of pH_i, whereas Bafilomycin A1 (0.1 µM, blocking V-ATPase) and EIPA (10 µM, inhibiting NHE) had no effect on pH_i. Interestingly, $ZnCl_2$ had a significant additive effect to the $0K^+$-induced acidification, in a similar way to the combination of Eso in $0K^+$ solution[1], suggesting that the unknown target of acute exposure to Eso could be HVCN1.

Conclusion: This study has identified a new potential H^+ transporter that is targeted acutely by Eso. Future work will evaluate the efficiency of other inhibitors such as the P-CABs on ASL pH homeostasis in CF hAECs as well as HVCN1 activity. Considering the potential benefits of PPIs against bacterial and viral infections, oxidative stress and inflammatory processes, we propose that the inhibition of multiple H^+ transporters by the use of PPIs (potentially combined with future identified inhibitors) in CF airways might help alleviate CF lung pathophysiology.

References: [1]Delpiano L et al Frontiers in Pharmacology 2018

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Evaluation of different growth conditions on acid-base homeostasis in non-CF and CF airway epithelial cells

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Introduction: Fully differentiated primary human Airway Epithelial Cells (hAECs) are currently an excellent in vitro model used in CF research. These cells form a pseudostratified epithelium that exhibits morphological and functional characteristics of human airways in vivo. Despite their widespread use, different protocols and growth conditions are used by different groups. This could potentially explain discrepancies in published results, such as differences in the pH of airway surface liquid (ASL). This is important, because ASL pH regulates epithelial function, mucin formation and antimicrobial activity, and is therefore a possible therapeutic target for CF airway disease. Our aim here was to compare three different commonly used media to establish if these impact on acid-base homeostasis in fully differentiated non-CF and CF airway cultures.

Methods: Primary hAECs were grown at air-liquid interface using three different conditions: (i) a medium developed by Dr Randell in Chapel Hill (UNC),

(ii) a medium containing the commercial synthetic serum Ultroser™ G (UG), and

(iii) the commercially available StemCell's PneumaCult™-ALI Medium.

pH homeostasis was studied by the measurement of ASL pH, intracellular pH (pHi) and short circuit current (Isc), in the presence or absence of chloride to discriminate bicarbonate from chloride transport.

Results: To date, sufficient independent experiments have so far been obtained using UNC and UG media. For both, resting ASL pH was not significantly different between CF and non-CF hAECs, but UG non-CF cells had a higher ASL pH than UNC non-CF cultures (7.22±0.14 vs 6.61±0.1). Furthermore, forskolin (Fsk) caused little change in ASL pH in UG non-CF cells, but significantly increased pH in UNC non-CF cells. These results were consistent with a higher 'resting' CFTR-dependent Isc in UG conditions. Moreover, the total Fsk-induced and CFTRinh172 (172)-sensitive chloride and bicarbonate Isc was larger in UNC compared to UG cultures. However, bicarbonate transport accounted for only 10% of the total Fsk-induced and 172-sensitive Isc in UNC conditions whereas it constituted 50% of the Fsk-induced and 20% of the 172-sensitive Isc in UG conditions. This suggests that UG medium increased either the bicarbonate permeability of CFTR or a CFTR-dependent electrogenic apical Anion Exchange (AE) activity. In contrast, the proportion of UTP-induced bicarbonate transport was similar in UNC and UG conditions for both CF and non-CF cells. Using intracellular pH measurements, UNC cultures had increased H+/K+-ATPase and basolateral AE activities in both CF and non-CF cells compared to UG cultures. Furthermore, although a resting apical AE activity was not detected under any growth conditions, in 3 out of 4 experiments on UG non-CF cells, the addition of Fsk led to the activation of apical AE activity.

Conclusion: Overall, growth conditions significantly impacted on the expression/activity of key ion channels and transporters involved in acid-base homeostasis in airway cells,which correlated with significant differences in resting ASL pH and the response to Fsk. This study therefore highlights the need for better awareness of the effects of growth conditions on ion transporters, and potentially the need for standardisation of this parameter in CF research.

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Assessment of alternative chloride channel-dependent fluid secretion in nasal and bronchial organoids of individuals with CF and control subjects

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An alternative method to restore airway epithelial fluid secretion in individuals with cystic fibrosis (CF) is to bypass the dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) protein by activation of alternative ion channels. TMEM16A/ANO1 represents such an alternative ion channel and is mainly expressed by secretory epithelial cells in the airways. This anion channel can be activated by the small molecule Eact. We developed a functional organoid swelling assay to study epithelial fluid secretion in airway organoids, which can be used to examine alternative ion channel activity in individuals with CF. Here, we examined Eact-induced fluid secretion in paired nasal and bronchial organoids from individuals with CF, and we compared CF with non-CF control subjects. The contribution of secretory cells on Eact-induced swelling was addressed by determining the effect of Notch signaling inhibition in CF nasal organoids. We observed higher fluid secretion upon Eact stimulation in nasal compared to bronchial organoids from CF subjects. Moreover, CF nasal organoids displayed higher Eact-induced fluid secretion compared to non-CF controls. Notch inhibition decreased the differentiation of secretory cells in CF nasal cell cultures, while enhancing ciliated cell numbers. This was paired with attenuated Eact-induced fluid secretion. These results suggest differences in alternative ion channel activity between nasal and bronchial epithelial cells from CF subjects and an effect of disease status on Eact-induced fluid responses. This might be explained by differences in epithelial cell differentiation, whereas depletion of secretory cells by Notch inhibition reduced Eact-induced fluid secretion. Further research is required to determine the contribution of TMEM16A, whereas inhibition of Eact-induced fluid secretion upon Notch inhibition was not paired with reduced TMEM16A mRNA expression levels.

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Synergistic inhibition of CFTR-dependent chloride secretion by urban air pollution particulate matter and oxidative stress in airway epithelial cells

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Introduction: The FDA recommends that clinically relevant drug-drug interactions be defined during drug development and assessed in prospective clinical studies prior to filing a new drug application. Consequently many interactions of approved CFTR modulators with other drugs are known. By contrast, the interactions of CFTR modulators with environmental factors have not been studied although they could impact efficacy. For example rescued F508del-CFTR is downregulated by lower concentrations of cigarette smoke extract compared to wild-type CFTR (1). Urban air pollution particulate matter (PM) induces ER stress and the unfolded protein response (2) and elevates oxidative stress that may contribute to the cellular response to PM (3), however the effect on rescued F508del-CFTR after corrector treatment is not known.

Objective: To examine the effects of PM and oxidative stress individually and in combination on airway epithelial cells, comparing responses of non-CF and CF cells.

Methods: Polarized CFBE cell monolayers and primary HBE cells mounted in Ussing chambers were used to measure chloride secretion. qPCR was performed on targets associated with oxidative stress. PM (standard reference material 1648) from the National Institute of Standards and Technology was used as PM.

Results: CFTR-dependent secretion was unaffected by overnight exposure to PM alone but was strongly inhibited when cells were treated with both PM and the oxidant stressors tertiary butylhydroquinone (tBHQ) or hydrogen peroxide (H_2O_2). CFBE cells expressing F508del-CFTR that had been partially rescued by the corrector drug VX-809 were more susceptible to PM + oxidant stress than cells expressing WT-CFTR. mRNA transcript levels for the antioxidant enzymes glutathione synthetase, superoxide dismutase 2, and catalase were elevated by oxidant exposure as expected, however this response was also impaired in cells expressing F508del-CFTR and further reduced by the presence of PM + oxidant stress. Immunoblots of primary human bronchial epithelial cell lysates revealed a slight decrease in CFTR protein expression, although a reduction in basolateral transport also contributed to the acute inhibition of CFTR-dependent secretion by oxidants.

Conclusions: We conclude that oxidant stress and PM cause synergistic inhibition of airway secretion and antioxidant defenses are compromised in CF cells. Finally, much of this inhibition of CFTR-dependent secretion may occur at the basolateral membrane.

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PLCβ3 is involved in forskolin-dependent CFTR activation

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Background: CFTR current is controlled by two processes: R domain phosphorylation by PKA and ATP binding on NBDs. Intracellular calcium leads to an indirect activation of CFTR through i) adenylyl cyclase and tyrosine kinases calcium-dependent activation and ii) effectors activation such as EPAC1 (Lérias et al., 2018). A direct activation of CFTR by intracellular calcium can even occur via an interaction between calmodulin and the R domain of CFTR (Bozoky et al., 2017). Consequently, signalling pathways increasing intracellular calcium, could modulate CFTR activity (Billet and Hanrahan, 2013). Phospholipases C (PLC) degrade PIP_2 to generate DAG and IP_3. The latter, upon binding to its receptor, leads to an intracellular calcium release. Our work shows that PLC inhibition decreases CFTR current activated by forskolin.

Objective: Among PLC isoforms expressed in pulmonary epithelial cells, we intend to identify which isoform(s) play(s) a part in CFTR current in order to modulate it by a specific activation.

Methods: The effects of silencing or pharmacological modulation of PLC on CFTR current was studied in Ussing chamber experiments in polarized non CF (16HBE), CF over-expressing F508delCFTR (F508delCFBE) and CF over-expressing wtCFTR (wtCFBE) cell lines. Expression of PLC and transfections efficiencies were confirmed by western blotting.

Results and discussion: In non CF and temperature corrected CF cell lines, the pharmacological and non specific inhibition of PLC leads to a decreased CFTR current previously activated by forskolin around 40%. Moreover, in 16HBE cell line, inhibiting PLC before forskolin activation also decreases CFTR current in a significant manner. We identified 3 isoforms of PLC expressed in 16HBE: PLCδ1, PLCγ1 and PLCβ3. The use of specific siRNA showed that PLCδ1 is not implicated in forskolin-dependent activation of CFTR, whereas PLCγ1 and especially PLCβ3 are involved in this process. PLC, by production of IP_3, increases intracellular calcium. It has been shown that production of IP_3 increases after forskolin stimulation on cells over-expressing PLC compared to non stimulated cells (Schmidt et al., 2001). But a direct interaction between PLCβ3 and CFTR within a macromolecular complex, that could potentiate CFTR current, cannot be excluded. Indeed, NHERF2 interacts with CFTR (Li et al., 2005) and also with PLCβ3 (Hwang et al., 2000).

Conclusion PLCβ3 acts as a potentiator of the forskolin-dependent CFTR activation. As we know that PLCβ3 is under-expressed in the CF cell line CFBE, it could be interesting to activate this PLC isoform in order to increase F508delCFTR current after trafficking correction.

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Autophagy in cystic fibrosis

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Introduction: The self-degradative process, termed autophagy, is an essential cellular process by which damaged organelles, as well as misfolded and aggregated proteins, are cleared from the cell in a non-inflammatory manner. However, this mechanism is postulated to be defective in cystic fibrosis (CF), as a result of abnormal function and production of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In CF the accumulation of defective CFTR in the cytosol, and a lack of expression of functional CFTR on the cell membrane, leads to perturbation of autophagy, defective bacterial clearance and amplified inflammation. In this study the state of autophagy was investigated using human bronchial epithelial cell (HBEC) lines with the main CFTR mutations.

Methods: HBEC lines Beas-2b (WT), IB3-1 (CF - ΔF508/W1282X), CUFI-1 (CF - ΔF508/ΔF508) were stimulated with the autophagy inducer Rapamycin (1 mM) and also with the autophagy inhibitor chloroquine (50 mM) for 24 hours. The autophagy proteins LC3B, p62, beclin1 and vps34 were assessed via western blot. HBECs were also stimulated with rapamycin and chloroquine for 24 hours and their autophagosomes quantified using autophagosome dye and flow cytometry assays.

Results: The CuFi-1 cell line showed an increased ratio of LC3BII/LC3BI under basal conditions in comparison to the WT cell line Beas-2b, this difference was increased greatly with the addition of rapamycin and chloroquine and was statistically significant ($p < 0.005$). Beclin1 was also increased in both IB3-1 and CuFi-1 cell lines, under basal conditions compared to Beas-2b, and again the difference became greater upon addition of rapamycin and chloroquine, however not significantly. The number of autophagosomes present in the CuFi-1 cell line under basal conditions was significantly greater ($p < 0.00005$) than Beas-2b, and remained so upon addition of rapamycin and chloroquine. The IB3-1 cell line, however, did not show any difference to Beas-2b under basal conditions after the addition of rapamycin and chloroquine.

Conclusion: These data indicate the upregulation of different components of the autophagy pathway and appear to be mutation specific, as the ΔF508 homozygous cell line demonstrated significant differences to the WT whereas the ΔF508 heterozygous did not.

P43

A new approach for cystic fibrosis diagnosis based on ion ratios from non-invasively obtained skin-wipe sweat samples analyzed by capillary electrophoresis with contactless conductometric detection

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Cystic fibrosis (CF) is caused by a malfunction in the CFTR chloride channel in sweat ducts impairing the reabsorption of chloride anions that results in high salt content of the sweat. In CF diagnostics, sweat testing using pilocarpine sweat induction (Macroduct) and analysis of chloride concentration is used. While chloride is the major ion, the transport of which is impaired, the related transport of other ions, such as sodium, potassium, calcium or bicarbonate has been also of interest. Two years ago, we have developed a skin wipe test, in which the sweat is wiped from a patients forearm using a cotton swab moisturized in deionized water and extracted, yielding a sample in which all ions (anions and cations) can be analyzed [1]. The samples are analyzed by capillary electrophoresis with contactless conductometric detection (CE-C4D) and the major ions such as chloride, potassium and sodium were simultaneously analyzed in a background electrolyte containing 20 mM 2-(N-morpholino)ethanesulfonic acid, 20 mM L-histidine and 2mM 18-crown-6. When ratios of chloride to potassium ($Cl^-/K^+ > 4$) were used, CF patients were clearly separated from healthy controls, with 100% sensitivity. We present here the updated data from a large cohort of CF patients , healthy controls and CF carriers (in total > 200 subjects), collected in multiple CF centers between 2016 and 2018. The skin wipe test combined with total ionic analysis by CE-C4D has a great potential to be a suitable surrogate to standard Macroduct sweat test in CF diagnostics because it is simpler, faster (ca 7 minutes total time), cheaper (0.1 EUR vs 30 EUR) and completely non-invasive. Although the analysis and the ratio method has been used primarily in CF diagnostics in an attempt to improve the sensitivity of the conventional Macroduct sweat test and to gain better diagnostics of the patients who have the sweat chloride concentration in the intermediate area (30-60 mmoL/L), we see the potential of the developed sampling and analysis in understanding the function of the CFTR channels and share some of our new ideas in the presentation.

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P44

Whole-cell patch-clamp recordings of CFTR-mediated chloride currents in native and cultured nasal epithelial cells from CF and non-CF subjects

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Primary Human Nasal Epithelial Cells (HNECs) are used for preclinical tests of CFTR modulators (Pranke *et al., Sci Rep.* 2017; 7:7375). Cell expansion is used to amplify the modest number of cells collected by nasal brushing. However, it may modify protein expression and it is not known whether CFTR activity in primary cultures of patient-derived HNECs accurately reflects its activity in freshly isolated cells.

We compared CFTR-mediated Cl^- currents using the whole-cell configuration of the patch-clamp technique in freshly sampled nasal cells and in primary cultures of HNECs derived from the same individual. Clumps of freshly brushed HNECs were dissociated by gentle mechanical agitation, while HNEC monolayers were dissociated using collagenase followed by gentle mechanical agitation. Using the whole-cell configuration of the patch-clamp technique, CFTR-mediated Cl^- currents were recorded in viable ciliated cells which retained their polarity. Cells were collected from 6 healthy controls (WT), 2 CF patients with severe mutations (F508del/2143delT; G85E/G85E) with minimal activity and 6 CF patients with residual CFTR function mutations (F508del/R117H; R258G/L997F; F508del/c.870-1113-1110del; G551D/L997F; G1244E/R352Q; G551D/5T). Amiloride (10 µM) was added to extracellular solutions to inhibit ENaC. The magnitude of CFTR-mediated Cl^- current was quantified following activation with forskolin (10 µM) and inhibition by $CFTR_{inh}$-172 (10 µM). Finally, using the nasal potential difference (NPD) and Ussing chamber assays, we compared CFTR activity in freshly isolated and cultured HNECs with that in epithelia *in vivo* and *in vitro*.

Gigaseals were obtained on 20% of freshly isolated and 50% of cultured HNECs. Two to ten individual whole-cell recordings of CFTR-mediated Cl^- currents were successfully acquired for each condition and patient.

In WT HNECs, the magnitude of CFTR-mediated Cl^- current (recorded at +60 mV) ranged from 15.2 to 20.9 pA/pF in freshly isolated cells and from 15.1 to 22.4 pA/pF in cultured cells. No CFTR-mediated Cl^- current was observed in HNECs from the 2 CF patients with severe mutations. By contrast, CFTR-mediated Cl^- currents were observed in HNECs from all CF patients with residual CFTR function (freshly isolated: 1.8 – 7.3 pA/pF; cultured: 2.6 – 11.2 pA/pF at +60 mV). First analyses performed on 4 individuals (1 WT and 3 CF) demonstrated that the magnitude of CFTR-mediated Cl^- current recorded in freshly isolated HNECs are not significantly different from those of cultured cells from the same individual, regardless of the genotype. Analysis of the entire cohort and full correlation with different CFTR biomarkers (NPD, short-circuit current) will be presented during the conference.

In conclusion, we demonstrate that CFTR activity can be successfully recorded by the whole-cell patch-clamp technique in both freshly isolated and cultured HNECs, and it discriminates cells expressing different CFTR genotypes. First analyses show that CFTR activity in HNECs reflects that of native nasal epithelial cells.

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P45
3D printed disposable ussing chambers

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Electrophysiological measurement of chloride transport by the cystic fibrosis transmembrane conductance regulator (CFTR) is used to provide an invaluable assessment of the residual and modulator-stimulated activity of mutant versions of CFTR in cells derived from people with CF. Ussing chamber analysis is the industry standard for establishing the in vitro efficacy for mutant CFTR drug discovery, thus an Ussing chamber setup and the accompanying instrumentation are a standard fixture in most electrophysiology labs. To date, the cost of a single Ussing chamber necessitates its repeated reuse, albeit requiring an extensive cleaning protocol between uses in order to remove residual compounds so as not to cross-contaminate experiments. Despite thorough cleansing, protocols may or may not effectively remove CFTR modulators employed in the previous run, and additional wear on the chambers can be observed, particularly for more effective cleaning strategies. This is particularly a problem for “sticky” modulators such as the potentiator ivacaftor and related molecules. An effective, but expensive solution for eliminating modulator carry-over risk is to use each Ussing chamber only once, i.e. treat them as single-use, disposable equipment. However, to make a disposable Ussing chamber approach cost effective, a new method of chamber production is required.

Three-dimensional (3D) printer technology has advanced to the point where complex 3D objects can be designed and printed from a variety of materials ranging from insulating plastics to metals, and multiple material sources can even be employed simultaneously. This greatly increases the versatility and functionality of the objects that can be created using the technology. The resolution of the print, printable size, printing material type, and number of print sources all affect the cost of the 3D printer and its outputs. Herein we report the development of a 3D printable Ussing chamber system from relatively inexpensive materials, while employing an inexpensive 3D printer. The total investment is anticipated to be within the budget of even a single academic electrophysiology lab, and thus provides a time-saving solution to achieve cross-contamination-free Ussing chamber measurements of CFTR or another channel’s function. Comparisons of the performance of the 3D printed chamber to a standard manufactured Ussing chamber will be provided.

P47

CFTR processing mutations cause distinct trafficking and functional defects

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Introduction: Approved and investigational CFTR correctors and potentiators have the possibility to treat up to 90% of CF patients. Still, 10% of patients remain in need of specific therapies. This group comprises patients with two minimal function alleles, including certain severe protein processing mutations, e.g. N1303K and G85E. We established a flow cytometry assay to quantify single cell CFTR plasma membrane (PM) density. After validating the assay using the well-characterized processing mutation F508del, we next aimed at studying mutations with a known or suggested processing defect, i.e. G85E, A455E and N1303K and their responses to corrector VX-809 and potentiator VX-770.

Methods: We generated HEK293T cell lines stably expressing mutant CFTR with a 3HA-tag in the 4th extracellular loop (Sharma et al, 2004) under control of the CMV promoter after puromycin selection. G551D and WT served as controls for normal trafficking. We assessed CFTR maturation by western blot (WB), CFTR expression by immunocytochemistry (ICC) and quantified PM density by flow cytometry (% gated * median fluorescence intensity, MFI). CFTR function was assessed by halide sensitive YFP quenching assay ($1-F/F_0$). In parallel, CFTR maturation and function were studied in primary intestinal organoids overexpressing the respective CFTR mutants. CFTR function was assessed by forskolin induced swelling.

Results: On WB, G85E, A455E, F508del and N1303K showed a similar defect in CFTR maturation, namely an absent or strongly reduced band C. However, quantitative analysis of PM density by flow cytometry revealed distinct differences between mutants, i.e. absence of PM CFTR for G85E (0.2% PM expression relative to WT), low levels of PM CFTR for F508del (2.4%) and A455E (4.3%) and medium levels for N1303K (12.3%). As a reference, gating mutant G551D resulted in 109.9% PM CFTR. VX-809 increased PM density by 6-fold for F508del, 2.7-fold for A455E and 1.7-fold for N1303K, but failed to correct G85E. CFTR function in the YFP assay was low to absent at baseline for all processing mutants (1-8%), compared to 42% for WT. Combined VX-809+VX-770 increased CFTR function by 8.3-fold for F508del over DMSO, 2.8-fold for A455E and 2.3-fold for N1303K. For G85E no functional rescue was observed. In transduced organoids, WB confirmed the maturation defect as in HEK293T cell lines (no band C for the processing mutants). Both endogenous and overexpressed G85E and N1303K failed to functionally respond to VX-809+VX-770, in contrast to F508del, resulting in ~70% organoid swelling. A455E analysis is ongoing.

Conclusion: The flow cytometry based PM density assay provides a first rapid and quantitative method using stable cell lines to identify trafficking defects of the many poorly studied rare *CFTR* mutations. Although mutants G85E, A455E, F508del and N1303K at first sight presented with a similar processing defect on WB, the flow assay identified distinct trafficking defects. Currently we are characterizing the rare mutations E60K and E92K. The findings obtained in cell lines will be fully compared to data in primary cell models to ensure maximal translational potential.

P48

Monitoring the phospho-occupancy of CFTR in respiratory epithelia using mass spectrometry

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Background: CFTR channel activity is regulated primarily by cAMP-dependent, protein kinase (PKA)-dependent phosphorylation of the regulatory domain (RD) and ATP-dependent dimerization of the nucleotide-binding domains (NBDs). It is established that the intrinsically unstructured RD phosphorylation is a prerequisite for pore opening upon ATP-induced dimerization of the NBDs. To prevent CFTR activation by PKA, fifteen consensus PKA phosphorylation sites had to be mutated in the RD and NBD1 (15SA), suggesting that the non-phosphorylated RD displacement is cooperatively regulated by multiple phosphosites. However, we still lack a quantitative method to monitor the dynamics of CFTR phosphosites occupancy in order to directly investigate its role as a functional determinant of channel gating in its native environment. To this end, we implemented a novel mass spectrometry-based (MS) workflow to quantify the phospho-occupancy of most of the PKA consensus sites in WT-CFTR in transduced human bronchial epithelia (CFBE).

Methods: We engineered a CFTR construct with His_6-biotin-His_6 (HBH) tag at the N-terminus and a 3HA-tag on the fourth extracellular loop (HBH-CFTR). The plasma membrane expression and the short circuit current measurements (I_{sc}) of the HBH-CFTR were similar to that of the CFTR-3HA variant. We performed a label-free, targeted MS data acquisition on the affinity-enriched CFTR by combining electron-transfer dissociation (ETD) and higher-energy collision dissociation (HCD), referred to as EThcD fragmentation.

Results: We measured the constitutive phospho-occupancy of ten CFTR PKA consensus sites (S422, S660, S670, S686, S700, S712, S737, S753, S768, and S795) in both resting and stimulated conditions in CFBE. Most sites registered at least 5% elevated phosphorylation levels upon forskolin stimulation, consistent with the model that phosphorylation of multiple sites synergistically contributes to the channel activation. Interestingly, S686, which was essential in CFTR activation based on previous mutational analysis, was not amenable to forskolin-induced phosphorylation. We also compared the phosphorylation levels between adenylyl cyclase (AC) stimulation and inhibition by forskolin and SQ22536, respectively. S660, S670, S700, S712, S737, S753 and S795 showed a significant increase in their phosphorylation level by PKA activation. Having established the phosphorylation levels in the basal and stimulated CFTR, we tested the acute activation mechanism of CFTR upon cigarette smoke condensate (CSC) exposure. The phospho-occupancy of the PKA sites, with the exception of two sites, was similar to that of the forskolin-stimulation, suggesting the involvement of PKA activation upon CSC exposure. Ongoing experiments compare the effect β2-adrenergic receptor, vasoactive intestinal peptide receptor and AC activation on the of phospho-occupancy of CFTR.

Conclusion: Our EThcD-MS technique helps to monitor the phospho-occupancy of multiple phosphosites simultaneously under resting and stimulated physiological and pathological conditions. This technique can further be implemented to study the phospho-occupancy of WT and mutant CFTRs in primary human bronchial epithelia.

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P49

Role of the proteasome in the biosynthetic arrest of SLC26A9 by F508del-CFTR

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Introduction: Currently available drugs that correct F508del-CFTR misfolding provide only modest clinical benefit for most CF patients, therefore alternative anion channels and other potentially druggable targets are being explored. SLC26A9 is a constitutively active anion channel expressed in human airways that modifies the severity of CF airway disease in patients with the G551D-CFTR mutation. The functional expression of complex glycosylated SLC26A9 is reduced in cells that express F508del-CFTR mediated in part by a PDZ- and CAL-dependent mechanism. SLC26A transporters also interact with the regulatory (R) domain of WT-CFTR through its Sulfate Transporter AntiSigma factor antagonist (STAS) domain, however the role of this interaction in SLC26A9 biosynthetic arrest is uncertain. Indeed, it is not known if the interaction with immature F508del-CFTR in the endoplasmic reticulum (ER) contributes to the retention and proteasomal degradation of SLC26A9.

Aim: To understand the interaction between SLC26A9 and CFTR and its impact on SLC26A9 expression.

Methods: BHK cells overexpressing wild-type (WT) or F508del-CFTR and parental BHK cells lacking CFTR were transiently transfected with SLC26A9 cDNA. SLC26A9 protein was quantified in lysates by immunoblotting and at the plasma membrane by cell surface biotinylation. SLC26A9 levels were assessed in well-differentiated CF and non-CF primary human bronchial epithelial cells (pHBEs) by immunofluorescence staining and confocal microscopy.

Results: Total and plasma membrane SLC26A9 expression were both lower in BHK cells when co-expressed with F508del-CFTR than when expressed alone or with WT-CFTR. Similar results were obtained when well-differentiated CF and non-CF primary human bronchial epithelial cells (pHBEs) were studied by confocal imaging. Since F508del-CFTR misfolding leads to its retention in the ER and subsequent proteasomal degradation, we examined the effects of proteasome inhibitors on SLC26A9 degradation. Inhibiting the proteasome increased SLC26A9 immunofluorescence in F508del-CFTR homozygous pHBE cells but not in non-CF pHBEs, suggesting there is enhanced proteasomal degradation of SLC26A9 with F508del-CFTR in pHBEs. This difference in proteasome inhibitor sensitivity was not observed in non-epithelial cells overexpressing SLC26A9 with F508del-CFTR or WT-CFTR. Apical SLC26A9 levels increased in pHBEs and in non-epithelial cells when F508del-CFTR was partially corrected using low temperature or VX-809, and this rescue was mimicked by co-transfecting cells with WT-CFTR. The rate of SLC26A9 degradation was measured when expressed alone or with WT-CFTR or F508del-CFTR. In the presence of the protein synthesis inhibitor cycloheximide, degradation of immature SLC26A9 was enhanced in F508del-expressing cellssuggesting there is CFTR-dependent, proteasome-mediated degradation of SLC26A9 at the ER.

Conclusions: These results suggest that ER retention of F508del-CFTR and SLC26A9 leads to premature degradation of both proteins and SLC26A9 biosynthetic arrest is due in part to an interaction between them. Disrupting this interaction will make SLC26A9 an exciting therapeutic target for most CF patients.

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P50

TGF-β1 recruits LMTK2 to inhibit CFTR-mediated chloride transport in human bronchial epithelial cells

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The Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated Cl^--selective ion channel that is expressed at the apical membrane of epithelial cells. In the human airway, CFTR regulates the mucociliary clearance. Mutations in the *CFTR*gene lead to Cystic Fibrosis (CF). The most common mutation, *F508del*, leads to CFTR retention in the endoplasmic reticulum, a defect that can be rescued by small molecule correctors, such as VX-809. Transforming Growth Factor (TGF)-β1 is a known genetic modifier associated with accelerated lung disease in patients homozygous for the *F508del*mutation and may also compromise the corrector-mediated rescue of F508del-CFTR (*1*). The cell surface abundance of CFTR depends on its biosynthetic processing and endocytic trafficking. CFTR endocytosis is stimulated by Lemur tyrosine kinase 2 (LMTK2), a transmembrane protein localized at the apical membrane in human bronchial epithelial (CFBE41o-) cells, by an inhibitory phosphorylation of CFTR-Ser^{737}, leading to reduced CFTR abundance at the plasma membrane (*2*).

TGF-β1 inhibits CFTR-mediated Cl^-transport by mechanisms that have not been completely elucidated. We hypothesized that TGF-β1 recruits LMTK2 to the plasma membrane to induce CFTR endocytosis.

Polarized CFBE41o- cells were treated with TGF-β1 (15 ng/mL) and the apical membrane LMTK2 abundance was examined by the biotinylation-based assay. LMTK2 density increased specifically at the apical plasma membrane, reaching its maximum after one hour of TGF-β1 treatment. In view of this timing, the following post-transcriptional mechanisms were considered: (i) LMTK2 transcytosis from the basolateral to apical membrane; (ii) increased LMTK2 protein translation; (iii) decreased LMTK2 protein degradation; (iv) increased biosynthetic processing of LMTK2; (v) inhibition of LMTK2 endocytosis, or (vi) increased LMTK2 recycling. Examination of the above mechanisms revealed that TGF-β1 specifically increased LMTK2 recycling from submembranous compartments in Rab11-positive recycling vesicles to the plasma membrane. By contrast, TGF-β1 did not affect LMTK2 transcytosis, LMTK2 protein translation, biosynthetic processing, or LMTK2 endocytosis.

Our data demonstrate that TGF-β1 recruits LMTK2 to the apical plasma membrane in human bronchial epithelial cells by increasing its recycling in Rab11-positive vesicles. We describe a novel mechanism of LMTK2-mediated CFTR inhibition by TGF-β1 in human airway epithelial cells. Since most CF patients homozygous for *F508del* have elevated TGF-β1 levels, our findings may lead to novel therapeutic targets blocking excessive TGF-β1 signaling.

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P51

Investigating the drug-drug plasma protein binding and cytochrome interactions of ivacaftor

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Currently, ivacaftor is the only transmembrane conductance regulator (CFTR) potentiator available that improves the pulmonary function for patients with gating mutations e.g. G551D CFTR-protein mutation. Because ivacaftor is highly bound (>97%) to plasma proteins, co-administered CF drugs are likely to compete for the same plasma protein binding pockets, therefore impacting on the unbound plasma concentration.

This, in turn, could majorly impact on the in vivo efficacy of ivacaftor and therapeutic outcomes. This biochemical study compares the binding affinity of ivacaftor, metabolites and co-administered CF drugs for human serum albumin (HSA) and α1-acid glycoprotein (AGP) using sur-face plasmon resonance and fluorimetric binding assays measuring the displacement of site-selective probes. Furthermore, the effect of ivacaftor, its major metabolites M1 and M6 on the enzymatic activity of the major xenobiotic metabolizing enzymes e.g. CYP1A2 and CYP3A4 as well as the minor enzymes e.g. CYP2B6 and CYP2C9 is assayed.

Due to their ability to strongly compete for the ivacaftor binding sites on HSA and AGP, drug–drug interactions between ivacaftor are to be expected with the following co-medications: ducosate, montelukast, ibuprofen, dicloxacillin, omeprazole, and loratadine. The significance of these plasma protein drug–drug interactions is also interpreted in terms of molecular docking simulations. This in vitro study provides valuable insights into the plasma protein drug–drug interactions of ivacaftor with co-administered CF drugs. All in all, present findings would furthermore suggest cytochrome interactions are to be expected under ivacaftor therapy.

The data may prove useful in future clinical trials for a staggered treatment that aims to maximize the effective free drug concentration and clinical efficacy of ivacaftor.

P52

HspB5, a phospho-regulated corrector of F508del-CFTR

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Background: Many studies have shown that chaperones are implicated in the synthesis and maturation of WT- and mutated CFTR, of which Hsp40, Hsc70 and Hsp90 have been the most studied. We focused on HspB5 to evaluate its role and effect in cystic fibrosis (CF). HspB5, also called αB-crystallin, is a member of the small Heat Shock Proteins (sHSPs) family. HspB5 is an ubiquitous protein which is mainly expressed in the heart, skeletal muscle, kidney, spleen, thymus, prostate, brain, crystalline and lung, therefore in tissues with a high level of oxidative metabolism.

Moreover, HspB5 presents, through its chaperon-like activity, the potential capacity to improve the stability, maturation and transport at the apical plasma membrane of F508del-CFTR protein.

Aims: To study the therapeutic potential of HspB5 in CF.

Methods: We used control and CF mice lungs and primary human nasal epithelial cells (HNEC) to assess HspB5 expression by ELISA. Phosphorylation status of HspB5 was determined in F508del- and WT-CFTR-CFBE41o- cells. Cell surface expression of F508del-CFTR after HspB5 and phosphomimetics expression was assessed by different technics: IF by confocal microscopy in BEAS-2B cells, cell surface ELISA in HEK293 MSR Grip Tite (293MSR-GT) cells expressing F508del-CFTR (Rotin's Lab, Toronto, Canada). Function of F508del-CFTR after HspB5 and phosphomimetics expression was measured by YFP assay in 293MSR-GT.

Results: In lung, endogenous HspB5 expression was increased in CF mice model compared to control mice. Interestingly, HspB5 is induced in control mice challenged with LPS but not in CF mice model. Endogenous HspB5 protein is detected in HNEC from CF and non-CF patients. We determined that the phosphorylation status of HspB5 was altered in F508del-CFTR-CFBE41o- in comparison to WT-CFTR-CFBE41o-. As HspB5 functions can be regulated by its phosphorylation, WT-HspB5 and phosphomimetic-HspB5 mutants (mutants that mimicked the presence or absence of phosphorylation) were used. We established that WT-HspB5 was able to partly correct the F508del-CFTR localization and function. The phosphorylation status of HspB5 was essential to regulate this phenomenon.

Conclusion: Our results support our hypothesis that HspB5 is a relevant target for the treatment of cystic fibrosis. The recent development of peptide aptamers and chemical molecules that modulate the sHsps intracellular activity and the clinical ongoing trials testing the safe *in vivo* delivery of recombinant **HspB5 for multiple sclerosis treatment and chronic obstructive pulmonary disease, allow us to consider the rapid development of *in vivo* tests in cystic fibrosis context.

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P53

Keratin 8-dependent regulation of CFTR and alpha-1-antitrypsin targeting

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Growing number of evidence indicate that protein-protein interactions regulate CFTR protein trafficking to its target site. We have previously reported that the intermediary filament protein keratin 8 (K8) affects the trafficking of misfolded F508del-CFTR from the endoplasmic reticulum (ER) to the plasma membrane by interacting directly with F508del-NBD1, and that disruption of this interaction with chemical compound c407 leads to partial correction of functional F508del-CFTR (Colas et al. 2012; Odolczyk et al. 2013). We hypothesized that K8 can interact with other misfolded proteins, preventing their correct targeting, and contributing to pathogenesis. To test this hypothesis we choose the misfolded Z mutant of alpha-1 antitrypsin (ZA1AT, mutation E342K) protein responsible for A1AT deficiency. A1AT is an inhibitor of inflammatory proteases (e.g. elastase) and protects the lungs from degradation. Misfolded but functional ZA1AT is degraded by the ubiquitin-proteasome or by autophagy resulting in reduced secretion, what leads ultimately to the lung and/or liver diseases.

The aims of the study are to verify whether (i) Z-A1AT/A1AT interacts with K8, (ii) K8 regulates Z-A1AT/A1AT secretion, (iii) c407 disrupts ZA1AT-K8 interaction and increases mutant secretion.

The ZA1AT/A1AT cDNA-transfected HeLa cells and primary human bronchial epithelial (HBE) cells, from healthy subjects and Z allele-homozygous patients, were used. The *in cellulo* possible interaction between K8 and ZA1AT/A1AT was tested by co-immunoprecipitation and proximity ligation assay (PLA). Direct interactions were tested by surface plasmon resonance (SPR), pull-down and hydrogen-deuterium exchange mass spectrometry (HDex-MS) on recombinant K8 and A1AT from human serum. ZA1AT/A1AT secretion under different treatment conditions was tested by WB on culture supernatants. The effect of K8 knock-down was investigated by stable transduction of HeLa cells with shRNA against K8.

ZA1AT secretion from both HeLa and HBE cells was weaker than A1AT secretion. As compared to A1AT-cells, in cells expressing ZA1AT, K8 filaments network was modified and phosphorylated. PLA has shown that ZA1AT preferentially localized in close proximity with K8 as compared to A1AT in HeLa and HBE cells, however, both A1AT and ZA1AT co-immunoprecipitated with K8. The increased number of potential interactions with K8 was observed previously for F508delCFTR. Biophysical analysis by SPR and pull-down confirmed that K8 physically interacts with ZA1AT/A1AT. Moreover, HDex-MS mapped the 92–96 amino acid region of A1AT helix D, as the site of interaction with K8. In HeLa cells, knock-down of K8 expression and c407 treatment significantly increased the secretion of ZA1AT. This observation reproduced the results obtained for F508del-CFTR.

In conclusion, the physical interaction with K8 regulates ZA1AT/A1AT secretion and its pharmacological disruption partially restores secretion of ZA1AT mutant. K8 is an important regulator of both, transmembrane (CFTR) and soluble (A1AT) proteins maturation and trafficking in cells. The misfolded protein-K8 complex represents a target for pharmacotherapy of A1AT deficiency and cystic fibrosis. We propose that the interaction with the intermediary filament protein, K8, is a general mechanism of misfolded proteins quality control and regulates protein targeting in multiple epithelial protein misfolding diseases.

P54

Hit optimization for the development of novel ubiquitin-ligase RNF5 inhibitors

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In cystic fibrosis (CF), deletion of phenylalanine 508 (F508del) in the gene encoding the CF transmembrane conductance regulator (CFTR) is associated to misfolding and premature degradation of this mutant anion channel protein. Among the known proteins associated with F508del-CFTR processing, particularly interesting is the ubiquitin ligase RNF5/RMA1. Recently it was demonstrated that genetic suppression of RNF5 *in vivo* is particularly effective in rescuing mutant CFTR at the functional level, leading to an attenuation of CF pathological phenotype, and validating the relevance of RNF5 as a drug target for CF. Therefore, by using a computational approach, based on ligand docking and virtual screening, our group reported the discovery of a drug-like small-molecule RNF5 inhibitor, named **inh-02**. *In vitro* ubiquitination experiments demonstrated that treatment with **inh-02** modulates the activity of two known RNF5 targets, ATG4B and paxillin, which confirmed that **inh-2** inhibits RNF5 ligase activity. Finally, long-term incubation with inh-02 caused significant F508del-CFTR rescue in immortalized and primary bronchial epithelial cells deriving from CF patients homozygous for the F508del mutation, thus validating RNF5 as drug target for FC, and providing evidences to support its druggability.

Therein, we focused on the chemical exploration of the RNF5 RING domain through the design and synthesis of a large library of **inh-2** analogues. The new derivatives were synthesized leaving the central thiadiazolidine ring and formamidine portion unaltered, while exploring different functional groups on the aromatics rings of the benzamidine region. Besides, the alkyl chain that links the thiadiazolidine ring to the phenyl group was modified by elongation, while the aromatic ring of the same region was decorated with different functional groups. At the same time *in vitro* experiments were carried out to investigate and compare the new derivatives activity to **inh-2** one. Finally, the obtained data were used to carry out structure-activity relationship studies (SAR), which may be important to investigate and confirm the druggability of the inhibition of this promising target.

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P55

SPLUNC1 peptidomimetics inhibit Orai1 to reduce pulmonary inflammation

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CF patients undergo cycles of chronic bacterial infection and inflammation that lead to persistent neutrophilia and bronchiectasis. Indeed, lung disease is the major cause of morbidity and mortality in CF patients. Therefore, one of the cornerstones of a comprehensive treatment plan is the use of anti-inflammatory drugs. However, beyond ibuprofen, there are currently no effective anti-inflammatory drugs to treat CF patients. Orai1 is a plasma membrane Ca^{2+} channel that contributes to the development of chronic inflammation by changing gene expression and stimulating cytokine secretion. The short palate lung and nasal epithelial clone 1 (SPLUNC1) is a highly abundant, multi-functional, secreted protein that plays a critical role in maintaining lung health. SPLUNC1 binds to and negatively regulates Orai1, thus inhibiting store operated Ca^{2+} entry[1]. Furthermore, SPLUNC1 knockout mice exhibit a hyperinflammatory phenotype[2]. While SPLUNC1 is present in airway secretions from healthy individuals, it is diminished in immunocompromised patients, such as those with CF[1,3]. We have identified the region of SPLUNC1 that binds to Orai1 and created a peptidomimetic of this region, called α6. Here, we have explored its ability to regulate Orai1. To determine if the α6 peptide could inhibit Orai1, HEK293T cells and CF primary human bronchial epithelial cells (HBECs) were cultured and thapsigargin-induced Ca^{2+} release ± SPLUNC1 or ± α6 was measured. The peptide inhibited Ca^{2+} influx in a similar fashion as SPLUNC1, with an IC_{50} of 920.1 nM. This inhibitory effect was replicated in multiple pulmonary cell types including neutrophils and T-cells. Since secretion of the chemoattractant IL-8 is Ca^{2+} dependent, α6 was then tested to determine whether it could prevent IL-8 secretion in CF HBECs. Supernatant of mucopurulent material (SMM) +/- α6 was added mucosally and serosal IL-8 secretion was measured. SMM significantly increased IL-8 secretion, however α6 reduced IL-8 secretion to baseline levels. Additionally, α6 retained efficacy in the proteolytic SMM environment, suggesting that α6 is anti-inflammatory and not immunosuppressive. In conclusion, the α6 peptide significantly inhibits Orai1 and reduces pulmonary inflammation, indicating that it may serve as a novel, inhaled anti-inflammatory peptide.

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P56

Metabolic reprogramming of cystic fibrosis macrophages by the IRE1a-XBP1 pathway leads to an exaggerated inflammatory response

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Introduction: Cystic fibrosis (CF) is a monogenic recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR). CFTR mutations cause dysregulation of membrane channel function with intracellular accumulation of misfolded proteins, particularly in DF508 mutations, leading to Endoplasmic Reticulum (ER) stress and activation of the IRE1a-XBP1 pathway. Activation of IRE1a leads to splicing of XBP1 (XBP1s), a transcription factor linked to production of proinflammatory cytokines and an important regulator of cellular metabolism. XBP1s is elevated in several CF immune cells with further implications in inflammation. Classically activated M1 macrophages are important initiators of the inflammatory response, and these proinflammatory cells exhibit high levels of glycolysis. There is evidence indicating that the IRE1a-XBP1 pathway regulates M1-M2 macrophage polarisation and controls mitochondrial activity and energy consumption in immune cells. We hypothesised that the exaggerated inflammatory response seen in CF, with high levels of XBP1s, can be associated with a metabolic dysfunction in M1 macrophages.

Aim: To investigate ER stress in specific immune cells from CF patients, the metabolic state of these cells and also to determine whether this is associated with a proinflammatory phenotype.

Methods: Peripheral blood mononuclear cells (PBMCs), isolated neutrophils, monocytes, lymphocytes, and human blood monocyte-derived macrophages (BMDM) from CF patients and healthy control (HC) volunteers were used to evaluate ER stress, using quantitative real-time PCR (qPCR). BMDM were differentiated for 7 days, in the presence of either GM-CSF or M-CSF, and then activated on the 6^{th} day with LPS and IFNg for M1 macrophages, or IL-4 and IL-13 for M2 macrophages, respectively. M1-M2 macrophage ratios were measured using flowcytometry. IL-6 and TNF levels were measured by qPCR and ELISA. The metabolic profiles of monocytes and M1 macrophages were analysed in real time by quantifying the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in the Seahorse XFe96 analyser. XBP1s was blocked using 4m8c and MKC-3946 inhibitors. Significant values ($P< 0.05$).

Results: Gene expression analysis revealed a significant increase in *BiP, IRE1a, XBP1s, ATF4, GADD34*, *IL-6* and *TNF* in PBMCs from CF patients. Further analyses revealed that only neutrophils and monocytes showed a significant increase of ER stress markers, more pronounced in monocytes. *BiP, IRE1a, ATF4,* and *CHOP expression* were significantly increased in neutrophils, while *IRE1a, ATF4, PERK, ERDJ4, CHOP, GADD34*, and *TNF* were all significantly increased in monocytes. The proportion of CF M2 macrophages was significantly lower; although, the proportion of CF M1 macrophages was unaffected, *IL-6* and *TNF* production were significantly higher, with significantly higher levels of *BiP, XBP1s, ERDJ4, TNF and IL-6.* Monocytes and M1 macrophages showed significantly higher levels of OCR and ECAR with an increased glycolytic reserve. Finally, inhibition of XBP1s, before macrophage polarisation, reduced the high levels of OCR and ECAR lowering the production of IL-6 and TNF in CF M1 macrophages.

Conclusions: These findings suggest that CF M1 macrophages undergo systematic metabolic reprogramming to cope with their increased energy demands. This hypermetabolic state is associated with high levels of XBP1s production, driving a proinflammatory phenotype in CF M1 macrophages.

P58

The PDZ containing domain protein, cal, links CFTR to the regulation of inflammation

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Patients with CF have a robust inflammatory response to bacterial infection. A number of studies have associated components of the immune system such as TNFα with the severity of lung disease. Functionally, the binding of TNFα to its receptor stimulates the transcription factor NF-κB and the downstream release of cytokines IL-6 and IL-8 into the airways of CF patients. Here we show that CAL binds to CFTR and to components of the NF-κB signal transduction pathway, namely, TRADD and TRAF2. CAL is localized to the trans-Golgi complex and ER and regulates the expression of WT mature CFTR by targeting CFTR for degradation in the lysosome. CAL also plays a role in the degradation of F508-del in the ER suggesting that CAL is important in both proteasomal and lysosomal pathways. TRADD is a scaffold protein that recruits TRAF2 upon stimulation of the TNFα receptor. TRAF2 is an E3 ligase, which functions as an additional scaffold protein recruiting two other E3 ligases. We found that WT-CFTR binds to and colocalizes with TRADD and that F508-del does not. NF-κB activation is higher in CFBE expressing F508-del cells compared to those expressing WT-CFTR. However, this differential effect is abolished when TRADD levels are knocked down. Consistently, transfecting wt-CFTR into CFBE cells reduces NF-κB activity. However, the reduction is abolished by the CFTR inhibitor-172. Transfecting the correctly trafficked CFTR conduction mutants G551D or S341A also fails to reduce NFkB activity. Thus, CFTR must be functional to regulate NF-κB activity. We also found that TNFα produced a greater increase in NF-κB activity in CFBE cells compared to wt CFTR-corrected cells. The effect is also abolished when TRADD is knocked down by shRNA. Thus, wt-CFTR's control of TRADD modulates the physiological activation of NF-kB by TNFα. We also found that the mechanism by which wt-CFTR, but not F508-del, suppresses TRADD is by lysosomal degradation and that CFTR-induced degradation of TRADD does not occur unless CFTR interacts with CAL, suggesting that CAL plays a key role in the ability of CFTR to regulate TRADD. In conclusion, we have uncovered a novel mechanism whereby wt-CFTR regulates TNFα signaling by enhancing TRADD degradation. Interestingly TRAF2 proteins form mushroom shaped trimers with the coiled-coil domains comprising the mushroom stalk. We suggest that the binding of CAL to TRAF2 occurs via its coiled coil domain. Thus by reducing the levels of TRADD and by potentially regulating TRAF2, wt-CFTR in concert with CAL suppresses downstream proinflammatory NFkB signaling. This mechanism fails in F508-del containing cells leading to enhanced NF-κB signal transduction in response to TNFα stimulation.

P59

The cellular prion protein (PrP^C) modulates CFTR-associated inflammation

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Chronic pulmonary inflammation is a hallmark of several lung diseases such as cystic fibrosis (CF). The CF lung inflammatory process relies on bacterial invasion and the long-lasting activation of neutrophils and on the remodeling of epithelial cells. These cells produce mediators, such as pro-inflammatory cytokines (TNFα) and interleukins (IL1β, IL6, and IL8) that contribute to the lung injury and respiratory failure.

The release of pro-inflammatory mediators by CF-epithelial cells (CFBE) during the early inflammatory process may favor the permeabilization of the lung epithelial barrier and the subsequent infiltration of peripheral neutrophils and pathogens that exacerbate the disease state. Though, the molecular mechanisms underlying the alteration of the permeability in human CF lung epithelial barrier and the triggering to the inflammation process remain elusive.

Recently, we demonstrated that the cellular prion protein (PrP^C) is a member of the lung junctional complexes that protect the junctional proteins against oxidative stress. PrP^C was also found to be highly expressed in CF cells and tissue compared to controls. This protein colocalizes with wt- and dF508-CFTR proteins without any biochemical interaction. Yet, the involvement of PrP^C in lung inflammation and pathogen invasion in normal and CF conditions is still to be investigated.

Aims: The aims of this study was to determine

i) the expression and the localization of PrP^C and CFTR proteins in the lung of wild type (wt), $PrP^{C-/-}$, and $CFTR^{-/-}$ mice models,

ii) the levels of expression and the stability of the junctional proteins,

iii) the regulation of the inflammatory profiles in normal and infected cells and mice models, using *Pseudomonas aeruginosa* (PA),

iv) the effect of inflammation on PrP^C expression and localization.

Results: At the tissue level, PrP^C protein showed an apical localization in epithelial bronchial cells, similar to what has been observed in HBE cells. The invalidation of PrP^C gene increased CFTR expression at mRNA and protein levels in HBE cells and in the mice model ($PrP^{C-/-}$). Interestingly, the invalidation of CFTR gene in mice ($CFTR^{-/-}$) increased the level of expression of PrP^C protein, suggesting the existence of a crosstalk between the two genes.

The invalidation of PrP^C in HBE cells increased ROS production, but decreased the secreted levels of IL6, IL10 and TNFα, suggesting that a new intracellular pathway, NFkB-independent, is involved in the regulation of cytokines secretion in a PrP^C-dependent manner.

In $PrP^{C-/-}$ mice, systemic TNFα decreased in a sex-dependent manner after acute (24h) infection with PA. After stimulation with LPS, we showed that the overexpression of PrP^C in HBE cells has an impact on the secretion of pro and anti-inflammatory cytokines, while the invalidation of PrP^C only reduced IL6 secretion. Similar results were observed after PA infection in $PrP^{C-/-}$ mice.

Conclusions: Altogether, our results demonstrate that PrP^C expression level plays an important role in CF disease through alterations of the lung epithelial barrier and modulation of epithelial cell's inflammatory responses.

However, it remains unknown whether PrP^C role in lung inflammation would also depend on PrP^C capacity to adjust the response of cells targeted by pro-infammatory factors.

P60

Cathepsin G activity reporters detect chronic airway inflammation by microscopy and flow cytometry

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Cystic fibrosis is characterized by extensive bronchiectasis due to the uncontrolled release of active neutrophil serine proteases (cathepsin G, neutrophil elastase and proteinase 3) in the airways. Recently, spatially localized FRET reporters able to visualize neutrophil elastase activity on patient cells highlighted the detrimental role that the protein plays in CF lung disease. However, the lack of knowledge about the mutual interactions among the three neutrophil serine proteases and the impossibility to translate confocal microscopy into the clinics render their validation as biomarkers arduous. In addition, chronic inflammated airways are often full of extracellular DNA originated from NETing, necrotic and apoptotic cells. Despite the neutrophil serine proteases high affinity for these DNA-web like structures, if and how they tune inflammation and damage tissues when bound to DNA is still obscure. To assess if cathepsin G (CG) is an additional key player in chronic lung inflammation we developed a new series of FRET reporters that we employed for the quantification of membrane-bound and soluble CG activity. If compared to healthy donors, CF neutrophils showed a 3-fold increase in their surface-associated activity and the constant secretion of CG resulted in a 3,5-fold change in the extracellular milieu. To overcome microscopy limitations, we implemented the application of a small molecule FRET reporter into the flow cytometry technology. As a proof of concept validation of the novel methodology, we correlated the measured activities on 11 human samples both via microscopy and flow cytometry obtaining clear results in terms of reproducibility. This latest technology opens the doors to longitudinal and in-depth clinical studies and within this context CG needs to be considered as a new potential biomarker. The employment of the novel tools will fuel a complete understanding of proteases pathophysiology not only in CF but whenever proteases-driven inflammation comes into the game.

P61

Involvement of the RhoGTPase activator Vav3 in the CF airway epithelium remodelling

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The airway epithelium integrity and the maintenance of its barrier function are vital for airway epithelial defense against infection. Cystic Fibrosis (CF) is associated with an increased airway epithelium susceptibility to chronic infections and exacerbated inflammation. The link between the CFTR dysfunction and these phenotypic deregulations remains poorly understood. Regulators of the cytoskeleton network are critical for cell-cell and cell-ECM interactions, which contribute to the barrier polarity and stability. In this study, we investigated the involvement of Vav3, a key GEF (Guanine nucleotide Exchange Factors) activator of RhoGTPases in the control and CF airway epithelial cells.

In primary cultures at the air-liquid interface, we observed increased expression of Vav3 in CF (F508delCFTR) human airway epithelial cells (HAECs) compared to normal HAECs. Similar increased expression of Vav3 was observed in the human Calu-3 airway epithelial cells line after CFTR knockdown (KD-CFTR) by CRISPR-Cas9. The role of Vav3 in airway epithelial cells was evaluated by knockdown in Calu-3 cells (KD-Vav3). KD-Vav3 cells showed remodeled actin cytoskeleton with a modification of the junctional network. Indeed, Vav3 knockdown induced zigzag shape of ZO-1, reduced E-cadherin and Cx43 expression. Interestingly, the expression and activity of β1-integrin were decreased in the KD-Vav3 cells, which led to a reduction in fibronectin-dependent cell adhesion.

An ectopic luminal accumulation of the β1-integrin in CF airway epithelium has been recently reported (Grassmé *et al*., Cell Host Microbe, 2017). Consistent with these results, we show that Vav3 overexpression through the CFTR knockdown in Calu-3 increased the apical expression of β1 integrin, associated with deposition of fibronectin at the luminal side. We also observed that the apical localization of Vav3 is increased in CF suggesting a luminal Vav3/β1-integrin complex. Indeed, co-immunoprecipitation studies revealed that Vav3 and β1 integrin are physically interacting and that this interaction is increased in CF. Our results suggest that Vav3 acts as a regulator of the β1 integrin activity and expression, and that its enhanced ectopic expression is a consequence of CFTR dysfunction.

Through its capacity to regulate the cytoskeletal and the junctional networks, and by forming a luminal complex with active β1-integrin and fibronectin that can favor bacteria trapping (Hoffmann *et al*., European Journal of Cell Biology, 2011), Vav3 may represent a new target in CF.

P62

Oxidative stress-associated lipid biomarkers in bronchoalveolar lavage correlate with CF lung disease severity and progression

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Introduction: CF infants show signs of lung disease before overt bacterial colonization, suggesting an intrinsic, CFTR-dependent mechanism as the trigger for early lung disease. We showed previously that the EGFR/ADAM17/AREG/IL8 axis, which controls the shedding of growth factors and cytokines involved in inflammation and tissue remodeling, is more active in primary differentiated CF than in non-CF airway epithelial cultures (HBEC-ALI), and is decreased by supplementing the medium with glutathione (GSH) [Stolarczyk M, 2018, AJP Lung]. In an accompanying paper we showed that both CF HBEC-ALI and CF-KO Pig BEC-ALI contain higher levels of oxidative stress markers. Here we hypothesize that oxidative stress in the bronchial epithelium contributes to the progression of early lung disease in CF children, via activation of pro-inflammatory and fibrotic signaling.

Methods:

Population: CF-infants were enrolled in the Rotterdam early CF monitoring program (I-BALL). Chest computed tomography (CT) scans and Broncho-alveolar lavage (BAL) were collected at 1, 3 and 5 years. Patients with at least 2 timepoints were included and measured. PRAGMA-CF was used to score chest-CT's for total disease (%Dis) and bronchiectasis (%Bx). BAL was analyzed for cell-count.

Inflammatory markers: cell-free BAL supernatants were analyzed for: a panel of bioactive lipids associated with oxidative stress, using quantitative HPLC-MS/MS (LACDR, Leiden), neutrophil elastase (NE) activity using a fluorometric assay (Cayman), and myeloperoxidase (MPO) activity and detection by immunocapture. Proteins involved in inflammation and tissue remodelling were measured using a Fluidigm-based protein array (Olink^{TM}). Spearman correlation was used to correlate markers with clinical outcomes.

CF HBEC-ALI culture: Primary human bronchial cells from surgical specimens of CF patients and controls were used for invitro experiments on membrane inserts at air-liquid interface.

Analyses of CF lung disease progression: Multivariate regression analysis was used to show whether these inflammatory markers relate to CF lung disease on chest-CT 2 years later,

Results: Oxidative stress-associated lipid markers measured in BALF, including isoprostanes (8-iso-PGE2) and lysolipids (lysolecithin and lysophosphatidic acid species), correlated positively (Spearman Rho>0.55, P< 0.01) with %Dis and %Bx, and inflammatory markers (% BALF neutrophils, NE and MPO activities) in our univariate analyses. More than half of 92 proteins involved in myeloid cell migration and proliferation measured by protein array correlated positively with both lysolipids and isoprostanes in BALF.

In CF HBEC-ALI culture, shedding of several factors were significantly reduced (>2-fold, P< 0.001; N=3) by GSH supplementation (AREG, CX3CL1, CCL20, CXCL5, CXCL1, IL8, CX3CL1, TNFRSF12A, VEGFA, CXCL10) or enhanced (>2-fold, P< 0.001; N=3) by a lysolipid receptor (LPAn) agonist (AREG, IL6, IL8, TRAIL, MCP-1, CCL20, VEFGA, CXCL10).

Importantly, lysoplipids and isoprostanes is higher in patients with high %Dis 2 years later. (R^2>0.6, P< 0.001), suggesting that these parameters relate to disease progression.

Conclusions: Oxidative stress-associated lipid markers in BAL from CF children correlate with early CF lung disease and inflammatory signaling. Our data suggest an active role of oxidative stress and lysolipid signalling in the onset and progression of CF lung disease. Lipidomic may prove a promising addition in monitoring disease progression and early intervention.

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P63

Oxidative stress and abnormal bioactive lipid metabolism in CF bronchial epithelial cells in air-liquid interface culture, partial correction by Glutathione, Fenretinide and Orkambi

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Introduction: Early CF lung disease is characterized by inflammation and tissue remodeling. Studies in F508del-CFTR mouse and human CF infant lung showed enhanced infiltration of hyperactive neutrophils, not explained by bacterial colonization. Moreover, this infiltration correlates with lipid markers of oxidative stress and abnormal metabolism of sphingosines and polyunsaturated fatty acids, suggesting a causative relationship between bioactive lipid metabolism and inflammation of the CF lung.

Aims:

1)To establish if abnormal bioactive lipid metabolism is a cell autonomous characteristic of CF airway epithelia which might be correctable pharmacologically.

2) To provide a possible explanation for the etiology of excessive inflammation in CF lung disease.

Methods: Primary bronchial epithelial cells from neonatal CFTR KO pig (PBEC-ALI) and CF patients (HBEC-ALI) were cultured at the air-liquid interface on Costar inserts in BEGM-ALI for 21 days. Wild-type neonate pig littermates and non-CF human surgical samples were used as controls. Lipids were extracted and analyzed by LC/MS mass spectrometry.

Results: We previously reported that the EGFR/ADAM17/AREG/IL-8 axis is hyperactive in CF HBEC-ALI compared to WT, which is partially corrected by extracellular reduced glutathione (GSH), suggesting that oxidative stress and pro-inflammatory and pro-fibrotic signaling is a cell-autonomous property of CF airway epithelia (Stolarczyk, M; AJP Lung 314:L555, 2018). Here we report that two biomarkers of oxidative stress, malonyl-dialdehyde (MDA) and Nitrotyrosine-(N-Tyr), are enhanced twofold ($P< 0.01$) in CF PBEC (N=6) and CF HBEC (3 patients, 3-6 samples each) compared to controls ($P< 0.01$). The ratio of ω-6 (Arachidonic acid, 20:4n-6) to ω-3 (DHA 22:6n-3) long chain polyunsaturated fatty acids (LCPUFA) is increased more than ten-fold in CF airway cells. Furthermore, the total ceramide pool is reduced by half in CF airway cells due to a decrease in very long chain ceramides (VLCC: C24:0, C24:1, and C26:0) and a reciprocal increase in long chain ceramides (LCC: Cer14;Cer16), resulting in a significant 4-fold increase of the LCC/VLCC ratio. Oxidative stress markers, LCPUFA and ceramide ratio are significantly (N=6-10, $P< 0,05$) but partially corrected by treatment with either the retinoid acid analogue Fenretinide, extracellular GSH (10 mM), or Orkambi™ (VX-809 plus VX-770). Transfection of CF epithelial cells with wtCFTR completely corrects their aberrant lipid profile, showing a direct link between CFTR expression and aberrant lipid profiles, consistent with our finding that partial correction of F508del CFTR activity by Orkambi (20% of WT) leads to partial correction of the aberrant lipid profile.

Conclusions: CF PBEC- and CF HBEC ALI have distinct lipidomic signatures that are consistent with elevated oxidative stress and inflammatory signaling, and which can be corrected by different interventions. Fenretinide affects ceramide metabolism, reduces inflammation in CF mutant mice, and is being tested in CF patients in a Phase II clinical trial (APPLAUD). Attenuation of oxidative stress may reduce inflammation and tissue remodeling. Future detailed studies of lipid metabolism in CF HBEC-ALI may reveal novel therapeutic targets. Our data suggest that a study of lipidomic markers is required to establish the efficacy of available and future CFTR targeted therapeutics.

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CFTR-F508del mutation affects differentiation and functional activity of bone-resorbing osteoclasts

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Objectives: Cystic Fibrosis Bone Disease (CFBD) is one of the major comorbidities with diabetes affecting patients with cystic fibrosis (CF). Bone homeostasis is depending on effective coupling between osteoblast activity (OBs – bone forming cells) and osteoclast activity (OCs – bone resorbing cells). OCs are differentiated from monocytic precursors under the action of several mediators (M-CSF, RANKL, sphingosine-1-phosphate (S1P)) whose expression is deregulated in CFBD (Velard F et al., AJRCCM, 2018). Our hypothesis is that the CFTR-F508del mutation could alter the differentiation and functional activity of OCs.

Methods: Differentiation of blood-derived monocytes from homozygous CF-F508 patients (n=7, aged 31 to 52 years), heterozygous CF-F508 (n=10, aged 28 to 58 years) and monocytes from healthy subjects (non-CF, n=8, aged 26 to 59 years) in the presence or absence of a CFTR inhibitor (CFTR-Inh172, 10 µM), was analyzed after 7-14-21 days of culture (Abdallah et al., 2018). The number of OCs formed, their size and their resorption activity (number of resorption trenches on dentin) were analyzed. Expression of the S1P receptor (S1PR1) gene was evaluated by RTqPCR. S1P concentration was analyzed using ELISA in the sera of CF-F508 patients (n=5) and healthy subjects (n=3) and in the culture supernatants of differentiated OCs.

Results: CFTR-F508del mutation significantly reduced OCs number formed from monocytes of homozygous and heterozygous CF-F508 patients compared to non-CF healthy monocytes. This reduction in differentiated OCs was associated with an increased OCs size and a decrease in their functional capacities to form resorption trenches, which is also found from healthy monocytes treated with the CFTR inhibitor Inh172. In CF-F508 patients, S1P levels in serum were increased (+ 50%) compared with healthy subjects. In contrast, S1P production by CF-F508 OCs was reduced (-25%) as well as for healthy OCs treated with Inh172, compared to untreated healthy OCs. The S1PR1 receptor was overexpressed in the presence of Inh172 at early time of differentiation (J7) and then is under-expressed at the late time (J21) compared to untreated healthy OCs.

Discussion: The S1P and the S1PR1 receptor play a major role in the circulation of OCs precursor monocytes from the serum compartment to the bone compartment via a S1P concentration gradient. S1P stimulates OBs bone forming activity (Meshcheryakova A, 2017). Our results highlight the critical role of CFTR function in S1P production, regulation of the S1PR1 receptor and the potential for differentiation and resorption activity of OCs.

Our data suggest the existence of a disruption in the communication between OBs and OCs, via a reduction in the number of formed OCs and a decreased production of S1P. This may result in a reduction in osteoblast activity and consequently a decrease in bone formation in CF patients.

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The normal and CF airway peptidomes regulate cell growth

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Airway secretions contain both proteins and peptides which play important roles in regulating lung homeostasis. In the CF lung, chronic neutrophilia and infection lead to an increase in free protease levels that induce protein/peptide cleavage, suggesting an impaired/altered functionality. Little is known about the peptidome of airway secretions. Here, we set out to define the normal and CF peptidomes and to determine some of their physiological roles. We size-fractionated sputum from normal and CF subjects and studied these fractions by LC-MS/MS without conventional tryptic digestion in order to detect endogenously produced peptides. We identified ~130 endogenous peptides in normal sputum and ~700 peptides that were common to all CF patients. Interestingly, only 60 peptides were seen in both normal and CF subjects. Using principle component analysis and k-means clustering, we found that the normal and CF peptidomes were $p < 0.00013$ different. Since little is known about the functionality of the normal and CF peptidomes, we utilized SPOT synthesis technology to make a library of 300 peptides that included the entire normal peptidome and a selection from the CF peptidome. We then performed high content imaging using 384 well plates as described[1] in order to determine whether these peptides could affect cell growth. We found that the normal peptidome contained 10 peptides that increased cell growth by 27±1.2%, while the CF peptidome had 3 independent peptides that increased cell growth by 24.8±2.8% compared to control. In both cases, the peptides ranged in size from 6-15 residues, with the mean being 11 and 9 for normal and CF peptides respectively. In contrast, the normal peptidome had 11 peptides that inhibited cell growth to 57.3±1.6% while the CF peptidome had 10 peptides that inhibited cell growth to 56±2.1% of control. The inhibitory peptides were smaller than the stimulatory peptides, and in both cases were all 5-6 residues long. Interestingly, all of the CF inhibitory peptides were derived from *Pseudomonas aeruginosa*. Thus, we hypothesize that the normal lung peptidome plays a role in modulating cell growth through an as-yet undetermined mechanism. Further, we speculate that a better understanding of peptides that can promote cell growth may lead to novel therapeutics to promote lung repair after injury.

1. Sassano MF et al, PLoS Biology, 16(3):e2003904 2018. Funded by UNC-Chapel Hill and St. George's, University of London

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The role of IL-17s/IL-17Rs immune axis in airway defense and immunopathology during *Pseudomonas aeruginosa* infection

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The exaggerated inflammation and tissue damage, associated to persistent infections such as *Pseudomonas aeruginosa*, are central pathological features of Cystic Fibrosis (CF) lung disease. The interleukin(IL)-17A/IL-17 receptors A (IL-17RA) axis represents an emerging key player in CF pulmonary disease. In this regard, the IL-17 pathway, through IL-17RA, has been shown to play a key role in host defence and in the modulation of immunopathology in a murine model of chronic respiratory infection by *P. aeruginosa* (Lorè et al., Scientific Reports, 2016).

Here, we aim at dissecting the pleiotropic activities of IL-17RA mediated by the interaction with other IL-17 receptors (IL-17RC, IL-17RB, IL-17RE) during chronic lung infection by *P. aeruginosa* in murine models.

In C57BL/6N, long term chronic infection by *P. aeruginosa* embedded in agar beads is mainly associated to chronic neutrophilic inflammation ($CD11b^{+}$, $GR\text{-}1^{hi}$) and T cell recruitment. In this model we found that IL-17A, IL-17F, IL-17E, IL-17B and IL-17C levels increased during the early (2-days) and late (28-days) phases of chronic respiratory infection, potentially modulating host response through different IL-17 receptors. Moreover, preliminary data by flow cytometry suggests that IL-17 receptors are differently expressed among stromal and immune cells in the lung.

To directly address the contribution of each IL-17R during chronic respiratory infections, three new knockout (KO) murine models for each IL-17R were generated by CRISPR/Cas9 indel-mediated gene KO technology. To date, functional validation of the three IL-17 receptors KO murine lines both *in vitro* and *in vivo* is in progress.

The constitutive activation of IL-17 cytokines suggests that the interaction of IL-17RA with other IL-17 receptors may selectively modulate host defense and immunopathology. Further mechanistic studies on IL-17s/IL-17Rs immune axis models might help the design of novel immunotherapeutic strategies limiting exaggerated inflammation and tissue damage during CF chronic respiratory infection.

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Effects of short-term Lumacaftor-Ivacaftor therapy on lung microbiome in Phe508del homozygous patients with cystic fibrosis

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The interplay between cystic fibrosis (CF) airways microbiota and the disease severity and CFTR malfunction were demonstrated by several studies. The discovery of CFTR correcotr and potentiator was a breakthrough discovery but the influence of those therapy on the microbiome of the lung is still not fully explored. The aim of our study was to analysed the short-term effects of lumacaftor-ivacaftor on the lung microbiome in Phe508del homozygous patients

15 patients were included in this study sputum was use to sampled the lung microbiota at baseline and 8 to 16 weeks after initiation of therapy with the approved dose of lumacaftor 400 mg in combination with ivacaftor 250 mg every 12 h. Samples were aliquoted (200 µL) and treated with PMA to avoids subsequent PCR amplification of extracellular DNA. DNA extractions were performed using the QIAamp Mini Kit. Microbiome was explored by amplifying the the V4 region of the 16S rRNA gene (515F and 806R) and PCR amplicons were sequenced in a Illumina Miseq sequencing system with 250 cycles. In parallel, the number of 16S copies was quantified by quantitative PCR (qPCR). Sequences were analyzed with the R package dada2. Raw sequences were filtered and trimmed for good quality and merge as contigs. Ribosomal sequence variants (RSV) were assigned to taxonomy using the Silva database (version 132). RSV assigned to eukaryotes, archae and chloroplast were removed from the analysis.

Pseudomonas aeruginosa was present in five patients at baseline and no change was seen through therapy with lumacaftor-ivacaftor. Other common genera found in the cohort were Staphylococcus, Neisseria, Veillonella and Prevotella, but no significant changes in single generas were observed under therapy. Nevertheless, treatment with lumacaftor-ivacaftor significantly increased the alpha-diversity of the microbiome of Phe508del homozygous patients. This effect was mostly due to a significant increase in the richness after lumacaftor-ivacaftor therapy. Dominance as the relative abundance of the most dominant RSV and the overall bacterial burden showed tendencies of reduction under therapy.

Those results indicates that the combination of lumacaftor and ivacaftor in Phe508del homozygous patients influence the microbiome of the lungs. This influencerelate in an increase of the α-diversity not due to the decrease of the most abundant RSV (dominance) but mostly to an improved colonization by the rare biosphere. This rare biosphere is mostly constituted of anaerobes like Prevotella, Veillonella and aerobic commensals like Neisseria. This would indicate that the CFTR restoration allow less stringent selection pressure on the migrants from the upper airways by a modification of the ecological niche. It was previously shown in several studies that a more diverse microbiome in the lower airways was correlated with a better lung function and milder disease. The absence of decrease in the pathogen load would indicate that CFTR modulation alone do not allow eradication of the infection however the increase in the diversity would indicate that an early CFTR modulation might prevent or delay the first infection by conserving a diverse microbiota in the lower airways offering potential competitors against the gram-negative pathogens.

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Identification of specific microRNAs in CF bronchial epithelial cells infected by *Pseudomonas aeruginosa*

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INTRODUCTION: Cystic Fibrosis (CF) is characterized by chronic infections mainly by *Pseudomonas aeruginosa*. The consequent inflammation results in a progressive decline of the lung function of CF patients. Drugs targeting specific mutations are now used to restore functional CFTR and have improved the lung function of some CF patients. However, all patients would not respond equally to these drugs that have not yet demonstrated a clear-cut effect on chronic infection. Thus, considerable effort is still needed to find alternative strategies to fight against infection. Host defense against *P. aeruginosa* is especially initiated by airway epithelial cells constituting not only a physical barrier against microorganisms but also orchestrating an adapted and efficient innate immune response. In CF, this immune response against *P. aeruginosa* is altered. In this context, we have previously shown by transcriptional analysis of mRNAs that primary bronchial epithelial cells from CF patients (F508del homozygous) exhibit a distinct inflammatory profile in response to *P. aeruginosa* infection compared to cells isolated from donors with no CF disease (Balloy *et al*. PlosOne, 2015). We hypothesized that this distinct inflammatory response may involve microRNA (miRNA). In this context, we aimed to know if primary CF bronchial epithelial cells, express a different pattern of miRNAs after infection with live *P. aeruginosa* in comparison of bronchial cells isolated from donors with no CF disease. Combinations with previous transcriptomic data will allow us to identify their mRNAs targets.

METHODS: miRNAs were isolated from non-CF (n=4) and CF bronchial epithelial cells (n=4, F508del homozygous) infected by *P. aeruginosa* (MOI 0.25) for 2, 4 and 6h. Libraries were prepared using a TruSeq Small RNA Sample Prep kit (Illumina). After purification, sequencing was realized using an Hiseq 2500 (Illumina). Sequences were aligned with bowtie1 against miRBase 21.0. Counts were analyzed using R and DESeq2. Raw data were corrected by the method of Benjamini et Hochberg and significant threshold $p \leq 0,05$ was chosen.

RESULTS: We found 13 miRNAs differentially expressed between CF and non-CF cells before infection including miRNAs already described in the regulation of lung inflammation and lung epithelial innate immunity. After infection by *P. aeruginosa*, only seven miRNAs were modulated. In CF samples, three miRNAs were found upregulated, and three downregulated. In non-CF samples, only one miRNA was found to be upregulated after infection. Overall, the biological role of these miRNAs is not known except their involvement in non-small cell lung cancer.

CONCLUSION: We identify a specific pattern of miRNAs significantly altered in CF cells. Further works are necessary to identify the function of these miRNAs in the excessive inflammatory response of CF bronchial cells. Complementary to the antibiotics, manipulation of the host response with these miRNAs could represent a new therapeutic option, allowing a better anti-inflammatory and anti-infective response of CF bronchial cells.

P70

Distinguishing active bacterial populations associated with clinical exacerbations in cystic fibrosis patients by culture and DNA and RNA based molecular profiling

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Background and objectives: Chronic airway infections, characterized by recurrent

pulmonary exacerbations, are responsible for >90% of morbidity and mortality in individuals with Cystic Fibrosis (CF). Although conventional CF pathogens (e.g. *P. aeruginosa*) are targets of antibiotic therapy, colonization of the lungs by complex polymicrobial communities is now recognized. Our previous research has observed novel pathogens in CF, and polymicrobial interactions, where increased P. aeruginosa virulence may contribute to airway infections. However, these culture-based findings are not recapitulated in culture-independent microbiome profiling studies. Free DNA in sputum may confound molecular profiling methods and viable but inactive cells may complicate quantitative culture data. Because expectorated sputum produces highly heterogeneous mucous plugs, we postulate that only a portion is representative of the active bacterial populations driving the disease. How well DNA is able to represent what is happening at a microbial level during exacerbations is unclear, thus more unconventional methods need to be used when profiling. To distinguish populations, culture will be compared with DNA and RNA based molecular profiling.

Methods: Spontaneous sputum produced by adult patients attending regular CF clinic visits were obtained within 30 minutes and processed. Aliquots were treated for quantitative extensive airway culture, done anaerobically and aerobically, and processed for DNA and RNA extraction. Homogenization of the sputum was done with a standard saline wash followed by three incremental treatments of DTT increasing in concentration and incubation. Bacterial composition was determined by profiling of the V3 or V3/V4 variable region of the 16S rRNA gene (DNA) and 16S rRNA (RNA), and sequenced on an Illumina MiSeq and processed through the sl1p bioinformatics pipeline.

Results: Quantitative culture, done anaerobically and aerobically, allows for recovery of majority of the CF airway microbiome and resulted in distinct cultured organisms for each patient. At each processing step of a sample, a portion of the sample was teased apart resulting in aliquots from the periphery to the core where the mucosal plugs reside. Individual specific bacterial compositions were recapitulated in molecular profiles; however, the RNA profiles were not congruent with DNA based profiling. Comparative profiling revealed bacterial spatial heterogeneity within the sputum microbiome.

Conclusions: Each bacterial profiling method resulted in distinct bacterial compositions. The microbiology of sputum is highly patient specific and thus very difficult to identify a unifying pattern. Future studies will address the temporal stability of each of the profiles to determine if the time between expectoration and sample processing affect composition.

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Novel compounds to enhance the eradication and reduce the virulence of respiratory infections

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Introduction: It is well established that bacterial biofilms limit the effectiveness of conventional antibiotics in patients with CF, leading to chronic respiratory infections that are difficult to eradicate. Virulence factors produced by bacteria have a key role in the establishment and progression of biofilm infections, as well as mediating host response and damaging the lung epithelium (e.g. Kadioglu et al., 2008).

Disrupting biofilms and reducing the production of virulence factors in P. aeruginosa provides a novel treatment approach for chronic infections and can reduce the impact of infection on the lung. Neem Biotech has established a screening cascade to identify novel compounds that can inhibit the formation of biofilms, increase the effectiveness of antibiotics and reduce virulence in CF relevant bacteria. Candidate compounds are now being tested across clinically relevant assays to select a lead candidate for development.

Methods: A P. aeruginosa biofilm model in Artificial Sputum Media (ASM) was used to investigate the effect of compounds across strains and with 3 different standard of care antibiotics. The effect of compound treatment on biofilms alone and in combination with antibiotic was monitored by counting colony forming units (CFU) and structurally assessed by confocal laser microscopy. Single and mixed species biofilms were grown using a drip flow reactor and stained using SYTO 9 and propidium iodide before visualisation.

Select bacterial isolates were inoculated (OD_{595} 0.7) on 0.4% agar plates containing increasing concentrations of compound or vehicle. Swarming and swimming motility was imaged and quantified using ImageJ analysis software. The production of virulence factors, pyocyanin, alginate and rhamnolipid, were assessed spectrophotometrically using the Carbazole method, methylene blue and the Orcinol assay respectively.

BEAS-2B cells were grown to 80-90% confluence and exposed to bacterial exudates treated with compound or vehicle. Cell death was compared between condition and the production of inflammatory cytokines was quantified using an ELISA assay (Abcam).

Results: Compounds enhanced the activity of multiple antibiotics against CF relevant clinical isolates in ASM. Confocal imaging demonstrated disruption of the biofilm matrix with an enhanced cell death when combined with a reduced (>2.5-fold) dose of antibiotic.

Treatment with compounds further showed a decrease in key virulence phenotypes that are important for the establishment and progression of respiratory infections. Quantification of exoproducts showed a 44-72% reduction in pyocyanin across P. aeruginosa isolates 48 hrs following treatment, with alginate and rhamnolipid showing similar patterns at 24 hrs.

Human bronchial epithelial cells showed reduced toxicity and a reduced inflammatory response when exposed to bacterial exudates from compound treated biofilms, compared to vehicle treated bacteria ($P < 0.05$). The effect on host cell response was correlated to virulence factor production.

Conclusions: Neem Biotech has developed a screening platform for compound testing against bacterial biofilms and virulence.

The screen has identified candidates that enhance the activity of standard of care antibiotics against difficult to eradicate clinical isolates and reduce the virulence of bacteria prevalent in respiratory infections.

The lead candidate is now being progressed to preclinical development.

P72

Respiratory epithelial cells can remember infection

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INTRODUCTION: Since a few years, the concept that innate immune cells are not capable of memory is questioned by publications demonstrating an innate immune memory response by monocytes-macrophages. Thus, following a first contact with a microorganism, these cells are able to increase or decrease their response several days later to a second stimulus. This is described as trained or tolerant innate immune memory, respectively. We hypothesized that respiratory epithelial cells which are the first cells of the respiratory tract to encounter pathogens were also capable of memory. Our aim was to study the memory of bronchial epithelial cells exposed to component of microorganisms recovered in expectorations of cystic fibrosis patients such as the bacteria, *Pseudomonas aeruginosa* and the fungus *Aspergillus fumigatus*.

METHODS: To this aim, non-confluent bronchial epithelial cells (BEAS-2B cell line) ($2.8x10^4$ cells/well into 96-well plate) were pre-stimulated for 48 hours by bacterial components. The microbial components were then removed, cells were washed and incubated in new culture medium for 4 days. After this resting phase, cells, reaching confluence ($10.3x10^4$ cells/well) were stimulated for 15 hours by other microbial components or by lived *Aspergillus fumigatus* conidia. The cellular response was quantified by measuring IL-8 release in the supernatants. In some experiments, a pharmacological inhibitor of histone acetyltransferase was incubated with cells during the pre-stimulation and the resting phase.

RESULTS: Our results showed that the bronchial epithelial cell response to the second stimulus varied, based on the initial microbial component the cells were pre-exposed with. *P. aeruginosa* flagellin pre-stimulation modified the capacity of the cells to respond to a second stimulus. These modifications were different according the agonists used during the second challenge as, on one hand, a trained response was observed when the cells were stimulated by *A. fumigatus* conidia, and on the other, a tolerant response was obtained when stimulated by a bacterial lipopolysaccharide (*E. Coli*). The study of the mechanisms underlying this phenomenon and particularly epigenetic modifications were searched. Preliminary experiments using acetyltransferase inhibitors led us to hypothesize that histone modifications were involved in this innate immune memory of epithelial cells.

CONCLUSION: In this study, we highlighted that bronchial epithelial cells can memorize the first encounter with a microbial component a phenomenon transmitted during cellular division. This results in modification of their response to a second stimulus occurring several days later. Further studies of the underlying mechanisms could allow identification of signaling and therapeutic pathways. Such a memory mechanism could be at play in multimicrobial infections of the lung as it occurs in CF patients.

P73

ASL contribution to airway epithelial protection against *Pseudomonas aeruginosa*

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Background: Cystic fibrosis (CF) induces respiratory disorders including an early and persistent bacterial colonization, determining the prognosis of the disease. The healthy airway epithelium is covered by an Airway Surface Liquid (ASL), first bulwark against infection, while the CF airway epithelium is dehydrated. From adolescence, *Pseudomonas aeruginosa* is the major pathogen found in the airway epithelium of CF patients. This infection turns to chronicity, the mechanism of which is poorly understood.

Objectives: Our study aims to evaluate the contribution of the ASL on the airway epithelium protection towards bacterial infection in a physiological ASL-producer Calu-3 cell model, and to determine the underlying mechanisms.

Methods: We generated CTL (Wild-Type *CFTR*) and *CFTR* knockdown (KD-CFTR) Calu-3 cell lines by a CRISPR-Cas9 approach, and polarized the cells by culturing in air liquid interface.

To establish a *Pseudomonas aeruginosa* (*Pa*) airway colonization, we apically infected both Calu-3 cell types with a *Pa* mutant deleted in the LasR quorum sensing regulator gene. This mutant was less virulent towards healthy epithelium than the WT PAO1 strain. Calu-3 cells were infected with 1000 CFU/mL of the LasR mutant for 24h and transepithelial resistance (TER) and epithelium surface injury (SI) were measured.

To evaluate the influence of ASL on TER, ASL was removed from CTL cultures and transferred to the apical surface of KD-CFTR cells for 24h.

Expression and localization of junctional proteins were investigated by Western Blot and immunostaining.

Results: Our preliminary data show that CTL ASL increased TER of KD-CFTR cells (KD-CFTR $^{+}/_{-}$ CTL ASL: 367 ± 11 *vs.* 314 ± 32 $\Omega.cm^2$) whereas removal of ASL from CTL cultures decreased TER (CTL $^{+}/_{-}$ ASL: 560 ± 4 *vs.* 406 ± 23 $\Omega.cm^2$).

Following *Pa* infection, we likewise observed that ASL removal from healthy epithelium decreased TER (CTL $^{+}/_{-}$ ASL: 187 ± 10 *vs.* 85 ± 5 $\Omega.cm^2$, $p< 0.001$) and slightly enhanced SI (CTL $^{+}/_{-}$ ASL: 0 ± 0% *vs.* 8 ± 3%, $p=0.02$) in CTL cells. In contrast, the addition of CTL ASL on the KD-CFTR epithelium had a protective effect from *Pa* infection as revealed by higher TER (KD-CFTR $^{+}/_{-}$ CTL ASL: 86 ± 7 *vs.* 54 ± 1 $\Omega.cm^2$, $p< 0.001$) and lower SI (KD-CFTR $^{+}/_{-}$ CTL ASL: 18 ± 6 *vs.* 58 ± 7, $p< 0.001$).

Interestingly, the expression of the cell adhesion molecules claudin-3 and α1-catenin was markedly reduced after *CFTR* knockdown. Moreover, ASL removal from CTL epithelium reduced claudin-3 expression, while CTL ASL addition on KD-CFTR cells leads to increased claudin-3 expression.

Conclusion: Our data suggest that the ASL contributes to the airway epithelium integrity by a sustained expression of a physiological junction network. We propose an ASL-dependent modulation of the junctional network linked to claudin-3 and α1-catenin.

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Set of preclinical mouse models of respiratory infection to evaluate antibiotic efficacy

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Objectives: Contrary to safety testing, evaluation of antibiotic efficacy in animal models is not specified in any guidelines. Most frequently, efficacy is tested in acute respiratory infection models with chronic pneumonia largely unexplored, generating serious concerns about animal models as predictive tools for treating chronically-infected patients. We tested and compared the predictive value of preclinical mouse models of acute and chronic *P. aeruginosa* respiratory infection by evaluating the efficacy of two marketed antibacterials -tobramycin (TOB) and colistin (COL) to clarify:

(1) whether and how efficacy testing differs in murine models of acute vs chronic respiratory infection;

(2) how different administration routes -aerosol vs intranasal (i.n.) vs subcutaneous (s.c.)- impact on the host response and pathogen clearance efficacy;

(3) how different treatment schedules -soon after infection vs during chronic colonization- affect the efficacy;

(4) whether the bacterial burden and host response to antibiotic treatment are correlated.

Methods: C57BL/6 mice were infected with planktonic *P. aeruginosa* reference PAO1 or clinical multidrug resistant RP73 strains embedded in agar beads by intratracheal instillation (i.t.). Treatment started soon or days after infection by Penn-Century aerosolizer (p.c.), i.n. or s.c. Bacterial burden, leukocytes recruitment and cytokine/chemokine levels were analysed.

Results: In acute infection, p.c. or s.c. administrations of TOB were the most effective in reducing bacterial load, while i.n. treatment showed modest efficacy. COL reduced the bacterial load less effectively in comparison to TOB. Chronically-infected mice treated daily for seven days by p.c. or s.c. TOB starting soon after infection showed significantly reduced bacterial load compared to the placebo-treated group. COL induced a modest bacterial load reduction only when administered by p.c.. Notably, TOB had generally greater efficacy in reducing bacterial load compared to COL. Differently, the inflammatory profile in the airways, the neutrophil load in particular, was significantly reduced to a greater extent by COL compared to TOB, indicating that the inflammatory response does not strictly reflect changes in the bacterial load. When the seven-day treatment schedule started days after infection, both TOB and COL failed to eradicate *P. aeruginosa* and reduce inflammation compared to placebo.

Conclusion: Our findings demonstrate the limited predictive value of acute *P. aeruginosa* infection model unless complemented with models of chronic infection designed to mimic the onset and progression of persistent infection in humans. Both models should be systematically and specifically implemented in a preclinical platform supporting antibacterial efficacy testing. We show that only a combination of these models may provide an essential link between preclinical testing and clinical efficacy.

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Primary human nasal epithelial cell air/liquid interface cultures: an *in vitro* model system for assessing CFTR function

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Traditional methods for primary culture of human airway epithelial cells rarely allowed more than 1-2 passages before significant loss of epithelial function. Recently developed approaches including irradiated fibroblast feeder layers and inclusion of rho-associated protein kinase inhibitor enable >6 passages (10^5 to 10^6 fold expansion of cell number) with good retention of epithelial function. The "gold standard" for in vitro testing of CFTR correctors and potentiators is well-differentiated primary human bronchial epithelial cells obtained from lungs transplanted from CF patients. The number of different patient samples obtained in this manner is small and the range of CFTR mutations available for study is limited. The goal of this work is to establish methods for collection, culture, and expansion of primary human nasal epithelial cells, derived from non-CF volunteers and CF subjects to examine of inter-subject variation in CFTR expression, function, and response to CFTR modulator drugs. Nasal brushings were obtained from >20 healthy, non-CF volunteers and >100 CF subjects. Nasal cells were collected a second time (>2 months later) from a subset of the subjects. Samples were expanded (to passage 2) and cryopreserved. For analysis of CFTR function and expression, cells from multiple passages were seeded onto filter supports for establishment of air/liquid interface culture. The epithelial monolayers were transferred to Ussing chambers 15-60 days after seeding for measurements of CFTR-dependent Cl secretion. Viable epithelial cell cultures were established from nearly all samples and cultures from CF and non-CF subjects exhibited large amiloride-sensitive sodium current. Forskokin-stimulated and I^{172}-inhibited current was readily observed in all non-CF monolayers, but was minimal in CF monolayers. CFTR mRNA expression across non-CF subjects varied by ~10-fold and was correlated to CFTR Cl current. Cultures derived from CF subjects exhibited a broad range of responses to CFTR modulators among different CFTR genotypes and within subjects homozygous for F508del CFTR. The response to VX-809/VX-770 varied ~5X between least and most responsive cultures from F508del/F508del cells. There is good group-wise correlation between in vitro responses in cultured cells and the FEV1 response obtained from clinical trials data. We are currently working to assess the correlation between individual patient clinical response to Kalydeco, Orkambi, and Symdeko and the in vitro nasal epithelial cell culture responses to these modulators. From studies of primary human nasal epithelial cell cultures we have also identified two rare mutations (L145H and L138ins) that respond to VX-809 and VX-661. These studies support the use of primary human nasal epithelial cell cultures for testing CFTR modulators.

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P76

Human induced pluripotent stem cell derived p63 expressing epithelial cells provide a personalized model for CF lung epithelium

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Introduction: hiPSC can be used to develop organotypic cell culture models to study the pathophysiology of rare and common variants of Cystic Fibrosis, and the efficacy of experimental therapeutics. In contrast to committed stem/progenitor cells, hiPSC can be clonally modified by gene editing using TALEN and CRISPR/CAS9, and differentiated to multiple epithelial lineages. Moreover, this allows the generation of CFTR corrected lines from CF patients, which provides a valid wild type control in comparative analysis, and future potential for cell replacement therapy (Merkert S et al: Stem Cell Res. 2017, 23:95-97). Developing robust differentiation protocols that allow large scale air-liquid interface (ALI) culture of hiPSC derived airway cells is still challenging.

Approach: We have generated F508del CFTR, corrected, and N1303K CFTR hiPSC, a folding mutant prominent in the Mediterranean population, for which no effective correctors are available. Fluorescent markers monitoring epithelial differentiation: NKX2.1 (NKX2.1eGFP), the airway basal cell marker p63 (p63mVenusnuc NeoR), and CFTR (CFTR-dTomato) were introduced by TALENs in the respective gene loci. In F508del CFTR lines used for HT screening a YFP halide sensitive probe was introduced.

Methods: hiPSC were subjected to a 20 day differentiation and selection protocol, which reproducibly yielded a population of NKX2.1-GFP/p63-Venusnuc neoR expressing cells (~85%). After expansion of p63+ hiPSC in media used to differentiate primary airway basal cells in ALI culture (BEGM, Pneumacult), cells were transferred to standard ECM coated membrane inserts (Costar). At different intervals, cells were analyzed by QPCR, electrophysiology, confocal and electron microscopy.

Results: Depending on the ECM coating, p63+ iPSC on inserts reached variable TEER values within five days before transfer to ALI (500-2500 Ohm/cm2.). QPCR analysis of ALI cultured p63+ hiPSC revealed expression of general airway markers (NKX2.1, SOX2, FOXA2, SOX9, NGFR, CK5), ciliated cells (FOXJ1, CFTR, SCNN1A/ENACa) and secretory cell markers (CCSP, MUC5AC). Microscopy showed clusters of high cuboid epithelial cells expressing lateral (ECAD) and tight junctions (ZO-1), airway markers (KRT5, KRT8, KRT14, FOXJ1, P63). Multi-ciliated (TUBIV+) and secretory cells (CCSP, MUC5AC) were observed at high frequency, confirmed by electron microscopy.

Conclusions: We report efficient generation of hiPSC derived basal airway progenitor cells using a selectable p63 marker, which can be used to generate airway epithelia at air-liquid interface. hiPSC p63+ derived F508del airway cells will be used to validate novel F508CFTR activating compounds from a high throughput screen on iPSC derived CFTR expressing intestinal cells. In F508del CFTR mutant mice, and human primary bronchial epithelial cells in air liquid interface culture we observe oxidative stress and abberant bioactive lipid metabolism, resulting in enhanced pro-inflammatory and pro-fibrotic signaling. hiPSC derived airway cells can be used to study this in an isogenic model not affected by previous pathology, that can be modified by gene editing.

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High throughput screening for modulators of p.Phe508del CFTR activity applying an organotypic functional assay based on genetically engineered CF disease-specific induced pluripotent stem cells (iPSCs)

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It is expected that the availability of patient-specific induced pluripotent stem cells (iPSCs) in conjunction with precise genome-engineering approaches will revolutionize human disease modelling and drug screening. Organotypic culture systems based on disease-specific iPSCs exhibit obvious advantages compared to immortalized cell lines and primary cell cultures, however, their implementation in high throughput (HT) assays is technically challenging and as yet there are very few examples for successful conduction of iPSC-based HT drug screens. Here we demonstrate the development and conduction of an organotypic Cl^-/I^- exchange assay based on Cystic Fibrosis (CF) disease-specific iPSCs for the application in HT drug screening.

CF-iPSCs (homozygous for p.Phe508del) were generated via reprogramming of $CD34^{pos}$ cells isolated from small volumes of non-mobilized peripheral blood. The resulting CF-iPSCs were analysed regarding their karyotype, pluripotency status and differentiation potential. TALEN-based genome engineering was applied for 'footprintless' correction of the p.Phe508del mutation, and targeted introduction of a fluorescence reporter into the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) locus facilitated the directed differentiation into intestinal epithelia. Moreover the introduction of a halide sensitive YFP into the AAVS1 safe harbour locus enabled automated quantitative measurement of CFTR function. Reporter iPSC-derived epithelium revealed a CFTR-channel specific response after Forskolin application, which was inhibited after CFTR(inh)-172 treatment. CFTR function in CF iPSC-derived epithelia was partially rescued by treatment with VX-770 and VX-809, and seamless gene correction of the p.Phe508del mutation resulted in full restoration of CFTR function. A functional screen of ~ 42.500 small molecule compounds for modulators of Phe508del CFTR on a 384-well scale was conducted. Verified candidate compounds are currently evaluated in secondary assays.

As one of the first functional HT screens utilizing disease-specific iPSC derivatives, our approach will also contribute to clarify whether the general hypothesis holds true that organotypic assays based on patient-specific iPSCs are better predictors of clinical efficacy than conventional cellular assays that typically apply immortalized cell lines.

P78

The CFIT Program iPSC resource can generate CF-relevant tissues for studies of drug responses

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Background: Variation in CF disease severity is conferred not only by different CFTR genotypes, but also by modifier genes and environmental factors. Therapies targeting certain CFTR mutants have been approved, yet variations in clinical response highlight the need for an individualized approach, utilizing robust patient specific genetic and tissue-based testing platforms that are predictive of therapeutic outcomes to the approved and next generation compounds advancing through the drug discovery pipeline.

Methods: CF Canada-Sick Kids Program in Individual CF Therapy (CFIT; http://lab.research.sickkids.ca/cfit/) is generating a “first of its kind”, comprehensive resource possessing subject-specific cell cultures and data that will enable modeling of therapeutic responses. This resource consists of:

1) nasal cells from drug naïve patients suitable for culture and the study of drug responses in vitro,

2) matched gene expression data obtained by sequencing the RNA from the primary nasal tissue

3) whole genome sequencing of blood derived DNA from each of the 100 participants,

4) induced pluripotent stem cells (iPSCs) generated from each participant´s blood sample,

5) CRISPR-edited isogenic control iPSC lines for a subset of the participants and

6) prospective clinical data from patients treated with CF modulators clinically.

Results: We have recruited close to 80 of 100 total patient donors to the CFIT Program and nasal and iPSCs are available to CF researchers for their owns studies. We have biobanked primary airway (nasal) cultures and iPS cell lines from individuals homozygous for F508del, heterozygous for F508del and a minimal function mutation, and several rare homozygous mutations. CF patient iPSCs can be differentiated into airway epithelium and other CF-relevant tissues. We have differentiated iPSCs from CFIT participants and isogenic CRISPR-edited iPSC control cells and examined their CFTR channel function by membrane potential dye assay. CFTR correctors and potentiators modulate CFTR channel function in a medium throughput assay of differentiated cells and we can quantitate localized cellular CFTR channel function across individual wells. Conclusions: Mid-to high-throughput assays of CFTR channel function in tissues differentiated from CF patient iPSCs enable in-vitro clinical trials to profile drug responses for multiple individuals simultaneously or the drug response across multiple tissues from the same individual. We anticipate that this resource will enable testing and comparison of novel therapeutic interventions targeting F508del-CFTR, therapy discovery for rare CF causing mutations and modeling of pathogenesis and therapy in tissues that were previously not easily accessible for study but can be generated from induced pluripotent stem cells.

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P79

iPS cells: relevant models and the road to cell-based therapies?

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Cystic Fibrosis (CF) is a monogenic disease caused by a mutation in the gene coding for the chloride channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Currently, more than 30,000 individuals in the US are living with CF and harbor over 1,700 reported mutations. Despite being monogenic, the large number of distinct and rare variants of CFTR presents both a serious therapeutic challenge and confounds new drug discovery efforts. First-generation CFTR modulators, targeting the defective protein, are now FDA-approved for 34 genetic variants comprising ~59% of the patient population. New highly efficacious triple modulator therapy is currently in clinical development for all patients with at least one CFTR F508del allele, but about 5-7% of people with CF are not likely to benefit from such treatment. These people typically carry two alleles that fall into class I or V mutations as well as some refractory missense mutations, e.g., N1303K.

Drug discovery for the CF-causing variants without effective disease-modifying therapy on the horizon will require better models than cell lines heterologously expressing CFTR cDNA constructs. E.g., in the context of PTC variants, i) transcriptional (Nonsense Mediated mRNA Decay, NMD) ii) translational (readthrough) and iii) post translational modifications (folding / glycosylation) all have been shown to effect the amount and function of CFTR.

Inducible pluripotent stem cells (iPS cells) offer a promising approach to generating physiologically-relevant systems to aid in CF drug development for all mutations. iPS cells expand nearly limitlessly in the undifferentiated state, are adaptable for gene editing approaches, and can be differentiated into multiple CFTR-expressing cell lineages. This study details how iPS-based protocols are being developed for drug screening. Currently, we are working on an iPS directed differentiation protocol designed to assess CFTR on multiple levels: CFTR responses to potential therapies can be assayed accurately utilizing Biochemistry (Western / qRT-PCR), and electrophysiology or organoid swelling for function.

Cell-based therapy approaches may also be built on iPS-derived human bronchial epithelial (hBE)-like cells. Here, we show the single cell transcriptional profile of primary hBE cells compared to differentiating iPS-derived hBE-like cells, data, that has and should continue to enhance our directed differentiation output to become more physiologically relevant.

P80

Gene and base edited cell models of cystic fibrosis-causing mutations

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To date three CFTR modulators have been developed however so far they only benefit patients bearing 37 class III/IV mutations and F508del homozygous. There are still hundreds of other disease-causing mutations in need of a targeted therapy. Although the use of cellular models provides insights into disease mechanisms most of the existing cell lines rely on overexpression of CFTR. Nevertheless, homozygous cell lines for three CF-causing variants were recently generated (Valley, 2018). Thus, we are using gene editing approaches to produce novel cell models based on endogenous expression of CFTR that may contribute to the definition of novel therapeutic approaches.

Here, we present data from two gene editing methods for making isogenic cellular models homozygous for different CF-mutations, using the bronchial epithelial cell line 16HBE14o$^-$. The first is a highly efficient method that uses Cas9/gRNA ribonucleoproteins (RNPs) and single strand DNA (ssDNA) oligonucleotide donor, with reduced off-target effects (Liang, 2015). Different RNA molecules were designed close to I507del and I1234V mutation sites; these were assembled with high fidelity Cas9 to form the RNPs, delivered into 16HBE14o$^-$ cells and successful validated by ICE (Inference of CRISPR Edits) webtool. The gRNA that showed highest double stranded break (DSB) formation was selected for the design and co-transfection with a ssDNA donor. For each of the mutations, co-transfection of Cas9 RNP and donor showed an efficiency of HDR and an indel formation both higher than 20% in the pool of edited cells.

The second method, called A to G base editing, allows the direct and irreversible conversion of an adenosine into a guanosine in a programmable manner, without requirement of DSB or a donor DNA template (Gaudelli, 2017). In this case, one gRNA has been designed for each of the mutations M1V and I1234V according to the base editing window 4 to 7 nt (counting PAM sequence 21-23). As a proof of principle experiment, both gRNA and base editor (ABE 7.10) plasmids were co-transfected into Calu-3 cells and successful validated using EditR (Edit Deconvolution by Inference of Traces in R) and BE-Analyser webtools. These results showed an editing efficiency comparable with the one achieved by HDR, without any previously selection, but importantly the indel formation was nearly 1%. Consequently, this method is being used to produce the desired mutations in 16HBE14o$^-$ cells.

In summary, we showed that the two different genome editing methods have been established for the generation of isogenic homozygous mutant CF cell lines. The next step will be isolation of single cell colonies which then are going to be genotyped and the off-target effects evaluated for the selected clones to guarantee specificity. Ultimately these cells will be characterized at the cellular and molecular level in order to find disease signatures for CF that may result in novel therapeutic outcomes.

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P81

Establishment and reproducible screening of a large-scale CF patient derived colon organoid biobank

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Pre-clinical disease models are essential for the development of novel therapies and help better understand the molecular processes that are deregulated by CFTR mutations. In recent years, a novel technology to generate patient specific in vitro cultures, the Organoids, has gained widespread interest for the development of new therapies and as a predictive diagnostic tool. After we developed the organoid technology we established a large biobank of over 400 CF patient derived rectal organoids. In this biobank, both common and rare CFTR mutations are captured.

The organoid biobank has been used to test the activity of several recently developed CFTR correctors and potentiators. The forskolin induced swelling (FIS) assay measures CFTR activity in a real time manner. Here we report the reproducibility of the FIS assay over several different microscope platforms. In addition, we have tested the stability of swelling of organoids in prolonged culture. This high level of performance of the assay opens the possibility to use organoids for clinical applications. Patient derived organoids provide a feasible mean to test the response of existing and developmental drugs for individual patients.

P82

Development of a steady-state lumen area screening assay to measure CFTR function in organoids of cystic fibrosis patients

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We have developed sensitive functional assays that utilize intestinal organoids of cystic fibrosis (CF) patients to measure (residual) CF transmembrane conductance regulator (CFTR) function and response to CFTR modulating therapies. CFTR function (restoration) using this preclinical model system can be determined by quantitation of luminal fluid secretion via measurement of (i) forskolin-induced swelling (FIS) or (ii) the forskolin-independent steady-state lumen area (SLA) of CF organoids. Importantly, the dynamic range of both assays varies in that FIS is more responsive for CFTR function measurements at low CFTR function, whereas at higher CFTR activity SLA is more responsive. The SLA assay has a reduced throughput when compared to the FIS assay, as organoid lumen segmentation has to be performed manually, whereas FIS can be quantified automatically.

Here, we developed our conventional SLA assay into a medium-to-high throughput screening format by using a fluorescent live cell imaging dye that labeled the lumen of organoids specifically, allowing for automatic lumen segmentation and calculation of the percentage of the lumen area of the total organoid area (SLA). We generated an optimized and efficient labeling protocol in intestinal organoid cultures and demonstrated that CFTR function, as measured by well-characterized FIS responses in reference organoids, was not negatively affected by the labeling procedure. CFTR function quantitation in organoids by this novel approach with varying CFTR activity (ranging from low to wild-type CFTR activity) was benchmarked to previously published (conventional) SLA measurements and corresponded well to this reference data set. In contrast to the conventional assay our new approach was able to measure lumens in CF organoids. Finally, to perform medium-to-high throughput SLA measurements we developed a CellProfiler and ImageJ analysis pipeline which allows for automatic image segmentation and removal and manual correction of incorrectly segmented objects. As a proof-of-concept we measured residual CFTR function in a small patient cohort of individuals with the R117H-CFTR mutation on at least one allele using our novel SLA assay and data analysis pipeline.

In conclusion, we successfully developed our conventional SLA assay into an automated medium-to-high throughput screening assay, which expands the toolbox of existing CFTR function measurements and will be especially useful to study CFTR function (restoration) in CF organoids with high (residual) CFTR activity by existing but also future (next-generation) CFTR modulators.

P83

High reproducibility of forskolin-induced swelling of organoids across three academic laboratories

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In the last five years patient-specific CFTR function measurements generated by the forskolin-induced swelling (FIS) assay in human colon organoid stem cell cultures have proven to be an effective scientific and predictive clinical model system for both diagnostic and drug assessment applications in the field of cystic fibrosis (CF). As such, there has been both a medical and scientific interest from the CF community to implement this model system internationally across laboratories in a validated and standardized manner.

The HIT-CF Europe Project (EU, Horizon 2020) is a unique collaboration between the academic institutes Katholieke Universiteit Leuven (KUL), the University of Lisboa (LIS) and the University Medical Center Utrecht (UMCU), the foundation Hubrecht Organoid Technology (HUB) and the pharmaceutical partners Flatley Discovery Lab, Proteostasis Therapeutics and Eloxx Pharmaceuticals. It offers both the unique opportunity

(i) of enabling access to the organoid model by European CF patients with an ultrarare *CFTR* genotype, as well as

(ii) drug assessment by these pharmaceutical companies of newly developed CFTR modulators not yet on the market. The primary goal of this project is to test the efficacy of these new CFTR modulators in organoid cultures derived from over 600 CF patients that have ultrarare genotypes with the ultimate goal to offer novel treatments for this group of patients that would otherwise be excluded from (lifesaving) therapies.

The first phase of the HIT-CF Europe project focuses on the alignment of the three research laboratories with respect to the usage of protocols, validation of FIS data and working towards international European standardization and implementation of this preclinical CF model system. Therefore, organoid lines derived from six CF patients with prevalent *CFTR* genotypes ranging from severe class I to 'mild' class V mutations (G542X/G542X, F508del/R1161X, F508del/F508del, F508del/ G551D, F508del/ S1251N and F508del/ R117H) were cultured and CFTR function was measured by FIS. Untreated and VX-770 and/or, VX-809-treated organoids were tested and data of 32 identical conditions of KUL, LIS and UMCU were analyzed and compared to each other. Statistical analysis of the generated datasets by KUL, LIS and UMCU demonstrated high agreement between the data, based on relative organoid swelling responses and despite variation in imaging equipment and image analysis software. Cross analysis of the data demonstrated that differences were mainly caused by experimental variations and not due to different image analysis methodologies. Additional criteria such as minimal amount of organoid surface area plated per well and the intensity of calcein green signal during image acquisition may further decrease the observed variation in the FIS experiments. Taken together, the agreement between sites indicates that FIS measurements can be standardized across different laboratories with high reproducibility.

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Development of a humanized CF mouse model

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Animal models are widely used for the study of the pathophysiology of human diseases and the efficacy of new therapies. In the context of cystic fibrosis (CF), several animal (mouse, pig or ferret) models have been developed. However, they show little or no severe respiratory phenotype typically associated with the high morbidity and mortality of the human disease. Based on the observation that the F508del mutation of CFTR leads to a more severe CF phenotype for the human CFTR (hCFTR) than the murine CFTR (mCFTR), this project aims at developing a CF mouse model expressing the hCFTR gene.

A KO-CFTR mouse line was generated using the CRISPR/Cas9 genome editing system. In this aim, a guide RNA targeting the exon 1 was used to produce mutations in the mCFTR gene. In parallel, a wild-type (WT) or mutated (F508del mutation) hCFTR overexpressing mouse line was created by transgene addition. For this purpose, a plasmid construct (pcDNA3.1-hCFTR, CMV promoter) was designed, amplified, purified and microinjected into C57BL/6NCrl mouse embryos. Regarding the development of the "knock-out" mCFTR line, 33 mice were generated. After comparing the WT mouse exon 1 sequences with those of the generated mice, 7 mice were shown to have at least one mCFTR mutation. Regarding the humanized mouse model, the constructs of pcDNA3.1-hCFTR for WT and for F508del were injected into 178 and 115 embryos of C57BL/6NCrl mice respectively. Twenty-seven newborns were obtained for the WT group and 14 for the F508del group. Of these, 5 transgenic mice (2 WT-hCFTR and 3 F508del-hCFTR) were obtained. Biochemical studies using immuno-tagging and western blotting are ongoing to check whether the generated founding mice of the KO-CFTR line are indeed devoid of the expression of the CFTR protein. Quantification of the WT or the F508del mutated CFTR is to be analyzed in the founding mice of the humanized lines to check protein over-expression. When these data were verified, the founding lines will be used to generate lineages of the humanized CF mouse model, which will then be characterized from organ and tissue phenotypic point of view. The model will hopefully allow a better understanding of the genotype/phenotype differences between the hCFTR and the mCFTR.

P85

Lack of cystic fibrosis transmembrane conductance regulator causes low cortical bone thickness and high cortical porosity in newborn pigs

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Background: People with cystic fibrosis exhibit growth defects and brittle bones. This observation has been attributed, in part, to malnutrition and chronic pulmonary inflammation. We tested the hypothesis that disruption of the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene directly affects bone microarchitecture and integrity by studying bone of newborn $Cftr^{-/-}$ pigs.

Methods: We examined femoral cortical and trabecular bones of $Cftr^{-/-}$ pigs less than 24h after birth using micro computed tomography (m CT, Skyscan 1076, Bruker). Scans were performed with the following settings: tube voltage, 80 kV; tube current, 0.125 mA; and voxel size, 17 x 17 x 17 mm (x, y, z). Three-dimensional images were rebuilt and analysed using the NRecon GPU version and CTAn (Bruker) software programs, respectively. The cortical bone porosity and structure were defined using a 3.4 mm wide region centered on the middle of the femur. A total of 37 newborn $Cftr^{-/-}$ piglets (24 males and 13 females) and 18 newborn $Cftr^{+/+}$ piglets (8 males and 10 females) was subjected to mCT scan.

Results: Compared to newborn $Cftr^{+/+}$ pig controls, $Cftr^{-/-}$ femoral bone exhibited significantly lower total volume (TV), bone volume (BV) and bone volume density (BV/TV) but only in females. However, the $Cftr^{-/-}$ bone mineral density (BMD) in trabecular and cortical tissues was significantly reduced in both sexes, compared to $Cftr^{+/+}$ piglets. Interestingly, focusing at the porosity of cortical bone in $Cftr^{-/-}$ pigs as a determinant of bone fragility associated with high fracture risk, we observed higher closed porosity with a marked increase of closed pore surface in cortical bone of $Cftr^{-/-}$ pigs (+18,7% for males and +48% for females). These results suggest a lower bone remodelling, lower interconnectivity within the vascular network, and increased bone fragility in $Cftr^{-/-}$ animals. No significant difference was observed in the open cortical porosity, whatever the gender.

Conclusion: Altogether, these data highlight the critical regulatory role of CFTR in bone development and maintenance, and suggest that some bone defects in people with cystic fibrosis are likely primary.

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Pancreatic ductal fluid and bicarbonate secretion of the ferret and pig models of cystic fibrosis (CF)

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Cystic fibrosis (CF) is a lethal genetic disease affecting several organs, including the pancreas. There are several animal models are available to study the CF related pancreatic tissue damage although they have limitations (e.g. the mouse model). Recently a cystic fibrosis transmembrane regulator (CFTR) knockout ferret and pig model have been generated.

Our aim was to investigate the fluid and bicarbonate secretion in the CF and wild type (WT) ferret and pig pancreatic ducts.

Intra/interlobular pancreatic ducts were isolated from newborn CF and WT ferret and pig pancreata. Expression of CFTR (cystic fibrosis transmembrane regulator) was detected by immunohistochemistry.Resting pH, buffer capacity and Cl^-/HCO_3^-exchange activity were evaluated by microfluorometry. Intracellular Cl^- efflux was measured by the Cl^- indicator MQAE ((*N*-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide). To inhibit the CFTR activity we used the CFTR inhibitior c2992-172 (Sigma) in 10 µM concentration in Cl^- free HCO_3^- solution. CFTR-dependent chloride efflux was calculated as the difference in alterations of Cl^- free HCO_3^- solution fluorescence in the absence and presence of CFTR selective inhibitor CFTRinh-172. The rate of chloride efflux induced by HCO_3^- after substitution of chloride by nitrate in the perfusion medium was measured by the change in fluorescence of MQAE. Fluid secretion of ducts were examined by videomicroscopy.

Resting intracellular pH of pancreatic ductal epithelial cells was 7.17±0.08 in ferrets. Functionally active sodium/hydrogen exchangers and sodium/bicarbonate cotransporters were detected in WT pancreatic ducts in ferrets. Intracellular Cl^- efflux measurements revealed a strong Cl^- efflux in pancreatic ducts isolated from WT ferrets ($\Delta F/F_0$=1.5±0.03) and pigs ($\Delta F/F_0$=1.6±0.04). Notably, bicarbonate secretion was significantly decreased in CF ferret ducts compared to WT ($p< 0.04$) Fluid secretion measurements revealed a significant increase in fluid secretion to HCO_3^- and 5µM forskolin and 100 µM IBMX in both WT pig and WT ferret ducts. In CF ferret and pig ducts increase of the fluid secretion were not detected during the stimulation period with 5µM forskolin and 100 µM IBMX, while in WT ferret and pig ducts stimulation of the fluid secretion occurred.

Absence of the CFTR leads to decreased or completely abolished pancreatic ductal fluid secretion both in ferrets and pigs. Pancreatic ductal bicarbonate secretion is also significantly decreased in ferrets.

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Genetic repair of CFTR function in cystic fibrosis organoids using CRISPR/Cas9 adenine base editing

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The monogenetic and autosomal recessive characteristics of cystic fibrosis (CF) allows for the use of gene editing techniques to restore defective CFTR function at its root. This is particularly interesting for patients with severe mutations that cannot be rescued by current small molecule therapies.

Previously, we showed that the CRISPR/Cas9 system could be used to genetically restore CFTR function in F508del/F508del organoids. However, conventional CRISPR/Cas9-mediated homology directed repair (HDR) has several disadvantages, such as undesirable off-target effects and the generation of indels or translocations at the target site. The recently developed novel CRISPR/Cas9 base editing technology circumvents these issues as this technique induces a permanent conversion of one base pair to another at a locus without generating double-stranded DNA breaks and could therefore potentially be a great tool to repair severe (class I) *CFTR* mutations.

Here, we used for the first time the newest adenine base editor to irreversibly convert specific A-T to C-G in patient-derived organoids harboring the R758X/R758X CFTR genotype. We also used an xCas9-adenine base editor to restore CFTR function in F508del/R553X organoids, thereby demonstrating the possibility for heterozygous repair by base editors. Furthermore, we showed for the first time that xCas9-adenine base editor can be used to repair a disease-causing mutation on an orthogonal (non NGG) PAM (NGT). In addition to the conventional adenine base editors, a recently published optimized version was also used to test the hypothesis if increasing expression of the base editor due to expression and nuclear localization optimization enhances gene editing efficiency. Transfection of organoids with the base editors was performed by electroporation, according to previously published protocols, with the exception that organoids were grown on Wnt surrogate instead of Wnt-conditioned medium 1 week prior to and 4 weeks after electroporation. Functional rescue of CFTR was assessed using the forskolin-induced swelling assay in these cultures. Multiple gene-corrected organoids per genotype were generated and identified by an increase in organoid swelling upon forskolin stimulation for one hour. These individual organoids were picked and passaged multiple times until a clonal organoid culture was developed. We demonstrated by Western blotting the production of full length CFTR protein and confirmed by Sanger sequencing the adenine base editing event in the CFTR gene in these clones. To assess potential off-target base editing, whole genome sequencing will be performed in the near future.

Altogether, we demonstrated proof of concept for gene correction by the newest adenine base editors in intestinal organoids derived from CF patients showing the potential of correcting the CFTR defect at the root of the disease in primary human CF cells.

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CFTR correction in primary airway epithelial cells with CRISPR/Cas9 as a proof of principle *ex vivo* gene therapy for Cystic Fibrosis

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Cystic fibrosis (CF) is the most common heritable disease among people with Northern European ancestry; however, there is still no available cure. Gene therapy is a promising approach to cure CF and CRISPR/Cas9 gene editing could provide precise and permanent correction of the CFTR gene. We aim to correct and expand epithelial progenitor cells *in vitro* and subsequently deliver them to the airways as a combined gene and cell therapy.

We have optimised the culture conditions for expansion of human airway epithelial cells by culturing them in the presence of an irradiated mouse fibroblast feeder layer. This allows us to expand adult primary cells for over 10 passages (and more than 40 population doublings) while maintaining their ability to differentiate *in vitro* in Air Liquid Interface (ALI) cultures. The differentiated ALI cultures were shown to be positive for airway epithelial cell markers and differentiation markers. The cells also demonstrated electrical responses and the expected chloride channel activity, according to their phenotype (CF and Normal cells), in Ussing Chambers.

We have optimised GFP transfections of primary CF epithelial cells with an efficiency of approximately 60%. CRISPR/Cas9 mediated double strand breaks were created in CFTR with optimal guide RNAs in 45% of cells. We have developed a donor repair plasmid with a puromycin selection cassette, which facilitates the correction of nasal epithelial cells with the most common CF mutation, ΔF508, exploiting the cells' homology directed repair pathway. Genotyping via Sanger sequencing confirmed the presence of the repair cassette and the absence of the ΔF508 mutation in the selected cell population. Scarless excision of the cassette at the flanking piggybac sites, followed by a second negative selection, results in cells with the corrected, healthy genotype. Corrected cells are being investigated for functionality and for their potential to repair the CF epithelium.

We have also created isogenic CFTR knockouts in Normal Human Bronchial Epithelial (NHBE) cells as a means of establishing the percentage of corrected versus mutated cells needed for successful CF treatment via cell therapy. Sequential rounds of transfection were used to achieve high percentages of indels in exon 2 of CFTR in NHBE. After 3 rounds, 80% of sequences appeared with indels in the NHBE cell population. These cells were single cell cloned, demonstrating efficacy of this technique in primary airway epithelial cells, and allowing the isolation of potential CFTR knockouts. The single cell clones are currently under investigation for functional knockouts.

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CFTR super exon partially corrects W1282X-CFTR

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Gene editing and gene therapy are promising therapeutic approaches for cystic fibrosis (CF). Strategies to correct individual CFTR variants are not feasible for most CF-causing genetic variants as more than 1,700 genetic variants of the CFTR gene have been suggested to cause CF. Insertion of a partial cDNA (also known as a super exon) into the native CFTR genomic locus can restore the CFTR gene to a wt coding sequence for all mutations downstream of the insertion site while retaining the endogenous CFTR promoter. However, the functional consequences of super exon insertion into CFTR are largely unexplored. Large fragment targeted insertion through gene editing, such as the introduction of a super exon, remains a technical challenge because it conventionally requires the low efficiency homology directed repair (HDR) pathway. As an alternative, homology independent targeted integration (HITI) is a recently developed technique for large fragment targeted insertion that bypasses the HDR pathway.

To explore the viability of the approach and functional consequences of partial cDNA insertion into CFTR, we employed both conventional HDR and HITI approaches for insertion of a small super exon into CFTR in gene edited 16HBE14o- cells expressing W1282X CFTR (cell line CFF-16HBEge CFTR W1282X). Previously, exonic sequences have been targeted for CFTR super exon insertion; however, we targeted intron 22 with CRISPR/Cas9 to avoid unproductive editing events (indels) in the CFTR-coding sequence. The inserted large fragment contains the ~250 distal nucleotides of intron 22 (to restore the natural splice donor of exon 23), a super exon coding for the native CFTR exons 23-27, and a bovine growth hormone (BGH) polyA sequence. Both the conventional HDR and HITI approaches yielded several clonal cell lines containing the desired genomic super exon sequence, which is predicted to yield a functional CFTR. As expected, the native genomic CFTR sequence distal to the intron 22 insertion site, including the W1282X mutation, was retained downstream of the inserted super exon and BGH polyA sequence.

Cells expressing the CFTR super exon allele no longer expressed truncated CFTR protein characteristic of the W1282X variant. Instead, Western blots revealed full length CFTR protein. Consistent with the Western analysis, we observed partially rescued CFTR function (~5-10% of WT function) in electrophysiological assays. Unexpectedly, the CFTR mRNA expressed from the super exon allele was subject to nonsense-mediated mRNA decay (NMD), very similar to the native W1282X-CFTR mRNA, as demonstrated by reduced mRNA levels that could be increased 5-8 fold with the NMD-inhibitor SMG1i.

In summary, we have demonstrated successful large fragment targeted insertion of a CFTR super exon through two editing strategies, resulting in partial functional rescue of CFTR. However, these studies also highlight the need for additional characterization to fully elucidate the functional consequences of super exons as a gene editing strategy for CF. Work is ongoing to further assess the impact and processing of super exon alleles, e.g., for different CFTR mutations and other insertion sites.

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CFTR protein detection in organoids from healthy and CF patients with nonsense mutations support using the organoid model to test ELX-02 mediated CFTR readthrough restoration

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Eukaryotic Specific Ribosome Glycoside (ESRG) modulators are a novel class of therapeutics that are currently being investigated as a treatment strategy in cystic fibrosis (CF) patients harboring at least one nonsense mutation allele. ELX-02 a next-generation ESRG was derived from a rational design approach to suppress premature termination codon (PTC) efficiently with less toxic effects than aminoglycosides. As such, it promotes ribosomal read-through of nonsense mutations and inhibits translation termination at in-frame premature stop codons. This results in restoration of full-length functional protein. We have previously demonstrated that ELX-02 rescues *CFTR* function in a dose-responsive fashion across a variety of *CFTR* nonsense mutation-harboring patient-derived organoids as well as leads to increases in *CFTR* mRNA. Intestinal stem cells organoids have been a reliable model system for examining response to CFTR modulators in CF patient derived cells with nonsense mutations because of their sensitivity in detecting CFTR function using a forskolin-induced assay. However, a limitation in the field has been consistent detection of endogenous CFTR protein including its detection in the organoid model. In our current work we have developed an approach to detect both the endogenous core glycosylated and mature form of CFTR protein (B and C form respectively) in healthy and patient derived organoids, using Simple Western capillary electrophoresis. In addition, we have demonstrated detection of apical CFTR localization in healthy organoids using confocal imaging. This approach demonstrates the utility of the 3D patient-derived organoid model to evaluate ELX-02 mediated readthrough restoration of *CFTR* function, mRNA and readthrough synthesis of CFTR protein. These studies and others support upcoming ELX-02 clinical trial in CF patients.

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Exploring splicing modulation approaches using antisense oligonucleotides in cystic fibrosis organoids

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Approximately 12% of the reported cystic fibrosis (CF) causing mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are splicing mutations. These splicing mutations are responsible for the generation of aberrantly spliced pre-mRNA, which leads to the production of incorrect mRNA transcripts and synthesis of non-functional CFTR. Although splicing mutations comprise a significant group of all known CFTR mutations, therapeutic strategies targeting this class of mutations remain largely unexplored.

Antisense oligonucleotides (AONs) are short synthetic chemically-modified single-stranded complementary RNA molecules that sterically occupy aberrant splice sites and/or regulatory splicing elements thereby restoring incorrect pre-mRNA splicing. This straightforward splicing modulation approach is an attractive therapeutic tool and has been successful for other diseases with the first therapies already having entered the market. Currently, AON-mediated splicing interventions do not exist for CF.

As such, we initiated the development of a preclinical subject-specific AON development pipeline using CF patient-derived intestinal organoid cultures harbouring splicing mutations. Our preliminary results demonstrated that 2′-O-methyl ribose and phosphorothioate backbone (2OMePS) chemically-modified AONs could be delivered into organoid cultures. Previously published 2OMePS-modified AON sequences were tested for AON-mediated splicing modulation in CF organoid cultures but CFTR function as measured by the forskolin-induced swelling (FIS) assay could not be observed in organoids, which was also confirmed by analysis of mRNA transcripts in these AON-treated organoids. We are currently investigating whether the lack of effective CFTR splicing modulation might be due to

(i) low levels of nucleus-localized AONs or

(ii) efficacy differences of AON chemistries between model systems or

(iii) identified AON sequences in continuous cell lines cannot be translated directly to patient-derived primary culture models.

In parallel, we are exploring a novel screening approach to identify AON sequences with the ability to restore aberrant CFTR splicing in organoids using U7-small nuclear RNA (snRNA)-based AONs in organoids. We have generated a control virus expressing the previously published CyPA-targeting U7-based AONs to validate splicing modulation effectivity in intestinal organoid cultures as proof of concept. An in silico coarse walk along the 3272-26A>G splice mutation in the CFTR gene was performed and we identified various possible splicing modulating target sequences. These sequences are currently being cloned into an U7-snRNA-expressing lentiviral plasmid of which lentiviruses will be generated which we will use in the future to quantify correctly and incorrectly spliced mRNA products together with CFTR function restoration using the FIS assay. Here, we will present our preliminary results of this novel approach to identify AON sequences effectively targeting splicing mutations in CF organoids.

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Restoration of CFTR function by antisense oligonucleotide splicing modulation in respiratory and intestinal primary model systems

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A significant proportion of disease-causing mutations in humans affects pre-mRNA splicing. Among these are mutations affecting non-canonical splicing motifs leading to both aberrantly and correctly spliced transcripts. Disease severity in patients carrying these mutations is highly variable and correlates with the level of aberrantly spliced transcripts. Antisense oligonucleotides (ASOs) were found to be highly efficient in modulation of the splicing pattern in several genetic diseases. We aim to develop a ASOs-based therapy to modulate the level of correctly spliced CFTR mRNA translated to normal functional CFTR proteins, in patients carrying non-canonical splicing mutations. As a model we first focused on a common CFTR splicing mutation, the 3849+10kb C-to-T, which leads to inclusion of an 84 bp cryptic exon between exons 22-23 in the mature mRNA. This cryptic exon contains an in-frame stop codon that leads to degradation of a significant fraction of the mRNA by the nonsense mediated mRNA decay (NMD) pathway as well as to the production of prematurely truncated nonfunctional proteins. We designed 2´-O-methyl phosphorothioate-modified (2'-OMe) ASOs, targeted to prevent the recognition of the cryptic exon. Screening of these ASOs led to the identification of several ASOs that significantly decrease the level of aberrantly spliced CFTR mRNA and increase the level of correctly spliced CFTR mRNAs. Importantly, we demonstrate that these ASOs restore the CFTR channel function in primary human nasal epithelial cells (HNEs). For improvement of free ASO uptake we are studying the effect of the chemical modification 2'-O-(2-methoxyethyl)-oligoribonucleotide phosphorothioate (2'-MOE). The results show a further improvement in the efficiency and potency of the ASO and enables the use of lower ASO concentrations to fully restore the CFTR function in HNEs. HNE cells have been shown to recapitulate the properties of lower airway epithelial cells as well as of primary human bronchial epithelial cultures. HNEs are easy to collect by simple nasal brushing and allow quantification of chloride transport as a marker of CFTR function. Most importantly, HNEs enable investigation of CFTR modulation in a system expressing the endogenous CFTR in its native context required for regulation of correct splicing, NMD etc. However, as expansionn of HNEs cultures is limited, we have extended our analyses to a complementary primary cellular system, patient-derived rectal organoids. Organoids provide a limitless resource enabling studying of the endogenous CFTR gene in its native context. We developed a protocol for efficient transfection of ASOs into intestinal organoids. The results of these experiments will be discussed. . Altogether, our results indicate that ASOs targeted to mask splicing motifs in the CFTR gene can increase the correct splicing of CFTR, leading to improvement of channel function and highlighting the potential of our lead ASO as a therapeutic approach for CF patients carrying splicing mutations.

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The effect of CF sputum on CFTR mediated Cl^- transport and ASL height. Is gene editing sufficient to restore function in the CF environment?

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CF lung disease is an obvious candidate for gene therapy due to its monogenetic nature and the accessibility of the airways to nebulisers. It has been suggested that as little as ~10% of endogenous CFTR expression *in vivo* is sufficient to prevent lung disease. However, this is a controversial topic with estimates ranging from 10-50% for restoration of ion channel activity in cells models with either endogenous expression of CFTR or overexpression of CFTR. The abnormal, chronically inflamed and infected luminal environment of the CF lung produces abnormal sputum (including upregulated cytokines, bacterial toxins, altered pH), shown to further disrupt ion channel activity. With gene editing technology continually improving, we want to identify how much functional CFTR is required to restore activity of CF cultures in the presence of CF sputum.

To answer this question we used two models: primary human bronchiolar epithelial cell cultures of mixed CF (CFBE):non-CF (NHBE; a model for gene editing) from alternate sexes and CFBE transduced with CFTR under the expression of a high activity promoter (a model for gene therapy). Cells were grown on semipermeable membranes and differentiated at airway-liquid interface. Cells were incubated with apically applied supernatant of non-CF or CF airway mucopurulent material pooled from 9 donors (CF or non-CF sputum) before functional analysis. Droplet digital PCR (ddPCR) and immunohistochemistry were performed for characterisation of cultures. Short circuit current (I_{sc}) and airway surface liquid (ASL) height were measured as functional read-outs.

We demonstrated a 41% decrease (n=5, $p< 0.001$) in CFTR-mediated I_{sc} and a loss of vasoactive intestinal peptide (VIP, activator of CFTR) stimulated ASL secretion (n=5, $p< 0.001$) after incubation with CF sputum compared to non-CF sputum. Using ddPCR analysis of AMEL-X and AMEL-Y to accurately determine the number of CFBE vs NHBE in the mixed cell cultures, we observed that CFTR activity increased in a linear fashion with the percentage non-CF cells ($CFTR_{inh}$Isc $\mu A/cm^2$: $R^2=0.91$, n=28). The VIP-stimulated increase in ASL height followed a similar trend further indicating an effect of CF sputum on CFTR stimulated I_{sc} and ASL height. In comparison, ~16% transduction of CF cultures with over-expressing CFTR was sufficient to fully restore Cl^- secretion and increase ASL height (n=6) in the presence of CF sputum.

Our data implies that overexpression rather than endogenous expression of CFTR is a more effective method for restoring function in a CF culture. However, this technology is limited by its transient and immunoreactive nature. Currently, permanent restoration of function in the CF lung would require an unachievable amount of gene editing events. Thus, we propose the use of an enhanced CFTR gene we have developed for gene editing. Codon optimisation and a substitution mutation in cytosolic loop 3 of CFTR produced ~7 fold greater processing in to protein and dramatically increased anion transport activity as shown by a YFP quenching assay in HEK293T cells (n=3). It is our hope that this enhanced gene could be used to reduce the amount of editing events necessary to restore function in the CF lung.

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Effective transduction of Rhesus macaque lung and human enteroids with AAV1-CFTR

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Several gene therapy human and non-human primate studies have shown that AAV delivery of CFTR is safe. A challenge is to deliver enough CFTR to be therapeutic. Given that the turnover of airway cells may make gene transfer with recombinant AAV-based vectors transient, repeat dosing of AAV1 will be required. The goal is to assess whether repeat dosing of AAV1CFTR leads to widespread gene transfer and CFTR expression. To test the ability of AAV1 to transduce human cells, normal human enteroids were grown on permeable supports, infected with AAV1 containing Δ264- or Δ27-264-CFTR and Isc measured. These versions of CFTR increased endogenous CFTR via transcomplementation. Significant increases in forskolin-activated currents were detected indicating that transduction occurred after apical and/or basolateral exposure. To test whether repeat dosing of AAV1 is effective, we sprayed into the airways of 4 healthy Rhesus monkeys, 2 doses of 10^{13} vg of AAV1Δ27-264-CFTR at 0 and 30 days, respectively, followed by a single dose of 10^{13} vg of AAV1GFP at day 60. Monkeys were sacrificed at day 90. There were no adverse events related to the study indicating that triple dosing with AAV1 vectors is safe. Sera from treated animals were analyzed for anti-AAV1 neutralizing antibodies. There was a significant rise in the titer after the first dose in all 4 animals as the animals transitioned from a preimmune state to post-vector exposure. An elevated anti-AAV1 titer was established in all treated monkeys 30 days after the first dose and increased further 30 days after the second dose. By the third dose all four monkeys had escalating titers. A positive T cell response was noted after the second dose in one animal and after the third dose in all the animals. Thus, AAV1 antibodies were induced in sera upon reexposures to vector but no adverse events occurred. Samples were taken from 17 different lung regions and the vector genomes measured in lung using vector-specific real-time PCR. rAAV1CFTR and rAAV1GFP vector DNA was detectable in all lung sections and animals respectively. The lung cells were estimated to contain between 1 and 60 particles per cell of AAV1CFTR. Interestingly, vector genomes were detectable in the liver with a tendency to detect more AAV1GFP containing genomes compared to those containing CFTR. Despite the presence of AAV1, there was no liver toxicity detected in any of the animals. GFP protein expression detected by western blot was detected in the lungs of all animals. CFTR protein expression detected by western blot was significant higher compared to an uninfected control. All lung sections assessed by confocal microscopy showed increased CFTR staining compared with uninfected monkey and were positive for GFP staining indicating widespread gene transduction by AAV1GFP. Our results show that the AAV1 serotype transduces both human and monkey airway cells. Given that significant numbers of vector genomes from AAV1CFTR virus were present in monkeys four months after the first instillation coupled with CFTR and GFP transduction suggests that repeat dosing of AAV1 based vectors is feasible.

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Prolonged incubation with CFTR modulators and forskolin induced swelling on organoids with N1303K mutation

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The response to CFTR modulators in rectal organoids from CF patients can help to understand which CFTR mutations can be rescued by CFTR modulators. In this study, the effect of CFTR modulators tezacaftor (teza) 3μM and ivacaftor (iva) 3 μM separately or in combination (combo) was assessed in organoids from 11 patients with different CF genotypes using the FIS assay. The relative increase in total area of the organoids after exposure to different forskolin (fsk) concentrations (0.008 to 5μM) was measured on images acquired at 10 min intervals for 1 hour using a confocal microscope. Then, we left the organoids to incubate with 5μM forskolin and the modulators for additional 24 hours and again acquired images. The increase in organoid area after 1 hour and 24 hours of incubation was calculated as a percentage of baseline area (t0 = 0%) for each forskolin concentration and test condition (combo, luma only, iva only or medium). Organoid swelling after one hour incubation with combo and 5μM forskolin in the different genotypes (legacy names) was as follows: F508del/3849+10kbC>T (89%±6.8), F508del/I507del (58%±3.8), F508del/G542X (48%±1.8), F508del/3272-26A>G (48%±1.9), F508del/ 2789+5G>A (39%±3.2) and 3272-16T>A/394delTT (35%±2.6). No swelling was observed for the organoids with the genotypes (N1303K/1717-1G>A, N1303K/delEx19, N1303K/N1303K, G542X/G542X and Q461X/Q401X). After 24 hours of incubation, all organoids that swelled after one hour showed an increase in organoid area (from new baseline) in a range between 17 to 210%. Interestingly, after 24 hour of incubation the organoids with at least one N1303K mutation (N1303K/1717-1G>A and N1303K/delEx19) showed small but consistent swelling when incubated with 5μM forskolin and combo (average 48%±15.6). The organoid culture homozygous for the N1303K mutation showed higher swelling for fsk-combo (101%±22.7) compared to the previous and swelling was also observed after 24 hours of incubation with the fsk-iva (50%±13). No swelling was observed, after 24 hours, for any of the N1303K organoids when incubated with 5μM fsk alone or fsk-teza nor with modulators without fsk. Moreover, no swelling was observed in organoids with the nonsense genotypes G542X/G542X and Q461X/Q401X using the same incubation conditions. These preliminary results obtained using a longer incubation with modulators and 5μM forskolin indicate that N1303K CFTR mutants maybe rescued by CFTR modulators. Furthermore, this swelling seems CFTR mediated since no swelling was observed in organoids with two nonsense mutations. The need of a longer incubation and the presence of forskolin may indicate the need for a more efficient potentiator or combination of potentiators. This is in accordance with Phuan PW et al1 stressing the need for combinations of potentiators N1303K airway cell cultures. In our experiments, prolonged incubation with forskolin-potentiator-corrector seemed to further increase CFTR function for this mutant. Further work needs to be done to study the rescue of N1303K and to verify if/how rescue can be improved to reach clinical significance. Acknowledgments: Work supported by the Belgian CF Patients´ association (Mucovereniging).

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Two novel corrector classes with the potential to be developed for new combination therapies

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There are over 2,000 mutations associated with the cystic fibrosis transmembrane conductance regulator (CFTR) gene and approximately 280 of them are confirmed to cause cystic fibrosis (CF). CF-causing mutations are divided into six mechanistic classes that may be amenable to a precision medicine approach. Deletion of phenylalanine at position 508 (F508del) is the most prevalent mutation, with over 85% of patients carrying at least one allele. The F508del mutation impairs the conformational maturation of the protein, attenuating trafficking of CFTR to the cell surface. Pharmacological chaperones known as correctors can partially rescue this trafficking defect; and the early first-to-market correctors, such as lumacaftor and tezacaftor, when combined with a potentiator, confer a modest clinical benefit to CF patients homozygous for the F508del mutation.

More recently, clinical-stage correctors such as VX-445 and VX-659, combined with a potentiator and an early corrector, demonstrated meaningful improvements in *in vitro* and early clinical efficacy. Similarly, we have described the novel clinical-stage corrector, PTI-801, that in combination with the potentiator, PTI-808, provided an absolute percent predicted FEV_1 improvement of 5.9% from baseline. Additionally, PTI-801 is distinct from the other correctors in its protection against *in vitro* chronic inhibition of F508del-CFTR stability and activity conferred by ivacaftor (VX-770). We sought to determine whether additional corrector mechanisms beyond those represented by these reported clinical-stage groups of correctors could be identified for F508del-CFTR.

A high throughput phenotypic screen to discover additional novel CFTR modulators was performed. We describe two novel classes of CFTR correctors with distinct characteristics from known clinical stage CFTR correctors. The first, represented by PTI-CCCN, is capable of synergizing with lumacaftor/tezacaftor and PTI-801. PTI-CCCN improves F508del-CFTR chloride transport activity, and nearly doubles this activity when combined with lumacaftor or tezacaftor and ivacaftor. PTI-CCCN in combination with a potentiator also shows the potential to correct other class II mutations as shown by *in vitro* efficacy in human bronchial epithelial (HBE) cells derived from donors harboring an N1303K mutation. Lastly, addition of PTI-CCCN to the triple combination of PTI-801, the amplifier PTI-428, and PTI-808 restored CFTR activity in homozygous F508del HBE cells to approximately 140% of normal CFTR levels.

The second corrector class, from a distinct chemical series and represented by PTI-CCCP, shows synergy with PTI-801 and similar molecules, but does not demonstrate additivity to lumacaftor/tezacaftor. PTI-CCCP activity in immunoblotting of F508del/F508del HBE cells shows that it increases both immature and mature F508del-CFTR species, and that these activities are synergistic both with PTI-801 and similar correctors, as well as with CFTR amplifier. This synergy is recapitulated in activity measurements in the Ussing chamber. Further characterization of the properties of PTI-CCCP will help elucidate its full overlap and distinctions from the lumacaftor/tezacaftor class of correctors.

The new modality of CFTR correction represented by PTI-CCCN provides a potential new advancement in the development of small molecule modulators to target the dysfunction of F508del-CFTR. These two novel corrector classes also expand the diversity of available combinations with known modulators with the aim of improving clinical efficacy for CF patients.

P98
Rescue of F508del-CFTR by Corrector RDR01752

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Background: Rescuing the functional expression of F508del-CFTR, the most common mutation in cystic fibrosis (CF), is the major goal in the development of new treatments for this disease. Despite the experimental progress obtained by VX-809 and VX-661 in rescuing F508del-CFTR, only modest clinical outcomes were observed in F508del-homozygous patients treated with these correctors in combination with potentiator VX-770. Therefore, there is still an unmet need for compounds that rescue more efficiently the functional expression of F508del-CFTR.

Aim: This study aims to evaluate the effects and mechanism of action (MoA) of a new compound, RDR01752 (5-(4-Nitrophenyl)-2-furaldehyde 2-phenylhydrazone [1]) in rescuing F508del-CFTR to the plasma membrane (PM) alone or combined with VX-809 or VX-661.

Methods: F508del-CFTR processing was evaluated by Western blot (WB) in CF bronchial epithelial (CFBE) cells expressing F508del-CFTR treated with increasing doses of RDR01752 (0.5 to 20 μM). PM expression was determined by immunofluorescence (IF) microscopy in CFBE cells stably expressing F508del-mCherry-Flag-CFTR under an inducible (Tet-On) promoter [2]. The mechanism of action (MoA) was also investigated by IF microscopy using F508del-CFTR genetic revertants (G550E, R1070W, 4RK) and wt-CFTR DD/AA. Additionally, rescue of F508del-CFTR function by RDR01752 was measured in CFBE cells in Ussing chamber by short-circuit current and in intestinal organoids from F508del/F508del patients by forskolin-induced swelling (FIS) assay.

Results and discussion: The maximal rescue of F508del-CFTR processing and PM expression obtained by RDR01752 was achieved at the 10 μM dose to similar levels of those under VX-809 (3.7 μM) or VX-661 (5 μM). However, both Ussing chamber data from F508del-CFTR expressing CFBE cells and F508del/F508del human intestinal organoids evidenced lower functional rescue by RDR01752 as compared to VX-809. Co-administration of RDR01752 with VX-809 or VX-661 did not additively enhance PM expression of F508del-CFTR. In fact, experimental data using CFTR genetic revertants indicate that RDR01752, VX-809 and VX-661 seem to share the same putative bind site at the NBD1:ICL4 interface.

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[1] PubChemID: 9566157

[2] Botelho et al (2015) *Sci Rep***5**: 9038.

P99

A novel amplifier-enhanced high throughput screen based on CFTR functional activity identifies read through modulators of G542X-CFTR

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Nonsense mutations in the CFTR gene prematurely terminate translation of the CFTR mRNA leading to the production of a truncated and likely non-functional CFTR protein. About 10% of persons with CF have at least one nonsense or premature termination codon (PTC) mutation. RNA transcripts bearing a PTC are generally targeted for degradation by a cellular quality-control mechanism referred to as nonsense-mediated mRNA decay (NMD). The efficiency of NMD is impacted by additional elements in its substrate transcripts beyond the PTC itself, such as a preference for a spacing of the PTC at more than 50 nucleotides upstream from the last exon-exon junction that results after splicing. Traditional approaches to identify read-through modulators have used reporters with a PTC in a cDNA version of CFTR, thus eliminating the additional signals that impart NMD sensitivity, and potentially changing the biological context under which read-through may be occurring. With this in mind, we created stable cell lines using HEK 293 cells transfected with a published G542X-CFTR minigene construct containing full length CFTR and intronic-like insertions at exon-exon junctions for exons 14, 15, and 16 (Masvidal et al., 2014). The inclusion of these spliced out sequences appropriately distanced downstream of the G542X PTC mutation enables the involvement of NMD, allowing for potential identification of NMD-modulating compounds as well as read-through modulators that rely on the NMD pathway or elements of its signaling.

Proteostasis Therapeutics, Inc.'s proprietary amplifier CFTR modulators confer increased immature CFTR protein levels and stabilized CFTR mRNA through a mechanism that is dependent on translation and independent of the mutation in the CFTR gene. Amplifiers require only the translated sequence of CFTR to confer this increase in protein abundance and mRNA stability. Amplifiers are complementary to other CFTR modulators, providing additional substrate upon which those modulators can act. This is true for read-through agents, such as the aminoglycoside G418, which also act co-translationally. The *in vitro* efficacy of G418 is enhanced in combination with the amplifier in cell line and primary human bronchial epithelial (HBE) cell models of PTC mutant CFTR. Specifically, the combination of an amplifier and G418 promotes functional rescue in G542X-CFTR expressing cell lines and in G542X homozygous patient-derived HBE cells.

To enhance the amount of functioning G542X-CFTR that would be detectable in the presence of read-through, we performed a novel high throughput screen (HTS) of 640,000 compounds in the presence of an amplifier and corrector. Read-through of G542X-CFTR was assessed by CFTR functional activity using a fluorescent membrane potential dye assay. The completed HTS was statistically robust with an average Z' of approximately 0.68 across the more than five-hundred 1536-well plates. The hit rate of about 1% yielded approximately 6,400 compounds that will now be confirmed, counter-screened and have dose-dependency determined. Confirmed actives will be prioritized for direct activity measurements in Ussing chambers using primary HBE cells derived from a G542X/G542X donor.

P100

Ganglioside GM1 improves the plasma membrane stabilization of F508del-CFTR rescued by the use of CFTR modulators

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The new challenge for the CF therapy is based on the development of pharmacological agents able to increase the surface level of mutated CFTR (correctors), as well as its plasma membrane (PM) activity (potentiators). Unfortunately, for the most common CF-causing mutation F508del, their efficacy seems to be time-limited due to a reduced stability of corrected protein at PM.

As known, CFTR stability at the PM is guaranteed by a precise coordination in PM microdomain between scaffolding proteins, such as ezrin, NHERF1 and a particular class of bioactive lipids called sphingolipids. Indeed, a perturbation of this equilibrium reduces the expression of rescued CFTR.

During the years an increasing number of proteins involved in the CFTR PM stabilization and function were described, but scant information are available regarding the lipid counterpart. For this reason, we focused on this aspect that led to identify the monosialoganglioside 1 (GM1) as lipid interactor of CFTR in bronchial epithelial cells.

A relationship between CFTR and GM1 has been suggested by the evidence that silencing CFTR expression is paralleled with a decreased content of this ganglioside in airways cells.

Since GM1 has been described to play an important role in the activity of some PM proteins, we investigated the effects of GM1 on the PM stability of F508del CFTR rescued by the use of Lumacaftor (VX-809) and Ivacaftor (VX-770).

To this purpose we analysed CFTR expression upon treatment of CF bronchial epithelial cells individually with VX-809 or VX-770, or with the combination of both drugs, in the presence of exogenous GM1. In CF cells, the treatment with the VX-809 induces an increase in the mature form of CFTR which is reverted by the combined treatment with the VX-770. Interestingly, when GM1 is exogenously administered to these cells, the amount of the mature form of CFTR remains high even after the combined drugs treatment. In addition, we demonstrated that the rescued F508del-CFTR in the cells directly interacts with the exogenously administered GM1. Consequently, we analysed the effect of VX-809, VX-770 and GM1 on CFTR scaffolding proteins NHERF1 and ezrin. We found that the expression of both proteins increased in cells treated with GM1 and subjected to double pharmacological treatment.

Finally, we analysed the effect of GM1 treatment on CFTR function by performing functional fluorescence assays. We observed that treatment with GM1, in combination with VX-770 and VX-809, increases CFTR function with respect to the treatment with only corrector and potentiator.

Taken together, our results support the role of GM1 in the stabilization and function of CFTR at PM level, suggesting GM1 as adjuvant for the CF therapeutic strategy based on the use of corrector and potentiators.

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P101
Rescue F508del CFTR with nanobodies

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As the F508del mutation causes destabilization of CFTR that leads to its misfolding and subsequent degradation, identification stabilizing molecules would provide a new therapeutic route. We isolated nanobodies that bind different epitopes of NBD1 and thermostabilize full-length CFTR (Sigoillot et al. under revision). We have initiated a large effort to investigate the potential of nanobodies to rescue F508del CFTR in living cells. HEK293 cells that stably express mutated CFTR with an extracellular HA tag were transfected with EGFP fused nanobodies. Using flow cytometry we can detect CFTR at the plasma membrane of non-permeabilized cells expressing nanobodies-EGFP. We now show that expressing a stabilizing nanobody leads to increased expression of F508del CFTR at the plasma membrane compared to a control nanobody. Moreover this effect is synergistic with the action of correctors (VX-809 and VX-661) but not with the rescuing effect of low temperature (cells incubated at 27 °C). In the future we will try other strategies to deliver nanobodies inside the cells such as fusion to a cell penetrating peptide (TAT) or osmocytosis and the use of a specific detergent (i.e. the iTOP method). We will also monitor the effect of nanobodies on CFTR function in cells expressing intracellular YFP whose decrease in fluorescence reveals entrance of the halide due to CFTR activity.

Our work strongly suggests that approaches aiming at stabilizing NBD1 have therapeutic potential for cystic fibrosis.

P102

The pharmacology of novel TMEM16A potentiator compounds

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TMEM16A was recently identified as a calcium-activated chloride conductance and a key orchestrator of anion secretion in the human airway epithelium (Caputo et al 2008; Schroeder et al 2008; Yang et al 2008). It is now clinically established that promoting anion secretion in the airway leads to enhanced mucus clearance and reduced exacerbation frequency in CF patients and as such TMEM16A represents an important target for the next generation of mucokinetics. Importantly, positive regulators of TMEM16A function will be expected to be of benefit in all CF patients, irrespective of their CFTR mutational status.

Using 4 parallel screening approaches, we identified several chemically diverse, low molecular weight compounds that potentiated TMEM16A function. These hit compounds were validated for TMEM16A function using a patch-clamp assay under conditions where $[Ca^{2+}]_i$ was tightly buffered at an EC_{20} for TMEM16A channel activity. This enabled hits that activated TMEM16A by non-specifically elevating $[Ca^{2+}]_i$ to be rapidly filtered out from the hit list.

The efficacy of *bona fide* TMEM16A potentiators translated through to function in ion transport studies in CF-HBE. Pre-treatment of CF-HBE with TMEM16A potentiators for between 5 min to 96h resulted in an enhancement of Ca^{2+}-mediated anion-secretory responses that were sensitive to the TMEM16A blocker, Ani9. Measurements of $[Ca^{2+}]_i$ confirmed that TMEM16A potentiators had no effect on calcium mobilization, consistent with a direct effect on the channel.

A Series 1 TMEM16A potentiator, ETX001, increased the secretion of airway surface liquid (ASL) in CF-HBE. The ETX001-driven increase in ASL height was further enhanced in cells that had been pre-treated with IL-13 to boost TMEM16A expression. A close structural analogue of ETX001, ETX002, that is inactive on TMEM16A, did not increase ASL height.

Together, these data support the concept that potentiators of the alternative airway chloride conductance, TMEM16A, can restore anion conductance and fluid secretion in both primary CF cells. Enterprise Therapeutics are advancing TMEM16A potentiators into clinical development.

Caputo et al (2008) Science 322(5901):590-594

Schroeder et al (2008) Cell 134(6):1019-1029

Yang et al (2008) Nature 455(7217):1210-1215

P103

ATP12A as an alternative therapeutic target in cystic fibrosis lung disease

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ATP12A is an emerging target in cystic fibrosis (CF). This membrane protein is expressed in the apical membrane of the airway epithelium where it is responsible for proton secretion coupled to K^+ absorption. In CF epithelia, ATP12A activity, combined with the defective bicarbonate secretion due to CFTR loss of function, causes abnormal acidification of airway surface liquid (ASL) that leads to impaired antimicrobial activity and increased mucus viscosity (Shah et al., Science 2016). The importance of ATP12A in CF is further supported by recent findings revealing an increased expression of this pump in the airways of CF patients (Scudieri et al., JCI Insight 2018).

Given the emerging role of ATP12A in CF lung pathogenesis, it would be highly desirable to find potent and specific pharmacological inhibitors of ATP12A. Indeed, compounds available so far to inhibit ATP12A, such as ouabain, lack specificity. Our aim is to develop reliable assays and cell models to measure ATP12A function and to initiate the search of new ATP12A inhibitors.

For this purpose, we are working on two different strategies: i) evaluation of ATP12A function in primary bronchial epithelial cells which endogenously express the proton pump and ii) generation of a heterologous cell line with stable expression of ATP12A.

For the first approach, well-differentiated CF bronchial epithelia grown on permeable supports adapted to 96-well plate were covered with a small volume of saline solution with low buffer capacity containing SNARF-1 as a ratiometric pH sensor. Cells were then incubated in a temperature-controlled plate reader and SNARF-1 fluorescence was monitored at different time points. With this system we confirmed the more acidic ASL pH of CF epithelia, the ASL alkalinization by IL-4, and the reduced ASL acidification in CF epithelia by ouabain.

For the second approach, we are working at the generation of a cell line with stable co-expression of ATP12A and ATP1B1. The latter is a β-subunit of the Na^+/K^+ pump, found to colocalize with ATP12A in the apical membrane of airway epithelial cells (Scudieri et al., JCI Insight 2018). The association between the two proteins was confirmed in different cell lines, such as CFBE41o-, HEK-293 and FRT. When ATP12A was transfected alone, we mostly found a perinuclear staining, whereas when both proteins were transfected together, we found appearance of ATP12A on the cell border, with a close overlap with ATP1B1 signal. To measure ATP12A function we are evaluating different chemical and genetically encoded pH-sensitive probes, and different experimental conditions.

In conclusion, we have setup the conditions for measuring ATP12A function in primary cultures of airway epithelial cells at a medium/low-throughput scale, and we have generated a FRT cell line with stable expression of ATP12A and ATP1B1, which will be more suitable for high-throughput screening of chemical libraries to find novel potent and specific ATP12A inhibitors. Pharmacological inhibition of ATP12A-mediated proton secretion could antagonize acidification in CF airways and help to normalize mucus properties and restore antimicrobial activity.

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P104

Novel therapeutic approaches based on small molecule transmembrane anion transporters to improve the mucociliary properties of cystic fibrosis airway epithelia

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The great effort from the cystic fibrosis (CF) research community has resulted in the approval of drugs addressing CF molecular basic defects such as Kalydeco(tm) and Orkambi(tm) developed by Vertex Pharmaceuticals. Despite these successes, the performance of these drugs is far from optimal for numerous patients and other therapeutic approaches are urgently needed. We have planned to develop a therapy applicable to all CF patients, regardless of the type of mutation they harbor. To do so, we have focused on anionophores, small molecules that facilitate the transmembrane transport of anions independently of the molecular characteristics of the CFTR defect. The nature of the idea overcomes the initial limitations of current strategies and represents a potential universal remedy for all CF patients. Inspired by the structure of natural products such as the prodiginines and tambjamines, a large collection of new molecules has been designed and synthesized. We have characterized the molecular mechanism of transport of anionophores and proved their ability to transport chloride and bicarbonate in artificial systems and in isolated cultured cells. Being pulmonary disease the main cause of morbidity and mortality in CF patients, we have assessed whether the treatment with anionophores results in a correction of the imbalance in ion transport of CF airway epithelia and thus in the mucociliary properties of the air surface fluid (ASF). We have observed that in differentiated bronchial epithelia obtained from CF patients, anionophores have improved the periciliar fluid composition either by reducing the fluid reabsorption and the viscosity of the secreted mucus. Moreover, we have demonstrated that the presence of these anionophores do not interfere with the activation of CFTR protein and do not modify the action of the CFTR modulators such as lumacaftor and ivacaftor. We conclude that the identified compounds capable to correct mucus viscosity and fluid transport in CF-ASF may represent a promising starting point for the development of drug candidates for CF therapy.

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P105

The attached stratified mucus in obstructive airway disease is detached by calcium removal

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Mucus accumulation is a hallmark of chronic airway diseases. To better understand the mechanism behind mucus accumulation in the airways, we have developed a mouse model of mucus obstruction by intranasal administration of elastase. In this model, goblet cell hyperplasia/metaplasia was observed and accompanied by mucus plugging with the main secreted gel-forming airway mucins Muc5ac and Muc5b. After washing, a mucus layer covering the epithelium remained attached to the surface goblet cells. Bacteria were trapped on and in this mucus but found on the epithelial cells in mice not exposed to elastase. Although the attached mucus layer essentially separates bacteria from the epithelium, bacterial retention would lead to lung deterioration.

In our previous work, we showed that calcium removal is required for normal mucus unfolding and expansion of the mucin polymers, thus facilitating its clearance. So, in order to find potential mucus-detaching drugs, we have designed and used a novel calcium-chelating peptide to treat the elastase-exposed airways. Common osmotic agents were used as controls. Our calcium-chelating peptide was effectively able to detach the mucus layer and significant reduction of airway obstruction. However, hypertonic saline or mannitol decreased airway mucus obstruction but failed to detach the attached mucus layer.

We conclude that in chronic lung disease, the mucus is transformed into a stratified, attached mucus layer resembling the protective colonic mucus and the airway mucus layer was detached by the calcium-chelating peptide.

P106

Inhaled therapy with a cell-permeable PI3Kgamma mimetic peptide to limit bronchoconstriction and lung inflammation in cystic fibrosis

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Background and rationale: The underlying cause of cystic fibrosis (CF) is a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP (cAMP)-stimulated chloride channel. The consequent CFTR dysfunction primarily affects the respiratory system, where the reduced activity of the channel results in the production of a thick, sticky mucus responsible for small airway obstruction and, together with airway inflammation and infections, eventually leads to respiratory failure. CF mucus is also responsible for resistance to drug diffusion, reducing the effectiveness of drug delivery to the lung. This is one of the major obstacles for the development of new inhalation CF therapeutics, together with aerosol particle size that affects both the dose deposited and the distribution of aerosol particles in the lungs.

Hypothesis and objectives: We previously showed that phosphoinositide 3-kinase γ (PI3Kγ) acts as a scaffold protein which negatively regulates cAMP, by favoring the activation of cAMP-degrading enzymes, phosphodiesterases 3 and 4. We designed a cell-permeable peptide that, by interfering with PI3Kγ scaffold activity (Patent n° WO/2016/103176), enhances cAMP levels and promotes CFTR function in normal and CF bronchial epithelial cells. Here, we intend to explore the ability of the compound to enhance cAMP in airway smooth muscle and immune cells, ensuring concomitant

(i) bronchodilation and

(ii) anti-inflammatory effects. Furthermore, we will verify that aerosolized formulation of the peptide possesses particle size and mucus permeability suitable for lung delivery.

Methods:

The permeability and the aerodynamic properties of the cell-permeable PI3Kγ peptide were assessed by a Parallel Artificial Membrane Permeability (PAMPA) assay and a Next Generation Impactor (NGI) study, respectively. The ability of the peptide to function as a

(i) bronchodilator and

(ii) anti-inflammatory agent was evaluated in a mouse model of chronic lung inflammation, ovalbumin (OVA)-sensitized mice.

Results: We found that, in PAMPA assay 4,2% of the peptide is able to cross the phospholipidic barrier in the presence of CF-mimicking mucus. In this assay, the retention factor is fully comparable to that of propranolol, a standard reference compound in permeability assays, and can therefore be considered permeable to cell membranes even in the presence of CF-like mucus. Analysis by NGI revealed that the peptide has aerodynamic mean dimensions suitable for inhaled delivery in humans when nebulized by three different devices (one jet nebulizer and two mesh nebulizers). Of note, when aerosolized with the two mesh nebulizers, the respirable fraction was higher than 90%. Finally, in vivo, we found that the peptide not only significantly elevates cAMP in the lungs without affecting cAMP homeostasis in distal organs, but it also limits methacholine-induced airway hyperresponsiveness and reduces neutrophilic lung inflammation in OVA mice.

Conclusions: Overall, the results of this study demonstrate that the peptide targeting PI3Kγ scaffold activity has optimal chemical and aerodynamic properties for lung delivery and can promote at least three independent therapeutic effects (bronchodilation, reduction of inflammation and CFTR modulation).

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P107

Exploiting a PI3Kγ mimetic peptide as a CFTR modulator in cystic fibrosis

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Background and rationale: The underlying cause of cystic fibrosis (CF) is a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP (cAMP)-stimulated chloride channel. Current treatment regimens for CF combine several agents delaying pulmonary dysfunction and, as a consequence, are complex and time consuming, with a tremendous impact on the quality of life of patients. The clinical approval of Orkambi®, a combined drug composed of a CFTR potentiator and a corrector, highlighted the possibility of pharmacologically targeting the basic molecular defect of CF. However, the efficacy of this treatment appears unsatisfactory, likely because these molecules have been identified without a mechanistic rationale.

Hypothesis and objectives: We previously showed that phosphoinositide 3-kinase γ (PI3Kγ) acts as a scaffold protein which negatively regulates cAMP by favoring the activation of cAMP-degrading enzymes, phosphodiesterases 3 and 4 (PDE3 and PDE4). Here, we hypothesize that targeting PI3Kγ scaffold activity enhances cAMP in epithelial cells, leading to CFTR modulation.

Methods: We explored the ability of a cell-permeable peptide targeting the scaffold activity of PI3Kγ (Patent n° PCT/IB2015/059880 - WO/2016/103176) to function as a CFTR modulator. HEK293T cells and human primary bronchial epithelial cells (WT and F508del-CFTR) were used, as well as intestinal CF patient-derived organoids.

Results: We found that, in vitro, the peptide stimulates PKA-mediated phosphorylation of both WT and F508del-CFTR. In VX-809-corrected primary epithelial cells, the peptide potentiates F508del-CFTR currents and synergizes with VX-770, by increasing its efficacy by 5 folds. The compound also mediates the opening of the channel in intestinal CF organoids in a forskolin-induced swelling assay. Unexpectedly, the PI3Kγ peptide stimulates F508del-CFTR activity in the absence of any corrector, suggesting that the peptide can also affect CFTR trafficking to the plasma membrane. Immunogold staining and biotinylation assay demonstrated that the peptide increases the amount of CFTR on the plasma membrane. Moreover, we demonstrated that the PI3Kγ peptide promotes the trafficking to the plasma membrane of core-glycosylated F508del-CFTR via the unconventional secretory pathway.

Conclusions: We validated PI3Kγ as a new pivotal regulator of the CFTR channel. By inducing the PKA-mediated phosphorylation of the CFTR, the cell-permeable peptide targeting the scaffold activity of PI3Kγ not only regulates CFTR channel activity, but can also enhance CFTR trafficking. The rationale-based and mechanism-driven approach of the current project will ultimately allow the development of a new more finely tuned therapy targeting CF basic defect.

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Characterization of biological activity of RNF5 inhibitors as potential drugs for cystic fibrosis basic defect

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In cystic fibrosis,deletion of phenylalanine 508 (F508del) in the CFTR anion channel is associated to misfolding and premature degradation of the mutant protein. Among the known proteins associated with F508del-CFTR processing, particularly interesting is the ubiquitin ligase RNF5/RMA1. Genetic suppression of RNF5 in vivo led to attenuation of pathological phenotypes due to intestinational malabsorption in CF mice, validating the relevance of RNF5 as a target for CF.

By using a computational approach, based on homology modeling / ligand docking and virtual screening, we discovered inh-2, a drug-like small molecule putative inhibitor of RNF5 ligase.

Our aim is now to identify optimized RNF5 inhibitors and evaluate their efficacy on rescuing CFTR activity in epithelia from CF patients (bearing F508del and other class II mutations). In addition, we will also investigate possible side effects in terms of global changes in cell proteome and cellular processes.

To test RNF5 inhibitors, we have performed a biological evaluation of activity by using the microfluorimetric YFP-based assay on CFBE41o-cells. The ability of inhibitors to rescue F508del-CFTR was assayed on well-differentiated primary cultures of human bronchial epithelial cells using two different electrophysiological techniques: the transepithelial electrical resistance and potential difference measurements and short-circuit current recordings in Ussing chamber. To study the side effects due to inhibition of RNF5 inhibitors we evaluated cell proliferation, viability and apoptosis.

Our results clearly demonstrate that RNF5 inhibition can rescue F508del-CFTR trafficking defect and that this mechanism is not only amenable in cell lines or in a murine CF model, but also in human primary bronchial epithelia, that are the main target tissue of CF treatment. These findings thus validate RNF5 as a drug target for CF, and provide evidences to support its druggability.

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LMTK2 inhibits PP1c to regulate the TGF-β1 signaling pathway in cystic fibrosis

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Genetic modifiers have considerable influence on lung function variation in cystic fibrosis (CF). Transforming Growth Factor (TGF)-β1 is a known modifier associated with accelerated lung disease in patients homozygous for *F508del*. TGF-β1 inhibits CFTR biogenesis and impairs the functional rescue of F508del-CFTR to corrector-mediated rescue in primary differentiated human bronchial epithelial cells (*1*). Increased TGF-β1 levels are associated with variants of the TGF-β1 gene and are present in ~40% of *F508del* homozygous patients. Understanding the regulation of TGF-β signaling in human airways is critical to design strategies to attenuate its effects, allowing the rescue of F508del-CFTR. We aimed to elucidate the mechanisms of TGF-β1 signaling via the proximal pathway at the level of TGF-β receptor (TβR)-I and TβR-II in human bronchial epithelial cells. TGF-β1 initiates a signaling cascade by binding to TβR-II, which phosphorylates and activates TβR-I. In non-stimulated cells, Protein Phosphatase (PP)1 dephosphorylates TβR-I, protecting it from constitutive activation by TβR-II; however, it is not known how TGF-β1 blocks the PP1-mediated inhibition of TβR-I to initiate its signaling. We hypothesized that PP1 is inhibited through PP1-T320 phosphorylation. Lemur tyrosine kinase-2 (LMTK2) mediates the inhibitory phosphorylation of the catalytic subunit of PP1 (PP1c) in HeLa cells; however, it is unknown whether LMTK2 also inactivates PP1 in human bronchial epithelial cells.

To better understand how PP1c is regulated after TGF-β1 stimulus, we examined mRNA expression of the PP1c- and LMTK2-interacting proteins by the Next Generation mRNA-seq in human bronchial epithelial (CFBE41o-) cells. Expression of 27 genes was affected, including GADD34 and p35. A further mRNA-seq analysis after LMTK2 knockdown demonstrated that TGF-β1 pathway was affected, mainly the expression of TβR-I and Smad4, confirming the involvement of LMTK2 in the regulation of the pathway. Western blot experiments demonstrated that TGF-β1 increased the inhibitory phosphorylation of PP1c, in a process mediated by LMTK2. Indeed, siRNA-mediated LMTK2 depletion promoted activation of PP1c, leading to a subsequent attenuation of the TGF-β1 signaling via R-Smads. In turn, abundance of the R-Smads proteins was increased to counteract these effects. These results were confirmed by using a selective PP1c activator, which also inhibited activation of R-Smads despite TGF-β1 treatment.

Our data demonstrate that PP1c prevents activation of the TGF-β1 pathway in the absence of the ligand while LMTK2 promotes signaling after TGF-β1 stimulus. Our studies may lead to novel therapeutic targets blocking abnormal TGF-β1 signaling, thereby improving the functional rescue of F508del-CFTR by CFTR correctors.

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Inhibition of autoinflammation in cystic fibrosis using small molecule therapy

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Introduction: In Cystic Fibrosis (CF), CFTR mutations lead to abnormal production of the CFTR anion channel, causing dysregulation of epithelial fluid transport and a dehydrated airway surface liquid in the lung with consequent mucus build up and hypoxia. Prolonged hypoxia causes increased cell death, release of cytokines and damage-associated molecular patterns (DAMPS), therefore attracting pro-inflammatory neutrophils and macrophages resulting in a hyper-inflammatory CF lung. The accumulation of misfolded CFTR protein in the cell, along with lack of expression of functional CFTR channel on the cell surface leads to excessive cellular stress, dysregulation of epithelial sodium channels (ENaC) and a consequent disruption in ionic balance, all of which contribute to this hyper-inflammatory state. Here we investigate the role that ENaC plays in modulating inflammasome activation in CF using ENaC inhibitors and CFTR modulators.

Methods: Serum cytokines were measured in patients with CF and controls. *In vitro*assays were used to characterise effects of small molecules (ENaC inhibitors and CFTR modulators) on NLRP3 inflammasome activation in peripheral blood mononuclear cells (PBMCs) and monocytes. Western blotting was used to detect b-ENaC in Human Bronchial Epithelial cells (HBECs) and monocytes.

Results: For the first time we show that b-ENaCexpression is increased in monocytes from CF patients relative to controls. We observed increased IL-1b and IL-18 cytokines in CF monocytes, which was blocked by the addition of inhibitors targeting the NLRP3 inflammasome. Activation of NLRP3 appeared to be mediated by increased K^+ efflux following dysregulation of ENaC and high sodium flux. Addition of Na^+ channel inhibitor, amiloride, and S18 (peptide based on endogenous ENaC inhibitor SPLUNK1) *in vitro*decreased NLRP3 inflammatory cytokines. Supporting this, overexpression of b-ENaC, in the absence of CFTR dysfunction, increased NLRP3-dependent inflammation, indicating a CFTR-independent ENaC-axis in CF pathophysiology. In addition we showed that CFTR modulators significantly reduced the levels of serum inflammatory cytokines in patients within three months of treatment as well as in PBMCs *in vitro.*

Conclusions: Collectively, we have shown that autoinflammation in CF can be reduced with the use of small molecule therapies which target CFTR and ENaC. Sodium channel modulation in combination with CFTR modulators is an important therapeutic strategy to combat lung inflammation in CF.

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Niclosamide repurposed for the treatment of inflammatory airway disease

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Inflammatory airway diseases such as asthma, cystic fibrosis (CF) and COPD are characterized by airway restriction and mucus hypersecretion. CF and asthma exhibit enhanced expression of the Ca^{2+} activated Cl^- channel TMEM16A in metaplastic club cells and airway smooth muscle, and contributes strongly to mucus hypersecretion and bronchoconstriction. Nonspecific inhibitors of TMEM16 proteins like niflumic acid (NFA), inhibit mucus production and bronchoconstriction. Here we demonstrate that the FDA-approved drug niclosamide is a highly potent inhibitor of TMEM16A and TMEM16F. In asthmatic mice, niclosamide inhibited mucus production and secretion, as well as bronchoconstriction, and also shows an anti-inflammatory effect. Both TMEM16A and TMEM16F are essential for mucus production/secretion that is explained by augmentation of intracellular Ca^{2+} signals. TMEM16A/Fsupport exocytic release of mucus and inflammatory mediators, which are both inhibited by niclosamide. The present results unmask TMEM16A/F as pathogenic factors during inflammatory airway disease, and suggest niclosamide as a potent drug for the treatment of asthma, CF, and COPD.

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Open randomised study on docosahexaenoic acid, 5-methyltetrahydrofolate and vitamin B12 supplementation in cystic fibrosis pediatric patients: focus on fatty acids, inflammation and blood cells membrane

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Background: An imbalance between omega-6 and omega-3 fatty acids (arachidonic acid (AA) and docosahexaenoic acid (DHA)) in subjects with cystic fibrosis (CF) has been reported. Abnormal phospholipid fatty acid amounts are reported in plasma and blood cells membranes from CF patients. Omega-3 fatty acids have been shown to play an important role in the integrity of cellular membranes, where they exert an anti-inflammatory effect consistent with decreased production of pro-inflammatory metabolites from the omega-6 fatty acids and increased biologically less-active omega-3 end products. Clinical trials performed on CF patients have revealed that oral supplementation with omega-3 polyunsaturated fatty acid (PUFA) modifies fatty acid profiles in plasma and cell membrane levels. Moreover, a number of clinical studies have demonstrated improvement of nutritional, clinical, and inflammatory parameters. However, effectiveness of omega-3 PUFA supplementation in CF remains controversial. It has been demonstrated that in CF children, 5-MTHF and vitamin B12 diet supplementation might ameliorate red blood cells membrane features.

Objectives: This work aims to evaluate in CF children the effects of DHA supplementation and compare to those of DHA+5-MTHF+vitamin B12 supplementation, on fatty acids profile, inflammatory status, blood cells membrane fluidity and microviscosity, and clinical outcomes.

Methods: 32 CF pediatric patients with pancreatic insufficiency (age between 7 and 14 years) were recruited and randomly assigned to receive

(i) group A: DHA (250mg/day up to 30kg body weight and 500mg/day over 30kg) and 5-MTHF (15mg every other day) and vitamin B12 (0,5mg every other day)

(ii) group B DHA (250mg/day up to 30kg body weight and 500mg/day over 30kg), for 6 weeks. Before and after treatment and one month later blood samples were collected to evaluate the fatty acid profile, folate and vitamin B12 levels in serum, fluidity and microviscosity of monocytes and granulocytes membrane, inflammatory markers in exhaled breath condensate (EBC) and in plasma. Furthermore lung function, as per cent predicted forced expiratory volume in one second (FEV1), were measured.

Results: A plasma DHA enrichment and a parallel increase of DHA/AA ratio were induced by both DHA and DHA+5-MTHF+vitamin B12 supplementation, but without significant difference between the two groups of treatments. Levels of inflammatory molecules (IL-1beta/IL-2/IL-4/IL-5/IL-6/IL-7/IL-8/IL-10/IL-12/IL-13/IFNgamma/GM-CSF/TNFalfa/MCP-1) were not significantly changed in plasma and in EBC after treatments. Interestingly, a decrease in the microviscosity of monocytes membranes after DHA and DHA+5-MTHF+B12 treatments were observed. No changes in membrane fluidity of monocytes and granulocytes emerged between the start and the end values in group A and group B. Levels of all these parameters were similar between groups before treatments. Both groups exhibited median FEV1 values >65% of predicted values at baseline. No between-group difference was observed in lung function parameter before and at the end of treatments.

Conclusions: Our results are consistent with previous studies showing no effects of increased plasma levels of DHA on inflammatory molecules and lung function. Addition of 5-MTHF+vitamin B12 to DHA supplementation does not affect any outcome evaluated in this study. Our findings of decreased microviscosity in monocytes membrane might suggest a possible anti-inflammatory effect that require further investigations.

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Intranasal *Lactobacilli* administration: a new way to prevent *Pseudomonas aeruginosa* respiratory tract infections

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Background:*Pseudomonas aeruginosa* (PA) is associated with respiratory tract infections, in cystic fibrosis (CF) patients. Chronic infection combined with acquisition of antibiotics resistance leads to therapeutic deadlock. Among non-antibiotic alternative, the use of *Lactobacilli* is promising. Oral administration of *Lactobacilli* stimulates immune system, decreasing the nosocomial pneumonia infections, incidence and severity of exacerbation in CF patients. The intranasal administration is interesting because it stimulates local respiratory immunity. Our team has identified a cocktail of 3 *Lactobacillus* strains (screened from a collection of 50 CF patient's respiratory strains) for its antipseudomonal properties *in vitro*. Therefore, we investigated the preventive effect of an intranasal administration of these 3 strains of *Lactobacillus* (alone or in cocktail) in a murine model of acute PA pneumonia.

Materials/methods: The *Lactobacillus* cocktail (named Lpsb) was made of 3 strains: *L. paracasei*, *L. salivarius* and *L. brevis*. Nasal delivery of each *Lactobacillus* strain (10^6CFU/mice) 18h prior nasal PAO1 instillation (10^6CFU/mice) was evaluated in 9 groups of C57BL/6 mice (Lpsb, Lp, Ls, Lb, PAO1, Lpsb+PAO1, Lp+PAO1, Ls+PAO1, Lb+PAO1).

At 24h PAO1 post-infection (p.i.), lung & serum were collected for bacterial load determination (enumerations and qPCR analysis (Héry-Arnaud et al., 2017)) and cytokines analysis.

Results:

- Our preliminary results showed that Lpsb cocktail delivery induced an increase of survival rate (100% $p< 0.001$) 7 days p.i. compared to the PAO1 group (11.7% survival).
- *Lactobacillus* administration induced a decrease in the PA lung load 24h p.i. compared to the PAO1 group with a higher effect in the presence of Lpsb cocktail and *L. salivarius* (reduction > 1 $\log_{10}$ $p< 0.05$) and a trend to decrease with *L. brevis* ($p=0.055$).
- Molecular analysis corroborate these results. The *L. salivarius* instillation led to a significant PA lung load reduction ($p< 0.05$) compared to the PAO1 group.
- Unexpectedly, 24h after PAO1 instillation, PA lung load was similar with *L. paracasei* compared to PAO1 group.

Conclusions: This is the first study examining the protective efficacy of live *Lactobacillus* intranasal administration against PA infection in mice. We identified one strain of beneficial bacteria, the *L. salivarius*, with high prophylactic antipseudomonal properties *in vivo*. Next step in our study is to decipher the involved mechanisms.

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Is cystic fibrosis related bone disease reversible? New data on CFTR potentiation and osteoclast precursor monocytes

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Rationale: Bone fragility and osteoporosis often affect children and young adults with cystic fibrosis (CF) disease and is associated with significant morbidity due to vertebral fractures and decreased lung function (Jacquot J et al., Osteoporos Int, 2016). We recently reported that bone demineralization is improved by the CFTR potentiator ivacaftor in young patients carrying the G551-D CFTR mutation (Sermet-Gaudelus I et al., J Cystic Fibros, 2016). Due to the presence of CFTR in monocytes, we hypothesized that ivacaftor may impact monocyte differentiation and activation. Monocyte osteoclast precursors fuse and differentiate to form bone-resorbing multinuclear osteoclasts upon stimulation by two essential factors, the monocyte/macrophage colony stimulating factor (M-CSF) and the receptor activator of NF-kB ligand (RANKL).

Methods: We analyzed RANK and M-CSFR receptors on peripheral blood monocytes by flow cytometry and clinical data in 11 G551D-bearing CF patients (5 females, 6 males, median age 27, range 18-39 years) and, evaluated potential changes following 9- and 12 months ivacaftor treatment.

Results: Ivacaftor improves sweat chloride and lung function. In addition, ten out of the 11 patients also improved or stabilized their bone mineral density (BMD) by ivacaftor (an improvement > 0.2 of BMD z-score). Ivacaftor does not change the number of blood monocytes. However, the average percentage of double $RANK^+/M\text{-}CSFR^+$ monocytes (91.6 ± 1.5%) in G551D patients was strongly higher (P=0.00017) compared with 18 healthy individuals (2.7 ± 0.8%, 4 females, 14 males, median age 37, range 20-65 years). Interestingly, this percentage decreased after 9- and 12 months ivacaftor treatment (n=6 and 4 patients, respectively), a reduction which was more pronounced in two patients. Flow cytometry data also demonstrates higher expression of both M-CSFR and RANK receptors (more predominantly for the RANK receptor), reported by unit of monocytic cells in G551D patients compared to 18 healthy donors.

Moreover, we examined ex vivo differentiation and activation of healthy monocytes into osteoclasts for a 21-days period with/or without the addition of Inh-172 drug, an inhibitor of CFTR chloride channel activity. Interestingly, multinuclear osteoclasts derived from Inh172-treated healthy monocytes were largest, more adherent, and were prone to generate large pits and trenches of dentin resorption. In addition, multinuclear osteoclasts derived Inh172-treated healthy monocytes released a lower level of bioactive lipid mediator sphingosine 1-phosphate (S1P), a key mediator in the directed migration of osteoblast/osteoclast precursors attached to the bone surface.

Conclusion: Altogether, these data highlight the critical regulatory role of CFTR in M-CSFR and RANK receptors expression in monocytes, and suggest CF bone disease as a new, cell-type-monocyte dysfunction disease, providing new insights into the pathogenesis of CF bone disease (Velard F et al. AJRCCM, 2018).

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Novel molecular imaging tools for cystic fibrosis

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Molecular imaging, defined as the *in vivo* characterization and measurement of biological processes at the cellular and molecular level, allows the non-invasive visualization of a target molecule *in vivo* by virtue of its interaction with an imaging probe. Nuclear imaging techniques as positron emission tomography (PET) and single photon emission computed tomography (SPECT) are the most sensitive modalities currently available, and are used clinically for the diagnosis and therapeutic follow-up in several diseases. In the field of CF, only a few PET and SPECT studies have been performed, mainly for the assessment of secondary pathological manifestations of lung disease, and not focused on assessing the basic cellular and molecular defect. The aim of the present work is to develop novel non-invasive imaging probes for the detection of normal (and rescued F508del-CFTR) at the plasma membrane of human cells. This will be accomplished through the isolation of CFTR-specific antibody fragments by the phage display technology and subsequent labelling with a useful radionuclide.

A synthetic phagemid library of human single-chain variable fragments (scFv) was panned for the isolation of plasma membrane-CFTR-binding clones. Firstly, the library was screened for scFv against the first extracellular loop (ECL1) of CFTR, synthesized under standard solid-phase conditions, with subsequent PEGylation and biotinylation to allow for phagemid selection using magnetic beads coupled to streptavidin molecules. In a second approach, selection was carried out in cells, and therefore, in the presence of a functional antigen. The BHK F508del-CFTR cell line was used for negative selection of plasma membrane CFTR, and the resulting phage library was then panned against a cell line with high levels of plasma membrane CFTR expression (BHK wt-CFTR). In both approaches, three rounds of phage binding to antigen, washing, elution, and reamplification of phage binders were performed. The DNA sequences from the scFv clones selected after three pannings were introduced by direct cloning into pET-scFv-T, an appropriate vector for scFv expression. Screening of scFv molecules by monoclonal enzyme-linked immunosorbent assay (ELISA) is currently underway to identify strong ECL1 binders. Flow cytometry studies are envisaged to confirm binding of the ELISA-selected clones to cells expressing CFTR.

After expression and validation by biochemical techniques, the scFv will be radiolabelled with technetium-99m (^{99m}Tc). Labelling will be performed directly through a hexahistidine tag present in the scFv or taking advantage of the recently developed strategy of click chemistry. The ability of the radiolabelled scFvs to detect CFTR at the cell surface will be assessed in human bronchial epithelial cells. Biodistribution assays with the most promising probes will be performed in normal mice and in CF mouse models. If successful, these non-invasive molecular imaging probes will have the potential to be a useful imaging biomarker in the assessment of early therapy response in drug evaluation.

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Sputum rheology: a robust biophysical marker for the monitoring of cystic fibrosis patients

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In cystic fibrosis (CF), the difficulty to expectorate is associated with abnormal mucus viscoelasticity. Sputum rheology has thus been proposed as a physical biomarker to quantitatively assess the patients' condition and the treatments' efficacy. However, checking the reproducibility of rheological measurements is a prerequisite to validate the potential of sputum rheology as biomarker.

In this study, we investigated the within-measurement and within-subject variabilities of sputum rheological measurements in healthy volunteers (n = 11), stable CF (n = 11), asthmatic (n = 12), and COPD patients (n = 11). We first quantitatively assessed the intrinsic reproducibility of sputum rheometry by performing consecutive (15 min apart) measurements on sputum samples split into two aliquots. We then analysed the within-subject reproducibility from sputa collected in consecutive visits (48 h apart).

In the linear regime (strain < 5%), the elastic (G') and viscous (G") moduli were measured constant, with G' > G", characteristic of an elastic solid behaviour as expected in both healthy and pathologic cases; CF patients sputa typically exhibit moduli higher by one order of magnitude. Beyond a critical strain (> 1000%), the mucus becomes more fluid-like as G' decreases below G", and starts to flow.

Viscoelastic moduli remained quantitatively comparable over consecutive measurements, both in the linear regime and at the crossover (when G' = G"), regardless of the subject condition. This correspondence was statistically confirmed by intra-class correlation, and validates the significance of the rheological measurement technique. On the longer term, the within-subject reproducibility deteriorated. Fair to good intra-class correlations were only found for the linear moduli. However, sputum linear rheology remains a relevant marker of the patient condition since healthy and pathologic patient populations remain clearly distinct ($p < 0.001$) beyond the within-subject variability. We also observed that administering CF patients rhDNase (Pulmozyme, 2.5 ml nebulised) results in a significant decrease of both the linear and crossover moduli. Taken together, these results confirm the robustness and the relevance of the rheological biomarker to assess both the patients' condition and the treatments' efficacy in cystic fibrosis.