2017

European Cystic Fibrosis Society

14th ECFS Basic Science Conference

Conference Programme & Abstract Book

Albufeira, Portugal

Chairpersons

Ineke Braakman, Marcus Mall and Tzyh-Chang Hwang

29 March – 01 April 2017

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CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to welcome you in Portugal to the 14th European Cystic Fibrosis Conference entirely dedicated to Basic Science.

This year we are delighted to welcome Prof. Braakman as the conference Chairperson who will be supported by Prof. Mall and Prof. Hwang 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 I hope productive collaborations.

I extend a very warm welcome to an exciting conference.

Kris De Boeck
President
European Cystic Fibrosis Society

Welcome from the conference chairpersons

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

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

In older times it was a fishing village and artists, painters, poets and writers fell in love with it. Albufeira has become one of the most popular and much-visited tourist centres in the South of Europe.

It is said, that it was here, looking at the intensity of the blue sea that Paul McCartney wrote "Yesterday", one of the most beautiful songs of the Beatles.

But let's come back to today and to the exciting next few days!

During this ECFS conference, you will hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment.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

This year's programme includes a number of symposia with international speakers covering topical aspects of high-quality basic research in cystic fibrosis together with invited talks from submitted abstracts. There are two keynote lectures, flash poster sessions, as well as a session from the ECFS Basic Science Working Group. On Saturday we will have two parallel sessions, one will be the Flash Paper session on the recent new CFTR structures and the other one will be dedicated on how to write a paper.

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

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The Netherlands

Marcus Mall
University of Heidelberg
Germany

Tzyh-Chang Hwang
University of Missouri-Columbia
United States

2017 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

29 March - 01 April 2017, Albufeira, Portugal
Programme

Chairpersons: Ineke Braakman (Utrecht, NL),
Marcus Mall (Heidelberg, DE), Tzyh-Chang Hwang (Missouri-Columbia, US)

Wednesday, 29 March 2017 (Day 1)	
13:30-17:00	**Pre-Conference Seminar "Gene editing & stem cells"** **Chairs: Patrick Harrison (IE) – Ulrich Martin (DE)**
	Coffee break – 15:05
17:30-18:00	**Official Opening of the Meeting by the Conference Chairpersons**
18:00-19:00	**Opening Keynote Lecture** Back to the Future - Ray Frizzell (US)
19:00-19:45	**Welcome Reception**
19:45-21:30	*Dinner*

Thursday, 30 March 2017 (Day 2)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Therapeutic approaches** **Chairs: László Csanády (HU) - Martin Mense (US)**
08:45-09:10	Discovery and Characterization of novel CFTR Modulators - Ashvani Singh (US)
09:10-09:35	Therapeutic potential of proteostasis modulation in cystic fibrosis - Nicoletta Pedemonte (IT)
09:35-10:00	The development of artificial anion transporters for cystic fibrosis - David Sheppard (UK)
10:00-10:10	Combined presentation Abstracts 83and 20 - SPX-101 is a novel ENaC-targeted therapeutic for cystic fibrosis that restores mucus transport - David Scott (US) / ENaC internalization by SPX-101 is a novel CF therapy for all CFTR mutation - Juliana Sesma (US)
10:10-10:20	Abstract 112 - Amplifiers co-translationally increase CFTR levels at the ER membrane by improving membrane targeting of CFTR - John Miller (US)
10:20-10:30	Abstract 14 - A common mechanism for CFTR potentiators - Han-I Yeh (US)
10:30-11:00	*Coffee break* & **Poster viewing**

11:00-12:45	**Symposium 2 – Model systems**
	Chairs: Hugo De Jonge (NL) - Sinead Weldon (UK)
11:00-11:25	Mechanisms of Pulmonary Disease and Diabetes in Cystic Fibrosis Ferrets - John Engelhardt (US)
11:25-11:50	BAC Transgenic Mouse Models Expressing Human CFTR - Lara R. Gawenis (US)
11:50-12:15	Model systems to study CFTR dysfunction - Marc Chanson (CH)
12:15-12:25	Abstract 76 - Profiling of CFTR modulators GLPG1837 and GLPG2222 using intestinal organoids - Sara Musch (BE)
12:25-12:35	Abstract 118 - Human periodontal ligament stem cells: A novel model for cystic fibrosis research and personalized medicine - Mario Romano (IT)
12:35-12:45	Abstract 78 - Evaluation of the primary human nasal epithelial cell cultures as a biomarker in personalized Cystic Fibrosis treatment - Iwona Pranke (FR)
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 – CFTR Structure**
	Chairs: TC Hwang (US) - Liz Miller (UK)
16:00-16:25	Calmodulin binding to CFTR R region and PKA-independent activation of CFTR - Julie Forman-Kay (CA)
16:25-16:50	3D structures of the full length CFTR protein: combining theoretical and experimental data - Isabelle Callebaut (FR)
16:50-17:15	The molecular structure of CFTR revealed by cryo-EM - Jue Chen (US)
17:15-17:25	Abstract 47 - The processing defect of ΔF508- and ΔY512-CFTR are generated by similar mechanisms with different initial flaws - Jeng-Haur Chen (HK)
17:25–17:35	Abstract 52 - Molecular dynamics flexible fitting (MDFF) simulations identify new models of closed state CFTR - Hanoch Senderowitz (IL)
17:35-17:45	Abstract 51 - Biosynthetic and functional correction of CF-causing NBD2 defects - Patrick Thibodeau (US)
17:45-18:00	*Break*
18:00-19:45	**ECFS Basic Science Working Group Session** ***Task Force on Personalised Medicine for CF*** Special discussion groups: 1. Validation/ optimization of novel biomarkers Discussion leader: Kris de Boeck 2. Assays for improved endpoints to evaluate novel drugs: intestinal and respiratory organoids Discussion leader: Jeff Beekman 3. Novel therapeutic approaches to CF therapies: alternative channels and gene/cell therapies Discussion leader: Marcus Mall
19:45-21:30	*Dinner*
21:30-23:00	**Evening Poster Session:** Posters with Even numbers

Friday, 31 March 2017 (Day 3)	
07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4 – Translational CF Research**
	Chairs: John Engelhardt (US) - Kris De Boeck (BE)
08:45-09:10	Intestinal Organoids for personalised medicine - Jeff Beekman (NL)
09:10-09:35	Non-viral CRISPR/Cas gene editing enabled by co-delivery of mRNA and sgRNA inside of synthetic lipid nanoparticles – Daniel Siegwart (US)
09:35-10:00	Genetic and functional evidence for SLC26A9 as a CF modifier - Lisa Strug (CA)
10:00-10:10	Abstract 84 - Transduction of Rhesus macaque lung by AAV1 – Liudmila Cebotaru (US)
10:10-10:20	Abstract 107 - Nanoparticle-Mediated Delivery of Modified tRNAs as a Therapeutic Approach for CF caused by a Nonsense Mutation - Michael Torres (US)
10:20-10:30	Abstract 79 - Unravelling the mechanisms of airway epithelium repair in cystic fibrosis - Aderonke Sofoluwe (CH)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 5 - Mucus and Mucins**
	Chairs: David Thornton (UK) - Marcus Mall (DE)
11:00-11:25	Synergistic stimulation of gland secretion and mucus clearance - Jeff Wine (US)
11:25-11:50	Mucus Clearance in Normal Airways and Conversion into Attached Mucus in Chronic Lung Disease - Gunnar C. Hansson (SE)
11:50-12:15	Optical coherence microscopy (OCM) for assessment of mucus transport - Peter König (DE)
12:15-12:25	Abstract 81 - Relative roles of reduced mucus clearance and mucus hypersecretion in the pathogenesis of airway mucus plugging in mice - Sandra Christochowitz (DE)
12:25-12:35	Abstract 82 - CFTR and bicarbonate as determinants of airway mucus secretion and microrheology - Pierre Lesimple (FR)
12:35-12:45	Abstract 26 - Identification of proteins associated with goblet cell hyperplasia - Ilaria Musante (IT)
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, 01 April 2017 (Day 4)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 6 – Cell Physiology and Ion transport**
	Chairs: David Sheppard (UK) - Luis Galietta (IT)
08:45-09:10	Studying asymmetric movements in CFTR's two nucleotide binding sites - László Csanády (HU)
09:10-09:35	Functional Integrity of CFTR in the Cell Membrane: Role of ATP binding in Site 1 – TC Hwang (US)
09:35-10:00	Novel protease inhibitors to balance ENaC function in CF airways - Lorraine Martin (UK)
10:00-10:10	Abstract 05 - The contribution of the paracellular pathway to HCO3- secretion by human airway epithelial cells - Vinciane Saint-Criq (UK)
10:10-10:20	Abstract 27 - Role and structure-function relationship of the TMEM16A chloride channel - Paolo Scudieri (IT)
10:20-10:30	Abstract 29 - The calcium-activated potassium channel KCa3.1 Inhibits sodium absorption in airway epithelial cells - Génesis Vega (CL)
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:45	**Symposium 7 – CFTR Folding, Trafficking and Activity**
	Chairs: Isabelle Callebaut (FR) - Margarida Amaral (PT)
11:00-11:25	Interactions and post-translational modifications: regulating CFTR trafficking - Carlos Farinha (PT)
11:25-11:50	A yeast model to understand biosynthesis, folding and traffic of ABC transporters - Liz Miller (UK)
11:50-12:15	Regulation of CFTR Channel Function by Lipids and Lipid-mediated Signaling - Nael McCarty (US)
12:15-12:25	Abstract 34 - Silent mutations affectings CFTRs translational landscape rescue folding mutations - Robert Rauscher (DE)
12:25-12:35	Abstract 102 - A high-content siRNA screen at near genomic scale reveals novel F508del-CFTR therapeutic targets - Hugo Botelho (PT)
12:35-12:45	Abstract 50 - Rescue Δ508-CFTR with nanobodies - Marie Overtus (BE)
12:45-14:30	*Lunch*
14:30-16:40	**Symposium 8 – Inflammation and Host Pathogen interaction**
	Chairs: Jeff Wine (US) - Martina Gentzsch (US)
14:30-14:55	Bioactive lipids and CF lung inflammation, innocent markers or guilty agonists? - Bob Scholte (NL)
14:55-15:20	Cathepsin S in CF lung disease - Sinead Weldon (UK)
15:20-15:45	Novel genetically-diverse mouse models to unravel the complexity of the lung infections Alessandra Bragonzi (IT)
15:45-16:10	The role of CFTR in the Neutrophil - Gerry McElvaney (IE)
16:10-16:20	Abstract 59 - Early alterations in airway mucins glycosylation and mucociliary clearance players in the development of cystic fibrosis lung disease - Ignacio Caballero (FR)

16:20-16:30	Abstract 63 - Altered Airway Macrophage Phenotype and Function in Mice with Mucociliary Clearance Dysfunction - Michelle Paulsen (DE)
16:30-16:40	Abstract 70 - SPLUNC1 is pH-dependent and reduces Burkholderia cepacia growth in airway surface liquid – Robert Tarran (US)
16:40-17:10	*Coffee Break*
17:10-18:10	**Flash Paper Session - CFTR structures** **Mike Gray (UK)** **Or** **How to write a successful paper** **Emma Grainger (Elsevier)**
18:10-18:15	*Break*
18:15-19:15	**Closing Keynote lecture** Studying CFTR: From Basic Science to Treatment of Cystic Fibrosis – Martina Gentzsch (US)
20:30	*Dinner / Social Event*

POSTER TITLES & AUTHORS

P1 Epithelial chloride transport by CFTR requires ANO1
Roberta Benedetto, Jiraporn Ousingsawat, Podchanart Wanitchakool, Yong Zhang, Michael J. Holtzman, Margarida Amaral, Rainer Schreiber, Karl Kunzelmann

P2 Anoctamin 1 traffic and function is enhanced by tethering proteins family of extended synaptotagmins
Joana R. Lérias, Madalena C. Pinto, Hugo M. Botelho, Rainer Schreiber, Margarida D. Amaral, Karl Kunzelmann

P3 Deregulation of planar Cell polarity and TGFβ signaling in the airways of cystic fibrosis mouse models
Sabrina Noel, Nadtha Panin, Mathilde Beka, Amandine Collin, Sophie Gohy, Marcus Mall, Teresinha Leal

P4 Anoctamins 1 and 6 – identification of novel traffic regulators and their significance to cystic fibrosis
Madalena C. Pinto, Joana R. Lérias, Hugo M. Botelho, Nikhil T. Awatade, Rainer Pepperkok, Karl Kunzelmann, Margarida D. Amaral

P5 The contribution of the paracellular pathway to HCO_3^- secretion by human airway epithelial cells.
Vinciane Saint-Criq, Eric S. Davis, Robert Tarran, Michael A. Gray

P6 CFTR regulates insulin secretion and calcium signalling in islet cells.
Fiona Manderson Koivula, Dawood Khan, Ryan Kelsey, Neville McClenaghan, Alan Harper, Catriona Kelly

P7 Involvement of sphingosine 1-phosphate in cystic fibrosis bone disease
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P8 Novel insight into the role of CFTR in the lacrimal functional unit and its influence on the fluid secretion of lacrimal gland ducts in mouse
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P9 Expression and function of Cl^- loaders, extruders and channels in pancreatic islet ß-cells: implications for intracellular Cl^- regulation, insulin secretion and CFRD
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P10 Peptide-based, ratiometric FRET probes visualize channel-activating protease (CAP) activity.
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P11 Intermediate conductance K^+ channel is required for parathyroid hormone-stimulated CFTR-mediated anion secretion by Caco-2 intestinal epithelia
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P12 Impaired PMCA function causes Ca^{2+} overload and cell damage in CFTR knock out pancreatic ductal cells
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P13 Primary human bronchial epithelial cells: electrophysiological experiments to compare impact of culture medium on CFTR activity
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P14 A common mechanism for CFTR potentiators
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P15 The *Slca4a4*$^{-/-}$ mouse displays a muco-obstructive lung phenotype resembling human cystic fibrosis.
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P16 Sodium in the apical solution mediates downregulation of ion transport in cultured pig tracheal epithelia
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P17 A paediatric primary nasal epithelial cell culture model system to investigate bypass channels for CF therapy
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P18 Involvement of the Na^+-bicarbonate-cotransporter in the airway surface liquid pH regulation of primary bronchial epithelial cells
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P19 A new animal model for epithelial ion transport modeling (focusing on CFTR) - characterization of pancreatic ductal fluid and bicarbonate secretion in wild type ferrets
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P20 ENaC internalization by SPX-101 is a novel CF therapy for all CFTR mutation.
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P21 Cigarette smoke-induced aggregation of CFTR into ceramide platforms is ROS-dependent
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P28 Insulin signaling via the PI3K/Akt pathway regulates airway glucose uptake and barrier function in a CFTR-dependent manner
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P65 Regulation of ferroportin expression in cystic fibrosis bronchial epithelial cells is possibly influenced by hypoxia, transforming growth factor-beta and epithelial sodium channel.
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Rebecca McElroy, Jason McGrath, Amal El Banna, Bettina Schock

P68 Adaptive microbial interactions between *P. aeruginosa* and *A. xylosoxidans*
Angela Sandri, Janus A.J. Haagensen, Alicia Jimenez Fernandez, Søren Molin, Helle Krogh Johansen, Maria M. Lleò

P69 Matrix metalloprotease inhibitors as anti-inflammatory therapy in *Pseudomonas aeruginosa* lung infection
Angela Sandri, Fabio Stellari, Federico Boschi, Alessia Ortombina, Gabriella Bergamini, Maria M. Lleò

P70 SPLUNC1 is pH-dependent and reduces burkholderia cepacia growth in airway surface liquid.
Saira Ahmad, William G. Walton, Matthew Redinbo, Robert Tarran

P71 Explosive cell lysis is involved in biofilm formation and the biogenesis of staphylococcal "public goods"
Davide Losa, Lynne Turnbull, Rosalia Cavaliere, Benjamin B.A. Raymond, Steven P. Djordjevic, Cynthia B. Whitchurch

P72 Successful gene editing of human embryonic stem cells to generate a novel CF airway epithelial model
Sara Cuevas Ocana, Magomet Aushev, Julia Reichelt, Neil Perkins, Michael Gray

P73 Characterization of transepithelial nasal potential difference in cystic fibrosis-like mouse
Teresinha Leal, Mathilde Beka, Nadtha Panin, Marcus Mall, Sabrina Noel

P74 The *Cftr*^{tm1eur}/F508del CFTR mouse model have reduced beta-cell mass due to reduced cell size and reduced number of docked insulin granules.
Anna Edlund, Michael Hühn, Emma Svedin, Ines Mollet, Mia Abels, Nils Wierup, Bob J. Scholte, Malin Flodström-Tullberg, Lena Eliasson

P75 Comparison of ex vivo and in vitro intestinal cystic fibrosis models to measure CFTR-dependent ion channel activity
Marne Hagemeijer, Domenique Zomer-van Ommen, Eyleen de Poel, Evelien Kruisselbrink, Hugo Oppelaar, Karin de Winter-de Groot, Kors van der Ent, Jeffrey Beekman

P76 Profiling of CFTR modulators GLPG1837 and GLPG2222 using intestinal organoids
Sara Musch, Anabela Santo Ramalho, Luc Nelles, Steven Van Der Plas, Martin Andrews, Thierry Christophe, Jan Van Der Schueren, Kris De Boeck, Katja Conrath

P77 Directed differentiation and genetic modification of novel iPS cell lines from CF patients
Daniel Bachiller, Aarne Fleischer, Esther Palomino, Ivan Lorenzo, Victor Galvez, Trond Aasen, Juan Carlos Izpisua-Belmonte, Fernando Gomez

P78 Evaluation of the primary human nasal epithelial cell cultures as a biomarker in personalized cystic fibrosis treatment
Iwona Pranke, Aurélie Hatton, Juliette Simonin, Jean Philippe Jais, Françoise Le Pimpec-Barthes, Myriam Mesbahi, Emanuelle Girodon-Boulandet, Alexandre Hinzpeter, Aleksander Edelman, Isabelle Sermet-Gaudelus

P79 Unravelling the mechanisms of airway epithelium repair in cystic fibrosis
Aderonke Sofoluwe, Joanna Bou Saab, Marc Bacchetta, Marc Chanson, Alice Zoso

P80 Generation of disease-specific iPSCs and development of transgenic reporter cell lines for cystic fibrosis disease modelling and drug screening
Sylvia Merkert, Madline Schubert, Alexandra Haase, Lena Engels, Saskia Ulrich, Nico Lachmann, Burkhard Tümmler, Luis Galietta, Ruth Olmer, Ulrich Martin

P81 Relative roles of reduced mucus clearance and mucus hypersecretion in the pathogenesis of airway mucus plugging in mice
Sandra Christochowitz, Simone Schmidt, Jolanthe Schatterny, Matthias Hagner, Marcus A. Mall

P82 CFTR and bicarbonate as determinants of airway mucus secretion and microrheology.
Pierre Lesimple, Maria Kilfoil, O'Grady M. Scott, Renaud Robert, Hanrahan W. John

P83 SPX-101 is a novel ENaC-targeted therapeutic for cystic fibrosis that restores mucus transport
David Scott, Matthew Walker, Juliana Sesma, Timothey Stuhlmiller, Bryant Wu, William Abraham, Juan Sabater, Robert Tarran

P84 Transduction of Rhesus macaque lung by AAV1
William B. Guggino, John Engelhardt, Ziying Yan, Liudmila Cebotaru

P85 An Investigation into novel inhibitors of channel activating proteases with implicators for cystic fibrosis lung disease
Lisa E.J. Douglas, Timothy E.G. Ferguson, James A. Reihill, S. Lorraine Martin

P86 Effect of brevenal as a single agent and combined with orkambi on airway surface liquid and mucociliary transport in CF primary human bronchial epithelial cells
Emily Falk Libby, Henry Fortinberry, Susan E. Birket, Carlos Milla, Daniel Baden, Andrea Bourdelais, Matthias Salathe, Isaac Cohen, William M. Abraham, Guillermo J. Tearney, Steven M. Rowe

P87 VX-770 potentiation of CFTR gating involves stabilisation of the pre-hydrolytic O_1 open state
Emily Langron, Paola Vergani

P88 Task force for cystic fibrosis (TFCF): discovery and characterization of potent F508del-CFTR modulators
Fabio Bertozzi, Tiziano Bandiera, Paolo Di Fruscia, Federico Sorana, Francesco Berti, Alejandra Rodríguez-Gimeno, Emanuela Caci, Loretta Ferrera, Valeria Tomati, Emanuela Pesce, Paolo Scudieri, Nicoletta Pedemonte, Luis J.V. Galietta

P89 CFTR gene editing with CRISPR/Cas9 in cultivated human tracheal epithelial cells for the development of cystic fibrosis therapies.
Afroditi Avgerinou, Demetra-Ellie Phylactopoulos, Ahmad Aldossary, Asllan Gjinovci, Chris O' Callaghan, Stephen Hart, Paola Bonfanti

P90 Novel miRNA inhibition strategy based on biodegradable polymers for CF gene therapy studies
Elena Fernandez Fernandez, Chiara de Santi, Joanne Ramsey, Sally-Ann Cryan, Catherine Greene

P91 The discovery of novel TMEM16A potentiator compounds
Henry Danahay, Sarah Lilley, Holly Charlton, Roy Fox, Clive McCarthy, Martin Gosling

P92 Characterisation and microparticle formulation of a novel CAP inhibitor, QUBTL1, with implications for CF lung disease.
Jessica R. Maye, S Lorraine Martin, James A. Reihill

P93 New trimethylangelicin analogues as modulators of defective CFTR
Adriana Chilin, Giovanni Marzaro, Ilaria Lampronti, Roberto Gambari, Anna Tamanini, Alessandra Santangelo, Giuseppe Lippi, Giulio Cabrini, Maria Cristina Dechecchi

P94 Validation of a CFTR alternative splicing reporter system for high-throughput microscopy and identification of novel CFTR regulators of alternative splicing
Susana Igreja, Hugo M. Botelho, Veronica Felicio, Giulia Pianigiani, Franco Pagani, Margarida D. Amaral

P95 Rescue of F508del-CFTR by corrector RDR01752
Miquéias Lopes-Pacheco, Nikhil T. Awatade, Margarida D. Amaral

P96 *In vitro* characterization and correction of splicing mutations in IVS 5 by an antisense oligonucleotide
Veronica Felicio, Arsénia J. Massinga, Nikhil T. Awatade, Luka A. Clarke, Susana Igreja, Margarida D. Amaral

P97 Ivacaftor and lumacaftor cooperatively amend the function of pathogenic mutations in cystic fibrosis
Ying-Chun Yu, Wen-Ying Lin, Tzyh-Chang Hwang

P98 Alkyl fluorination enhances the efficacy and potency of tris-thiourea artificial anion transporters
Hongyu Li, Michael J. Spooner, Xin Wu, David N. Sheppard, Philip A. Gale

P99 A High-Throughput Screening assay to identify factors correcting CFTR mutations bearing premature termination codons (PTCs).
Verónica Felício, Hugo M. Botelho, Susana Igreja, Margarida D. Amaral

P100 Effects of increasing levels of gamma-glutamyltransferase in cystic fibrosis airways on glutathione inhalation therapies.
Alessandro Corti, Paola Melotti, Claudio Sorio, Gabriella Bergamini, Andreas Hector, Matthias Griese, Alfonso Pompella

P101 Combination of gene therapy with CFTR modulators for enhanced therapeutic efficacy
Marjolein Ensinck, Liesbeth De Keersmaecker, Anabela S. Ramalho, Kris De Boeck, Rik Gijsbers, Frauke Christ, Zeger Debyser, Marianne S. Carlon

P102 A high-content siRNA screen at near genomic scale reveals novel F508del-CFTR therapeutic targets
Hugo M. Botelho, Sang H. Lim, Christian Tischer, Rainer Pepperkok, Igor Stagljar, Margarida D. Amaral

P103 High throughput microscopy screens identify F508del-CFTR correctors and ANO1 traffic modulators in a unique portuguese natural compound library
Catarina Baptista, Helena Vieira, Rogério Tenreiro, Nikhil T. Awatade, Margarida D. Amaral, Hugo M. Botelho

P104 Assay development and high-throughput screen installation for identifying SLC26A9 chloride channel activators
Anita Balazs, Aliaksandr Halavatyi, Johanna J. Salomon, Marko Lampe, Rainer Pepperkok, Marcus A. Mall

P105 Derivatives of esculentin-1a: promising frog-skin peptides for the development of new antipseudomonal drugs with expanding properties
Bruno Casciaro, Maria Rosa Loffredo, Vincenzo Luca, Maria Luisa Mangoni

P106 Targeting PI3Kγ scaffold function to activate airway CFTR, limit lung inflammation and promote bronchorelaxation in cystic fibrosis
Alessandra Ghigo, Alessandra Murabito, Kai Ren, Flora Pirozzi, Alessio Montresor, Wito Richter, Daniela Wenzel, Michaela Matthey, Nancy L. Quinney, Deborah M. Cholon, Bernd Fleischmann, Martina Gentzsch, Carlo Laudanna, Emilio Hirsch

P107 Nanoparticle-mediated delivery of modified tRNAs as a therapeutic approach for CF caused by a nonsense mutation.
Michael J. Torres, Jason B. Miller, Jennifer R. Peters-Hall, Linda Millen, Linde De Keyzer, Zhiwei Cai, Qin Li, Jamie Wangen, Jan Harrington, Amita Patel, Carla Oliveira, Andrew P. Feranchak, David N. Sheppard, Scott H. Randell, Rachel Green, Hermann Bihler, Martin Mense, Robert J. Bridges, Jerry W. Shay, Arthur E. Johnson, Philip J. Thomas, Daniel J. Siegwart

P108 Correcting the ΔF508 mutation of the CF gene by CRISPR/Cas9
Ahmad Aldossary, Aristides Tagalakis, Deborah Baines, Christopher O'Callaghan, Stephen Hart

P109 Curated database of candidate therapeutics for the activation of CFTR-mediated ion conductance (CandActCFTR)
Manuel Manfred Nietert, Sylvia Hafkemeyer, Frauke Stanke

P110 Mesoangioblasts -vessel associated progenitor cells as a novel cell-based therapy for cystic fibrosis
Chiara Vezzali, Giuseppe Celesti, Chiara Bonfanti, Stefania Antonini, Valeria Tomati, Emanuela Pesce, Christina Barone, Marie E. Egan, Nicoletta Pedemonte, Emanuela Bruscia, Graziella Messina

P111 Stepwise generation of CFTR-expressing airway epithelial cells from human pluripotent stem cells
Saskia Ulrich, Sandra Baus, Sylvia Merkert, Lena Engels, Ruth Olmer, Ulrich Martin

P112 Amplifiers co-translationally increase CFTR levels at the ER membrane by improving membrane targeting of CFTR
Danijela Dukovski, David C. Kombo, Adriana Villella, Nipul Patel, Matthew D. Cullen, Cecilia M. Bastos, Soheil Aghamohammadzadeh, Ben Munoz, John Preston Miller

P113 CFTR superexon homology-independent targeted integration to correct CF-causing variants in and downstream of Exon 23
Karen Mention, David Sanz, Kader Cavusoglu-Doran, Jennifer Hollywood, Martina Scallan, Patrick Harrison

P114 Development of CRISPR Cpf1 editing for the CFTR gene
Kader Cavusoglu-Doran, David Sanz, Karen Mention, Martina Scallan, Patrick Harrison

P115 Receptor-targeted nanocomplex delivery of siRNA silences αENAC expression in vitro and in vivo and improves mucociliary function: a therapeutic strategy for cystic fibrosis
Aristides Tagalakis, Mustafa Munye, Rositsa Ivanova, Hanpeng Chen, Claire Smith, Ahmad Aldossary, Luca Rosa, Stuart Jones, Guy Moss, Christopher O'Callaghan, Robin McAnulty, Stephen Hart

P116 High-throughput forskolin-induced swelling assay to identify and/or repurpose old and new cftr-restoring drug compounds using intestinal organoids of subjects with cystic fibrosis.
Eyleen de Poel, M.C. Hagemeijer, A.M. Vonk, H. Oppelaar, G. Berkers, P. van Mourik, C.K. van der Ent, J.M. Beekman

P117 Personalized cell-based therapy for cystic fibrosis-related lung disease
Kim Goh, Ludovic Vallier

P118 Human periodontal ligament stem cells: a novel model for cystic fibrosis research and personalized medicine
Sara Patruno, Eleonora Cianci, Oriana Trubiani, Francesca Diomede, Laura Pierdomenico, Veronica C. Mari, Antonio Recchiuti, Giuseppina Bologna, Ilaria Merciaro, Matteo Serroni, Paolo Moretti, Marco Marchisio, Mario Romano

P119 Impact of ASL acidification on bacterial killing capacity in cystic fibrosis airways
Juliette Simonin, Emmanuelle Bille, Aurélie Hatton, Iwona Pranke, Xavier Nassif, Aleksander Edelman, Isabelle Sermet-Gaudelus

P120 Non-viral CRISPR/Cas gene editing in mouse lungs enabled by co-delivery of mRNA and sgRNA inside of synthetic lipid nanoparticles
Daniel J. Siegwart, Jason B. Miller, Shuyuan Zhang, Petra Kos, Hu Xiong, Kejin Zhou, Hao Zhu, Robert J. Bridges, Philip J. Thomas, Arthur E. Johnson, Michael J. Torres

P121 Role of CFTR Cl^- channel in the exocrine/endocrine function of the pancreas
Emese Tóth, Petra Pallagi, Péter Hegyi, Viktória Venglovecz

P122 LncRNAs: emerging players in *CFTR* gene regulation
Jessica Varilh, Alexandra Pommier, Magali Taulan-Cadars

P123 Unsolved severe chronic rhinosinusitis elucidated by extensive *CFTR* genotyping
Virginie Prulière-Escabasse, Fanny Degrugillier, Stéphanie Simon, Natascha Remus, Chadia Mekki, Laurence Bassinet, Xavier Decrouy, Isabelle Sermet-Gaudelus, Aurélie Hatton, Brice Hoffmann, Isabelle Callebaut, Alexandre Hinzpeter, Pascale Fanen

P124 miRNA profiling identifies new CF regulators
Alexandra Pommier, Jessica Varilh, Jennifer Bonini, Michel Koenig, Mireille Claustres, Magali Taulan-Cadars

P125 Trypsin-like protease activity predicts disease severity and patient mortality in adults with cystic fibrosis
James A. Reihill, Kelly L. Moffitt, Andrew M. Jones, J. Stuart Elborn, S. Lorraine Martin

P126 PTC mutations in CFTR: effect on messenger RNA abundance
Luka A. Clarke, Veronica M. Felicio, Nikhil T. Awatade, Silvia Gartner, Carmen Bertuzzo, Jeff Beekman, Margarida D. Amaral

P127 Beyond the ussing chamber: MTECC-24-96
Robert J. Bridges, Willy Van Driessche

P128 Cystic fibrosis infant airways harbor a pathogenic subset of live neutrophils
Camilla Margaroli, Hamed Horati, Bob J. Scholte, Mieke Veltman, Limin Peng, Harm A.W.M. Tiddens, Hettie M. Janssens, Rabindra Tirouvanziam

P129 Characterization of Q1412X-CFTR, a severe form Class VI pathogenic mutation
Jiunn-Tyng Yeh, Tzyh-Chang Hwang

AWARD WINNERS

ECFS Young Fellows Travel Award

Emily Langron (UK)
Michelle Paulsen (DE)
Stella Prins (UK)
Robert Rauscher (DE)
Daniel Scholl (BE)
Anastasia Tchoukaev (FR)
Han-I Yeh (US)

Student Helper Award

Sara Cuevas Ocana (UK)
Madalena Pinto (PT)
Aderonke Sofoluwe (CH)
Marcel van Willigen (NL)

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Massimo Aureli
Giuseppe Celesti
Alessandra Ghigo
Maria Rosa Loffredo
Ilaria Musante
Paolo Scudieri

Mukoviszidose eV Travel Award

Roberta Benedetto
Sandra Christochowitz
Ayca Seyhan Agircan

Vaincre La Mucoviscidose Travel Award

Pauline Bardin
Marie-Laure Jourdain
Alexandra Pommier
Iwona Pranke
Juliette Simonin
Jessica Varilh

29 March — 18:00–19:00

Opening Keynote Lecture

Back to the Future

Raymond A. Frizzell

University of Pittsburgh, Dept of Pediatrics, 7116 Rangos Research Bldg, 4401 Penn Ave, Pittsburgh, PA 15224 USA.

Programme note: the details are not authorised for inclusion

30 March — 08:45–10:30

Symposium 01: Therapeutic Approaches

S1.1 Discovery and Characterization of Novel CFTR Modulators

Ashvani Singh

AbbVie, 1 North Waukegan Road, North Chicago, IL 60064, USA

Cystic fibrosis is a multisystem disease of the lungs, sinuses, pancreas, and gastrointestinal tract that is caused by dysfunction or deficiency of the cystic fibrosis transmembrane conductance regulator protein (CFTR), the epithelial anion channel that regulates salt and water balance in tissues and maintains homoeostasis of the airway surface liquid layer of the lungs. To address the most prevalent defects leading to Cystic Fibrosis (CF), two biomolecular activities are required, namely correctors to increase the "amount" of properly folded F508delCFTR levels at the cell surface, and potentiators to allow the effective opening, *i.e.* "function" of the F508delCFTR channel. Combined, these activities allow chloride ion transport yielding improved hydration of the lung surface and subsequent restoration of mucociliary clearance. To yield an enhanced clinical benefit to CF patients, a complementary "triple combination" therapy consisting of two corrector molecules, type 1 (C1) and type 2 (C2) with additive mechanisms along with a potentiator (P) are being developed for maximum restoration of mutated CFTR function. AbbVie-Galapagos have generated a suite of potential "best-in-class" molecules representing the complementary components, including ABBV/GLPG2222 (C1), GLPG2737 (C2) and GLPG1837, ABBV/GLPG2451, ABBV/GLPG3067 (P). *In* vitro biological characterization data of these novel CFTR modulators will be presented.

S1.2 Therapeutic potential of proteostasis modulation in cystic fibrosis

Nicoletta Pedemonte

U.O.C. Genetica Medica – Istituto Giannina Gaslini – Genova (Italy)

Deletion of phenylalanine 508 (F508del) in the CFTR chloride channel causes a processing defect of the mutant protein resulting in its ritention 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, it is common thought that 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 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.

Particularly interesting is the E3 ubiquitin ligase RNF5, a known CFTR interactor whose silencing in immortalized F508del human bronchial cells causes a marked CFTR rescue and shows a strong additive effect with corrector VX-809. The therapeutic potential of this target was validated in CF mice homozygus for F508del mutation, demonstrating that the genetic suppression of RNF5 in vivo leads to an attenuation of intestinal pathological phenotypes. These findings supported the possibility to use RNF5 as a novel drug target for CF therapy.

S1.3 The development of artificial anion transporters for cystic fibrosis

Hongyu Li[1], Hennie Valkenier[2], Xin Wu[3,4], Sabir Hussain[2], Christopher M. Dias[2], Luke W. Judd[2], Jean-Baptiste Joos[2], Germinal Magro[2], Peter R. Brotherhood[2], Michael J. Spooner[3], Ethan N. W. Howe[3,4], Philip A. Gale[3,4], David N. Sheppard[1], Anthony P. Davis[2]

[1]University of Bristol, School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences Building, University Walk, Bristol BS8 1TD, UK, [2]University of Bristol, School of Chemistry, Cantock's Close, Bristol BS8 1TS, UK, [3] University of Southampton, Chemistry, Southampton SO17 1BJ, UK, [4]University of Sydney, School of Chemistry, Syndey, NSW 2006, Australia

Programme note: the details are not authorised for inclusion

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S1.4.a SPX-101 is a novel ENaC-targeted therapeutic for cystic fibrosis that restores mucus transport

David Scott[1], Matthew Walker[1], Juliana Sesma[1], Timothey Stuhlmiller[1], Bryant Wu[1], William Abraham[2], Juan Sabater[2], Robert Tarran[1,3]

[1]Spyryx Biosciences, Durham, United States, [2]University of Miami, Mount Sinai Medical Center, Miami, United States, [3]University of North Carolina Chapel Hill, Chapel Hill, United States

Background: Airway epithelial cells secrete Short Palate, Lung, and Nasal Clone 1 (SPLUNC1) to regulate epithelial sodium channel (ENaC) activity by lowering the number of channels on the cell surface. However, SPLUNC1 loses this regulatory function at the acidic pH of the cystic fibrosis (CF) airway leading to hyperactivation of ENaC. When ENaC becomes hyperactivated, the increased sodium transport creates an osmotic gradient that pulls fluid out of the airway. Loss of airway fluid causes mucus dehydration, decreased mucociliary clearance, and chronic bacterial infections which account for the majority of morbidity and mortality associated with CF. ENaC represents a unique therapeutic target capable of treating all CF patients independent of their underlying CFTR mutation. We have developed a peptide mimetic of SPLUNC1, SPX-101, that maintains ENaC regulatory function even at acidic pH.

Objectives: To determine if SPX-101 increases mucus transport in CF-like lung disease and to assess the safety of the peptide when delivered by nebulization.

Results: SPX-101 increases mucus transport in mouse and sheep models of CF lung disease. A single intranasal dose of SPX-101 significantly increased mucus transport compared to control peptide in βENaC transgenic mice. This resulted in increased survival of βENaC mice to >90% with once-daily intranasal administration. Using a new sheep model where CFTR inhibitor is delivered to the upper airway via nebulization, thereby decreasing mucus movement by ~50%, SPX-101, but not small molecule inhibitors of ENaC such as amiloride, fully restored mucus transport. This effect of SPX-101 lasted >8 hours with a single dose. In 28-day GLP toxicology assessments of nebulized SPX-101 there were no adverse events. Importantly, there was no observed diuretic or hyperkalemic effect of SPX-101 up to the maximum deliverable dose.

Conclusion: SPX-101 increases mucus transport in multiple models of CF-like lung disease with efficacy achieved by once-daily dosing. The peptide has no adverse systemic effects. Taken together, SPX-101 represents a novel peptide-based therapy to treat all patients with CF regardless of the CFTR mutation.

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S1.4.b ENaC internalization by SPX-101 is a novel CF therapy for all CFTR mutation.

Juliana Sesma[1], Matthew Walker[1], Timothy Stuhlmiller[1], Bryant Wu[1], Timothy Crowder[1], Robert Tarran[1,2], David Scott[1]

[1]Spyryx Biosciences, Durham, United States, [2]Marsico Lung institute and University of North Carolina, Chapel Hill, United States

Background: Hyperactivation of the epithelial sodium channel (ENaC) in the respiratory airways strengthens the pathophysiology of CF. In normal lungs, short palate lung and nasal epithelial clone 1 (SPLUNC1) is secreted to the airway surface liquid and binds to ENaC inducing its internalization. This mechanism regulates ENaC concentration in the plasma membrane. In CF, the acidic pH impedes the binding of SPLUNC1 to ENaC resulting in hyperabsorption of Na^+, dehydration of the airways, and impaired mucociliary clearance. Here we present SPX-101, a SPLUNC1-derived peptide that internalizes ENaC regardless of the pH.

Objectives: To investigate the in vitro and in vivo effectiveness of SPX-101.

Methods: ENaC internalization by SPX-101 in primary human bronchial epithelial cells (HBEC) from healthy and CF donors was assessed by surface biotinylation and western blot analysis. SPX-101 specificity was measured by TAMRA-tagged SPX-101 binding to βENaC-expressing cells vs mock transfected or expressing ASIC1/2 (most homologous ENaC ion channel) cells. Amiloride sensitive current was determined in healthy and CF HBEC by transepithelial voltage (Vt) and resistance (R) EVOM measurements. SPX-101's in vivo therapeutic effect was assessed by mouse survival curves and by analysis of leukocyte distribution in broncho-alveolar lavage fluids of a CF-like mouse model, the βENaC transgenic mice.

Conclusions: SPX-101 binds selectively to ENaC and promotes internalization of the alpha, beta, and gamma subunits. SPX-101 -promoted ENaC internalization decreases amiloride-sensitive current. Once-daily intranasal dosing of SPX-101 to the βENaC transgenic mice increases survival to more than 90% and also reduces neutrophil and eosinophil infiltration into the lungs. Taken together, SPX-101 represents a novel peptide-based therapy to treat all patients with CF regardless of the CFTR mutation.

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S1.5 Amplifiers co-translationally increase CFTR levels at the ER membrane by improving membrane targeting of CFTR

Danijela Dukovski, David C. Kombo, Adriana Villella, Nipul Patel, Matthew D. Cullen, Cecilia M. Bastos, Soheil Aghamohammadzadeh, Ben Munoz, John Preston Miller

Proteostasis Therapeutics, Inc., Cambridge, United States

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is an inefficiently synthesized transmembrane chloride channel. Its first transmembrane helix (TM1) is unique, and contains two charged residues that are conserved across its orthologs. TM1 acts as an inefficient signal sequence directing the ribosome-nascent chain complex (RNC) to the ER with modest efficiency (Lu Y, et al., J Biol Chem. 1998 Jan 2;273(1):568-76.; Schlebach JP, Sanders CR. J Membr Biol. 2015 Jun;248(3):371-81.). In addition, a significant portion of the immature CFTR molecules that manage to overcome this inefficiency and are synthesized in the ER are still co-translationally targeted for degradation (Lukacs GL, et al., EMBO J. 1994 Dec 15;13(24):6076-86.). Because these inefficiencies are inherent in CFTR, modulators that address them would be expected to be mutation agnostic and to complement downstream therapeutic modalities such as correctors and potentiators.

Proteostasis Therapeutics, Inc. is developing amplifiers, a novel class of CFTR modulator that act early in CFTR synthesis to increase the levels of immature CFTR protein. Amplifiers selectively stabilize CFTR mRNA, act co-translationally and their mechanism of action works through the translated portion of CFTR mRNA.

The observed increase in steady state CFTR mRNA levels due to amplifier occurs in the ER-localized population of CFTR mRNA, consistent with the co-translational targeting of CFTR to the ER membrane. Mutations that impact membrane targeting of CFTR also impact the amplifier response, suggesting a model in which amplifier is acting to improve the inefficiency of this process for CFTR. Based on our experimental evidence, we performed *in silico* modeling of TM1 of CFTR and amplifier molecules binding to the signal recognition particle (SRP), and were able to identify good poses. Inactive compounds and non-responsive related proteins were used as negative controls. Based on this, we hypothesized and demonstrated that inactive amplifier analogs are non-competitive for binding.

Taken together, our results identify a mechanism through which amplifiers are able to selectively overcome the inefficiencies inherent in CFTR synthesis. Targeting an early step in CFTR biosynthesis, amplifiers act independent of specific CF-causing mutations, and thus have the potential to increase the levels of CFTR protein for all patient genotypes. In addition, the increase in immature CFTR protein provides more substrate for downstream CFTR modulators, and has the potential to complement upstream gene therapy based approaches.

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S1.6 A common mechanism for CFTR potentiators

Han-I Yeh, Tzyh-Chang Hwang

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Cystic fibrosis (CF) is one of the most common life-shortening genetic diseases, affecting 1 in every 2500 newborns of Caucasian origin. CF is caused by loss-of-function mutations in the gene Cystic Fibrosis Transmembrane conductance Regulator (CFTR), which encodes a phosphorylation-activated but ATP-gated chloride channel. Gating defects are present in many pathogenic mutations including deltaF508 and G551D, two common mutations associated with severe form of CF. In the past decade, high-throughput drug screening has discovered a CFTR potentiator, VX-770 (Ivacaftor), which is now an FDA-approved treatment for a fraction of patients with CF. Lately, GLPG1837, a novel and potentially more effective CFTR potentiator was identified by Conrath et al. (Pediatric Pulmonology. 2016), but its mechanism of action remains unknown. Here, we demonstrate that GLPG1837 enhances macroscopic wild-type CFTR (WT-CFTR) current by 2.06 ± 0.08 fold (n = 19) in a dose-dependent manner with $K_{1/2}$ ~ 0.4 μM. Microscopic kinetic analysis shows that the open probability of WT-CFTR in the presence of 2 mM ATP is boosted to 0.71 ± 0.01 (n = 9) with open time and closed time constants of 851 ± 55 ms and 328 ± 24 ms, respectively. Interestingly, macroscopic current relaxation upon ATP washout in the continuous presence of GLPG1837 yields a double exponential current decay (t_1 = 1.51 ± 0.36 s, 78 ± 3% and t_2 = 16.23 ± 4.97 s, 22 ± 3 %; n = 7), suggesting a significant proportion of GLPG1837-bound channels undergo ATP-independent opening. Accordingly, a higher activity and prolonged open time (642 ± 121 ms, n = 8; c.f. 150 ± 27 ms without GLPG1837, n = 4) are observed in microscopic currents even tens of seconds after removal of ATP when the channels are treated continuously with GLPG1837. This effect of GLPG1837 on both ATP-dependent and ATP-independent gating leads us to examine if GLPG1837 affects CFTR mutants with distinct gating properties. We observed a deceleration of the non-hydrolytic closing rate on hydrolysis-deficient mutant E1371S-CFTR (t = 129.7 ± 10.5 s and 59.8 ± 9.8 s with and without GLPG1837 respectively; n = 4; $p < 0.05$), and potentiation of mutants with defective NBD-dimerization such as G551D-CFTR (19.6 ± 0.8-fold increase of macroscopic current; n = 18) and deltaNBD2-CFTR (18.0 ± 2.4-fold increase, n = 4). Similar results have been reported previously for VX-770 (Jih et al., PNAS, 2013; Yeh et al., JGP 2015). To test if GLPG1837 and VX-770 may share the same mechanism of action, we carried out experimental protocols that combine VX-770 and GLPG1837. The macroscopic current of G551D-CFTR in the presence of GLPG1837 is 1.89 ± 0.14 fold of that with VX-770 (n = 5). However, in the continuous perfusion of VX-770, addition of GLPG1837 fails to increase the current, whereas acute application of VX-770 to the current potentiated by GLPG1837 decreases the current to the level achieved by VX-770 alone. These results suggest that GLPG1837 and VX-770 may compete for a same or structurally closely related binding site.

30 March — 11:00–12:45

Symposium 02: Model Systems

S2.1 Mechanisms of Pulmonary Disease and Diabetes in Cystic Fibrosis Ferrets

John F. Engelhardt

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New animals models of cystic fibrosis (CF) are rapidly improving our understanding of CF pathophysiology. In this presentation, we will discuss recent studies in the CF ferret model that have shed light on disease processes in the lung and pancreas. Lung infections in CF are classically thought to initiate an abnormal inflammatory response, mucus obstruction, and chronic airway remodeling. Bacteria adapt to the changing environment in the CF lung by evolving phenotypes that provide a growth advantage (e.g. mucoid and antibiotic resistance). We sought to model the chronic evolution CF lung disease in the ferret model by maintaining CF and non-CF control animals on sustained antibiotics from birth. These animals were longitudinally evaluated by bronchoalveolar lavage and high-resolution CT imaging. Surprisingly, CF ferrets maintained on multiple synergistic antibiotics from birth were protected from lung infections for several years. CF animals lacked detectable bacterial and fungal infections using both culture and DNA methods. Despite this protection from infection, CF animals still developed hallmarks of structural bronchiectasis, neutrophil-dominated inflammation, and mucus accumulation in the lung. Proteomics analysis of the bronchoalveolar lavage fluid from CF animals demonstrated significant enrichment in pathways controlling neutrophil and leukocyte functions including adhesion, migration, and chemotaxis, as compared to non-CF controls. These findings implicate mucoinflammatory processes in the CF lung as etiologically pathogenic in the absence of clinically apparent microbial infections. While beta-cell dysfunction in CF leads to diabetes, the mechanism by which CFTR influences islet insulin secretion remains debated. We investigated the CFTR-dependent mechanisms impacting islet insulin secretion in CF ferret islets. Interestingly, neonatal CF ferret islets exhibited elevated basal insulin secretion in low glucose under static, but not perfusion conditions, suggesting a CFTR-dependent diffusible factor alters beta-cell glucose sensitivity. Enhanced glucose sensitivity of neonatal CF ferret islets was supported by elevated SLC2A1 transcripts, enhanced basal inhibition of K-ATP, and elevated intracellular calcium under low glucose conditions. CFTR expression also impacted the expression of INS, CELA3B, and several β-cell maturation and proliferation genes. Pharmacologic inhibition of CFTR reduced glucose-stimulated insulin secretion by wild-type ferret, but similarly reduced insulin secretion and intracellular calcium in CFTR-knockout ferret islets, suggesting the mechanism of action is not through CFTR. Single molecule fluorescent in situ hybridization demonstrated that CFTR RNA co-localized within KRT7+ ductal cells, but not endocrine cells, of ferret and human islets and ferret pancreas. These results suggest that CFTR may impact β-cell function via a paracrine mechanism through islet-associated exocrine-derived cell types.

S2.2 BAC Transgenic Mouse Models Expressing Human CFTR

Lara R. Gawenis[1,2], Craig A. Hodges[3,4], Jinghua Liu[1,2], Mitchell L. Drumm[3,4], Lane L. Clarke[1,2]

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Studies using cell lines have demonstrated that defective protein processing of human (h) DF508 CFTR persisted when hDF508 CFTR was expressed in mouse cells (Ostedgaard et al., *PNAS (USA),* 2007;104:15370-5). Therefore, we reasoned that the development of a bacterial artificial chromosome (BAC) transgenic mouse expressing h*CFTR* under control of its promoter elements in a mouse (m) Cftr-null background would allow for the creation of models expressing specific mutations of hCFTR for use in in vivo testing of small molecule modifiers of CFTR processing/function and genome editing therapeutics. Previous studies of a similar yeast artificial chromosome transgenic in *Cftr*-/- mice found conservation of expression by h*CFTR* regulatory elements (Manson et al., *EMBO J*, 1997;16:4238-49). We utilized a ~260 kb BAC construct encompassing 40.1 kb 5' to the h*CFTR* transcriptional start site, the h*CFTR* locus, and 25 kb 3' to generate transgenic founders. Nine h*CFTR* founders were evaluated for germ-line transmission by PCR. One male mouse exhibiting robust germ-line transmission of the BAC transgene was used in subsequent breeding. A Taqman(r) assay indicated the hCFTR mRNA was intact at the exon 26-27 splice site and expressed in various tissues including intestine and airway. The BAC inserted into a single integration site on mouse chromosome 8 and has been fully sequenced. h*CFTR*+ offspring were crossbred with a *Cftr*-/- mouse line for the generation of h*CFTR*+ or h*CFTR*-/m*Cftr*- mice. h*CFTR*+/m*Cftr*- mice have higher body weights relative to sex-matched h*CFTR*-/m*Cftr*- siblings at 3 and 6 weeks of age (3 wks.: male, +34.3%; female, +25%; 6wks: male, +57.2%, female, +33.7%; n = 10). Freshly-excised jejuna from these mice were mounted in Ussing chambers for measurement of short-circuit current (Isc). Treatment with the cAMP-stimulant forskolin (10 μM) elicited a robust and sustained increase of Isc in the h*CFTR*+/m*Cftr*- jejuna as compared to hCFTR-/mCftr- jejuna (DIsc, in μA/cm²: □87.5±8.8 vs. □5.6±2.1, respectively; n=3+). Furthermore, h*CFTR*+/m*Cftr*- mice exhibited significantly greater nasal potential differences (NPD) as compared to hCFTR-/mCftr- littermates (NPD: □8.43±2.4 mV vs. +1.0±0.6 respectively; n=6). We are utilizing the genome editing system CRISPR/Cas9 (Wang et al., *Cell,* 2013;153:910-8) to generate five human CFTR mutations (F508del, G542X, W1282X, G551D and 3849+10kb C>T) in the h*CFTR* mouse strain that will allow various CFTR-directed therapies to be tested. Single guide RNAs (sgRNAs) for all five of the desired CFTR mutations have been injected, along with Cas9 nuclease and oligonucleotides carrying the desired *CFTR* mutation, into single-cell mouse embryos from the C57Bl/6J background. Currently, we are sequencing DNA from the resulting pups of these five lines to verify the mutations present. Once each desired *CFTR* mutation is identified in founder mice, they will be crossed to a m*Cftr*-/- strain and characterized for loss of CFTR function and responsiveness to specific therapies. Successful generation of these models will provide novel tools for the wide-spread testing of new pharmacological and gene-editing therapies for the treatment of CF disease. Funded by CFF Therapeutics, Inc.

S2.3 Model systems to study CFTR dysfunction

Marc Chanson

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Our objectives are to develop and characterize three model systems to study the molecular consequences of CFTR dysfunction *in vitro* and *in vivo*. The first model is based on lentiviral transduction of human airway epithelial cells (HAECs) with CFTR targeting CRISPR-CAS9 complex. CFTR knocked-down-HAECs are polarized on Transwell filters to allowing study of basal and stimulated responses. The second model aims to identify the intracellular signaling pathways that regulate HAEC differentiation in non-CF and CF primary cultures. RNA-Seq has been performed on primary HAECs at different stages of repair after mechanical injury. The third model that we have developed is a CFTR knockout rat. Two rat strains (MUKORATs) have been generated by targeting exon2 of CFTR using CRISPR-CAS9 technology. Progress on these projects will be presented with emphasis on the phenotype of the MUKORATs.

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S2.4 Profiling of CFTR modulators GLPG1837 and GLPG2222 using intestinal organoids

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In order to improve the folding/maturation and functional properties of CFTR and thus address the main defects leading to cystic fibrosis (CF), we are developing different compound series. Each of these series contains either correctors that increase CFTR levels at the cell surface, or potentiators that allow the effective opening of the CFTR channel. Combined, these compounds are able to restore chloride ion transport yielding improved hydration of the lung surface and subsequent restoration of mucociliary clearance. We previously described the characterization of our potentiator compound GLPG1837 and our corrector compound GLPG2222. Here, the further profiling of the compounds using patient derived intestinal organoids is shown.

By measuring the forskolin-induced swelling (FIS) of CF intestinal organoids, a purely CFTR-driven process, the activity of GLPG2222 and GLPG1837 was determined. It was demonstrated that the corrective effect of GLPG2222 on F508del CFTR can be translated into an increase in FIS of patient derived organoids when combined with the potentiator GLPG1837. The ability of the potentiator GLPG1837 to open CFTR channels harbouring different mutations was evaluated by plotting concentration-response data obtained using organoid samples derived from donors with different genotypes. It was shown that GLPG1837 is more potent in restoring CFTR activity in S1251N/F508del organoids than in CFTR F508del/F508del organoids corrected with GLPG2222 and, especially, CFTR G551D/F508del organoids. The data obtained correlated well with results generated using YFP halide assays and, in the case of CFTR G551D/F508del and CFTR F508del/F508del, also with electrophysiology data obtained using patient derived primary bronchial epithelial cells.

In summary, we present the profiling of our CFTR modulators GLPG1837 and GLPG2222 using patient-derived organoids and the comparison with other assays.

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S2.5 Human periodontal ligament stem cells: a novel model for cystic fibrosis research and personalized medicine

Sara Patruno[1], Eleonora Cianci[1], Oriana Trubiani[1], Francesca Diomede[1], Laura Pierdomenico[1], Veronica C. Mari[1], Antonio Recchiuti[1], Giuseppina Bologna[1], Ilaria Merciaro[1], Matteo Serroni[1], Paolo Moretti[2], Marco Marchisio[1], Mario Romano[1]

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Primary cells from cystic fibrosis (CF) patients are paramount to study CF pathogenesis and develop personalized therapeutics. Airway epithelial cells isolated from explanted lungs or nasal brushing, intestinal organoids and peripheral blood leukocytes, are largely used for these purposes. In alternative, somatic cells from CF patients can be reprogrammed to obtain inducible pluripotent stem cells (iPSC) that can be differentiated into airway epithelial cells. These models, however, suffer of some limitations related to complex isolation procedures, limited lifespan and availability over time. We developed an additional cellular model for CF studies based on the observation that human periodontal ligament stem cells (hPDLSC) express a biologically active CFTR. hPDLSC are adult mesenchymal stem cells, easy to isolate and to propagate in vitro with excellent yield and maintenance of the original phenotype for a large number of passages. We tested the suitability of these cells for in vitro prediction of clinical response to CFTR potentiators/correctors. To this end, we measured CFTR activity in hPDLSC collected by gingival curettage performed during dental hygiene from 6 CF patients with varying genotypes, before and after exposure in vitro to VX-770/VX-809 (Orkambi) for 72 h. We observed a variable degree of increment in CFTR activity, from no variation to a 30-50% increase. We tested the reproducibility of these measurements after several passages and after storage in liquid nitrogen. No significant changes were recorded, indicating that hPDLSC represent a very stable and reliable model for in vitro drug testing and discovery. Two of the donor patients, both homozygous for the ΔF508 genotype, were enrolled in a clinical trial with Orkambi and CFTR activity was also measured in peripheral blood monocytes before and after 3 month therapy. Remarkably, monocytes from the patient whose hPDLSC did not respond to Orkambi also showed no response, whereas the other patient showed a comparable increase in CFTR activity in hPDLSC and monocytes.

We previously documented that hPDLSC can undergo osteogenic and adipogenic differentiation (Cianci et al., Stem Cell Transl. Med., 2016). Therefore, we asked whether these cells could serve as a model to investigate on the bone and adipose tissue defects, frequently observed in CF patients. For this purpose, we compared the osteogenic and adipogenic differentiation pattern of hPDLSC from 4 CF patients (3 ΔF508/ΔF508 and 1 ΔF508/R1070Q) and 4 healthy age- and sex-matched donors. CF cells manifested severely impaired adipogenic (P = 00002) and significantly delayed osteogenic (P = 0.018) differentiation.

In summary, the advantages of hPDLSC for CF studies are: 1. Easy collection by minimally invasive procedures; 2. No need for reprogramming; 3. No need for differentiation into epithelial cells for drug screening; 4. Almost 100% success with the explants; 5. Excellent proliferation rate; 6. Long term storage and availability, ideal to make a repository for drug testing on rare mutations as well as for HT screening for drug discovery; 7. Valuable tools to investigate on osteogenesis and adipogenesis in CF.

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S2.6 Evaluation of the primary human nasal epithelial cell cultures as a biomarker in personalized cystic fibrosis treatment

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Clinical studies with CFTR modulators have demonstrated that functional restoration of the mutated CFTR can be translated into benefit for patients. However, high variability of patients' responses highlights the importance of finding relevant biomarkers to predict the clinical responses. Primary human nasal epithelial (HNE) cells are easy to collect by nasal brushing and allow quantification of cAMP-mediated chloride transport as an indicator of CFTR function.

CFTR activity in vitro was assessed in primary HNE cultures, in comparison to human bronchial epithelial cells, by short circuit-current (Isc) measurements. Variation of Isc after application of Forskolin/IBMx and CFTR potentiator VX-770 ($\Delta Isc_{Fsk/IBMx+VX-770}$ in $\mu A/cm^2$) was an index of the CFTR-dependent chloride secretion. CFTR function in vivo was assessed via nasal potential difference (NPD) measurements in patients. CFTR expression in the apical surface of reconstituted epithelia was assessed by CFTR immunostaining and semi-quantification of the apical fluorescence.

Isc measurements in HNE cultures recapitulated the results obtained in HBE cultures from CF patients and healthy controls, and were accurate enough to distinguish different levels of CFTR function. CFTR activity in HNE cultures from F508del homozygous patients ($\Delta Isc_{Fsk/IBMx+VX-770}$=0.2 (0.1)) was significantly lower than that found in patients with genotypes associated with a residual CFTR function or a wide spectrum genotype ($\Delta Isc_{Fsk/IBMx+VX-770}$=8.8 (7)). In cultures from F508del healthy carriers, CFTR activity was significantly lower than that of healthy donors ($\Delta Isc_{Fsk/IBMx+VX-770}$= 5.1 (1.4) versus 12.6 (2.6); p=0.03). These results were correlated to apical CFTR expression (R^2=0.9, p< 0.0001) and in vivo CFTR activity as measured by nasal potential difference (R^2=0.82, p< 0.0001).

We then evaluated the correction of CFTR function by VX-809 in HNE cultures issued from patients homozygous for the F508del mutation or carrying CFTR genotypes displaying a wide spectrum of CFTR activity. VX-809 treatment of F508del homozygous cells significantly increased the average $\Delta Isc_{Fsk/IBMx+VX-770}$ as compared to DMSO by a mean of 1.8 $\mu A/cm^2$ (0.6-3.1), reaching an average of 23.6% (1-69%) of the WT-CFTR level (p< 10^{-6}). This change was correlated to an increase in the CFTR apical expression (R^2=0.6, p< 0.001). Finally, we assessed the predictive value of the primary HNE cell cultures by comparing the in vitro pharmacological rescue by CFTR modulators to the clinical efficacy of this treatment in patients. In vitro correction levels were significantly correlated to respiratory function improvement in 8 F508del homozygous patients who had initiated the combination of VX-809 and VX-770 (Orkambi(r)) treatment. Patients whose FEV1 improved by more than 5% displayed a mean change of Fsk/IBMx+VX-770 response upon 10% of the average WT level.

We provide the first evidence that correction of CFTR function and expression in HNE cell cultures can reliably predict respiratory improvement in patients to be treated with CFTR modulators. Therefore, it may be used as a surrogate biomarker to preselect responder patients for personalized therapy.

30 March — 16:00–17:45

Symposium 03: CFTR Structure

S3.1 Calmodulin binding to CFTR R region and PKA-independent activation of CFTR

Zoltan Bozoky[1], Saumel Ahmadi [1], Tal Milman[1], Tae Hun Kim[1], Kai Du [1], Jacob Keller[2], Christine E. Bear[1,3,4], Julie D. Forman-Kay[1,3]

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Cystic fibrosis results from mutations and loss of function in the CFTR chloride channel. CF patients also experience perturbations in homeostasis of other ions, including increased calcium signalling linked to inflammation. The intrinsically disordered regulatory R region of CFTR is a protein interaction hub, with both intra- and inter-molecular binding partners. We have found that calmodulin interacts with the R region, with binding dependent on calcium loading of calmodulin and inhibited by phosphorylation of the R region by PKA. There is also competition between calmodulin binding and PKA phosphorylation. In cells, we observe PKA-independent activation of CFTR by calcium-calmodulin, with differential effects for wild type CFTR and the F508del mutant. Three R region elements can bind to each of the two calmodulin lobes, enabling multivalent interactions that could synergize with calmodulin-binding sites of other ion channels and transporters to facilitate CFTR clustering. These results suggest a mechanism for CFTR's role in regulating cellular ion homeostasis.

Reference: Bozoky et al, Synergy of cAMP and calcium signaling pathways in CFTR regulation, PNAS, in press (2017).

S3.2 3D structures of the full length CFTR protein: combining theoretical and experimental data.

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In 2016, after many years of efforts, experimental 3D structures of the full-length CFTR have been obtained using cryo-electron microscopy. On the one hand, the 3D structure of the zebrafish and human CFTR proteins were solved in a quiescent form (without ATP and without phosphorylation), revealing an "inward-facing" conformation where the nucleotide binding domains (NBDs) are dissociated (1). On the other hand, the 3D structures of human G551D CFTR were also obtained in the absence and presence of ATP, showing a closed dimeric configuration of the NBDs (2).

This novel, experimental information now complements the structural view that has been obtained using molecular models, which have been constructed on the basis of the experimental 3D structures from distant ABC exporters. These models have anticipated some striking features of the channel, among which, for one of them (3), the presence of cytosolic lateral accesses in the open form, that were also supported by experimental data (4). Moreover, the structural superimposition of the membrane-spanning domains (MSDs) using rigid blocks (4 x 3 helices) led to a quasi perfect match between our human CFTR model and the recent experimental 3D structures of the full length protein in a quiescent form, supporting afterwards the accuracy of the model even in these regions, which are particularly difficult to handle due to the low sequence identity existing between CFTR and the ABC exporter templates. Interestingly, the transition between the apo conformer and the open form appears to be allowed by the movement of only two large rigid blocks of the MSDs relatively to each other.

Outside the well-folded MSDs and NBDs are sequences that are generally designated as intrinsically disordered, corresponding to the regulatory domain (R domain), as well as the N- and C-terminal extensions. In the experimental 3D structures of the full length CFTR in a quiescent form (1), the N-terminal region forms a "lasso"-shaped domain, whereas the R region revealed a weak density, lying between the nucleotide-binding domains and preventing their association. Considering here our new model of the open form of the CFTR protein, now including the N- and C-ter extensions, as well as the R domain, we propose a structural view of the dynamic behavior of these regions, accommodating the different states of the channel.

This work is supported by the French Association Vaincre La Mucoviscidose.

(1) Zhang Z, Chen J. Atomic structure of the cystic fibrosis Transmembrane Conductance Regulator. Cell (2016) 167:1586-97.

(2) Ford RC, Collins RF, Meng X. G551D CFTR 3D structure determined by cryo-electron microscopy. NACFC 2016 (Orlando).

(3) Mornon JP, Hoffmann B*, Lehn P, Callebaut I. Full-open and closed CFTR channels, with lateral tunnels from the cytoplasm and an alternative position of the F508 region, as revealed by molecular dynamics. Cell Mol Life Sci. 2015 72:1377-403.*

(4) ElHiani Y, Linsdell P. Functional Architecture of the Cytoplasmic Entrance to the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore. J Biol Chem 290 :15855-65 ; El Hiani Y, Negoda A, Linsdell P. Cytoplasmic pathway followed by chloride ions to enter the CFTR channel pore. Cell Mol Life Sci. 2016 73:1917-25.

S3.3 The molecular structure of CFTR revealed by cryo-EM.

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel evolved from the ATP-binding cassette (ABC) transporter family. We determined the structures of human and zebrafish CFTR, both in the absence of ATP, by electron cryo-microscopy. Comparison of the two CFTR structures establishes the architecture of the ion pore, illuminates the functions of many positively charged residues, and identifies the structural bases for several aspects of CFTR's channel activity. The structures also reveal why many cystic fibrosis-causing mutations would lead to defects either in folding, ion conduction, or gating and suggests new avenues for therapeutic intervention.

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S3.4 The processing defect of ΔF508- and ΔY512-CFTR are generated by similar mechanisms with different initial flaws

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Mutations on the cystic fibrosis transmembrane conductance regulator (CFTR) may impair its physiological function leading to a genetic disease cystic fibrosis (CF). The most common CF mutation ΔF508, missing a phenylalanine at position 508, markedly disrupts protein processing of CFTR from ER to Golgi apparatus. Whether ΔF508-CFTR processing defect is associated with dysfunction of neighbor residues is unclear. To test this hypothesis, serial single-residue deletions from position 503 to 513 were constructed into human CFTR plasmids for transient expression in HeLa cells. The immunoblotting data demonstrate that fully glycosylated CFTR were abundantly present in the cells expressing wild-type, ΔV510- and ΔS511-CFTR, whereas other deletion mutants including ΔF508-CFTR exhibited mostly less glycosylated form of the proteins, indicating defective protein processing. Among all deletion mutants, only ΔF508- and ΔY512-CFTR showed increases in expression of fully glycosylated proteins by low-temperature culture, CFTR corrector C18 or combination of two treatments. These data suggest that the ΔF508 and ΔY512 mutations may diminish CFTR processing by similar mechanisms. The experiments with alanine substitutions on the loop between the F508 and Y512 residues demonstrate that fully glycosylated ΔF508-CFTR was not significantly altered by introduced G509A mutation, but enhanced by the V510A or V510G mutation and further markedly elevated by the double mutation G509A/V510A. Conversely, introduced mutations V510A, S511A or V510A/ S511A did not promote expression of fully glycosylated ΔY512-CFTR. Our data suggest that loop dysfunction is important for causing ΔF508-CFTR processing defect. These data also suggest that ΔF508- and ΔY512-CFTR may share multiple abnormalities leading to the processing defect, but these abnormalities could be originated from different local flaws.

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S3.5 Molecular dynamics flexible fitting (MDFF) simulations identify new models of closed state CFTR

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The search for CF therapies will be greatly advanced by high resolution structures of CFTR. Recently, a 3.7Å resolution structure for a CFTR ortholog (ABCC7, *Danio rerio* (zebrafish)) was published in *Cell* (PDB code 5TSI) and the coordinates of three other CFTR structures were deposited in the PDB (5UAR: zebrafish CFTR at 3.7Å resolution; 5UAK and 5U71: human CFTR structures both at 3.9Å resolution). All these structures share high structural similarity and all correspond to one conformational state (closed, inward-facing) of the channel. On the other hand, lower resolution structural data (at 9Å maximal resolution) are available for a different state of CFTR (outward-facing) that has been associated with the activated form of the channel. Multiple homology models of CFTR corresponding to the active state have been developed, yet their structures are different. Thus, there is a need to validate and refine these models against additional experimental data. In this work we refine two previously described, outward-facing CFTR models (Mornon et al., *Cellular and Molecular Life Sciences***2015,***72* (7), 1377-1403; Dalton et al., *Journal of chemical information and modeling***2012,***52* (7), 1842-53) using the available cryo-EM map of the *human* wild-type protein, by means of molecular dynamics flexible fitting (MDFF) simulations (a total of 120 ns per model). Despite different starting points, the final models share many common features. Analyzing the resulting MD trajectories (and not just the final models as is usually the case), we demonstrate that the refined models have good stereochemical properties and are in favorable agreement with multiple experimental data (e.g., cysteine cross linking data, salt bridge data, probe accessibility data). Moreover these new models correspond to a closed state of the channel with no membrane traversing pore yet with the two NBDs coupled in a dimeric conformation. Accordingly, analyzing the MDFF trajectories reveals open probabilities of the channel which are significantly lower than those obtained from control MD simulations performed without the EM map constraints. We propose that these models correspond to a CFTR conformation which to date was largely unexplored yet one that is supported by experimental data and forms part of the channel´s gating cycle. Finally we suggest that the combination of high resolution cryo-EM maps which are currently emerging from multiple labs and MDFF simulations will be of value for the development of yet more reliable CFTR models as well as for the identification of binding sites for CFTR modulators.

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S3.6 Biosynthetic and functional correction of CF-causing NBD2 defects

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While the majority of cystic fibrosis patients have at least one copy of the F508del allele, a significant number of CF patients are compound heterozygotes and/or harbor two non-F508del alleles that affect protein folding, trafficking and function. Drawing on CFTR and ABC-transporter mutation databases, we have identified and profiled a library of mutations in the cytosolic and transmembrane domains of CFTR for their effects on CFTR protein folding and structure. Previous studies have demonstrated that these mutations provide additional insight into the folding pathways of ABC-transporters, including CFTR. Specifically, these analyses provide evidence for hierarchical folding and assembly and suggest specific biophysical mechanisms by which these processes are disrupted. Importantly, these studies also suggest potential mechanisms of correction for CFTR variants that are not adequately addressed by clinically available therapeutics. Building off these observations, we have explored the biosynthetic and functional correction of severe NBD2 mutants, including the N1303K allele. These studies show that stabilization of the NBD-NBD interaction, mediated by specific second-site suppressors in the ATPase active sites, provides biosynthetic and functional correction for multiple mutations in NBD2. Protein trafficking and function are restored when evaluating cell surface localization biochemically and by electrophysiological studies. In addition, this mechanism of correction is independent of those suggested for clinically available therapeutics and suggests that combination therapies, including those currently available, could augment this mechanism to increase correction and potentiation of these CFTR variants. To complement these biophysical and cell biological studies, we have leveraged the recently solved structures of CFTR to evaluate physical insights for rare mutations and their impacts on CFTR. The available structures provide novel insight into mutational clustering within CFTR, suggesting key regions of the protein that should be evaluated in more detail. Together, these studies provide insight into many of the understudied mutations in CFTR and suggest new approaches for small molecule screening and development.

30 March — 18:00–19:15

ECFS Basic Science Working Group Session

Task Force on Personalised Medicine for CF

Special discussion groups:

1. Validation/ optimization of novel biomarkers

Discussion leader: Kris de Boeck

2. Assays for improved endpoints to evaluate novel drugs: intestinal and respiratory organoids

Discussion leader: Jeff Beekman

3. Novel therapeutic approaches to CF therapies: alternative channels and gene/cell therapies

Discussion leader: Marcus Mall

31 March — 08:45–10:30

Symposium 04: Translational CF Research

S4.1 Intestinal organoids for personalized medicine

J.M. Beekman

Pediatric Pulmonology, Department of Pediatrics, Wilhelmina Children's hospital and Regenerative Medicine Center Utrecht, University Medical Center Utrecht.

We explore the utility of in vitro culture approaches to stratify subjects with CF for disease severity or drug sensitivity. One such approach encompasses the use of forskolin-induced swelling in intestinal organoids as individual biomarker of CFTR function or response to therapy. An overview of ongoing projects aiming to quantitate relations between in vitro observation and in vivo disease markers or responses to therapy is provided, which support the use of organoids for CF drug development and patient stratification. In addition, data will be presented of new assay platforms including 384 wells screening technology and the use of 3D organoids to establish 2D filters for electrophysiological experiments. This expanding toolbox of organoid-based functional assays will help to more quickly stratify people with CF for therapy, and further study relations between fluid secretory phenotypes and electrophysiological properties of intestinal epithelium

S4.2 Non-viral CRISPR/Cas gene editing enabled by co-delivery of mRNA and sgRNA inside of synthetic lipid nanoparticles

Daniel J. Siegwart

University of Texas Southwestern Medical Center, Simmons Comprehensive Cancer Center, Department of Biochemistry, Dallas, Texas, United States

S4.3 Genetic and Functional evidence for SLC26A9 as a CF modifier

Lisa Strug

The Hospital for Sick Children and The University of Toronto

Cystic fibrosis (CF) is realizing the promise of personalized medicine. Recent advances in drug development that target the causal CFTR directly result in lung function improvement, but variability in response is demanding better prediction of outcomes to improve management decisions. CF gene modifiers may contribute to useful tools for predicting patient-specific therapeutic response. One proof-of-concept is the genetic modifier SLC26A9. The SLC26A9 modifier locus contributes to disease severity in the CF pancreas and intestine at birth, yet no association of SLC26A9 was seen with lung function in CF patients, even with stratification by age or in a recent meta-analysis of the largest CF population studied for genetic modification to date. This finding is inconsistent with observations that SLC26A9 is expressed in the human and mouse adult lung.

Here we present evidence for the role of SLC26A9 as a modifier in the CF pancreas, intestine and lung using data from the International CF Gene Modifier consortium. We (1) assess the evidence for whether associated genetic variation influences SLC26A9 gene expression, and in which CF-relevant tissues, and (2) determine the relationship of SLC26A9 with disease severity and therapeutic response in the CF lungs.

Genome-wide association analysis (GWAS) of intestinal obstruction in CF from the international CF Gene Modifier consortium indicates additional support for association with variants 5' of SLC26A9. We apply novel statistical genetics methodology that integrates GWAS evidence and public gene expression data from the Genotype-Tissue Expression project. Results indicate that variants associated with intestinal obstruction at the SLC26A9 locus are enriched with variants that also display evidence of expression quantitative trait loci for SLC26A9 in the pancreas.

SLC26A9 association with lung disease was assessed in individuals from the Canadian and French CF Gene Modifier consortia with CFTR-gating mutations and in those homozygous for the common Phe508del mutation. Variability in response to a CFTR-directed therapy attributed to SLC26A9 genotype was assessed in Canadian patients with gating mutations. A primary airway model system determined if SLC26A9 shows modification of Phe508del CFTR function upon treatment with a CFTR corrector.

In those with gating mutations that retain cell-surface localized CFTR we show that SLC26A9 modifies lung function while this is not the case in individuals homozygous for Phe508del where cell surface expression is lacking. Treatment response to ivacaftor, which aims to improve CFTR-channel opening probability in patients with gating mutations, shows substantial variability in response, 28% of which can be explained by rs7512462 in SLC26A9 ($p=0.0006$). When homozygous Phe508del primary bronchial cells are treated to restore surface CFTR, SLC26A9 likewise modifies treatment response ($p=0.02$).

Our latest findings provide additional support for the SLC26A9 locus as a modifier in CF, and indicate that the influence of SLC26A9 on intestinal obstruction may be through variation in expression in the pancreas. SLC26A9 airway modification findings suggest CFTR is required at the cell surface, and that modifiers such as common variants at the SLC26A9 locus may predict response to CFTR-directed therapeutics.

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S4.4 Transduction of Rhesus macaque lung by AAV1

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[1]Johns Hopkins U, Baltimore, United States, [2]U of Iowa, Iowa City, United States

The major hurdle with gene therapy is expression of enough CFTR protein to be therapeutic. In order to address this, we utilized a dual reporter assay based upon firefly (FL) and renilla (RL) luciferase cloned into AAV1 & 5 vectors. Two male and two female healthy Rhesus monkeys were exposed by trans-oral Penn Century microsprayer delivery to an aerosol containing both AAV1-CB-FL and AAV5-CB-RL. CB refers to the chicken β-actin promoter. The studies were sponsored by NHLBI Gene Therapy Resource Program (GTRP) and conducted at the Lovelace Respiratory Research Institute. The vector combination was formulated on the day of dosing by combining, as provided by the U. of Mass, Vector Core, 13.5 ml of the AAV5-CB-RL (1.2×10^{13} gc/ml) with 16.2 ml of the AAV1-CB-FL (1.0×10^{13} gc/ml), to provide a mixture with 0.54 gc/ml of each vector. Animals were observed for clinical signs of toxicity, and body weight. The animals were euthanized at 45 days post-exposure. Seventeen lung samples were collected for analysis of luciferase reporter gene expression (RL versus FL) at the U of Iowa and for vector expression by PCR (U of Florida). Importantly, the only significant clinical observation was a foot pad laceration, which was not a test article-related finding. All animals gained weight, as expected, during the study. These results show again that AAV1 vector delivery is safe. The vector genomes measured in each lung region at necropsy showed that the vector was widely distributed throughout the lung by the microsprayer demonstrating conclusively that our droplet size is sufficient to support widespread distribution. Average PCR data for 17 lung samples from four monkeys show clearly that there is a dramatic difference between the ability of AAV1 and 5 to infect the lung with AAV1 being approximately 10-fold more effective than AAV5. Despite the small number of monkeys, the data also clearly show better luciferase transduction with AAV 1 than with AAV5. The levels of neutralizing antibody (measured at U. Penn) increased dramatically in all animals between the pre-study time point (monkeys chosen for the study had undetectable titers) and the time of necropsy. Considerably higher titers were observed for AAV5 than for AAV1. These results provide justification for our choice of AAV1 for lung delivery given that it had a greater infection efficiency and transduction and lesser propensity to induce neutralizing antibodies than did AAV5. Funded by CFF and NHLBI.

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S4.5 Nanoparticle-mediated delivery of modified tRNAs as a therapeutic approach for CF caused by a nonsense mutation.

Michael J. Torres[1], Jason B. Miller[2], Jennifer R. Peters-Hall[3], Linda Millen[4], Linde De Keyzer[4,5], Zhiwei Cai[6], Qin Li[7], Jamie Wangen[8], Jan Harrington[9], Amita Patel[10], Carla Oliveira[11], Andrew P. Feranchak[7], David N. Sheppard[6], Scott H. Randell[12], Rachel Green[8], Hermann Bihler[9], Martin Mense[9], Robert J. Bridges[10], Jerry W. Shay[3], Arthur E. Johnson[1], Philip J. Thomas[4], Daniel J. Siegwart[2]

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Nonsense mutations represent nearly 10% of cystic fibrosis (CF)-causing alleles, including the opal codons G542X, W1282X, R553X, and R1162X which are the top four nonsense mutations and second, sixth, seventh, and twelfth most common disease mutations overall in CF. These mutations result in the premature termination of translation of the cystic fibrosis transmembrane conductance regulator (CFTR) and attendant degradation of CFTR mRNA. Current strategies in development include suppression of the nonsense-mediated mRNA decay (NMD) pathway and/or induction of readthrough of the premature termination codon (PTC) by altering the translation machinery to allow misincorporation of an amino acid at the site of the nonsense codon.

Taking advantage of a tRNA's ability to direct specific amino acids to the growing nascent chain, we modified an arginine tRNA to recognize an opal termination codon (Arg/Op) while maintaining aminoacylation activity. The Arg tRNA was selected as it would be predicted to act on both the R553X and R1162X alleles to restore wild type protein and because site-directed mutagenesis indicates that the G542R substitution maintains significant maturation and function in heterologous expression systems. Thus, a single suppressor tRNA might have utility for three of the four most common CF-causing nonsense mutations. Delivery of this modified tRNA to affected cells was accomplished with a unique non-viral nanoparticle formulation optimized for delivery of long nucleic acids. The efficacy of the approach was assessed by measuring CFTR dependent chloride secretion (Ieq) using differentiated primary G542X/G542X and F508del/G542X CFTR HBEs. Nanoparticle delivery of the modified Arg/Op suppressor tRNA restored significant CFTR function on both genotypes. Interestingly, treatment of heterozygotes with both the Arg/Op tRNA nanoparticles and VX809 resulted in strong synergy. This suggests that the G542R CFTR protein is a partial maturation mutant which can be restored with VX809, consistent with results in the heterologous expression system. Toxicity of the tRNA-loaded nanoparticles was assessed in mice. No overt toxicity as measured by body weight was observed. Furthermore, biodistribution studies suggest significant lung accumulation of the Arg/Op tRNA.

These data suggest that nanoparticle delivery of modified tRNA can improve CFTR function in primary HBEs and may offer an alternative therapeutic approach for the treatment of CF caused by nonsense mutations. Moreover, a single tRNA may be employed to treat multiple disease-causing alleles.

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S4.6 Unravelling the mechanisms of airway epithelium repair in cystic fibrosis

Aderonke Sofoluwe, Joanna Bou Saab, Marc Bacchetta, Marc Chanson, Alice Zoso

University of Geneva, Department of Physiology and Metabolism, Geneva, Switzerland

Pathological remodelling 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 constitute the 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.

We performed RNA sequencing of bronchial AECs in primary cultures from 4 CF (F508del/F508del) and 3 non-CF (NCF) patients. Well-differentiated cultures at the air-liquid interface were mechanically wounded and mRNA was isolated at different time points of the repair process: before wound (BW, control), 24h post wound (24hPW, dominated by migration and proliferation of progenitor cells), wound closure (WC) and 48h post WC (48hPWC, initiation of differentiation). To simulate *Pseudomonas aeruginosa* infection, we treated both CF and NCF AECs with 1µg/ml flagellin for 24h BW and at WC. 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. Differentially expressed genes were analysed for Gene ontology (GO) terms using METACORE analysis database. At first glance, analysis revealed a divergent pattern of differentially expressed genes between CF and NCF cultures during the repair process. Comparison of 24hPW with BW showed in NCF that 409 genes (662 in CF) were upregulated and 30 genes (71 in CF) were downregulated. Interestingly, we observed a switch in gene expression between WC and 48hPWC; in NCF, there were 32 upregulated genes (11 in CF) and 320 downregulated genes (only 42 in CF). GO analysis between CF and NCF cultures at each time point confirmed an imbalanced repair process. We observed that the most represented pathways include "cytoskeletal remodelling" and "cell cycle" categories. At 24hPW, analysis showed that the differently expressed genes belong to the "cell locomotion" and "cell migration" categories. Specifically, the highlighted pathways were "signal transduction", "cell adhesion" and "extracellular matrix remodelling". At WC, there were major differences in "response to wounding", "development", "cell cycle" and "cell migration" categories. At 48hPWC, the key terms identified were "development", "differentiation" and "cell migration". Upon flagellin stimulation, comparisons between CF and NCF samples BW showed pathways related to "cytoskeletal remodelling", "cell adhesion" and "development". At WC, differences in "cell adhesion", "signal transduction" and "innate and adaptive immune response" were observed.

Preliminary transcriptomic analysis of wound repair in CF AECs reveals an impairment in the regulation of cell migration and proliferation during injury, and in the initiation of differentiation. METACORE analysis indicates a delay in cell cycle exit and abnormal actin cytoskeleton remodelling. Flagellin treatment also confirms the abnormal immune response of CF AECs. Although confirmation at the protein level is required, this analysis provides a holistic view of the repair process in an *in vitro* human model of the CF airway epithelium.

31 March — 11:00–12:45

Symposium 05: Mucus and Mucins

S5.1 Synergistic stimulation of gland secretion and mucus clearance

Jeffrey J. Wine, Nam Soo Joo

Cystic Fibrosis Research Laboratory, Stanford University, Stanford, CA 94305

Normal airways maintain a sterile environment via a multi-pronged innate defence system that includes multiple antimicrobials, mucociliary clearance, macrophages and neutrophils. Loss of CFTR function compromises these defences, and with present standard of care virtually all people with CF eventually develop chronic infections. We hypothesize that if CF lungs are kept clear of chronic bacterial infections and mucus accumulation, nearly normal structural integrity and function can be maintained. Although sterile mucus plugging occurs very early in some small airways, evidence suggests this need not be progressive; but bacterial infections, which typically start in the nasal cavities and upper airways, are relentlessly progressive unless prevented/eradicated with exogenous antimicrobials and interventions to enhance mucociliary clearance.

As the field progresses toward effective and universal restoration of CFTR function, a niche remains for strategies that can augment impaired CF airway defences. The development of effective strategies can proceed efficiently to the extent that we understand the panoply of airway defence defects that result from lost CFTR function. We have had a long term interest in defective submucosal gland function. More recently, we have been studying mucociliary clearance (MCC), focusing on MCC velocity (MCCV) as a readout for efficient clearance.

While studying the control of MCCV in *ex vivo* ferret tracheas, we discovered that stimulation with combined agonists that increased both $[Ca^{2+}]_i$ and $[cAMP]_i$, which we had previously shown to produce synergistic increases in gland secretion, also produce a remarkably robust increase in MCCV. The increase, which can be produced with 0.3 mM carbachol + 10 mM forskolin, produces what appears to be a maximal level of MCCV (~40 mm/min) that is more the 2X the expected additive effects of the agonists used separately. We have termed this a 'synergy paradigm'.

In addition to dramatically increasing MCCV, the synergy paradigm has another beneficial effect: it eliminates the bronchoconstriction that usually occurs with cholinergic agonists.

At least four components contribute MCC in the upper airways: 1) secretion by submucosal glands; 2) secretion by surface epithelia; 3) ciliary beat frequency (CBF) and 4) absorption by surface epithelia. Increases of the first 3 and decreases in absorption are predicted to increase MCCV. So far we have evidence that the synergy paradigm increases gland and surface secretion and decreases surface absorption. We are in the process of measuring CBF and predict it will also be increased; if so, it suggests that the synergy paradigm produces a coordinated response in all 4 components that contribute to MCCV. Three lines of work are ongoing. One seeks to determine the role that CFTR plays in synergistic MCCV increases, another seeks details about the composition and properties of mucus secreted during synergy, and a third seeks to understand mechanisms for inhibition of absorption. If some degree of synergy persists in CF airways, this paradigm could have therapeutic potential. We are encouraged in this hope because, at least for glandular mucus secretion, two kinds of synergy operate, and the one with parameters similar to those used in our ferret experiments persists in $CFTR^{-/-}$ piglets.

S5.2 Mucus Clearance in Normal Airways and Conversion into Attached Mucus in Chronic Lung Disease

Gunnar C.Hansson[1], Joan Antoni Fernández-Blanco[1], Liisa Arike[1], Lauren N. Meiss[1], Harriet E. Nilsson[1], Dahlia Fakih[2], Andrea Bähr[2], Sergio Trillo Muyo[2], Ana M. Rodriguez-Pineiro[2], Jeffrey J. Wine[2], David J.Thornton[3], Philip J. Koeck[1], Hans Hebert[1], Nikolai Klymiuk[2], Anna Ermund[1]

[1]Department of Medical Biochemistry, University of Gothenburg, SE-405 30 Gothenburg, Sweden; Department of Biosciences and Nutrition, Karolinska Institutet, and School of Technology and Health, KTH Royal Institute of Technology, Novum, SE-141 57 Huddinge, Sweden, [2]Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-University Munich, Feodor-Lynen-Straße 25, 81377 Munich, Germany, [3]Wellcome Trust Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

Human and pig have in contrast to mice a high number of tracheobronchial submucosal glands producing the MUC5B mucin. How this mucin is packed in the submucosal gland mucus cells due to low pH and high Ca2+ has been studied by low resolution single particle electron microscopy. Upon secretion MUC5B is pulled out by the serous cell fluid into long linear polymers that form Alcian blue stained mucus strands appearing from the submucosal gland openings. In contrast, without glands as in mice the mucus appears as clouds. The mucus strands are transported cephalically and ventrally by the cilia in wild type (WT) piglet trachea, but are essentially unmovable in pigs lacking a functional CFTR channel (CF piglets) or in WT tracheas analyzed in buffer lacking bicarbonate. The mucus strands had a core of the MUC5B mucin, but were also coated with MUC5AC mucin from the goblet cells in the gland ducts and on the tracheal surface. The MUC5AC mucin is suggested to trap the CF mucus strands.

High amounts of mucus accumulated are key features of cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). This is associated with goblet cell metaplasia and mucin overexpression. Such alterations can also be induced by neutrophil elastase, the activity of which is increased in both diseases. Using a mouse model we have now induces mucus-mediated airway obstruction. The mucus plugs contain high levels of Clca1 and Fcgbp and by this resemble the physiologic situation in colon where mucus is separating bacteria from the epithelium. The mucus accumulated contained increased levels of the MUC5AC mucin and was well attached to epithelial cells not allowing its removal. Old and new therapeutic agents helped to remove the attached mucus.

S5.3 Optical coherence microscopy (OCM) for assessment of mucus transport

Peter König

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Cystic fibrosis (CF) is realizing the promise of personalized medicine. Recent advances in drug development that target the causal CFTR directly result in lung function improvement, but variability in response is demanding better prediction of outcomes to improve management decisions. CF gene modifiers may contribute to useful tools for predicting patient-specific therapeutic response. One proof-of-concept is the genetic modifier SLC26A9. The SLC26A9 modifier locus contributes to disease severity in the CF pancreas and intestine at birth, yet no association of SLC26A9 was seen with lung function in CF patients, even with stratification by age or in a recent meta-analysis of the largest CF population studied for genetic modification to date. This finding is inconsistent with observations that SLC26A9 is expressed in the human and mouse adult lung.

Here we present evidence for the role of SLC26A9 as a modifier in the CF pancreas, intestine and lung using data from the International CF Gene Modifier consortium. We (1) assess the evidence for whether associated genetic variation influences SLC26A9 gene expression, and in which CF-relevant tissues, and (2) determine the relationship of SLC26A9 with disease severity and therapeutic response in the CF lungs.

Genome-wide association analysis (GWAS) of intestinal obstruction in CF from the international CF Gene Modifier consortium indicates additional support for association with variants 5' of SLC26A9. We apply novel statistical genetics methodology that integrates GWAS evidence and public gene expression data from the Genotype-Tissue Expression project. Results indicate that variants associated with intestinal obstruction at the SLC26A9 locus are enriched with variants that also display evidence of expression quantitative trait loci for SLC26A9 in the pancreas.

SLC26A9 association with lung disease was assessed in individuals from the Canadian and French CF Gene Modifier consortia with CFTR-gating mutations and in those homozygous for the common Phe508del mutation. Variability in response to a CFTR-directed therapy attributed to SLC26A9 genotype was assessed in Canadian patients with gating mutations. A primary airway model system determined if SLC26A9 shows modification of Phe508del CFTR function upon treatment with a CFTR corrector.

In those with gating mutations that retain cell-surface localized CFTR we show that SLC26A9 modifies lung function while this is not the case in individuals homozygous for Phe508del where cell surface expression is lacking. Treatment response to ivacaftor, which aims to improve CFTR-channel opening probability in patients with gating mutations, shows substantial variability in response, 28% of which can be explained by rs7512462 in SLC26A9 ($p=0.0006$). When homozygous Phe508del primary bronchial cells are treated to restore surface CFTR, SLC26A9 likewise modifies treatment response ($p=0.02$).

Our latest findings provide additional support for the SLC26A9 locus as a modifier in CF, and indicate that the influence of SLC26A9 on intestinal obstruction may be through variation in expression in the pancreas. SLC26A9 airway modification findings suggest CFTR is required at the cell surface, and that modifiers such as common variants at the SLC26A9 locus may predict response to CFTR-directed therapeutics.

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S5.4 Relative roles of reduced mucus clearance and mucus hypersecretion in the pathogenesis of airway mucus plugging in mice

Sandra Christochowitz[1], Simone Schmidt[1], Jolanthe Schatterny[1], Matthias Hagner[1], Marcus A. Mall[1,2]

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Background: Reduced mucociliary clearance (MCC) and type 2 mediated airway inflammation triggering goblet cell metaplasia (GCM) and mucus hypersecretion are key features of chronic obstructive airways diseases including asthma, cystic fibrosis (CF) and COPD. However, the relative roles of increased mucin/mucus production vs. reduced mucus clearance in the pathogenesis of airway mucus obstruction characteristic of these diseases remain poorly understood.

Methods: To determine the relative roles of reduced MCC vs. type 2-mediated mucus hypersecretion, we generated i) βENaC-Tg mice lacking IL-13 (βENaC-Tg/IL-13$^{-/-}$) to study the impact of airway surface dehydration and impaired MCC in the absence of this key type 2 cytokine; ii) mice with lung-specific overexpression of IL-13 (IL-13-Tg/IL-13$^{-/-}$) to determine the impact of IL-13 mediated GCM and mucus hypersecretion alone; and iii) βENaC-Tg mice with lung-specific overexpression of IL-13 (βENaC-Tg/IL-13-Tg/IL-13$^{-/-}$) to study combined effects of reduced MCC and mucus hypersecretion.

Results: We found that reduced MCC due to airway surface dehydration is sufficient to induce chronic airway inflammation and airway mucus plugging in juvenile βENaC-Tg/IL-13$^{-/-}$ mice. However, expression of Muc5ac, Muc5b and GCM were reduced. In IL-13-Tg/IL-13$^{-/-}$ mice, eosinophilic inflammation, Muc5ac and Muc5b transcript levels and GCM were increased, but mucus plugging was reduced compared to βENaC-Tg/IL-13$^{-/-}$ mice. In neonatal βENaC-Tg/IL-13-Tg/IL-13$^{-/-}$ mice, Muc5ac and Muc5b transcript levels and GCM were elevated to similar levels compared to IL-13-Tg/IL-13$^{-/-}$ mice, but all mice died in the first week of life due to severe airway mucus plugging.

Conclusions: We demonstrate that reduced MCC is sufficient to produce mucus plugging in the absence of IL-13. However, IL-13 mediated GCM and elevated mucin expression aggravate mucus plugging leading to invariable death in neonatal βENaC-Tg mice with reduced MCC. These results indicate that impaired MCC and mucus hypersecretion act synergistically in the in vivo pathogenesis of airway mucus plugging.

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S5.5 CFTR and bicarbonate as determinants of airway mucus secretion and microrheology.

Pierre Lesimple[1], Maria Kilfoil[2], O'Grady M. Scott[3], Renaud Robert[4], Hanrahan W. John[4,5]

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Many tissues that are affected in CF have defects in HCO3- transport and mucus. It has been suggested that these abnormalities may be related, and evidence linking them has come from recent studies of isolated, perfused mouse intestine and cervix. Normal mouse tissues release more mucus than those from CF mice, but can be made to resemble CF tissues if exposed to HCO3-free medium or the anion transport inhibitor DIDS. Reduced mucus discharge during these manoeuvres may result from increased adhesion of secreted mucus to the epithelial surface due to alterations in its biophysical properties. Whether human airway epithelia show these differences in CF is not known, and the influence of the cellular HCO3- homeostasis on mucus biophysical properties has not been studied.

We examined the possible role of CFTR in mucus organization and rheology in airway epithelial mucous granules.

Using microrheology, spectrophotometry, immunostaining and western blotting, we compared the properties of secretions from non-CF vs CF human airway epithelial cells cultured in air / liquid conditions and examined CFTR-mediated transport in isolated mucin granules.

We found that CFTR is expressed in mucin-containing vesicles, where it is able to mediate anion currents towards the lumen. Secretions from CF cultures have elevated viscoelastic properties and are more adherent to the cell surface.

Taken together, our results suggest that deficient CFTR-mediated HCO3- secretion in the lumen of mucous granules could participate in the abnormal mucus properties in CF.

Aknowledgements : we thank the North American Cystic Fibrosis Foundation and Vaincre La Mucoviscidose for their financial support.

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S5.6 Identification of proteins associated with goblet cell hyperplasia

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Mucus accumulation in the airways is a feature of cystic fibrosis (CF), asthma and other respiratory diseases. Mucus hypersecretion and goblet cell hyperplasia are often driven by inflammatory stimuli, particularly the Th-2 cytokines IL-4 and IL-13. Interestingly, these cytokines are also modulators of ion transport in bronchial epithelia. Gene expression profiling on cultured human bronchial epithelial cells treated with IL-4 revealed a profound change in expression and function in multiple ion channels and transporters. IL-4 treatment markedly increased the expression of SLC26A4, TMEM16A, SLC12A2, ATP12A, CA2 and other genes involved in ion transport. Upregulation of these genes was confirmed at the protein level by immunofluorescence combined with confocal microscopy. Interestingly, some of the proteins induced by IL-4 (e.g. SLC12A2, ATP12A) were found in both ciliated and non-ciliated cells. Instead, other proteins (TMEM16A, CA2) were predominantly expressed in goblet cells. At the functional level, IL-4 treatment resulted in enhanced bicarbonate transport ability, as indicated by short-circuit current recordings and intracellular pH measurements. Furthermore, analysis of the composition of the apical fluid collected from cells treated with IL-4 revealed a marked accumulation of bicarbonate and, accordingly, a more alkaline pH compared to control cells. Importantly, we found that the enhanced bicarbonate transport in IL-4 treated epithelia has a relevant role in the mechanism of mucus release. Indeed, incubation of IL-4 treated cells in a bicarbonate-free basolateral solution strongly reduced mucus release in response to purinergic stimulation. A similar inhibition was also observed in CF cells, suggesting that CFTR activity is particularly important under goblet cell hyperplasia conditions. Among the proteins induced by IL-4, the role of ATP12A is particularly intriguingly. ATP12A is the non-gastric form of H^+/K^+-ATPase, responsible for proton secretion at the apical membrane of airway epithelial cells. Its upregulation in cells treated with IL-4 appears to be in contrast with the high bicarbonate levels and the alkaline pH measured in the apical fluid. However, we also found a low K^+ concentration in the apical fluid of cells treated with IL-4, a finding that could be due to K^+ reabsorption mediated by ATP12A. In conditions of reduced bicarbonate secretion, as in cystic fibrosis airways, ATP12A activity may causes abnormal acidification of airway surface liquid. Since CF is also characterized by goblet cell hyperplasia, we asked whether ATP12A is upregulated in CF conditions. We studied ATP12A expression in freshly excised bronchi, obtained at the time of lung transplant from CF and non CF patients. Immunofluorescence experiments carried on histological sections of non-CF bronchi revealed a very low expression of ATP12A. Instead, in CF samples, ATP12A was consistently detected on the surface epithelium, particularly on the apical membrane of non-ciliated cells and in submucosal glands. This finding, together with the observed upregulation of ATP12A under goblet cell hyperplasia conditions, suggests a link between ATP12A and mucus. Pharmacological inhibition ATP12-mediated proton pump could antagonize acidification in CF airways and help to normalize mucus properties and restore antimicrobial activity.

01 April — 08:45–10:30

Symposium 06: Cell Physiology and Ion Transport

S6.1 Studying asymmetric movements in CFTR's two nucleotide binding sites

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The CFTR chloride channel pore opens upon dimerization of its cytosolic nucleotide binding domains (NBD1 and 2), and closes upon disruption of this dimer following ATP hydrolysis. In the tight NBD dimer canonical motifs of both NBDs form two non-equivalent composite ATP binding sites. Composite site 2, formed by Walker motifs of NBD2 and the signature sequence motif of NBD1, is catalytically active, and hydrolyses ATP in each gating cycle. In contrast, composite site 1, formed by Walker motifs of NBD1 and the signature sequence motif of NBD2, is catalytically inactive, and keeps ATP bound throughout several gating cycles. Previous work employing the Rate-Equilibrium Free Energy Relationship (REFER) approach has shown that the pore opening conformational transition is initiated by tightening of the site-2 NBD interface, and that movements in this composite site are already completed in the opening transition state (Sorum et al., 2015, Cell 163:724-733). In contrast, little is known about the extent and precise timing of molecular motions in composite site 1, even though profound effects on channel gating kinetics of site-1 pertubations suggest gating-associated motions also in this site. Analysis of energetic profiles of channels gating in the absence and presence of ATP indicate that ATP bound at the dimer interface stabilizes the open state relative to the opening transition state, suggesting that this interface undergoes rearrangements between the transition state and the open state (Mihályi et al., 2016, Elife, 5. pii:e18164). Insofar as motions at the site-2 interface are likely completed in the transition state, one possible explanation is that these further rearrangements occur at the site-1 interface. Using the non-hydrolytic CFTR background construct cut-DR(D1370N), here we employ the REFER approach to compare relative timing of motions in sites 1 and 2 during channel opening. Relative timing of opening-associated motions in a particular protein position is reported by the thermodynamic parameter F, obtained from a Brønsted plot of the respective position. The F values of positions 1246 (site 2), 275 (coupling helix 2), and 348 (pore) have been published in the past. To obtain a more detailed map of F values, we are currently studying positions on both faces of both composite ATP binding sites, positions in the four coupling helices that link NBD conformational changes to movements in the pore, as well as further positions in the pore. Support: HHMI, CFF, MTA-Momentum Program

S6.2 Functional Integrity of CFTR in the Plasma Membrane: Role of ATP Binding in Site 1

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Introduction: CFTR is a phosphorylation-activated but ATP-gated anion channel. For CFTR to function in the cell membrane, its regulatory (R) domain has to be phosphorylated by protein kinas A (PKA); then opening of phosphorylated CFTR is controlled by ATP binding-induced dimerization of its two nucleotide-binding domains (NBD1 and NBD2) in a head-to-tail configuration; closing of the channel is facilitated by ATP hydrolysis at the catalysis-competent site (or site 2) and subsequent partial separation of the NBD dimer. Biochemical studies have demonstrated a fast turnover of ATP at site 2 due to ATP hydrolysis, but a tight binding of ATP in site 1.

Results: In numerous previous reports, we noted that wild-type CFTR currents in excised inside-out patches are not stable; i.e., the current decreases over time (aka "rundown"). Moreover, in many real-time recordings, the current rise upon sudden addition of ATP follows a time course that is inconsistent with microscopic kinetic parameters. Here we report two types of rundown that cannot be accounted for by dephosphorylation of CFTR since similar observations were made in ΔR-CFTR whose function does not depend on phosphorylation. First, reversible rundown: At a macroscopic level, addition of ATP after a brief washout of ATP increases the current monotonically, but causes a biphasic ($t1 < 1$ s, $t2 > 10$ s) rise of the current with a prolonged depletion of ATP, suggesting the presence of two populations of closed channels with different responsiveness to ATP (one poorly responding "rundown" channels, and the other normal channels). At a microscopic level, following a prolonged depletion of ATP, single-channel Po at millimolar ATP is low with long closed times. The recovery of the channel to normal Po of .4 takes tens to hundreds of seconds to complete, indicating that the rundown state is extremely stable but a complete recovery of normal function is possible (thus reversible rundown). Second, irreversible rundown: once the depletion of ATP is longer than ~60 s, the slow rising phase fails to attain the original level. The longer the ATP depletion, the smaller the fraction of recovered current. At a microscopic level, the Po did not recover over the experimental time (hence irreversible rundown). Interestingly, by introducing mutations at residues that are known to be involved in ATP binding at site 1, such as W401G and K464A, both reversible and irreversible rundowns are exacerbated. However, N6-phenylethyl-ATP (P-ATP), a high-affinity ATP analog, partially rectifies this defect in K464A, but not in W401G. These results can be explained by a simple idea that tight binding of ATP at site 1 stabilizes channel function by keeping the NBDs in a partially dimeric form. Intriguingly, a severe rundown was noted for delF508-CFTR treated with VX-770, but P-ATP dramatically stabilizes delF508-CFTR function.

Conclusions: The current studies demonstrate two types of phosphorylation-independent but site 1-dependent rundown in CFTR. This disruption of functional---and likely structural---integrity may partly explain the shorter half-life of delF508-CFTR in the cell membrane. Structural interpretations of our results will be discussed. ~0

S6.3 Novel protease inhibitors to balance ENaC function in CF airways

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The conducting airways are lined by a thin film of fluid known as airway surface liquid (ASL), which contributes to the removal of inhaled pathogens by mucociliary clearance, a critical component of innate immune defense. An optimal ASL volume is regulated by oppositely directed Cl^- and Na^+ flux (caused by osmotic gradients established by active ion transport) across the airway epithelium. In cystic fibrosis (CF) airways this regulation is lost because of the absence of CF transmembrane conductance regulator (CFTR)-mediated Cl^- secretion and the concomitant hyperabsorption of Na^+ via the epithelial sodium channel (ENaC). Proteolysis of the endogenous α and γ subunits of ENaC by apical membrane-bound Channel Activating Proteases (CAPs) is a fundamental regulatory mechanism for channel activity. In the CF lung however, a stark imbalance between the levels of CAPs and their natural inhibitors drives the activation of normally inactive ENaC which contributes to dehydration of the ASL layer, the depletion of which is regarded as an initiating factor for CF lung pathophysiology. On this basis, inhibition of CAPs-ENaC signalling represents a potential therapeutic intervention. We have developed a number of novel cell impermeable active-site directed compounds, of which QUB-TL1 is one, designed to inactivate key trypsin-like CAPs which are highly relevant in this regard.

Differentiated non-CF and CF human airway epithelial cells were used to assess the impact of inhibitors on a range of parameters including surface CAP activities, ENaC subunit processing/channel activity, ASL height and mucociliary clearance.

Treatment of airway epithelial cells with QUB-TL1 results in the significant downregulation of key endogenous CAP activities found to be excessively active at the surface of CF cultures. QUB-TL1-mediated CAP inhibition subsequently causes the internalisation of a pool of processed (active) ENaCγ prominent at the apical surface of CF cultures which correlates with a decline in channel activity. This downregulation of ENaC activity results in an increase in ASL height and improved mucociliary clearance in CF cells. We further find QUB-TL1 uniquely inhibits the ENaC activating enzyme furin, which is in contrast to the alternate trypsin-like CAP inhibitors camostat mesylate and aprotinin. QUB-TL1-mediated furin inhibition correlates with a reduction in neutrophil elastase-induced ENaC activation. Moreover, we find QUB-TL1 treatment protects CF cultures from *Pseudomonas aeruginosa* (Pa)exotoxin A-induced cytotoxicity. Paexotoxin A is a major toxic product activated by furin and positively associated with mortality. A novel trypsin-like inhibitor, NAP-858 will also be reported for the first time. QUB-TL1 and NAP-858****dampen CAPs-ENaC signalling which improves hydration status and mucociliary clearance in CF airway epithelial cell cultures. This may provide a mechanism to delay or prevent the development of CF lung disease in a manner independent of CF transmembrane conductance regulator mutation.

P5

S6.4 The contribution of the paracellular pathway to HCO_3^- secretion by human airway epithelial cells.

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Background: Epithelial cells secrete and absorb ions and water through the transcellular as well as the paracellular pathway and both these pathways potentially control the composition of the airway surface liquid (ASL). Recent studies have shown that the ASL is acidic in Cystic Fibrosis (CF). Although some of the molecular entities, responsible for this decreased pH, have been identified by different groups as (i) CFTR only[1] and (ii) the H^+/K^+-ATPase ATP12A[1,2], the contribution of the paracellular pathway to the pH defect has not been established. In order to determine if this pathway could be involved in the decreased ASL pH in CF, we compared the relative permeability of the paracellular pathway to chloride and bicarbonate in CF and non-CF airway epithelial cells.

Methods: Polarized epithelia (Calu-3 and CF and non-CF primary bronchial cells), grown on semi-permeable supports, were mounted in Ussing chambers and bathed bilaterally with modified Krebs solution (KRB). Basolateral and apical solutions were replaced sequentially by a low Cl^-/high HCO_3^- KRB to create an equal but oppositely directed transepithelial concentration gradients (5:1) for Cl^- and HCO_3^-, and the changes in potential difference (PD) were recorded to measure transcellular and paracellular HCO_3^- to Cl^- permeability. In order to eliminate the transcellular component and therefore measure the relative HCO_3^- to Cl^- paracellular permeability alone, $CFTR_{Inh172}$ and bumetanide and DIDS were added apically and basolaterally, respectively, and the same sequence of equal but opposite gradients was applied to the epithelia.

Results: In Calu-3 cells, when a basolateral to apical (B->A) Cl^- gradient was applied in the absence of inhibitors, the epithelium hyperpolarised (ΔPD=-3.98±0.21). This was also the case in the presence of inhibitors but the ΔPD was significantly reduced compared to the absence of inhibitors (ΔPD=-3.20±0.12, p=0.045, n=4). Expectedly, the opposite gradient (A->B Cl^- gradient) produced a depolarisation, the amplitude of which was unchanged when compared to the B->A Cl^- gradient (no inhibitor: 2.86±0.26, p=0.12, n=4; with inhibitor: 2.81±0.16, p=0.11, n=4). In non-CF primary bronchial cells, the B->A Cl^- gradient also induced an hyperpolarisation (ΔPD=-3.87±2.30, n=3) whereas it induced a depolarisation in CF primary bronchial cells (ΔPD=1.36±0.40, n=3). Interestingly, the depolarisation was also observed in the presence of channel and ion transporter inhibitors (non-CF: ΔPD=0.63±0.08; CF: ΔPD=1.18±0.42; n=3). In these cases, the opposite gradient produced hyperpolarisations of similar amplitudes in both CF and non-CF epithelia (non-CF: ΔPD=1.12±0.31; CF: ΔPD=1.29±0.41; n=3).

Conclusion: These results indicate that, in Calu-3 cells, the paracellular pathway is more permeable to Cl^- than HCO_3^-. However, in primary bronchial epithelial cells, we have found that the paracellular pathway is more permeable to HCO_3^- than Cl^-. Finally, the selectivity of the paracellular pathway for Cl^-/HCO_3^- does not appear to be different between non-CF and CF primary bronchial epithelial cells and therefore this suggests that the paracellular pathway is unlikely to contribute to the acidic ASL pH in CF airways.

Work supported by the CF Trust Strategic Research Centre grant SRC003

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S6.5 Role and structure-function relationship of the TMEM16A chloride channel

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TMEM16A protein (also known as anoctamin-1) is a calcium-activated chloride channel expressed in epithelial cells of different organs. In the airways, TMEM16A is localized on the surface epithelium and the sub-mucosal glands, where it is abundantly expressed in mucous cells, particularly under inflammatory conditions. TMEM16A may represent an alternative therapeutic pathway to circumvent the reduced epithelial anion transport in cystic fibrosis (CF). This approach could be essential for those cystic fibrosis patients expressing undruggable CFTR mutants but could also be useful as an adjuvant therapy supporting the effect of CFTR rescue maneuvers. However, a conclusive proof that TMEM16A may replace CFTR in its ability to control airway surface liquid properties is missing.

We found that modification of TMEM16A C-terminus leads to proteins with potentiation of channel activity. More precisely, replacement of the entire C-terminal region of TMEM16A with the equivalent domain of TMEM16B confers constitutive activity to the chimeric channel, as evaluated with the patch clamp technique or the HS-YFP. In our experiments, shortening of the chimeric region restricted the "activating domain" to a short sequence close to the last transmembrane domain and led to TMEM16A channels with high activity at very low intracellular calcium levels. To elucidate the molecular mechanism underlying this effect, we carried out experiments based on double chimeras, Forster resonance energy transfer, and intermolecular cross-linking. We also modeled TMEM16A structure using the Nectria haematococca TMEM16 protein as template. Our results indicate that the enhanced activity in chimeric channels is possibly due to altered interaction between the carboxy-terminus and the first intracellular loop in the TMEM16A homo-dimer. Mimicking this perturbation with a small molecule could be the basis for a pharmacological stimulation of TMEM16A-dependent anion transport. Experiments are in progress to express genetically-activated TMEM16A channels in cultured CF bronchial epithelia to assess the effect on airway surface liquid.

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S6.6 The calcium-activated potassium channel KCa3.1 Inhibits sodium absorption in airway epithelial cells

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Airway mucociliary clearence (MCC) is the main mechanism of lung immune innate defence, where its activity maintains the airways free of infection and obstruction. Defects in MCC are present in chronic lung diseases, such as cystic fibrosis, chronic obstructive pulmonary disease, and asthma. Adequate hydration of airways, maintenance of ciliary beating frequency (CBF) in epithelial cells and mucus homeostasis compromise MCC function, and are critically regulated by sodium absorption and chloride secretion in airway epithelial cells. The KCa3.1 potassium channel is essential for chloride secretion in the mouse intestine and its inactivation reduces the faecal water content. However, its role in respiratory epithelium is poorly understood. Therefore, we propose to elucidate the role of KCa3.1 in airway epithelial cells.

Ussing chamber experiments showed reduced amiloride sensitive short-circuit current (I_{sc}) in freshly isolated tracheas from *Kca3.1*$^{-/-}$ mice compared to wild type samples (-3.7 ± 4 vs -19.4 ± 5 μA * cm^{-2} respectively, p = 0.006). This effect was accompanied by a significant change in transepithelial voltage (V_{te}, -3.8 ± 0.8 vs. -2.0 ± 0.4 mV, p = 0.042), leaving basal I_{sc} and transepithelial tissue resistance unaffected. The observed reduction in amiloride sensitive I_{sc} in the *Kca3.1*$^{-/-}$ mice was not due to a downregulation of expression of ENaC subunits in epithelial cells from the mouse trachea evaluated by qRT-PCR. CBF measurements in tracheal epithelial cells were greatly increased in response to UTP in cells isolated from *Kca3.1*$^{-/-}$ mice compared to those isolated from WT. Such increase was also observed when WT cells were incubated with the KCa3.1 inhibitor TRAM-34 or the ENaC inhibitor amiloride previous to UTP stimulation. Similar results were obtained in human bronchial epithelial cells (HBEC) incubated with TRAM-34, as we observed a reduction in amiloride sensitive currents (3.1 ± 0.5 vs 1.6 ± 0.3 μA * cm^{-2} for control and TRAM-34, respectively). Incubation of HBEC with TRAM-34 reduced Il-4 induced goblet cell metaplasia, similar to that observed in a mouse model of chronic asthma.

These results suggest an important and novel role for KCa3.1 epithelial function. Our results demonstrate that KCa3.1 inhibition reduces sodium absorption in both mouse and human epithelium. This effect is independent on changes in ENaC expression and might be explained by changes in cell membrane potential that do not favour the electrochemical gradient for sodium entry. In addition, inhibition of KCa3.1 or direct inhibition of ENaC increased CBF, which could benefit MCC. The mechanistics of such increase are being explored. Inhibition of Il-4 induced goblet cell metaplasia indicates that KCa3.1 function is not restricted to maintenance of electrochemical gradients for ion movement but is also part of the signalling of relevant cytokines. In summary, inhibition of KCa3.1 could be a therapeutic strategy for the management of inflammatory lung diseases with decreased MCC.

Acknowledgment: Funded by FONDECYT 1151142 and CONICYT BFP.

01 April — 11:00–12:45

Symposium 07: CFTR Folding, Trafficking and Activity

S7.1 Interactions and post-translational modifications: regulating CFTR trafficking

Carlos M. Farinha

Understanding CFTR folding and trafficking pathways has been a matter of great focus in order to find improved therapeutic approaches to patients with CF.

After co-translational insertion in the membrane of the endoplasmic reticulum (ER) and core-glycosylation, CFTR undergoes a complex succession of steps with the main goal of checking the overall quality of its conformation that will ultimately lead to ER exit and traffic through the secretory pathway. The process is mediated by specific sorting motifs that include the four retention sequences AFTs (arginine-framed tripeptides) and the diacidic (DAD) exit code that mediates interaction with the COPII machinery [1-3]. Failure to pass the various checkpoints of the ER quality control targets the most frequent disease-causing mutant protein (F508del-CFTR) for premature degradation.

Once at the cell surface, CFTR stability is also controlled by multiple protein interactors, regulating not only anterograde traffic to the cell surface, but also its endocytosis and recycling thus achieving a fine and tight modulation of CFTR membrane levels. Among the different interactos, some protein kinases have been identified as key regulators, including spleen tyrosine kinase (SYK) or lemur tyrosine kinase 2 (LMTK2) [4, 5]. Recently, we have also shown that cAMP-dependent activation of the guanine nucleotide exchange factor EPAC1 (exchange protein directly activated by cAMP) stabilizes CFTR at the plasma membrane by a mechanism involving the PDZ adaptor NHERF1 [6].

We have employed pull-down approaches followed by mass spec identification of proteins in order to characterize the complexes that regulate CFTR exit from the ER and its membrane stability. Among the identified interactors, several are involved in protein trafficking and processing as well as in cell integrity and homeostasis and not previously directly associated with CFTR regulation, being currently under validation.

The identification of the specific CFTR interactors/regulators will likely identify novel therapeutic targets that could be ultimately used to promote mutant CFTR rescue and its stabilization at the membrane to the benefit of CF patients.

Acknowledgements

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S7.2 A yeast model to understand biosynthesis, folding and traffic of ABC transporters.

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The mechanistic basis for quality control surveillance within the secretory pathway remains poorly understood. We have used a systematic approach to discover proteins that influence the biogenesis of a model misfolded protein, the yeast ABC transporter, Yor1. Broadly analogous to human CFTR, mutations in which cause cystic fibrosis, Yor1 acts at the plasma membrane as a drug pump to confer resistance to oligomycin. Misfolding mutations cause ER retention and proteasomal degradation, leading to oligomycin sensitivity. This phenotype affords a rapid and robust screen for mutations that enhance or suppress the ability of cells to tolerate increasing concentrations of drug. Using a high throughput genetic screen, we measured the effect of each non-essential yeast gene on growth conferred by Yor1-ΔF, equivalent to the predominant disease-causing allele in CFTR. We discovered novel regulators of membrane protein biogenesis, including a pathway that seems to detect protein misfolding at the earliest stages of protein synthesis. We propose misfolding events can generate feedback to the ribosome that halts or slows translation, serving to either promote folding or prevent aberrant proteins from entering the secretory pathway. We also discovered an unexpected role for an ER export receptor, Erv14, which seems to increase the affinity of cargo proteins for the vesicle coat proteins, thereby enhancing ER egress. Genetic and physical interactions between the various regulators we identified suggest that these events are coordinated to promote efficient protein synthesis, folding and forward traffic.

S7.3 Regulation of CFTR Channel Function by Lipids and Lipid-mediated Signaling

Brandon B. Stauffer, Guiying Cui, Daniel T. Infield, Nael A. McCarty

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Little is known about the regulation of CFTR by lipids and lipid-mediated signaling pathways. Interactions between CFTR and cholesterol have been described, and these interactions regulate distribution and dynamics of CFTR in plasma membranes; however, it is not clear whether interactions with cholesterol impact CFTR channel function. The impact of annular lipids, which comprise the bulk of membrane in which the protein sits, also has not been determined. However, a role for regulation of CFTR activity by sphingomyelin, and/or sphingomyelin-mediated signaling pathways, has been suggested previously. Several types of bacteria that infect the lungs of CF patients, including Pseudomonas aeruginosa and Staphylococcus aureus, express sphingomyelinase C (SMase) as a virulence factor. SMase inhibits CFTR chloride channel activity in multiple cell systems, an effect that could exacerbate disease in CF and COPD patients. However, the mechanism by which sphingomyelin catalysis inhibits CFTR is not known. In this study we performed experiments in the Xenopus oocyte expression system to shed light on the molecular basis of inhibition. We found that the pathway leading to inhibition is not membrane delimited and that inhibited CFTR channels remain at the cell membrane, indicative of a novel silencing mechanism tied to channel gating. Evidence suggests that inhibition is state dependent, but occurs through a mechanism independent of CFTR's regulatory "R" domain. Consistent with an effect on CFTR gating behavior, we found that altering gating kinetics influenced the sensitivity to inhibition by SMase. Specifically, increasing channel activity by introducing the mutation K1250A or pretreating with the CFTR potentiator VX-770 (Ivacaftor) imparted resistance to inhibition. Some mutations that impede CFTR gating led to an increase in sensitivity suggesting that SMase targets a subset of closed states. Finally, we found that SMase-inhibited currents could not be restored by VX-770. Taken together, these data suggest that SMase inhibits CFTR currents by stimulating a cytosolic pathway that locks channels into a closed state at the cell membrane.

(Support: CFFdn MCCART15R0)

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S7.4 Silent mutations affectings CFTRs translational landscape rescue folding mutations

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The rate of protein synthesis modulates the folding efficiency of proteins. Differences in translation rates have been observed, in particular, in multi-domain proteins from different kingdoms of live. The uneven translation speed was found to be crucial for the folding of individual domains and overall functionality of the protein. During evolution transcript sequences have evolved to coordinate translational velocity and cotranslational folding. To gain deeper understanding whether mRNA sequence of the 5-domain protein CFTR, implicated in the pathology of cystic fibrosis, potentially regulates local translation rates, we employed ribosome profiling to learn about codon-dependent ribosomal velocity in CFBE cells. To gain insights into the effect of local alterations of translation velocity on CFTR biogenesis and cotranslational folding, we combined this powerful approach with biochemical assessment of disease relevant mutations combined with a silent polymorphism, T2562G; the latter locally decreases ribosomal speed over the mutated codon.

Investigating CFTR codon usage, we found that codons were not randomly distributed but rather clustered into regions of rare codons and regions of fast codons. Intriguingly, regions composed of rare codons were often arrayed down-stream of domain boundaries or transmembrane helices, regions that likely depend on reduced translation rates. Hence, we determined the velocity of ribosomes on individual codons and observed well-defined regions of slow translation. Thus, the CFTR transcript does not only encode vectorial information for amino acid sequence, but in a second dimension coordinates ribosomal velocity to optimally fine tune cotranslational folding.

Next, we combined the T2562G sSNP with a set of misfolding mutations. While CFTR levels where reduced in WT (e.g. containing only T2562G mutation), combination with disease relevant mutations (ΔF508, G85E, D579G, D614G and N1303K) augmented the steady state protein levels. Limited proteolysis experiments revealed a differential protease susceptibility suggesting an increased stability of the double mutants. Metabolic half-life measurements showed a slight increase in cellular stability.

In conclusion, the cotranslational folding of CFTR is coordinated by the presence of regions of slow translation along the transcript and mutation-induced alterations in the velocity adds to the effect of altered amino acid.

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S7.5 A high-content siRNA screen at near genomic scale reveals novel F508del-CFTR therapeutic targets

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Background: The F508del-CFTR mutation underlies about 85% of all cystic fibrosis (CF) cases by preventing cell surface expression of CFTR in many tissues, most notably in the airways. This is due to F508del-CFTR misfolding which leads to its retention in the endoplasmic reticulum (ER) and premature degradation by the ER quality control (ERQC). Nevertheless, F508del-CFTR can be rescued to the plasma membrane (PM) by chemical correctors, such as VX-809 or VX-661. F508del-homozygous patients can benefit from a combination drug of corrector VX-809 and potentatior VX-770 -- albeit with modest lung function improvement. There is still an unmet need for more potent F508del-CFTR traffic correctors.

Objective: We hypothesize that unknown cellular factors retain F508del-CFTR in the ER and our goal here is to identify such factors as possible drug targets.

Methods: To this end, we have previously generated a F508del-CFTR traffic reporter consisting in an mCherry-Flag-F508del-CFTR construct which was stably expressed CFBE cells under the control of a Tet-ON promoter [1]. Using fluorescence microscopy, F508del-CFTR traffic efficiency can be measured in each individual cell by calculating the fluorescence ratio of PM CFTR (given by immunodetected Flag) versus total CFTR (given by mCherry). We used this system to screen Ambion's Extended Druggable Genome siRNA library (Ref. AM80991V3.1), composed of 27,312 siRNAs targeting 9,128 human genes, i.e, about half of the genes in the human genome.

Results and Discussion: The primary siRNA screen revealed 78 genes whose knock-down significantly increased F508del-CFTR traffic over the negative control (more than two standard deviations). We defined F508del-CFTR traffic regulator genes by applying a lower stringency threshold to the raw screen data. Among such regulator genes, 39 are also CFTR interactors [2] and none are ENaC inhibitors [3], suggesting that the screening assay is pinpointing CFTR-specific regulatory partners. A gene ontology hit classification revealed an enrichment in PM proteins, including other ionic channels and proteins involved in epithelial homeostasis, differentiation and development. The Mammalian Membrane Two-Hybrid (MaMTH) system is being used as recently described [4] to map interactions between wt-CFTR/F508del-CFTR and 39 genes which are simultaneously F508del-CFTR traffic regulators and CFTR interactors. Ultimately, the most promising hits will be selected for rational inhibitor development.

Acknowledgment:*Work supported by UID/MULTI/04046/2013 centre grant from FCT, Portugal (to BioISI) and CFF-USA grant Ref: 7207534 from (to MDA). HMB is the recipient of post-doctoral fellowship SFRH/BPD/93017/2013 from FCT, Portugal.*

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S7.6 Rescue Δ508-CFTR with nanobodies

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The deletion of the phenylalanine 508 of CFTR leads to misfolding of the channel and prevents its translocation to the plasma membrane. In order to rescue expression of mutant CFTR we propose to develop CFTR-specific nanobodies, which are obtained by isolating the single variable fragment of heavy chain only antibodies found in camelids. Nanobodies have the peculiarities to bind to conformational epitopes and stabilize proteins. After immunization of a llama we obtained nanobodies against the nucleotide binding domain 1 (NBD1) of CFTR. We showed by ELISA that they recognize isolated NBD1 but also the full length CFTR obtained from solubilized membranes. Furthermore they are able to bind cellular CFTR, as shown by FACS experiments on permeabilized BHK21 cells expressing the wt-CFTR. Remarkably, the ability of nanobodies to bind ΔF508-CFTR is strongly modulated by correcting treatment such as VX-809 and decrease of cultivation temperature down to 27°C. Interestingly we show that this effect is not due to a rise of the expression of CFTR but rather to a conformational rescue of the mutated protein. We are able to correlate those data with the trafficking of ΔF508-CFTR at the plasma membrane by using non permeabilized BHK21 cells that express CFTR with an external HA epitope tag (Extope CFTR). Further studies will investigate the potential of the nanobodies themselves to modulate expression of ΔF508. This will be tested by expressing them directly in living cells by transfection or by using exogenously produced nanobodies fused to a cell penetrating peptide (CPP)

01 April — 14:30–16:40

Symposium 08: Inflammation and Host Pathogen Interaction

S8.1 Bioactive lipids and CF lung inflammation, innocent markers or guilty agonists?

Bob J. Scholte[1,2], Hamed Horati [2], Mieke Veltman[1,2], Marta Stolarczyk[1], Hettie Janssens[2], Danuta Radzioch[3], Juan Juan Bautista De Sanctis[4], Steve Stick[5]

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Abnormal lipid metabolism has been observed in CF patients and model systems. *In vivo* this can be in part attributed to intestinal lipid and bile acid malabsorption. However, it has also been reported that lipids with signaling properties (bioactive lipids), are abnormal in CF tissues, possibly affecting inflammatory and tissue remodeling signaling pathways. Touqui et al provided evidence that phospholipase A (cPLA2) activity is enhanced in CFTR deficient epithelia, affecting the production of Arachidonic Acid, and downstream receptor binding inflammation agonists (prostaglandins etc). An inhibitor of cPLA2 reduced mucus production in CF mutant mice. Several groups reported changes in ceramide metabolism in CF lung, which is involved in the resolution of lung inflammation. Experimental therapeutics affecting ceramide metabolism reduce lung inflammation in animal models, clinical trials are ongoing. We have recently reported on abnormal Sphingosine-1-phosphate (S1P) metabolism in the *Cftr*^{tm1EUR} F508del CFTR mouse model, correlating with lung inflammation, which could be partially corrected with an inhibitor of S1P katabolism (M. Veltman et al 2016), indicating an additional therapeutic option. These data suggest that the bioactive lipids involved are not merely markers of inflammation but play an active part in the progression of CF lung disease.

A****comprehensive mass spectrometry lipidomics analysis of bronchial lavage fluid (provided by the Australian AREST CF team) of infant (1-6 year) CF patients (N=40) compared to age matched non-CF patients (N=20) with comparable level of inflammation showed a difference in several classes of bioactive lipid species. In particular we observe a distinct increase of Long chain ceramides (Cer18:1/16:0) compared to Very Long Chain (Cer18:1/24:0) in CF patient with a high bronchiectasis score compared to non-CF. Further, lysolecithin species (LPC) were high in CF BALF. LPC is the second product of phospholipase A2 activity and a precursor of the pro-inflammatory agonist Lysophosphatidic acid (LPA). In addition to sphingosines and prostaglandins (PGA1, PGA2), LPC and LPA species correlate positively with inflammatory markers (IL8, % Neutrophils) in the BALF of CF infants. Furthermore, LPA species correlate with the lung CT (PRAGMA) score, which quantitates not only acute inflammation, air trapping and mucus plugging, but also progression of irreversible lung remodeling (bronchiectasis). this suggests that the cPLA2/LPC/LPA pathway is part of the NE activating pathway, and plays a role in the progression of CF lung disease. Cluster (PCA) analysis shows that in general lipid markers associated with oxidative stress, including isoprostanes, are able to distinguish patients with high and low CT scores, suggesting that this is a CFTR related factor in the progression of lung disease and an important therapeutic target.

Our current studies are aimed at defining the molecular relationship between CFTR deficiency and bioactive lipid metabolism in advanced cell culture models of bronchial epithelial cells. We have found that in differentiated human bronchial cells a LPA receptor agonist strongly activates basolateral secretion of multiple signaling proteins involved in inflammation and tissue remodeling, providing a mechanism for enhanced neutrophil infiltration in response to LPA signaling in CF lungs, and a potential therapeutic target.

Supported by NCFS, EU ERARE-INSTINCT, NIH, ZONMW, Longfonds.

S8.2 Cathepsin S in CF lung disease

Sinéad Weldon

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Cystic Fibrosis (CF) lung disease accounts for the majority of the mortality associated with this genetic disorder. Although infection is thought to be a primary driver of the lung damage associated with CF lung disease, recent research suggests that lung remodelling is evident soon after diagnosis even in children diagnosed with CF following newborn screening. CFTR mutations may impair not one but several aspects of lung defense mechanisms including the protease/antiprotease balance. The serine protease neutrophil elastase is recognised as a key mediator of lung destruction and inflammation especially during chronic *Pseudomonas aeruginosa* infection. In contrast to elastase, cysteine proteases are a family of proteases that have been relatively unexplored in the area of CF lung disease. However, following the demonstration of increased cathepsin S in the lungs of patients with CF in the early 2000's, interest in the cysteine protease, cathepsins S, has increased as the evidence supporting a pathogenic role for this protease in CF lung disease accumulates. The potential role of cathepsin S in the pathogenesis of CF lung disease as well as its value as a target for the treatment of chronic lung disease will be discussed.

S8.3 Novel genetically-diverse mouse models to unravel the complexity of the lung infections

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Most of our understanding of the host-pathogen interaction originates from studies on populations with redundant genotypes. In reality, highly adaptable bacteria are engaged in complex interactions with genetically diverse hosts. This complexity cannot not reproduced by the available models of respiratory infection in mice engineered for CF transmembrane conductance regulator (CFTR) mutation, as it fails to adequately replicate the clinical problem and, consequently, are unserviceable for pre-clinical purposes. One of the biggest challenges we face is to change the approach in modeling infection and move forward to a population-based model that may reproduce the broad spectrum of clinical respiratory disease observed in CF patients. Recently, a new mouse genetic reference population, named Collaborative Cross (CC), has been generated to mimic the genetic diversity of the human population. High allelic diversity has been achieved by systematic crossing of eight inbred founder mouse strains. Three are common laboratory strains - A/J, C57BL/6J, 129S1/SvImJ; three are wild-derived - CAST/Ei, PWK/PhJ, WSB/EiJ - ensuring maximum genetic diversity; and two strains - NOD/LtJ, NZO/HiLtJ - introduced genetic variants predisposing to medically important conditions. Consequently, the CC mice have greater recombination and genetic variation compared to other reference panels. High mapping resolution and sample sizes sufficient to drive phenotypic diversity in almost any trait of interest are hallmarks of CC lines. After *Pseudomonas aeruginosa* infection, CC mice exhibit distinct disease phenotypes ranging from total resistance to the lethal disease characterized by high bacterial burden. Most importantly, the widely marked differential response to *P. aeruginosa* infection in CC lines greatly expanded the range of disease phenotypes relative to classical mouse models and has closer similarity to what is observed in humans. This novel population represents a more predictive mouse model and can foster the investigation on pathophysiological mechanisms including genetic mapping of respiratory infection and translational studies.

S8.4 The role of CFTR in neutrophils

Noel G. McElvaney

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Cystic fibrosis (CF) is characterized by sustained neutrophil recruitment and neutrophil dominated inflammation from a very young age. Unfortunately these recruited neutrophils are ineffective in the clearance of bacteria and play a significant role in the pathogenesis of the lung disease associated with CF. Up until recently, neutrophil dysfunction in CF airways has been linked with necrosis and release of proteolytic enzymes, the latter playing a pivotal role in the destruction of lung tissue by overwhelming the anti-protease defenses of the lung. In addition these proteases impaired host defense by cleaving complement, complement receptors and immunoglobulins thus impairing the innate and adaptive immune responses. Our understanding of the pathophysiology of CF is undergoing a significant reassessment and there has been increasing interest in dysregulated neutrophil activity in CF. One of the key questions is whether this dysfunction is brought about by the severe inflammatory processes seen in CF or the intrinsic CFTR defect or a combination of both. In our work with circulating neutrophils from peripheral blood we have observed that intrinsic lack of CFTR activity is associated with decreased degranulation of secondary and tertiary neutrophil granules with significant implications for host defense. Decreased secretion of LL-37, lactoferrin and other secondary and tertiary granule components adversely affect the ability of neutrophils to exert an effective anti-microbial response. This dysregulation is replicated in normal neutrophils exposed to a CFTR inhibitor and is reversed in individuals with the G551D mutation who are receiving Kalydeco. Conversely, primary granule degranulation is increased in peripheral blood CF neutrophils with significantly increased release of neutrophil elastase and myeloperoxidase. This is, at least partly under the control of pH mediated processes which are likely influenced, by both intrinsic and extrinsic mechanisms. Conversely the abnormalities we have observed in lipid rafts in CF neutrophils are mediated largely by inflammatory process and are not reproduced in control neutrophils exposed to a CFTR inhibitor. This has major implications for cell stability and chemotaxis. Interestingly, medications such as Kalydeco which, in addition to having a potentiator effect on CFTR, also have an anti-inflammatory effect in CF, have been shown to reverse some of the lipid raft abnormalities seen. In summary the CF neutrophil is abnormal even in the relatively benign environment of the circulation. The abnormalities seen are attributable to both intrinsic CFTR defects and to the intense systemic and local inflammation seen in CF. By understanding these mechanisms we can attempt to restore the normal protective activities of these neutrophils and we can also use them as an index of efficacy in treatments aimed at either the intrinsic CFTR defect or inflammation.

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S8.5 Early alterations in airway mucins glycosylation and mucociliary clearance: key players in the development of cystic fibrosis lung disease

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Background: Cystic fibrosis (CF) disease is a recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) affecting the rheology of secretions, which become thick and difficult to clear from respiratory airways. It is characterized by chronic bacterial infection, often dominated by *Pseudomonas aeruginosa* (PA), persistent airway inflammation and lung tissue destruction. Debate remains concerning the mechanisms behind this exacerbated inflammation in CF lungs, whether innate or related to persistent bacterial infection. The development of the $CFTR^{-/-}$ pig model, which closely mimics CF human disease, has become an important tool to address this question. Here, we aimed to determine changes in the lung environment that may predispose to PA infection using a pig model of CF.

Methods: Male and female $CFTR^{+/-}$ pigs were mated and the progeny genotyped by PCR. Newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ were sacrificed before and 6h after inoculation with 2 mL of luminescent PA (5×10^6 cfu/mL) into the carina of the trachea. The upper and lower trachea, the proximal and distal bronchi and the bronchoalveolar lavage (BAL) fluid were collected to determine the level of mucins O-glycosylation, PA binding to mucins and the airways transcriptome. Disturbances in mucociliary transport were determined by ex-vivo imaging of luminescent PA (IVIS Spectrum, PerkinElmer).

Results: Our results showed an increased rate of sialylated O-glycans in BAL mucins from newborn non-infected $CFTR^{-/-}$ piglets. Mapping the rate of mucin sialylation through different segments of the airways showed that the highest differences in sialic acid were present in the upper airways. Interestingly, mucins isolated from non-infected $CFTR^{-/-}$ piglets strongly bound PA as opposed to those from non-infected $CFTR^{+/+}$. RNA-seq analysis showed no differences in the expression of most inflammatory genes, ruling out a pre-existing inflammatory condition.

Data from infected animals showed an increased hyper-sialylation in BAL mucins from both $CFTR^{+/+}$ and $CFTR^{-/-}$ piglets. RNA-seq analysis did not show major differences between the genotypes of infected piglets. However, when taken together, the cumulative score of 80 of the transcripts that were upregulated during infection was increased in the $CFTR^{-/-}$ piglets compared to controls. Ex-vivo imaging showed striking differences in PA localization. Six hours after inoculation, PA was mainly found in the larynx, pharynx and nose of $CFTR^{+/+}$ piglets while $CFTR^{-/-}$ piglets were unable to clear PA from the lungs, which suggests a defect on mucociliary clearance.

Conclusion: We observed an increased sialylation of mucins that result in enhanced PA adherence to the CF lung in the absence of any previous inflammation. Increase sialic acid occurs during inflammation, and do not represent a pathological condition by itself, since this increased bacterial adherence to mucins may help to clear the airways under normal conditions. However, we observed a major defect in the mucociliary transport that together with mucin sialylation may contribute to longer time of residency of bacteria on the CF lungs and development of lung inflammation.

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S8.6 Altered airway macrophage phenotype and function in mice with mucociliary clearance dysfunction

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Background: Airway macrophages are important contributors to lung homeostasis through the clearance of surfactants, apoptotic cells and cell debris. Positioned at the interface of host tissue and environment they mediate key responses to inhaled pathogens, through the release of various cytokines. Airway macrophages show a remarkable plasticity with phenotype and function being tightly regulated by the airway microenvironment. In a recent study, we demonstrated that impaired mucus clearance in *Scnn1b*-Tg mice with CF-like lung disease is associated with i) morphological macrophage activation, ii) expression of signatures of alternative macrophage activation, and iii) macrophage elastase (MMP12)-dependent structural damage (Trojanek JB, et al. AJRCMB 2014; 51:709-720). However, the functional role and phenotype of airway macrophages in CF-like lung disease needs further investigation.

Objective: The aim of this study was to characterize the phenotypes of airway macrophages isolated from lungs of *Scnn1b*-Tg mice and wild-type (wt) controls, and to compare responses to stimulation with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* ex vivo. To characterize functional properties of airway macrophages we measured the clearance of apoptotic cells from the lungs.

Methods: To achieve this goal, primary airway macrophages from *Scnn1b*-Tg mice and wt controls were isolated by bronchoalveolar lavage (BAL). Macrophage cultures were exposed to lipopolysaccharide (LPS; 100 ng/ml) or medium alone, supernatants and cells were collected 6, 12 and 24 hours post treatment and expression of macrophage signatures and proinflammatory mediators were determined. To determine the in vivo efferocytosis capacity of airway macrophages, apoptotic cells were delivered intratracheal into mouse lungs and efferocytosis of macrophages was measured by flow cytometry.

Results: In AMs isolated from *Scnn1b*-Tg mice we identified significant changes in baseline expression of genes associated with alternative activation, like Arginase 1, MMP12, CCL17 and CCL22. In addition the M1-associated surface marker CD86 was downregulated in macrophages from *Scnn1b*-Tg mice. Whereas increasing expression of the receptor Dectin-1 and TREM2 further emphasizes M2 polarization in *Scnn1b*-Tg mice compared to wt mice. Exposure of AMs to LPS upregulated key genes involved in proinflammatory and anti-bacterial responses in *Scnn1b*-Tg and wt macrophages. However, expression levels of LPS-induced proinflammatory mediators, like IL-1α, IL-6, IL-12p40, CCL2 and NOS2 were significantly enhanced in AMs isolated from *Scnn1b*-Tg mice. Impaired macrophage clearance of apoptotic cells is associated with inflammatory lung diseases. Here we show that the in vivo efferocytosis capacity of AMs was markedly decreased in *Scnn1b*-Tg mice in comparison to wt controls.

Conclusions: Our findings indicate that a mucostatic airway environment triggers polarization of airway macrophages toward alternative activation at baseline and primes them for augmented cytokine responses when challenged with bacterial-derived LPS. Moreover, defective efferocytosis may result in increased inflammation and pathogenesis. These results suggest that alternative airway macrophages may play an important role in the in vivo pathogenesis of chronic airway inflammation, and that a better understanding of the mechanisms underlying macrophage activation may lead to novel anti-inflammatory therapies for CF.

P70

S8.7 SPLUNC1 is pH-dependent and reduces burkholderia cepacia growth in airway surface liquid.

Saira Ahmad, William G. Walton, Matthew Redinbo, Robert Tarran

University of North Carolina at Chapel Hill, Chapel Hill, United States

Introduction: Cystic fibrosis (CF) is a genetic multi-organ disease caused by absent or dysfunctional CFTR mediated anion secretion, which in the lung, leads to mucus obstruction and chronic infection/inflammation. The *Burkholderia cenocepacia* complex (Bcc) is a group of 18 genetically distinct species. Although generally harmless to humans, they are extremely pathogenic to CF patients, and acquisition of Bcc is associated with a significant increase in mortality. Treatment of Bcc infections are difficult as the bacteria are multidrug resistant. Short Palate Lung and Nasal Epithelial Clone 1 (SPLUNC1) is secreted by the airway epithelia and serves as a multifunctional innate defense protein. SPLUNC1 is known to have antimicrobial functions against many gram-negative bacteria including Bcc. Additionally, knockdown/knockouts of SPLUNC1 from normal (NL) airways has resulted in increased *Pseudomonas aeruginosa* growth. Our data indicate that CF airway surface liquid (ASL) is characterized by a decrease in pH which renders SPLUNC1 inactive and unable to regulate the epithelial Na^+ channel (ENaC). We therefore tested the hypotheses that SPLUNC1 is needed in the airways to reduce Bcc growth and its antimicrobial activity is pH-sensitive.

Methods: SPLUNC1 was knocked down in NL primary human bronchial epithelia cells (HBECs) using shRNA. Lavages were collected, incubated with Bcc clinical isolate J2315 at MOI of 30 for 2 h, and colony forming units (CFUs) were determined for bacterial growth. We have recently elucidated the crystal structure of SPLUNC1 and screened novel mutants based on this structure for pH-sensitivity of antimicrobial activity. J2315 was incubated with 0.4 μM SPLUNC1 mutants for 2 h at pH range of 6 to 7.5, and CFUs were determined for SPLUNC1's antimicrobial activity. ASL from NL and CF HBECS were then incubated with 4 μM SPLUNC1 mutants for 2 h. Lavages were collected, incubated with J2315 at MOI of 30 for 2 h, and CFUs were determined for bacterial growth.

Results: Knockdown of SPLUNC1 in NL HBECs showed increased J2315 growth in the ASL compared to wildtype NL HBECs. Under acidic conditions, SPLUNC1's antimicrobial activity against J2315 was impaired, but was restored at pH 7.5. However, SPLUNC1 mutants that are pH-independent ENaC regulators (Q140E, K138D, and K156C) reduced J2315 bacterial growth at a low pH of 6.5. Additionally, while SPLUNC1's antimicrobial activity in CF ASL was impaired, Q140E, K138D, and K156C reduced J2315 growth in the CF ASL to similar levels as seen in the NL ASL.

Conclusions: Our data suggest that SPLUNC1 is needed in the airways to reduce Bcc growth. Additionally, SPLUNC1's antimicrobial activity against Bcc is pH-dependent and SPLUNC1 mutants, that are pH-independent ENaC regulators, also retained pH-independent antimicrobial activity. Whilst the impact of CF on SPLUNC1-Bcc antimicrobial interactions is currently under investigation, our data suggest that understanding this interaction may have important therapeutic applications for CF lung disease.

Acknowledgment: Funded by the NIH and the UK CF Trust.

01 April — 17:10–18:10

Parallel sessions

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1. Flash Paper Session - CFTR Structures

Moderator: Mike Gray (UK)

2. How to write a successful paper

Moderator: Emma Grainger (Elsevier)

01 April — 18:15–19:15

Closing Keynote Lecture

Studying CFTR: From Basic Science to Treatment of Cystic Fibrosis

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P1

Epithelial chloride transport by CFTR requires ANO1

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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is the secretory chloride/bicarbonate channel in airways and intestine that is activated through ATP binding and phosphorylation by protein kinase A, but fails to operate in cystic fibrosis (CF). Anoctamin 1 (ANO1, also known as TMEM16A) is thought to function as the Ca^{2+} activated secretory chloride channel independent of CFTR. Here we report that tissue specific knockout of the *Ano1* gene in mouse intestine and airways not only eliminates Ca^{2+}- activated Cl^- currents, but also abrogates CFTR-mediated Cl^- secretion and cAMP-activated whole cell currents. The data suggest new roles of ANO1 in differentiated epithelial cells: ANO1 provides a mechanism for enhanced ER Ca^{2+} store release, possibly engaging Store Operated cAMP Signaling (SOcAMPS) and activating Ca^{2+} regulated soluble adenylyl cyclases (sACs). This regulatory relationship may be the explanation for the functional overlap of CFTR and Ca^{2+}-dependent chloride transport, suggesting a fundamental role of ANO1 for

proper activation as well as membrane expression of CFTR.

Supported by DFG SFB699, Cystic Fibrosis Trust grant SRC003.

P2

Anoctamin 1 traffic and function is enhanced by tethering proteins family of extended synaptotagmins

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Background: Anoctamins are a family of 10 proteins of which at least one member, anoctamin 1 (ANO1) was demonstrated to be a Ca^{2+} activated Cl^- channel (CaCC). ANO1 homologue in yeast, Ist2, is a cortical ER protein involved in ER-plasma membrane tethering.

In addition, extended synaptotagmins (ESYTs) family comprise ER proteins that participate in the tethering function via C2 domain-dependent interactions with the plasma membrane (PM) that require PIP_2 in the case of ESYT2 and ESYT3 and also elevation of cytosolic Ca^{2+} in the case of ESYT1.

Aim: The main goal of this work is to determine the relationship between ESYT family and ANO1, since ESYT1 siRNA was found to decrease ANO1 traffic to PM.

Methods: Two cell lines were used, CFBE parental (to evaluate endogenous ANO1) and CFBE stably overexpressing a double-tagged 3HA-ANO1-GFP construct under an inducible promoter. Cells were transfected with siRNA against ESYT1, ESYT2 or ESYT3 for 72h and characterised for ANO1 function by patch-clamp and ANO1 PM expression by immunofluorescence. All results were compared to control (siRNA scrambled transfected cells).

Results and Discussion: We observed that the knockdown of ESYT1 and ESYT2 significantly decreased ANO1 PM traffic. Although ESYT3 knockdown did not cause a significant change in ANO1 PM levels, this is likely due to the very low levels of ESYT3 in CFBE cells. Patch-clamp data showed that knockdown of ESYT1, ESYT2 and ESYT3 significantly decreased ANO1 current density, in comparison to scrambled siRNA transfected cells. Moreover, additional experiments in CFBE parental cells with knockdown of ESYT1 showed similar results, confirming that ESYT1 played a similar role on endogenous ANO1 as that observed for 3HA-ANO1-GFP, being thus physiologically relevant.

Our observation that GPCR-mediated activation of ANO1 is compromised in cells with knockdown of ESYTs, suggests that the Ca^{2+} dependent lipid transfer between ER membrane and PM is also compromised.

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P3

Deregulation of planar Cell polarity and TGFβ signaling in the airways of cystic fibrosis mouse models

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As part of CF airway remodeling associated with chronic infection and inflammation, deregulation of planar cell polarity (PCP), an intracellular protein network controlling the orientation of epithelial cells in the plane of the tissue, ciliogenesis and cilia motion, is poorly understood. We have previously demonstrated that the expression of several genes encoding components of the PCP network is altered in human CF bronchial epithelial (HBE) cells CF cells. We have also shown that knocking-down the expression of the PCP core protein CELSR3 promotes TGFβ-induced epithelial to mesenchymal transition (EMT) in normal HBE cells.

In naïve conditions, we did not find any difference in CELSR3 expression between CF and wild-type mouse lungs. In CF primary mouse nasal epithelial cell 3-D cultures (MNECs), neither CELSR3 nor any of the PCP genes studied were deregulated compared to wild-type. Endotracheal administration of bleomycin, a chemotherapeutic agent inducing lung inflammation and remodeling (Huaux, Noel et al, PLoS One 2013), induced a decrease in CELSR3 expression, significantly more pronounced in CF than in wild-type mice. In both genotypes, CELSR3 expression correlated with the levels of inflammatory and fibrosis markers (IL-6, TGFβ, collagen content and TIMP-1).

Scnn1b-tg/+ (βENaC-overexpressing; Tg/+) mice display a spontaneous CF-like lung disease. In Tg/+-MNECs, expression of CELSR3 and other PCP genes was significantly deregulated in comparison with wild-type cells. Exposing MNECs to TGFβ (15ng/ml, 6 days) induced EMT, characterized by down-regulation of epithelial cell markers such as zonula occludens-1, and upregulation of α-smooth muscle actin, fibronectin and vimentin gene expression. TGFβ also decreased the expression of CELSR3. In Tg/+ cells, TGFβ-induced EMT was more marked than in wild-type cells and was not fully reversed by the TGFβ-receptor type I and II inhibitor, GW-788388. In both genotypes, GW-788388 alone clearly promoted apicobasal polarity and mucociliary differentiation of MNECs: it increased transepithelial electrical resistance and expression of epithelial cell markers, and it decreased expression of mesenchymal cell markers. Again, the effect was more pronounced in Tg/+ than in wild-type cells. Moreover, GW-788388 normalized PCP gene expression in Tg/+ but not in wild-type cells. These data suggest that the TGFβ-pathway is intrinsically upregulated in βENaC mouse cells.

Taken together, our results support the view that PCP is deregulated in airways of CF mouse models. The deregulation, likely contributing to the pulmonary phenotype in CF, seems to be linked, at least partly, to loss of CFTR function or to over-expression of ENaC. Both intrinsic and extrinsic factors impair PCP signaling in CF and render airway epithelial cells more susceptible to remodeling and defective mucociliary differentiation.

P4

Anoctamins 1 and 6 – identification of novel traffic regulators and their significance to cystic fibrosis

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Background: An attractive possibility to treat Cystic Fibrosis (CF) is through the activation of alternative (non-CFTR) anion channels. Since the identification of anoctamin 1 (ANO1) as a calcium (Ca^{2+})-activated Cl^- channel (CaCC), anoctamins have raised interest as possible target channels that can compensate for the absence of epithelial anion secretion in CF. The anoctamin protein family is composed of 10 members in mammals (ANO1-10) which are involved in a variety of functions including ion transport, phospholipid scrambling, cell volume regulation, proliferation, and apoptosis. Among the anoctamin family members, ANO1 and ANO6 were shown to mediate/regulate ion transport in the airway epithelium. Moreover, a functional relationship between these two anoctamins and CFTR has already been proposed.

Aim: The main goal of our work is to identify regulators of ANO1 and ANO6 and unveil the relationship between these anoctamins and CFTR, in order to develop novel therapies for CF.

Methods: We have generated CF Bronchial Epithelial (CFBE) cells stably expressing double-tagged ANO1 or ANO6 (GFP at the C-terminus and a triple hemagglutinin at an extracellular loop) under an inducible promoter. These cells were used to establish a microscopy-based assay to assess ANO1/ANO6 traffic to the plasma membrane (PM).

Results and Discussion: The novel cellular systems were found to be functional and cell-based ANO1/6 traffic assays were optimized for high-throughput microscopy (HTM). A HTM siRNA screen was performed for ANO1 traffic using a library of siRNAs targeting 503 genes previously identified as regulators of wt-CFTR or F508del-CFTR traffic. This screen revealed 68 genes that, when knocked down, enhance ANO1 PM traffic and 56 genes that, when knocked down, decrease ANO1 traffic. These traffic hits are involved in pathways including G-protein coupled receptor (GPCR) signalling, cell signalling, ion transport, cellular Ca^{2+} homeostasis, phosphorylation, regulation of cell proliferation and cell cycle. Data from a similar high-throughput siRNA screen for ANO6 are currently being analysed to identify regulators of this anoctamin. The top traffic hits will be validated and further characterized using biochemical and functional assays to identify genes that modulate ANO1/6 traffic/function. These results will then be compared with CFTR traffic hits to identify ANO1/6 regulators that operate independently of CFTR. These may reveal possible alternative drug targets for CF that could lead to a more sustained Cl^- secretion in patients, thus correcting the basic defect of the disease.

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P5

The contribution of the paracellular pathway to HCO_3^- secretion by human airway epithelial cells.

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Background: Epithelial cells secrete and absorb ions and water through the transcellular as well as the paracellular pathway and both these pathways potentially control the composition of the airway surface liquid (ASL). Recent studies have shown that the ASL is acidic in Cystic Fibrosis (CF). Although some of the molecular entities, responsible for this decreased pH, have been identified by different groups as (i) CFTR only[1] and (ii) the H^+/K^+-ATPase ATP12A[1,2], the contribution of the paracellular pathway to the pH defect has not been established. In order to determine if this pathway could be involved in the decreased ASL pH in CF, we compared the relative permeability of the paracellular pathway to chloride and bicarbonate in CF and non-CF airway epithelial cells.

Methods: Polarized epithelia (Calu-3 and CF and non-CF primary bronchial cells), grown on semi-permeable supports, were mounted in Ussing chambers and bathed bilaterally with modified Krebs solution (KRB). Basolateral and apical solutions were replaced sequentially by a low Cl^-/high HCO_3^- KRB to create an equal but oppositely directed transepithelial concentration gradients (5:1) for Cl^- and HCO_3^-, and the changes in potential difference (PD) were recorded to measure transcellular and paracellular HCO_3^- to Cl^- permeability. In order to eliminate the transcellular component and therefore measure the relative HCO_3^- to Cl^- paracellular permeability alone, $CFTR_{Inh172}$ and bumetanide and DIDS were added apically and basolaterally, respectively, and the same sequence of equal but opposite gradients was applied to the epithelia.

Results: In Calu-3 cells, when a basolateral to apical (B->A) Cl^- gradient was applied in the absence of inhibitors, the epithelium hyperpolarised (ΔPD=-3.98±0.21). This was also the case in the presence of inhibitors but the ΔPD was significantly reduced compared to the absence of inhibitors (ΔPD=-3.20±0.12, p=0.045, n=4). Expectedly, the opposite gradient (A->B Cl^- gradient) produced a depolarisation, the amplitude of which was unchanged when compared to the B->A Cl^- gradient (no inhibitor: 2.86±0.26, p=0.12, n=4; with inhibitor: 2.81±0.16, p=0.11, n=4). In non-CF primary bronchial cells, the B->A Cl^- gradient also induced an hyperpolarisation (ΔPD=-3.87±2.30, n=3) whereas it induced a depolarisation in CF primary bronchial cells (ΔPD=1.36±0.40, n=3). Interestingly, the depolarisation was also observed in the presence of channel and ion transporter inhibitors (non-CF: ΔPD=0.63±0.08; CF: ΔPD=1.18±0.42; n=3). In these cases, the opposite gradient produced hyperpolarisations of similar amplitudes in both CF and non-CF epithelia (non-CF: ΔPD=1.12±0.31; CF: ΔPD=1.29±0.41; n=3).

Conclusion: These results indicate that, in Calu-3 cells, the paracellular pathway is more permeable to Cl^- than HCO_3^-. However, in primary bronchial epithelial cells, we have found that the paracellular pathway is more permeable to HCO_3^- than Cl^-. Finally, the selectivity of the paracellular pathway for Cl^-/HCO_3^- does not appear to be different between non-CF and CF primary bronchial epithelial cells and therefore this suggests that the paracellular pathway is unlikely to contribute to the acidic ASL pH in CF airways.

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P6

CFTR regulates insulin secretion and calcium signalling in islet cells.

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Background: Cystic Fibrosis-related diabetes (CFRD) is the most frequently observed extra-pulmonary co-morbidity in CF patients, and significantly accelerates lung decline. It is accepted that CF may result in fibrotic progression in the pancreas, which eventually destroys both the exocrine and endocrine tissue. However, emerging evidence implicates CFTR in the regulation of insulin secretion from the pancreatic islet itself. Indeed, several recent studies have confirmed CFTR expression in primary human and mouse beta cells [Edlund *et al*, 2014; Guo *et al*, 2014], and in beta cell lines [Guo *et al,* 2014, NTimbane *et al,* 2016]. Impaired first phase insulin responses and glucose homeostasis are observed in people with CF [De Schepper *et al,* 1992], while functional CFTR channels are required for insulin exocytosis [Edlund *et al*, 2014] and the regulation of membrane potential [Guo *et al*, 2014]. These novel insights suggest that the pathogenesis of CFRD is more complicated than originally thought, with implications for diabetes treatment and screening in the CF population.

Aims: The aims of this study were to investigate the effect of CFTR deficiency on insulin secretion and intracellular calcium signaling.

Methods: Two CFTR expressing rodent beta cell lines (BRIN-BD11 and MIN6) were used for all experiments with selected data confirmed in primary mouse islets. CFTR was inhibited using CFTRinh172 or GlyH101 whilsts DIDs was used as an alternative chloride channel blocker. Insulin secretion in response to various stimuli was measured using insulin ELISA. Intracellular calcium was imaged and quantified using spectrophotometry and fluorescence microscopy.

Results: Insulin secretion was impaired at 16.7 mM glucose in cell lines, whilst insulin release in response to cAMP agonist GLP-1 was also reduced in both cell lines and primary islets following CFTR inhibition. Conversely, basal (non-stimulated) intracellular calcium concentrations were significantly higher in CFTR-inhibited cells even in the presence of EGTA. Preliminary data suggest that the increase in intracellular calcium flux results from differences in calcium uptake into mitochondrial stores in CFTR-inhibited cells.

Conclusion: Our results reveal a role for CFTR in the maintenance of islet function. Consistent with prior reports, we confirm a reduction in insulin secretion from CFTR deficient beta cells and primary islets, particularly in response to cAMP agonists such as GLP-1. This work highlights the importance of chloride channels in the maintenance of insulin secretion from the pancreatic beta cell.

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P7

Involvement of sphingosine 1-phosphate in cystic fibrosis bone disease

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These last years, the increasing life expectancy of patients with cystic fibrosis (CF) has been associated with the emergence of co-morbidities such as diabetes and CF-related bone disease (CFBD). This latter reached 50% of CF adults. The bone formation and bone remodelling are ensured through an effective coordination between two cell types: the osteoblasts and osteoclasts. The presence of F508del mutation in *Cftr* gene is part of recognized etiologies of the bone loss observed in CF patients through a deficit of osteoblastic maturation process in F508del osteoblasts (Velard *et al.,* 2014, AJRCCM). Bone homeostasis is dependent of the communication between the osteoblasts and osteoclasts which involves key cytokines (RANKL and OPG) and lipid mediators such as the prostaglandin E2 (PGE2) and the sphingosine 1-phosphate (S1P). S1P is produced by two sphingosine kinases (the SphK1 and SphK2) from sphingosine and ceramides and is implicated in the control of bone formation (Keller *et al*., 2014, Nature Com). The diversity in cell responses elicited by S1P (cell differentiation, increased PGE2 production by osteoblasts, apoptosis,...) may be attained in part by its dual characteristics of being both an intracellular second messenger and an extracellular signal through five identified S1P receptors (S1P1-5); S1P is also a powerful regulator of the CFTR activity (Malik et al., 2015, PLoS One). Previously, we have showed that a CFTR corrector and potentiator, the C18 (Vertex) is able to improve the osteogenic activity of F508del osteoblasts via a regulation of COX-2/PGE2 and RANKL expression (Velard *et al.*, 2014, ERJ; Delion *et al.,* 2016, J Pathol). All these results have opened new domains to explore for further biochemical characterization of the actors of bone metabolism in CF patients including the S1P/S1P1-5/COX-2-PGE2 axis (Jacquot *et al*., 2016, Osteoporos Int).

To investigate the role of the S1P/S1P1-5 receptors signalling in CFBD, we first evaluated the involvement of defective CFTR in the mRNA expression level of SphK1, 2 and S1P1-5 receptors in primary F508del CFTR osteoblasts obtained from 5 patients compared to primary normal osteoblasts (n=6). The effect of the addition of the CFTR corrector C18 (10 µM, 48h) in primary cultures of F508del osteoblasts was also evaluated.

Our results showed that the F508del mutation in osteoblasts reduced SphK2 mRNA expression, but not SphK1 expression. Normal and F508del osteoblasts expressed the S1P1, 2, 3, 4 but rarely the S1P5 receptor (present in 2 of 11 samples). The S1P4 receptor mRNA expression was slightly upregulated in F508del osteoblasts compared to normal osteoblasts. Addition of C18 in F508del osteoblasts restored the SphK2 mRNA expression, increased S1P2 and S1P3 mRNA expression and reduced the S1P4 mRNA expression. These preliminary data encourage us to now explore the production level of sphingosine kinases and receptors at protein level and the S1P production in F508del osteoblasts compared to normal osteoblasts. We also need to more investigate the S1P/S1P1-5/COX-2/RANKL axis in osteoclast-osteoblast interactions in the context of CFBD.

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P8

Novel insight into the role of CFTR in the lacrimal functional unit and its influence on the fluid secretion of lacrimal gland ducts in mouse

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Lacrimal gland (LG) is the major source of tears that bathe the ocular surface and its secretion is mediated by an array of ion transporters. Cystic fibrosis transmembrane conductance regulator (CFTR), while has been shown to play a critical role in exocrine glands, its role in LG secretion is unknown. Availability of transgenic mouse models carrying genetic defects in CFTR allows direct examination of its role in lacrimal secretion. Therefore the aim of this study is to explore potential structural and functional changes in the lacrimal functional unit in CFTR knock out (KO) mice, as compared to that from wild types (WT) and to investigate fluid secretion of isolated LG duct segments.

Tear production was measured by using phenol red impregnated cotton threads, with wetting length measured by a dissecting microscope.

Ocular surface integrity was investigated by applying 5% fluorescein sodium into the conjunctival sac, followed by corneal evaluation by a slit lamp biomicroscope equipped with cobalt-blue light, with the staining assessed by using the 2007 Dry Eye Work Shop (DEWS) grading system.

Immunofluorescence was used to confirm the presence and localization of CFTR protein.

LG interlobular and intralobar ducts were isolated as previously described by our group. Fluid secretion into the closed intraluminal space of duct segments, as swelling response was analyzed using bright field video-microscopy.

Intracellular Ca^{2+} level underlying cholinergic stimulation was measured with microfluorometry using FURA 2AM dye. Data was presented as means ± SEM.

Tear secretion from mice at 8-10 weeks of age were 2.47±0.43 mm/5 min (n=11) in WT mice and 1.27±0.19 mm/5 min (n=11) in KO mice (p=0.000). In the 14-16 weeks group, tear secretion was 4.43±0.96 mm/5 min (n=12) in WT mice and 2.19±0.74 mm/5 min (n=7) in KO mice (p=0.000). For those mice at 22-24 weeks, tear secretion was 3.86±0.91 mm/5 min (n=11) in the WT group and 1.36±0.14 mm/5 min (n=10) in KO group (p=0.000).

Corneal staining score was 1.5±1.16 in WT mice (n=6) and 9.16±1.83 in KO mice (n=6, p=0.00008) in animals at 8-10 weeks, while in the 22-24 weeks group, the staining was 7.16 ±1.5 in WT mice (n=6) and 15.33±1.66 in KO animals (n=6, p=0.0018).

Immunofluorescence confirmed the presence of CFTR in apical membranes of ductal cells.

Forskolin (10 μM) stimulation caused a rapid fluid secretion in ducts from WT mice (176.6±5.6 pl/min/mm^2); in contrast to ducts from KO animals which secretion remained unchanged during stimulation (1.9±5.6 pl/min/mm^2). Carbachol (100 μM) caused a biphasic secretory response consisting of a rapid pulse-like secretion in the first 5 min, followed by a plateau phase with similar secretory patterns both in WT (132.7±6.1 pl/min/$mm^{2)}$ and in KO ducts (130.1±5.2 pl/min/mm^2)(p=0.763).

No significant differences were observed in carbachol-stimulated cytosolic Ca^{2+} signaling in ducts from WT and KO mice.

These data demonstrated significant decreases in tear secretion, marked defects in ocular surface integrity and the lack of forskolin-evoked fluid secretion in KO mice, suggesting the key role CFTR may play in LG fluid secretion and in the maintenance of ocular surface integrity.

P9

Expression and function of Cl^- loaders, extruders and channels in pancreatic islet ß-cells: implications for intracellular Cl^- regulation, insulin secretion and CFRD

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Currently, patients with Cystic Fibrosis (CF) are the population with the highest risk for developing age-dependent diabetes, affecting ~50% of the cases by the 4th decade of life. Hyperglycemia in these patients worsens lung function and nutritional status and increases the number of hospitalizations resulting in higher mortality. Recent data suggests that patients with pancreatic insufficiency are at a higher risk to develop CF-related diabetes (CFRD), due to β-cell dysfunction. Glucose metabolism and plasma membrane depolarization are responsible for the initial secretory response in pancreatic β-cells and regulated primarily, though not exclusively, by the ATP-sensitive K^+ channel (K_{ATP}). In these cells, the intracellular chloride concentration ($[Cl^-]_i$) is kept above thermodynamic equilibrium providing an additional driving force for the depolarization and prolongation of action potentials necessary for sustained insulin secretion. In other cell types, at least seven cation-chloride cotransporters of the solute carrier protein family 12 group A (*Slc12a*) and some members of the *Slc4a* and *Slc26a* families are considered regulators of $[Cl^-]_i$. Whilst some of these transporters actively accumulate Cl^- ions, others extrude them, creating a Cl^- gradient across the plasma membrane which in turn is dissipated by functioning anion channels. The net functional balance between Cl^- loaders, extruders and channels determines whether Cl^- has a depolarizing, hyperpolarizing or no action in a given cell. Insulin-secreting β-cells express *Slc12a1* and *Slc12a2* the prototypical Cl^- loaders. However, the current knowledge regarding expression of Cl^- extruders such as *Slc12a4*, *Slc12a5, Slc12a6* and *Slc12a7* and channels such as *Cftr*, *Ano1* and *Vrac* is controversial or negligible. We have used conventional and quantitative RT-PCR, Western blotting and immunolocalization studies in human and rodent pancreatic islets and clonal β-cell lines to confirm the expression of these modulators of $[Cl^-]_i$. We also used available pharmacological tools to dissect their role in the secretory response. Our experiments suggest that insulin-secreting β-cells tightly maintain an outwardly directed Cl^- gradient and that impairing its dissipation through inhibition of *Cftr, Ano1* and *Vrac* blunts insulin secretion in response to glucose.

P10

Peptide-based, ratiometric FRET probes visualize channel-activating protease (CAP) activity.

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The epithelial sodium channel (ENaC) and the proteolytic processing of its alpha- and γ-subunits by channel-activating proteases (CAPs) play a crucial role in sodium transport across different epithelial membranes, including the airways, and in airway surface liquid (ASL) homeostasis. It was shown that CAP expression is elevated in primary bronchial epithelial cultures of Cystic Fibrosis (CF) patients compared to tissue from healthy subjects. However, the impact of CAPs on ENaC activity, ASL regulation and CF lung disease is unknown.

In order to study the role of CAPs to CF lung disease and their regulation in the disease context, substrate-based Förster Resonance Energy Transfer (FRET) probes were developed for measuring protease activity. Proteolytic cleavage of the probe resulted in a loss of FRET, giving a ratiometric readout reflecting enzyme activity. The attachment of a lipid anchor enabled the detection of membrane-associated enzyme activity in a spatially resolved manner. In heterogeneous cell populations, it is possible to distinguish cell types with proteolytic activity on their surface from cells without significant CAP activity, representing a strong advantage of our probes over commercial substrates.

Probes based on different substrate sequences have been evaluated with respect to selectivity and biophysical properties, namely solubility and maximal dynamic range upon cleavage. *In vitro* characterization using recombinant enzymes as well as cell-based characterization using broad-spectrum inhibitors identified the probe based on the CAP cleavage site in the human ENaC γ-subunit as the most selective one towards CAP activity.

As proof of concept, the probes were successfully tested in primary human nasal epithelial cells and primary murine tracheal epithelial cells. Both experimental sets suggested that our FRET probes are potential biomarkers for human specimen as well as research tools for further investigation of CAP activity in chronic lung diseases, including CF, idiopathic pulmonary fibrosis and lung cancer.

P11

Intermediate conductance K^+ channel is required for parathyroid hormone-stimulated CFTR-mediated anion secretion by Caco-2 intestinal epithelia

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Parathyroid hormone (PTH) plays an important role in calcium and phosphate homeostasis in the body. In previous work, we demonstrated that PTH rapidly stimulated HCO_3^- secretion by cystic fibrosis transmembrane conductance regulator (CFTR) in Caco-2 epithelia (1). Here, we investigate the underlying cellular mechanisms of PTH-stimulated anion secretion by Caco-2 intestinal epithelia with the Ussing chamber and patch-clamp techniques. Both recombinant PTH 1--34 (10 nM) and forskolin (10 μM) induced a transient peak within 1--3 min (early phase), followed by a sustained short-circuit current (ΔI_{sc}) lasting > 1 h (late phase) of comparable magnitudes (PTH: early phase, ΔI_{sc} = 18.28±0.89 μA/cm^2; late phase, ΔI_{sc} = 5.00±1.13 μA/cm^2; n = 10; forskolin: early phase, ΔI_{sc} = 18.09±0.74 μA/cm^2; late phase, ΔI_{sc} = 3.78±0.83 μA/cm^2; n = 10; P > 0.3). To identify the anion species involved, the effect of PTH was investigated in HCO_3^--free and/or Cl^--free solutions. After PTH activation, the ΔI_{sc} for both the early and late phases were reduced ~50% in either HCO_3^-- or Cl^--free solution, while it was almost absent during the late phase in Cl^-- and HCO_3^--free solution. To verify HCO_3^- secretion by CFTR, we pre-treated Caco-2 epithelia with $CFTR_{inh}$-172 (20 μM), acetazolamide (100 μM), or tenapanor (100 nM) and observed similar reductions in the PTH-induced ΔI_{sc}. We interpret these data to suggest that both Cl^- and HCO_3^- are involved in PTH-stimulated CFTR-mediated anion secretion by Caco-2 epithelia.

To investigate the role of basolateral K^+ channels in CFTR-mediated anion secretion (2), we pre-treated Caco-2 epithelia with the non-specific K^+ channel blocker ($BaCl_2$) and small molecule inhibitors of small-conductance K^+ channels (UCL-1684), intermediate-conductance K^+ channels (clotrimazole and TRAM-34), large-conductance K^+ channels (penitrem A), ATP-sensitive K^+ channels (glibenclamide) and cAMP-activated K^+ channels (chromanol 293B). Only $BaCl_2$ (5 mM), clotrimazole (50 μM) and TRAM-34 (20 μM) suppressed the PTH-induced ΔI_{sc} early and late phases. To verify the effects of TRAM-34 on intermediate-conductance K^+ channel activity, we tested the effects of TRAM-34 (20 μM) on the single-channel behaviour of recombinant wild-type human CFTR using excised inside-out membrane patches. TRAM-34 (20 μM) was without effect on the single-channel current amplitude (n = 5) and open probability of CFTR (n = 5) (P > 0.25). We interpret these results to suggest that PTH-stimulated CFTR-mediated anion secretion requires basolateral membrane K^+ recycling through intermediate-conductance K^+ channel. Supported by grants from Mahidol University, Thailand Research Fund, Newton Fund and CF Trust.

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P12

Impaired PMCA function causes Ca^{2+} overload and cell damage in CFTR knock out pancreatic ductal cells

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Introduction: The cystic fibrosis transmembrane conductance regulator (CFTR) has a major role in pancreatic ductal secretion and it's genetic defects significantly damage the pancreas. The exact mechanism of this pancreatic damage is only partially known. In cystic fibrosis airway epithelial cells, the intracellular Ca^{2+} homeostasis is disturbed, whereas the intracellular Ca^{2+} overload is a hallmark of acute pancreatitis. However, the connection of cystic fibrosis and intracellular Ca^{2+} signaling has never been suggested in pancreatic epithelial cells before. Therefore, our **aim** was to characterize the Ca^{2+} homeostasis of CFTR-deficient pancreatic ductal epithelial cells (PDEC).

Materials & methods: Wild type (WT) and CFTR knockout (KO) mouse pancreatic ductal and acinar cells and human cystic fibrosis pancreatic cell line (CFPAC-1; ΔF508 mutant) were used for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements. Changes of the mitochondrial membrane potential and mitochondrial morphology was assessed in isolated pancreatic ducts. Immunofluorescent staining and quantitative PCR measurements from whole pancreatic tissue of WT and CFTR KO mice were performed to detect the changes of protein expressions.

Results: Maximal $[Ca^{2+}]_i$ release upon carbachol stimulation showed no difference in WT and CFTR KO PDEC. Notably, the plateau phase of the Ca^{2+} signal was significantly elevated in CFTR-deficient PDEC, but was completely normal in CFTR KO pancreatic acinar cells. The functional inhibition of CFTR with 10μM CFTR(inh)-172 had no effect on the Ca^{2+} signals. The elevated plateau phase in CFTR KO PDEC was caused by the impaired Ca^{2+} extrusion due to the decreased function of the plasma membrane Ca^{2+} pump (PMCA). The same PMCA dysfunction was present in CFPAC-1 cells, which was restored by the corrected CFTR expression by Sendai virus transfection. In mice pancreas PMCA1 and PMCA4 isoforms were expressed, which was not different in WT and CFTR KO mice as revealed by qPCR. The sustained elevation of $[Ca^{2+}]_i$ caused a drop in mitochondrial membrane potential in CFTR KO PDEC without significant morphological alterations. Immunofluorescent staining of WT and CFTR KO pancreatic ducts showed elevated cytochrome c levels suggesting a higher rate of apoptosis.

Conclusion: Dysfunction of PMCA leads to disturbed Ca^{2+} homeostasis in CFTR- deficient PDEC and the consequent cellular Ca^{2+} overload impairs mitochondrial function. These changes might contribute to the impaired ductal function and pancreatic damage in cystic fibrosis.

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P13

Primary human bronchial epithelial cells: electrophysiological experiments to compare impact of culture medium on CFTR activity

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Cystic fibrosis (CF) is the most prevalent genetic disease in the Caucasian population and is caused by a defect in the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein. CFTR is an epithelial ion channel, expressed mainly on the apical site of the membrane, which enables anions to cross the cell membrane. Mutations in the CFTR gene cause the disease and CFTR-F508del is the most common mutation found in patients.

To determine the impact of CFTR modulators on F508del CFTR, many assays are available looking at cell surface expression and/ or functional activity. However, the golden standard methodology shown to be useful to predict clinical efficacy to some extent, is electrophysiological recording on primary human bronchial epithelial cells (hBEC). For this, primary bronchial epithelial cells are seeded on a porous support and allowed to differentiate for 3-4 weeks. The chloride conductance mediated by CFTR can be assessed using the transepithelial clamp circuit technique (TECC), and compounds that improve CFTR-F508del function can be evaluated by measuring resistance or current changes. To measure reproducible effects, it is essential to have a high quality layer of differentiated epithelial cells. Several different protocols to generate such cell layers are available and here we present the comparison of two frequently used differentiation protocols. The effect on the layer resistance, histology, currents and extent of effect of CFTR modulators and epithelial sodium channel (ENAC) inhibitors are presented. In a first protocol, UltroserG is a crucial component of the differentiation medium while the second protocol uses a defined medium that does not contain serum, or serum substitute. Similar H &E stained morphology is obtained with both differentiation media while some differences are observed when comparing the electrophysiological properties. Larger baseline currents were present in cells differentiated with UltroserG, with a strong reduction of this baseline current upon addition of amiloride, suggesting that the higher baseline is in part due to stronger ENAC activity in these cells. In addition, treatment of F508del cells with CFTR modulators (corrector(s) and/ or potentiators) give higher absolute forskolin (FSK)-induced current increases in the UltroserG differentiated cells. However, untreated control cells (containing DMSO alone) showed a significant increase in FSK-induced current in UltroserG differentiation medium while nearly absent in the serum-free medium. Even though the size of current is significantly different in the cells differentiated with the two protocols, upon normalization to the untreated control, comparable effects were measured for dual or triple combinations of CFTR modulators. Moreover, the potency of the CFTR modulator(s) was independent of the differentiation media.

Taken together, these data indicate that, although differences between the two differentiation protocols are observed, similar results are obtained when comparing compounds. As it is unknown which of these culture conditions resemble lung epithelia of human most, it is reassuring that both conditions lead to similar interpretations.

P14
A common mechanism for CFTR potentiators

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Cystic fibrosis (CF) is one of the most common life-shortening genetic diseases, affecting 1 in every 2500 newborns of Caucasian origin. CF is caused by loss-of-function mutations in the gene Cystic Fibrosis Transmembrane conductance Regulator (CFTR), which encodes a phosphorylation-activated but ATP-gated chloride channel. Gating defects are present in many pathogenic mutations including deltaF508 and G551D, two common mutations associated with severe form of CF. In the past decade, high-throughput drug screening has discovered a CFTR potentiator, VX-770 (Ivacaftor), which is now an FDA-approved treatment for a fraction of patients with CF. Lately, GLPG1837, a novel and potentially more effective CFTR potentiator was identified by Conrath et al. (Pediatric Pulmonology. 2016), but its mechanism of action remains unknown. Here, we demonstrate that GLPG1837 enhances macroscopic wild-type CFTR (WT-CFTR) current by 2.06 ± 0.08 fold (n = 19) in a dose-dependent manner with $K_{1/2}$ ~ 0.4 μM. Microscopic kinetic analysis shows that the open probability of WT-CFTR in the presence of 2 mM ATP is boosted to 0.71 ± 0.01 (n = 9) with open time and closed time constants of 851 ± 55 ms and 328 ± 24 ms, respectively. Interestingly, macroscopic current relaxation upon ATP washout in the continuous presence of GLPG1837 yields a double exponential current decay (t_1 = 1.51 ± 0.36 s, 78 ± 3% and t_2 = 16.23 ± 4.97 s, 22 ± 3 %; n = 7), suggesting a significant proportion of GLPG1837-bound channels undergo ATP-independent opening. Accordingly, a higher activity and prolonged open time (642 ± 121 ms, n = 8; c.f. 150 ± 27 ms without GLPG1837, n = 4) are observed in microscopic currents even tens of seconds after removal of ATP when the channels are treated continuously with GLPG1837. This effect of GLPG1837 on both ATP-dependent and ATP-independent gating leads us to examine if GLPG1837 affects CFTR mutants with distinct gating properties. We observed a deceleration of the non-hydrolytic closing rate on hydrolysis-deficient mutant E1371S-CFTR (t = 129.7 ± 10.5 s and 59.8 ± 9.8 s with and without GLPG1837 respectively; n = 4; $p < 0.05$), and potentiation of mutants with defective NBD-dimerization such as G551D-CFTR (19.6 ± 0.8-fold increase of macroscopic current; n = 18) and deltaNBD2-CFTR (18.0 ± 2.4-fold increase, n = 4). Similar results have been reported previously for VX-770 (Jih et al., PNAS, 2013; Yeh et al., JGP 2015). To test if GLPG1837 and VX-770 may share the same mechanism of action, we carried out experimental protocols that combine VX-770 and GLPG1837. The macroscopic current of G551D-CFTR in the presence of GLPG1837 is 1.89 ± 0.14 fold of that with VX-770 (n = 5). However, in the continuous perfusion of VX-770, addition of GLPG1837 fails to increase the current, whereas acute application of VX-770 to the current potentiated by GLPG1837 decreases the current to the level achieved by VX-770 alone. These results suggest that GLPG1837 and VX-770 may compete for a same or structurally closely related binding site.

P15

The *Slca4a4*$^{-/-}$ mouse displays a muco-obstructive lung phenotype resembling human cystic fibrosis.

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A failure in optimal bicarbonate supply, as seen in the airways of patients with cystic fibrosis, produces severe lung disease due to abnormal mucus maturation and acidic pH of airway surface liquid in the airways. The membrane proteins responsible for bicarbonate movement across airway epithelium include CFTR and TMEM16A channels in the apical membrane and a series of transporters of the SLC4 family in both apical and basolateral membranes. While most of these exchangers are electroneutral, the SCLA4A4 or NBCe1 Sodium/Bicarbonate cotransporter moves a ratio of 2-3 bicarbonate and 1 sodium ion into the cell. The aim of our study was to evaluate if the NBCe1 cotransporter is necessary for electrogenic bicarbonate transport in mouse airways.

Ussing chamber experiments of freshly isolated mouse trachea showed that roughly half of the UTP-evoked anionic current is lost when bicarbonate is replaced with HEPES in the bath solution (-105±11 vs -47±3 $\mu A*cm^{-2}$ respectively). To determine if UTP induces HCO_3^- exit, acutely isolated airway cells from mouse trachea were loaded with BCECF to track pH changes. UTP induced rapid acidification of airway cells which was fully abolished when using a CO_2/HCO_3^- free bath solution (ΔpH -0.21 ± 0.003 vs -0.016 ± 0.005, respectively).

Using a specific NBCe1 inhibitor (S0859) we observed that both UTP-evoked short-circuit current and intracellular acidification were significantly decreased, supporting the involvement of NBCe1 in epithelial anionic secretion. Nevertheless, Ussing chamber experiments in the *Slca4a4*$^{-/-}$ mice could not be performed due to low tissue resistance ($32.7\pm8.7\Omega cm^2$). Preliminary observations of histological tissue samples obtained from 3 week old *Slca4a4*$^{-/-}$ mice showed signs of mucus accumulation in bronchi and lung emphysema.

In summary, our electrophysiological and imaging data show that bicarbonate secretion occurs in mouse airways and appears to be dependent on NBCe1 cotransporter function. Genetic silencing of NBCe1 produces a lung phenotype with features often observed in cystic fibrosis human patients and could be due to restricted bicarbonate delivery and acidification of airways.

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P16

Sodium in the apical solution mediates downregulation of ion transport in cultured pig tracheal epithelia

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Respiratory epithelium controls transepithelial ion and fluid transport for maintaining homeostasis of airway surface. Whether the respiratory epithelium could sense and respond to surface fluid changes is vastly unknown. To test this hypothesis, the apical surface of cultured and differentiated pig tracheal epithelia in Millicell inserts was gently washed with the Krebs solution for 3 times, followed by adding 40 µl the Krebs solution for 1 to 24 hr incubation. Our data demonstrate that the short-circuit currents (Isc) of epithelia at basal condition (Isc_{-Basal}) and Isc changes by amiloride (ΔIsc_{-Amil}) due to inhibition of the epithelial sodium channel (ENaC) both were gradually decreased at 4, 7 and 10 hr of incubation, compared to that of the control epithelia with no treatment. However, Isc_{-Basal} and ΔIsc_{-Amil} were recovered back to the control values after 24 hr of incubation. Moreover, the Isc changes by forskolin and IBMX or by Cl^- transporter inhibitors GlyH-101, bumetanide and DPC all displayed similar time-dependent attenuation but with mild effects. These data suggest that ion transport of tracheal epithelia was reduced in response to wash and incubation of the apical Krebs solution. To further explore the mechanism leading to decreased Isc_{-Basal} and ΔIsc_{-Amil}, we either washed or directly incubated the apical side of tracheal epithelia with the Krebs solution or with the salt-free mannitol solution. The data indicate that after 7 hr, wash or incubation with the Krebs solutions all decreased Isc_{-Basal} and ΔIsc_{-Amil}, but with the mannitol solutions both treatments were without effect. By removal of individual Na^+, Cl^- or HCO_3^- in the Krebs solution, our data demonstrate that only the Na^+-free solution did not decrease Isc_{-Basal} and ΔIsc_{-Amil}. These data suggest that the underlying mechanism requires apical Na^+ and could be initiated quickly during the time of surface wash. Our further experiments found that pretreatment of cultured epithelia with the ERK inhibitor U0126 or the AMPK inhibitor compound C in the bottom medium largely attenuated the reductions in Isc_{-Basal} and ΔIsc_{-Amil} of tracheal epithelia by the apical Krebs solutions. In addition, the mRNA levels of ENaC-β and -γ subunits rather than other ion transporters were significantly reduced after 7 hr incubation and returned to normal at 24 hr, compared to that of the control with no treatment. Taken together, our data suggest that fluid challenge on the luminal surface of the tracheal epithelium may reduce transepithelial Na^+ transport, which is mediated by Na^+ in the apical solution resulting in activation of the ERK and AMPK signaling pathways and downregulation in the mRNA levels of ENaC-β and -γ subunits.

P17

A paediatric primary nasal epithelial cell culture model system to investigate bypass channels for CF therapy

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In cystic fibrosis (CF), dysfunctional CF transmembrane conductance regulator (CFTR) protein leads to defective airway apical chloride and bicarbonate transport, resulting in a dehydrated and acidic airway surface liquid (ASL), impaired mucociliary transport and progressive lung disease. An alternative chloride/bicarbonate channel, anoctamin 1 (ANO1), was recently identified as an essential component of the calcium activated chloride channel. Modulation of ANO1 could mitigate ASL defects and represents a promising target for CF therapy.

Robust experimental models are required to investigate this in the paediatric CF population, where it is recognised that disease processes occur early in life. Although primary bronchial epithelial cells (PBECs) have been pivotal in CF research, they are sampled using methods that require a general anaesthetic in children. Careful consideration and limitation of invasive procedures in children is of paramount importance. Primary nasal epithelial cells (PNECs) can be cultured from nasal mucosal brushings and are more accessible. However, characterisation of paediatric CF PNEC cultures has been limited to date.

To assess paediatric PNEC ion transport properties and determine if they are a representative research tool, we sampled nasal brushings from children with and without CF. These were used to establish PNEC air liquid interface cultures to provide a differentiated, pseudo-stratified epithelium. Histological assessment and immunostaining of relevant epithelial markers demonstrated typical epithelial characteristics.

Ussing chamber techniques were used to investigate short circuit current (I_{sc}) responses in paediatric PNECs. All CF PNEC donors displayed an amiloride-sensitive I_{sc} (ΔI_{sc}=14.53 μA/cm^2, ±10.8; mean ±SD n=5 donors) indicating inhibition of the epithelial sodium channel, ENaC. Addition of UTP, an activator of calcium activated chloride secretion, increased I_{sc} (ΔI_{sc}=11 μA/cm^2, ±7.9, n=5 donors). Responses were similar in wild-type PNEC donors, however in these, but not CF PNEC donors, the CFTR activator, forskolin, produced a robust I_{sc} increase (ΔI_{sc}=13.94 μA/cm^2, ±8.4, n=2 donors), which was inhibited by the CFTR inhibitor, CFTR(inh)-172 (ΔI_{sc}=11.48 μA/cm^2, ±4.7, n=2 donors).

Preliminary work was performed to characterise ANO1 activity in these CF donors. The UTP-senstive I_{sc} was reduced by the ANO1 inhibitor, CaCCinhA01, in 2 donors, indicating presence of ANO1 activity. The ANO1 activator, Eact, induced an I_{sc} increase in 4 donors.

Cytokines such as IL-4 have been shown to modify ion transport in adult PBECs, increase mucin secretion and play a key role in innate airway defense. Our preliminary investigation in CF PNECs of IL-4 basolateral application showed a 7-fold increase in UTP-induced I_{sc} (n=1 donor) and a 2-fold elevation in Eact-induced I_{sc} (n=1 donor).

These findings have shown that paediatric PNECs display analogous ion transport properties to adult PBECs and suggest a valid experimental model. This has significant implications for the future of CF therapeutic evaluation and development in children. Furthermore, our preliminary work has demonstrated activation of ANO1 with Eact. Compounds that target ANO1 may therefore ameliorate CF ASL defects. Future work will determine the effects of ANO1 activators using this model as a potential therapeutic avenue to improve ASL homeostasis.

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P18

Involvement of the Na^+-bicarbonate-cotransporter in the airway surface liquid pH regulation of primary bronchial epithelial cells

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Background: There is growing evidence for an acidic airway surface liquid (ASL) pH in CF and although, both CFTR and the H^+/K^+-ATPase ATP12A have been implicated in pH regulation, other transporters and ion channels might also be involved. Moreover, it has been shown in other epithelia that the Na^+-Bicarbonate-Cotransporter (NBC) is involved in transcellular HCO_3^- secretion but little is known about the potential role for this transporter in ASL pH regulation. Therefore, the aims of this study were to (i) develop a method allowing us to make dynamic measurements of the ASL pH in ALI cultures of bronchial epithelial cells in order to (ii) evaluate the role of the NBC in ASL pH regulation.

Methods: The ASL of CF and non-CF primary bronchial epithelial cells, grown on semi-permeable supports, was stained with a mixture of pHrodo, a pH-sensitive dye (0.1mg/ml, ex: 565, em: 585) and AlexaFluor488 (0.1 mg/ml, ex: 490, em: 525), diluted in a modified Krebs solution (KRB), overnight at 37°C, 5% CO_2. The following day, the plate was transferred to a temperature and CO_2 equilibrated plate-reader (TECAN Spark 10M) and readings were performed every 5 min. After 2hr of baseline readings, cells were then treated basolaterally with forskolin (10µM) for another 2hr before an NBC inhibitor (S0859, 30µM) was added to the cultures. At the end of the experiment, a standard curve was established, using the same mixture of dyes, in order to calculate ASL pH from fluorescence values. The standard curve, obtained with highly buffered solutions, was linear between pH5.5 and pH8.

Results: Our results show that non-CF primary epithelial cells responded to forskolin (FSK, 10µM) by a rapid increase ($t_{1/2}$=25min, ΔpH=0.38±0.09, p=0.034, n=5 donors) in ASL pH that was sustained over at least 6 hours. Expectedly, the cAMP agonist had no effect on the ASL pH of primary CF cells (ΔpH=0.05±0.02, p=0.142, n=5 donors). When non-CF cells were pre-treated with S0859 for 2hr, FSK-induced increase in ASL pH was profoundly reduced. However, S0859 alone did not have any effect on ASL pH in non-CF and CF primary epithelial cells. Finally, the addition of S0859, after FSK-induced alkalinisation, caused a 25 ± 9.7% decrease in ASL pH (ΔpH=-0.07±0.02, p=0.037 compared to FSK-induced alkalinisation, n=5 donors).

Conclusion: Our new technique provides stable ASL pH measurements in primary airway epithelial cells under thin film conditions and gives an insight into the kinetics of the responses to different agonists and inhibitors. Here, we show that forskolin induced an alkalinisation of the ASL in non-CF but not in CF primary epithelial cells. More importantly, we show for the first time the involvement of NBC in this response. Our method will allow us to identify new molecular entities involved in the regulation of ASL pH and potentially new therapeutic targets.

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P19

A new animal model for epithelial ion transport modeling (focusing on CFTR) - characterization of pancreatic ductal fluid and bicarbonate secretion in wild type ferrets

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Introduction: Cystic fibrosis (CF) is a lethal genetic disease affecting several organs, including the pancreas. Several animal models are available to study the CF related tissue damage although they have clear limitations. Recently a cystic fibrosis transmembrane regulator (CFTR) knock out ferret model was generated. The KO ferrets born with a normal pancreas, however, within a short period they develop a CF related pancreatic damage. This model would be the first available one to study pharmacological prevention of the disease development.

Aim: We aimed to characterize the fluid and bicarbonate secretion of wild type (WT) ferret pancreatic ducts.

Methods: Intra/interlobular pancreatic ducts were isolated from the WT ferret pancreas. Expression of CFTR was detected by immunohistochemistry. Resting pH, buffer capacity and Cl^-/HCO_3^- exchange activity were evaluated by microfluorometry. To measure the resting intracellular pH of pancreatic ductal epithelial cells (PDEC) were exposed to standard HEPES solution (pH 7.4), followed by an exposure to a high-K^+-HEPES solutions with the concentration of 10μM Nigericin Buffer capacity was calculated by measuring ΔpH_i in response to different concentrations of NH_4Cl^-/HCO_3^- pulses in Na^+-free solutions. Fluid secretion was examined by video microscopy.

Results: CFTR was expressed on the luminal membrane of ferret pancreatic ducts. The resting intracellular pH of pancreatic epithelial cells is lower (7.17±0.08) in ferrets compared to mice (7.31) or to guinea pigs (7.36). Concerning the bicarbonate influx mechanisms, functionally active sodium/hydrogen exchanger and sodium/bicarbonate cotransporter were detected. Anion exchanger activity measured by $NH4Cl^-$technique, Cl^- removal and inhibitory stop methods indicated that ferret pancreatic ducts secrete similar amount of bicarbonate as mice and guinea pigs. Video microscopy revealed a significant increase in fluid secretion to HCO_3^- and to 5μM forskolin stimulation.

Conclusion: Major epithelial ion transporters are expressed in the ferret pancreatic ductal epithelial cells. Our results indicate that ferret could be a suitable model organism to study the CF style pancreatic damage. Moreover this model could open up the possibilities to test pharmacological interventions in the disease development.

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P20

ENaC internalization by SPX-101 is a novel CF therapy for all CFTR mutation.

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Background: Hyperactivation of the epithelial sodium channel (ENaC) in the respiratory airways strengthens the pathophysiology of CF. In normal lungs, short palate lung and nasal epithelial clone 1 (SPLUNC1) is secreted to the airway surface liquid and binds to ENaC inducing its internalization. This mechanism regulates ENaC concentration in the plasma membrane. In CF, the acidic pH impedes the binding of SPLUNC1 to ENaC resulting in hyperabsorption of Na^+, dehydration of the airways, and impaired mucociliary clearance. Here we present SPX-101, a SPLUNC1-derived peptide that internalizes ENaC regardless of the pH.

Objectives: To investigate the in vitro and in vivo effectiveness of SPX-101.

Methods: ENaC internalization by SPX-101 in primary human bronchial epithelial cells (HBEC) from healthy and CF donors was assessed by surface biotinylation and western blot analysis. SPX-101 specificity was measured by TAMRA-tagged SPX-101 binding to βENaC-expressing cells vs mock transfected or expressing ASIC1/2 (most homologous ENaC ion channel) cells. Amiloride sensitive current was determined in healthy and CF HBEC by transepithelial voltage (Vt) and resistance (R) EVOM measurements. SPX-101's in vivo therapeutic effect was assessed by mouse survival curves and by analysis of leukocyte distribution in broncho-alveolar lavage fluids of a CF-like mouse model, the βENaC transgenic mice.

Conclusions: SPX-101 binds selectively to ENaC and promotes internalization of the alpha, beta, and gamma subunits. SPX-101 -promoted ENaC internalization decreases amiloride-sensitive current. Once-daily intranasal dosing of SPX-101 to the βENaC transgenic mice increases survival to more than 90% and also reduces neutrophil and eosinophil infiltration into the lungs. Taken together, SPX-101 represents a novel peptide-based therapy to treat all patients with CF regardless of the CFTR mutation.

P21

Cigarette smoke-induced aggregation of CFTR into ceramide platforms is ROS-dependent

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The cystic fibrosis transmembrane conductance regulator (CFTR) anion channel is required for cAMP-stimulated fluid secretion across airway epithelia and enables inhaled substances to be removed from the lung by mucociliary clearance. Airway epithelia are exposed to many environmental contaminants during normal breathing including cigarette smoke. To examine the acute effects of cigarette smoke on the behavior of CFTR at the airway surface, we examined the distribution, aggregation state, and mobility of fluorescently-tagged CFTR (GFP-CFTR) on primary human bronchial epithelial (pHBE) cells that had been exposed to cigarette smoke extract (CSE). We showed previously that some CFTR occurs in sub-resolution clusters which are homogeneously distributed on the surface of pHBEs under control conditions and obtained evidence for two dynamically-distinct populations. One CFTR population had small spatial scale transport dynamics and confinement that was dependent on membrane cholesterol, consistent with localization within lipid rafts. The other population had larger spatial scale dynamics indicating lateral mobility both outside and within rafts. Here we report that acute exposure to CSE causes a significant redistribution of CFTR between these two populations, which is mediated by recruitment into nano-scale clusters and also fusion of the clusters into large platforms (0.5-5 μm).

Acute CSE exposure caused the appearance of conspicuous platforms along with 3.5-fold increases in CFTR aggregation and confinement and a 2-fold increase in total surface expression. Since cigarette smoke contains oxidants and can potentially stimulate the intracellular production of reactive oxygen species (ROS), we examined the role of ROS in the CSE-induced changes in CFTR distribution, aggregation state, and mobility by pre-treating cells with the antioxidant N-acetylcysteine (NAC). NAC prevented platform formation and abrogated increases in CFTR aggregation and confinement, suggesting that CSE effects on surface CFTR are strongly dependent on ROS, most likely through stimulation of membrane ceramide production by acid sphingomylinase (aSMase) and formation of ceramide platforms.

To further examine the role of ROS in the response of CFTR to CSE, cells were loaded with a newly developed, cell-permeant ROS indicator and studied by confocal microscopy. A gradual increase in probe fluorescence requiring ~1 h was detected during CSE exposure indicating a slow increase in ROS concentration. Pre-treatment with NAC prevented this increase in fluorescence, confirming that the probe provides a measure of intracellular ROS concentration. By contrast, acute H_2O_2 addition to the bath solution caused immediate, step-like increase in probe fluorescence as predicted since cell membranes are highly permeable to H_2O_2. Together these results imply that the slow accumulation of ROS observed during CSE exposure reflects intracellular production by NADPH oxidases and/or mitochondria.

In summary, acute CSE exposure induces ROS production and the aggregation and confinement of CFTR within ceramide platforms. This transient increase in surface expression may enhance the secretory response that rids the epithelial surface of noxious substances.

P22

The Ca^{2+}-activated Cl^- channel anoctamin 1 (ANO1/TMEM16A) mediates Cl^- secretion in rat airway epithelia

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There is a variety of animal models available to study cystic fibrosis, a disease caused by mutations in the CFTR gene leading to impaired Cl^- secretion. However, the applicability of these models is limited as CFTR knockout mice and also CFTR-deficient rats fail to recapitulate phenotypic features of the human disease in the lung. One reason may be compensatory effects of Ca^{2+}-mediated Cl^- secretion in airway epithelia. The purpose of this study was to investigate whether the chloride channel ANO1 accounts for the molecular basis of Ca^{2+}-activated Cl^- conductance in rat airway epithelia. Furthermore, the spatial expression of the chloride channels, ANO1 and CFTR, and the sodium channel ENaC was studied in tissues of the respiratory system of rats.

For immunohistochemical stainings, respiratory epithelia of the nose, trachea and lungs of rats were co-stained using anti-ANO1, anti-CFTR and anti-ENaC antibodies in combination with markers for different epithelial cell types (e.g. ciliated cells, secretory cells). Rat tracheal epithelial cells were cultured under air-liquid culture conditions for at least 14 days and the short circuit current (I_{SC}) was subsequently measured in Ussing chambers.

Immunohistochemical stainings in rats showed a colocalization of ANO1 and CFTR in secretory epithelial cells of the lower airways. Both chloride channels were not expressed in ciliated cells. While ANO1 was expressed in secretory cells throughout the whole conducting airways, CFTR was only expressed in the lower airways. The sodium channel ENaC was exclusively expressed in ciliated cells. Bioelectrical studies revealed that the UTP-induced increase in I_{SC} reflecting the Ca^{2+}-activated Cl^- secretion was significantly inhibited by 95% after preincubation with the specific ANO1 inhibitor Ani9 (I_{SC} values of 0,6 ± 0,5 µA/cm² (pretreated) vs.12,6 ± 4,3 µA/cm² (non-treated), $p < 0.003$) in rat tracheal epithelial cells.

In conclusion, chloride channels and sodium channels are expressed in different cell types, suggesting a separation of the pathways of Cl^- secretion and Na^+ absorption in rats. ANO1 inhibitor Ani9 selectively inhibits UTP-induced Ca^{2+}-activated Cl^- currents. We thereby show, that Ca^{2+}-activated Cl^- currents are generated by ANO1 conductance, suggesting a significant contribution of ANO1 to chloride transport in rat airway epithelia.

P23

Confocal reflection microscopy is a promising novel method for the study of airway surface liquid dysregulation in cystic fibrosis

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Background: Measurements of airway surface liquid (ASL) on primary airway epithelial cultures (AECs) grown at air-liquid interface (ALI) identified ASL depletion as a characteristic abnormality in cystic fibrosis (CF) and may be used as an endpoint for preclinical testing of strategies to improve airway surface hydration in patients with CF. Traditionally, ASL height has been determined by confocal fluorescence microscopy after addition of fluorescently labelled liquid to the apical side of the epithelium, however, several hours are required to restore steady state ASL depth after this volume challenge.

Objective: To determine the suitability of confocal reflection microscopy as a novel approach to study ASL depth and regulation without the need of adding fluorescently labelled liquid to the apical side of primary airway cultures.

Methods: Primary AECs were isolated from βENaC-overexpressing mice as a model of CF lung disease and their wild-type littermate controls and cultured at an air-liquid interface. ASL height was measured simultaneously by confocal reflection microscopy and fluorescent microscopy as standard of reference and results were compared.

Results: Directly after the addition (t=0h) of the fluorescent dye in a volume of 20 µl, ASL height on AECs from wild-type mice determined by confocal reflection microscopy did not differ from values obtained by fluorescence microscopy (reflection: 32.6±5 µm vs. fluorescence: 30.6±4 µm; n=10; p=0.8). A t=2h after volume challenge, ASL height was reduced (reflection: 5.2±0.4 µm vs. fluorescence: 5.3±0.2 µm; n=10; p=0.9) and at t=24h ASL height recovered to normal levels (reflection: 5.8±0.3 µm vs. fluorescence: 6.1±0.2 µm; n=10; p=0.2) that did not differ between the two techniques. In AECs from βENaC-overexpressing mice, ASL height was similar at early time points (t=0 and t=2h), but remained reduced at t=24h (reflection: 4.4±0.2 µm vs. fluorescence: 4.7±0.4 µm; n=10; p=0.5). Similar to studies in wild-type AECs, ASL height data did not differ between reflection and fluorescence based measurements. Moreover, continuous measurements of unperturbed steady state measurements using reflection confocal microscopy revealed a stable ASL height measured in both wild-type and βENaC-overexpressing AECs for 6 hour. Similar to comparison studies wild type mice derived AECs shows higher ASL height than βENaC-overexpressing cells. In addition to murine primary AECs, measurements are performed also with human primary AECs from healthy patients. Steady state measurements of human AECs showed an ASL height of 12.8±0.9 µm and with the basolateral addition of Benzamil, due to blocked Na^+ and liquid absorption ASL height was increased to 15.3±1.9 µm (n=4) showing that reflection confocal microscopy is capable of measuring even the small changes in ASL height.

Conclusions: Our results support that reflection confocal microscopy can be used for accurate measurements of ASL height (dys)regulation without the need of addition of fluorescently labelled dye. This approach may facilitate preclinical evaluation of novel drugs designed to improve airway surface hydration in patients with CF under more physiological steady state conditions.

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P24

Do SK and IK channel subunits form heteromeric channels?

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Potentiation of basolateral membrane K^+ channels is a potential therapeutic strategy to enhance transepithelial ion transport in cystic fibrosis. However, the molecular identity of basolateral membrane K^+ channels in epithelia remains incompletely understood. Channel subunits encoded by the KCNN family of genes have been identified in epithelia, including IK (KCNN4) and SK1-3 (KCNN1-3) (1). Although there is evidence for different SK subtypes to co-assemble to form heteromeric channels (2), the possibility of IK-SK heteromers has not been explored.

HEK293 cells were transiently transfected with plasmids encoding human IK and either rat SK1 or human SK2 channel subunits, together with eGFP as a marker for transfection. Cells were bathed in an extracellular solution containing 140 mM K^+ and expressing cells were whole-cell voltage-clamped at 0 mV using electrodes containing 140 mM K^+ and 1 µM Ca^{2+} (2). Expressed currents were revealed by using voltage ramps from -100 to +100 mV (1 second duration) to generate current-voltage (I-V) relationships.

We chose a mutant form of rat SK1, which produces a channel that is sensitive to inhibition by extracellular apamin. Homomeric mutant rat SK1 channel current is blocked by both apamin and UCL1684, while being insensitive to the IK channel blocker TRAM-34. By contrast, homomeric IK channels are insensitive to both apamin and UCL1684, but blocked by extracellular TRAM-34. Cells expressing both mutant rat SK1 and human IK channel subunits exhibited near linear I-V relationships that were insensitive to supramaximal concentrations of apamin or UCL1684. However, these expressed currents were completely blocked by extracellular TRAM-34, with an IC_{50} of ~ 170 nM. This value is clearly different from the sensitivity of homomeric IK channel current (IC_{50} ~ 20 nM) (3), suggesting that heteromeric channels were formed. Because all expressed current was blocked by TRAM-34, the data suggest that no homomeric rat SK1 channels were present. Co-expression of human SK2 channel subunits with IK produced a current with similar biophysical and pharmacological properties. The finding that all current was sensitive to TRAM-34 suggests that heteromeric channels were preferentially formed.

In conclusion, we propose that an IK-SK heteromer can be formed in HEK293 cells under the experimental conditions used, raising the possibility that the same heteromic channels exist in native cells. Future studies should seek to identify IK-SK heteromultimers in epithelial cells using a similar pharmacological strategy.

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P25

CFTR regulates morphology and cell-cell signaling in islet cells.

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Background: Cystic Fibrosis-related diabetes (CFRD) significantly accelerates lung decline. Autopsy reports from the 1980s of children who died from CF, with and without diabetes, revealed that those with diabetes had significantly altered islet morphology, even in the absence of exocrine fibrosis [Iannucci *et al.* 1984; Soejima *et al.* 1986]. Additionally, CF-deficient ferrets have hampered endocrine pancreatic function at birth with an increase in the number of small islets present in the pancreas [Olivier *et al.* 2012]. Since islet architecture is central to the maintenance of normal patterns of insulin secretion [Halban *et al*. 1982], CFTR-driven alterations in islet morphology may contribute to the impairments in first phase insulin secretion that have been reported in CF patients [De Schepper *et al.* 1992).

Aims: The aim of this study was to investigate if CFTR inhibition altered the way in which islet cells interacted with each other and whether this could potentially lead to alterations in islet function.

Methods: Two CFTR expressing beta cell lines (BRIN-BD11 and MIN6) were used for all experiments with selected data confirmed in primary mouse islets. Cell lines were configured into pseudoislets using established protocols [Kelly *et al.* 2010]. CFTR was inhibited using CFTRinh172 or GlyH101 whilst DIDs was used as an alternative chloride channel blocker. Key gap junction proteins responsible for the maintenance of cell-cell communication within the islet were measured using qPCR. Lucifer Yellow staining was used as an initial measure of the presence of gap junctions..

Results: Pseudoislets grown under control conditions (vehicle) displayed clearly defined borders with little evidence of fragmentation. However, BRIN-BD11 and MIN6 pseudoislets displayed evidence of fragmentation and were smaller in size in the presence of either CFTR-inh^{172} (BRIN-BD11: 43.3 ± 5.4%; MIN6: 53.3 ± 4.1%). or GlyH-101 (BRIN-BD11: 26.7 ± 3.9%; MIN6: 54.2 ± 3.2%). This was accompanied by significant reductions in the expression of gap junction proteins Connexin 36 and E-Cadherin, and reductions in Lucifer Yellow staining in CFTR inhibited cells.

Conclusion: Our results reveal a role for CFTR in the maintenance of islet architecture and the expression of gap junction molecules in the islet. Disruptions of these processes may, in part, contribute to altered islet function in CF.

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P26

Identification of proteins associated with goblet cell hyperplasia

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Mucus accumulation in the airways is a feature of cystic fibrosis (CF), asthma and other respiratory diseases. Mucus hypersecretion and goblet cell hyperplasia are often driven by inflammatory stimuli, particularly the Th-2 cytokines IL-4 and IL-13. Interestingly, these cytokines are also modulators of ion transport in bronchial epithelia. Gene expression profiling on cultured human bronchial epithelial cells treated with IL-4 revealed a profound change in expression and function in multiple ion channels and transporters. IL-4 treatment markedly increased the expression of SLC26A4, TMEM16A, SLC12A2, ATP12A, CA2 and other genes involved in ion transport. Upregulation of these genes was confirmed at the protein level by immunofluorescence combined with confocal microscopy. Interestingly, some of the proteins induced by IL-4 (e.g. SLC12A2, ATP12A) were found in both ciliated and non-ciliated cells. Instead, other proteins (TMEM16A, CA2) were predominantly expressed in goblet cells. At the functional level, IL-4 treatment resulted in enhanced bicarbonate transport ability, as indicated by short-circuit current recordings and intracellular pH measurements. Furthermore, analysis of the composition of the apical fluid collected from cells treated with IL-4 revealed a marked accumulation of bicarbonate and, accordingly, a more alkaline pH compared to control cells. Importantly, we found that the enhanced bicarbonate transport in IL-4 treated epithelia has a relevant role in the mechanism of mucus release. Indeed, incubation of IL-4 treated cells in a bicarbonate-free basolateral solution strongly reduced mucus release in response to purinergic stimulation. A similar inhibition was also observed in CF cells, suggesting that CFTR activity is particularly important under goblet cell hyperplasia conditions. Among the proteins induced by IL-4, the role of ATP12A is particularly intriguingly. ATP12A is the non-gastric form of H^+/K^+-ATPase, responsible for proton secretion at the apical membrane of airway epithelial cells. Its upregulation in cells treated with IL-4 appears to be in contrast with the high bicarbonate levels and the alkaline pH measured in the apical fluid. However, we also found a low K^+ concentration in the apical fluid of cells treated with IL-4, a finding that could be due to K^+ reabsorption mediated by ATP12A. In conditions of reduced bicarbonate secretion, as in cystic fibrosis airways, ATP12A activity may causes abnormal acidification of airway surface liquid. Since CF is also characterized by goblet cell hyperplasia, we asked whether ATP12A is upregulated in CF conditions. We studied ATP12A expression in freshly excised bronchi, obtained at the time of lung transplant from CF and non CF patients. Immunofluorescence experiments carried on histological sections of non-CF bronchi revealed a very low expression of ATP12A. Instead, in CF samples, ATP12A was consistently detected on the surface epithelium, particularly on the apical membrane of non-ciliated cells and in submucosal glands. This finding, together with the observed upregulation of ATP12A under goblet cell hyperplasia conditions, suggests a link between ATP12A and mucus. Pharmacological inhibition ATP12-mediated proton pump could antagonize acidification in CF airways and help to normalize mucus properties and restore antimicrobial activity.

P27

Role and structure-function relationship of the TMEM16A chloride channel

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TMEM16A protein (also known as anoctamin-1) is a calcium-activated chloride channel expressed in epithelial cells of different organs. In the airways, TMEM16A is localized on the surface epithelium and the sub-mucosal glands, where it is abundantly expressed in mucous cells, particularly under inflammatory conditions. TMEM16A may represent an alternative therapeutic pathway to circumvent the reduced epithelial anion transport in cystic fibrosis (CF). This approach could be essential for those cystic fibrosis patients expressing undruggable CFTR mutants but could also be useful as an adjuvant therapy supporting the effect of CFTR rescue maneuvers. However, a conclusive proof that TMEM16A may replace CFTR in its ability to control airway surface liquid properties is missing.

We found that modification of TMEM16A C-terminus leads to proteins with potentiation of channel activity. More precisely, replacement of the entire C-terminal region of TMEM16A with the equivalent domain of TMEM16B confers constitutive activity to the chimeric channel, as evaluated with the patch clamp technique or the HS-YFP. In our experiments, shortening of the chimeric region restricted the "activating domain" to a short sequence close to the last transmembrane domain and led to TMEM16A channels with high activity at very low intracellular calcium levels. To elucidate the molecular mechanism underlying this effect, we carried out experiments based on double chimeras, Forster resonance energy transfer, and intermolecular cross-linking. We also modeled TMEM16A structure using the Nectria haematococca TMEM16 protein as template. Our results indicate that the enhanced activity in chimeric channels is possibly due to altered interaction between the carboxy-terminus and the first intracellular loop in the TMEM16A homo-dimer. Mimicking this perturbation with a small molecule could be the basis for a pharmacological stimulation of TMEM16A-dependent anion transport. Experiments are in progress to express genetically-activated TMEM16A channels in cultured CF bronchial epithelia to assess the effect on airway surface liquid.

P28

Insulin signaling via the PI3K/Akt pathway regulates airway glucose uptake and barrier function in a CFTR-dependent manner

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Cystic fibrosis-related diabetes (CFRD) is the most common co-morbidity associated with cystic fibrosis (CF) and also correlates with increased rates of lung function decline. Since glucose is present in the airways of patients with bacterial airway infections and since insulin controls glucose metabolism, the effect of insulin on CF airway epithelia was investigated to determine the role of insulin receptors and glucose transport in regulating glucose availability in the airway. The response to insulin in human airway epithelial cells was characterized by qPCR, immunoblot, immunofluorescence, and glucose uptake assays. PI3K/Akt signaling and CFTR activity were analyzed by pharmacological and immunoblot assays. Application of insulin stimulated glucose uptake in normal primary human airway epithelial cells, which correlated with organization of the Glut-4 glucose transporter toward the apical membrane. Insulin also promoted airway barrier function as demonstrated by increased transepithelial resistance and decreased paracellular flux of small molecules. This provides the first demonstration that airway cells express insulin-regulated glucose transporters and tight junctions that act in concert to form an "airway glucose barrier." Insulin failed to increase glucose uptake or decrease paracellular flux of small molecules in human airway epithelia expressing F508del-CFTR. Insulin stimulation of Akt1 and Akt2 signaling in CF airway cells was diminished compared to that observed in airway cells expressing wild-type CFTR. These results indicate that the airway glucose barrier is regulated by insulin and is dysfunctional in CF.

P29

The calcium-activated potassium channel KCa3.1 Inhibits sodium absorption in airway epithelial cells

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Airway mucociliary clearence (MCC) is the main mechanism of lung immune innate defence, where its activity maintains the airways free of infection and obstruction. Defects in MCC are present in chronic lung diseases, such as cystic fibrosis, chronic obstructive pulmonary disease, and asthma. Adequate hydration of airways, maintenance of ciliary beating frequency (CBF) in epithelial cells and mucus homeostasis compromise MCC function, and are critically regulated by sodium absorption and chloride secretion in airway epithelial cells. The KCa3.1 potassium channel is essential for chloride secretion in the mouse intestine and its inactivation reduces the faecal water content. However, its role in respiratory epithelium is poorly understood. Therefore, we propose to elucidate the role of KCa3.1 in airway epithelial cells.

Ussing chamber experiments showed reduced amiloride sensitive short-circuit current (I_{sc}) in freshly isolated tracheas from *Kca3.1*$^{-/-}$ mice compared to wild type samples (-3.7 ± 4 vs -19.4 ± 5 μA * cm^{-2} respectively, p = 0.006). This effect was accompanied by a significant change in transepithelial voltage (V_{te}, -3.8 ± 0.8 vs. -2.0 ± 0.4 mV, p = 0.042), leaving basal I_{sc} and transepithelial tissue resistance unaffected. The observed reduction in amiloride sensitive I_{sc} in the *Kca3.1*$^{-/-}$ mice was not due to a downregulation of expression of ENaC subunits in epithelial cells from the mouse trachea evaluated by qRT-PCR. CBF measurements in tracheal epithelial cells were greatly increased in response to UTP in cells isolated from *Kca3.1*$^{-/-}$ mice compared to those isolated from WT. Such increase was also observed when WT cells were incubated with the KCa3.1 inhibitor TRAM-34 or the ENaC inhibitor amiloride previous to UTP stimulation. Similar results were obtained in human bronchial epithelial cells (HBEC) incubated with TRAM-34, as we observed a reduction in amiloride sensitive currents (3.1 ± 0.5 vs 1.6 ± 0.3 μA * cm^{-2} for control and TRAM-34, respectively). Incubation of HBEC with TRAM-34 reduced Il-4 induced goblet cell metaplasia, similar to that observed in a mouse model of chronic asthma.

These results suggest an important and novel role for KCa3.1 epithelial function. Our results demonstrate that KCa3.1 inhibition reduces sodium absorption in both mouse and human epithelium. This effect is independent on changes in ENaC expression and might be explained by changes in cell membrane potential that do not favour the electrochemical gradient for sodium entry. In addition, inhibition of KCa3.1 or direct inhibition of ENaC increased CBF, which could benefit MCC. The mechanistics of such increase are being explored. Inhibition of Il-4 induced goblet cell metaplasia indicates that KCa3.1 function is not restricted to maintenance of electrochemical gradients for ion movement but is also part of the signalling of relevant cytokines. In summary, inhibition of KCa3.1 could be a therapeutic strategy for the management of inflammatory lung diseases with decreased MCC.

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P30

CFTR modulators enhance function R334W-CFTR both in intestinal organoids and conditionally reprogrammed human nasal epithelial cells

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Background: More than 2,000 mutations have been reported in the CFTR gene, albeit most still of unknown functional impact [1]. R334W is classified under Class IV mutation which has defective conductance and this mutation has residual activity as reported before [2].

Objective: The main goal of this work is to assess CFTR activity on rectal biopsies, human intestinal organoids, and conditionally reprogrammed (CR) human nasal epithelial (HNE) cells from three patients with the R334W/F508del genotype and to determine the efficacy of CFTR modulators on the latter two models.

Methods:CFTR activity was investigated on CFTR activity in different tissues/cells from three CF patients with the F508del/R334W genotype, namely in: *i)* rectal biopsies by Ussing chamber using a standard diagnosis protocol [3]; *i)* intestinal organoids by: the forskolin-induced swelling (FIS) assay [4] and quantified by fluorescence microscopy and imaging software; and *ii)* CR-HNE cells by open-circuit measurements in Ussing chamber. The response to CFTR modulators --potentiators VX-770 and Genistein (Gen) alone or with corrector VX-809 -- was also assessed but just on the latter two models, since rectal biopsies cannot be used to study CFTR modulators.

Results and Discussion: Residual CFTR activity was detected in tissues/cells from these three patients, and an increase in CFTR function was also observed both in organoids and CR-HNECs systems by VX-770 and Gen (See Table 1).[tab_01] (n=3, * indicates statistically significant from activity under DMSO, $p \leq 0.05$)

This increase by VX-770 and Gen potentiators was further enhanced in the presence of VX-809. All three biomarkers measuring CFTR basal activity in different tissues from patients with the R334W/F508del genotype (rectal biopsies, intestinal organoids and CR-HNECs) evidenced CFTR residual activity. Moreover, effect to CFTR corrector VX-809 and potentiators Gen and VX-770 evidenced a good correlation between responses to in intestinal and respiratory tissues but also variable responses among different patients with the same genotype.

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P31

Development of automated patch clamp technique to investigate CFTR function

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In order to improve the screening of new pharmacological compounds in CF field, we developed automated patch clamp technique. Indeed, "high throughput screening" techniques are often fast and sensible techniques but with lack of specificity (no discrimination between CFTR and other chloride channels) or without access to specific properties of channel function. To complete these screenings we are developing a planar automated patch clamp (APC) method using the Patchliner NPC-16 system from Nanion Technologies.

Planar automated patch clamp (APC) is a "medium throughput screening" technique with higher resolution than other screening methods, allowing access to accurate and complete electrophysiological properties of ion channels with faster rates than whole cell conventional patch clamp (CPC).

The general principle of APC is identical to CPC but the experimental conditions present some differences. Briefly, in APC experiments, the patch pipette is replaced by a 8 wells borosilicate microchip with two compartments (for extra- and intra-solutions) separated by a small hole. Cells in suspension are applied in the wells and one is retained by aspiration. After giga-seal formation, the whole cell configuration is obtained and recording starts. 8 experiments can be conducted in parallel.

In the present work, we first established the best experimental conditions to record both wt and corrected (Vx809 or 27°C 24h) F508del-CFTR chloride currents with the Patchliner system.

We tested several adherent cell lines stably expressing human wt- or F508del-CFTR. Then we optimized the conditions to reach the best success rate, acting on cells confluence, cells detachment protocol, patch clamp medium used and patch clamp configuration (whole cell or perforated) on the different cell types. Finally, results were compared with CPC results.

The best recordings were obtained using CHO and BHK cells stably expressing CFTR-wt. The activation cocktail Fsk 10µM + Gst 30µM elicited a time- and voltage- independent current fully inhibited by 10µM of $CFTR_{inh}172$. Usingoptimized conditions, we reached a success rate of almost 35% with no significant difference in cell capacitance and series resistance between APC and CPC (respectively for BHK-CFTR cells: Cp=17.6 ± 5.1 pF and Rs=18.8 ± 2.5 mΩ n=11; Cp=23.1 ± 2.8 pF and Rs=12.4 ± 1.4 mΩ n=6). No significant difference in current density was recorded at 0 mV (in presence of Fsk+Gst: 87.01 ± 15.9 pA/pF n=11 for APC and 80.5 ± 14.7 pA/pF n=6 for CPC with a theoretical equilibrium potential for chloride (E_{Cl^-}) of -40 mV).

To conclude, we developed a new tool for medium throughput screening. Automated patch clamp will provide us detailed and accurate informations during compound screening. Parallel patch clamp recording allows rapid and efficient investigation of ion currents with a variety of tests available: precise control of temperature, classic I/V relationship, time-course of current recorded for more than 30 min.

P32

A molecular switch in the scaffold NHERF1 enables misfolded CFTR to evade the peripheral quality control checkpoint

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The peripheral protein quality control (PPQC) checkpoint removes improperly folded proteins from the plasma membrane through a mechanism involving the E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70 interacting protein). PPQC limits the efficacy of some cystic fibrosis (CF) drugs, such as VX-809, that improve trafficking to the plasma membrane of misfoldedmutants of the CF transmembrane conductance regulator (CFTR), including F508del-CFTR, which retains partial functionality. We investi- gated the PPQC checkpoint in lung epithelial cells with F508del-CFTR that were exposed to VX-809. The conformation of the scaffold protein NHERF1 (Na+/H+ exchange regulatory factor 1) determined whether the PPQC recognized "rescued" F508del-CFTR (the portion that reached the cell surface in VX-809--treated cells). Activation of the cytoskeletal regulator Rac1 promoted an interaction between the actin-binding adaptor protein ezrin and NHERF1, triggering exposure of the second PDZ domain of NHERF1, which interacted with rescued F508del-CFTR. Because binding of F508del-CFTR to the sec- ond PDZ of NHERF1 precluded the recruitment of CHIP, the coexposure of airway cells to Rac1 acti- vator nearly tripled the efficacy of VX-809. Interference with the NHERF1-ezrin interaction prevented the increase of efficacy of VX-809 by Rac1 activation, but the actin-binding domain of ezrin was not required for the increase in efficacy. Thus, rather than mainly directing anchoring of F508del-CFTR to the actin cytoskeleton, induction of ezrin activation by Rac1 signaling triggered a conformational change in NHERF1, which was then able to bind and stabilize misfolded CFTR at the plasma mem- brane. These insights into the cell surface stabilization of CFTR provide new targets to improve treat- ment of CF.

P33

The NBD2 mutant N1303K associates with and influences the functioning of the autophagosome

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The missing phenylalanine at position 508 located NBD1 of CFTR is the most common mutation but severe disease-causing mutations also occur in NBD2. To provide information on potential therapeutic strategies for mutations in NBD2, we studied, using a combination of biochemical, cell biological and electrophysiological approaches and the newly created CFBE 41o$^-$ cell line, the disease-causing NBD2 mutant, N1303K. Adding the combination of correctors C4+C18 produces a large increase in the steady state levels of B band of N1303K indicating that they stabilize the B band. To evaluate the effect of C4+C18 on function, we measured short circuit currents for N1303K and found that C4+C18 increases the currents approximately 4 fold. To study the mechanism of correction, we applied E64, a cysteine protease lysosomal inhibitor, and observed that N1303K was not sensitive to E64. But further investigation showed that when treated with a combination of correctors, C4+C18, N1303K became sensitive to E64. To assess whether autophagy is involved with N1303K degradation, we evaluated the ratio LC3-II/I and the absolute amount of LC3-II. We found that there is no statistically significant increase in either the ratio of LC3-II/I or the absolute amount of LC3-II when E64 is applied in the absence of the corrector combination. This surprising result indicates that the rate of autophagy is slow in the cells containing N1303K. A large increase in the ratio of LC3-II /I and the absolute amount of LC3-II in the presence of the corrector combination C4+C18 indicated that the correctors are increasing autophagy. Next, we performed confocal experiments to evaluate the degree of colocalization between the NBD mutants and the LC3 as a marker for autophagosome. Because LC3-II is associated with the autophagosome and LC3-1 is cytosolic we argued that colocalization between LC3 and N1303K, a membrane protein would represent the degree to which both proteins reside in the autophagosome membrane. We found significant overlap in location between LC3 and N1303K in untreated cells. The colocalization is reduced by the combination of correctors. Taken together these data indicated that the combination of correctors in increasing the level of the B band is creating a form of N1303K that is more susceptible to degradation by the autophagosome and by themselves are increasing autophagy. We conclude that correctors have a dual effect particularly on N1303K. They improve trafficking and function at the plasma membrane, but at the same time increase degradation via the autophagosome and lysosome system. Thus, mutations in NBD2 of CFTR when compared to ΔF508-CFTR may require personalized strategies to rescue them.

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Silent mutations affectings CFTRs translational landscape rescue folding mutations

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The rate of protein synthesis modulates the folding efficiency of proteins. Differences in translation rates have been observed, in particular, in multi-domain proteins from different kingdoms of live. The uneven translation speed was found to be crucial for the folding of individual domains and overall functionality of the protein. During evolution transcript sequences have evolved to coordinate translational velocity and cotranslational folding. To gain deeper understanding whether mRNA sequence of the 5-domain protein CFTR, implicated in the pathology of cystic fibrosis, potentially regulates local translation rates, we employed ribosome profiling to learn about codon-dependent ribosomal velocity in CFBE cells. To gain insights into the effect of local alterations of translation velocity on CFTR biogenesis and cotranslational folding, we combined this powerful approach with biochemical assessment of disease relevant mutations combined with a silent polymorphism, T2562G; the latter locally decreases ribosomal speed over the mutated codon.

Investigating CFTR codon usage, we found that codons were not randomly distributed but rather clustered into regions of rare codons and regions of fast codons. Intriguingly, regions composed of rare codons were often arrayed down-stream of domain boundaries or transmembrane helices, regions that likely depend on reduced translation rates. Hence, we determined the velocity of ribosomes on individual codons and observed well-defined regions of slow translation. Thus, the CFTR transcript does not only encode vectorial information for amino acid sequence, but in a second dimension coordinates ribosomal velocity to optimally fine tune cotranslational folding.

Next, we combined the T2562G sSNP with a set of misfolding mutations. While CFTR levels where reduced in WT (e.g. containing only T2562G mutation), combination with disease relevant mutations (ΔF508, G85E, D579G, D614G and N1303K) augmented the steady state protein levels. Limited proteolysis experiments revealed a differential protease susceptibility suggesting an increased stability of the double mutants. Metabolic half-life measurements showed a slight increase in cellular stability.

In conclusion, the cotranslational folding of CFTR is coordinated by the presence of regions of slow translation along the transcript and mutation-induced alterations in the velocity adds to the effect of altered amino acid.

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Role of CFTR in the maintenance of airway epithelial cell differentiation

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Background: There is significant evidence that CFTR expression levels and its plasma membrane traffic are deeply connected to the differentiation status of the cells. Indeed, CFTR localization varies during airway development, from diffusely expressed in the cytoplasm (in immature lungs) to the apical compartment of cells (in mature/adult lung) [1]. Lack of functional CFTR, in turn, influences development (e.g. resulting in tracheal malformations and CBAVD), potentiates tissue fibrosis, leads to impaired secretory cell and epithelial differentiation and delays wound healing [2]. On the other hand, epithelial cell proliferation has been found to be upregulated in CF cells [3]. Consistently, CF patients are described to have an increased risk of cancer [4]. More recently, a dedifferentiation gene expression signature overlapping with epithelial to mesenchymal transition (EMT) was found in the CF epithelium [5].

Objective: We aimed to assess whether absence of functional CFTR disrupts epithelial differentiation, using the airways as a model.

Methods: Cryocuts of human CF (F508del/F508del) and non-CF airway tissue were characterized by immunofluorescence (IF) regarding expression and localization of E- and N-cadherin, cytokeratins 14 and 18, vimentin, ZO-1, SNAIL and TWIST, CFTR. Polarized and non-polarized cellular models (CFBE and HBE) were tested using a similar panel of markers (IF and Western blot). The differentiation state of the cells was also monitored (TEER and calcium-switch assay).

Results and Discussion: IF of airway tissue evidenced downregulation in the epithelial gene signature and an upregulation in the mesenchymal gene signature in CF lung tissue, consistent with loss of differentiation and occurrence of EMT in CF. A proliferation marker (KI67) also showed increased expression in the CF epithelia. In agreement, TEER measurements were lower for CF vs non-CF cells. Overall, the data supports an association between CFTR dysfunction and perturbed airway differentiation pathways.

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P36

Phosphorylation-dependent effect of cigarette smoke extract on CFTR function in respiratory epithelia

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Background: Loss of function of CFTR at the apical plasma membrane (PM) of respiratory epithelia leads to airway surface liquid dehydration, decreased mucociliary clearance, and sustained inflammation, which ultimately result the loss of lung function and constitutes the primary cause of mortality in cystic fibrosis (CF). Chronic obstructive pulmonary disease (COPD), the 4th leading cause of mortality worldwide, considered as an acquired form of CF. COPD is also associated with the functional expression defect of CFTR, which is primarily attributed to cigarette smoke (CS) exposure. Inhibition of constitutive and phosphorylated channel activity was reported by CS- or cigarette smoke extract (CSE) exposure but its mechanism remains obscure. Our goal was to elucidate the molecular basis of the smoke elicited loss-of-function phenotype of CFTR in respiratory epithelia, a prerequisite for designing more effective therapies for COPD.

Methods: CFTR plasma membrane (PM) expression and transport activity were measured in CF human bronchial epithelial cells (CFBE) and papillary lung adenocarcinoma (NCL-H44), expressing WT-CFTR-3HA under the control of tetracycline-controlled transcriptional activation, as well as in conditionally reprogrammed non-CF human bronchial epithelia (CR-HBE). CFTR PM expression and CFTR-dependent chloride transport were determined by cell surface biotinylation or PM ELISA and short circuit current (Isc), respectively. Cellular cAMP level was measured by cAMP ELISA kit.

Results: Unexpectedly we found that acute CSE exposure of CFBE, NCL-H441N and CR-HBE stimulates CFTR-dependent chloride secretion. The CSE mediated activation of the Isc required CFTR expression and was sensitive to the CFTR inhibitor, Inh_{172}. Adenylate cyclase (AC) inhibitors KH7 and SQ22536 significantly decreased the CSE mediated channel activation, implying that the CFTR stimulation by CSE requires the presence of functional AC. In accord, the cAMP-dependent protein kinase A (PKA) -insensitive 15SA-CFTR variant, lacking 15 consensus PKA phosphorylation sites, was resistant to stimulation by CSE. Surprisingly, while the cytosolic cAMP concentration was elevated by >100 fold after forskolin exposure, CSE exposure failed to elicit detectable elevation of the global cytoplasmic cAMP level in CFBE. This latter observation suggests that the elevation of the cAMP concentration may be localized to the vicinity of CFTR due to compartmentalized production and/or impeded elimination of cAMP, thus selectively activating PKA in the macromolecular CFTR signaling complex. In sharp contrast, CSE strongly inhibited the forskolin or isoproterenol (b-adrenergic receptor agonist) activated CFTR Isc. The inhibition but not the activation of CFTR, was reversible by CSE removal. Preliminary data suggest that biochemical downregulation of CFTR from the apical PM cannot account for the acute CSE inhibitory effect of the phosphorylated channel, measured by cell surface ELISA and biotinylation assays.

Conculsion: Acute stimulation of CFTR activity by CSE exposure likely represents a protective mechanisms of the airway to attenuate the toxicity of inhaled particles/chemicals by inducing their elimination through increased fluid secretion. In contrast, the CSE-induced acute inhibition of phosphorylated CFTR, which is likely the predominant form of CFTR in tissue, could contribute to the deterioration of lung function in COPD by impairing MCC and increasing the pro-inflammatory chemokine secretion.

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Characterization of a CFTR-mutant frequently found in Japanese cystic fibrosis patients

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Cystic Fibrosis (CF) is reported to be very rare among Asians (approximately three per million in Japan) and previous reports suggested that the profiles of CF-caused CFTR mutations found in Japanese CF patients are different from Caucasians.

Recently a novel massively deleted mutation lacking the coding sequences along three exons without frameshift (dele 16-17b mutation) has been found in Japanese CF patients with high frequency (13 alleles out of 28 CF alleles) (Nakakuki *et al*. J Hum Genet 2012; 57: 427-433). CFTR protein derived from the dele 16-17b CFTR gene is expected to lack 153 amino acids from Gly970 to Thr1122 (G970-T1122del-CFTR). Most importantly two non-consanguineous CF patients with homozygous dele 16-17b mutation have already been found out of all 29 Japanese CF patients, which suggests a small but significant population with heterozygous dele 16-17b mutation in Japanese. Furthermore, this mutation was found in only Asian patients (Korean and Japanese).

In this study, we attempted to characterize effects of the dele 16-17b mutation on CFTR protein. Our results revealed that the G970-T1122del-CFTR protein was synthesized albeit the massively deleted form, but not be delivered to the plasma membrane. Therefore, this mutation was considered to be Class II defective protein processing mutation. Unlike the F508del-CFTR, representative Class II mutation, a low-temperature rescue and the Corrector VX-809 treatment were not effective against the G970-T1122del-CFTR protein.

There is no CF screening system in Japan and many of Japanese pedestrians have little experience and knowledge about CF, which should lead to an underestimation of CF population in Japan. Our research will improve the understanding of CF and CFTR-related disease in Japanese.

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Identification of traffic factors involved in AFT-dependent CFTR exit from the ER

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Background: The most common Cystic Fibrosis (CF) disease-causing mutation (F508del, present in 70% of CF chromosomes) leads to CFTR misfolding which is recognized by the endoplasmic reticulum (ER) quality control (ERQC) resulting in ER retention and early degradation. The retention of misfolded CFTR at ER is mediated by the exposure of arginine-framed (RXR) tripeptides (AFTs), as previously described [1].

Objective: We aim to identify AFT interactor proteins involved in CFTR exit from the ER that may be used as novel therapeutic targets in the rescue of F508del-CFTR.

Methods: Pull-down assays using Cystic Fibrosis Bronchial Epithelial (CFBE) parental cells were performed to isolate AFT specific interactors. Synthetic peptides conjugated to agarose beads were specifically designed and used to mimic mutated or non-mutated AFT regions in CFTR. Samples were analyzed by LC-MS/MS method and proteins showing differential interactions with the two sets of peptides were selected. The Database for Annotation, Visualization and Integrated Discovery (DAVID - v6.7) and a Gene Set Enrichment Analysis (GSEA) were used to analyzes AFT interactors.

Results and Discussion: A high number of AFT interactors (>1K proteins) was identified. An increase in the interaction fold range were detected for the non-mutated AFT interactors, being 19 gene sets detected as upregulated for non-mutated AFT interactors, in contrast to only 2 gene upregulated for mutated AFT interactors. ER/Ribossomal, cytoskeleton and epithelial mesenchymal transition proteins are the most common pathway categories detected among the identified AFT interactors, with several of them not previously directly associated with CFTR regulation. The identification of the specific CFTR interactors/regulators, and its validation which is in progress, is a promising approach in the identification of novel therapeutic targets that could be ultimately used to the benefit of CF patients.

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P39

Validation of an automated live-cell wound healing assay to identify genes connecting CFTR traffic with epithelia differentiation

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Background: Upon injury, a healthy lung rapidly regenerates and restores epithelial integrity in order to maintain effective lung function. However, in patients with CF these repairing mechanisms are not sufficient to cope with the continuous and extensive damage due to recurrent bacterial infections and associated inflammation. It has been shown that CFTR itself is involved in airway epithelial repair. In previous wound healing experiments, we confirmed a delay in wound closure in CFBE cells expressing F508del-CFTR compared to wt-CFTR expressing cells.

Objective: This project aims to identify CFTR traffic factors which also affect the wound healing process.

Methods: We developed a triple reporter system to monitor CFTR traffic, proliferation and early differentiation to be used in a high-throughput (HT) microscopy wound healing siRNA screen (96-well). We used previously described human bronchial epithelial (CFBE) cell lines stably expressing either wt- or F508del-mCherry-Flag-CFTR under an inducible (TetON) promoter [1]. We stably transfected these cells with 2 different constructs: *i)* the promoter region of Ki67 (pKi-67) fused to GFP and a nuclear localization signal; and *ii)* the promoter region of Cytokeratin 18 (pKRT18) fused to CFP and a mitochondria translocation signal, as markers of cell proliferation and epithelial differentiation, respectively to monitor changes in their promoter activity. The timing of Ki67-signal disappearance/KRT18-signal appearance (i.e., the proliferation-to-differentiation, or "PTD switch") is the readout of the assay which will be validated here to be used in a HT microscopy siRNA screen besides the wound closure rate.

Results and Discussion: We first tested the new reporter cell lines in a pilot screen using control conditions, namely siRNAs targeting genes [2] or compounds previously described to affect cell migration in a scratch-wound assay. We identified that mitomycin C and Rho-kinase inhibitor (Y-27632) act as inhibitor and as stimulator, respectively of wound closure in wt-CFTR expressing CFBE cells. Wound closure rates for mitomycin C and Y-27632 were 2.7%/h and 8.5%/h, respectively, compared to 3.6%/h for control. Furthermore, CSNK1G2, a gene described to accelerate migration, increased (3.7%/h) the wound closure and PRKCE, described as migration inhibitor, also impaired (2.1%/h) gap closure in our system.

As to the triple reporter we confirmed by fluorescence microscopy the expression and correct intracellular localization of the two new reporters GFP-pKi67 and CFP-pKRT18, in parallel with the mCherry-CFTR. We observed GFP fluorescence in the nuclei and CFP in the mitochondria, thus enabling us to distinguish between proliferating and differentiating cells. The impact of expressing wt- or F508del-CFTR on the PTD switch is currently ongoing and will be further validated in a control screen. Moreover, the effect of CFTR traffic hits (» 195 genes) on the PTD switch will also be assessed to define which ones play a role in epithelial differentiation and correct the CF differentiation delay.

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vx-809 and vx-770 modulate the sphingolipid pattern of bronchial epithelial cell lines: effect on CFTR plasma membrane stabilization

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Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel expressed at the apical surface of epithelial cells. Mutations in CFTR gene cause Cystic Fibrosis (CF), an autosomal recessive disease characterized by severe lung disease due to the loss of CFTR at the cell plasma membrane (PM). Many pharmacological agents have been designed to increase the surface level of mutated CFTR (correctors), as well as its PM stability and activity (potentiators), even if their efficacy seems to be time-limited in particular for the most common CF-causing mutation: F508del. Several factors contribute to the PM CFTR stability, including its compartmentalization in the sphingolipid (SL)-enriched lipid rafts and the formation of the multiprotein complex involving ezrin and NHERF-1. Indeed NHERF-1 overexpression increases the wild type (WT)-CFTR apical expression and rescues the F508del CFTR surface expression; on the other hand, NHERF-1 knockdown reduces surface expression of WT-CFTR.

Based on these findings, we investigated the effects of potentiators and correctors on CFTR PM microenvironment.

We analysed the SL composition and NHERF-1 expression in CF and non-CF bronchial epithelial cell lines in relation to the treatment with VX-809 (corrector) and VX-770 (potentiator). In addition, in both cell lines we evaluated the SL pattern of lipid rafts.

The treatment with VX-809 and VX-770 induces in both cell lines an important reduction of phosphorylated ezrin, a reduction of NHERF1 expression and only modest differences in the SL pattern. Interestingly, in lipids rafts from both treated cells, we found a marked increase of all SL species, in particular ceramide, glucosylceramide and ganglioside GM3 that could be responsible for the ezrin dephosphorylation and reduction of NHERF1 expression.

These results indicate that combined treatment with corrector/potentiator induces modification in lipid rafts organization in terms of proteins and lipids, which could be responsible for the limited stability of mutated CFTR at PM level. The results of this study could permit the development of new therapeutic strategies for CF treatment.

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P41

CFTR folding and domain assembly in missense patient mutations

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Correct folding and assembly are required for Cystic fibrosis transmembrane conductance regulator (CFTR) to properly function as an ion channel. This process is disrupted by disease-causing mutations. Problems in folding are relatively well understood for some mutations such as F508del, whereas for most missense mutations this is largely unknown. Understanding the impact on folding of missense mutations is vital for patients, but also will provide crucial information on the folding of wild-type CFTR and related proteins.

Our aim is to exploit the CFTR2 missense mutation database to investigate the effects of mutations on CFTR folding and domain assembly. "CFTR2" provides a broad range of mutations spread throughout the molecule and with a range of defects, from misfolding and rapid degradation to subtle channel-function defects.

As approach we use a combination of biosynthetic radiolabeling and a protease-sensitivity assay in combination with domain-specific antibodies. The results include biochemical conformational parameters on the transmembrane domains (TMDs) and nucleotide-binding domains (NBDs) of CFTR for each missense mutant, which we combined with published functional parameters such as conductance or P_o, and clinical parameters such as sweat chloride levels and FEV1. Correlations between parameters as well as exceptions uncovered how CFTR folds and assembles its domains, and what the requirements are for exit from the endoplasmic reticulum (ER) and for function.

We discovered that mutations in TMD1 or NBD1 were more likely to affect exit from the ER than mutations in the other domains. The median percentage of molecules that had left the ER in the two hours after synthesis was 35% and 5% for TMD1 and NBD1 mutants, respectively, whereas this was 75%, 73%, and 78% for wild-type CFTR, TMD2 and NBD2 mutants.

Although folding of NBD1 and NBD2 did correlate in most mutants, there are notable exceptions in the two TMDs, which did have some form of native-like NBD1 but never acquired the native conformation of NBD2. One fascinating exception is a mutation in NBD1 that destabilized NBD1 and TMD2 more than NBD2 and TMD1.

Overall, we established the folding characteristics of several missense mutations, thereby uncovering the wild-type CFTR folding pathways.

P42

Investigating the effects of second-site mutations in F508del-CFTR on channel function and trafficking.

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Mutations in the CFTR gene, encoding an anion channel, are the cause of cystic fibrosis. The most common mutation is F508del. F508del-CFTR is unable to fold correctly. This causes two problems. Firstly, F508del-CFTR is prevented from trafficking to the cell membrane. Secondly, the function of F508del-CFTR is almost totally impaired. Because of these problems, F508del-CFTR cannot do what it is supposed to do, namely regulate the movement of salt and water in and out of cells that line various ducts in the body. As a result, dehydrated secretions are produced on the surface of these cells, creating a range of problems in the lungs, pancreas, intestines, and other parts of the body.

How can we restore the trafficking and function of F508del-CFTR? It has been shown that the defects caused by F508del, can be partially rescued by other mutations at a second site in the CFTR gene. One of these second-site mutations is R1070W, located in the ICL4 at its interface with NBD1 (Thibodeau *et al.* 2010. *J Biol Chem*. 285(46):35825-35835). In ongoing research we are systematically replacing residues in the ICL4 with tryptophan, tyrosine, phenylalanine, methionine, glutamine and histidine to investigate whether other second-site mutations in the ICL4 can also rescue F508del-CFTR.

To determine the effects of these second-site mutations on F508del-CFTR, we use two assays. The first assay makes use of YFP (H148Q/I152L), a yellow fluorescent protein whose fluorescence is quenched in the presence of I^- (Galietta *et al*. 2001. *Am. J. Physiol. Cell. Physiol.* 281:C1734-C1742). We tagged the YFP (H148Q/I152L) to the N-terminal of the CFTR. The YFP-CFTR fusion protein makes it possible to normalize fluorescence reading to CFTR expression and to relate quenching to the concentration of I^- located at the intracellular side of the CFTR pore.

The second assay is a novel optical assay capable of measuring cellular localization of CFTR. This assay exploits a pH-sensitive red fluorescent protein, pHTomato, fused within an extracellular domain of CFTR. Only when CFTR is localized at the plasma membrane, pHTomato is exposed to the extracellular buffer. Increasing extracellular pH results in an immediate increase in fluorescence, which represents CFTR membrane density. A subsequent NH_4^+ wash neutralizes pH of internal vesicles, inducing a further fluorescence increase, related to the amount of CFTR present in biosynthetic vesicles. Coexpression of eGFP allows normalisation for transfection efficiency.

With these assays we hope to identify second-site mutations in the ICL4 that correct the trafficking and function defects of F508del-CFTR. Together with ever improving structural models/crystals of CFTR, knowledge of which alterations effectively rescue F508del-CFTR might prove valuable insight for mechanistic studies and *in silico* compound screening. Eventually this could lead to the development of better drugs to treat the most common form of cystic fibrosis.

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Inhibition of F508del-CFTR/EDEMs interaction restores a functional but immature F508del-CFTR to the plasma membrane

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ER degradation-enhancing α-mannosidase-like proteins (EDEMs) comprising EDEM1, EDEM2 and EDEM3 belong to the glycosyl hydrolase 47 (GH47) family and play a key role in recognition and sorting to the ER- Associated Degradation pathway (ERAD) of misfolded proteins. To date, no studies have shown the implication of these lectins in the retention of CFTR carrying the most common cystic fibrosis-causing mutation F508del (F508del-CFTR). We studied the interaction of the three EDEMs and showed by a siRNA strategy that decreasing the interaction of EDEM1/ or EDEM2/F508del-CFTR restores a CFTR-chloride activity. Moreover we showed a corrector effect of the deoxymannojirimycin (DMJ), an inhibitor of the GH47 family (Olivari and Molinari, 2007) and their multivalent derivatives, the trivalent-DMJ being the best candidate. Here we further studied the effect of the trivalent-DMJ and its EDEM-dependent mechanism of action on F508del-CFTR correction.

First, using a proximity ligation assay and western-blot on Hela F508del-CFTR cells we showed that the trivalent-DMJ decreases the interaction of F508del-CFTR with EDEM1 and EDEM2 variants, without altering their expression level. Then, we confirmed the corrector effect of the trivalent-DMJ on human polarized bronchial epithelial cells (CFBE) with a restoration of its apical membrane localization by immunofluorescence and confocal imaging and an Isc current of 20 $\mu A.cm^2$ using Ussing chamber techniques. With the fluorescent probe oxonol, we observed a corrector effect of the trivalent DMJ on human respiratory epithelial cells freshly isolated from CF patients. Surprisingly, using western blot technique we did not observe a restoration of the complex glycosylated form of CFTR on treated-Hela F508del-CFTR cells and hypothesized that our corrector restores a functional F508del-CFTR to the plasma membrane in its immature form. To confirm that, we studied the effect of the trivalent-DMJ on F508del-CFTR/ERGIC53 and F508del-CFTR/VIP36 interactions, lectins markers of ER-Golgi intermediate compartment (ERGIC) and Golgi apparatus, respectively. We measured an increase of about 1.7 times of both F508del-CFTR/ERGIC53 and F508del-CFTR/VIP36 interactions in cells treated with the trivalent-DMJ, suggesting the exit of F508del-CFTR from the ER and its transport to the Golgi apparatus. Furthermore, we analyzed surface CFTR using cell surface biotinylation and western blot assays, and confirmed the presence in the plasma membrane of immature form of F508del-CFTR in trivalent-DMJ-treated cells.

To conclude, the trivalent-DMJ corrects the defective trafficking of F508del-CFTR with an EDEM-dependent mechanism of action. Because DMJ is an inhibitor of Golgi-mannosidases enzymes involved in the maturation pattern of glycoproteins, it could explain the absence of the mature form of F508del-CFTR observed on our western-blot. The lack of this N-glycan motif is without effect on a F508del-CFTR activity restoration since the absence of the oligosaccharide moieties is already demonstrated as not essential for channel function (Morris et al., 1993). Finally, our work highlights EDEM1 and EDEM2 as pharmacological targets to develop correctors for F508del-CFTR.

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Understanding the conformational dynamics of CFTR

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Background: A unique feature of CFTR is the presence of the regulatory insertion (RI) in the first nucleotide binding domain (NBD1); a 32 -residue long segment predicted to be intrinsically disordered and extremely mobile, whose physiological role is unknown. Studies have shown that deletion of the RI does not only preserve channel function but also enhances maturation. Furthermore, deletion of the RI thermally stabilizes CFTR allowing the channel to overcome the deleterious effects of the cystic fibrosis causing mutation ΔF508 that is also located in NBD1. Indeed, removal of RI allows cell-surface expression of the DF508 mutant.

These observations lead to immediate questions. What are the structural and functional roles of the RI? Why is a destabilizing and apparently disordered segment conserved in all CFTR orthologs?

Objectives: We aim to identify the conformational landscape of the RI to understand its native function and its relation to NBD1 dynamics, stability and F508. We want to confirm whether or not the RI is unstructured or if it adopts specific conformations that are associated with different functions.

Methods: In order to do so we introduced engineered pairs of cysteines in a cys-less background of NBD1, one of the cysteines located in the RI. We labeled the purified protein with spin labels and used double electron-electron resonance (DEER) spectroscopy to characterize the distance distributions between the cys pairs. To corroborate and complement the obtained data we will also label the protein with fluorophore pairs and perform single molecule FRET measurements.

Results: Initial DEER measurements between 2 cys pairs show the existence of several distances thus indicating that the RI adopts specific discrete conformations. Furthermore, we are attempting to isolate these distinct conformations by using NBD1-specific nanobodies known to significantly influence NBD1 thermodynamic stability. These nanobodies are conformationally selective and their interaction with NBD1 is well characterized by ITC, crystallography and ATP binding assays, allowing for an extensive interpretation of the DEER and smFRET results.

Conclusion: Our preliminary results indicate that the RI is not unstructured but rather a mobile segment that tends to adopt different but distinct conformations. By investigating the modulation of these states by different approaches (F508 deletion, phosphorylation, binding of nanobodies,...) we intend to uncover the allosteric network linking RI to F508 and other parts of the protein.

P45

SLC26A9 is prematurely degraded along with misfolded F508del-CFTR

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Currently available drugs for Cystic Fibrosis (CF) that correct F508del-CFTR misfolding provide only modest clinical benefit, therefore other anion channels such as SLC26A9 are being explored as potential therapeutic targets. SLC26A9 is constitutively active however its channel function is modulated by CFTR and its current is significantly reduced in cells that express F508del-CFTR. Wild-type CFTR is known to interact with the Sulphate Transporter and AntiSigma factor antagonist (STAS) domain of SLC26A9 through its regulatory (R) domain, therefore we examined whether a similar interaction with F508del-CFTR might lead to retention of SLC26A9 in the endoplasmic reticulum and premature degradation by the proteasome.

BHK cells overexpressing wild-type (WT) or F508del-CFTR were transiently transfected with SLC26A9 cDNA and steady-state SLC26A9 protein expression was assessed by immunoblotting cell lysates and by use of a cell surface biotinylation assay. The amount of SLC26A9 protein in whole cell lysates was reduced in cells that co-express F508del-CFTR when compared with those expressing WT-CFTR. SLC26A9 surface expression was also reduced by 4 fold in F508del-CFTR cells expressing cells compared to WT-CFTR cells or control parental BHK cells which are devoid of CFTR. This suggests that co-expression of F508del-CFTR has a more pronounced negative effect on SLC26A9 expression than the complete absence of CFTR. In addition, SLC26A9 expression in whole cell lysates was increased by 1.5 fold in WT-CFTR expressing cells compared to parental BHK cells, and this difference was further enhanced by cAMP/PKA stimulation. Partial correction of F508del-CFTR trafficking by incubation at low temperature or by pre-treatment with the corrector VX-809 elevated both total and cell surface expression of SLC26A9. Expression of SLC26A9 in F508del-CFTR cells was also restored by transfecting cells with WT-CFTR cDNA, and preliminary data indicate this could be mimicked by co-expressing only the R domain of CFTR. Finally, we also found that inhibiting the proteasome pathway increased SLC26A9 whole cell expression in parental BHK cells, similarly to CFTR. These results indicate that SLC26A9 surface expression depends on the trafficking and surface expression of CFTR, and support the notion that CFTR and SLC26A9 physically interact via the phosphorylated R domain of CFTR. This interaction probably causes SLC26A9 to be degraded prematurely in CF cells that express F508del-CFTR. Fully understanding the mechanism of the interaction between CFTR and SLC26A9 will be important for future investigations into the potential use of SLC26A9 as a therapeutic target in CF treatment.

P46

Phosphorylation effects on the solubility and stability of F508del CFTR.

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The F508del mutation results in instability in CFTR leading to premature degradation of the protein and reduced residence at the epithelial membrane. Pharmacological correction of the instability is required in order to provide enough protein for function. Two recent studies of CFTR stability have highlighted the instability of F508del CFTR in cells, macroscopic membrane patches and single channel recordings finding that a sub-population of F508del-CFTR molecules can be rescued from the instability defect whilst the majority cannot [1,2]. Other recent studies of the structure of CFTR have drawn a structure/function link between phosphorylation and the transition from an inactivate/inward-facing conformation to an active, open-ready/outward-facing conformation [3,4].

Here we have extended these studies by examining the effects of phosphorylation on the solubility and stability of the protein. Data has been shown that phosphorylation, the presence of ATP and current Vertex compounds VX-661 and VX-809 increase the solubility of the F508del-CFTR construct in DDM detergent micelles. Phosphorylated F508del-CFTR can be purified in sufficient quantities that stability assays can be conducted on the protein in the presence of compounds such as ivacaftor and lumacaftor. Data has been generated by the use of a CPM dye as a screening component for new compounds aimed at correcting the instability of F508del.

Methods: Expression of a SUMO and GFP-tagged F508del-CFTR construct was done in *Saccharomyces cerevisiae* grown at 20^{o}C. Solubilisation of the protein was carried out in a range of mild and harsh detergents including n-Dodecyl β-D-maltoside (DDM) and lysophatidylglycerol (LPG) . Following solubilisation, purification of the protein can occur using affinity chromatography, either via Nickel-NTA chromatography or GFP affinity. This yields a variety of purity and yield.

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The processing defect of ΔF508- and ΔY512-CFTR are generated by similar mechanisms with different initial flaws

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Mutations on the cystic fibrosis transmembrane conductance regulator (CFTR) may impair its physiological function leading to a genetic disease cystic fibrosis (CF). The most common CF mutation ΔF508, missing a phenylalanine at position 508, markedly disrupts protein processing of CFTR from ER to Golgi apparatus. Whether ΔF508-CFTR processing defect is associated with dysfunction of neighbor residues is unclear. To test this hypothesis, serial single-residue deletions from position 503 to 513 were constructed into human CFTR plasmids for transient expression in HeLa cells. The immunoblotting data demonstrate that fully glycosylated CFTR were abundantly present in the cells expressing wild-type, ΔV510- and ΔS511-CFTR, whereas other deletion mutants including ΔF508-CFTR exhibited mostly less glycosylated form of the proteins, indicating defective protein processing. Among all deletion mutants, only ΔF508- and ΔY512-CFTR showed increases in expression of fully glycosylated proteins by low-temperature culture, CFTR corrector C18 or combination of two treatments. These data suggest that the ΔF508 and ΔY512 mutations may diminish CFTR processing by similar mechanisms. The experiments with alanine substitutions on the loop between the F508 and Y512 residues demonstrate that fully glycosylated ΔF508-CFTR was not significantly altered by introduced G509A mutation, but enhanced by the V510A or V510G mutation and further markedly elevated by the double mutation G509A/V510A. Conversely, introduced mutations V510A, S511A or V510A/ S511A did not promote expression of fully glycosylated ΔY512-CFTR. Our data suggest that loop dysfunction is important for causing ΔF508-CFTR processing defect. These data also suggest that ΔF508- and ΔY512-CFTR may share multiple abnormalities leading to the processing defect, but these abnormalities could be originated from different local flaws.

P48

Dissecting the role of Hsp90 cochaperones in folding of CFTR in Saccharomyces cerevisiae

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Introduction: Maintaining proteome homeostasis is a fundamental and major challenge for the cell. Proteins must fold into their unique, native conformation to function properly. Many human disorders arise from misfolded proteins. Molecular chaperone networks assist folding and mediate retention and degradation upon misfolding, thereby ensuring quality control and protecting proteome integrity. This proteostasis network hence is a therapeutic target for diseases caused by protein misfolding, such as cystic fibrosis caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR).

Heat shock protein 90 (Hsp90) is one of the most abundant chaperones in the cell; it acts in conjunction with Hsp70 and cochaperones, which regulate its ATP-driven cycle and provide substrate specificity. Because Hsp90 facilitates folding of a wide array of cellular substrates, it controls a large number of cellular processes.

CFTR consists of two Trans Membrane Domains (TMD1 and TMD2), two Nucleotide-Binding Domains (NBD1 and NBD2) and regulatory region R. The most common disease mutation is the deletion of phenylalanine at position 508 (ΔF508) in NBD1, which results in CFTR misfolding, retention in the ER and impaired function. CFTR folds its domains mainly co-translationally, while ΔF508 impairs NBD1 folding before CFTR synthesis is complete. Folding of the cytosolic NBD1 domain is limiting in CFTR folding and is a likely target for Hsp90.

Here we aim to identify which molecular cochaperones are involved in Hsp90-assisted folding of the NBD1 domain in yeast, *Saccharomyces cerevisiae.*

Methods:*S. cerevisiae* strains deleted for selected (co)chaperones were transformed with wild-type or ΔF508-CFTR-encoding plasmids. Phenotypic characterization on NBD1 folding was scored by protease-susceptibility assay followed by Western blotting.

Results: We showed that in full-length CFTR NBD1 folds and ΔF508-NBD1 misfolds in yeast as in a mammalian cell. Initial experiments with deleted similar cochaperones Hch1 or Aha1 suggest that they display different roles: Hch1 deletion is immaterial, whereas deletion of Aha1 mildly promotes ΔF508-NBD1 folding.

Discussion and future prospects: Although intensely studied, the precise molecular mechanisms of how chaperones assist in folding the cytosolic domain of multispanning membrane proteins in living cells remains poorly understood. These results indicated that our approach is suitable to dissect the co-chaperone networks involved in NBD1 folding of CFTR in yeast. Further screening is in progress to reveal additional molecular co-chaperones involved in this process. Since NBD1 folding is at the core of the ΔF508-CFTR misfolding cascade, this approach may offer an alternative strategy to identify potential cellular drug targets.

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Biochemical and functional analysis of the cystic fibrosis mutations S549N- and S549R-CFTR and their rescue by CFTR modulators

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S549N- and S549R-CFTR are two CF mutations associated with a severe disease phenotype, which affect a key residue of the LSGGQ motif in the first nucleotide-binding domain of CFTR. To understand better how these mutations disrupt CFTR function and investigate their rescue by small molecules, we expressed them in mammalian cells and performed Ussing chamber, Western blotting and patch-clamp experiments. Fischer rat thyroid (FRT) epithelia expressing S549N- and S549R-CFTR generated residual cAMP-stimulated Cl^- currents that were reduced 2- and 3-fold compared to those of wild-type CFTR. Although S549N-CFTR was without effect on CFTR expression, S549R-CFTR reduced noticeably the amount of mature, fully glycosylated CFTR protein, albeit, the reduction was not as severe as that of F508del-CFTR. Single-channel studies of S549N- and S549R-CFTR in excised inside-out membrane patches from CHO cells at 37 °C demonstrated that these mutations were without effect on current flow through open CFTR Cl^- channels. They also revealed that both mutations were without effect on CFTR stability unlike the impact of F508del-CFTR. However, both mutations disrupted severely CFTR channel gating with S549R-CFTR reducing both the ATP affinity and gating efficacy of CFTR. In the presence of ATP (1 mM) and PKA (75 nM), the open probability (P_o) of S549N- and S549R-CFTR were 10- and 6-fold lower than that of wild-type CFTR (wild-type: $P_o = 0.52 \pm 0.04$, n = 6; S549N-CFTR: $P_o = 0.05 \pm 0.01$, n = 7; S549R-CFTR: $P_o = 0.09 \pm 0.02$, n = 6; $P < 0.05$). To understand better how the mutations disrupt channel gating, we performed an analysis of bursts. Although both S549N- and S549R-CFTR reduced greatly the frequency of channel opening (interburst interval was prolonged 50-fold by both mutations), they also slowed channel closure (mean burst duration was increased by 53 and 93% for S549N- and S549R-CFTR, respectively). These results contrast to the actions of the gating mutations G551D- and G1349D-CFTR, which decrease greatly the frequency and shorten the duration of channel openings (1). Because S549R-CFTR affected both CFTR expression and channel gating, we investigated the rescue of mutant Cl^- channels with the CFTR corrector lumacaftor and the CFTR potentiator ivacaftor. Lumacaftor (3 μM) restored wild-type levels of mature CFTR protein to S549R-CFTR, while ivacaftor (< 1 μM) increased the frequency and duration of S549N- and S549R-CFTR channel openings. Together, lumacaftor (3 μM) and ivacaftor (5 μM) restored wild-type levels of CFTR-mediated Cl^- current to S549N-CFTR, while that of S549R-CFTR was 67% that of wild-type CFTR. In conclusion, S549N-CFTR behaves as a class III mutation that affects channel gating only, whereas S549R-CFTR behaves as a class II-III mutation that impacts both CFTR processing and channel gating. We propose that combination therapy with lumacaftor and ivacaftor likely represents a better treatment strategy than monotherapy with ivacaftor, particularly for S549R-CFTR. Supported by CFFT, NIH and the Research Council of Oman.

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P50
Rescue Δ508-CFTR with nanobodies

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The deletion of the phenylalanine 508 of CFTR leads to misfolding of the channel and prevents its translocation to the plasma membrane. In order to rescue expression of mutant CFTR we propose to develop CFTR-specific nanobodies, which are obtained by isolating the single variable fragment of heavy chain only antibodies found in camelids. Nanobodies have the peculiarities to bind to conformational epitopes and stabilize proteins. After immunization of a llama we obtained nanobodies against the nucleotide binding domain 1 (NBD1) of CFTR. We showed by ELISA that they recognize isolated NBD1 but also the full length CFTR obtained from solubilized membranes. Furthermore they are able to bind cellular CFTR, as shown by FACS experiments on permeabilized BHK21 cells expressing the wt-CFTR. Remarkably, the ability of nanobodies to bind ΔF508-CFTR is strongly modulated by correcting treatment such as VX-809 and decrease of cultivation temperature down to 27°C. Interestingly we show that this effect is not due to a rise of the expression of CFTR but rather to a conformational rescue of the mutated protein. We are able to correlate those data with the trafficking of ΔF508-CFTR at the plasma membrane by using non permeabilized BHK21 cells that express CFTR with an external HA epitope tag (Extope CFTR). Further studies will investigate the potential of the nanobodies themselves to modulate expression of ΔF508. This will be tested by expressing them directly in living cells by transfection or by using exogenously produced nanobodies fused to a cell penetrating peptide (CPP)

P51

Biosynthetic and functional correction of CF-causing NBD2 defects

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While the majority of cystic fibrosis patients have at least one copy of the F508del allele, a significant number of CF patients are compound heterozygotes and/or harbor two non-F508del alleles that affect protein folding, trafficking and function. Drawing on CFTR and ABC-transporter mutation databases, we have identified and profiled a library of mutations in the cytosolic and transmembrane domains of CFTR for their effects on CFTR protein folding and structure. Previous studies have demonstrated that these mutations provide additional insight into the folding pathways of ABC-transporters, including CFTR. Specifically, these analyses provide evidence for hierarchical folding and assembly and suggest specific biophysical mechanisms by which these processes are disrupted. Importantly, these studies also suggest potential mechanisms of correction for CFTR variants that are not adequately addressed by clinically available therapeutics. Building off these observations, we have explored the biosynthetic and functional correction of severe NBD2 mutants, including the N1303K allele. These studies show that stabilization of the NBD-NBD interaction, mediated by specific second-site suppressors in the ATPase active sites, provides biosynthetic and functional correction for multiple mutations in NBD2. Protein trafficking and function are restored when evaluating cell surface localization biochemically and by electrophysiological studies. In addition, this mechanism of correction is independent of those suggested for clinically available therapeutics and suggests that combination therapies, including those currently available, could augment this mechanism to increase correction and potentiation of these CFTR variants. To complement these biophysical and cell biological studies, we have leveraged the recently solved structures of CFTR to evaluate physical insights for rare mutations and their impacts on CFTR. The available structures provide novel insight into mutational clustering within CFTR, suggesting key regions of the protein that should be evaluated in more detail. Together, these studies provide insight into many of the understudied mutations in CFTR and suggest new approaches for small molecule screening and development.

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Molecular dynamics flexible fitting (MDFF) simulations identify new models of closed state CFTR

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The search for CF therapies will be greatly advanced by high resolution structures of CFTR. Recently, a 3.7Å resolution structure for a CFTR ortholog (ABCC7, *Danio rerio* (zebrafish)) was published in *Cell* (PDB code 5TSI) and the coordinates of three other CFTR structures were deposited in the PDB (5UAR: zebrafish CFTR at 3.7Å resolution; 5UAK and 5U71: human CFTR structures both at 3.9Å resolution). All these structures share high structural similarity and all correspond to one conformational state (closed, inward-facing) of the channel. On the other hand, lower resolution structural data (at 9Å maximal resolution) are available for a different state of CFTR (outward-facing) that has been associated with the activated form of the channel. Multiple homology models of CFTR corresponding to the active state have been developed, yet their structures are different. Thus, there is a need to validate and refine these models against additional experimental data. In this work we refine two previously described, outward-facing CFTR models (Mornon et al., *Cellular and Molecular Life Sciences***2015,***72* (7), 1377-1403; Dalton et al., *Journal of chemical information and modeling***2012,***52* (7), 1842-53) using the available cryo-EM map of the *human* wild-type protein, by means of molecular dynamics flexible fitting (MDFF) simulations (a total of 120 ns per model). Despite different starting points, the final models share many common features. Analyzing the resulting MD trajectories (and not just the final models as is usually the case), we demonstrate that the refined models have good stereochemical properties and are in favorable agreement with multiple experimental data (e.g., cysteine cross linking data, salt bridge data, probe accessibility data). Moreover these new models correspond to a closed state of the channel with no membrane traversing pore yet with the two NBDs coupled in a dimeric conformation. Accordingly, analyzing the MDFF trajectories reveals open probabilities of the channel which are significantly lower than those obtained from control MD simulations performed without the EM map constraints. We propose that these models correspond to a CFTR conformation which to date was largely unexplored yet one that is supported by experimental data and forms part of the channel´s gating cycle. Finally we suggest that the combination of high resolution cryo-EM maps which are currently emerging from multiple labs and MDFF simulations will be of value for the development of yet more reliable CFTR models as well as for the identification of binding sites for CFTR modulators.

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Transfer of hematopoietic stem cells improved outcome of *P. aeruginosa* lung infection in a cystic fibrosis mouse model

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Introduction: Lungs of patients with cystic fibrosis (CF) are challenged by recurrent infections and chronic inflammation. For a long time, CF research focused primarily on the epithelial dysfunction caused by the defect in the CFTR ion channel and expressed as surface dehydration, impaired mucociliary cleaning and mucus plugging. However, the question to what extent a *CFTR* based malfunction in professional phagocytes contributes to chronic lung infection and hyperinflammatory immune response is still unsolved. In our current project, we improved the genetically predisposed immune response of CF mice to airway infection with *P. aeruginosa* via transplantation of *Cftr* wild type hematopoietic stem and precursor cells (HSPCs) and started to evaluate macrophages of wild type and CF mice regarding their phagocytic capabilities.

Methods: In transplantation experiments, four classes of mouse chimeras (

Results: Genotyping the *Cftr* locus in PBMCs with a set of informative genetic markers allowed quantification of wild type and mutated *Cftr* in mouse chimeras up to a few percent. Infection experiments revealed reduced lung bacterial numbers as well as increased survival in CF mice which were transplanted with wild type HSPCs

Conclusions: First, we could demonstrate a genotype conversion of more than ninety percent in almost all CF^{B6}, CF^{CF}, $B6^{CF}$ and $B6^{B6}$) were generated by intravenous transfusion of freshly isolated HSPCs. We analysed the *Cftr* locus in blood samples of chimeric mice with wild type and CF sensitive markers to check for genotype conversion due to HSPC transplantation. Mice were then infected in a standardized airway infection model with *P. aeruginosa*, the key organism for CF lung disease using a disease causing, but sublethal infection dose. *In vitro* phagocytosis assays with macrophages isolated from CF and wild type mice were carried out to investigate differences in uptake and killing of bacteria.(CF^{B6}) compared to CF mice which received isogenic cells (CF^{CF}). Cytokine analysis of macrophages harvested from infected lungs by bronchoalveolar lavage fluid (BALF) showed reduced levels of inflammatory cytokines in CF^{B6} mice as well. Vice versa experiments in $B6^{CF}$ chimeras and $B6^{B6}$ controls did not display significant differences in the clinically apparent infection at all. However, looking more closely at the cellular level, we were able to detect different activation profiles$B6^{CF}$ and $B6^{B6}$mice. In contrast, *in vitro* analysis of macrophages in phagocytosis assays did not reveal impaired uptake or killing in CF macrophages compared to macrophages derived from their wild type littermates.CF^{B6} as well as in $B6^{CF}$ chimeras. We therefore conclude that the improved outcome seen in infection experiments was due to HSPC transplantation. Cytokine levels and activation marker profiles of macrophages clearly showed differential activation of macrophages *in vivo*, a finding which was not mirrored by differential phagocytic activity of wild type and CF macrophages *in vitro*. Albeit cellular mechanisms still have to be identified, the transfer of HSPCs significantly improved the immune response towards *P. aeruginosa* infection in CF mice and therefore may even be a therapeutic approach in treating lung infections in CF patients.

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Macrophages from cystic fibrosis patients have normal oxidative status

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Backgrounds: Oxidative stress has been shown to play a significant role in cystic fibrosis (CF), and it is now clearly established that CF patients present high systemic levels of oxidative stress. Nevertheless, even though we know that macrophages play a crucial role in the host defense system against microorganisms, all of their features haven't been studied. More specifically, the oxidative status of macrophage from patients with CF is still unclear.

Methods: Peripheral blood mononuclear cells obtained from healthy donors (non-CF) and stable patients with CF were separated and monocytes were differentiated into macrophages. Cellular oxidants were assessed with the probes H_2DCFDA and BodipyC11. Gene expression of antioxidant enzymes was evaluated by RT-qPCR. Mitochondrial membrane potential (Δψm) was assessed with the probe DIOC6.

Results: There was no difference between reactive oxygen species level in CF macrophages compared to non-CF cells, and lipid peroxidation was significant lower levels in CF macrophages. SOD1, SOD2, GPX1, GPX4 and catalase gene expressions were the same or higher in CF macrophages compared to non-CF macrophages. Finally, there was no change in the Δψm level in CF macrophages compared to non-CF.

Conclusions: Macrophages from patients with CF revealed no exacerbated oxidative stress. The normal gene expression of antioxidant enzymes suggested efficient defense systems. Standard Δψm advocated intact functions of the mitochondria, which would therefore not be responsible for a ROS over-production. CF macrophage may consequently not be involved in the exacerbated oxidative stress commonly observed in CF patients.

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Implication of miR-199A in bronchial inflammation in cystic fibrosis patients

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INTRODUCTION: Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population, caused by CFTR (Cystic Fibrosis Transmembrane conductance Regulator) gene mutations. CFTR encodes a chloride channel, which is essential for the osmotic balance of airway surface liquid and mucus clearance. The most common mutation is F508del, resulting in the lack of the channel at the apical membrane and leading to an ionic imbalance and a thickened mucus. Hyperviscosity resulting leads to an impaired mucociliary clearance promoting bacterial colonization and establishment of infection/inflammation cycles, which in the long term degrade the pulmonary epithelium. Chronic inflammation is the hallmark of CF lung disease. Indeed, it was reported in bronchoalveolar lavage fluids, sputum and in primary airway epithelial cells of CF patients a polynuclear neutrophil invasion and an interleukin-8 (IL-8) hypersecretion. Whereas inflammation origin's is still discussed, previously works have demonstrated that this chronic inflammation is mainly caused by the alteration of the NF-κB pathway (Tabary and al., 1998) which can be regulated by microRNA (miR). MicroRNAs are a class of non-coding small RNA, which most often bind to the 3'UTR of target genes mRNAs and thereby repress their translation and/or induce their degradation and are little studied in cystic fibrosis.

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

METHODS: By an overall analysis of all miR (miRNome), we demonstrated that miR-199a expression was decreased in the bronchial epithelial cells of CF patients. miR-199a is predicted to target the 3´UTR of IKKβ, one of the proteins of the NF-κB pathway.

RESULTS: In this study, we showed on bronchial explants from patients that miR-199a is decreased in CF patients compared to non-CF patients, thus confirming miRNome data. By in vitro studies, we have shown that miR□199a modulates the expression of IKKβ by a direct interaction at its 3´UTR in bronchial epithelial cells from CF patients (CFBE41o). By miR-199a overexpression experiments', we showed that miR-199a decreased the expression of the IKKβ protein, the activity of NF□κB and the secretion of IL-8 in the CF cells culture supernatants.

CONCLUSION: We demonstrated that miR-199a 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. However, from a fundamental point, the origin of miR-199a expression deregulation remains to be studied.

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Inflammasome activation in cystic fibrosis bronchial epithelial cells is exacerbated in hypoxia

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Background: Pulmonary inflammation in cystic fibrosis (CF) is driven by innate-immune cells and the airway surface epithelium. Infiltration of innate immune cells may be orchestrated by a hypoxic bronchial epithelial cell environment, caused by mucous plugging. Prolonged hypoxia causes increased cell death with release of cytokines and danger associated molecular patterns (DAMPs), therefore attracting proinflammatory neutrophils and macrophages. The inflammation may be sterile in nature, but is often exacerbated by persistent pulmonary infections, leading to a high concentration of both pattern associated molecular patterns (PAMPs) and DAMPs in the local environment.

Aim: This project investigated the inflammatory response of bronchial epithelial cell lines, when stimulated with bacterial components in hypoxia. In particular, this project focusses on inflammasome-mediated inflammation.

Methods: Human bronchial epithelial (HBE) cell lines Beas-2b (WT) and IB3-1 (ΔF508/ W1282) were cultured under normoxic and hypoxic conditions, using a hypoxystation (1% oxygen). Cells were stimulated with bacterial LPS (10ng/ml 4h) to prime the inflammasome by inducing pro-cytokine production. The NLRP3, pyrin and NLRC4 inflammasomes were then activated and assembled using specific stimulants; ATP (1mM 1h), *Clostridium difficile* toxin B (TcdB), and *Pseudomonas aeruginosa* flagellin, respectively. Gene expression of inflammasome-related genes (*NLRP3, pyrin, caspase-1, ASC, IL-1b* and *IL-18*) was performed using SYBR-Green RT-qPCR. IL-18 secretion was detected using ELISA.

Results: The inflammasomes were activated at baseline in CF-HBE cells, with increased expression of inflammasome-related genes, whereas there was no increase in WT-HBE cells. IL-18 secretion was also increased at baseline. Furthermore, stimulation with bacterial components resulted in significantly greater increases in inflammasome-related genes and IL-18 secretion in CF-HBE in comparison to WT-HBE cells, suggesting that CF-HBE are hyperresponsive to bacterial stimuli. Both inflammasome gene expression and IL-18 secretion were further elevated in CF-HBE cells when cultured under hypoxic conditions.

Conclusions: Collectively, these data suggest that inflammasome signalling is a key component of the inflammatory phenotype seen in the CF lung and may be a result of the intrinsic defect in CF indirectly influencing inflammasome priming and activation and subsequent IL-18 release. Furthermore, the hypoxic environment caused by mucous plugging, exacerbates inflammasome activation and assembly within CF-HBE cells as well as IL-18 secretion.

P57

The role of low-oxygen-activated (LXA) locus encoded proteins in the pathogenesis of Burkholderia cepacia complex.

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Burkholderia cepacia complex (Bcc), is a group of 20 closely related species of Gram negative bacteria and a clinically important opportunistic pathogen that causes chronic lung infections in people with cystic fibrosis (CF). Bcc is highly antibiotic resistant and Bcc infections are rarely eradicated. One species in particular, *B. cenocepacia* is associated with poor clinical outcomes and an increased rate of mortality. Bcc has been shown to survive and persist in host cells during chronic infection through many mechanisms that are not fully understood.

We have previously found that a series of Irish sequential clinical *B. cenocepacia* isolates from two chronically infected CF patients increased their ability to attach to CF lung epithelial cells over time of chronic infection (Cullen et al., AJRCCM in press). An in-depth proteomic analysis showed that 20 proteins encoded within a cluster of 50 genes on chromosome 2 were consistently up-regulated in the sequential isolates. This cluster was previously designated as the low-oxygen-activated (*lxa)* locus due to its upregulation in response to low oxygen conditions (Sass *et al.* 2013). The consistent up-regulation during chronic infection suggests that this locus is not exclusively associated with low oxygen conditions and may play an important role in chronic infection. Two upregulated proteins of interest were a universal stress protein (pUSP, BCAM0276) and a phospholipid binding protein (PBP, BCAM0280). Single targeted gene deletion mutants of the *pusp* and *pbp* genes were developed in the *B. cenocepacia* strain K56-2, and both mutants showed 90% reduction in attachment to CF lung epithelial cells (CFBE41o$^-$) compared with the K56-2 ($p < 0.005$). Virulence of the *pbp* mutant in *Galleria mellonella* infection model was reduced by over 5-fold relative to K562 wild type ($p < 0.005$), while the *pusp* mutant showed no impairment in virulence. The *pusp* mutant was also more sensitive to peroxide-induced oxidative stress ($p < 0.0001$), and low pH ($p < 0.05$), both of which are relevant to the CF lung environment. These results demonstrate that both proteins previously associated with adaptation to low oxygen conditions may also play a considerable role in Bcc pathogenesis and particularly in the adaptation of Bcc to chronic infection of the CF lung.

Sass, A. M., Schmerk, C., Agnoli, K., Norville, P. J., Eberl, L., Valvano, M. A. & Mahenthiralingam, E. 2013. The unexpected discovery of a novel low-oxygen-activated locus for the anoxic persistence of Burkholderia cenocepacia. *ISME J,* 7**,** 1568-81.

P58

CFTR regulates PTEN-dependent immunity: a role in the cystic fibrosis pathology

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Mutations that compromise either normal protein levels, membrane expression or activity of the Cystic Fibrosis Transmembrane Regulator Channel (CFTR) result in progressive airway damage, recurrent bacterial infection and hyper-inflammation (Cystic Fibrosis, CF). However, the reasons why dysfunctional CFTR is linked to an excessive host immune response remain unknown. In this work we show that CF patients harboring mutations that either reduce de novo CFTR production or the proper folding and transport of the channel to the membrane express significantly reduced amounts of the Phosphatase and Tensin homolog (PTEN), a well-known tumor suppressor and phosphatase. We have confirmed *in vivo* and *in vitro* that PTEN deficiency predisposes to increased airway infection and excessive amounts of pro-inflammatory cytokine production after *P. aeruginosa* challenge, the major bacterial pathogen in CF. By using bioinformatics and biochemical approaches, we predicted and demonstrated by immune precipitation experiments, a molecular interaction between PTEN and the cytoplasmic C-terminal domain of CFTR suggesting that this channel is a scaffolding protein for the phosphatase. *In vitro* functional experiments, were performed to confirm a link between CFTR levels and the anti-bacterial properties of PTEN in human monocytes. The clinical significance of this interaction was demonstrated as PBMCs from CF patients on CFTR potentiators/correctors, as compared with non-treated controls had recovered PTEN levels similar to normals. Our results suggest that the inflammatory damage and the recurrent airway infection associated with *P. aeruginosa* in CF patients is, at least in part, due to diminished association of the tumor suppressor PTEN and CFTR and increasing PTEN levels in the airway epithelium could be a therapeutic target in this disease.

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Early alterations in airway mucins glycosylation and mucociliary clearance: key players in the development of cystic fibrosis lung disease

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Background: Cystic fibrosis (CF) disease is a recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) affecting the rheology of secretions, which become thick and difficult to clear from respiratory airways. It is characterized by chronic bacterial infection, often dominated by *Pseudomonas aeruginosa* (PA), persistent airway inflammation and lung tissue destruction. Debate remains concerning the mechanisms behind this exacerbated inflammation in CF lungs, whether innate or related to persistent bacterial infection. The development of the $CFTR^{-/-}$ pig model, which closely mimics CF human disease, has become an important tool to address this question. Here, we aimed to determine changes in the lung environment that may predispose to PA infection using a pig model of CF.

Methods: Male and female $CFTR^{+/-}$ pigs were mated and the progeny genotyped by PCR. Newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ were sacrificed before and 6h after inoculation with 2 mL of luminescent PA (5 $x10^6$ cfu/mL) into the carina of the trachea. The upper and lower trachea, the proximal and distal bronchi and the bronchoalveolar lavage (BAL) fluid were collected to determine the level of mucins O-glycosylation, PA binding to mucins and the airways transcriptome. Disturbances in mucociliary transport were determined by ex-vivo imaging of luminescent PA (IVIS Spectrum, PerkinElmer).

Results: Our results showed an increased rate of sialylated O-glycans in BAL mucins from newborn non-infected $CFTR^{-/-}$ piglets. Mapping the rate of mucin sialylation through different segments of the airways showed that the highest differences in sialic acid were present in the upper airways. Interestingly, mucins isolated from non-infected $CFTR^{-/-}$ piglets strongly bound PA as opposed to those from non-infected $CFTR^{+/+}$. RNA-seq analysis showed no differences in the expression of most inflammatory genes, ruling out a pre-existing inflammatory condition.

Data from infected animals showed an increased hyper-sialylation in BAL mucins from both $CFTR^{+/+}$ and $CFTR^{-/-}$ piglets. RNA-seq analysis did not show major differences between the genotypes of infected piglets. However, when taken together, the cumulative score of 80 of the transcripts that were upregulated during infection was increased in the $CFTR^{-/-}$ piglets compared to controls. Ex-vivo imaging showed striking differences in PA localization. Six hours after inoculation, PA was mainly found in the larynx, pharynx and nose of $CFTR^{+/+}$ piglets while $CFTR^{-/-}$ piglets were unable to clear PA from the lungs, which suggests a defect on mucociliary clearance.

Conclusion: We observed an increased sialylation of mucins that result in enhanced PA adherence to the CF lung in the absence of any previous inflammation. Increase sialic acid occurs during inflammation, and do not represent a pathological condition by itself, since this increased bacterial adherence to mucins may help to clear the airways under normal conditions. However, we observed a major defect in the mucociliary transport that together with mucin sialylation may contribute to longer time of residency of bacteria on the CF lungs and development of lung inflammation.

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Pseudomonas aeruginosa infection induces alterations in plasma membrane lipid composition in cystic fibrosis airway cells: molecular mechanisms and therapeutic options for CF lung pathology

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Several studies indicate that sphingolipids (SL) play a regulatory role in airway inflammation, the most critical aspect of CF lung disease. Recently, Ceramide derived from glycosphingolipids (GSL) has gained more interest. Indeed, the inhibition of glucocerebrosidase GBA2 is associated with a significant reduction of IL-8 after infection with *P. aeruginosa* strain PAO1. Moreover, GBA2 down-regulation by siRNA causes a reduction of the intrinsic inflammatory state in CF human epithelial bronchial cells (CFhEBC) (Aureli M, Chem Phys Lipids, 2016).

We speculated that in CFhEBC the alterations in lipid composition of specific plasma membrane (PM) complexes, mediated by GBA2 and other GSL-hydrolases, lead to the aberrant inflammatory response to *P. aeruginosa*. To figure out the possible molecular mechanism, we investigated the effect of *P. aeruginosa* infection on specialized membrane area called lipids rafts. Moreover, in order to develop new anti-inflammatory strategies we tested the inhibition effect of different deoxynojirimycin (DNJ) iminosugar derivatives) on GBA2 and their inflammatory properties upon *P. aeruginosa* infection. In CFhEBC and control cells we evaluated the effect of PAO1 infection on: i) activity of SL hydrolases, ii) SL pattern, and iii) lipid rafts organization. The iminosugars used to inhibit GBA2 were characterized in terms of: i) EC50, ii) cell toxicity, and iii) effect on IL-8 expression, as read-out of anti-inflammatory activity. We found that in CFhEBC, *P. aeruginosa* infection causes a recruitment of PM-associated GSL-hydrolases into lipids rafts. At this site, the enrichment of the enzymes involved in the GSL catabolism causes a reduction of the ganglioside GM1, which is followed by an increased levels of Glucosylceramide and Ceramide. Both these events are responsible for the activation of the inflammatory response.

We identified multivalent DNJ C9 iminosugars and racemic mixtures of N-alkylated iminosugars as powerful inhibitors of GBA2. Moreover, these compounds show anti-inflammatory activity at very low dosage (nM), without inducing apoptosis or affecting cell viability.

Summarizing, our data further support the role of GBA2 in the inflammatory response to *P. aeruginosa* infection. The development of iminosugar DNJ derivatives for its inhibition could provide real therapeutic options for CF lung disease.

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Linking cystic fibrosis lung disease pathophysiology with ceramide accumulation and inflammation

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The genetic and functional basis of cystic fibrosis (CF) is well described. However, the exact pathogenesis of CF lung disease (CFLD), the major cause of morbidity and mortality, remains an area of great interest and investigation.

Several previous studies have demonstrated an accumulation of the sphingolipid ceramide in airway tissue from people with CF, and in CF murine models. Ceramide is a key lipid component of the plasma membrane but also plays an important role in numerous cellular signalling pathways. Accumulation of ceramide has been linked with the development of inflammation in several disorders, including CF; although the mechanism linking CFTR dysfunction with ceramide accumulation and its role in driving inflammation remains unknown.

Using differentiated air liquid interface (ALI) cultures of primary human bronchial epithelial cells (PBECs) from people with CF and non-CF controls, we aim to understand the links between CF, ceramide accumulation and inflammation.

Ceramide was elevated in CF PBEC-ALI cultures compared to non-CF controls as assessed by immuno-histochemistry (IHC) and thin layer chromatography (TLC), with a noticeable increase observed in the apical plasma membrane (IHC) and plasma membrane as a whole (TLC).

Sphingomyelinase activity, which catalyses the breakdown of sphingomyelin into ceramide, was increased in CF PBEC-ALI cultures compared to non-CF controls. This effect was observed in both whole cell enzyme assays (4.4% increase in activity), and a novel assay measuring enzyme activity at the apical surface of PBEC-ALI cultures (5.1% increase in activity, n=3, $p< 0.01$). Conversely ceramidase activity, which catalyses the breakdown of ceramide into sphingosine, was decreased in CF PBEC-ALI cultures compared to non-CF controls (Whole cell assay; 3.8% reduction in activity. Apical assay; 3.6% reduction in activity, n=3, $p< 0.01$).

Preliminary data showed a reduction in airway surface liquid (ASL) pH in CF PBEC-ALI cultures compared to non-CF controls (pH 7.5 vs pH 7.2, n=3, $p< 0.01$), which we hypothesise may be a major driver in the above enzyme dysfunction. Other early stage data showed a twofold increase in IL-8 secretion in CF PBEC-ALI cultures compared to non-CF controls as measured by IL-8 ELISA (204 pg/mL vs 424 pg/mL, n=3, $p< 0.01$).

To summarise, we have demonstrated that ceramide accumulation in CF PBEC-ALI cultures is driven by an alteration in sphingomyelinase and ceramidase activity at the apical surface. We propose that this aberrant activity is controlled by alterations in ASL pH arising from CFTR dysfunction. Furthermore, we describe an innate inflammatory environment in CF cells which we propose is driven by ceramide accumulation

Our future work will explore several distinct strands in order to specifically identify the mechanisms linking CFTR dysfunction, ceramide accumulation and inflammation in CFLD; with an overall aim of identifying novel therapeutic targets.

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Regulation of serpin A1 (α1-antitrypsin) and A6 (corticosteroid-binding globulin) in the inflammatory context of cystic fibrosis

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Background: Cystic fibrosis (CF) is characterized by a chronic pulmonary inflammation, which is the main cause of morbidity and mortality. The inflammation is defined by an important recruitment of neutrophils, which secrete high levels of elastase. Some serpins (serin protease inhibitors), produced mainly by the liver, could help to fight lung inflammation: the serpin A1 (α1□antitrypsin, AAT) which inhibit elastase, and the serpin A6 (corticosteroid-binding globulin, CBG), the protein able to address glucocorticoids (GC) to the inflammatory site. AAT is also produced by the lung and several preliminary studies have suggested an expression of a pulmonary CBG, but the nature and the function of this CBG have yet to be defined. The aim of the work is to study both serpins regulation in liver and lung of CF patients in order to optimize their treatment.

Methods: Hepatic biopsies of cirrhotics (cir) CF and non cir CF patients: analysis of CBG and AAT expression; Biopsies of bronchus, bronchioles and parenchyma from CF and non CF patients: measurements of CBG and AAT transcripts; Study of plasmatic levels of serpins from CF vs non CF patients; Pulmonary cell lines: analysis of the regulation of CBG in inflammatory and anti-inflammatory context.

Results: We show an up-regulation of hepatic CBG in CF cirrhotic patients, independently of the cirrhotic status, whereas AAT expression was not changed. At the pulmonary level, CBG and AAT expression patterns in bronchus, bronchioles and parenchyma were different. Furthermore, we demonstrate, in CF patients, a decrease of CBG expression and an increase of AAT expression in the different parts studied. The study of CBG in non CF and CF pulmonary cell lines shows that the protein is regulated by both pro- and/or anti-inflammation, without any difference between CF and non CF cell lines.

Conclusions: Together, the results show a specific increase of hepatic CBG in CF patients. If this up-regulation is confirmed in the plasma, the CBG could potentially represent a way to optimize the use of GC in CF patients. Furthermore, we demonstrate an expression of CBG in lung, with an overexpression in CF patients. This local production of CBG could inhibit GC intracellular actions. Through binding of the GC, the pulmonary CBG would confine the GC and thus, block their anti-inflammatory effects. The decrease of this CBG, observed in CF lung suggests an optimization of GC effects locally. The increase of pulmonary AAT could also participate to the resolution of the inflammation, which is not observed in CF patients.

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Altered airway macrophage phenotype and function in mice with mucociliary clearance dysfunction

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Background: Airway macrophages are important contributors to lung homeostasis through the clearance of surfactants, apoptotic cells and cell debris. Positioned at the interface of host tissue and environment they mediate key responses to inhaled pathogens, through the release of various cytokines. Airway macrophages show a remarkable plasticity with phenotype and function being tightly regulated by the airway microenvironment. In a recent study, we demonstrated that impaired mucus clearance in *Scnn1b*-Tg mice with CF-like lung disease is associated with i) morphological macrophage activation, ii) expression of signatures of alternative macrophage activation, and iii) macrophage elastase (MMP12)-dependent structural damage (Trojanek JB, et al. AJRCMB 2014; 51:709-720). However, the functional role and phenotype of airway macrophages in CF-like lung disease needs further investigation.

Objective: The aim of this study was to characterize the phenotypes of airway macrophages isolated from lungs of *Scnn1b*-Tg mice and wild-type (wt) controls, and to compare responses to stimulation with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* ex vivo. To characterize functional properties of airway macrophages we measured the clearance of apoptotic cells from the lungs.

Methods: To achieve this goal, primary airway macrophages from *Scnn1b*-Tg mice and wt controls were isolated by bronchoalveolar lavage (BAL). Macrophage cultures were exposed to lipopolysaccharide (LPS; 100 ng/ml) or medium alone, supernatants and cells were collected 6, 12 and 24 hours post treatment and expression of macrophage signatures and proinflammatory mediators were determined. To determine the in vivo efferocytosis capacity of airway macrophages, apoptotic cells were delivered intratracheal into mouse lungs and efferocytosis of macrophages was measured by flow cytometry.

Results: In AMs isolated from *Scnn1b*-Tg mice we identified significant changes in baseline expression of genes associated with alternative activation, like Arginase 1, MMP12, CCL17 and CCL22. In addition the M1-associated surface marker CD86 was downregulated in macrophages from *Scnn1b*-Tg mice. Whereas increasing expression of the receptor Dectin-1 and TREM2 further emphasizes M2 polarization in *Scnn1b*-Tg mice compared to wt mice. Exposure of AMs to LPS upregulated key genes involved in proinflammatory and anti-bacterial responses in *Scnn1b*-Tg and wt macrophages. However, expression levels of LPS-induced proinflammatory mediators, like IL-1α, IL-6, IL-12p40, CCL2 and NOS2 were significantly enhanced in AMs isolated from *Scnn1b*-Tg mice. Impaired macrophage clearance of apoptotic cells is associated with inflammatory lung diseases. Here we show that the in vivo efferocytosis capacity of AMs was markedly decreased in *Scnn1b*-Tg mice in comparison to wt controls.

Conclusions: Our findings indicate that a mucostatic airway environment triggers polarization of airway macrophages toward alternative activation at baseline and primes them for augmented cytokine responses when challenged with bacterial-derived LPS. Moreover, defective efferocytosis may result in increased inflammation and pathogenesis. These results suggest that alternative airway macrophages may play an important role in the in vivo pathogenesis of chronic airway inflammation, and that a better understanding of the mechanisms underlying macrophage activation may lead to novel anti-inflammatory therapies for CF.

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The IL-9/Th9 pathway promotes pathogenic inflammation in cystic fibrosis

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In patients with cystic fibrosis (CF), the expression of IL-9 and IL-9R is increased and is associated with mucus overproduction (*Hauber HP et al. Laryngoscope. 2003. 113:1037-42*). In these patients, the primary source of morbidity and mortality is due to a vicious cycle of airways infection and inflammation eventually resulting in lung damage. The inflammatory response in CF is dysregulated at several levels, resulting in inefficient microbial clearance and contributing to lung damage (*Cohen TS & Prince A. Nat. Med. 2012.18:509--519*). This is supported by several studies that have documented an altered balance of inflammatory/anti-inflammatory cytokines in CF, providing evidence that targeting specific inflammatory/anti-inflammatory pathways is a valid therapeutic strategy in CF *(Iannitti RG et al. AJRCCM 2013. 187:609-620*).This balance is essential for the efficient control of *Aspergillus fumigatus* diseases in CF, where the colonization by the fungus is common and may lead to fungal sensitization, bronchitis and allergic broncho-pulmonary aspergillosis (ABPA) as well as worse FEV1 (*Cowley AC, Pediatr Pulmonol. 2016. doi: 10.1002/ppul.23618*). Whether and how IL-9 contributes to immunity and pathology in response to the fungus in CF is not known. Th9 cells, as sources of IL-9, contribute to lung inflammation and allergy (*Koch S et al. Semin Immunopathol. 2017. 39:55-68*). However, the mechanisms behind the IL-9/Th9-mediated immunopathology in the lung is not known. We find that IL-9-driven IL-2 production by mast cells expanded CD25+ILC2 activating Th9 cells that in turn amplified allergic inflammation. By producing IL-9, Th9 cells in turn served as a positive loop amplifying the IL-9/MC/ILC2 axis, promoting a deleterious vicious circle in which the production of profibrotic TGF-β by IL-9-stimulated mast cells plays a plausible important role. Blocking IL-9 or inhibiting CD117 (c-Kit) signaling counteracted the pathogenic potential of the IL-9-mast cells-IL-2 axis. Overproduction of IL-9 was observed in expectorates from cystic fibrosis (CF) patients and a sex-specific variant of IL-9 was predictive of allergic reactions in female patients. This study provides a therapeutic angle to ameliorate the pathological consequences of microbial colonization in the lung and offers a plausible explanation for gender differences in outcomes of patients with CF.

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Regulation of ferroportin expression in cystic fibrosis bronchial epithelial cells is possibly influenced by hypoxia, transforming growth factor-beta and epithelial sodium channel.

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Altered expression of hypoxia and their inducible pathways exert severe damaging effects on airway epithelial cells. There exists a long standing relation between hypoxia and iron homeostasis. We reported that impaired stabilization of hypoxia inducible factor-1α in cystic fibrosis (CF) bronchial epithelial cell lines (CFBE41o-) may contribute towards increased cellular levels of iron by involving HMOX1. However the underlying molecular mechanisms are still elusive. Thus, in this study we quantified the expression of two hypoxia inducible genes transforming growth factor beta (TGF-β) and ferroportin1 (FPN1). Altered expression of TGF-β and FPN1 might potentially be involved in altered iron homeostasis in CFBE41o- cell lines. We identified a significantly decreased expression of TGF-β and FPN1 mRNA and protein levels in CFBE41o- cells. By rescuing the stability of HIF-1α using iron chelators we could restore TGF-β and FPN1 expression in CFBE41o- cells. On the other hand we observed that expression of TGF-β positively correlated with the expression of FPN1 in CF cell lines. We could also link expression of ENaC with ferroportin expression. Moreover inhibition of ENaC restored altered TLR4 and HO-1 expression in CF cell lines. Together, these data demonstrate that regulation of intracellular iron in airway epithelial cells play an important role in maintaining cellular homeostasis.

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Unravelling the DREAMing in cystic fibrosis airway inflammation

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Airway epithelial cells in Cystic Fibrosis (CF) have a pro-inflammatory phenotype facilitated in part by a lack of A20 (Kelly C *et al.* Eur Respir J 2013), which contributes to persistent NF-κB activation. A20 transcription is regulated by DREAM, the **D**ownstream **R**egulatory **E**lement **A**ntagonist **M**odulator. DREAM functions as a transcriptional repressor of A20 through binding to the downstream regulatory elements (DREs) and CF airway epithelial cells (CFBEo- and primary nasal epithelial cells) show increased mRNA and nuclear expression of DREAM.

Hypothesis: In CF, increased DREAM expression contributes to chronic inflammation through repression of the NF-kB regulator A20.

Methods: We used bronchial epithelial cells (16HBE14o- and CFBE41o-)

stimulated with LPS from *P. aeruginosa* (10 µg/ml, 0-24h) and (**a**) determined DREAM-DRE interaction (ChIP assay, ab500) and (**b**) investigated DREAM protein expression in in nuclear extracts (Western Blotting, reducing conditions) using a DREAM specific antibody (ab61770, 1/500, 5% milk).

Results: Basal DRE3-DREAM interaction was significantly increased CFBE41o- compared to non-CF control cells ($p < 0.01$, all Kruskal-Wallis, n=3). 16HBE14o- cells show a significant decrease of DRE3-DREAM interaction 60 min after LPS exposure ($p < 0.05$), but there was no change in the interaction in CF cells. Nuclear expression of DREAM in CF cells was significantly higher than in non-CF cells ($p < 0.001$).

Western Blotting of nuclear cell lysates revealed the presence of DREAM positive bands with a molecular weight of 30 kD and 60 kD, but CFBE41o- cells also showed DREAM positive bands with a molecular weight of 120 kD.

Conclusion: Tiruppathi C *et al*. (Nat Immunol. 2014) showed that following stimulation with bacterial LPS, DREAM is removed from the DRE3 site allowing the transcription of A20. However, our data suggests that in CF airways cells, DREAM remains bound to DRE3, which may result in A20 not being transcribed.

Moreover, when DREAM binds to the DNA it forms dimers, but at high concentrations DREAM tetramers are formed (Osawa *et al*. JBC 2001), which have a higher binding capacity to the DNA than the DREAM dimers. Our data suggest increased presence of DREAM positive tetramers in CF airway cells, which may contribute to the increased DREAM-DRE3 interaction, reduced A20 transcription and subsequent increased NF-κB and inflammation.

P67

Presenilin – a DREAM binding protein - in airway epithelial cells in patients with CF

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In Cystic Fibrosis (CF), an apparent lack of basal and inducible A20 levels is coupled with heightened and sustained inflammatory responses mediated through increased expression of DREAM (Downstream Regulatory Element Antagonist Modulator), a multifunctional protein that behaves as an A20 repressor. Presenilin proteins (PS1, PS2) were first discovered in association with early onset familial Alzheimer's Disease (AD) and have been shown to interact with DREAM. Our previous bioinformatic approach to identify A20 inducing Drug (Malcomson *et al*. PNAS 2016) indicated PS1 as a significant CF disease related gene in air epithelial cells. However, to date, the function of presenilins in airways inflammation remains elusive.

Hypothesis: Elevated PS1 levels in CF epithelial cells contribute to the high nuclear DREAM expression and subsequent repression of A20 transcription.

Methods: We used bronchial epithelial cells (16HBE14o- and CFBE41o-)

stimulated with LPS from *P. aeruginosa* (10 µg/ml, 0-24h) and (**a**) determined PS1 mRNA (by qPCR, Roche) and (**b**) investigated PS1 protein expression (Western Blotting, whole cell lysate reducing conditions) and its subcellular distribution (Immunocytochemistry) using a PS1 specific antibody (ab71181, 1/500, 5% milk).

Results: PS1 mRNA expression was significantly increased in CFBE14o- compared to 16HBE14o- 8h after LPS stimulation ($p < 0.01$, Kruskal-Wallis, n=6), which was confirmed in primary nasal epithelial cells from patients with CF (Phe508del homozygous). Western Blotting of whole cell lysate detected a band of approximately 60kDa (predicted MW: 53kDa) in both cell types. In 16HBE14o- exposure to LPS for 24h increased PS1 in whole cell lysate compared to non-stimulated cells, but this did not reach significance. CFBE14o- showed overall lower PS1 expression than 16HBE14o-, especially after LPS stimulation ($p < 0.05$, Kruskal-Wallis, n=4).

Immunocytochemistry in 16HBE14o-revealed a strong PS1 staining at the cell membrane and intracellularly, which, upon stimulation with LPS, accumulated in and around the nuclear membrane (starting at 10 min post LPS, reversing to control levels at 4h). CFBE41o- showed a similar, but much stronger intra/peri-nuclear staining for PS1 which remained throughout the study period (4h).

Conclusion: The work described here is one of the first to explore the expression of presenilins in CF airway epithelial cells. To date the function of PS1 in airway epithelial cells is unknown, but future work will elucidate potential co-localisation with DREAM or TRAF6. Furthermore, the expression and function of PS1 in primary airway cell and the effect of a lack of PS1 (siRNA) on inflammatory responses will be investigated.

P68

Adaptive microbial interactions between *P. aeruginosa* and *A. xylosoxidans*

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Background: Given the polymicrobial nature of CF lung infection, *Pseudomonas aeruginosa* is often co-isolated with other microbial species sharing the lung environment. In the case of chronic infections, this cohabitation can last for long time; thus, it is likely that *P. aeruginosa* and its neighbors can engage interactions, potentially influencing the course of infection. *Achromobacter xylosoxidans* recently gained attention as an important emerging pathogen that can cause severe chronic infection associated with lung inflammation and decline in respiratory function, further complicated by multidrug resistance. Despite the frequent co-isolation of the two microorganisms, studies about possible interactions lack. We investigated the behavior of co-isolated clinical strains of *P. aeruginosa* and *A. xylosoxidans* with respect to growth and adhesion capability, and their evolution during chronic co-infection.

Methods: We selected *P. aeruginosa* and *A. xylosoxidans* strains longitudinally collected from a chronically co-infected CF patient followed at Rigshospitalet, Copenhagen, Denmark. Genotypic relatedness of longitudinal isolates was verified by pulse field gel electrophoresis and whole genome sequencing profiles. Colony morphology, growth and adhesion were evaluated. Biofilms were grown in flow-chamber system up to 5 days and monitored by confocal microscopy.

Results: The patient was colonized with the same *P. aeruginosa* and *A. xylosoxidans* genotypes for 3 years. During this time, *P. aeruginosa* underwent phenotypic evolution in terms of different colony morphology, lower growth rate and increased adhesion, while *A. xylosoxidans* isolates showed no phenotypic changes and low surface adhesion capability. *A. xylosoxidans* cells or their products released in the culture supernatant could affect growth and adhesion of the co-isolated *P. aeruginosa* strain: an inhibitory effect was observed between the first co-isolates but was lost over time. Although unable to adhere on abiotic surfaces, *A. xylosoxidans* showed adhesion on *P. aeruginosa* cells/extracellular matrix, forming mixed biofilm communities. Inhibitory activity of *A. xylosoxidans* against *P. aeruginosa* biofilm formation was observed only in the first stages of infection.

Conclusions: During long term cohabitation in CF lungs, initial competitive interactions between *A. xylosoxidans* and *P. aeruginosa* seem to evolve towards reciprocal adaptation, which might represent a survival advantage. Although the occurrence of close microbial interactions within the CF lung is not yet clear, our study might help to understand the role of bacterial interactions on survival and persistence in in the human host.

P69

Matrix metalloprotease inhibitors as anti-inflammatory therapy in *Pseudomonas aeruginosa* lung infection

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Background: Excessive and dysregulated secretion of host and bacterial proteases in cystic fibrosis (CF) lung strongly contribute to exacerbation of the inflammatory response and lung damage. *Pseudomonas aeruginosa* secreted proteases interfere with key host processes and degrade lung tissue, thereby promoting lung disease. We evaluated broad spectrum human matrix metalloprotease (MMP) inhibitors as *P. aeruginosa* protease inhibitors, and the effect of bacterial protease inhibition on mice inflammatory response.

Methods: Protease activity was measured in *P. aeruginosa* culture supernatant by azocasein assay. BalbC mice and congenic CFTR deficient and homozygous C57BL/6 mice expressing luciferase gene under the control of bovine IL-8 promoter (bIL-8-Luc) were intratracheally challenged with culture supernatant pre-treated or not with different MMP inhibitors. Activation of exogenous IL-8 promoter was monitored by in vivo bioluminescence imaging.

Results: Hydroxamate-based MMP inhibitors Ilomastat and Marimastat, but not Batimastat, inhibited proteases secreted by a virulent *P. aeruginosa* CF clinical isolate expressing high protease activity. In bIL-8-Luc-transgenic mice, intratracheal challenge with *P. aeruginosa* culture supernatant containing active proteases induced lung inflammatory response in terms of bioluminescence emission in the lungs area. When culture supernatant was pre-treated with Ilomastat or Marimastat before the intratracheal challenge, a lower bioluminescence emission was recorded in all mouse strains, indicating a reduced inflammatory response.

Conclusions: Proteases secreted by *P. aeruginosa* seem to play an important role in the exacerbation of lung inflammatory response. Therefore, their inhibition by protease inhibitors such as Ilomastat and Marimastat might represent a useful additional anti-inflammatory therapy in *P. aeruginosa*-infected patients. Future studies will further evaluate the anti-inflammatory properties of these molecules and the involvement of *P. aeruginosa* proteases in CF lung disease.

P70

SPLUNC1 is pH-dependent and reduces burkholderia cepacia growth in airway surface liquid.

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Introduction: Cystic fibrosis (CF) is a genetic multi-organ disease caused by absent or dysfunctional CFTR mediated anion secretion, which in the lung, leads to mucus obstruction and chronic infection/inflammation. The *Burkholderia cenocepacia* complex (Bcc) is a group of 18 genetically distinct species. Although generally harmless to humans, they are extremely pathogenic to CF patients, and acquisition of Bcc is associated with a significant increase in mortality. Treatment of Bcc infections are difficult as the bacteria are multidrug resistant. Short Palate Lung and Nasal Epithelial Clone 1 (SPLUNC1) is secreted by the airway epithelia and serves as a multifunctional innate defense protein. SPLUNC1 is known to have antimicrobial functions against many gram-negative bacteria including Bcc. Additionally, knockdown/knockouts of SPLUNC1 from normal (NL) airways has resulted in increased *Pseudomonas aeruginosa* growth. Our data indicate that CF airway surface liquid (ASL) is characterized by a decrease in pH which renders SPLUNC1 inactive and unable to regulate the epithelial Na^+ channel (ENaC). We therefore tested the hypotheses that SPLUNC1 is needed in the airways to reduce Bcc growth and its antimicrobial activity is pH-sensitive.

Methods: SPLUNC1 was knocked down in NL primary human bronchial epithelia cells (HBECs) using shRNA. Lavages were collected, incubated with Bcc clinical isolate J2315 at MOI of 30 for 2 h, and colony forming units (CFUs) were determined for bacterial growth. We have recently elucidated the crystal structure of SPLUNC1 and screened novel mutants based on this structure for pH-sensitivity of antimicrobial activity. J2315 was incubated with 0.4 μM SPLUNC1 mutants for 2 h at pH range of 6 to 7.5, and CFUs were determined for SPLUNC1's antimicrobial activity. ASL from NL and CF HBECS were then incubated with 4 μM SPLUNC1 mutants for 2 h. Lavages were collected, incubated with J2315 at MOI of 30 for 2 h, and CFUs were determined for bacterial growth.

Results: Knockdown of SPLUNC1 in NL HBECs showed increased J2315 growth in the ASL compared to wildtype NL HBECs. Under acidic conditions, SPLUNC1's antimicrobial activity against J2315 was impaired, but was restored at pH 7.5. However, SPLUNC1 mutants that are pH-independent ENaC regulators (Q140E, K138D, and K156C) reduced J2315 bacterial growth at a low pH of 6.5. Additionally, while SPLUNC1's antimicrobial activity in CF ASL was impaired, Q140E, K138D, and K156C reduced J2315 growth in the CF ASL to similar levels as seen in the NL ASL.

Conclusions: Our data suggest that SPLUNC1 is needed in the airways to reduce Bcc growth. Additionally, SPLUNC1's antimicrobial activity against Bcc is pH-dependent and SPLUNC1 mutants, that are pH-independent ENaC regulators, also retained pH-independent antimicrobial activity. Whilst the impact of CF on SPLUNC1-Bcc antimicrobial interactions is currently under investigation, our data suggest that understanding this interaction may have important therapeutic applications for CF lung disease.

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P71

Explosive cell lysis is involved in biofilm formation and the biogenesis of staphylococcal "public goods"

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The Gram-positive *Staphylococcus aureus* is a major cause of nosocomial and community-acquired infections, as well as one of the main pathogen colonizing the airways of cystic fibrosis patients. Many of those develop into chronic infections and are difficult to eradicate due to the formation of biofilm - complex multi-cellular structures that enhance antibiotic resistance and evasion of human defences. Biofilms are characterized by cell attachment to a surface and encapsulation in self-produced extracellular polymeric substances, comprised of extracellular DNA (eDNA), membrane vesicles (MVs) and bacterial cytosolic proteins referred as "public goods". These extracellular products enhance the structural integrity of biofilm matrix, protect the community from antimicrobial agents and contribute to virulence and chronic infection.

Since the mechanism of "public goods" production by *S. aureus* has not been fully elucidated yet, we monitored the expansion of Staphylococcal interstitial biofilms by time-lapse microscopy and the release of eDNA using a cell impermeant fluorescent dye specific for DNA. We found that occasionally individual cells within a cluster lysed and at the same time eDNA was produced. Two type of cell lysis were observed: slow lysis, in which DNA remained partially trapped inside dead cells and after several minutes it was released into the extracellular space; and explosive cell lysis, a rapid event occurring within seconds characterized by efficient eDNA released in bursts. Super-resolution microscopy (OMX 3D-SIM) also revealed the presence of MVs at the sites of eDNA smears. The main enzyme involved in cell lysis in *S. aureus* is the peptidoglycan hydrolase Atl. We found that mutants lacking *atl* showed significantly impaired explosive cell lysis, and were abrogated in the production of eDNA and MVs in interstitial biofilms. Finally, we also found that staphylococcal cell lysis accounts for MV production in response to antibiotic stress and is important for biofilm formation. This is the first time that the peptidoglycan hydrolase Atl has been shown to account for MV release and that Gram-positive cell lysis has been monitored live at the single cell level. Deciphering these mechanisms driving biofilm formation is essential to develop new approaches for treating bacterial infections and fighting antibiotic resistance.

P72

Successful gene editing of human embryonic stem cells to generate a novel CF airway epithelial model

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Despite recent discoveries, such as Ivacaftor and Orkambi, targeting the basic cystic fibrosis (CF) defect, the vast majority of CF patients lack an effective treatment. Therefore, there is still a need to find more effective agents for all CF patients. However, further CF drug discovery is impeded by the limited availability of primary human airway epithelial material and their high background variability between donors. To overcome the availability and variability issues and to improve drug discovery, we aim to develop a novel CF cellular model. An established human embryonic stem cell (hESC) line, CA1, was used to perform genome editing techniques that allow the introduction of desired CF-causing mutations, such as F508del, into the cell's genome. A recently published directed differentiation protocol will then be used for the production of fully differentiated CFTR-expressing airway epithelial cells from CF-hESCs *(Wong et al, 2015)*.

Several genome editing technologies, such as TALENs, have successfully introduced disease-causing mutations, allowing the investigation of numerous diseases in vitro. We have designed a TALEN that specifically targets the *CFTR*-gene (*CFTR*-TALEN) and introduces a double strand break (DBS). TALEN plasmids were engineered using the Golden-gate assembly method and correct assembly was detected by restriction digestion and sequencing analysis. In order to perform successful genome editing, CA1 cells were adapted to grow as single-cells rather than the standard clump-culture, under feeder-free conditions. To efficiently deliver plasmid DNA into the CA1 cells, lipid-based transfection reagents, electroporation and nucleofection were tested using an eGFP DNA plasmid. Transfection efficiency ranged from 9-90% as assessed by flow cytometry, with the best results given by nucleofection. Moreover, transfection of the *CFTR*-TALEN into the CA1 cells was confirmed by immunofluorescence and 15% *CFTR*-TALEN activity was detected by the T7 endonuclease I assay. To generate F508del-cells and to repair the introduced DBS, a single-stranded oligodeoxynucleotide, harbouring the desired F508del mutation, was designed and transfected with the *CFTR*-TALEN into the CA1 cells. PCR analysis and sequencing showed integration of the F508del mutation at the correct site within the pool of transfected CA1 cells.

Our next aim is to clonally select and expand correctly modified and unmodified CA1 cells, creating a model consisting of CF-hESC and an isogenic hESC control, respectively. We will then perform directed differentiation of these hESCs into airway epithelial cells, under ALI-conditions. This CF-airway epithelial model, including the isogenic control, will be biochemically and functionally characterized to assess whether these cell cultures represent a sustainable, physiologically relevant, CF model that will enable the study of ion transport and CF drug evaluation.

These gene edited hESCs may be used to model other affected-tissues in CF patients, as hESCs can theoretically be differentiated into any cell type of interest. Furthermore, this approach can also be exploited to produce CF models due to other mutations. Alternatively, the approach can be used to modify other genes that might be of interest for CF research, such as modifier genes.

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P73

Characterization of transepithelial nasal potential difference in cystic fibrosis-like mouse

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Normal airways display coordinated regulation of anion efflux through CFTR and Na^+ influx through the epithelial sodium channel ENaC. Tight regulation of epithelial ion transport is required for airway surface liquid (ASL) homeostasis and mucus clearance. In CF airway epithelium, lack of CFTR-dependent anion transport and elevated ENaC-dependent Na^+ entry are the main causes of ASL dehydration, which has been postulated to be the initiating event of CF airways disease pathogenesis. The *scnn1b-Tg* (βENaC overexpressing) mouse was engineered to mimic the hyperactivity of ENaC channels observed in CF airways. It supports the idea of a critical role of increased sodium conductance in the development of CF lung disease.

We used transepithelial potential difference (PD) *in vivo* to measure sodium and chloride transports across the nasal mucosa of wild-type and *scnn1b-Tg* heterozygous (Tg/+) mice. In basal conditions, more polarized PD values were recorded in Tg/+ than in wild-type mice. Perfusion of buffered Ringer's solution at pH 7.4 did not modify basal PD in wild-type mice. However, in Tg/+ mice, Ringer's-pH 7.4 rapidly induced a small depolarization (+4mV). The effect was prevented by adjusting the pH of the solution to 6.5. Depolarization in response to perfusion of Ringer's solution containing amiloride (10^{-4} M) was significantly increased (+50%) in Tg/+ compared to wild-type mice. Perfusion of chloride-free Ringer's containing forskolin (10^{-5} M) induced a hyperpolarization of the nasal epithelium of similar magnitude in Tg/+ as in wild-type mice, suggesting that the CFTR-dependent chloride conductance is not affected by ENaC hyperactivity in Tg/+ mice.

To test the hypothesis that elevated Na^+ transport in Tg/+ mouse airways is due to an increased density of αβγENaC channels or a possible change in subunit stoichiometry, we quantified ENaC subunit transcripts in mouse nasal epithelium and in primary nasal epithelial cells in 3D cultures. Both α and βENaC transcript levels were elevated in Tg/+ mice while no significant change in γ-subunit expression was observed.

Our results show a pH-sensitive hyperactivity of ENaC in the nasal epithelium of *scnn1b-Tg* mice, possibly associated with a switch in subunit stoichiometry of the ENaC channel without ruling out the possible modulation by inflammation.

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The *Cftr* ^{tm1eur}/F508del CFTR mouse model have reduced beta-cell mass due to reduced cell size and reduced number of docked insulin granules.

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Cystic fibrosis (CF) -related diabetes (CFRD) is the most common complication in CF, yet the etiopathology of CFRD is largely unknown. Loss of islet cell mass has been proposed as mechanism in CFRD. Primary beta-cell defects have also been suggested since a delayed and/or absent first phase insulin secretion during glucose challenge is commonly observed in CF patients. First phase insulin secretion has been correlated to release of granules already docked and primed at the plasma membrane. We have previously shown that CFTR is present in both human and mouse pancreatic beta-cells (Edlund A et.al., 2014) and that inhibition of CFTR reduce insulin secretion by direct effects on exocytosis of insulin containing granules. The *Cftr* $^{tm1/eur}$mouse model of CF (F508del) carries the F508del mutation and have been reported to have reduced beta-cell mass (Fontes G et.al., 2015). Here, we have investigated islet morphology in the F508del mouse model. More specifically, alpha- beta- and delta-cell mass was investigated and the total number of insulin stained cells was counted. Moreover, total number of insulin granules and the number of docked granules at the plasma membrane was determined in beta-cells.

Methods: CFTR$^{+/+}$ (WT) and ^{F508del}CFTR$^{+/+}$ (*Cftr* $^{tm1/eur}$, F508del) female and male mice aged 7-9 weeks were used in this study. CFTR was detected in isolated beta-cells using confocal immunocytochemistry and expression analyzed using Zen Software. Islet cell mass was investigated with immunohistochemistry and beta-cell morphology was investigated with transmission electron microscopy. Islet insulin and glucagon content was measured with RIA. The total number of insulin granules (granules/μm^3) and number of docked granules at the plasma membrane (granules/μm^2) where calculated by an in-house MatLab program.

Results: CFTR protein was present in both WT and F508del beta-cells and CFTR plasma membrane expression was reduced in F508del beta-cells compared to WT ($p< 0.05$). Loss of beta-cell mass has been proposed as a mechanism in CFRD. Pancreatic islets from F508del mice had reduced beta-cell mass (N=21 pancreatic sections from 7 mice of each genotype, $p< 0.01$) and the number of beta-cells within the insulin stained area was similar in F508del and WT, suggesting that F508del have smaller beta-cells. In support of that, insulin content was also reduced in isolated islets from F508del compared to WT. Alpha- and delta-cell mass was spared and there was no difference in glucagon content between isolated islets from F508del and WT. We also investigated beta-cell morphology and found no difference in the number of insulin granules per beta-cell. However, the number of granules docked at the plasma membrane was reduced in F508del ($N_{F508del}$=33 beta-cells from 2 mice, N_{WT}=29 beta-cells from 2 mice, $p< 0.01$), indicating defective priming as we previously suggested (Edlund A et.al., 2014).

Conclusions: We conclude that the *Cftr* ^{tm1eur}/F508del CFTR mouse model has reduced beta-cell mass due to reduced beta-cell size and that alpha- and delta-cell mass is spared. Moreover, the number of docked granules at the plasma membrane is reduced in F508del beta-cells. Hence, CFRD is most likely due to a combination of reduced beta-cell mass and impaired beta-cell function.

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Comparison of ex vivo and in vitro intestinal cystic fibrosis models to measure CFTR-dependent ion channel activity

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The cystic fibrosis transmembrane conductance regulator (CFTR) protein is an anion channel that regulates transport of chloride and bicarbonate ions and fluid homeostasis across mucosal epithelium. As such, CFTR ion channel activity can be directly measured by electrophysiological techniques using Ussing chambers in short-circuit (voltage-clamped) or open circuit settings. These intestinal current measurements (ICM) on rectal biopsies obtained from cystic fibrosis (CF) subjects are used for CF diagnosis, studying individual CFTR function and in vivo impact of CFTR modulators.

We developed an in vitro model to study CFTR function in human intestinal stem cells that self-organize into organoids. CFTR function in this model system can be measured by performing a relatively simple but sensitive fluid secretion assay, termed the forskolin-induced swelling (FIS) assay. Importantly, the FIS assay recapitulates essential features of CF disease and in vivo response to therapy, and swelling of the organoids is fully dependent on CFTR functioning.

At the moment, it remains difficult to compare CFTR function measurements of the fluid secretion (FIS) assay directly to the electrophysiological (ICM) readouts in intestinal epithelium from CF subjects. Here, we compared existing functional CFTR assays that utilize rectal biopsies and intestinal organoids with electrophysiological measurements in 2D organoid-derived intestinal monolayers. We generated 2D monolayers from twelve different CF donors with six different *CFTR* genotypes (ranging from wild-type to severe CF phenotypes) and extensively characterized these 2D intestinal monolayers at the protein and mRNA expression levels for selected cell markers and ion channels. We performed electrophysiological CFTR function measurements of these monolayers under open-circuit conditions using a Multi-Trans Epithelial Current Clamp (MTECC) system. Based on the equivalent short-circuit current (Ieq) values we were able to discriminate the different *CFTR* genotypes into three distinct classes: high (WT/WT and carrier), moderate (R117H/F508del and S1251N/F508del) and low or no CFTR function (F508del/F508del and class I/class I). We correlated the 2D intestinal monolayer responses with functional data from donor-matched rectal biopsies (ICM) and intestinal organoid (FIS) responses and demonstrated that for both comparisons a good correlation exists (Pearson's $r = 0.73$ and $r = 0.89$, respectively).

In conclusion, our data indicate that 2D organoid-derived monolayers can be used as an additional electrophysiological tool for CFTR function measurements with the advantage that primary intestinal stem cell cultures possess long-term expansion and culturing capacity.

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Profiling of CFTR modulators GLPG1837 and GLPG2222 using intestinal organoids

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In order to improve the folding/maturation and functional properties of CFTR and thus address the main defects leading to cystic fibrosis (CF), we are developing different compound series. Each of these series contains either correctors that increase CFTR levels at the cell surface, or potentiators that allow the effective opening of the CFTR channel. Combined, these compounds are able to restore chloride ion transport yielding improved hydration of the lung surface and subsequent restoration of mucociliary clearance. We previously described the characterization of our potentiator compound GLPG1837 and our corrector compound GLPG2222. Here, the further profiling of the compounds using patient derived intestinal organoids is shown.

By measuring the forskolin-induced swelling (FIS) of CF intestinal organoids, a purely CFTR-driven process, the activity of GLPG2222 and GLPG1837 was determined. It was demonstrated that the corrective effect of GLPG2222 on F508del CFTR can be translated into an increase in FIS of patient derived organoids when combined with the potentiator GLPG1837. The ability of the potentiator GLPG1837 to open CFTR channels harbouring different mutations was evaluated by plotting concentration-response data obtained using organoid samples derived from donors with different genotypes. It was shown that GLPG1837 is more potent in restoring CFTR activity in S1251N/F508del organoids than in CFTR F508del/F508del organoids corrected with GLPG2222 and, especially, CFTR G551D/F508del organoids. The data obtained correlated well with results generated using YFP halide assays and, in the case of CFTR G551D/F508del and CFTR F508del/F508del, also with electrophysiology data obtained using patient derived primary bronchial epithelial cells.

In summary, we present the profiling of our CFTR modulators GLPG1837 and GLPG2222 using patient-derived organoids and the comparison with other assays.

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Directed differentiation and genetic modification of novel iPS cell lines from CF patients

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Background: Cystic fibrosis (CF) is the main genetic cause of death among Caucasian children. It is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. *CFTR* encodes a cyclic adenosine monophosphate (c-AMP)-dependent, phosphorylation-regulated chloride channel required for transport of chloride and other ions through cell membranes. The disease is characterized by abnormal fluid and electrolyte mobility across the epithelia of numerous tissues. The first manifestations occur in early childhood, generally affecting the respiratory tract, and later extending to other organs. Although several animal models exist, human genetic variability cannot be reproduced in them. Therefore, the generation of patient-specific iPS cells would be an important tool that can be used in disease modeling and drug discovery.

Methods: We reprogrammed human skin fibroblasts and keratinocytes from two CF patients carrying the p.F508del mutation and one healthy donor. Reprogramming was achieved by retroviral transduction with four (c-MYC, KLF4, OCT4 and SOX2), or three (KLF4, OCT4 and SOX2) of the classic Yamanaka's factors. The resulting iPS cells were karyotyped and tested by immunocytochemistry for stemness. Differentiation was induced by embryoid body formation and by treatment with a defined combination of growth factors.

Results: A total of 30 independent CF and 18 wild type hiPSC lines were obtained from the three donors' fibroblasts and keratinocytes. The lines with better morphology and proliferation rates were further characterized. All of them showed strong expression of stemness markers (NANOG, OCT4, SSEA4 and TRA-1-60) and, following embryoid body formation, were able to differentiate into ectoderm (NESTIN, TUJ1), mesoderm (GATA4, a-ACTININ) and endoderm (a-FETOPROTEIN (AFP), SOX17). In addition, directed differentiation into foregut and hindgut endoderm, the progenitors of lung, liver, and intestine endoderm, was confirmed by the presence of the FOXA2, SOX2 and CDX2 cell markers.

Conclusions: Our hiPSC lines can be used for disease modeling and drug testing, as well as for the development of new cell-based regenerative therapies. In addition, CF hiPSC directed differentiation into fore- and hindgut endoderm demonstrates the dispensability of the *CFTR* gene during *in vitro* early endoderm development.

Keyword: Cystic fibrosis, patient-specific induced pluripotent stem cells, *in vitro* endoderm differentiation.

P78

Evaluation of the primary human nasal epithelial cell cultures as a biomarker in personalized cystic fibrosis treatment

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Clinical studies with CFTR modulators have demonstrated that functional restoration of the mutated CFTR can be translated into benefit for patients. However, high variability of patients' responses highlights the importance of finding relevant biomarkers to predict the clinical responses. Primary human nasal epithelial (HNE) cells are easy to collect by nasal brushing and allow quantification of cAMP-mediated chloride transport as an indicator of CFTR function.

CFTR activity in vitro was assessed in primary HNE cultures, in comparison to human bronchial epithelial cells, by short circuit-current (Isc) measurements. Variation of Isc after application of Forskolin/IBMx and CFTR potentiator VX-770 ($\Delta Isc_{Fsk/IBMx+VX-770}$ in $\mu A/cm^2$) was an index of the CFTR-dependent chloride secretion. CFTR function in vivo was assessed via nasal potential difference (NPD) measurements in patients. CFTR expression in the apical surface of reconstituted epithelia was assessed by CFTR immunostaining and semi-quantification of the apical fluorescence.

Isc measurements in HNE cultures recapitulated the results obtained in HBE cultures from CF patients and healthy controls, and were accurate enough to distinguish different levels of CFTR function. CFTR activity in HNE cultures from F508del homozygous patients ($\Delta Isc_{Fsk/IBMx+VX-770}$=0.2 (0.1)) was significantly lower than that found in patients with genotypes associated with a residual CFTR function or a wide spectrum genotype ($\Delta Isc_{Fsk/IBMx+VX-770}$=8.8 (7)). In cultures from F508del healthy carriers, CFTR activity was significantly lower than that of healthy donors ($\Delta Isc_{Fsk/IBMx+VX-770}$= 5.1 (1.4) versus 12.6 (2.6); p=0.03). These results were correlated to apical CFTR expression (R^2=0.9, p< 0.0001) and in vivo CFTR activity as measured by nasal potential difference (R^2=0.82, p< 0.0001).

We then evaluated the correction of CFTR function by VX-809 in HNE cultures issued from patients homozygous for the F508del mutation or carrying CFTR genotypes displaying a wide spectrum of CFTR activity. VX-809 treatment of F508del homozygous cells significantly increased the average $\Delta Isc_{Fsk/IBMx+VX-770}$ as compared to DMSO by a mean of 1.8 $\mu A/cm^2$ (0.6-3.1), reaching an average of 23.6% (1-69%) of the WT-CFTR level (p< 10^{-6}). This change was correlated to an increase in the CFTR apical expression (R^2=0.6, p< 0.001). Finally, we assessed the predictive value of the primary HNE cell cultures by comparing the in vitro pharmacological rescue by CFTR modulators to the clinical efficacy of this treatment in patients. In vitro correction levels were significantly correlated to respiratory function improvement in 8 F508del homozygous patients who had initiated the combination of VX-809 and VX-770 (Orkambi(r)) treatment. Patients whose FEV1 improved by more than 5% displayed a mean change of Fsk/IBMx+VX-770 response upon 10% of the average WT level.

We provide the first evidence that correction of CFTR function and expression in HNE cell cultures can reliably predict respiratory improvement in patients to be treated with CFTR modulators. Therefore, it may be used as a surrogate biomarker to preselect responder patients for personalized therapy.

P79

Unravelling the mechanisms of airway epithelium repair in cystic fibrosis

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Pathological remodelling 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 constitute the 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.

We performed RNA sequencing of bronchial AECs in primary cultures from 4 CF (F508del/F508del) and 3 non-CF (NCF) patients. Well-differentiated cultures at the air-liquid interface were mechanically wounded and mRNA was isolated at different time points of the repair process: before wound (BW, control), 24h post wound (24hPW, dominated by migration and proliferation of progenitor cells), wound closure (WC) and 48h post WC (48hPWC, initiation of differentiation). To simulate *Pseudomonas aeruginosa* infection, we treated both CF and NCF AECs with 1µg/ml flagellin for 24h BW and at WC. 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. Differentially expressed genes were analysed for Gene ontology (GO) terms using METACORE analysis database. At first glance, analysis revealed a divergent pattern of differentially expressed genes between CF and NCF cultures during the repair process. Comparison of 24hPW with BW showed in NCF that 409 genes (662 in CF) were upregulated and 30 genes (71 in CF) were downregulated. Interestingly, we observed a switch in gene expression between WC and 48hPWC; in NCF, there were 32 upregulated genes (11 in CF) and 320 downregulated genes (only 42 in CF). GO analysis between CF and NCF cultures at each time point confirmed an imbalanced repair process. We observed that the most represented pathways include "cytoskeletal remodelling" and "cell cycle" categories. At 24hPW, analysis showed that the differently expressed genes belong to the "cell locomotion" and "cell migration" categories. Specifically, the highlighted pathways were "signal transduction", "cell adhesion" and "extracellular matrix remodelling". At WC, there were major differences in "response to wounding", "development", "cell cycle" and "cell migration" categories. At 48hPWC, the key terms identified were "development", "differentiation" and "cell migration". Upon flagellin stimulation, comparisons between CF and NCF samples BW showed pathways related to "cytoskeletal remodelling", "cell adhesion" and "development". At WC, differences in "cell adhesion", "signal transduction" and "innate and adaptive immune response" were observed.

Preliminary transcriptomic analysis of wound repair in CF AECs reveals an impairment in the regulation of cell migration and proliferation during injury, and in the initiation of differentiation. METACORE analysis indicates a delay in cell cycle exit and abnormal actin cytoskeleton remodelling. Flagellin treatment also confirms the abnormal immune response of CF AECs. Although confirmation at the protein level is required, this analysis provides a holistic view of the repair process in an *in vitro* human model of the CF airway epithelium.

P80

Generation of disease-specific iPSCs and development of transgenic reporter cell lines for cystic fibrosis disease modelling and drug screening

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The genetic disorder Cystic Fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene coding for a cAMP-activated chloride-channel. So far, immortalized cell lines overexpressing mutant CFTR-variants have been used to screen compound libraries. In fact, CFTR-modulators have been identified, but show modest effects at best. Obviously, the complexity of the mutant CFTR-maturation and turnover kinetics including the influence of genetic modifiers require the use of advanced personalized cellular models. To address these unmet needs we focus on the generation of induced pluripotent stem cell (iPSC) lines from CF-patients homozygous for F508del mutation. CF-iPSCs 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 potential to differentiate. TALEN-based genome engineering was applied for targeted introduction of reporter transgenes. Several pluripotent stem cell lines were generated expressing a tomato-fluorescence-reporter under control of the CFTR-locus. Moreover, a halide sensitive yellow fluorescent protein (YFP) was introduced into the AAVS1-locus to monitor CFTR-function. Finally, the F508del mutation in the CF-iPSC reporter line was genetically corrected using an oligonucleotide-based 'footprintless' approach. The stable expression and the functionality of the YFP-reporter could be demonstrated. Directed differentiation of reporter iPSCs towards intestinal/biliary epithelium revealed YFP^{pos}/$tomato^{pos}$ cells, displaying CFTR-channel specific response after Forskolin application, which was inhibited after CFTR(inh)-172 treatment. Furthermore, heterozygous correction of the F508del mutation resulted in recovery of CFTR function comparable to wild type CFTR. These results represent a proof of concept for the applicability of genetically engineered patient-specific iPSC lines in disease modelling with regard to the individual genetic context. Furthermore, we are preparing for first iPSC-based high-throughput screens aiming at the identification of novel correctors and potentiators of CFTR-trafficking-mutations.

P81

Relative roles of reduced mucus clearance and mucus hypersecretion in the pathogenesis of airway mucus plugging in mice

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Background: Reduced mucociliary clearance (MCC) and type 2 mediated airway inflammation triggering goblet cell metaplasia (GCM) and mucus hypersecretion are key features of chronic obstructive airways diseases including asthma, cystic fibrosis (CF) and COPD. However, the relative roles of increased mucin/mucus production vs. reduced mucus clearance in the pathogenesis of airway mucus obstruction characteristic of these diseases remain poorly understood.

Methods: To determine the relative roles of reduced MCC vs. type 2-mediated mucus hypersecretion, we generated i) βENaC-Tg mice lacking IL-13 (βENaC-Tg/IL-13$^{-/-}$) to study the impact of airway surface dehydration and impaired MCC in the absence of this key type 2 cytokine; ii) mice with lung-specific overexpression of IL-13 (IL-13-Tg/IL-13$^{-/-}$) to determine the impact of IL-13 mediated GCM and mucus hypersecretion alone; and iii) βENaC-Tg mice with lung-specific overexpression of IL-13 (βENaC-Tg/IL-13-Tg/IL-13$^{-/-}$) to study combined effects of reduced MCC and mucus hypersecretion.

Results: We found that reduced MCC due to airway surface dehydration is sufficient to induce chronic airway inflammation and airway mucus plugging in juvenile βENaC-Tg/IL-13$^{-/-}$ mice. However, expression of Muc5ac, Muc5b and GCM were reduced. In IL-13-Tg/IL-13$^{-/-}$ mice, eosinophilic inflammation, Muc5ac and Muc5b transcript levels and GCM were increased, but mucus plugging was reduced compared to βENaC-Tg/IL-13$^{-/-}$ mice. In neonatal βENaC-Tg/IL-13-Tg/IL-13$^{-/-}$ mice, Muc5ac and Muc5b transcript levels and GCM were elevated to similar levels compared to IL-13-Tg/IL-13$^{-/-}$ mice, but all mice died in the first week of life due to severe airway mucus plugging.

Conclusions: We demonstrate that reduced MCC is sufficient to produce mucus plugging in the absence of IL-13. However, IL-13 mediated GCM and elevated mucin expression aggravate mucus plugging leading to invariable death in neonatal βENaC-Tg mice with reduced MCC. These results indicate that impaired MCC and mucus hypersecretion act synergistically in the in vivo pathogenesis of airway mucus plugging.

P82

CFTR and bicarbonate as determinants of airway mucus secretion and microrheology.

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Many tissues that are affected in CF have defects in HCO3- transport and mucus. It has been suggested that these abnormalities may be related, and evidence linking them has come from recent studies of isolated, perfused mouse intestine and cervix. Normal mouse tissues release more mucus than those from CF mice, but can be made to resemble CF tissues if exposed to HCO3-free medium or the anion transport inhibitor DIDS. Reduced mucus discharge during these manoeuvres may result from increased adhesion of secreted mucus to the epithelial surface due to alterations in its biophysical properties. Whether human airway epithelia show these differences in CF is not known, and the influence of the cellular HCO3- homeostasis on mucus biophysical properties has not been studied.

We examined the possible role of CFTR in mucus organization and rheology in airway epithelial mucous granules.

Using microrheology, spectrophotometry, immunostaining and western blotting, we compared the properties of secretions from non-CF vs CF human airway epithelial cells cultured in air / liquid conditions and examined CFTR-mediated transport in isolated mucin granules.

We found that CFTR is expressed in mucin-containing vesicles, where it is able to mediate anion currents towards the lumen. Secretions from CF cultures have elevated viscoelastic properties and are more adherent to the cell surface.

Taken together, our results suggest that deficient CFTR-mediated HCO3- secretion in the lumen of mucous granules could participate in the abnormal mucus properties in CF.

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P83

SPX-101 is a novel ENaC-targeted therapeutic for cystic fibrosis that restores mucus transport

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Background: Airway epithelial cells secrete Short Palate, Lung, and Nasal Clone 1 (SPLUNC1) to regulate epithelial sodium channel (ENaC) activity by lowering the number of channels on the cell surface. However, SPLUNC1 loses this regulatory function at the acidic pH of the cystic fibrosis (CF) airway leading to hyperactivation of ENaC. When ENaC becomes hyperactivated, the increased sodium transport creates an osmotic gradient that pulls fluid out of the airway. Loss of airway fluid causes mucus dehydration, decreased mucociliary clearance, and chronic bacterial infections which account for the majority of morbidity and mortality associated with CF. ENaC represents a unique therapeutic target capable of treating all CF patients independent of their underlying CFTR mutation. We have developed a peptide mimetic of SPLUNC1, SPX-101, that maintains ENaC regulatory function even at acidic pH.

Objectives: To determine if SPX-101 increases mucus transport in CF-like lung disease and to assess the safety of the peptide when delivered by nebulization.

Results: SPX-101 increases mucus transport in mouse and sheep models of CF lung disease. A single intranasal dose of SPX-101 significantly increased mucus transport compared to control peptide in βENaC transgenic mice. This resulted in increased survival of βENaC mice to >90% with once-daily intranasal administration. Using a new sheep model where CFTR inhibitor is delivered to the upper airway via nebulization, thereby decreasing mucus movement by ~50%, SPX-101, but not small molecule inhibitors of ENaC such as amiloride, fully restored mucus transport. This effect of SPX-101 lasted >8 hours with a single dose. In 28-day GLP toxicology assessments of nebulized SPX-101 there were no adverse events. Importantly, there was no observed diuretic or hyperkalemic effect of SPX-101 up to the maximum deliverable dose.

Conclusion: SPX-101 increases mucus transport in multiple models of CF-like lung disease with efficacy achieved by once-daily dosing. The peptide has no adverse systemic effects. Taken together, SPX-101 represents a novel peptide-based therapy to treat all patients with CF regardless of the CFTR mutation.

P84

Transduction of Rhesus macaque lung by AAV1

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The major hurdle with gene therapy is expression of enough CFTR protein to be therapeutic. In order to address this, we utilized a dual reporter assay based upon firefly (FL) and renilla (RL) luciferase cloned into AAV1 & 5 vectors. Two male and two female healthy Rhesus monkeys were exposed by trans-oral Penn Century microsprayer delivery to an aerosol containing both AAV1-CB-FL and AAV5-CB-RL. CB refers to the chicken β-actin promoter. The studies were sponsored by NHLBI Gene Therapy Resource Program (GTRP) and conducted at the Lovelace Respiratory Research Institute. The vector combination was formulated on the day of dosing by combining, as provided by the U. of Mass, Vector Core, 13.5 ml of the AAV5-CB-RL (1.2×10^{13} gc/ml) with 16.2 ml of the AAV1-CB-FL (1.0×10^{13} gc/ml), to provide a mixture with 0.54 gc/ml of each vector. Animals were observed for clinical signs of toxicity, and body weight. The animals were euthanized at 45 days post-exposure. Seventeen lung samples were collected for analysis of luciferase reporter gene expression (RL versus FL) at the U of Iowa and for vector expression by PCR (U of Florida). Importantly, the only significant clinical observation was a foot pad laceration, which was not a test article-related finding. All animals gained weight, as expected, during the study. These results show again that AAV1 vector delivery is safe. The vector genomes measured in each lung region at necropsy showed that the vector was widely distributed throughout the lung by the microsprayer demonstrating conclusively that our droplet size is sufficient to support widespread distribution. Average PCR data for 17 lung samples from four monkeys show clearly that there is a dramatic difference between the ability of AAV1 and 5 to infect the lung with AAV1 being approximately 10-fold more effective than AAV5. Despite the small number of monkeys, the data also clearly show better luciferase transduction with AAV 1 than with AAV5. The levels of neutralizing antibody (measured at U. Penn) increased dramatically in all animals between the pre-study time point (monkeys chosen for the study had undetectable titers) and the time of necropsy. Considerably higher titers were observed for AAV5 than for AAV1. These results provide justification for our choice of AAV1 for lung delivery given that it had a greater infection efficiency and transduction and lesser propensity to induce neutralizing antibodies than did AAV5. Funded by CFF and NHLBI.

P85

An Investigation into novel inhibitors of channel activating proteases with implicators for cystic fibrosis lung disease

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Introduction: Cystic fibrosis (CF) is an autosomal recessive disease, characterised by chronic lung infections, inflammation and irreversible lung damage. ENaC, a sodium channel found within the lung epithelia can be activated through proteolytic cleavage by channel activating proteases (CAPs) (1), leading to upregulated sodium absorption from the airways, contributing to the dehydration and thick mucus observed in CF (2,3). The inhibition of these CAPs presents a promising target for therapy with a hope of rehydrating the airways through reduced ENaC activation, thereby restoring airway surface liquid and effective mucociliary clearance.

Aims and Objectives: Previous work by Reihill et al shows that QUB-TL1, an active site-directed protease inhibitor, effectively inhibits trypsin and trypsin-like enzymes, which reduces ENaC-mediated sodium absorption (4). The objective of this study was to build upon this knowledge base by kinetically evaluating the mode of action of other novel protease inhibitors, NAP858, NAP1099, NAP1127 and NAP743 and investigating their downstream effects on a number of inflammatory markers.

Methods: The novel inhibitors' activity against a range of proteases were profiled using fluorogenic substrates, followed by calculation of second order rate constants in the case of NAP858 and NAP1099. Further to this, in order to visualize the binding of each novel inhibitor to recombinant trypsin, western blotting techniques were utilized. The cytotoxicity of each of the inhibitors at a range of concentrations through treatment of a CuFi cell line for 24 hours was observed by monitoring the release of the cytosolic enzyme lactate dehydrogenase (LDH). Downstream effects on expression levels of proteinase activated receptor 2 (PAR-2) and cytokines, IL-8 and IL-6, in CuFi cells treated with our inhibitors were investigated using real-time quantitative PCR (qPCR).

Results and Discussion: We report NAP858, NAP1099, NAP1127 and NAP743 to be effective irreversible inhibitors of trypsin, matriptase, prostasin and human airway trypsin-like protease (HAT). Additional kinetic studies, calculating second order rate constants for NAP858 and NAP1099 allowed direct comparison to QUBTL1 and shows these two compounds to be equal in inhibition of trypsin to QUBTL1 and superior against matriptase and HAT. Cytotoxicity studies performed on CuFi cells indicated that treatment of these cells with our compounds for 24 hours did not increase LDH release from cells when compared with vehicle alone. Initial qPCR studies have indicated that CuFi cell treatment with NAP858 and NAP1099 causes a reduction in IL-8 expression, a potentially beneficial outcome as this pro-inflammatory cytokine is found in elevated concentrations in the CF lung (5).

Conclusion: These novel inhibitor compounds are promising follow-up compounds to QUBTL1, especially regarding efficacy against a number of key trypsin-like proteases in CF. They, therefore, present a promising future therapeutic option to inhibit the activation of ENaC by CAPs.

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P86

Effect of brevenal as a single agent and combined with orkambi on airway surface liquid and mucociliary transport in CF primary human bronchial epithelial cells

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In cystic fibrosis (CF), airway surface liquid (ASL) volume is depleted. This leads to an unfavorable environment that impairs mucociliary clearance, a key innate defense mechanism in the lung. Brevenal, a compound isolated from the dinoflagellate, *Karenia Brevis*, is a potent voltage-sensitive sodium channel (VSSC) modulator. Although VSSCs are not yet known to be expressed in epithelial cells, we have shown in CF human bronchial epithelial (HBE) cells that brevenal increases ASL secretion and abrogates increased TGFß1-induced ASL absorption (Abraham W, Salathe M, NACFC 2016, 80, p.223). We also have reported that aerosol brevenal restores mucociliary transport (MCT) dose-dependently when nebulized in sheep with slowed mucus clearance due to aerosol challenge with an inhibitor of cystic fibrosis transmembrane conductance regulator ($CFTR_{Inh}$-172) or $CFTR_{Inh}$-172 with human neutrophil elastase. Furthermore, when used in combination with the CFTR potentiator, ivacaftor, these agents demonstrate an additive effect. Brevenal does not affect the epithelial sodium channel (ENaC) or CFTR, suggesting it may alter the function of other ion transporters to change ASL and MCT, and has the potential for synergistic effects when combined with known CFTR or ENaC modulators. We used one-micron resolution optical coherence tomography (µOCT) to examine the effect of brevenal alone (1 µM) and in combination with CFTR corrector-potentiator therapy (lumacaftor (3 µM, VX-809) and ivacaftor (10 µM, VX-770); luma/iva) on ASL depth, MCT rate, and ciliary beat frequency (CBF) in primary CF HBE cells homozygous for F508del CFTR. At 24 hrs, brevenal-treated (basolateral) cells exhibited higher ASL depth (10.25±0.8mm; P=NS) vs. vehicle (8.08±0.5mm; P=NS), and compared favorably to luma/iva (9.23±0.5mm). Compared to baseline, brevenal elicited a significant change in ASL (2.90±0.7mm; $P< 0.05$) similar to luma/iva (2.52±0.4mm; P=NS) that was not seen with vehicle (1.14±0.7mm; P=NS). While brevenal had no effect in augmenting CBF (0.29±0.3Hz), in preliminary studies it significantly increased MCT rate from baseline (2.02±0.4mm/min; $P< 0.05$) at levels greater than luma/iva (1.24±0.3mm/min; P=NS) or vehicle (0.82±0.2mm/min; P=NS). Studies evaluating brevenal-luma/iva combination treatment, with which we have observed ~2-fold greater ASL change than with either compound alone, are ongoing. Results indicate that brevenal augments ASL depth and MCT rate without altering ciliary beating in F508del primary HBE monolayers, and suggest the need for further studies to determine its molecular target and benefit as an adjuvant therapy to luma/iva.

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VX-770 potentiation of CFTR gating involves stabilisation of the pre-hydrolytic O_1 open state

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Cystic fibrosis (CF) is a debilitating disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which codes for the CFTR anion channel.

VX-770 (Ivacaftor, Vertex Pharmaceuticals) was the first drug approved to treat the cause of the disease, by improving ion channel function of certain CFTR mutants. It has been hypothesised that VX-770 achieves this mainly by stabilisation of the post-hydrolytic open state, O_2 (Jih et al. 2013. *Proc. Natl. Acad. Sci.* 110:4404-9). However, an increased opening rate was also found to be critical in VX-770's potentiation of F508del-CFTR (Kopeikin et al. 2014. *J. Cyst. Fibros.* 13: 508-14).

The vast majority of WT-CFTR opening events are terminated following hydrolysis of ATP at the catalytic ATP-binding site, i.e. following a first transition from a pre-hydrolytic open state, O_1, to the post-hydrolytic O_2 state. D1370N is located in the Walker B motif of NBD2, in the catalytic ATP-binding site. Open dwell-time distribution data are consistent with all ATP-dependent opening events terminating via the non-hydrolytic closing pathway (Csanády et al. 2010. *Proc. Natl. Acad. Sci.* 107:1241-6). Patch clamp experiments, in conditions in which the O_1 and O_2 states could be differentiated by conductance, also showed that D1370N-CFTR only entered the O_1 state (Gunderson and Kopito 1995. *Cell.* 82:231-9).

We used a YFP-CFTR fusion gating probe, developed in our lab, to obtain information on the VX-770 mechanism. Halide sensitive YFP (Galietta et al. 2001. *FEBS Lett.* 499:220-4), tagged to the intracellular N-terminal of CFTR, is quenched in the presence of cytosolic iodide. When expressed in cells bathed in high extracellular iodide, the rate of YFP quenching reports on CFTR activity. HEK293 cells expressing YFP-WT-CFTR and YFP-D1370N-CFTR were used to compare potentiation by VX-770. VX-770 was able to effectively potentiate both channels. In fact, regardless of phosphorylation level, the fold-increase induced by VX-770 was extremely similar on both forms of CFTR. In the presence of saturating ATP, as occurs in the cytosol of live cells, it is unlikely that D1370N-CFTR would ever enter the O_2 state. The similarity of potentiation of WT-CFTR and D1370N-CFTR cannot be explained by stabilisation of only the O_2 state, and is more consistent with VX-770 acting by stabilisation of the opening transition state, and, to a greater extent, the pre-hydrolytic open state O_1, as likely occurs in F508del-CFTR.

Single channel patch clamp experiments to further investigate the VX-770 mechanism of action are ongoing.

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Task force for cystic fibrosis (TFCF): discovery and characterization of potent F508del-CFTR modulators

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The most frequent mutation among patients with cystic fibrosis (CF), deletion of phenylalanine 508 (F508del), causes a defective maturation and impaired gating of the CFTR chloride channel. The maturation defect can be treated with compounds known as *correctors*, whereas the gating defect can be overcome by compounds called *potentiators*.

To identify new molecules that rescue mutant CFTR function, we screened our compound collection of more than 11,000 diverse, drug-like compounds in two different cell types, FRT and CFBE41o- expressing F508del-CFTR. We used two functional assays, based on the halide-sensitive YFP (HS-YFP), designed to identify correctors and potentiators. A number of hits, belonging to various chemical classes, were identified in both the corrector and potentiator screenings. After confirmation of activity in secondary assays, we selected two classes of correctors and one class of potentiators for chemical expansion and investigation of the Structure-Activity Relationships (SARs). So far, more than 600 hit analogues have been synthesized to explore the SARs and improve drug-like properties.

As for correctors, several compounds with improved potency and efficacy with respect to the initial hits were discovered. We have identified a set of very active correctors with comparable or higher efficacy than VX-809 in different assays. In particular, a set of compounds showed high efficacy and potency in the low nanomolar range when tested with the HS-YFP and the Trans-Epithelial Electrical Conductance (TEEC) assays run on cell lines expressing F508del-CFTR. Rescue activity was confirmed in primary bronchial epithelial cells from F508del/F508del CF patients, with one compound being fully active at 10 nM.

Chemical expansion of the potentiator hits led to compounds displaying nanomolar potency in the F508del-CFTR FRT cell assay. Considering that ivacaftor (VX-770) has been reported to decrease the activity of corrector VX-809 upon chronic co-incubation (Cholon et al., *Sci Transl Med* 6: 246ra96, 2014; Veit et al., *Sci Transl Med* 6: 246ra97, 2014), we performed combination studies of our most promising correctors and potentiators. Gratifyingly, chronic incubation of one of our most active potentiators with both VX-809 and a TFCF corrector did not decrease correctors' activity in the TEEC assay performed in F508del-CFTR FRT cells.

The *in vitro* data of the most promising TFCF correctors and potentiators will be presented and discussed.

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CFTR gene editing with CRISPR/Cas9 in cultivated human tracheal epithelial cells for the development of cystic fibrosis therapies.

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Despite cystic fibrosis (CF) being the most common heritable disease among people with Northern European ancestry and affecting around 10,000 people in the UK alone, no cure is currently available. With drugs for its treatment showing limited efficacy and working only on patients with specific mutations, we believe that gene therapy is a promising approach to cure CF. CRISPR/Cas9 is a revolutionary gene editing system that provides a relatively easy way for precise and permanent correction of the CFTR gene.

However, since CRISPR/Cas9 delivery is still inefficient for correction of sufficient airway epithelial cells in vivo, we aim to select and expand corrected cells *in vitro* suitable for cell therapy.

Here, we isolate, characterise and cultivate human tracheal epithelial cells, which may display an extensive growth potential *ex vivo*, from a variety of sources: healthy and CF fetal and adult cells from bronchoscopies and nasal brushings. Preliminary data demonstrate that we can expand cells for which we are optimising transfection approaches and single-cell cloning methods as well as CRISPR designs for CFTR correction.

In addition, we are developing a bioengineered system for assessing functionality and engraftment capacity of cultivated epithelial cells, which will additionally serve as a testing model to increase cell transfection efficiency for potential *in vivo* correction CF therapies.

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Novel miRNA inhibition strategy based on biodegradable polymers for CF gene therapy studies

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Cystic fibrosis (CF) is a life-limiting inherited disease caused by genetic defects in the *cystic fibrosis transmembrane conductance regulator* (CFTR) gene. In CF 90% of mortalities are a result of pulmonary disease, and therefore targeting the lung of affected patients is one of the main priorities to correct CF symptoms in the airways. CFTR is known to be regulated by a set of lead miRNAs which are overexpressed in the lungs of CF suffers. Therefore, a strategy that can specifically interfere with miRNA-mediated inhibition of CFTR expression in CF epithelial cells has the potential to enhance CFTR expression and function. CFTR-specific Target Site Blockers (TSBs) are miRNA inhibitors that can block endogenous miRNA binding to specific miRNA responsive elements in the 3' untranslated region of CFTR mRNA. However, lung gene therapy for CF has not been successful due to several challenges such as the absence of an appropriate vector for airway gene expression, the stimulation of the immune system, and the difficulty in maintaining long-term therapeutic gene expression in airways. Hence, the successful therapeutic delivery of CFTR-specific TSBs to airway epithelial cells requires the design of suitable carriers at the expense of minimal toxicity. The present study aims to optimize the utilization of polymeric nanoparticles (NP) as an alternative method for the efficient and safe delivery of CFTR-specific TSBs. To this end, we describe the formulation and physicochemical characterization of TSB/NPs using two different biocompatible and biodegradable polymers; the FDA approved poly lactide-co-glycolide (PLGA) and the natural polymer chitosan (CS). PLGA-TSB/NPs were produced at (N/P) charge ratio of 4, while electrostatic self-assembled CS-TSB/NPs from two sources (animal and vegetal) were complexed in a range of (N/P) charge ratios (1.5-15) with various degrees of acetylation and molecular weight. Size distribution and polydispersity index of the TSB-NPs were determined by dynamic light scattering with non-invasive back scattering showing an average diameter ~ 200 nm and a monomodal distribution of particle size. The zeta potential was determined from the electrophoretic mobility by mixed-laser Doppler electrophoresis and phase analysis light scattering revealed a neutral surface charge. The physicochemical characteristics affect the delivery, efficiency and toxicity of NPs and consequently, their synthesis was optimized to produce TBS-loaded, PLGA and CS NPs that complexed and condensed CFTR-specific TSBs. In conclusion, biodegradable polymeric NPs offer a promising platform for the delivery of CFTR-specific TSBs to CF airway epithelial cells.

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The discovery 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.

To date a small number of tool compounds have been described in the literature as TMEM16A activators (Namkung et al. 2011) although we have previously demonstrated that these compounds can all directly elevate intracellular Ca^{2+} $[Ca^{2+}]_i$ levels. Our goal has been to identify novel potentiators of TMEM16A function that work independently of Ca^{2+} elevation. Using FRT-cells stably expressing TMEM16A, we have utilised a number of screening platforms including: membrane potential, halide quench and automated patch clamp electrophysiology (Q-Patch) to identify hit compounds. The Q-Patch assay was also used to validate hits identified in the membrane potential and halide quench formats and thereby remove false positives. The Q-Patch assay enabled compounds to be tested under conditions where both the membrane voltage and $[Ca^{2+}]_i$ could be clamped (260 nM) thereby eliminating compounds that elevated $[Ca^{2+}]_i$.

Using these approaches, we have identified a number of chemical series which can potentiate TMEM16A activity in FRT cells under conditions where $[Ca^{2+}]_i$ is clamped. We then addressed whether these compounds could translate into primary cultures of CF-HBEs. CF-derived HBE, cultured at air-liquid interface for 14-21 days were exposed to IL-13 (10 ng/mL) to upregulate TMEM16A expression. We then tested the potency and efficacy of the validated hit compounds in 2 ion transport formats. In format #1, CF-HBE were voltage clamped to 0 mV and amiloride added to the apical chamber. The SERCA pump inhibitor cyclopiazonic acid (CPA) was then added at an EC_{20} concentration to partially activate the channel followed by test compound. Finally, CaCC-A01 was added to confirm the magnitude of the TMEM16A-mediated short-circuit current (ISC). Under these conditions, test compounds induced a concentration-dependent increase in ISC that was blocked by CaCC-A01. In format #2, CF-HBE were again voltage clamped to 0 mV and amiloride added to the apical chamber followed by test compound or vehicle. Following incubation with test compound HBE were stimulated with an EC_{20} concentration of UTP (200 nM) and the increase in ISC was measured. In this assay format, test compound enhanced both the magnitude and duration of the secretory ISC response to UTP.

To our knowledge, these compounds represent the first description of TMEM16A potentiators that can lead to channel activation independent of an elevation in $[Ca^{2+}]_i$. Drug discovery efforts are ongoing to establish clinical candidates for testing in CF patients.

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P92

Characterisation and microparticle formulation of a novel CAP inhibitor, QUBTL1, with implications for CF lung disease.

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Introduction: Impaired mucociliary clearance (MCC) in cystic fibrosis leads to chronic cycles of infection and inflammation, which in turn causes impairment of respiratory processes and damage to the lung tissue (1). MCC may be corrected through inhibition of trypsin-like, channel activating proteases (CAPs) such as trypsin, furin and prostasin, which activate the epithelial sodium channel (ENaC), dysregulation of which causes dehydration of the airways surface liquid (2). A novel CAP inhibitor, QUB-TL1, has demonstrated efficacy in restoration of mucocilliary clearance (3). It is prudent to consider that inhibition of CAPs may have a synergistic effect on inflammatory pathways closely linked within the CF lung.

Aims and Objectives: The objective of this study was to investigate the effects of QUB-TL1 upon inflammation and develop a potential delivery method for the peptide.

Methods: CF airway epithelial cells were treated with the novel compound (QUBTL1), in the presence and absence of known immunostimulant, Tumour Necrosis Factor alpha (TNFα) for 24 hrs (4). Supernatant from each treatment was analysed for concentration of cytokines IL-6 and IL-8 by enzyme-linked immunosorbent assay (ELISA). RNA harvested from the treated cells was assayed for its effects on cytokines, in addition to a number of other receptors involved in inflammatory processes such as PAR-2, NFκB, TLR4 and A20, at the level of expression by both qPCR and RT-PCR methods. The biotinylated compound was then bound to streptavidin-coated microparticles, applied to cells and assayed by fluorigenic assay and ELISA. In addition, the cytotoxic effect of particles on cells was assessed via LDH assay.

Results and Discussion: QUB-TL1 showed efficacy in the reduction of TNFα mediated IL-6 and IL-8 release ($n=3$, $p< 0.05$), with an approximate two-fold reduction observed for both. Through qPCR, the compound showed a significant effect in reducing expression of PAR-2, IL-6, NFκB and A20 in particular. The streptavidin coated microparticles were found to retain the ability to inhibit trypsin and reduce cytokine release, similar to free inhibitor. The particles also exhibited a negligible amount of toxicity *in vitro*.

Conclusion: The incorporation of the compound onto microparticles has promise as a means of delivery, which presents an opportunity to locally deliver CAP inhibitors to the lung surface via inhalable formulations. QUB-TL1 appears to have multiple effects *in vitro*, not only in the suppression of ENaC and rehydration of the airways but in enabling modulation of the enhanced cycle of inflammation which causes overt lung damage in CF. Modulation of this sort could in turn improve quality of life and reduce hospitalization. Thus this inhibitor demonstrates great potential in treatment of CF lung disease.

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P93

New trimethylangelicin analogues as modulators of defective CFTR

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TMA (trimethylangelicin) is a small-molecule modulator of CFTR function, exhibiting both corrector and potentiator activities (Favia, 2014). Recent evidence was brought that TMA binds directly to CFTR and shares mechanisms to VX-770 and VX-809 for potentiating and stabilizing CFTR, respectively (Laselva, 2016). TMA also demonstrated anti-inflammatory properties by reducing IL-8 expression (Tamanini, 2011), so making TMA a promising agent for treatment of cystic fibrosis. Unfortunately, TMA was also shown to have potential phototoxicity and mutagenicity, despite the photoreactivity is absent when the compound is not directly irradiated with UVA light. Due to concerns about these toxic effects, a new research project was started with the aim to synthesize and test new generation TMA analogues with identical or better activity profile but reduced or absent side effects. We synthesized some derivatives by modifying specific structural features on the TMA scaffold, in order to understand which structural determinants are responsible for anti-inflammatory properties, CFTR modulatory properties and dual anti-inflammatory/CFTR modulatory activity, as well as which substituents allow minimization or abolishment of photoreactivity.

These new compounds were then tested for: a) anti-inflammatory activity, based on inhibition of NF-kB/DNA interaction and expression of pro-inflammatory genes in CF bronchial cell lines ;b) effects as CFTR function modulation, based on analysis of CFTR-mediated ion transport in FRT-YFP cells expressing human F508del CFTR or G551D CFTR and CF human bronchial epithelial cell lines; c) evaluation of phototoxicity (test of DNA damage upon UVA irradiation), cellular toxicity (MTT, cell cycle and apoptosis analyses), and mutagenicity (Ames test).

Some analogues demonstrated good corrector and potentiator activities with no mutagenicity and phototoxicity. In particular, two lead compounds were identified (one exhibiting high CFTR correction activity and poor NF-kB inhibition and one exhibiting good NF-kB inhibition), thus demonstrating that anti-inflammatory, CFTR potentiator and CFTR corrector activities could be split in TMA analogues.

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Validation of a CFTR alternative splicing reporter system for high-throughput microscopy and identification of novel CFTR regulators of alternative splicing

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Background: Splicing mutations represent ~11% off all CFTR mutations reported at the CFTR Mutation Database [1]. Such mutations affect mRNA processing abolishing or reducing normal CFTR mRNA and protein expression. To date, there are no CFTR mutation-specific therapies to treat patients carrying splicing mutations. There is thus an unmet need to find novel therapeutic strategies that correct splicing defects. We have previously described the effect of the CFTR splicing mutation 2657+5G>A (legacy name 2789+5G>A) in IVS16 (IVS14b), showing that it originates transcripts lacking exon 16 (exon 14b) and wild-type transcripts leading to reduced CFTR at the plasma membrane - a bona fide splicing mutation [2].

Objective: Our aim here is to validate a novel alternative splicing reporter system for high-throughput (HT) microscopy and to identify splicing factors that correct CFTR splicing defects at the mRNA level to become novel drug targets for CF.

Methods: We generated a CFTR mini-gene reporter construct containing the 2657+5G>A mutation as a model to develop a double-tagged CFTR splicing reporter system suitable to be used in HT microscopy. This reporter system (mCherry-Flag-Mut-CFTR) consists of a pcDNA5/FRT/CFTR vector containing a fluorescent mCherry tag fused to the N-terminus of the CFTR mini-gene that comprises full-length CFTR cDNA with introns 14, 15 and 16 and a Flag epitope tag inserted at the 4th extracellular loop at exon 17, i.e. downstream of the 2657+5G>A mutation. This construct was inserted into HEK293 Flp-In cells to stably express it, in parallel with a similar wt-CFTR construct. To validate this novel splicing reporter we used RNA-based strategies to establish suitable controls for the HT screening assay, including: *(i)* antisense oligonucleotide 1 (AON1), previously shown to rescue this mutation [2]; *(ii)* a panel of exon-specific U1 small nuclear RNAs (ExSpeU1s) cloned into the pLVTHM lentiviral vector aiming to correct exon skipping caused by the 2657+5G>A mutation; and *(iii)* scrambled shRNA (Scr shRNA) as negative control. The correction of splicing was assessed by: *(i)* semi-quantitative RT-PCR; *(ii)* Western-blot (WB) to assess the appearance of full-length and fully-glycosylated (band C) CFTR; and (iii) HT microscopy analysis using a ratiometric fluorescence readout.

Results and Discussion: RT-PCR, WB and fluorescence ratiometric data in AON1-treated cells showed correction as follows: 87%, 48% and 24% of wt levels, respectively (vs 56%, 23% and 6% for Scr AON). From the panel of ExSpeU1-treated cells correction was as follows: 81-87% and 27-46%, respectively (vs 53% and 23% for Scr shRNA); fluorescence microscopy experiments are ongoing. We are currently using this reporter system to screen a lentiviral human library (shRNA library) subset of The RNAi Consortium which is enriched in a large collection of genes known or predicted to be involved in splicing - 425 genes - using HT screening microscopy. With this strategy, we expect to identify gene knock-downs increasing normal vs alternative CFTR splicing, i.e. potential drug targets for CF patients with alternative splicing mutations.

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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 (lumacaftor) in rescuing F508del-CFTR, only modest clinical outcomes were observed in F508del-homozygous patients treated with this corrector in combination with potentiator VX-770 (ivacaftor). Therefore, there is still an unmet need for more potent compounds that rescue more efficiently the functional expression of F508del-CFTR.

Objective: 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 when administrated individually or in combination with VX-809 and/or VX-770.

Methods: Towards this aim, F508del-CFTR rescue to the plasma membrane (PM) was evaluated by Western blot (WB) in CF bronchial epithelial (CFBE) cells stably expressing F508del-CFTR treated with incremental doses of the RDR01752 compound (1, 3, 5, 10 and 20μM) or 3.7 μM VX-809 for comparison (both for 24h). PM traffic efficiency was determined by immunofluorescence (IF) microscopy in CFBE cells stably expressing F508del-mCherry-Flag-CFTR under an inducible (Tet-On) promoter [2]. Additionally, rescue of F508del-CFTR function by RDR01752 was measured in intestinal organoids derived from F508del/F508del patients bearing F508del-CFTR.

Results and Discussion: Our WB data indicate that the maximal rescue of F508del-CFTR by RDR01752 is achieved at the 20μM dose. Data also indicate that the amount of processed form of F508del-CFTR at this dose is similar of that observed under 3.7 μM VX-809. Traffic efficiency by IF confirmed these data. However, in terms of function, preliminary data in F508del/F508del human intestinal organoids evidenced lower functional rescue by RDR01752 as compared to VX-809.

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In vitro characterization and correction of splicing mutations in IVS 5 by an antisense oligonucleotide

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Background: Until now, more than 2,000 alterations have been described in the CFTR gene, most presumed to be pathogenic [1]. A significant fraction of these (~11%) affects pre-mRNA splicing. Novel therapeutic approaches to correct splicing mutations have been described using antisense oligonucleotides (AONs) that correct splicing defects, indicating that CF patients carrying such mutations may benefit from AON treatment [2]. We have recently reported a novel splicing mutation - 711+3A>T in IVS5 -- found in a CF patient [3]. The functional consequences of this mutation have not been described.

Objective: Herein, we aim to determine the impact of the 711+3A>T splicing mutation and to correct the aberrant splicing caused by this mutation as well as by other previously reported splicing mutations in the same splicing consensus (711+1 G>T, 711+3 A>G and 711+5 G>A), using an RNA-based AON strategy.

Methods: To this end, we designed one single AON complementary to the pre-mRNA area of interest in IVS5 and tested its effect in correcting the splicing defects caused by the above splicing mutations using a mini-gene consisting in full-length CFTR cDNA plus the intronic regions IVS4 and IVS5 cloned into pCDNA5 vector and stably expressed in HEK293 Flp-In cells. The correction of splicing was assessed by quantitative RT-PCR (qRT-PCR).

Results and Discussion: Our qRT-PCR data show that all the above splicing mutations lead to skipping of IVS5 and very little production of wt transcripts, namely: 21% (711+3A>T), 2% (711+1 G>T), 9% (711+3 A>G) and 11% (711+5 G>A). Our data also show that for all the splicing mutations the AON significantly restored exon 5 inclusion in CFTR mRNA in cells transfected with this AON, namely to the following levels: 52%, 12%, 19% and 50%, respectively.

In conclusion, our *in vitro* studies revealed that four splicing mutations in IVS5, 711+1 G>T, 711+3 A>G, 711+3A>T and 711+5 G>A lead to aberrant transcripts lacking exon 5 and very low levels of wt transcripts. However, exon skipping can be corrected by a single AON in all 4 mutations, thus suggesting that this AON has some therapeutic potential for CF patients carrying any of these mutations.

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Ivacaftor and lumacaftor cooperatively amend the function of pathogenic mutations in cystic fibrosis

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Cystic fibrosis (CF), one of the most common lethal genetic diseases, is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene encoding a chloride ion channel. Dysfunctional CFTR led to variable disease severity based on a range of cell biological and functional defects manifested in CFTR mutants. As the overall CFTR-dependent chloride flux ($\boldsymbol{I} = N \cdot P_o \cdot$ i) is the product of *N*, number of channels in the cell membrane, P_o, the open probability of individual channels, and i, single-channel current amplitude, great efforts have been placed on finding reagents that can increase *N* (i.e., correctors such as lumacaftor) or P_o (i.e., potentiators such as ivacaftor). Indeed, Orkambi (combined ivacaftor and lumacaftor) has been approved recently by the U.S. FDA for the treatment of patients carrying the most common pathogenic mutation ΔF508. Theoretically, as ivacaftor rectifies the gating defects while lumacaftor increases *N* by improving protein trafficking, a dramatic therapeutic effect is anticipated. However, the full potential of this combination regiment for ΔF508 mutation has not been realized due to an undesirable influence of chronic exposure to ivacaftor on the effect of lumacaftor. Here, we propose that the negative impact of combination on ΔF508 is mutant-specific. Indeed, our western blot analysis shows that lumacaftor alone significantly increases the surface expression (i.e., band C) of wild-type CFTR by ~2-fold (237 ± 31.5 %, n = 5), and that this enhancement remains steady even after a 24-hour treatment with ivacaftor (223 ± 32.9 %, n = 5). As a control, the effect of lumacaftor on ΔF508 is diminished with ivacaftor as reported previously. Consistent with the western blot results, patch-clamp recordings of WT-CFTR in excised inside-out patch show a ~4 -fold increase of the protein kinas A (PKA)-activated CFTR currents by lumacaftor treatment (I_{DMSOWT} = 80.03 ± 16.5 pA, n = 26; $I_{\_\,LumacaftorWT}$= *536.8 ± 143.5 pA, n = 26). Again, this effect of lumacaftor is minimally affected by ivacaftor (*IOrkambi_WT = 340.3 ± 119.0 pA, n = 26). These results with WT-CFTR encouraged us to test possible synergism between lumacaftor and ivacaftor on pathogenic mutations that do not exhibit trafficking defects. Indeed, results similar to those observed for WT-CFTR were obtained for G551D (severe-CF, Class III), R117H (mild-CF, Class IV) and R352Q (severe-CF. Class IV). We therefore conclude 1) the negative impact of ivacaftor on lumacaftor is mutant-specific; 2) the combined therapy should not be limited to patients carrying the ΔF508 mutation, and could benefit a broad spectrum of CF patients; 3) combining western blot and patch-clamp recording provides a straightforward in vitro assay to materialize precision medicine in the field. While carrying out patch-clamp recordings, we observed significant "irreversible run-down" of ΔF508-CFTR currents (i.e., a time-dependent decrease of the current that cannot be recovered by PKA and ATP), but not with WT-, G551D-, R117H- or R352Q-CFTR. This instability of ΔF508-CFTR in the cell membrane may partly account for the negative drug-drug interaction seen with lumacaftor and ivacaftor.

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Alkyl fluorination enhances the efficacy and potency of tris-thiourea artificial anion transporters

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Artificial anion transporters (anionophores) are a potential treatment for cystic fibrosis. Although many classes of anionophores have been developed and tested, transporter design is still being optimised. Here, we use fluorination to modulate the properties of tris-thiourea anionophores, including lipophilicity and acidity. As controls, we studied alkylated tri-thioureas.

Four fluorinated tris-thiourea anionophores (**5**-**8**) and their alkyl counterparts (**1**-**4**) were designed. Chloride transport by the compounds was measured in lipid vesicles and intact cells using pH-driven vesicle assays and Fischer rat thyroid epithelial cells expressing the halide-sensitive yellow fluorescent protein (YFP-FRT cells)[1,2]. As some tripodal thioureas demonstrate Cl^-/OH^- selectivity[1], the 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) assay was modified to determine the relative selectivity of the fluorinated compounds. To interpret the data, we also studied the anion-binding strength and lipophilicity of the test small molecules.

Both the lipid vesicle and YFP-FRT cell assays demonstrated that fluorinated anionophores had higher efficacy and potency compared with their alkyl counterparts. Fluorination of the ethyl and butyl analogues **5** and **7** yielded an order of magnitude improvement in activity over their alkyl counterparts, while the most active fluorinated propyl analogue **6** enhanced anion transport more than two orders of magnitude over its alkyl analogue. Based on these and other results, we suggest that fluorine atoms spread the negative charge of Cl^- to reduce energy barriers and enhance anion transport. However, compound **8** with the longest fluorinated chain was ineffective in the YFP-FRT assay and had limited enhancement over compound **4** in the lipid vesicle assay. The high lipophilicity of **8** might explain these results because we found that compounds with longer chain lengths, which provide better Cl^- shielding, were more effective anion transporters. Interestingly, we also found that compounds with lower Cl^-/OH^- selectivity were better anion transporters, particularly in YFP-FRT cells, which suggests that H^+/OH^- transport mechanisms, including free fatty acid, which is abundant in cell membranes, might facilitate Cl^- transport[3]. We conclude that fluorination enhances the efficacy and potency of anionophores, but reduces selectivity for Cl^- over H^+/OH^-. Future studies should seek to identify potent efficacious anionophores with high Cl^- selectivity[4]. Supported by the EPSRC.

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P99

A High-Throughput Screening assay to identify factors correcting CFTR mutations bearing premature termination codons (PTCs).

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Background: Nonsense mutations, which introduce premature stop codons account for about 8% of the ~2,000 CFTR gene variants reported at CFTR Mutation Database [1]. These mutations usually lead to extensive transcript degradation by nonsense-mediated mRNA decay (NMD) thus preventing protein production. Such mutations are therefore associated with severe CF phenotypes. Several drugs specifically promoting PTC suppression (or readthrough) are currently under trial for CF. Indeed, aminoglycoside antibiotics and Ataluren have shown some *in vitro* ability to suppress PTCs but are unspecific, show low efficacy and toxicity. There is thus an unmet need to treat patients with these mutations and novel therapeutic strategies should be explored.

Objective: Our aim here is to identify novel factors that correct the defective processing of these transcripts and to validate them as novel drug targets.

Methods: To achieve this goal we are using a CFTR mini-gene model (i.e., full-length CFTR cDNA with introns IVS14, 15 and 16; legacy name IVS13, 14a and 14b) harbouring the G542X mutation (at exon 12; legacy name 11), which not only leads to the production of significantly lower levels of CFTR transcripts but also recapitulates the full NMD process. The mini-gene features mCherry and eGFP tags at the CFTR N- and C-termini, respectively which allow determining the total CFTR levels (mCherry) and only of full-length protein (eGFP) i.e., if there is no read-through, there is no eGFP fluorescence. The construct also has a Flag-tag which becomes extracellular after G542X correction, originating mature mCherry-Flag-G542X-CFTR-eGFP. This construct was inserted into HEK293 Flp-In cells to stably express it, in parallel with a similar wt-CFTR construct. To validate this NMD reporter assay, we used an NMD inhibitor - SMG-1 inhibitor (kindly provided by M. Mense, CFFT labs) as a positive control on mCherry-Flag-G542X-CFTR-eGFP cells. The NMD correction was assessed by: *(i)* semi-quantitative RT-PCR; *(ii)* high-throughput (HT) microscopy analysis using a ratiometric readout (eGFP/mCherry).

Results and Discussion: Our RT-PCR data show that levels of G542X-CFTR transcripts in non-treated and SMG-1 inhibitor treated cells were: 20% and 60%, respectively. By fluorescence microscopy we could not determine presence of eGFP fluorescence in SMG1-treated cells because SMG-1 inhibition only prevents NMD but has no readthrough activity. However, we observed a 5-fold increase in mCherry fluorescence, indicating a good dynamic range for detection of NMD suppression, thus validating the reporter assay for HT microscopy. We are currently using this CFTR-NMD reporter to identify novel NMD factors by screening a previously validated shRNA library -- a subset of The RNAi Consortium (TRC) -- which is enriched in shRNAs targeting genes known or predicted to be involved in transcript processing (425 genes), using HT microscopy.

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P100

Effects of increasing levels of gamma-glutamyltransferase in cystic fibrosis airways on glutathione inhalation therapies.

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Background: Glutathione (GSH) is a primary antioxidant whose levels are significantly decreased in lungs during inflammation. Inhalation treatments with GSH have become popular among cystic fibrosis (CF) patients, but the results attained so far are disappointing. One of the possible reasons may lie in the fact that CF lungs often present with increased levels of gammaglutamyltransferase (GGT), an enzyme secreted by inflammatory cells, capable of degrading GSH.

Methods: We analyzed GGT activities in CF sputum samples obtained from a previously published clinical trial and we re-evaluated the clinical and biochemical responses in relation to GGT activities. A CF mouse model transiently transgenized with a human IL-8 promoter/luciferase reporter gene was set up.

Results: GGT activity correlated with neutrophil elastase. GSH treatment produced a significant improvement in the level of inflammatory cytokines (IL-8, TNF-α, IL-1β) in sputum, but only in subjects with a reduction in inflammation. Increased GGT levels during GSH therapy were associated with higher levels of an oxidative stress marker (protein carbonyls). The in vivo model allowed us to study the activation status of NF-κB factor.

Conclusions: Our data demonstrate that sputum GGT correlates with neutrophilic inflammation in CF airways. Differentiating patients with decreasing GGT activity may discriminate subjects with resolving inflammation --- more likely profiting from inhaled GSH --- from those with exacerbation of inflammation (and increasing GGT activity) --- in which GSH might even produce aggravation of the damage. These results suggest that recommendation of therapeutic GSH inhalation should not be further cautioned and that the actual inflammatory status of the airways should be considered when antiinflammatory treatments are targeted.

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P101

Combination of gene therapy with CFTR modulators for enhanced therapeutic efficacy

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Introduction: Gene therapy provides a mutation-independent approach to tackle the underlying defect in cystic fibrosis. In previous work, we have shown that adeno-associated viral vector (AAV) delivery of a truncated CFTR (CFTRΔR, deletion of AA 708-759) not only resulted in improved CFTR activity in the nasal mucosa of CF mice, but also partially rescued CFTR function in patient derived intestinal organoids (Vidović, Carlon *et al*., AJRCCM, 2016). One reason accounting for this partial rescue could relate to overexpression of CFTR. This might lead to a saturation of the processing or trafficking machinery, resulting in a pool of intracellular non-functional CFTR. Combining gene therapy with CFTR modulators for promoting CFTR processing or function, could potentially improve the therapeutic potential of gene therapy.

Methods: To allow sensitive and quantitative detection of CFTR at the plasma membrane (PM), we introduced a 3HA-tag into the 4th extracellular (EC) loop (Sharma *et al*., J Cell Biol, 2004) of CFTR variants (WT, CFTRΔR, F508del) and generated stable cell lines using lentiviral vectors in HEK293T cells. PM CFTR was detected by immunocytochemistry (ICC) or FACS by staining for the EC3HA-tag without permeabilizing the cells. Total CFTR was analyzed after permeabilization. Western blotting (WB) was performed for semi-quantitative assessment of (im)mature CFTR. The effect of corrector VX809 (2,5 µM, 24 h incubation) was assessed on PM and total CFTR, and strong vs. weak overexpression (CMV or CMV173-driven CFTR), determined by ICC, WB and/or FACS. Values are reported as mean ± SD.

Results: Introduction of the EC3HA-tag allowed nice PM visualization of WT-CFTR and CFTRΔR, which was absent for F508del-CFTR. FACS quantification confirmed these findings. To determine the sensitivity of the different assays, we first validated the effect of VX809 on F508del cells. In the FACS-based PM density assay, this resulted in a 4,5 (± 0,3)-fold increase in PM fraction compared to DMSO-control. A rescue in F508del-CFTR maturation (band C on WB) confirmed this. For WT-CFTR, the increase after VX809 treatment was ~45% both for PM and total CFTR (48,1 ± 1,7% and 42,5 ± 10,0%, respectively). The effect of VX809 on PM density for strong vs. weak overexpression of WT-CFTR was comparable in relative fold-increase (~50%), although the absolute increase was larger for the strong CMV promoter. Functional studies in intestinal organoids are ongoing to address a potential increase in CFTR function after a combination of viral vector delivery and CFTR modulators.

Conclusions: We have developed a sensitive and quantitative FACS-based PM density assay to detect changes in the amount of membrane localized (mutant) CFTR by using extracellular tagged CFTR variants. We validated this assay using VX809 on F508del-CFTR expressing cells. VX809 treatment of WT-CFTR overexpressing cells resulted in a ~50% increase in PM fraction. Functional studies will determine the relevance of this PM increase. In that regard, experiments are planned to address the effect of CFTR modulators on gene therapy mediated CFTR overexpression in intestinal organoids and CF animal models.

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A high-content siRNA screen at near genomic scale reveals novel F508del-CFTR therapeutic targets

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Background: The F508del-CFTR mutation underlies about 85% of all cystic fibrosis (CF) cases by preventing cell surface expression of CFTR in many tissues, most notably in the airways. This is due to F508del-CFTR misfolding which leads to its retention in the endoplasmic reticulum (ER) and premature degradation by the ER quality control (ERQC). Nevertheless, F508del-CFTR can be rescued to the plasma membrane (PM) by chemical correctors, such as VX-809 or VX-661. F508del-homozygous patients can benefit from a combination drug of corrector VX-809 and potentatior VX-770 -- albeit with modest lung function improvement. There is still an unmet need for more potent F508del-CFTR traffic correctors.

Objective: We hypothesize that unknown cellular factors retain F508del-CFTR in the ER and our goal here is to identify such factors as possible drug targets.

Methods: To this end, we have previously generated a F508del-CFTR traffic reporter consisting in an mCherry-Flag-F508del-CFTR construct which was stably expressed CFBE cells under the control of a Tet-ON promoter [1]. Using fluorescence microscopy, F508del-CFTR traffic efficiency can be measured in each individual cell by calculating the fluorescence ratio of PM CFTR (given by immunodetected Flag) versus total CFTR (given by mCherry). We used this system to screen Ambion's Extended Druggable Genome siRNA library (Ref. AM80991V3.1), composed of 27,312 siRNAs targeting 9,128 human genes, i.e, about half of the genes in the human genome.

Results and Discussion: The primary siRNA screen revealed 78 genes whose knock-down significantly increased F508del-CFTR traffic over the negative control (more than two standard deviations). We defined F508del-CFTR traffic regulator genes by applying a lower stringency threshold to the raw screen data. Among such regulator genes, 39 are also CFTR interactors [2] and none are ENaC inhibitors [3], suggesting that the screening assay is pinpointing CFTR-specific regulatory partners. A gene ontology hit classification revealed an enrichment in PM proteins, including other ionic channels and proteins involved in epithelial homeostasis, differentiation and development. The Mammalian Membrane Two-Hybrid (MaMTH) system is being used as recently described [4] to map interactions between wt-CFTR/F508del-CFTR and 39 genes which are simultaneously F508del-CFTR traffic regulators and CFTR interactors. Ultimately, the most promising hits will be selected for rational inhibitor development.

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P103

High throughput microscopy screens identify F508del-CFTR correctors and ANO1 traffic modulators in a unique portuguese natural compound library

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Background: Although ~45% of cystic fibrosis (CF) patients worldwide are eligible to be prescribed with either Orkambi (~40% of patients are F508del homozygous) or Kalydeco (~5% of patients with rare gating mutations) [1], it is widely accepted that treatment of CF is still an unmet need and that CF pharmacotherapy must be improved. Alternative approaches such as the pharmacological activation of non-CFTR chloride channels, like ANO1, deserve further exploring as they can compensate for the loss of CFTR-dependent ion transport in all CF patients, as a "mutation-agnostic" approach. Thus, novel sources of pharmacophores and drug discovery screening programs are needed. Natural derived products (NP) have long been a traditional source of medicines, and are still considered a successful supply of potential novel drug leads with more than 1 million new NP discovered so far [2].

Objective: Herein, we aim to identify correctors of F508del-CFTR traffic by screening a collection of unique marine and terrestrial macro and microbial organisms extracts (LUSOEXTRACT) as new potential drug leads for CF.

Methods: We used a previously described microscopy-based F508del-CFTR traffic assay [3] to screen the total LUSOEXTRACT collection, containing 3591 extracts originated from 1206 organisms isolated from Portuguese terrestrial and aquatic ecosystems.

Results and Discussion: This screen identified a total of 290 extracts causing a traffic efficiency of F508del-CFTR significantly greater than the negative control (Z-score ≥ 2), which were thus classified as hits. Among these, a subset of 38 extracts (top 19 marine and top 19 terrestrial extracts) was selected for dose-response and toxicity studies using the same screening platform. Results were analysed using a Principal Component multivariate analysis (PCA) that allowed defining extract clusters according to their dose-dependent effect on traffic efficiency and cell viability. A final validated set of 33 extracts was selected and ranked according to their potency in rescuing F508del-CFTR localization. Of these, 27 extracts also enhanced ANO1 traffic. Hits origin is as diverse as corals, sponges, fungi and bacteria. The determination of F508del-CFTR chloride transport activity rescue by the most potent hit extract in polarized bronchial epithelial cells was precluded by extract cytotoxicity. Fractionating and purifying active compounds from extract is ongoing.

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P104

Assay development and high-throughput screen installation for identifying SLC26A9 chloride channel activators

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Introduction: The SLC26A9 chloride channel represents a promising candidate to provide apical chloride transport in the absence of functional CFTR, thus circumvent the primary defect in cystic fibrosis. Recent evidence suggests that SLC26A9 Cl^- channel function may be activated therapeutically by compounds that increase translocation of the protein into the apical plasma membrane.

Aims:To systematically identify therapeutic target genes and lead compounds promoting trafficking of SLC26A9, we aim to perform high-throughput siRNA and chemical library screens. For that we are developing two complimentary, cell-based assays to measure: (i) apical membrane localization of SLC26A9 and (ii) SLC26A9 function using a membrane potential sensitive (FLIPR) dye.

Methods: We generated CFBE41o- cells with stable expression of HA-tagged SLC26A9. Association of SLC26A9 to the plasma membrane is estimated using 3D confocal fluorescence microscopy. The apical membrane is labelled with Concavalin-A and SLC26A9 is stained via its HA-tag. Co-localisation of SLC26A9 with Concavalin-A is quantified as a correlation between 3D voxel intensities.

Changes in membrane potential are measured by live-cell FLIPR time-lapse imaging in 96-well format. After baseline measurements inhibitor is added manually. Intensity time-traces for individual cells are quantified after segmentation and image quality control.

Results: We established an initial image analysis pipeline to measure fraction of cytoplasmic protein co-localizing with plasma membrane. Currently we are optimizing sample preparation protocol in order to increase dynamic range of the traffic assay.

Using the FLIPR assay we quantified the baseline and the response upon adding inhibitor. We were able to detect significant difference between SLC26A9 over-expressing and control cells.

Conclusions & Perspectives: We are developing robust assays to monitor SLC26A9 function and membrane localisation. Currently we are adapting these assays for high-throughput experiments (high-throughput data processing and automated reagent addition synchronised with time lapse imaging). This platform will enable us in the future to identify therapeutic strategies to activate SLC26A9.

P105

Derivatives of esculentin-1a: promising frog-skin peptides for the development of new antipseudomonal drugs with expanding properties

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Naturally-occurring antimicrobial peptides (AMPs) represent an interesting class of molecules for the development of new anti-infective agents with multiple properties. The discovery of new antimicrobials is highly demanded, due to the increasing number of microorganisms that are resistant to the currently-used drugs. Remarkably, one of the most feared opportunistic pathogens, especially in the lungs of cystic fibrosis (CF) sufferers is the gram-negative bacterium *Pseudomonas aeruginosa*, because of its intrinsic low susceptibility to antibiotics and ability to form biofilm-communities that are resistant to a variety of environmental insults. Recently, we discovered that a derivative of the frog-skin AMP esculentin-1a, esculentin-1a(1-21)NH2 [Esc(1-21) GIFSKLAGKKIKNLLISGLKG-$_{NH2}$ had potent antimicrobial activity against both free-living and biofilm forms of *P. aeruginosa* with minimal concentrations inhibiting microbial growth (MICs) ranging from 4 to 8 μM (1). Subsequently, a diastereomer of Esc(1-21), Esc(1-21)-1c, carrying two D-amino acids was designed. It resulted to be slightly weaker than the all-L peptide against the planktonic form of *P. aeruginosa* (with a concentration causing 99.9% killing of bacterial cells equal to 4 μM *versus* 1 μM of the parent peptide). However, it showed a higher bactericidal activity against the more dangerous biofilm phenotype; a lower cytotoxicity and higher biostability (2). In this work, further biological properties were investigated. Among them: (i) the ability of Esc(1-21) and its diastereomer to induce bacterial resistance after multiple cycles of treatment at sub-MICs in comparison with conventional antibiotics; (ii) the peptides' effect on the motility of the planktonic form of CF clinical isolates of P. aeruginosa and (iii) the effect of the diastereomer on preformed Pseudomonas biofilm in the presence of artificial sputum medium (ASM) that better simulates the lung environment (i.e. sputum composition) of CF patients. The results of these experiments have indicated that in contrast with aztreonam, colistin, tobramycin and ciprofloxacin, both peptides do not induce resistance after a prolonged exposure (~ 1 month) of the bacterial culture to them, as pointed out by the invariant MIC. In addition, the diastereomer was found to have a higher ability than the all-L Esc(1-21) to inhibit the bacterium's motility and presumably its ability to reach and colonize a biological surface (e.g. airways epithelium) switching to the biofilm form. Finally, when the diastereomer was added to Pseudomonas biofilm in ASM it was found to cause 20 % killing of the biofilm after 6 h treatment at the MIC, while only 2% reduction of viable biofilm was found for the conventional antibiotic aztreonam. All these data suggest that the two frog-skin derived AMPs represent very interesting candidates for the development of new antipseudomonal drugs for treatment of lung infections.

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P106

Targeting PI3Kγ scaffold function to activate airway CFTR, limit lung inflammation and promote bronchorelaxation 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 obstruction of small airways and, together with airway inflammation and infections, eventually leads to respiratory failure. A number of CFTR correctors and potentiators, improving membrane expression and gating of the channel respectively, have been developed, but their ability to rescue the basic defect of CF is still unsatisfactory.

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 key cAMP-degrading enzymes, phosphodiesterases 3 and 4 (PDE3 and PDE4). Here, we hypothesize that targeting PI3Kγ scaffold activity enhances cAMP in airway smooth muscle, immune and epithelial cells, leading to concomitant (i) bronchodilation, (ii) anti-inflammatory effects and (iii) CFTR potentiation.

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 (i) bronchodilator, (ii) anti-inflammatory agent and (iii) CFTR potentiator. A mouse model of chronic lung inflammation (OVA-sensitized mice) and human primary bronchial epithelial cells (F508del) were used.

Results: We found that, in vivo, the peptide: i) can be efficiently delivered to the lower airways by intratracheal instillation in mice; ii) significantly elevates cAMP in the lungs and iii) is devoid of the typical side effects of PDE inhibitors, such as cardiac arrhythmias and emesis. Notably, the peptide limits methacholine-induced airway hyperresponsiveness and reduces neutrophilic lung inflammation in OVA mice. In vitro, the peptide potentiates F508del-CFTR currents upon pharmacological correction with VX-809 and, unlike the gold-standard potentiator VX-770, does not interfere with channel stability in the chronic setting. Finally, in VX-809-corrected primary cells, the peptide synergizes with VX-770 by increasing its efficacy by 5 fold.

Conclusions: Overall, the results of this study demonstrate that the peptide targeting PI3Kγ may be exploited therapeutically to provide (i) bronchodilation, (ii) anti-inflammatory effects and (iii) CFTR potentiation. This will eventually allow maximizing patients' benefit. Chemical optimization of the peptide is ongoing and will eventually pave the way to a peptide-based aerosol therapy to be used in combination with standard-of-care CFTR correctors/potentiators.

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P107

Nanoparticle-mediated delivery of modified tRNAs as a therapeutic approach for CF caused by a nonsense mutation.

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Nonsense mutations represent nearly 10% of cystic fibrosis (CF)-causing alleles, including the opal codons G542X, W1282X, R553X, and R1162X which are the top four nonsense mutations and second, sixth, seventh, and twelfth most common disease mutations overall in CF. These mutations result in the premature termination of translation of the cystic fibrosis transmembrane conductance regulator (CFTR) and attendant degradation of CFTR mRNA. Current strategies in development include suppression of the nonsense-mediated mRNA decay (NMD) pathway and/or induction of readthrough of the premature termination codon (PTC) by altering the translation machinery to allow misincorporation of an amino acid at the site of the nonsense codon.

Taking advantage of a tRNA's ability to direct specific amino acids to the growing nascent chain, we modified an arginine tRNA to recognize an opal termination codon (Arg/Op) while maintaining aminoacylation activity. The Arg tRNA was selected as it would be predicted to act on both the R553X and R1162X alleles to restore wild type protein and because site-directed mutagenesis indicates that the G542R substitution maintains significant maturation and function in heterologous expression systems. Thus, a single suppressor tRNA might have utility for three of the four most common CF-causing nonsense mutations. Delivery of this modified tRNA to affected cells was accomplished with a unique non-viral nanoparticle formulation optimized for delivery of long nucleic acids. The efficacy of the approach was assessed by measuring CFTR dependent chloride secretion (Ieq) using differentiated primary G542X/G542X and F508del/G542X CFTR HBEs. Nanoparticle delivery of the modified Arg/Op suppressor tRNA restored significant CFTR function on both genotypes. Interestingly, treatment of heterozygotes with both the Arg/Op tRNA nanoparticles and VX809 resulted in strong synergy. This suggests that the G542R CFTR protein is a partial maturation mutant which can be restored with VX809, consistent with results in the heterologous expression system. Toxicity of the tRNA-loaded nanoparticles was assessed in mice. No overt toxicity as measured by body weight was observed. Furthermore, biodistribution studies suggest significant lung accumulation of the Arg/Op tRNA.

These data suggest that nanoparticle delivery of modified tRNA can improve CFTR function in primary HBEs and may offer an alternative therapeutic approach for the treatment of CF caused by nonsense mutations. Moreover, a single tRNA may be employed to treat multiple disease-causing alleles.

P108

Correcting the ΔF508 mutation of the CF gene by CRISPR/Cas9

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Cystic Fibrosis (CF) is one of the most common recessive genetic diseases. At present, for most patients, there is no clinically effective drug able to treat CF lung disease manifestations. Several clinical trials have been performed to date assessing the potential of gene therapy to limit the progression of CF lung disease, but a clinically relevant treatment has yet to emerge. The major challenges in gene therapy for CF relate to the limited levels of gene transfer achieved in lung airway epithelium and persistence of transgene expression.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system emerged as a powerful tool for gene editing. This system has certain advantages over previous gene editing technologies including zinc finger nucleases (ZnFN) and TALENs. It is simple, inexpensive, easily programmed and highly efficient.

Here, we are investigating the potential of genome editing to develop genetic therapies for CF using the CRISPR/Cas9 system delivered by Receptor-Targeted Nanocomplexes (RTN). We aim to correct the CFTR ΔF508 mutation which is the most common CF mutation, affecting more than 70% of patients. Initially, multiple CRISPR/Cas9 constructs were screened for double strand breaks (DSB) targeting the CFTR gene near the ΔF508 mutation in the CFBE41o- cell line. The efficiency of DSB formation was evaluated by the T7 endonuclease I assay. The efficiency of this system incorporating a DNA plasmid produced approximately 20% of insertions/deletions (Indels) in the CFTR gene.

Co-transfection of CRISPR/Cas9 and CFTR donor template was then performed to mediate homology directed repair (HDR) of the ΔF508 mutation. To enhance the HDR efficiency, the donor template had a puromycin encoding gene incorporated within, which can be removed if desired as it is flanked with LoxP sequences. Cells resistant for puromycin were selected and expanded in order to confirm the correction. The results showed correction of the ΔF508 mutation in 25% of the clones. In addition, we showed that changing the Protospacer Adjacent Motif (PAM) which Cas9 uses to recognise the targeting sequence had a great impact in preventing re-cutting of the corrected allele. Polarised monolayers of the corrected cells are growing on an air-liquid interface culture to determine the CFTR chloride channel activity.

In conclusion, the CRISPR/Cas9 system is a great tool for gene editing. CRISPR/Cas9 mediated gene correction was pronounced only after selection. The potential advantage of the CRISPR/Cas9 system is that correction will be permanent in cells where the life span is quite long preserving the native regulatory elements. In addition, if the correction happens in progenitor cell types this could potentially allow for long term, stable correction. However, there are many challenges including achieving more efficient levels of gene repair and methods of delivery. Non-immunogenic, non-viral delivery platform allow potentially for repeated delivery leading to an accumulation of therapeutic gene repair. It is reasonable to anticipate that these gene editing systems coupled with effective delivery offer new prospects to CF gene therapy.

P109

Curated database of candidate therapeutics for the activation of CFTR-mediated ion conductance (CandActCFTR)

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Cystic Fibrosis (CF) is an autosomal-recessively inherited disease with an incidence of 1:2000 in Europe, caused by two defective chromosomal copies of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Substances that increase the function of mutant CFTR in CF patients are at the core of CF research for several years. The candidate therapeutics are recognized by systematic screening of substances or directly tested because of their promising molecular characteristics. Based on the experiences of the last decade, the field agrees that in order to reach real benefit for cystic fibrosis patients (improvement of well-being, quality of life and survival in cystic fibrosis) causative therapies will require a combination of substances which can activate CFTR-mediated ion conductance in a synergistic fashion.

Due to the heterogeneity of the research initiatives and due to the gap between data acquisition and publication, as well as due to the publication bias that favors positive findings, some substances (even inactive agents) are currently evaluated in duplicate. The present research infrastructure cannot effectively assist in the identification of those small molecule therapeutics which are most suitable for such a synergistic combination therapy, or which can be ruled out for further testing.

A curated database of candidate therapeutics for the activation of CFTR-mediated ion conductance can effectively merge the data from publicly available sources, unpublished primary data and findings derived from screening efforts. The acquired information on all tested substances will allow the identification of the most promising candidates for future development as therapeutics.

Here we present the newly started CandActCFTR database project, which focuses on curating a database of candidate therapeutics for the activation of CFTR-mediated ion conductance. CandActCFTR aims to demonstrate that the joint analysis of data for a well-focused pharmacological application can be successfully realized while respecting each researchers intellectual property rights. To achieve this task we will combine and extend the well established IT-structures from the Chemoinformatics and Systems Biology research fields (CDK - Chemical Development Kit and openBIS -- Open Source Biology Information System) with services such as `Persistent Identifiers´ for citable research data. The data structure of our database will allow users to apply different search strategies: e.g. searching for specific compounds or widen the search for similar chemical compounds and see how they where tested; or to search for compounds grouped by similar screening conditions of interest.

This project is funded by the Deutsche Forschungsgemeinschaft (DFG), the self-governing organisation for independent science and research in Germany.

More information can be found @ http://candactcftr.ams.med.uni-goettingen.de

P110

Mesoangioblasts -vessel associated progenitor cells as a novel cell-based therapy for cystic fibrosis

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Cystic Fibrosis (CF) is caused by mutations in the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which functions mainly as a cAMP-dependent chloride channel and is primarily expressed in the apical membrane of secretory epithelia. Lung disease, characterized by airway obstruction, inflammation and bacterial infection is the leading cause of death in CF patients. At variance with some pharmacological approaches, no efficacious gene and cell therapy have been proved to date. We are developing a cell therapy based on transplantation of mouse mesoangioblasts (mMABs) in mouse models of CF. MABs are vessels-associated progenitor cells that are able to cross the vessel wall upon intra-arterial injection, undergo skeletal muscle differentiation and rescue skeletal muscle dystrophy in mice and dogs. Our results show that mMABs, upon a systemic delivery, engraft lung, tracheal and intestinal epithelium up to 2 months in *wt* mice and up to 4/6 months in the CF mouse models *KOCftr*^{tm1UNC} and *F508del CFTR,* respectively. We also measured, by cell sorting, the percentage of engrafted mMABs into the lung overtime which was from 0.1% at 1 month to 0.4% after 6 months from a single cell injection in CF mouse models. MAB injection leads to a partial rescue in the expression of the fully glycosylated CFTR protein in *F508del CFTR* mice up to 4 months from their single transplantation. Donor engrafted mMABs, re-isolated from *F508del CFTR* transplanted mice, express the epithelial markers E-cadherin and CC10 a specific marker of Club Cells, but they do not differentiate in SPC+ alveolar epithelial type II cells or Acetylated a-Tubulin+ ciliated cells. *In vitro*, mMABs cultured with bronchial epithelial medium show a *de novo* expression of epithelial markers (e.g. E-cadherin, Adam10) and a decrease of mesenchymal and myogenic ones (e.g. N-cadherin, Fibronectin, Vimentin, MyoD, Myogenin), as well as of SNAIL, a transcription factor associated with the epithelial to mesenchymal transition, suggesting the activation of the reverse mesenchymal transition. mMABs can also engraft nasal and intestinal epithelium of *KOCftr*^{tm1UNC} mice, restoring some CFTR-dependent chloride secretions up to 4 months and expressing the epithelial marker E-cadherin. In a preliminary study on human MABs (hMABs) we demonstrated that, *in vitro*, hMABs express a mature form of CFTR. hMABs co-cultured with human bronchial epithelial cells (HBE) derived from CF patients harboring the F508del-CFTR mutation are able to integrate the epithelium and to restore CFTR activity. The extent of the correction was similar to the one observed upon rescue of F508del-CFTR trafficking defect by treatment with VX-809. This is the first study investigating the potency of MABs to treat CF disease as innovative cell-based therapy.

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P111

Stepwise generation of CFTR-expressing airway epithelial cells from human pluripotent stem cells

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Pluripotent stem cells (PSCs), like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer promising new options for the treatment of lung diseases like cystic fibrosis (CF) by cellular/tissue replacement therapies, disease modelling and drug screening. These approaches crucially rely on the efficient differentiation of PSCs into respective lung epithelial cells, whereby most promising differentiation strategies rely on the stepwise imitation of human lung development. On that account, the presented study aimed at the establishment of an efficient differentiation protocol for the generation of CFTR-expressing airway epithelial cells as a cell population of high value for CF research.

We made use of the human ESC reporter line hES3*NKX2.1-eGFP (kindly provided by A. Elefanty) expressing eGFP under the endogenous NKX2.1 promoter. Furthermore, we took advantage of a lab-internal generated double transgenic cell line based on the hES3*NKX2.1-eGFP cells additionally expressing dTomato controlled by the endogenous CFTR promotor. The established differentiation protocol comprises the stepwise differentiation to definitive endoderm, its patterning to anterior foregut endoderm, the induction of NKX2.1-eGFP^{pos} lung progenitor cells and the generation of CFTR-dTomato^{pos} airway epithelial cells.

Using the commercially available STEMdiff(tm) Definitive Endoderm Kit by STEMCELL Technologies resulted in the robust and efficient generation of a highly enriched DE population of ~ 98 % CXCR4^{pos}/C-Kit^{pos} cells and CXCR4^{pos}/EpCAM^{pos} cells demonstrated by flow cytometric analysis and additionally verified by co-expression of the transcription factors FOXA2 and SOX17 on mRNA and protein expression level. Further differentiation resulted in a distinct FOXA2^{pos}/SOX2^{pos} anterior foregut endoderm population of ~ 76 % quantified by flow cytometric analysis and verified on protein and mRNA expression level, which gave rise to ~ 55 % NKX2.1-eGFP^{pos} lung progenitor cells. Lung cell fate was verified by exclusion of the thyroid marker PAX8 and the neuroectoderm markers PAX6 and TUBB3 on mRNA and protein expression level. Interestingly, the generated NKX2.1-eGFP^{pos} lung progenitor cells co-expressed SOX2, FOXA2 and EpCAM which is a described marker panel for a proximal lung cell fate specification of the cells. Subsequent maturation of purified NKX2.1-eGFP^{pos} cells under air-liquid-interface conditions demonstrated the formation of ~ 30 % CFTR-dTomato^{pos} airway epithelial cells comprising club cells, which has been demonstrated by co-expression with SP-B and CCSP on protein expression level and further visualized using transmission electron microscopy. The differentiation cultures showed further mature airway cell markers on mRNA expression level comprising MUC5AC (goblet cells) and p63 (basal cells), whereas markers for distal alveolar epithelial cells (SP-C and AQP5) and ciliated cells (FOXJ1) were absent.

In summary, we were able to stepwise generate almost pure definitive endoderm followed by the induction of a distinct anterior foregut endoderm population resulting in a decent percentage of NKX2.1-eGFP^{pos} lung progenitor cells. Further maturation demonstrated the generation of CFTR-dTomato^{pos} airway epithelial cells comprising club cells. Future work will focus on the further optimization of the established differentiation strategy and its adaption to in-house generated CF patient-specific (genetically corrected) iPSCs to provide a suitable cell source for high throughput screening approaches for the identification of new therapeutic drugs to treat CF.

P112

Amplifiers co-translationally increase CFTR levels at the ER membrane by improving membrane targeting of CFTR

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The cystic fibrosis transmembrane conductance regulator (CFTR) protein is an inefficiently synthesized transmembrane chloride channel. Its first transmembrane helix (TM1) is unique, and contains two charged residues that are conserved across its orthologs. TM1 acts as an inefficient signal sequence directing the ribosome-nascent chain complex (RNC) to the ER with modest efficiency (Lu Y, et al., J Biol Chem. 1998 Jan 2;273(1):568-76.; Schlebach JP, Sanders CR. J Membr Biol. 2015 Jun;248(3):371-81.). In addition, a significant portion of the immature CFTR molecules that manage to overcome this inefficiency and are synthesized in the ER are still co-translationally targeted for degradation (Lukacs GL, et al., EMBO J. 1994 Dec 15;13(24):6076-86.). Because these inefficiencies are inherent in CFTR, modulators that address them would be expected to be mutation agnostic and to complement downstream therapeutic modalities such as correctors and potentiators.

Proteostasis Therapeutics, Inc. is developing amplifiers, a novel class of CFTR modulator that act early in CFTR synthesis to increase the levels of immature CFTR protein. Amplifiers selectively stabilize CFTR mRNA, act co-translationally and their mechanism of action works through the translated portion of CFTR mRNA.

The observed increase in steady state CFTR mRNA levels due to amplifier occurs in the ER-localized population of CFTR mRNA, consistent with the co-translational targeting of CFTR to the ER membrane. Mutations that impact membrane targeting of CFTR also impact the amplifier response, suggesting a model in which amplifier is acting to improve the inefficiency of this process for CFTR. Based on our experimental evidence, we performed *in silico* modeling of TM1 of CFTR and amplifier molecules binding to the signal recognition particle (SRP), and were able to identify good poses. Inactive compounds and non-responsive related proteins were used as negative controls. Based on this, we hypothesized and demonstrated that inactive amplifier analogs are non-competitive for binding.

Taken together, our results identify a mechanism through which amplifiers are able to selectively overcome the inefficiencies inherent in CFTR synthesis. Targeting an early step in CFTR biosynthesis, amplifiers act independent of specific CF-causing mutations, and thus have the potential to increase the levels of CFTR protein for all patient genotypes. In addition, the increase in immature CFTR protein provides more substrate for downstream CFTR modulators, and has the potential to complement upstream gene therapy based approaches.

P113

CFTR superexon homology-independent targeted integration to correct CF-causing variants in and downstream of Exon 23

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Gene editing of individual CF-causing variants by homology-directed repair (HDR) is precise, but efficiency is rarely above 1% of transfected cells without drug selection (McNeer, 2015; Hollywood, 2016). Moreover, editing each of the 272 known CF-causing mutations individually is not a feasible therapeutic approach. To address this, we and others successfully incorporated a CF Superexon$^{11\text{-}27}$ construct into the CFTR gene using ZFNs (Hollywood, 2014; Bednarski, 2014, 2016) and Cas9 (Hollywood, 2015) but efficiency was even lower.

Here we describe the efficient incorporation of a CF Superexon$^{23\text{-}27}$ construct into the CFTR gene using homology-independent targeted integration (HITI), a recently described technique (Suzuki et al., 2016) to massively boost stable transgene incorporation with editing efficiency of up to 60% in cells and >10% *in vivo*. The major advantage of HITI is that if the insert is integrated in the wrong orientation, then in ~90% of cases, the gRNAs used will excise the insert recreating the target site for a subsequent attempt at correct integration without the need for additional manipulation. In addition to higher editing efficiency, HITI allows editing of slow/non-dividing cells such as long-lasting lung epithelial cells.

We have designed and synthesized a 1.78kb CF Superexon comprising a splice acceptor site followed by Exons 23-27 fused together and a 2A-mCherry reporter gene. The construct is designed to be incorporated into intron 22 using Cas9 gRNAs previously validated in our lab for this region (Sanz, 2016). Successful integration of the CF Superexon$^{23\text{-}27\text{-}2A\text{-}mCherry}$ construct should result in a wild-type mRNA expressed under the control of the endogenous CFTR promoter with many of the normal regulatory features of the gene intact. Thus, with a single Cas9 gRNA and one relatively small donor plasmid, it should be possible to correct ~5% of all CF-causing variants including the currently non-druggable W1282X and N1303K variants, and due to the site of integration in intron 23, the 3849+10kbG>T should also be corrected.

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P114
Development of CRISPR Cpf1 editing for the CFTR gene

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We have previously described the use of CRISPR/Cas9 editing to correct the F508del variant using CRISPR Cas9 by homology directed repair (Hollywood et al., 2016) and correction of several deep-intron CF-causing variants such as 3849+10kB C>T by non-homologous end joining (Sanz et al., 2016). In this study, we describe two assay systems to optimise the use of the recently described CRISPR Cpf1/gRNA system (Zetsche et al., 2015) for gene editing. Cpf1 has several potential advantages relative to Cas9 including both a smaller protein and gRNA size which is useful for delivery, and an AT-rich PAM sequence which are more common in the CFTR gene than the G-rich PAM sequences of Cas9. Another interesting feature of Cpf1 is that it generates 4/5 bp overhangs which could be used for directional incorporation of CF superexon or whole cDNA sequences. These overhangs could potentially increase the efficiency of the pseudoexon excision we have previously reported for Cas9, if the target sites are designed are selected to generate compatible overhangs, a strategy successfully used for in vitro manipulation of DNA by Cpf1/gRNA (Li et al., 2016).

We designed and synthesised two sets of four Cpf1 gRNAs to cut either side of a genomic pseudoexon sequence to identify the most effective pair for genomic excision. However, initial experiments of all sixteen possible combinations of Cpf1 gRNA failed to successfully excise the target region; a similar observation has been reported in two studies in murine cells, namely that use of two gRNAs causes two localised indel regions either side of the target region, but the target region itself is not excised (Hur 2016; Kim 2016). To further evalute the activity of Cpf1, we developed two plasmid-based reporter assays. The first measures Cpf1 cleavage activity using a disrupted Luciferase reporter gene with sufficient homology (583 bp) to allow recombination by single stranded annealing which restores the luciferase open reading frame with concomitant restoration of reporter function -- we have previously validated Cas9 gRNA cleavage using this assay (Healy 2015). The second assay disrupts the luciferase open reading frame by insertion of two artificial intron sequences (with suitable splice donor and acceptor sites) flanking a pseudoexon containing an in-frame stop codon. The artificial introns each contain a control Cas9 gRNA target and four different Cpf1 gRNA targets to allow multiple combinations of Cas9/gRNAs and Cpf1/gRNAs to be evaluated, including a formal evaluation of the effect of the relative orientation of the PAM sequences on the excision of target sequences. Data from these studies should allow design of Cpf1 gRNAs to successfully excise the 3849+10kbC>T mutation and possibly incorporate CF superxons by utilising the overhang sequences generated by Cpf1.

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P115

Receptor-targeted nanocomplex delivery of siRNA silences αENAC expression in vitro and in vivo and improves mucociliary function: a therapeutic strategy for cystic fibrosis

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Cystic Fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), encoding a cyclic AMP-activated chloride channel. CFTR also regulates the epithelial sodium channel, ENaC, leading to increased sodium and water absorption resulting in depletion of the overlaying airway surface liquid (ASL), thickened mucus and impaired mucociliary clearance allowing bacterial infections that contribute to the CF lung pathology. We are investigating RNA inhibition (RNAi) of ENaC as a therapy for CF to restore airway hydration and mucociliary function. We describe here development of targeted nanocomplexes optimised for transfection of epithelial cells in vitro and in vivo, evaluate molecular and functional effects of ENaC silencing in a cellular model of the human airway epithelium and then assessment of their translational potential in transfections of normal mice lungs.

The nanocomplexes (called RTNs) comprise formulations of liposomes and an epithelial receptor-targeting peptide which self-assemble on mixing with siRNA. We showed that the biophysical properties and transfection efficiency of RTNs were unaltered by nebulisation offering a convenient route for delivery. The mucus lining of the epithelium presents potentially a significant barrier to transfection, particularly in CF, so we assessed RTN diffusion rates compared to naked siRNA. Interestingly, the diffusion coefficients of RTNs and naked siRNA were similar in CF mucus, despite their enormous size differences, while in porcine mucus the diffusion rates of RTNs (1.38×10^{-8} cm^2 s^{-1}) were significantly faster than naked siRNA at 3.75×10^{-9} cm^2 s^{-1}. The surface properties of the RTNs appear to facilitate transit through mucus and so are compatible with airway delivery.

RTNs achieved 30% silencing of the αENaC subunit after a single transfection of fully differentiated and ciliated primary CFBE cells grown in Air-Liquid Interface (ALI) cultures which was increased to 50% after three sequential transfections. We then assessed whether these levels of silencing were sufficient to anticipate therapeutic efficacy. The amiloride --responsive short-circuit current (I_{sc}) analysis in an Ussing chamber indicated reduced ENaC transport activity. In addition, mucus protein concentration was reduced and depth of the airway surface liquid (ASL) was increased indicating improved hydration. The restoration of a normal cilia beating frequency (CBF) indicated that ENaC silencing by siRNA led to restoration of mucociliary clearance in ALI cultured CF cells. In vivo transfections in normal mice showed single doses of siRNA delivered by oropharyngeal instillation in mouse lung silenced α ENaC by ~30%, while three doses of siRNA delivered at 48 h intervals resulted in ~60% silencing, similar to ALI transfection data. Silencing persisted for at least 7 days and all the mice tolerated well the dosing with RTNs.

In conclusion, the siRNA nanoparticles described here are compatible with airway delivery and possess properties that enable them to overcome mucociliary barriers leading to efficient silencing of ENaC. The restoration of epithelial hydration and ciliary function support the notion of ENaC silencing as a therapy for CF. Transfection in vivo achieved similar levels of efficiency to in vitro and persisted for at least one week indicating therapeutic potential.

P116

High-throughput forskolin-induced swelling assay to identify and/or repurpose old and new cftr-restoring drug compounds using intestinal organoids of subjects with cystic fibrosis.

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More than 2,000 mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene have been identified until now of which approximately 85% are CF-disease causing. Novel mutation-specific therapies that repair CFTR protein function have yielded impressive clinical improvements, however, only for a minority of all subjects with CF. This highlights the need for identification of additional CFTR-modulating drugs, and thus also for assays that provide sufficient throughput and translational value to assist in preclinical drug development and patient stratification. We have previously developed a CFTR function readout in patient-derived rectal organoids, termed the forskolin-induced swelling (FIS) assay that has high translational value and considerable throughput. The aim of this study was to increase the throughput of the FIS assay by developing a 384-wells plate assay, while retaining assay characteristics of the current 96 wells plate assay. To confirm feasibility of the novel HTS assay, FIS responses to ivacaftor and/or lumacaftor treatment in previously well-characterized organoids harboring homozygous F508del and F508del/S1251N mutations were measured using this approach. Our HTS measurements showed to be reproducible among experiments and to be of high quality as indicated by Z'-factor values. Consequently, we started to implement our HTS approach, among others, for the identification and/or repurposing of old and new CFTR-restoring compounds in Dutch CF subjects diagnosed with rare, uncharacterized *CFTR* mutations (prevalence of < 0.5% in the Dutch CF population; the 'Rainbow' project). We will screen more than 1,000 FDA-approved compounds in a total of 150 Dutch CF patients. Our preliminary data obtained from our initial toxicity screens (organoid morphology and live-dead staining) and the CFTR-modulating potential of some of these compounds in organoids harboring homozygous F508del, F508del/S1251N and rare missense mutations indicate the feasibility and strength of our HTS drug discovery platform. As such, we have recently started screening of the FDA-approved drug library in these 150 Dutch CF subjects that harbor rare uncharacterized *CFTR* mutations and we anticipate to identify novel and/or repurpose old CFTR-restoring compounds for this specific group of patients.

P117

Personalized cell-based therapy for cystic fibrosis-related lung disease

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Although significant advances have been made in the identification and use of small molecules in the treatment of Cystic Fibrosis (CF), a large proportion of CF patients will not be able to benefit from these therapeutic strategies as these patients include patients with Class I mutations in the CFTR gene which disrupts expression of the CFTR protein and patients already in the advanced stages of lung disease. Lung transplantation remains one of the key treatment options however this option is not readily available due to organ shortage, surgical risks and long-term immunosuppression. It is therefore imperative that alternative treatment strategies are developed to address these issues. Human pluripotent stem cells (hPSCs) could be an extremely valuable source of lung cells given their ability to propagate themselves in culture indefinitely and their ability to generate all cell types. Our group has generated induced pluripotent stem cells (iPSCs) using dermal fibroblasts from CF patients under Good Manufacturing Practice (GMP)-compliant conditions to allow the transfer and use of these cell lines in the clinic in the future. The CRISPR/Cas genome editing system has been successfully applied to correct the mutations present in the CFTR gene in the CF iPSC lines. These CF iPSC lines will be differentiated into lung cells which will be assessed functionally in vitro, particularly the expression and function of the CFTR protein. We are also developing a protocol to derive basal stem cells, a cell type implicated in lung repair and regeneration and could therefore be useful for cell-based therapies. These cells will then be transplanted into animal models of lung disease/injury to assess their ability to ameliorate the lung disease/injury phenotype.

P118

Human periodontal ligament stem cells: a novel model for cystic fibrosis research and personalized medicine

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Primary cells from cystic fibrosis (CF) patients are paramount to study CF pathogenesis and develop personalized therapeutics. Airway epithelial cells isolated from explanted lungs or nasal brushing, intestinal organoids and peripheral blood leukocytes, are largely used for these purposes. In alternative, somatic cells from CF patients can be reprogrammed to obtain inducible pluripotent stem cells (iPSC) that can be differentiated into airway epithelial cells. These models, however, suffer of some limitations related to complex isolation procedures, limited lifespan and availability over time. We developed an additional cellular model for CF studies based on the observation that human periodontal ligament stem cells (hPDLSC) express a biologically active CFTR. hPDLSC are adult mesenchymal stem cells, easy to isolate and to propagate in vitro with excellent yield and maintenance of the original phenotype for a large number of passages. We tested the suitability of these cells for in vitro prediction of clinical response to CFTR potentiators/correctors. To this end, we measured CFTR activity in hPDLSC collected by gingival curettage performed during dental hygiene from 6 CF patients with varying genotypes, before and after exposure in vitro to VX-770/VX-809 (Orkambi) for 72 h. We observed a variable degree of increment in CFTR activity, from no variation to a 30-50% increase. We tested the reproducibility of these measurements after several passages and after storage in liquid nitrogen. No significant changes were recorded, indicating that hPDLSC represent a very stable and reliable model for in vitro drug testing and discovery. Two of the donor patients, both homozygous for the ΔF508 genotype, were enrolled in a clinical trial with Orkambi and CFTR activity was also measured in peripheral blood monocytes before and after 3 month therapy. Remarkably, monocytes from the patient whose hPDLSC did not respond to Orkambi also showed no response, whereas the other patient showed a comparable increase in CFTR activity in hPDLSC and monocytes.

We previously documented that hPDLSC can undergo osteogenic and adipogenic differentiation (Cianci et al., Stem Cell Transl. Med., 2016). Therefore, we asked whether these cells could serve as a model to investigate on the bone and adipose tissue defects, frequently observed in CF patients. For this purpose, we compared the osteogenic and adipogenic differentiation pattern of hPDLSC from 4 CF patients (3 ΔF508/ΔF508 and 1 ΔF508/R1070Q) and 4 healthy age- and sex-matched donors. CF cells manifested severely impaired adipogenic (P = 00002) and significantly delayed osteogenic (P = 0.018) differentiation.

In summary, the advantages of hPDLSC for CF studies are: 1. Easy collection by minimally invasive procedures; 2. No need for reprogramming; 3. No need for differentiation into epithelial cells for drug screening; 4. Almost 100% success with the explants; 5. Excellent proliferation rate; 6. Long term storage and availability, ideal to make a repository for drug testing on rare mutations as well as for HT screening for drug discovery; 7. Valuable tools to investigate on osteogenesis and adipogenesis in CF.

P119

Impact of ASL acidification on bacterial killing capacity in cystic fibrosis airways

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Background: Respiratory failure determines prognosis in Cystic Fibrosis (CF). CF infants are first colonized with *Staphylococcus aureus*. This colonization turns to chronicity, the mechanism of which is unknown.

It has been shown in the pig CF model that pH can influence bacterial killing capacity by acting on Airway Surface Liquid (ASL) antimicrobial peptides activity (Pezzulo *et al.* Nature, 2012). Our study aims to determinate the influence of pH on *S. aureus* bacterial killing capacity on physiological conditions in human respiratory cell models.

Methods: pH was measured using a microelectrode (Thermo Scientific Orion 9810BN) in WT and F508del bronchial cells lines (CFBE 41o$^-$) and in bronchial primary cells from healthy controls and F508del homozygous patients after apical addition of 50µL of ringer (25mM HCO_3^-, pH=7.4) in a controlled atmosphere box (5% CO_2 and 37°C, Tech Systemes).

To evaluate bacterial killing capacity, epithelium was apically infected with an inoculum of 300 CFU/ml (CFBE) or 3000 CFU/ml (primary cells) of *S.aureus* CIP 76.25 (Collection de l´Institut Pasteur). ASL was collected after 1h30 (CFBE) or 6h (primary cells) incubation to count survival bacteria.

Adhesion and invasion of *S.aureus* were assessed by confocal microscopy.

Results: A significant difference in ASL pH was found between WT and CF cell lines (WT: 7.42±0.02 *versus* CF: 7.15±0.01; ($p< 0.001$). The same pattern of data was observed in primary cells (WT: 7.43±0.006 *versus* CF: 7.36±0.01; ($p=0.002$).

Bacterial killing capacity is significantly higher in the WT epithelium *vs* the CF epithelium, by 20 fold in the primary cells ($p=0.03$) and by 2 fold in the CFBE cells ($p=0.03$).

We determine in CFBE cells that *S.aureus* remains in ASL and does not display any adherence nor invasion of WT or CF epithelium.

Conclusion: Our data confirm that F508del ASL is acidified and that the bacterial killing capacity of the CF epithelium is significantly decreased.

P120

Non-viral CRISPR/Cas gene editing in mouse lungs enabled by co-delivery of mRNA and sgRNA inside of synthetic lipid nanoparticles

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CRISPR/Cas is a revolutionary gene editing technology with wide-ranging utility, including potential correction of the mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) that cause CF. Future therapeutic use depends on the ability to safely and effectively deliver CRISPR/Cas components to relevant cells. To date, delivery has largely been accomplished using viruses, which have limited translational potential.

We will present and report the synthesis and development of zwitterionic amino lipids (ZALs) that are uniquely able to co-deliver long RNAs (Cas9 mRNA and targeted sgRNA) from ZAL nanoparticles (ZNPs) to enable gene editing. Delivery of low sgRNA doses (15 nM) can reduce reporter protein expression by >90% in multiple cell types, including lung. In contrast to transient therapies (e.g. RNAi-mediated mRNA degradation),[1,2] we will show that ZNP delivery of sgRNA results in permanent DNA modification, where the 95% decrease in protein expression is sustained indefinitely even after multiple rounds of cellular division. Sequence specific editing was confirmed by the Surveyor assay. ZNP delivery of mRNA results in high protein expression at low doses *in vitro* (< 600 pM) and *in vivo* (1 mg/kg). We administered Luc mRNA ZNPs intravenously (i.v.) to multiple strains of mice and quantified high expression of luciferase in liver, lung and spleen tissue 24 hours after injection. In mice, intravenous co-delivery of Cas9 mRNA and sgLoxP (4:1 wt ratio; 5 mg/kg total RNA) induced expression of floxed tdTomato in the liver, kidneys, and lungs of genetically engineered mice. The effectiveness of ZNPs for delivery of long RNAs provides a chemical guide for the rational design of future carriers. Moreover, this development of gene editing using synthetic nanoparticles is a promising step towards improving the safety, efficacy, and utility of CRISPR/Cas.[3]

The ability to deliver long mRNAs to relevant organs suggest potential to deliver CFTR mRNA *in vivo* to lungs. Preliminary data on this topic, including mRNA delivery to primary human bronchial epithelial (HBE) cells derived from CF patient explants that harbor nonsense mutations in CFTR, will also be presented.[4] Cumulatively, these results show that CRISPR/Cas editing is possible using non-viral carriers. The use of scalable and translatable technologies, such as ZNPs, will provide powerful tools for *in vivo* gene editing.

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P121

Role of CFTR Cl^- channel in the exocrine/endocrine function of the pancreas

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Introduction: Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Cl^- channel controls the flow of anions through the apical membrane of pancreatic ductal epithelial cells (PDECs). Dysfunction of the channel causes abberant fluid secretion and leads to the lethal genetic disease, cystic fibrosis (CF). Previous data indicate that approximately 50% of CF patients develop diabetes during their lifetime. However, the relation between CF and diabetes is not exactly known. Our hypothesis is that changes in exocrine fluid secretion may plays role in the development of endocrine dysfunction. Therefore, our aim in this study was to investigate how CFTR influences the exocrine/endocrine function of the pancreas using CFTR knock out (KO) mice.

Methods: Intra/interlobular pancreatic ducts were isolated from the pancreas of wild type (WT) and CFTR KO mice on a FVB/N. Immunostaining of CFTR, insulin- and glucagon-secreting cells were performed by immunohistochemistry. Pancreatic ductal HCO_3^- secretion was measured by the inhibitory stop methods using fluorescence microscopy. Pancreatic ductal fluid secretion was examined by videomicroscopy, whereas, patch clamp technique was used to detect the CFTR activity in the isolated PDECs

Results: Strong CFTR expression was detected on the luminal membrane of PDECs in WT, but not in CFTR KO mice. The absence of CFTR activity in KO animals was also confirmed by patch clamp technique. Using immunohistochemistry we have shown that the number of α and β cells significantly decreased in KO mice compare to WT mice. Investigation of the exocrine function has shown that HCO_3^- secretion reduced by 57.1 ± 5.8%, whereas fluid secretion almost completely abolished in ducts isolated from CFTR KO animals.

Conclusion: In this preliminary study we have shown that the absence of CFTR Cl^- channel decreases pancreatic exocrine function and also influences the endocrine part of the pancreas. Further investigations are needed to identify how CFTR affect the endocrine function of the pancreas.

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P122

LncRNAs: emerging players in *CFTR* gene regulation

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Objectives: *CFTR* gene displays a tightly tissue specific and temporal expression pattern. Recently, we showed that transcription factors and small non-coding RNAs (miRNAs) act in synchrony to explain the weak *CFTR* mRNA level in mature lung cells. Other non-coding RNAs such as long non-coding RNAs (lncRNAs) have been investigated as regulatory players in the control of genes showing tissue-specific expression. These lncRNAs could operate locally near their sites of synthesis. In this work, as we predicted the presence of nine lncRNAs within the *CFTR* locus, we decided to define their potential role on the *CFTR* expression.

Materials and Methods: To identify lncRNAs within *CFTR* locus and adjacent regulatory sequences, we used databases collecting known lncRNAs and conservation-related information (LNCipedia, UCSC, Ensembl...). Expression analysis by RT-qPCR has been conducted in 20 different human tissues from several donors and in cell lines (BEAS-2B, 16HBE14o-, Caco-2). For selected non-coding RNAs, antisense oligonucleotides (named Gapmers) have been designed to inhibit their expression. Gapmers have been transfected at different concentrations in bronchial cells and lncRNAs or *CFTR* mRNA level were analyzed by RT-qPCR.

Results: By using *in silico* tools, we identified 9 putative non-coding RNAs located within the *CFTR* locus, 2 exonic overlapping, 2 natural antisense, 1 promoter-associated and 3 intergenic transcripts. Prediction tools confirmed the good conservation of their canonic splicing sites and polyadenylation signals in mammalians. Quantitative analysis showed a detectable expression of 6 non-coding RNAs (Ct from 27 to 32) underlying their putative biological existence. Interestingly these lncRNAs display a tissue-specific expression profile. To assess whether these lncRNAs could exert *cis*-acting role locally or on neighbouring protein-coding genes, we used Gapmers to inhibit their expression. RNA silencing of one lncRNA induced a two-fold increase in *CFTR* mRNA level, indicating a potential implication in the repression of *CFTR* gene expression. An exonic overlapping lncRNA induced a decrease in the level of *CFTR* mRNA suggesting an activating role in *CFTR* transcription. To confirm our data, CRISPR-Cas9 approach designed to knock-out these lncRNAs is in progress.

Conclusion: In this study, we characterized new lncRNAs transcribed in the *CFTR* locus. These non-coding RNAs exhibit a tissue-specific expression and our findings suggest that these regulatory players might contribute to *CFTR* gene expression. These new partners could represent promising targets for future therapeutic in CF.

P123

Unsolved severe chronic rhinosinusitis elucidated by extensive *CFTR* genotyping

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Pulmonary and sinonasal (chronic rhinosinusitis, CRS) involvements occur in up to 86% of affected cystic fibrosis (CF) children. Chronic pulmonary infection with *Pseudomonas aeruginosa* (*Pa*) is associated with accelerated decline in lung function and earlier mortality in CF. Whereas carriers of a single *CFTR* mutation show higher prevalence of CRS than the general population, *Pa*-positive sinus culture is not an indication for exploration for *CFTR* gene mutations when sweat test is normal.

Newborn screening detects children with a single *CFTR* mutation escaping from periodic CF centre survey because they do not fulfil the diagnostic criteria for CF. Raman *et al*. found *Pa*-positive cultures from the sinuses of p.Phe508del-heterozygous children in a group of children with CRS who underwent genotyping for *CFTR* mutation using an assay that detects 90% of mutations.

In the present study, we performed extensive *CFTR* genotyping in a child with CRS, *Pa*-positive culture and p.Phe508del-heterozygote mutation. We identified the p.(Phe1099Leu) mutation (F1099L), inherited from his mother of Martinican origin. *In vivo* functional tests and *in vitro* assays were performed. Transepithelial ion transport measurements in nasal mucosa showed ENaC channel hyperactivity, as assessed by the high response to amiloride while cAMP-dependent chloride secretion was normal, as assessed by the response to isoproterenol and low chloride solution. In the rectal mucosa, forskolin, carbachol and histamine responses were at the lower limit of normal, indicating moderate defect in CFTR dependent Cl^- conduction. Biochemical analysis showed that F1099L-CFTR was not fully mature, this decreased processing was further confirmed by inspection of its subcellular localization on confocal microscopy, showing that F1099L-CFTR was weakly expressed at the plasma membrane. Treatment with VX-809 alone or in combination with VX-770 completely corrected the processing defect.

Phe1099 is located within the membrane-spanning domains, in transmembrane helix TM11, as predicted from a recent model of CFTR´s 3D structure. In this model, mutation of Phe1099 into a leucine is predicted to diminish TM10-TM11-TM12 bundle stability and to impair protein folding. The position of Phe1099 is distinct from that of Phe508, which is located at the interface between NBD1 and ICL4. Correction of F1099L-CFTR by VX-809 may thus correct the stability of the MSD2 TM assembly, following a mechanism which may be similar to that suggested for the correction of the F508del mutation by VX-809 through stabilization of MSD1.

We propose extensive genotyping in CRS with *Pa*-positive cultures to discover rare mutations which could be treated by CFTR correctors to postpone pulmonary infection and before onset of pulmonary degradation.

P124
miRNA profiling identifies new CF regulators

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Objectives: Changes in microRNAs (miRNAs) expression profile in CF cells (bronchial cells in monolayer or in Air-Liquid Interface (ALI), or nasal and bronchial brushings) have been described in the literature, mainly by quantification of miRNAs preselected by the TLDA profiling platform (TaqMan Low Density Array). Despite the high sensitivity of this platform, the catalogue of miRNAs generated is not exhaustive (742 miRNAs /2588 miRNAs known). In order to obtain an overview, we studied the expression profile of miRNAs by an unbiased sequencing approach (MiSeq, Illumina) on the ALI bronchial cultures (5 non-CF and 5 CF). The aim of this work is to define the genes and / or signaling pathways targeted by these deregulated miRNAs.

Materials and methods: RNAs or proteins were extracted from ALI cultures (5 non-CF and 5 CF p.Phe508del/p.Phe508del), or isolated from bronchial cells (CFBE41o-, 16HBE14o- and Beas-2B). To study the effect of miRNAs or miRNAs inhibitors, the expression level of miRNAs, transcripts or proteins of interest was evaluated by RT-qPCR or immunoblot.

Results: In CF bronchial cells, we showed the deregulation of 35 miRNAs, which mainly target mRNAs involved in PI3K-Akt pathway (21 out of 35 deregulated miRNAs) and MAPK pathway (18 out of 35 deregulated miRNAs). First, to validate the role of these genes in CF, we quantified by qPCR, the expression of more than 100 genes related to PI3K-Akt and MAPK pathways (by using a dedicated PCR array, PI3K-Akt Signaling Pathway RT2 profiler, or by using lab-designed primers). Of these genes, 15 are over- or under-expressed in CF ALI cultures compared with the non-CF ALI cultures. Next, to confirm whether dysregulated miRNAs participate in post-transcriptional regulation of these deregulated genes, we decided to use inhibitors (for 5 up-regulated miRNAs) or mimics (for one down-regulated miRNA) in CF ALI cultures. So far, we showed that 3 miRNAs contribute to mRNA stability of 5 genes. As a crosstalk between PI3K-Akt and MAPK pathways, RNA-BPs are good candidates. We next focused on the TTP protein (Tristetraprolin), a RNA-BP targeted by 6 deregulated miRNAs (predicted by TargetScan, Diana...) and regulated by PI3K-Akt and MAPK pathways. This RNA-BP, deregulated in CF cultures, binds to 3´UTR and orchestrates stability in competition or in synergy with miRNAs of many mRNAs. The TTP non-phosphorylated form modulates the stability of many interacting partners including pro-inflammatory proteins such as IL-8. We showed by immunoblot an accumulation of the TTP phosphorylated form in CF cells, its inactive form. In order to define which proteins participate in TTP phosphorylation in CF cells, the expression level of different proteins related to PI3K-Akt and/or MAPK pathways is being evaluated. The effect of the overexpression or silencing of TTP is also underway.

Conclusion: The miRNA signature in CF context led to focus on a complex molecular circuitry including the RNA-BPs, new molecules regulating CF physiopathology. Decoding miRNAs/TTP partners' map could be of great interest in CF.

P125

Trypsin-like protease activity predicts disease severity and patient mortality in adults with cystic fibrosis

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Background: Serine trypsin-like (TL) proteases, which are excessively active in CF airways, promote activation of the epithelial sodium channel (ENaC) and airways dehydration; a key initiating factor for CF lung disease pathogenesis. Furthermore, TL- proteases enhance mucin gene expression and mucus hypersecretion, yet whether there is any relationship between the activity of these enzymes and CF pulmonary disease is unknown.

Objective: To determine whether TL-protease activity, measured in adult CF sputum sol, correlates with lung disease and patient outcome (survival).

Methods: In this cross-sectional, retrospective study we analysed CF sputum sol collected from 30 clinically stable adult CF patients. Protease activity was measured by monitoring the hydrolysis of a peptide-based fluorogenic substrate (QAR-AMC). Biomarkers of inflammation (IL-8 and TNF-α) were measured by ELISA. Lung function was assessed by spirometry (FEV_1). Mortality data was retrospectively obtained and time in months until death or transplantation used for subsequent survival analysis.

Results: TL-protease activity inversely correlated with lung function (FEV_1) (r=-0.4, p=0.031) however, no relationship with IL-8 and TNF-a was observed. Kaplan-Meier analysis demonstrated significantly reduced survival for those individuals with above median TL-protease activity. Using a multivariate Cox regression analysis (adjusted for age and BMI) a significantly increased mortality hazard (HR 1.028, 95% CI 1.007-1.049; p=0.009) was also identified.

Conclusions: TL-protease activity inversely correlates with lung function and patient survival. As such tryptic activity may warrant consideration when modelling CF survivorship and should be investigated further as a biomarker of CF lung disease and as a potential therapeutic target.

P126

PTC mutations in CFTR: effect on messenger RNA abundance

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Background: Of approximately 2,000 potentially disease causing CFTR variants described [1], about 8% are nonsense mutations which introduce premature termination codons (PTCs) into the CFTR messenger RNA (mRNA). Furthermore, frameshift mutations, which represent nearly 16% of the total, may also have this effect. The presence of a PTC is commonly associated with mRNA degradation of the mRNA via nonsense mediated decay (NMD) [2], leading to reduced transcript abundance in the cell.

Objective: Our aims here are: *i)* to determine the extent to which individual PTC mutations affect mRNA abundance in an allele specific manner; *ii*) to describe whether mRNA levels vary either due to mutation position or among individuals; and *iii*) to investigate the suitability of intestinal organoids and conditionally reprogrammed (CR) human nasal epithelial cells (HNECs) as models for assessing this phenomenon.

Methods: We measured the relative abundance of allele specific CFTR mRNA transcripts in native (HNECs, rectal biopsies) and cultured tissues (primary CR-HNECs and intestinal organoids) collected from CF patients with F508del/PTC CFTR genotypes. We applied the ddCT qRT-PCR method in the exon 10-11 region to compare expression of the PTC-bearing CFTR allele against that of the F508del allele, using primer pairs that reliably distinguish the 3-bp difference between the F508del and non-F508del sequences as previously described [3]. Other primer pairs have also been also tested and optimized for discrimination between PTC and non-F508del alleles in some cases (W1282X, G542X, E60X).

Results and Discussion: Our data show that relative abundance of PTC-derived mRNAs (nonsense mutations S4X, G542X, Y1092X and W1282X, and frameshift mutations 2183AA>G and 3866insA) varies between 21% and 33% of the total CFTR transcript levels in native HNECs, with the exception of S4X (47%), thereby giving evidence for NMD for the majority of PTC bearing CFTR transcripts. By contrast, transcripts bearing non-PTC mutations (P205S, R334W, A561E) were slightly more abundant (50%-61% of total) than F508del transcripts, supporting our previous data [4]. Data were also obtained in intestinal organoids for nonsense mutations (eg, E60X, G542X, Y1092X, R1162X, W1282X) and frameshift mutations (eg, 365insT and 2183AA>G), demonstrating similarly and consistently reduced levels of PTC bearing transcripts (17%-39% for PTC mutations, 20%-27% for frameshift mutations) compared to non-PTC transcripts (eg, A455E: 49%, 711+1G>T: 51%), and validating the use of organoids as a surrogate for the respiratory epithelium in this study, a conclusion which was also extended to CR-HNECs for some genotypes. Our work provides a basis for future studies on the possible benefits of NMD suppression in combination with PTC read-through therapies.

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P127

Beyond the ussing chamber: MTECC-24-96

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The Ussing chamber, named after Hans Ussing, has been the main means of evaluating the electrophysiological properties of epithelia for over 65 years. Epithelia are clamped between two half chambers with two electrodes placed on each side. One electrode on each side is used to measure the transepithelial voltage (Vt) and the second pair of electrodes is used to pass current across the epithelium. When Vt is clamped to zero the epithelium is said to be under closed circuit, or short circuit current conditions and the applied current is referred to as the short circuit current (Isc). Isc is a measure on net electrolyte transport. The epithelium may also be studied under open circuit conditions and an equivalent current (Ieq) can be calculated from the measured Vt and the transepithelial resistance (Rt) using Ohm's law (Ieq = Vt/Rt). In symmetric solutions Isc will equal Ieq. Indeed many of the early studies on airway epithelia were performed under open circuit conditions and reported Ieq values as a measure of net electrolyte transport. Thus the Ussing chamber holds an important place in advancing our understanding of transpithelial electrolyte transport. One limitation of the Ussing chamber methodology is that it is essentially limited to performing studies in a single chamber format and thus only a modest through-put can be achieved even when running multiple chambers in parallel. Here we report the development of multi-channel systems that can be used with 24 and 96 well filter plates to obtain Ieq and transepithelial conductance (Gt) measurements. The electronics uses a 24 or 96 channel current clamp (MTECC-24 or MTECC-96) together with electrode manifolds with 96 or 384 electrodes allowing for two voltage and two current passing electrodes across each filter. The entire system can be used manually on a bench top or mounted to a robotic platform for the automated movement of the electrode manifold to several filter plates on a workstation deck. In a typical experiment we use five 24 well filter plates allowing for measurements on 120 filters. Our typical experiments run for two hours and two experiments can be performed per day. Everything, except the electrodes, is disposable minimizing the chance of compound carry over between experiments. The system has been valadiated using primary CF hBE cells, nasal cells derived from non-CF and CF donors, CFTR and ENaC expressing FRT cells, T84 cells and CFBE expressing CFTR cells. Well-to-well and plate-to-plate variations in the responses were remarkably low with coefficients of variation (CV) of less than 10% and Z'-factor values of greater than 0.75. Automated Ieq measurements with this 24 well format can now be used to evaluate up 500 compounds per week further accelerating the discovery of drugs for the treatment of CF. Supported by CFFT BRIDGE07XX0

P128

Cystic fibrosis infant airways harbor a pathogenic subset of live neutrophils

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Objectives: In cystic fibrosis (CF) adults, we showed that a subset of airway polymorphonuclear neutrophils (dubbed "A2 PMNs") releases the toxic protease elastase actively, a pathological process that correlates negatively with lung function. Here, we assessed whether the pathogenic A2 subset of PMNs was present in CF infants, and whether the presence of this subset correlated with the severity of airway damage at an early disease stage.

Methods: This study was approved by the ErasmusMC and Emory University Institutional Review Boards. Ten CF patients (age: 1-5 y, mean age: 4.2 y) were enrolled via the surveillance program at the Sophia Children's Hospital. Blood and bronchoalveolar lavage fluid (BALF) were collected and analyzed by flow cytometry. The PRAGMA CF method was used to score CF lung disease on chest CT-scans.

Results: PRAGMA total disease (%Dis) and bronchiectasis (%Bx) scores ranged from .9-9.9 (median: 3.2) and 0-3.8 (median: 1.1), respectively. PMN% of total cell count in BALF ranged from 3-66. Irrespective of %Dis and %Bx scores, and of PMN% in BALF, CF infant airways harbored a distinct population of activated PMNs. Airway PMNs showed increased surface expression of CD66b compared to blood PMNs (CD66b median fluorescence intensity -MFI-: 1,947 vs. 354, respectively, P< .0001), as expected upon blood PMN recruitment to airways. Remarkably, in all CF infants, a significant fraction (20-71%) of these activated airway PMNs showed the characteristic features of pathogenic A2 PMNs that we previously identified in CF adults, namely: (i) increased surface expression of the elastase-rich granule marker CD63, and of the homing receptor CXCR4; and (ii) decreased expression of the phagocytic receptor CD16. Consequently, total airway PMNs differed significantly from blood PMNs in this infant cohort, in similar ways to the changes observed in adults (CD63 MFI: 1,182 vs. 246, P=.004; CXCR4 MFI: 2,607 vs. 420, P=.0003; CD16 MFI: 5,062 vs. 11,700, P=.03, respectively). In addition, the median fluorescence intensity of CD63 in airway PMNs, which is a compound measure of the A2 subset frequency and of the degree of elastase release by that subset (independent of total count) correlated significantly with the %Dis score (Rho=.65, P=.04), suggesting an association between the cellular anomaly of airway PMNs and the severity of airway damage in CF infants.

Conclusion: This pilot cross-sectional study supports the notion that CF airways harbor a subset of pathogenic PMNs from the earliest stage of disease, and that this subset plays a key role in the progression of CF lung disease, likely via the active release of elastase and other toxic mediators. Parallel tracking of lung disease by chest CT and of PMN dysfunction by flow cytometry is a powerful approach to monitor the course of CF lung disease in infants, paving the way for future longitudinal and interventional studies.

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Characterization of Q1412X-CFTR, a severe form Class VI pathogenic mutation

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Cystic fibrosis (CF) is caused by loss-of-function mutation of cystic fibrosis transmembrane regulator (CFTR), a phosphorylation activated but ATP-gated chloride channel. Based on the underlying molecular mechanism, disease-associated mutations can be categorized into six classes with respective deficits in protein production (Class I and V), intracellular trafficking (Class II), channel gating (Class III), conductance (Class IV), and stability in the plasma membrane (Class VI). Previous studies showed that Class VI mutations caused by frameshift mutation (e.g., 4236delTC, 4279insA) or premature stop codons (e.g., Q1412X, S1455X) result in faster degradation of the mature, complex-glycosylated protein in the cell membrane. While it is unlikely a frameshift mutation may be functional, mutations that keep a large fraction of the protein may maintain some degree of function. Indeed, it was reported that Q1412X-CFTR assumes normal gating function.

We first used Western blot experiments to confirm a decreased level of mature Q1412X-CFTR in the cell membrane (~25 fold lower than WT-CFTR). Importantly, the CFTR corrector VX-809 (Lumacaftor) increases the expression of mature Q1412X by 4.03 ± 0.6 folds (n = 3). While it is known that the combination of CFTR potentiator VX-770 (Ivacaftor) and VX-809 reduces the efficacy of the corrector for ΔF508, this negative drug-drug interaction is not observed for Q1412X-CFTR (5-fold increase of band C in the presence of VX-809 and VX-770), suggesting that the antagonistic interaction between VX-770 and VX-809 is mutation-specific.

Since a 25-fold decrease of mature proteins in the cell membrane may not be sufficient to account for the severe phenotype in patients carrying the Q1412X mutation, patch-clamp electrophysiological studies were carried out to assess the functional properties of Q1412X-CFTR. In inside-out patches, 200 nM VX-770 increased the macroscopic current of Q1412X by 6.7 ± 0.5 fold (n = 6). The channel activity can be further enhanced by nitrate with an overall 13.6 ± 1.5 fold increase of the macroscopic current (n = 3). Thus, the maximal possible Po of Q1412X-CFTR is 1/14 or 0.07, which is > 6 fold lower than that of WT channels. Together, we conclude that the severe phenotype caused by the Q1412X mutation results from a combination of defects in membrane stability and gating. Functional studies also revealed interesting interaction between VX-809 and VX-770, although the nature of this interaction is unclear. For cells pretreated with VX-809, potentiation by VX-770 was increased to 12.3 ± 1 folds (n = 3). These results predicts a ~50-fold improvement of Q1412X-CFTR channel function by combination VX-809/VX-770. Indeed, preliminary data by comparing overall currents between the control with no treatment and conditions with both drugs show a 20-fold increase. Thus our studies support the treatment regiment of combination Lumacaftor/Ivacaftor for patients carrying the Q1412X mutation.