2013
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

Malaga, Spain

Chairpersons

Aleksander Edelman, Bob Ford and Rob Tarran

20 - 23 March 2013

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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 to the 10th European Cystic Fibrosis Conference entirely dedicated to Basic Science which, this year, takes place in Torremolinos-Malaga, Spain. This year we are delighted to welcome Prof. Aleksander Edelman as the conference Chairperson who will be supported by Prof. Robert Ford and Prof. Robert Tarran 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.

Stuart Elborn
President
European Cystic Fibrosis Society

Conference Chairpersons' Welcome

We are very happy to welcome you to the 10th Basic Science of CF conference 2013, in Torremolinos-Malaga, Spain.

Among the foothills of the Andalusian mountains, in an area of gentle terrain that decreases in altitude as it approaches the seacoast, lies the territory of Torremolinos, formerly a district of Málaga
In the 50's Torremolinos was still a tranquil village, which sprang up around a watchtower and some mills exploiting the abundant spring water.
Over the years, Torremolinos has evolved as an attractive and appealing resort.

With this conference, you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment. A key characteristic of the conference is the high attendance of young participants, both PhD students and post-docs, who contribute enormously to the success of this annual event with their enthusiasm and vivid discussions. Here you will have excellent opportunities to discuss your data and interact in a great atmosphere with the best of European and International experts in this field.
This year's programme includes a number of symposia with international experts covering topical aspects of basic research in cystic fibrosis together with invited talks from submitted abstracts.There are also two keynote lectures, two workshops as well as two interactive special interest group discussions. On Thursday and Friday evening we will finish the day with poster sessions and a chance to win the best poster prize! This combination has been extremely successful in the past and the conference offers a forum for informal brain-storming-type discussions.

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

Aleksander Edelman
University of Paris Descartes
France

Bob Ford
University of Manchester
United Kingdom

Rob Tarran
University of North Carolina
Chapel Hill,
United States

2013 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

20 March- 23 March 2013, Malaga (Torremolinos), Spain

Programme

Chairpersons: Aleksander Edelman (Paris, France), Bob Ford (Manchester, United Kingdom), Rob Tarran (Chapel Hill, United States)

Wednesday, 20 March 2013 (Day 1)	
10:00-16:00	**Pre-Conference Seminar - Animal Models**
17:30-18:00	**Official Opening of the Meeting by the Conference Chairpersons** – Room Malaga
18:00-19:00	**Opening Keynote Lecture** **I**nnate Defense, Lung Infection and Cystic Fibrosis – Michel Chignard (FR)
19:00-19:45	**Welcome Reception**
19:45-21:30	*Dinner*

Thursday, 21 March 2013 (Day 2)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – Pathophysiology Related to CF**
	Chairs: Michael Gray (UK) / Miguel Valverde (ES)
08:45-09:10	Inflammatory Responses in the Lungs of newborn cf and non-cf Pigs challenged with Bacteria Paul McCray (US)
09:10-09:35	Ethanol and Fatty Acids Strongly Inhibit the Activity of the CFTR Chloride Channel and Anion Exchangers in Pancreatic Ductal Epithelial Cells - Peter Hegyi (HU)
09:35-10:00	TRP Channels relevant to epithelial Physiology - Miguel Valverde (ES)
10:00-10:10	Abstract 25 : Planar Cell Polarity protein network, which controls ciliogenesis and cilia function, is altered in Human Cystic Fibrosis Bronchial Epithelial cells - Sabrina Noël (BE)
10:10-10:20	Abstract 28：TMEM16A/ANO1 expression in human bronchial epithelia – P.Scudieri (IT)
10:20-10:30	Abstract 62：Correction of Bone Defect in Cystic Fibrosis: Studies from F508del in CFTR Human and Mouse Models - Jacky Jacquot (FR)
10:30-11:00	*Coffee break* & **Poster viewing** – Room Granada
11:00-12:45	**Symposium 2 – Cell Biology of CF: Autophagy and Degradation**
	Chairs: Margarida Amaral (PT) / Bruce Stanton (US)
11:00-11:25	Targeting autophagy as a new strategy to circumvent ΔF508CFTR defect - Luigi Maiuri (IT)
11:25-11:50	A *Pseudomonas aeruginosa* toxin enhances the degradation of CFTR and disrupts MHC class I antigen presentation in respiratory epithelial cells - Bruce Stanton (US)
11:50-12:15	CFTR as a signal generator, new evidence.- Anil Mehta (UK)
12:15-12:25	Abstract 13：Palmitate accumulates within phosphatidylcholine of cystic fibrosis human bronchial epithelial cells: impact on F508del-CFTR trafficking.- Laurie-Anne Payet (FR)
12:25-12:35	Abstract 24 : Modulation of epithelial repair and autophagy by female hormones in normal and cystic fibrosis human epithelial bronchial cells - Vinciane Saint Criq (IE)
12:35-12:45	Abstract 14：Exchange Proteins Directly Activated by Cyclic AMP: Novel CFTR Interactors? - João Fernandes (PT)
12:45-14:30	*Lunch*
14:30-16:40	**Symposium 3 – Infection, Inflammation and Immunity - I**
	Chairs: Michel Chignard (FR) / Giulio Cabrini (IT)
14:30-14:55	Pulmonary Phage Therapy: efficiency of bacteriophages to treat bacterial infections of cystic fibrosis patients - Laurent Debarbieux (FR)
14:55-15:20	Type IIA secretory phospholipase A2 plays a key role in elimination of *Staphylococcus aureus* from cystic fibrosis lung - Lhousseine Touqui (FR)

15:20-15:45	Living without oxygen: anaerobiasis in the CF Airway - Stuart Elborn (UK)
15:45-16:10	Mucus Hyperconcentration as an initiator of infection and inflammation in CF - Ric Boucher (US)
16:10–16:20	Abstract 31: Lipoxin A4 Delays the Invasion of Cystic Fibrosis Bronchial Epithelial Cells by the pathogen *Pseudomonas aeruginosa* - Gerard Higgins (IE)
16:20-16:30	Abstract 40 **:** Anti-inflammatory effect of FDA approved drugs in CF cells - Pascale Fanen (FR)
16:30-16:40	Abstract 39 **:** Potential role of expression of E3 Ubiquitin Ligases in regulating the inflammatory phenotype of Cystic Fibrosis - Sara Canato (PT)
16:40-17:10	*Coffee Break* & **Poster Viewing** – Room Granada
	Rooms : Malaga + Cordoba II
17:10-18:40	**Special Group Discussion-I – CFTR Gene Expression / Modifiers**
	Moderators: Harriet Corvol (FR) / Paul McCray (US)
17:10-18:40	**Workshop-I – Expression and Purification of CFTR**
	Moderators: Christine Bear (CA) / Bob Ford (UK)
20:00-21:30	*Dinner*
21:30-23:30	Flash Poster Session Odd Numbers
	Evening Poster Session – Posters with Odd Numbers - Room: Granada

Friday, 22 March 2013 (Day 3)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4 – CFTR Folding: Towards Correcting the Defect**
	Chairs: Christine Bear (CA) / Ineke Braakman (NL)
08:45-09:10	Lesson and tools from a decade of biophysical studies of CFTR nucleotide-binding domains - John Hunt (US)
09:10-09:35	Towards a rational design of combination corrector strategy to restore ΔF508-CFTR coupled domain folding - Gergely Lukacs (CA)
09:35-10:00	Folding and assembly of MSD1 and NBD1 during CFTR - Ineke Braakman (NL)
10:00-10:10	Abstract 7 : Mechanistic Studies of CFTR Correctors - André Schmidt (US)
10:10-10:20	Abstract 4 : The Corrector C18 Modulates The Channel Activity Of Purified And Reconstituted CFTR Independent Of ATP - Paul Eckford (CA)
10:20-10:30	Abstract 12 : The iminosugar IsoLAB corrects the defective trafficking of F508del-CFTR by prevention of Hsp90/CFTR interaction - Johanna Bertrand (FR)
10:30-11:00	*Coffee break* & **Poster viewing** – Room Granada

11:00-12:45	**Symposium 5 – Infection, Inflammation and Immunity - II**
	Chairs: Stuart Elborn (UK) / Jean-Michel Sallenave (FR)
11:00-11:25	Phospholipase C beta and pro-inflammatory signaling in bronchial epithelial cells - Giulio Cabrini (IT)
11:25-11:50	Modulation of inflammatory signaling by Nrf2 mediated antioxidant responses - Assem Ziady (US)
11:50-12:15	The neutrophil in cystic fibrosis: dysregulation and inflammation - Gerry McElvaney (IE)
12:15-12:25	Abstract 36 : Neutrophil Elastase-mediated Increase in Airway Temperature during Inflammation - Annika Schmidt (DE)
12:25-12:35	Abstract 33 **:** Classically activated macrophages promote inflammation in response to *Pseudomonas aeruginosa* but do not provide an effective barrier against bacterial growth - Luisa Martinez-Pomares (UK)
12:35-12:45	Abstract 38 : Residence in Biofilms allows *Burkholderia cepacia* Complex (Bcc) Bacteria to Evade Neutrophil Anti-microbial Activities - Mark Murphy (IE)
12:45-14:00	***Lunch***
14:00-18:00	**Free Afternoon**
18:00-20:10	**Symposium 6 – CFTR Functional Interactome**
	Chairs: Ray Frizzell (US) / Aleksander Edelman (FR)
18:00-18:25	Small heat shock proteins target mutant CFTR for degradation via a SUMO-dependent pathway - Ray Frizzell (US)
18:25-18:50	CFTR interactions studied in live cells - Peter Haggie (US)
18:50-19:15	Potentiating and correcting double effect of snake venom Phospholipase A2 on ΔF508-CFTR - Grazyna Faure (FR)
19:15-19:40	cAMP compartmentalisation and regulation of CFTR - Manuella Zaccolo (UK)
19:40-19:50	Abstract 10 : Interaction between keratin 8 and Cystic Fibrosis Transmembrane Regulator (CFTR): structural studies – Anna Kupniewska (FR)
19:50-20:00	Abstract 59 : A functional CFTR assay using primary cystic fibrosis intestinal organoids - Florijn Dekkers (NL)
20:00-20:10	Abstract 23 : Signaling detected at ENaC N-termini controls ENaC proteolysis.- Jack Stutts (US)
20:15-21:30	*Dinner*
21:30-23:30	Flash Poster Session Even Numbers Evening Poster Session - Posters with Even Numbers – Room Granada

Saturday, 23 March 2013 (Day 4)

07:30-08:45	Breakfast
08:45-10:30	**Symposium 7 – Mucus and CF**
	Chairs: Steve Ballard (US) / Gerd Döring (DE)
08:45-09:10	Airway mucin organisation and maturation - David J.Thornton (UK)
09:10-09:35	Bicarbonate, a functional CFTR channel, and enzymatic activities are required for proper mucin secretion and link Cystic Fibrosis to its mucus phenotype - Gunnar C. Hansson (SE)
09:35-10:00	Genesis of Thick Mucus in Cystic Fibrosis Airways - Steve Ballard (US)
10:00-10:10	Abstract 44 **:** Modification of the salivary secretion assay in F508del mice – Salivary Chloride Quantification - Peter Sandner (DE)
10:10-10:20	Abstract 60 : Treatment with VX-809 and VX-770 in rectal CF organoids expressing uncommon CFTR genotypes - Evelien Kruisselbrink (NL)
10:20-10:30	Abstract 26 **:** SLC26A9-mediated Cl Secretion Is Induced In Airway Inflammation And Prevents Mucus Obstruction In Mice - Julia Duerr (DE)
10:30-11:00	*Coffee break* & **Poster viewing –** Room Granada
	Rooms : Malaga + Cordoba II
11:00-12:30	**Special group discussion-II – Correctors / Potentiators / New Treatments**
	Moderators: Christine Bear (CA) / John Hanrahan (CA)
11:00-12:30	**Workshop II – Imaging – Microscopy**
	Moderators: Peter Haggie (US) / Rob Tarran (US)
12:30-14:00	*Lunch*
14:00-15:45	**Symposium 8 – CFTR Structure and Function**
	Chairs: Oscar Moran (IT) / Robert Ford (UK)
14:00-14:25	Chimeric constructs endow the human CFTR Cl^- channel with the sub-conductance state behaviour of murine CFTR - David Sheppard (UK)
14:25-14:50	Insight into the structure, function and energetics of CFTR and its domains gained from atomic-level molecular simulations - Hanoch Senderowitz (IL)
14:50-15:15	P-glycoprotein, CFTR, and domain unfolding/stability studies: what can we learn about membrane protein structural responses to ligand binding and interdomain cooperativity? - Christie Brouillette (US)
15:15-15:25	Abstract 2 : Generation and Characterization of The G551D Mutation in CFTR Expressed in *S. cerevisiae* – Xin Meng (UK)
15:25-15:35	Abstract 3 : Purification and biophysical characterization of full-length CFTR expressed in *Saccharomyces cerevisiae* – Natasha Cant (UK)
15:35-15:45	Abstract 8 : ICL1 Facilitates the Conformational Maturation of CFTR Through Interdomain Interfaces with NBD1 and ICL3 – Steven Molinski (CA)

15:45-16:15	*Coffee Break & Poster Viewing* –Room Granada
16:15-18:00	**Symposium 9 – CFTR and COPD / Smoking**
	Chairs: Hugo De Jonge (NL) / Gerry McElvaney (IE)
16:15-16:40	Cigarette Smoke Exposure and the Failure of Airway Surface Liquid Homeostasis - Rob Tarran (US)
16:40-17:05	Acquired CFTR Dysfunction in Chronic Obstructive Pulmonary Disease - Steven Rowe (US)
17:05-17:30	CFTR modulates onset and severity of chronic obstructive lung disease in ENaC-overexpressing mice - Marcus Mall (DE)
17:30-17:40	Abstract 21 : SLC6A14 enhances constitutive CFTR-mediated Cl^- secretion in Human non-CF and CF Primary Bronchial Epithelia - Tanja Gonska (CA)
17:40-17:50	Abstract 55 : Small Molecule Inhibitors of GSNOR Possess Anti-inflammatory and Bronchodilatory Actions in Mouse Models of Inflammatory Lung Disease and Modulate CFTR Function in F508del CFTR Mice.- Sherif Gabriel (US)
17:50-18:00	Abstract 5 : Functional Consequences of the I507ATC➔ATT Silent Codon Change in ΔF508 CFTR - Zsuzsa Bebok (US)
18:00-18:15	*Break*
18:15-19:15	**Closing Keynote Lecture** - Room: Malaga
	Pharmacological Strategies to Rescue Chloride Transport in Cystic Fibrosis - Luis Galietta (IT)
20:30	*Dinner / Social Event*

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Florijn Dekkers, C.L. Wiegerinck, H.R. de Jonge, I Bronsveld, H.M. Janssens, Karin M de Winter-de Groot, A.M. Brandsma, N. W. M. de Jong, M.J.C. Bijvelds, B. J. Scholte, E.E.S. Nieuwenhuis, S. van den Brink, H Clevers, C.K. van der Ent, S. Middendorp, Jeffrey Beekman

P.60 **Treatment with VX-809 and VX-770 in rectal CF Organoids expressing uncommon CFTR Genotypes**
Evelien Kruisselbrink, Florijn Dekkers, Karin M de Winter-de Groot, H.M. Janssens, C.K. van der Ent, Jeffrey Beekman

P.62 **Correction of Bone Defect in Cystic Fibrosis: Studies from F508del in CFTR Human and Mouse Models**
Carole Le Henaff, Eric Häy, Olivier Tabary, Frédéric Velard, Françoise Barthes, Isabelle Sermet-Gaudelus, Pierre Marie, Jacky Jacquot

P.63 **Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) 186-8T/C Allele in a Patient with Cystic Fibrosis (CF) diagnosis.**
Sara Caldrer, Claudio Sorio, Chiara Angiari, Jan Johansson, Genny Verzè, Michele Ettorre, Mario Buffelli, Baroukh Assael, Paola Melotti

P.64 **Comparative Study For The Evaluation Of A New Technology For Cystic Fibrosis Screening**
Mauro CA Rongioletti, Fabrizio Papa, Cinzia Vaccarella, Maria B Majolini, Alessandra Luciano1, C Centrone, B Minuti, Valentina Mazzucchi, M Belli, I Giotti, C Giuliani, F Torricelli, Giancarlo M Liumbruno

P.65 **Spatial cAMP/PKA Compartmentalization and Activity in Primary Airway Cells**
Anna C Abbattiscianni, Stefania Monterisi, Maria Favia, Rosa A Cardone, Stephan J Reshkin, Manuela Zaccolo, Valeria Casavola

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20 March – 18:00-19:00 **Room: Malaga**

Opening Keynote Lecture

Innate Defense, Lung Infection and Cystic Fibrosis

Michel Chignard

Défense innée et Inflammation – InsermU874
Institut Pasteur, Paris - France

Innate immunity is the host first line of defense against infection. Its role is to sense and eliminate pathogens. Pathogen recognition involves cellular receptors called 'pattern recognition receptors' or PRRs. These receptors include different groups of specialized microbial and danger signal sensors among which the most thoroughly studied to date are Toll-like receptors (TLRs). These receptors recognize conserved motifs expressed by families of microorganisms that are sometimes essential for their survival, the 'pathogen-associated molecular patterns' or PAMPs. Interactions between PAMPS and TLRs trigger a host response with the aim of suppressing infections.

Experimental models of lung infection using knock out mice for different TLRs clearly demonstrated their requirement for the eradication of pathogens frequently encountered in cystic fibrosis, such as *Pseudomonas aeruginosa, Burkholderia cenocepacia, or Aspergillus fumigatus*. Nonetheless, the continuum, pathogen infection-TLR recognition-host cell activation-pathogen eradication-host survival, is not always the outcome. First of all several PAMPs-TLRs could be at play for the recognition of a single microorganism such as *Pseudomonas aeruginosa*, i.e. LPS-TLR4 and flagellin-TLR5. TLR9 is also participating but in an unexpected manner, being detrimental by dampening the innate response of cells. Moreover, the same cells, molecules and mechanisms involved in the protective process can also be involved in a deleterious inflammatory process. In fact, the host response to an invading pathogen should be proportional to the "attack" and should not be either overzealous or modest, under threat of inflammation or infection, respectively. For example, excessive inflammatory responses has been evidenced in the case of lung infection by *Burkholderia cenocepacia*. Paradoxically, in cystic fibrosis, both chronic inflammation and chronic infection are present concomitantly in the lung without eradication of the organisms. The question that arises is "are TLR expressed and do they work the same way in CF cells?". The answer is not clear-cut when looking at TLR2, 4, 5 and 9, the most important ones for fighting bacterial infection. More knowledge should be acquired as ways of manipulating TLR activation in order to potentially dampen inflammation or enhance innate defense.

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Thursday 21 March – 08:45-10:30 **Room: Malaga**

SYMPOSIUM 1

Pathophysiology Related to CF

Chairs: Michael Gray (UK) / Miguel Valverde (ES)

S1.1 Inflammatory responses in the lungs of newborn cf and non-C pigs challenged with bacteria

Bartlett, J.A. [1], Wohlford-Lenane, C. [1], Barker, C.K. [1], Ramachandran, S. [1,2], Manzel, L. [3], Pezzulo, A. [3], Zabner, J. [3], Welsh, M.J. [2,3,4,5], Colby, J. [6], Levy, B.D. [6], Look, D.C. [3], Nauseef, W.M. [3], Meyerholz, D.K. [7], Stoltz, D.A.[3], McCray, P.B. Jr.[1,2]

[1]Department of Pediatrics,[2] Interdisciplinary Program in Genetics,[3] Departments of Internal Medicine, 4 Molecular Physiology and Biophysics,[7] Pathology,[5] Howard Hughes Medical Institute, Carver College of Medicine, University of Iowa, Iowa City, IA [6] Pulmonary and Critical Care Medicine at Brigham and Women's Hospital and Harvard Medical School, Boston, MA

There is broad consensus that inflammation is an early and important contributor to lung disease in individuals with cystic fibrosis (CF). The CF pig model offers a unique opportunity to study the timing of the onset of infection and inflammation in the CF lung. We previously showed that newborn CF pigs exhibit impaired innate immunity in the absence of pulmonary inflammation and a reduced ASL pH. This observation suggests that a host defense defect precedes the onset of inflammation in the CF lung; however, it does not address the possibility that CF lung disease may be further complicated by an altered ability to respond to inflammatory stimuli. Here we test the hypothesis that acute inflammatory responses are perturbed in the absence of functional CFTR. We first investigated the responses of newborn CF and non-CF pigs to an inflammatory stimulus, heat-killed Staphylococcus aureus (SA). SA was aerosolized into the lungs of newborn CF pigs and non-CF littermate controls. At 4 hours post-instillation, animals were euthanized and pulmonary inflammation was assessed. We found that, for both CF and non-CF pigs, a SA dose of approximately 109 CFU equivalents/animal was sufficient to elicit measurable changes in BAL inflammatory cell counts and IL-8 secretion. Overall, total inflammatory cells, PMN counts, and IL-8 production were similar in BAL fluid collected from CF and non-CF lungs after SA exposure, as were levels of the anti-inflammatory mediator lipoxin A4 (LXA4). Histopathological analysis confirmed that PMN infiltration in CF lung and trachea tissue was similar to non-CF littermate controls. Furthermore, we failed to detect significant differences in tissue myeloperoxidase activity in CF and non-CF pigs in response to SA challenge. In parallel, we studied well-differentiated primary airway epithelial cultures derived from CF and non-CF pigs. In agreement with our in vivo results, CF and non-CF epithelia treated with inflammatory stimuli (including TNF, heat-killed SA and gentamicin-killed *Pseudomonas aeruginosa*) produced similar levels of IL-8 and exhibited similar levels of NF-kappaB activation at 1 and 20 hours post stimulation. Transcriptome array analysis of CF and non-CF pulmonary tissue and primary cultures is ongoing. In sum, we failed to find evidence that acute inflammatory responses are altered in newborn CF pigs. Our study does not rule out CF-associated problems with the in vivo resolution phase of the inflammatory response or genotype specific differences in the response to chronic inflammatory stimuli. These combined in vitro and in vivo approaches provide new insights into the complex relationship between loss of CFTR function and pulmonary inflammation.

S1.2 – Ethanol and fatty acids strongly inhibit the activity of the CFTR chloride channel and anion exchangers in pancreatic ductal epithelial cells

Péter Hegy

First department of Medicine, University of Szeged, Szeged, Hungary

Background. Excessive ethanol (EtOH) consumption is one of the most common causes of acute pancreatitis, which has no specific treatment. Sarles observed that sweat Cl^- concentration was elevated in alcoholic patients, which suggests decreased function of cystic fibrosis transmembrane conductance regulator Cl-channel (CFTR). Pancreatic ductal epithelial cells (PDEC) secrete HCO3- rich pancreatic fluid via Cl^-/HCO3-exchanger (CBE) and CFTR, which prevents acinar damage. Since, no information is available about the effects of EtOH and EtOH metabolites (fatty acid ethyl esters and fatty acids) on PDEC, we aimed to characterize the effects of these factors on PDEC.

Methods. In our experiments human pancreatic epithelial cell line (Capan-1), guinea pig PDEC and human pancreatic tissue were used. Changes of intracellular pH (pHi), Ca2+ concentration ([Ca2+]i), ATP [(ATP)i] and mitochondrial membrane potential (ΔΨ) of PDEC were measured by microfluorometry or confocal microscopy. We measured the effects of ethanol metabolites on CFTR Cl^- current of PDEC with patch clamp. The expression and localization of CFTR were analysed in PDEC and in human pancreatic tissue samples (from patients with alcohol induced acute (AP) or chronic pancreatitis (CP) or without pancreatic disease (NP)) with immunohistochemistry and RT-PCR.

Results. The administration of low EtOH concentration (10mM) stimulated pancreatic epithelial HCO3-secretion in vitro via IP3 mediated [Ca2+]i elevation. In contrast, both high concentration of EtOH (100mM) and palmitoleic acid (POA) (200µM) inhibited the HCO3- secretion of PDEC. Both the activities of the apical CBE and CFTR were decreased by 100mM EtOH or 200µM POA. The administration of 200µM POA induced sustained [Ca2+]i elevation by releasing Ca2+ from the endoplasmic reticulum via IP3 and ryanodin receptor activation and extracellular Ca2+ influx. Moreover, 100mM EtOH and 200µM POA depleted the (ATP)i and decreased ΔΨ. The inhibitory effects of EtOH and POA were mediated by sustained [Ca2+]i elevation. We also showed that EtOH, POAEE and POA significantly decreased the expression of CFTR after 48h in PDEC. The expression of CFTR was significantly decreased on the luminal surface of pancreatic ducts in AP and CP patients.

Conclusions. These results suggest that one of the main targets of EtOH and fatty acids is CFTR. Both toxic factors inhibit pancreatic ductal HCO3- secretion as well. Restoration of CFTR function and the HCO3-secretion may be potential therapeutic possibilities in alcohol induced AP and CP.

The authors have no disclosures to declare. This work was supported by OTKA, Hungarian Academy of Sciences and National Development Agency.

S1.3 – TRP channels relevant to epithelial physiology

Miguel A. Valverde

Laboratory of Molecular Physiology and Channelopathies, Universitat Pompeu Fabra, Barcelona, Spain

Clearance of mucus and pathogenic agents from lungs and the transport of gametes and embryos in the female reproductive organs are key functions of ciliated epithelia such as those present in the airways and the oviduct. Mucus secreted by specialized cells coats the epithelial surfaces that may be exposed to the external environment, thereby providing protection against pathogens and other forms of cellular abuse. The signaling events that lead to mucin secretion in the airways and intestine involve mainly, but not exclusively, P2Y purinergic and muscarinic receptor activation by ATP and acetylcholine, respectively. The subsequent generation of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3), the activation of protein kinase-C (PKC) and the release of Ca2+ from the ER as well as Ca2+ influx through the plasma membrane altogether cooperate to produce mucus secretion. Mucus transport depends mainly on the generation of Ca2+ signals and the beating of cilia. Transient receptor potential cation channels (TRP) participate in the generation of Ca2+ signals at different locations of epithelial systems thereby controlling their correct functioning. My talk will focus on two TRP channels with relevance to the control of ciliary beat frequency and mucus secretion

S1.4 Planar Cell Polarity Protein Network, Which Controls Ciliogenesis and Cilia Function, Is Altered in Human Cystic Fibrosis Bronchial Epithelial Cells

Wendy Delbart[1], Barbara Dhooghe[1], Pierre Wallemacq[1], Patrick Lebecque[2], Teresinha Leal[1], Sabrina Noel[1]

[1]Université catholique de Louvain, Louvain Centre for Toxicology and Applied Pharmacology, Brussels, Belgium, [2]Cliniques Universitaires St Luc, Pediatric Pulmonology & Cystic Fibrosis, Brussels, Belgium

Mucociliary clearance (MCC), abnormal in CF, is physiologically regulated by epithelial ion transport together with epithelial cilia movement and mechanical phenomenon such as cough and sneezing. Recently, new insights on ciliogenesis and cilia function have emerged. Planar Cell Polarity (PCP) is a tightly controlled protein network which drives the orientation of specialized structures (such as fly wings, mammal furs or fish scales) within the plane of the epithelial tissue. PCP has been described in mammal lung epithelial cells as a crucial mechanism controlling ciliogenesis and cilia function. Cilia structure and function have been studies in CF. Although the majority of these studies showed no structural abnormality and a normal cilia beat frequency (CBF), it has also been showed that ciliary disorientation, rather than ultrastructural abnormalities or slow CBF, may occur secondary to lung inflammation and result in delayed MCC. We hypothesized that CF HBEs may display abnormalities in PCP network which could further impair coordinated cilia function in the plan of the epithelium.

We demonstrated that HBEs expressed several PCP genes. Among them, expression of CELSR3 (Cadherin EGF LAG seven-pass G-type receptor 3) and Vangl-1 (Van-Gogh like 1) was down-regulated in CF (F508del/F508del) HBEs as compared to non-CF cells. In contrast, Fz3 (Frizzled 3), Fz6, Pk1 (Prickle 1) and Vangl-2 were upregulated in CF cells. Very low levels of CELSR3 protein were found in CF cells; moreover we observed that CELSR3 does not undergo autoproteolytic cleavage (in the endoplasmic reticulum) nor N-glycosylation (in the Golgi apparatus) as it normally does in normal HBEs.

These results suggest that expression and processing of PCP proteins is abnormal in CF-HBEs and may altered coordinated function of cilia within the bronchial epithelium in Cystic Fibrosis.

S1.5 TMEM16A/ANO1 Expression in Human Bronchial Epithelia

Paolo Scudieri[1], Emanuela Caci[1], Ambra Gianotti[1], Patrizia Morelli[1], Luis J.V. Galietta[1]

[1]Istituto G. Gaslini, U.O.C. Genetica Medica, Genova, Italy

TMEM16A protein, also known as anoctamin-1, is an important component of calcium-activated chloride channels (CaCCs). TMEM16A expression and function in human bronchial epithelia is strongly upregulated by IL-4 and IL-13, Th-2 cytokines that induce mucus hypersecretion and goblet cell hyperplasia. We have recently reported that TMEM16A hyperexpression induced by IL-4 occurs mostly in goblet cells whereas CFTR is expressed in ciliated cells (Scudieri et al., J Physiol 590: 6141-6155, 2012). Since cystic fibrosis (CF) is also characterized by mucus hypersecretion, we studied TMEM16A expression in freshly excised CF bronchi and in cultured bronchial epithelia under CF-like conditions. Histological sections of CF bronchi, obtained at the time of lung transplant, were studied by immunofluorescence. We found no evidence of TMEM16A hyperexpression in the surface epithelium despite the abundant presence of goblet cells (detected by MUC5AC staining). We also investigated TMEM16A expression in cultured bronchial epithelia incubated for 24 hours with the supernatant of *P. aeruginosa* cultures or with single bacterial components. Under this condition, we found little increase in TMEM16A expression and function. The most effective stimulus, pyocyanin (60 µM), increased the percentage of TMEM16A-expressing cells and calcium-dependent chloride secretion by only two-fold. In contrast, incubation with IL-4 (10 ng/ml) increased TMEM16A cells and chloride secretion by more than ten-fold. Our results in cultured cells indicate that strong TMEM16A hyperexpression in goblet cells is a phenomenon specifically evoked by Th-2 cytokines and not by conditions mimicking CF airway infection. These results appear to be consistent with the relatively low expression of TMEM16A in CF airways in vivo. Pharmacological stimulation of TMEM16A expression in vivo could be a possible approach to enhance anion transport in CF airways.

S1.6 Correction of Bone Defect in Cystic Fibrosis: Studies from F508del in CFTR Human and Mouse Models

Carole Le Henaff[1], Eric Häy[2], Olivier Tabary[3], Frédéric Velard[1], Françoise Barthes[4], Isabelle Sermet-Gaudelus[5], Pierre Marie[2], Jacky Jacquot[1]

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In patients with cystic fibrosis (CF), bone fragility is recognized as a severe complication of the disease and is a real problem of management of patients. Vertebral and rib fractures are particularly harmful to the respiratory function in CF decreasing their ventilatory capacity and promoting lung infections. Whether the role of F508del mutation in the dysfunction of bone metabolism remains to be elucidated, its involvement in this dysfunction has been demonstrated in KO-CFTR and F508del-CFTR mouse models. We have showed that young and adult F508del *Cftr*^{tm1Eur} mice, in two sexes, have a severe osteopenia characterized by a decrease in bone mass and bone formation accompanied with reduced level of serum insulin-like Growth Factor 1 (IGF-1) (Le Henaff et al., 2012). Miglustat (N-butyldeoxynojyrimicin, NB-DNJ, Zavesca®), an orally approved drug by the US Food and Drug Administration and European Union for type I Gaucher disease and for children and adults with Niemann-Pick type C disease, was reported to normalize sodium and CFTR-dependent chloride transport in human F508del CFTR lung cells and in nasal mucosa of F508del mice.

Here, we investigated the bone mass and microarchitecture of F508del mice relative to wild type (WT) littermates after oral administration of 120 mg/kg/day miglustat for 28 days using *in vivo* micro-CT, bone mass and dynamic parameters of bone formation. Levels of two serum growth factors, insulin-like growth factor 1 (IGF-1) and 17b-estradiol (E_2), were also determined. Once-day treatment with miglustat over 4 weeks normalized trabecular bone volume and improved bone microarchitecture in the lumbar spine of F508del mice. This increase in bone volume and structure was associated with enhanced new bone formation and significantly higher serum levels of E_2, but without changes in IGF-1 levels in miglustat-treated F508del mice. Interestingly, in the miglustat-treated F508del mice, the body weight attained at the end of the treatment were 10 % higher compared to untreated F508del mice. The miglustat-treated F508del mice had no diarrhea at any stage of the study.

Human F508-CFTR osteoblasts (cells that form bone) were cultured from breastbone fragments of a 20-year-old CF male homozygous for the F508del-CFTR mutation undergoing lung transplantation. Compared to normal human osteoblasts, we found a lower CFTR-dependent chloride conductance in F508del-CFTR osteoblasts. The exposure of F508del-CFTR osteoblasts to 10 nM E_2 for 48 hours restored a CFTR-dependent chloride current. F508del-CFTR osteoblasts produced a lower amount of osteoprotegerin (OPG, a key regulator of bone turnover) compared to normal osteoblasts. Interestingly, 10 nM E_2 for a 48 hour-period markedly increased OPG secretion in F508del-CFTR osteoblasts.

The implication of our work is to propose, in the short term, the first treatment to fight against the CF bone disease and, thus allowing for longevity and a better quality of life for patients with cystic fibrosis.

Supported in part by grants from the French Cystic Fibrosis Association Vaincre la Mucoviscidose and an Inserm-Region Champagne Ardenne research program, France. We would like to express our gratitude to Dr. Olivier Morand (Actelion Pharmaceuticals Ldt, Switzerland) for the generous gift of miglustat.

Thursday 21 March – 11:00-12:45

SYMPOSIUM 2
Cell Biology of CF: Autophagy and Degradation
Chairs: Margarida Amaral (PT) / Bruce Stanton (US)

S2.1 – Targeting autophagy as a new strategy to circumvent ΔF508CFTR defect

Luigi Maiuri

European Institute for Research in Cystic Fibrosis, Division of Cell Biology, San Raffaele Scientific Institute, 20132 Milan, Italy

Mismanaged protein trafficking by the proteostasis network contributes to several conformational diseases including cystic fibrosis (CF). Autophagy, a regulated pathway involving the lysosomal degradation of cytoplasmic organelles or cytosolic components, has also emerged as a cellular mechanism essential for the maintenance of cellular homeostasis. Disabled autophagy is directly involved in the pathogenesis of multiple diseases including cancer, viral infection, neurodegenerative diseases and chronic inflammatory diseases. Human and mouse CF airways are autophagy deficient, with highly reduced autophagosome formation and SQSTM1/p62 accumulation. Increased levels of reactive oxygen species (ROS), induced by defective CFTR function, lead to tissue transglutaminase-2 (TG2) activation driving cross-linking and aggresome accumulation of several TG2-substrate proteins, among which the sequestration of the essential autophagy protein BECN1. The functional sequestration of BECN1 dislodges the PtdIns3K complex III away from the endoplasmic reticulum (ER), thus inhibiting autophagosome formation.

Rescuing autophagy is an emerging approach to treat several human conformational diseases. Restoring BECN1 and autophagy, either by BECN1 overexpression or by TG2 inhibitors as cystamine, or depleting p62 can blunt inflammation in ΔF508-CFTR homozygous airways, rescue ΔF508-CFTR trafficking to the plasma membrane (PM) of airway epithelia and prolong ΔF508-CFTR PM residence beyond drug washout. The effects of cystamine in damping down lung inflammation extend well beyond cystamine withdrawal unless CFTR is depleted during washout, indicating that these effects rely on PM residence of rescued ΔF508-CFTR. Such a prolonged PM residence after rescue enables the beneficial action of CFTR potentiators on ΔF508-CFTR. These results underscore a functional link between BECN1, p62 and ΔF508-CFTR residence at the surface epithelia of mouse and human ΔF508-CFTR homozygous airways.

Altogether, these findings demonstrate that targeting the cellular environment, instead of specifically targeting the misfolded ΔF508-CFTR, could represent an attractive option to circumvent ΔF508-CFTR defect.

Manipulating proteostasis by proteostasis regulators has emerged as a novel therapeutic approach for the treatment of conformational diseases. CF constitutes the quintessential example of a conformational disease. These results provide pre-clinical evidences that prior restoration of proteostasis in CF airways could enable the action of CFTR potentiators in reducing lung inflammation. They delineate a novel strategy for the treatment of ΔF508-CFTR homozygous patients in which patients are first treated with cystamine and subsequently pulsed with CFTR potentiators.

Rescuing autophagy may represent a novel approach in human inherited loss-of-function diseases in which protein misfolding compromises the relocation of functional mutants to their site of activity. Manipulating the cellular mechanisms that ultimately link protein misfolding to protein malfunction, might constitute a therapeutic strategy for the treatment of a range of conformational diseases.

S2.2 - A *Pseudomonas aeruginosa* toxin enhances the degradation of CFTR and disrupts MHC class I antigen presentation in respiratory epithelial cells.

Bruce A. Stanton[1], Kenneth H. Ely[1], Brent L. Berwin[1], Naveen Bangia[3], Siying Ye[1], Kathy A. Green[1], William R. Green[1], Richard I. Enelow[1] and Jennifer M. Bomberger[2]

[1]Department of Microbiology and Immunology, The Geisel School of Medicine at Dartmouth, Hanover, NH 03755 USA, [2] Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219 USA, [3]Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 12345 USA

Previous work from our laboratory identified a secreted protein from P. aeruginosa, Cif (PA2934) that selectively decreases the expression and function of several ABC transporters in the plasma membrane of human airway epithelial cells, including CFTR. Cif is secreted from P. aeruginosa in bacterial-derived outer membrane vesicles, which enhance the delivery of bacterial toxins across mucus plugs and over long distances in the airways, and fuse with host cell lipid raft domains and thereby deliver Cif to the cytosol. Cif is highly expressed by clinical isolates of P. aeruginosa and is detected in sputum samples from CF and pseudomonal pneumonia patients. In the current study, we tested the hypothesis that Cif regulates the transporter associated with antigen processing (TAP), an ABC transporter that plays a key role in bacterial and viral antigen presentation via MHC class I molecules and pathogen clearance by CD8+ cytotoxic T cells. Our studies demonstrate that Cif inhibits CD8+ T cell viral antigen recognition by increasing the ubiquitination and proteasomal degradation of TAP1, resulting in a reduction in antigen transport into the endoplasmic reticulum and a 83% decrease in both MHC class I cell surface expression and viral antigen presentation at the plasma membrane of airway epithelial cells. Cif is the first example of a virulence factor secreted by bacteria, or any non-viral pathogen, that down regulates MHC class I antigen presentation. In conclusion, our data provide a novel explanation for the clinical observation that bacterial infection exacerbates viral infections-we propose that infection with Pseudomonas down-regulates viral antigen presentation by decreasing TAP1 levels, which results in less viral clearance by CD8+ cytotoxic T cells, thereby exacerbating viral infections.

Supported by the NIH (P20-RR018787/GM103413 and R01HL074175) and the CF Foundation

S2.3 - CFTR as a signal generator, new evidence.

Mehta A on behalf of PIs in Regensberg, Pittsburgh, Lisbon, Sheffied, Manchester and Padua

Division of Medical Science, University of Dundee.

CELL_ENERGY: A hidden relationship exists between two very different kinases that act on adjacent enzymes controlling fat metabolism and cell energy (JBC:284:5645-53/J.Bioenerg.Biomembr:38:181-187). The first kinase, AMP-activated kinase (AMPK) senses low-energy through basal, AMP-activated and ADP-augmented activities and the second, nucleoside-diphosphate-kinase (NDPK) is a high-energy phosphoryl-transferase. NDPK transfers the terminal-phosphate from ATP [~30 kJ /mol; 1 attojoule/NDPK] to its own labile P-N-link (phospho-histidine; p-his-118) and makes that available at 1000 cycles per second to drive cellular signalling cascades, thus shuttling NDPK-generated GTP [via p-his-118] to G-proteins (such as Rac bound to the NADP-oxidase system during assembly of the bacterial killing complex, JBC. 2005 80; 3802-11). Membrane-local cAMP generation is an essential NDPK-requiring process because NDPK his-phosphorylates G-beta to drive cAMP synthesis (PNAS, 2009;106;16269-74).

NDPK_AMPK_CFTR: NDPK, when p-his kinase dead, negates AMPK signalling towards CFTR (King/Hallows/Mehta in JBC, 2012). Hallows (Pittsburgh), Muimo (Sheffield) and Kunzelmann (Regensburg) together demonstrate that AMPK inhibits CFTR but needs p-his-118 on NDPK to negate CFTR function. That AMPK requires NDPK to signal its intent is consistent with knock-down data from Dictyostelium (Annesley/Fisher/Mehta in PLosOne/Arch Pharmacol). Dicty AMPK overactivity renders Dicty blind to light migration that is reversed by NDPK knock-down suggesting a broad phylogenetic conservation of the (energetic) link between NDPK and AMPK. NDPK-KD also reverses AMPK-induced abnormalities in the Dicty cell cycle. Nearby to p-his-118 in NDPK lies serine-120, an in vitro target for a third kinase, CK2 (casein kinase 2). CK2 is permissive for PKA to act on CFTR. Teams led by Sheppard, Kunzelmann and Gray together showed that CK2 activity is required for CFTR to stay open after a PKA stimulus using three independent model systems (mammalian cells, oocytes and sealed pancreatic ducts; (Treharne et al 2009 Cell-Physiol-Biochem. 24:347-60). CK2 is an essential, constitutively active and acid-residue directed serine/threonine kinase that prevents apoptosis by rendering its (thousands of) phosphorylated targets blind to Caspases (calcium/aspartate directed proteases [linked to calpain actions]). CK2, also being acid-residue directed, masks Caspase recognition sites through nearby p-serine generation thus blocking the homing signal in the Caspase cleavage site.

FRACTURE OF CFTR: Having localised the sites of CK2-CFTR interaction near F508, we recently linked mutation of one of the sites to altered CFTR cleavage patterns (Tosoni et al 2013, BJ 449:295-305). For example CFTR-S511A mutation changed the pattern of CFTR fragmentation (new ~100kDa band). Separately, ~15-mer peptides corresponding to the F508-site in CFTR were complex, ATP-dependent, allosteric controllers of CK2 activity. It follows that should we demonstrate either abnormal NDPK or CK2 activity in cells bearing CFTR mutations, we would predict that thousands of cellular processes would go awry: exactly what we see in CF. Specifically, membrane-disrupted, F508-deleted loss of CFTR is predicted to disturb our proposed CK2-NDPK-AMPK-LIMBO (local intra-membrane-membrane-organisation) signalling hub. This model predicts abnormal bacterial killing and widespread G protein dysfunction, phenomena that are well recognised in CF pathophysiology. The supporting data for this model will be presented.

Supported by the Wellcome Trust and many grants to my collaborators ranging from DFG, Portugal Government grants, CFF and others

S2.4 – Palmitate Accumulates within Phosphatidylcholine of Cystic Fibrosis Human Bronchial Epithelial Cells: Impact on F508del-CFTR Trafficking

Laurie-Anne Payet[1], Linette Kadri[1], Jean Marc Berjeaud[2], Sandra Mirval[1], Frédéric Becq[1], Clarisse Vandebrouck[1], Thierry Ferreira[1]

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The most common mutation in cystic fibrosis (CF), *F508del-CFTR* (CF transmembrane conductance regulator) results in the retention of the protein in the endoplasmic reticulum (ER), which could be corrected by pharmacological agents. Lipid homeostasis is also altered in CF. In fact, few studies showed lower levels of unsaturated fatty acids (UFA) in CF cells and plasma (Anderson *et al.*, 2008; Kuo *et al.*, 1962; Freedman et *al.*, 2004). This defect also appears to impact the overall fatty acyl content of phospholipids, with a decrease of five phosphatidylcholine (PC) UFA-containing species in the plasma of CF patients (Guerrera *et al.*, 2009).
In the present study, lipid analyses by HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry) show that palmitate, a saturated fatty acid (SFA), accumulates within PC of bronchial epithelial cells from CF patient lungs, as compared to non-CF patients. In a parallel study on a simple eukaryotic model, *Saccharomyces cerevisiae*, we demonstrated that SFA accumulation within PC, by altering membrane fluidity, impacts several important cellular processes such as protein folding in the ER and vesicular budding from the Golgi apparatus. These observations led us to postulate that palmitate accumulation observed *in vivo* in CF patient cells could alter F508del-CFTR trafficking correction by pharmacological means.
Surprisingly, contrary to human bronchial epithelial cells, this accumulation was not observed in a CF bronchial epithelial cell line, CFBE41o$^-$. Thus, CFBE41o$^-$ cells were incubated with exogenous palmitate to mimic what is seen *in vivo* in CF patient cells. In this context, we are presently investigating the possible effects of palmitate accumulation within PC in CFBE41o$^-$ cells on F508del-CFTR trafficking correction by pharmacological agents. Preliminary data concerning these aspects will be presented.

This work is supported by “Vaincre La Mucoviscidose”, MESR, CNRS and EFSD.

S2.5 - Modulation of Epithelial Repair and Autophagy by Female Hormones in Normal and Cystic Fibrosis Human Epithelial Bronchial Cells

Vinciane Saint-Criq[1], Lea Baudoin[1], Natalia Lajczak[1], Brian J Harvey[1]

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Background: Cystic fibrosis (CF) is the most frequent recessive disease that can impact directly on an individual's longevity among Caucasians. In the lungs, the perturbation of Cl^- efflux leads to an abnormal thick mucus that cannot be removed by mucociliary clearance. The trapped mucus favours bacterial colonisation, promoting chronic infection and inflammation resulting in lung destruction. It has been proven that CF affects women more seriously than men. Indeed, female CF patients have shorter life expectancies than males [1] and frequency of lung exacerbations in CF females correlates with fluctuations of the concentration in estrogen (E2) in plasma during the menstrual cycle [2]. Epithelial repair plays a key role in CF because of the numerous lesions in the CF airways epithelia. This wound healing process involves different mechanisms such as cell migration, proliferation, apoptosis and autophagy. Autophagy is a process involving the degradation of a cell´s own components through the lysosomal machinery, via the ATG (AuTophagy related Gene) pathway. It has been shown recently that there is a lack of this process in CF [3]. In this context, the aim of this study was to test the effect of female hormones (17β-estradiol, E2, and Progesterone, P4) on epithelial repair and autophagy in CF and non-CF human bronchial epithelial cells

Methods: The effect of female hormones (E2 10 nM, P4 10 nM) on epithelial repair of a non-CF (NuLi-1) and a CF (CuFi-1) cell lines was determined by wound healing assay. Cells were grown on millicell transparent inserts and pictures taken every 3 hours. The modulation of autophagy by these female hormones was studied by immunoblotting and immunofluorescence using LC3B-II, p62 and Beclin-1 as autophagy markers.

Results: We show that, contrary to what has been published previously [5] there is no difference in the rate of wound repair after injury between CF and non-CF cells. This result can be explained by the conditions in which cells were grown i.e. our cells were differentiated on inserts. Performing wound healing assay, we show that female hormones did not affect the rate of wound healing in CF cells but E2 only can decrease epithelial repair in non-CF cells. As previously shown by Luciani *et al.*, there is less LC3B-II in CF than in non-CF cells, strongly suggesting a lack in autophagy in CF airway cells. E2 decreased the LC3B-II and increased p62 expression levels indicating that E2 is able to decrease autophagy in non-CF cells only. In confocal microscopy experiments, E2 seemed to inhibit non-CF and CF cell migration by modulating actin reorganization and decreasing the number of pseudopodes.

Conclusion: These results indicate that E2 can decrease lung repair after injury and autophagy in non-CF bronchial epithelial cells.

Acknowledgements: Supported by EU FP7 Marie Curie CEMP Fellowship (to VS-C), Higher Education Authority of Ireland (PRTLI4 NBIP to BJH)

References:

[1] Rosenfeld *et al.,* Am J Epidemiol. 1997;145:794-803 Chotirmall *et al.*; 2012; N Engl J Med. 24;366(21)
[3] Luciani *et al*., Autophagy. 2011;7(1):104-6

S2.6 – **Exchange Proteins Directly Activated by Cyclic AMP: Novel CFTR Interactors?**

João Fernandes[1], Margarida Amaral[1], Carlos Farinha[1]

[1]Center for Biodiversity, Functional and Integrative Genomics (BioFIG), Membrane Protein Disorders Unit, Lisbon, Portugal

CFTR is stimulated when cyclic AMP levels near the plasma membrane increase enough for protein kinase A (PKA) to be activated, triggering CFTR phosphorylation and channel opening. However, PKA is not the sole cyclic AMP sensor in the cell: the family of exchange proteins directly activated by cyclic AMP (Epacs) also responds to direct binding of this second messenger. Epacs are guanine nucleotide exchange factors for small GTPases of the Rap family, which are involved in the control of cell-cell and cell-matrix adhesion, cytoskeleton rearrangements and cell polarization, processes which in cystic fibrosis are dysregulated. Moreover, Epacs are reported to localize to the plasma membrane by the same scaffolding proteins that bind CFTR and PKA, namely ezrin-radixin-moesin (ERM) proteins. This suggests that activation of both CFTR and Epac might be spatially and temporally coincident. However, little is known concerning the involvement of Epacs in CFTR biology. Our aim was thus to elucidate the impact of Epacs in CFTR biogenesis, processing and trafficking.
In this report, we show that both Epacs and their effector Rap1 are expressed in the A549 expressing CFTR under a Tet-ON promoter and CFBE lung epithelial cell lines, as well as in primary cultures of human bronchial epithelial cells. We also show by co-immunoprecipitation that Epac physically interacts with CFTR in A549 cells, supporting a functional interaction between the two proteins. Lastly, preliminary data suggest a decrease in CFTR steady-state levels when A549 cells are treated with 8-pCPT-2'-O-Me-cAMP-AM (a membrane-permeable Epac-selective cyclic AMP analogue that does not activate PKA). Taken together, our data support the hypothesis that Epacs may have a negative impact on CFTR membrane traffic.

Work supported by CFF (USA) 7207534 grant, PTDC/BIA-BCM/112635/2009 and BioFig (PEst-OE/BIA/UI4046/2011) grants from FCT.

Thursday 21 March – 14:30-16:40

SYMPOSIUM 3
Infection, Inflammation and Immunity - I
Chairs: Michel Chignard (FR) / Giulio Cabrini (IT)

S3.1 – Pulmonary Phage Therapy: efficiency of bacteriophages to treat bacterial infections of cystic fibrosis patients

Emilie Saussereau[1,2], Isabelle Sermet[2], Aleksander Edelman[2] and Laurent Debarbieux[1]

[1]Institut Pasteur, Department of Microbiology, Paris, France
[2]Faculté de Médecine Paris-Descartes, INSERM, U845, Paris, France

Bacteriophages, viruses infecting exclusively bacteria, have been used to treat human bacterial infections in the early 20th century, before antibiotics were discovered. However, antibiotics, molecules by definition, rapidly supplanted bacteriophages, viruses by definition. The past two decades, the search for new antibiotics was less successful than expected while the threat of multidrug resistant bacteria continuously increased. In this particular context, there is currently a worldwide renewed interest for phage therapy, the therapeutic application of virulent bacteriophages. Several publications have shown convincing results on the overall efficiency of bacteriophages to treat various bacterial infections, nevertheless, only few have been deeply investigated. Our team is particularly involved in the application of bacteriophages to treat lung infections. We demonstrated that bacteriophages can both cure and prevent lung infections caused by *Pseudomonas aeruginosa*, using a mouse model. Our studies cover bacteriophages isolation and characterization, including full genome sequencing, in vitro and in vivo safety studies towards the immune system and cystic fibrosis context, real-time monitoring of infected animals, as well as pre-clinical studies using the sputum from cystic fibrosis patients. Starting with a brief overview of the challenges that the development of Pulmonary Phage Therapy is confronted to, my presentation will end by uncovering our most recent unpublished data.

S3.2 - **Type IIA secretory phospholipase A2 plays a key role in elimination of staphylococcus aureus from cystic fibrosis lung**

Erwan Pernet[1,2], Domonique Leduc[1,2], Laurent Guillemot[1,2], Pierre-Régis Burgel[3], Isabelle SERMET-Gaudelus[4], Michel Chignard[1,2], Yongzheng WU[1,2]* Lhousseine Touqui[1,2]*

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At early stage of cystic fibrosis (CF), patient lungs are mainly colonized by *Staphylococcus aureus* (SA) while *Pseudomonas aeruginosa* (PA) becomes predominant at late stage. Type IIA secretory phospholipase A2 (sPLA2-IIA) is a bactericidal enzyme produced by mammalian cells. We show here that this enzyme kills laboratory and clinical strains of SA with an EC 50 at 10 ng/ml but had no effect on PA strains even at concentrations above 10 µg/ml. This killing is due to selective hydrolysis by sPLA2-IIA of phosphatidylglycerol, the major membrane lipid of SA. Transgenic mice over-expressing human sPLA2-IIA are protected from SA, but not PA lung infection. In lung co-infection animal models, SA is eradicated faster than PA that enhances SA clearance. This clearance is abolished by pharmacological inhibition of PLA2-IIA. PA but not SA induces sPLA2-IIA expression in human bronchial CF epithelial cells IB3 and alveolar macrophages (AMs) by different mechanisms. In AMs, PA induces sPLA2-IIA via LPS/TLR4/NF-κB pathway. In IB3, this induction occurs via the type-III secretion system. Among the proteins of this system ExoS toxin plays the major role in sPLA2-IIA induction. The transcription factor KLF seems to play a key role in ExoS-induced sPLA2-IIA expression in IB3 cells. Expectorations from adult CF patients exhibit high levels of sPLA2-IIA that play a major role in the killing of SA, but not PA, by these expectorations. Our results suggest that PA-induced sPLA2-IIA expression plays a role in the elimination of SA from CF lung. This highlights a new mechanism by which a pathogen can eliminate another pathogen by using the innate immunity of the host.

S3.3 – Living without oxygen: anaerobiasis in the CF Airway

Stuart Elborn

Queen's University Belfast, Centre for Infection and Immunity, Belfast, United Kingdom

The airways mucus in CF is mostly in anaerobic conditions. This has a significant impact on the composition of the microbiota, the metabolism of bacteria and the regulation of host responses.
Pseudomonas aeruginosa behaves differently when cultured aerobically compared with anaerobically with different metabolic needs and different resistance patterns to antibiotics. Most antimicrobial testing is undertaken in aerobic conditions and might be one of the reasons why antimicrobial resistance correlates so poorly with clinical outcome.
The appreciation that there is an anaerobic environment has also led to the identification of obligate anaerobic and microaerophilic bacteria in the airway. A range of studies using direct culture and T-RFLP, PCR and pyro-sequencing have all identified an abundance of anaerobic bacteria in the airways of people with cystic fibrosis. The pattern of the microbiota suggests that less diversity is associated with more severe disease. This may be driven by our current antibiotic practices. Many anaerobic bacteria are resistant to common antimicrobials used in cystic fibrosis and an understanding of the microbiota may change treatment approaches to infection in CF.
Host inflammatory responses to infection may also be impacted by anaerobic environments through importandt regulatory pathways such as HIF.
Understanding the interactions in this complex microbiota and how it drives virulence and antibiotic resistance and how this drives inflammation and remodelling is an important area for further research.

S3.4 – Mucus Hyperconcentration/Increased Osmotic Pressure: Role in CF Pathogenesis

Richard C. Boucher, M.D.

University of North Carolina at Chapel Hill

Cystic fibrosis lung disease reflects a heterogeneous defect in host defense against bacterial bronchial infection. A major component in innate host defense of the airways is the mucus clearance system. Recently, a new description of the cell biologic and biophysical regulation of mucus transport in the human lung has emerged. In contrast to the classical model of a mucus layer ("gel") moving atop a watery layer in which the cilia beat (the periciliary liquid layer), the airway surface is best described as comprised of two gels, i.e., a mucus layer gel and a dense, tethered mucin gel in the periciliary environment. For mucus transport to proceed normally, the periciliary gel must be well hydrated so it can perform its lubricant functions. Importantly, the distribution of water on the airway surface compartment between the two gels is governed by their relative osmotic pressures/moduli. The osmotic pressure/moduli are power functions of the mucin concentration and, hence, are very sensitive to the relative hydration of the two airway surface gels. Quantitative measurements now can predict when the hydration of the airway surface is sufficient to mediate flow versus the degree of dehydration that will cause flow to cease. An increase in mucin concentration and mucus layer osmotic pressure can reflect both an absolute depletion of airway surface liquid and/or an increase in mucin secretion. The biologic consequences of increased mucin concentration/osmotic moduli have been observed in the βENaC mouse. In this mouse, an increase in mucus concentration produces airways muco-obstructive lung disease, including microaerophilic/anaerobic infection, inflammation characterized by a neutrophilic intraluminal accumulation, activation of macrophages within mucus, and airways remodeling. Data with respect to mucin concentrations in normal and CF airways mucus are sparse. One report, using immunologic measurement techniques, reported that mucin concentrations were absolutely reduced in CF compared to normals. Utilizing biophysical techniques independent of potential proteolytic degradation of mucin epitopes, we observed that the mucin concentration in CF subjects airway secretions was ~3x higher than normal. These measurements were confirmed by direct measurements of airway mucus osmotic pressures that revealed CF subjects exhibited raised osmotic pressures in ranges that may exceed the PCL osmotic pressure and produces mucus stasis/adhesion. In summary, it appears that the CF lung is subject to heterogeneous environmental insults, e.g., viruses and aspiration, that produce localized increases in mucin concentration, that lead to mucus adhesion, inflammation, and ultimately infection of adherent mucus.

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S3.4 - Lipoxin A_4 Delays the Invasion of Cystic Fibrosis Bronchial Epithelial Cells by the Pathogen *Pseudomonas aeruginosa*

Gerard Higgins[1,2], Brian J Harvey[2], Paul McNally[1], Valerie Urbach[1,2]

[1]National Children's Research Centre, Dublin, Ireland, [2]Royal College of Surgeons in Ireland, Dublin, Ireland

Cystic fibrosis (CF) caused by the mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene results in a decreased Cl^- secretion and hyperabsorption of Na^+ in the airways leading to dehydration of the Airway Surface Liquid (ASL) layer. The reduction in ASL height impairs mucocillary clearance and favours lung infection and inflammation. The eicosanoid lipoxin A_4 (LXA_4) described as a signal for the resolution of inflammation, is decreased in the lungs of patients with CF (*Karp et al, 2004*). We have previously shown that LXA_4 stimulates Cl^- secretion *(Bonnans et al, 2003)*, increases ASL height in Human Bronchial Epithelial (HBE) and Cystic Fibrosis Bronchial Epithelial (CFBE) cells (*Verriere et al, 2012*) and stimulates ZO-1 expression and transepithelial electrical resistance (TER) in Human Airway Epithelial Cells (*Urbach et al, 2008*). We hypothesize that decreased levels of LXA_4 in CF airways could favour the invasion of HBE and CFBE cells by microorganisms. CF lungs are chronically infected by *P. aeruginosa* by adolescence and this leads into the progressive lung destruction. Using a gentamyicin invasion assay and confocal microscopy we investigated the role of LXA_4 on the epithelial integrity of HBE and CFBE when infected with *P. aeruginosa*. LXA_4 (1nM) alone did not affect *P. aeruginosa* growth but prevented the invasion of HBE and HCFBE cells by *P. aeruginosa* within the first 4 hours after inoculation. These results were confirmed by visualisation using confocal microscopy. We report a novel role for LXA_4 in delaying the invasion of CFBE by a microorganism, which may lead to a new therapeutic route for CF patients.

Acknowledgements: Funded by the *Children´s Medical and Research Foundation in Ireland*, *The Health Research Board of Ireland* and *INSERM*

S3.5 - Anti-Inflammatory Effect of FDA Approved Drugs in CF Cells

Jérémy Rocca[1,2], Agathe Tarze[1,2], Virginie Prulière-Escabasse[1,2,3], Alix de Becdelièvre[1,2,4], Anne Hulin[4], Natascha Remus[1,5], Ralph Epaud[1,2,5], Pascale Fanen[1,2,4]

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Pulmonary disease is the main cause of morbidity and mortality in cystic fibrosis (CF) patients due to exacerbated inflammation. Loss of CFTR activity is associated with dysregulation of transcription factors implicated in the inflammatory pathway such as NF-κB, AP-1 and PPARγ and the abnormal activation of NF-κB leads to increased Interleukin-8 (IL-8) secretion.
Our aim is to test FDA approved drugs having yet undefined anti-inflammatory effect in CF airway epithelial cells.
We first tested the effect of three molecules codified ATB-3A, FLJ-1 and JR-01 and compared them to ibuprofen, on HeLa cells overexpressing wild-type or F508del-CFTR and Calu-3 cells. We performed luciferase reporter gene assays in order to measure i) IL-8 promoter activity, and ii) the activity of synthetic promoters containing either NF-κB or AP-1 or PPARγ responsive elements. We then assess IL-8 secretion by immunodetection with Immulite®. Finally, the effect of these molecules was studied on CFTR expression, and chloride channel activity by western blot and the fluorescent chloride indicator N(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) respectively.
We observe a significant inhibition of IL-8 secretion in airway epithelial cells after ATB-3A treatment, which was associated to a reduced IL-8 promoter activity in the different cell lines. At the molecular level, we showed an inhibition of NF-κB and AP-1 transcriptional activity, whereas PPARγ activity was enhanced. We then obtained similar results with FLJ-1 and JR-01 on NF-κB and AP-1 activity in addition to a huge response to FLJ-01 on PPARγ activity. Treatment with ibuprofen or ATB-3A induces a significant increase of mature form of CFTR in wt-CFTR HeLa cells. In the case of ATB-3A, this enhancement was also associated with a higher chloride channel activity in wt-CFTR HeLa cells. An increase of CFTR activity was also observed in human primary nasal epithelial cells as assessed by short-circuit measurement. These data suggest that ATB-3A could also act as a CFTR potentiator.
Altogether, these results are consistent with a global anti-inflammatory effect of these drugs relevant in the CF context. Further experiments are ongoing on CF and non CF hNEC cultured at the air-liquid interface and CF mouse model.

Supported by Vaincre la Mucoviscidose

S3.6 - Potential Role of Expression of E3 Ubiquitin Ligases in Regulating the Inflammatory Phenotype of Cystic Fibrosis

Sara Canato[1], Margarida Telhada[1], Margarida Amaral[1], Luka Clarke[1]

[1]Center for Biodiversity, Functional & Integrative Genomics (BioFIG), Lisbon, Portugal

Cystic Fibrosis (CF) disease is characterized by an aggressive inflammatory response and chronic infection in the airway. One important marker of CF lung disease, the pleiotropic cytokine TGF-beta[1,2], is negatively regulated by E3 ubiquitin ligases, which have been found to be dysregulated in previous studies of F508del-CFTR-related gene expression[3].

To understand the role of E3 ubiquitin ligases in CF, we studied the effects of: 1) F508del mutation and 2) exposure to TGF-beta and TNF-alpha cytokines on mRNA and protein expression of the E3 ubiquitin ligases SMURF1, SMURF2 and NEDD4L in polarized CF bronchial epithelial cell models.

Using real-time quantitative PCR, we demonstrated that the F508del mutation is not sufficient to induce significant differential expression of E3 ubiquitin ligases. However, the F508del-CFTR genotype altered the responsiveness of E3 ubiquitin ligases to both inflammatory cytokines. Our results showed that both TGF-beta and TNF-alpha increased the expression of *SMURF2* mRNA in F508del-CFTR CFBE cells, suggesting an up-regulation of this E3 ubiquitin ligase under inflammatory status. In addition, this increased expression was consistent with an observed decrease in *SMAD2* and *SMAD3* mRNA expression.

These results suggest that increased expression of E3 ubiquitin ligases in CF under inflammation could partly be responsible for the increased pro-inflammatory mediators that characterize CF disease, via an inhibition of the Smad-dependent anti-inflammatory effects of TGF-beta.

Our preliminary data strengthen the potential implications of differential expression of enzymes involved in ubiquitination on modulation of inflammatory responses in CF.

Supported by PEst-OE/BIA/UI4046/2011 grant BioFIG.

1. Arkwright PD *et al* (2000) *Thorax* **55**: 459-62.
2. Bartlett JR *et al* (2009) *JAMA* **302**:1076-83.
3. Hampton TH *et al* (2010) *Am J Physiol Lung Cell Mol Physiol* **298**: 473-482.

Thursday 21 March – 17:10-18:40

SPECIAL GROUP DISCUSSION I
CFTR Gene Expression / Modifiers
Moderators: Harriet Corvol (FR) / Paul McCray (US)

A focus of this session will be to have an interactive discussion regarding the limitations in knowledge in our understanding related to the regulation of CFTR expression and modifiers of CFTR expression and function. From this discussion we will generate suggestions from future research directions and priorities. The goals include improved understanding of factors influencing CFTR expression and function, and how genetic modifiers influence phenotype. Concepts that may lead to new therapies will be discussed. Our emphasis will be driven by audience participation and input.

Thursday 21 March – 17:10-18:40

WORKSHOP - I
Expression and Purification of CFTR
Moderators: Christine Bear (CA) / Bob Ford (UK)

This workshop will look at new developments in the expression and purification of CFTR. This will include discussion of the domains of CFTR (nucleotide binding domains 1 and 2, R region) as well as for the full-length protein which has to be solubilised from the membrane and purified in the presence of a detergent. The workshop will take the form of short 'current state of the art' methodology presentations, describing a range of the available expression systems and the particular nuances of optimising the expression of CFTR domains or full length proteins. The workshop will then discuss the latest methods for purification of CFTR domains and full length protein and the barriers that remain to be overcome. Both parts of the workshop will be followed by question and answer and troubleshooting sessions. The presenters will be encouraged to use movies or videos to illustrate the methodology as well as handout sheets and slides. Postdoctoral researchers and PhD students from various labs will be encouraged to present and act as discussants in the workshop.

Friday 22 March – 08:45-10:30 **Room: Malaga**

SYMPOSIUM 4
CFTR Folding: Towards Correcting the Defect
Chairs: Christine Bear (CA) / Ineke Braakman (NL)

S4.1 - Lesson and tools from a decade of biophysical studies of CFTR nucleotide-binding domains

John F. Hunt

Columbia University Department of Biological Sciences and CFTR 3D Structure Consortium, 702A Farichild Center, MC2434, New York, NY 10027

While it has not been possible to date to obtain a high-resolution structure of full-length CFTR, important insights and tools have emerged from studies of its isolated nucleotide-binding domains (NBD1 and NBD2). This presentation will outline the history of these studies and emphasize their technical and conceptual highlights. Topics will include: (i) the surprising connection between the problems encountered in the purification of NBD1 and the molecular etiology of the disease caused by the F508del mutation; (ii) tools for high-throughput screening developed based on thermodynamic and biophysical analyses of human NBD1; and (iii) the ostensibly catalytically inactive conformation of human NBD2 observed in its crystal structure.

S4.2 - Towards a rational design of combination corrector strategy to restore ΔF508-CFTR coupled domain folding

Tsukasa Okiyoneda[1], Guido Veit1, Johanna F. Dekkers[2,3,4], Miklos Bagdany[1], Naoto Soya[1], Haijin Xu[1], Ariel Roldan[1], A. S. Verkman[5], Mark Kurth[6], Agnes Simon[7], Tamas Hegedus[8], Jeffrey M. Beekman[2,3,4], Gergely L. Lukacs[1]

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CFTR, a member of the ABC transporter family, consists of five major domains and undergoes inefficient co- and posttranslational domain folding during ER biogenesis, assisted by molecular chaperone networks. Individual missense mutations cause conformational defects in multiple domains, revealed by limited proteolysis and domain specific immunoblotting. Likewise, deletion of F508 (ΔF508) in the NBD1 primarily impairs the NBD1 thermodynamic and kinetic stability, and disrupts the NBD1-MSD1/2 interface, leading to global conformational defect of the ΔF508-CFTR at multiple domain levels (1-3). Past efforts to achieve correction of ΔF508-CFTR misfolding by individual or combination of correctors had limited success, conceivable due to correction of only one of the primary folding impairment. This inference is in line with the finding that genetic stabilization of both NBD1 and the NBD1/MSD2 interface is critical to restore ΔF508-CFTR folding (2,3). Therefore, we postulate that corrector combinations targeting the primary structural defects may synergistically counteract ΔF508-CFTR misfolding. To test this hypothesis first the mechanism of action of available ΔF508-CFTR correctors was examined. Based on experimental and in silico data, correctors are predicted to target the NBD1/MSD interface or the NBD2. Currently, only chemical chaperones stabilize the ΔF508-NBD1. In line with previous observations, individual corrector compounds, including the investigational drug VX-809, or chemical chaperones rescue the ΔF508-CFTR expression <15% of its WT counterpart. In contrast, combinations of compounds and correctors targeting distinct structural defects are able to synergistically restore the ΔF508-CFTR processing and cell surface function in multiple model systems, including intestinal organoids, obtained from homozygous ΔF508 patients. These findings reinforce the CFTR coupled domain folding model and suggest a novel, rational design for combination therapy in CF. Supported by CFC and CFFTI.

1) Protasevich I et al. Thermal unfolding studies show the disease causing F508 deletion mutation in cystic fibrosis transmembrane conductance regulator (CFTR) thermodynamically destabilizes nucleotide-binding domain 1. Protein Sci 2010, 19: 1917–1931

2) Rabeh WM et al. Correction of both NBD1 energetics and domain interface is required to restore δF508 CFTR folding and function. Cell 2012, 148: 150–163.

3) Mendoza JL et al. 2012. Requirements for efficient correction of δF508 CFTR revealed by analyses of evolved sequences. Cell 2012, 148: 164–174..

S4.3 – Folding and assembly of MSD1 and NBD1 during CFTR biosynthesis

Ineke Braakman, Bertrand Kleizen, Floor Peters, Marjolein Mijnders

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

Programme Note: Abstract details are not authorised for publication

S4.4 - Mechanistic Studies of CFTR Correctors

André Schmidt[1], Linda Millen[1], Juan L. Mendoza[1], Margaret Fuller[1], Yi Cheng[2], Greg A. Miller[3], W. Christian Wigley[3], Robert J. Bridges[2], Philip J. Thomas[1]

[1]University of Texas Southwestern Medical Center, Physiology, Dallas, United States, [2]Chicago Medical School, Physiology and Biophysics, North Chicago, United States, [3]Reata Pharmaceuticals Inc., Irving, United States

Cystic Fibrosis (CF) is the most common monogenic, recessive lethal disease in populations of European descent, caused by dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), an anion-channel normally expressed in the apical membrane of some epithelial cells. Defective CFTR leads to multi-organ disease, the most important patho-physiological consequence being impaired mucus clearance from the lung. The most common mutation, found in 90% of CF patients, is a deletion of phenylalanine at position 508, termed F508del-CFTR. This mutation prevents the protein from efficiently folding, and, hence, accumulation in the endoplasmic reticulum prior to degradation. Importantly, the mutant protein is appropriately translated in the physiologically relevant cells. Thus, a possible strategy to treat the disease is to discover small molecules, which correct the impaired maturation of the mutant protein and thereby restore its normal cellular function. A number of small molecules have been identified that partially restore F508del-CFTR Cl^--channel function; some of these are represented in the Cystic Fibrosis Foundation Therapeutics (CFFT) panel. Recent work in our lab (Mendoza *et al.,* Cell 2012), indicates that at least two critical steps are defective in the F508del mutation: 1) decreased stability and folding yield of nucleotide binding domain 1 (NBD1), the domain containing the site of the deletion, and 2) poor assembly of the native multi-domain structure. To determine if CFFT panel small molecules act on these or different steps in CFTR folding, we assessed them in the context of second-site suppressor mutations, which have been demonstrated to counteract each of the F508del defective steps, domain folding and domain assembly. First, we assessed the isolated NBD1: To evaluate domain stability, *in vitro* thermal melts of the purified NBD1 protein were performed. To evaluate domain folding yield a cell-based assay was employed. Second, in combination with step-specific second-site suppressor mutations, we assessed full-length maturation using biochemical methods. Third, we performed assays on these combinations to evaluate their Cl^--channel function. The results suggest that some of the CFFT panel compounds target CFTR directly and act preferentially on one of the biosynthetic steps, but not the other, consistent with their limited efficacy. Importantly, a combination of assembly step specific compounds identified from the CFFT panel, and a compound which acts by a different mechanism of action, is able to affect efficient rescue of F508del-CFTR maturation and function. Understanding of the mechanism of action of these small molecules provides a rational path to effective combinations and to genotype specific treatment of other CFTR folding mutations.

We thank CFFT for providing us with the compounds for this study (Kirsten Hencken, khencken@cff.org or by phone (301) 841-2609).

Mendoza *et al.* (2012): Requirements for efficient correction of ΔF508-CFTR revealed by analyses of evolved sequences. *Cell* **148**(1-2), 164-74.

S4.5 The Corrector C18 Modulates the Channel Activity of Purified and Reconstituted CFTR Independent of ATP

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We recently developed a rapid purification and reconstitution method for Wt-CFTR and clinically relevant mutants F508del- and G551D-CFTR. Using this system we developed a halide efflux assay to monitor directly the functional activity of phosphorylated CFTR in a defined lipid membrane environment. The measured activity was dependent on the amount of protein reconstituted, genotype, and phosphorylation status, as well as the presence of ATP. Activity in the assay was sensitive to inhibition by the CFTR-specific inhibitor CFTRinh-172, and potentiated by well-known CFTR potentiators such as VRT-532 and VX-770 (ivacaftor). For VX-770 we noted a similar relative potentiation for Wt-, G551D- and F508del-CFTR. Interestingly, the VX-770 response was both independent and additive with ATP, suggesting that VX-770 binds to all genotypes of CFTR and at a site distinct from the ATP binding pocket to promote significant channel opening even in the absence of ATP. In contrast to potentiators, CFTR correctors are designed to rescue trafficking of mutant CFTR to the cell surface. However it appears that F508del-CFTR has defects in biosynthetic processing and trafficking to the cell surface, functional activity at the cell surface, and resident time at the membrane. An ideal corrector may improve all of these activities.

Since C18 is a highly effective corrector in other assays, it is an analog of VX-809 which is in clinical trials, and it is readily accessible, we probed its direct interaction with CFTR. Direct binding of C18 to CFTR modulates the halide flux activity of the purified and reconstituted protein in our assay, which is independent of membrane trafficking defects. At a concentration of 10 μM, C18 increases CFTR activity in this assay >2-fold for F508del-CFTR over vehicle treated control samples. It produced a similar 2.5-fold increase in Wt-CFTR flux activity.

The G551D mutant has little flux activity due to a defect in the ATP binding-hydrolysis cycle thought to be linked to channel opening and closing. In this clinically-relevant mutant, C18 induced a 3-fold increase in halide flux activity. VX-770 potentiates G551D-CFTR similarly, and in fact VX-770 can potentiate both Wt- and F508del-CFTR in the complete absence of ATP. In light of our previous VX-770 results and our new data on C18 treatment of G551D-CFTR, we were prompted to examine the effect of C18 on the activity of Wt-CFTR in the nominal absence of ATP. We examined a >2-fold increase in the flux activity of this phosphorylated protein in the absence of added nucleotide relative to a control sample in the absence of ATP but in the presence of the DMSO vehicle. These data suggest that like VX-770, C18 can induce channel function independent of ATP-mediated channel opening. There is no significant difference in ATPase activity for purified CFTR protein in the presence of 2 μM C18 or vehicle over a range of ATP concentrations. Taken together, our data suggest that C18 binds directly to CFTR and modulates its channel activity by an ATP-independent mechanism, at a site distinct from the canonical ATP-binding site.

S4.6 - The Iminosugar Isolab Corrects the Defective Trafficking of F508del-CFTR by Prevention of Hsp90/CFTR Interaction

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Cystic fibrosis (CF) is an autosomal and recessive disease caused by mutations in Cystic Fibrosis Transmembrane conductance Regulator gene (*CFTR*). Approximately 90% of CF patients have the F508del mutation in one or both *CFTR* alleles. This mutation leads to the retention of F508del-CFTR protein in the endoplasmic reticulum (ER), to abnormal gating of CFTR channel and endocytosis. One of the major therapeutic strategy in Cystic Fibrosis aims at developing correctors of CFTR channels. In previous study we identified an iminosugar, IsoLAB, as a new corrector of the F508del-CFTR defective trafficking independently of the ER-α-glucosidase inhibition (Best et al., 2010). In the present work we studied the mechanism of action of IsoLAB.

Firstly, we investigated the effect of IsoLAB on CF tracheal human epithelial cells (CF-KM4) in competition with several inhibitors of the biosynthetic pathway and of the ER quality machinery using the iodide efflux technique. We observed a lack of correction by IsoLAB in presence of brefeldin A, a vesicular ER/Golgi-intermediate compartment traffic inhibitor. This result indicates that IsoLAB-corrected F508del-CFTR follows a conventional trafficking pathway. We observed that thapsigargin, tunicamycin and miglustat, known to alter the F508del-CFTR/calnexin interaction, potentiate the F508del-CFTR rescue induced by IsoLAB. We also found that a treatment with MG132, a proteasome inhibitor, potentiates the IsoLAB effect. Altogether, these results suggest that calnexin and/or proteasome are not the molecular targets of IsoLAB.

On the contrary, when cells were incubated at low-temperature, a procedure known to rescue F508del-CFTR proteins via the inhibition of heat shock proteins (Hsp), we did not observe potentiation of the correction by IsoLAB, whereas it is the case with other correctors. This result suggests a potential effect of IsoLAB on Hsp proteins. To test this hypothesis we conducted additional experiments. While 4-PBA, an Hsp70 inhibitor, slightly potentiated the effect of IsoLAB, we observed no potentiation when IsoLAB was associated in co-treatment with geldanamycin or ansamycin, two Hsp90 inhibitors. Thus, we focused on the Hsp90-dependent pathway and used a Duolink “Proximity ligation Assay” to detect and quantify Hsp90/CFTR interaction. We observed that transfected CF-KM4 cells with Hsp90 siRNA (1) decreased the Hsp90/CFTR interaction and (2) corrected the abnormal trafficking of F508del-CFTR. Similar results were obtained after a pharmacological treatment by geldanamycin or ansamycin. Finally, IsoLAB decreased by 90% the Hsp90/CFTR interaction resulting in the restoration of a functional F508del-CFTR to the plasma membrane.

In conclusion, we propose that the F508del-CFTR corrector IsoLAB act as an Hsp90-dependent pathway inhibitor. Further experiments will be conducted to strengthen this hypothesis and to determine whether IsoLab is a direct inhibitor of Hsp90.

Supported by «Groupama Centre-Atlantique » and « Fondation pour la Recherche Médicale».

Friday 22 March – 11:00-12:45

SYMPOSIUM 5
Infection, Inflammation and Immunity - II
Chairs: Stuart Elborn (UK) / Jean-Michel Sallenave (FR)

S5.1 - Phospholipase C beta and pro-inflammatory signaling in bronchial epithelial cells

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Excessive lung inflammation, depending on the intrinsic CFTR protein defect(s), is strongly amplified and worsened by recurrent and chronic bacterial infection, leading to progressive tissue damage and respiratory insufficiency. Understanding the pro-inflammatory signaling elicited by bacterial infection in bronchial epithelial cells is one of the strategies aimed to provide insights for innovative CF-tailored anti-inflammatory therapies, to help the effect of novel correctors and potentiators of mutant CFTR.

Based on phosphorylation analyses, transcription factor "decoy" molecules and chromatin immmunoprecipitation, we recently confirmed and extended the major signaling components involved in P.aeruginosa-dependent transcription of the key inflammatory marker IL-8 [1]. Thus, Mitogen-Activated Protein Kinases ERK1/2, p38 and JNK, together with RSK1/2 and HSP27 kinases, downstream Toll-like Receptors (TLR) 2 and 5, activate transcription factors NF-kB, NF-IL6, AP-1, CREB and CHOP, that are all relevant to the modulation of IL-8 gene transcription.

Considering the redundancy of this pro-inflammatory signaling and trying to focus on those signaling components that could be more relevant to pharmacological modulation of CF inflammation, we went back to obtain insights from clinics and modifier genes. Starting from a gene association study searching within 721 variants from 135 candidate genes of the immune response which could be the most relevant to influence the clinical progression of lung disease in F508del CFTR homozygous patients, we found on top of the ranking list the phospholipases C beta (PLCB) of the isoforms 3, 1 and 4 [2]. Testing the role of PLCB3 in IL-8 gene transcription, we found that the intracellular calcium-activating PLCB3 pathway is not sufficient to induce IL-8 gene transcription but potentiates the MyD88 pathway downstream TLR2/5. In parallel, strong inhibition of expression of PLCB3 significantly reduces, but does not abolish, IL-8 expression, making PLCB a potential target to mitigate excessive inflammation without completely inactivating the anti-infective defences. As the gene variant most significantly associated with a better clinical course of lung disease in CF patients encodes for a Ser845 to Leu variation in PLCB3, we are presently investigating whether Leu845 is associated with a loss-of-function of PLCB3, which could validate the role of PLCB isoforms as potentially relevant anti-inflammatory targets. In conclusion, to test this hypothesis and the role of the intracellular calcium-dependent pro-inflammatory signaling in CF lung pathology, different approaches based on downmodulation of endogenous PLCB3 and expression of PLCB3 variants in vitro are presently compared with in silico structural analyses.

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[2] Bezzerri V, d'Adamo P, Rimessi A, Lanzara C, Crovella S, Nicolis E, Tamanini A, Athanasakis E, Tebon M, Bisoffi G, Drumm ML, Knowles MR, Pinton P, Gasparini P, Berton G, Cabrini G. Phospholipase C-{beta}3 Is a Key Modulator of IL-8 Expression in Cystic Fibrosis Bronchial Epithelial Cells. J Immunol. 2011;186:4946-58.

Supported by the Italian Cystic Fibrosis Research Foundation grant FFC#19/2011 and the “Delegazione FFC Belluno” and grant FFC#5/2011 and Fondazione Cariverona.

S5.2 – Modulation of inflammatory signaling by Nrf2 mediated antioxidant responses

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While the notion that an innate hyper-inflammatory phenotype exists in CF epithelia is supported by a number of studies in cell line and some studies in primary epithelia, it remains controversial. However, most clinical studies have found that in the complete context of the CF lung, inflammatory cytokines and other markers of inflammation are markedly elevated. Controlling inflammation with anti-inflammatory agents, such as ibuprofen, has been beneficial and has resulted in a significant improvement of lung function, as assessed by FEV1. Therefore, at the signaling level, pathways that control inflammation deserve investigation. Pathways that regulate redox signaling are of interest, as they play a key role in modulating inflammatory cascades. For example, IL-1β and TNF-α mediated activation of NF-κB occurs via H2O2 dependent signaling. H2O2 is also associated with the activation of a number of other pro-inflammatory pathways, such as MAPK and JNK.

Antioxidants, which control steady state H2O2, inhibit IL-1β and TNF-α receptor mediated cytokine production. In cells, the chief mechanism for the control of oxidants is the antioxidant response element (ARE), which is under the regulation of the transcription factor Nrf2. Studies of Nrf2 have demonstrated that it plays a key role in controlling inflammatory pathways in cells. Furthermore, Nrf2 deficient mice are more susceptible to pulmonary infection and prone to bowel inflammation and obstruction, both of which are characteristic of CF. We investigated the expression and activity of Nrf2 in a primary site of dysfunction and inflammatory signaling in CF, epithelial cells. For our studies, we use up to 6 models of CF epithelia, 2 cell line pairs, 2 primary cell models, as well as excised nasal epithelia from 2 CF mutant mouse models. Our studies are the first to reveal that Nrf2 expression and activity are significantly dysregulated in CF models. This decrease in Nrf2 activity results in decreases of antioxidant proteins, including peroxidases such as catalase and PRDX1, 3, 5, and 6. A functional consequence is the accumulation of intracellular H2O2, which results in a significant increase in inflammatory cytokine production by CF versus non-CF controls. Furthermore, rebound in GSH following oxidation was diminished in CF versus non-CF cells, with a drop of ~63% in GSH levels following 5 min of oxidation which only returns to 86% of pretreatment levels in CF cells by 90 min. Conversely, nonCF cells exhibit an increase in GSH of 87% following oxidation, which remains elevated for 90 min. Correction of Nrf2 dysfunction, by a variety of approaches, in CF cells significantly reverses increases in oxidant and cytokine levels, and increases levels of GSH while reducing levels of GSSG. The functional consequences of the correction of Nrf2 dysregulation in CF cells include the reduction of protective protein oxidation and the significant reduction of inflammatory signaling.

Taken together our studies indicate that Nrf2 dysregulation in CF epithelial cells positively contributes to increased inflammatory signaling, and that correction of Nrf2 dysfunction in CF may be a beneficial strategy. However, any Nrf2-targeted therapy in CF should investigate the safety of chronic Nrf2 activation.

S5.3 - The neutrophil in cystic fibrosis: dysregulation and inflammation

N Gerry McElvaney

Division of Respiratory Research, RCSI Beaumont Hospital, Dublin, Ireland

The neutrophil is the major inflammatory cell in the lungs of people with cystic fibrosis (PWCF). Despite this large neutrophil presence, the airways in PWCF are also chronically colonized with bacteria suggesting that while neutrophils are present in large numbers they are singularly ineffective. In this presentation we will discuss how neutrophils are attracted to the lung in PWCF in such large numbers and how this is dysregulated, evaluating the roles of toll like receptor agonists, interleukin-8, interleukin-17, glycosaminogycans and neutrophil elastase and other proteases. We will also elucidate how even when the neutrophil reaches the lung its actions are altered and in some cases incapacitated by the effects of proteases. We will discuss the abnormalities in degranulation found in CF neutrophils partly due to inflammatory processes but also due to intrinsic defects in the CF neutrophil. This dysregulation of the neutrophil will be discussed in the context of the enormous inflammatory and infectious burden present in the CF lung and how the ineffective neutrophil not only fails to clear infection but also contributes to the inflammatory process. Finally we will evaluate the effects of therapies such as hypertonic saline, ion channel modulators and other new therapies on neutrophil promulgated inflammation.

S5.4 - Neutrophil Elastase-mediated Increase in Airway Temperature during Inflammation

Gerd Döring[1], Annika Schmidt[1], Rosi Bissinger[1], Garrit Koller[2], Laurette Malleret[3], Ciro Dorazio[4], Baroukh Assael[4], Martino Facchinelli[5], Giorgio Piacentini[5], Bernhard Schulte-Hubbert[6], Jutta Hammermann[7], Monika Schniederjans[8], Susanne Häußler[8], Keith C. Meyer[9], Dieter Worlitzsch[10], Soeren Damkiaer[11], Kenneth Bruce[2], Azzaq Belaaouaj[3], John J. Lipuma[12], Joachim Seelig[13]

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Objectives: *P. aeruginosa* is the dominant pathogen in chronic lung infections in CF The reason(s) for this selection is unclear.
Methods: In CF patients and healthy controls we determined the exhaled airwaytemperature using PLET and the temperature in the airway lumen and in mucus plugs using a probe inserted into a flexible bronchoscope.We determined the enthalpy of the binding reaction between purified human neutrophil elastase (NE) and its endogenous inhibitor α_1-PI using isothermal titration calorimetry. In infected wildtype and isogenic $NE^{-/-}$ mice, we determined the pouch airspace temperature 4 days after *P. aeruginosa* challenge.We cultured bacterial pathogens at 30°C and 38°C for 96 h anaerobically *in vitro*, and measured their density. We compared the transcriptome of *P. aeruginosa* after anaerobic growth at 30°C and 38°C and sequenced the microbiota in 8 paired early and late CF sputum specimens.
Results: PLET temperatures in 56 CF patients were inversely correlated to lung function,suggesting that bronchial obstruction increases airway temperature perhaps due to obstruction. A significantly higher mean temperature of 37.98±0.80°C was measured within mucus plugs in the anterior upper lobe segmental bronchi of 5 CF patients as compared to the airway lumenal temperatures (36.62±0.91°C) ($p< 0.05$) which were similar to that of 3 healthy individuals (36.2±0.66°C). When a 150 µM α_1-PI solution was titrated into a dilute (15 µM) NE solution, the initial injection peaks revealed an exothermic reaction with a binding enthalpy of -18.5±1.3 kcal/mol. Temperatures in pouch airspaces of infected WT mice were significantly higher compared to controls, implicating an exothermic NE:α1-PI complex formation as a relevant mechanism for the local temperature rise. After 96 h at 38°C, the densities of *S. epidermidis, S. aureus, S. maltophilia, P. putida, B. cenocepacia* and *M. abscessus* were significantly lower (or remained constant) vs. 30°C while only *P. aeruginosa* grew at 38°C. At 39°C this difference became even more pronounced. In contrast, *P. aeruginosa* grew at both elevated temperatures. Growth of *P. aeruginosa* was not dependent on the production of alginate because a mucoid *P. aeruginosa* $mucA^{-/-}$deletion mutant grew similarly well as the non-mucoid parent strain and the isogenic non-mucoid variant *P. aeruginosa* $mucA^{-/-}/algT^{-/-}$. Furthermore, loss of the Type 6 secretion systems (T6SS) did not affect growth. Collectively the data show that from the investigated bacterial strains, only *P. aeruginosa* is capable to grow at elevated temperature. We identified 858 differentially expressed genes at 38°C vs 30°C. Virulence genes, including components of the T3SS and components of the LasR regulated quorum sensing system were significantly up-regulated at 38°C compared to 30°C, suggesting that *P. aeruginosa* particularly activates its protective armory at higher environmental temperatures. In addition, a Mg^{2+} transporter known to enhance the thermotolerance by stabilizing proteins or protein-nucleic acid interactions was highly up-regulated at 38°C.The mean *P. aeruginosa* relative abundance was 0.191 in early sputum samples and 0.592 in late samples.
Conclusion: NE mediates a temperature increase in mucus plugs of CF patients which favours the selection of *P.aeruginosa.*

S5.5 - Classically Activated Macrophages Promote Inflammation in Response to *Pseudomonas aeruginosa* but Do Not Provide an Effective Barrier against Bacterial Growth

Sonali Singh[1], Helen Barr[1], Andrew Fogarty[1], Paul Williams[1], Miguel Camara[1], Luisa Martinez-Pomares[1]

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Pseudomonas aeruginosa (PA) is an opportunistic pathogen that causes severe respiratory infections in susceptible individuals (e.g. cystic fibrosis (CF) patients). Th1-dominated responses are considered protective against PA infection. CF patients have been proposed to have a Th2-biased immune response suggesting that lack of a Th1 response could contribute to the establishment of chronic infection. We have compared IFN-g production by CF and healthy peripheral blood mononuclear cells (PBMCs) in response to several stimuli. In agreement with these original observations we have detected a significant reduction in IFN-g production by CF PBMCs in response to the mitogen phytohaemagglutinin (PHA) and the superantigen Staphylococcal enterotoxin B (SEB) and established a positive correlation between IFN-g production and lung function measured as FEV_1.

Because classically activated macrophages (M1) are considered key effector cells in Th1 responses we investigated how a Th-1-dominated immune response would affect the interaction of PA with macrophages. Our results show that classical activation in the presence and absence of the pro-inflammatory cytokine GM-CSF did not enhance bacterial clearance or macrophage survival significantly and these were not affected by bacteria opsonisation. On the other hand, IFN-g shifted the cytokine response in response to PA towards increased inflammation. In particular, it caused a dramatic up-regulation of MCP-1 and TNF-a expression and down-regulation of IL-10 expression by PA-infected macrophages. Comparison of TNF-a vs IL-10 and IL-6 vs IL-10 ratios revealed the following hierarchy: M-CSF < M-CSF + IFN-g ≤ GM-CSF < GM-CSF + IFN-g. This shift would result in the recruitment and activation of a variety of host cells including antigen-presenting cells, neutrophils, and lymphocytes. These results indicate that rather than exerting a major microbicidal function, macrophages, even when classically activated, act as regulators of the inflammatory response to PA.

S5.6 - Residence in Biofilms allows *Burkholderia cepacia* Complex (Bcc) Bacteria to Evade Neutrophil Anti-microbial Activities

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The primary morbidity of Cystic fibrosis (CF) -the generation of unusually viscous pulmonary mucous- greatly diminishes mucociliary clearance from the lung. This engenders an environment well-suited to colonisation by microorganisms. However, despite the continuous recruitment of neutrophils into the airway lumen, many of these microorganisms persist.

Bacteria of the *Burkholderia cepacia* complex (Bcc) are such persistors. Though less prevalent among CF patients than *Pseudomonas aeruginosa*, Bcc members are disproportionally associated with patient mortality owing to a unique morbidity they may induce, termed '*cepacia* syndrome', which is characterised by an acute-onset deterioration of lung function with associated septicaemia. Treatment is a challenge as most members of Bcc are multi-drug-resistant and display an ability to form biofilms, further reducing their antibiotic susceptibility.

Therefore, we examined the role of biofilm formation in protecting Bcc bacteria from neutrophil anti-microbial activity. The nature of the physical biofilm-neutrophil interaction was visualised using confocal laser-scanning microscopy (CLSM). In addition, the impact of neutrophils, or components thereof, on biofilm development was investigated using microtiter plate assays of biofilm density. We also investigated the effect of culture in the presence of Bcc biofilm on neutrophil anti-microbial activity and on the fate of the neutrophil.

CLSM confirmed that differentiated, neutrophil-like HL60 cells (dHL60s) remained at the exterior surface of established *B. multivorans* LMG 13010 biofilms (72 h) up to 2 hours after inoculation, impeding phagocytosis. This was despite limited migration and the appearance of pseudopodia.

The CF-associated species LMG 13010, *B. cenocepacia* K56-2 and *B. dolosa* LMG 18941 were grown as static biofilms for 4, 24 or 48 hours, at which point dHL60 cells were added. Following 24 and 48 hours co-incubation, biofilm biomass was determined using crystal violet.

Culture in the presence of dHL60 cells lead to reduced initial formation of biofilm with respect to untreated biofilms, however, during 72 h maturation, greater biofilm biomass was formed by LMG 13010 and LMG 18941 in the presence of dHL60s ($p < 0.01$).

As neutrophils undergo apoptosis and disintegrate shortly after recruitment to the lung, this experiment was repeated with whole-cell lysates of dHL60 cells in order to investigate the effect of neutrophil components on biofilm development. Again, biofilm density increased after incubation with lysed dHL60s for LMG 13010 and LMG 18941($p < 0.001$).

These results suggest that Bcc members produce denser biofilms when incubated with dHL60 cells, either through interaction with viable neutrophils or components thereof, or incorporation of exogenous molecules such as DNA or actin, and that this impedes phagocytosis.

Furthermore, dHL60s secreted disproportionately low amounts of IL-8 when exposed to biofilms relative to planktonic bacteria ($p < 0.001$) and extracellularly degranulated less myeloperoxidase enzyme when added to more mature biofilms (48-72 h).

Hence, biofilms facilitate Bcc species' persistence in the CF lung by masking them from detection and acting as a barrier, and the presence of neutrophils reinforces that biofilm. Therefore, strategies to improve neutrophil efficacy in clearing colonising Bcc must focus on overcoming biofilm-mediated resistance.

This work has been carried out with funding from Science Foundation Ireland (SFI)

Friday 22 March – 18:00-20:10

SYMPOSIUM 6
CFTR Functional Interactome
Chairs: Ray Frizzell (US) / Aleksander Edelman (FR)

S6.1 - Small heat shock proteins target mutant CFTR for degradation via a SUMO-dependent pathway

Raymond A. Frizzell[1], Annette Ahner[1], Xiaoyan Gong[1], Gergely L. Lukacs[2] and Patrick H. Thibodeau[1]

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Small heat shock proteins target mutant CFTR for degradation via a SUMO-dependent pathway
Annette Ahnera, Xiaoyan Gonga, Patrick H. Thibodeaua, Gergely L. Lukacsb and Raymond A. Frizzella
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Defining the significant checkpoints in CFTR biogenesis should identify targets for therapeutic intervention for CFTR folding mutants like F508del. Small heat shock proteins (sHsps) bind destabilized proteins during cell stress and disease, but their physiological functions are less clear. We found that small heat shock proteins (sHsps) selectively facilitate the degradation of F508del with little effect on the WT protein. We chose Hsp27 for further study (1) based on its expression in airway cells, its involvement in protein folding diseases and its identification as a member of the CFTR interactome (2).
Hsp27 interacted physically with Ubc9, the SUMO E2 conjugating enzyme, implying that F508del SUMOylation may lead to its sHsp-mediated degradation. The selective interaction of Hsp27 with F508del CFTR could then recruit Ubc9 to the complex to promote CFTR SUMOylation. The SUMO cascade is organized like that for ubiquitin, but transfer of SUMO to substrate is the job of the single SUMO E2, Ubc9, whereas SUMO E3s usually assist with catalysis and substrate selection (3). As for the ubiquitin cascade, SUMO conjugates are cleaved by specific cysteine proteases (SENPs) that remove SUMO. In keeping with the model: Hsp27/Ubc9/SUMO-F508del, we found that enhancing or disabling the SUMO pathway increased or blocked, respectively, Hsp27's ability to degrade mutant CFTR. In vitro, Hsp27 promoted the preferential SUMOylation of F508del NBD1 using purified SUMO cascade components.
sHsps are ATP-independent chaperones that generally do not interact with native structures or completely denatured proteins, but with proteins having an intermediate, foldable conformation. With increasing denaturant, NBD1 undergoes transition to an intermediate conformation, detected as an increase in intrinsic tryptophan fluorescence. F508del NBD1 undergoes this transition at lower denaturant concentration than the WT NBD, reflecting its conformational instability. The time-course of this transition to the non-native conformation was paralleled by SUMOylation of the mutant NBD in vitro, behavior similar to the protein recognition properties of sHsps.
Hsp27 promoted the SUMOylation of full-length F508del CFTR in vivo, which preferred endogenous SUMO-2/3 paralogs that form poly-chains. The SUMO-targeted ubiquitin ligase (STUbL), RNF4, contains tandem SUMO interacting motifs (SIMs), and can facilitate nuclear protein degradation (4). Over-expression of RNF4 elicited F508del degradation, whereas Hsp27 knockdown blocked RNF4's impact on mutant CFTR expression. Similarly, the ability of Hsp27 to degrade F508del CFTR was lost during over-expression of dominant-negative RNF4. These findings link sHsp-mediated F508del degradation to its SUMOylation and its to STUbL-mediated targeting to the ubiquitin-proteasome system, thereby implicating this pathway in ERAD of a cytosolic, transmembrane protein.

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S6.2 - CFTR INTERACTIONS STUDIED IN LIVE CELLS

Peter M. Haggie, Ph.D

Division of Nephrology, Department of Medicine, University of California, San Francisco

Although the CFTR gene was discovered more than twenty years ago, many aspects of CFTR biology remain unresolved and controversial. Research in the CFTR field has been dominated by biochemical and electrophysiological approaches that have undoubtedly advanced our understanding of CFTR biology. Biochemical techniques, however, typically generate ensemble-averaged results that are characterized by low spatial and temporal resolution. Similarly, electrical measurements generate average results in whole cell configurations or investigate isolated membrane regions in single channel recordings; albeit with excellent temporal resolution. In contrast, live cell fluorescence imaging approaches have the potential to generate direct and dynamic experimental data that cannot be obtained with alternative methods. Single molecule fluorescence imaging approaches in particular yield information on large numbers of individual proteins with nanometer spatial resolution and millisecond time resolution. Our recent studies have applied live cell fluorescence microscopy to investigate the role of scaffold protein interactions in the surface stability of rescued mutant CFTR molecules. Deletion of phenylalanine 508 (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR) plasma membrane chloride channel is the most common cause of cystic fibrosis (CF). Though several maneuvers can rescue endoplasmic reticulum-retained ΔF508CFTR and promote its trafficking to the plasma membrane, rescued ΔF508CFTR remains susceptible to quality control mechanisms that lead to accelerated endocytosis, ubiquitination and lysosomal degradation. To directly assess the role of scaffold protein interactions in rescued ΔF508CFTR surface instability, the plasma membrane mobility of ΔF508CFTR was measured in live cells by quantum dot single particle tracking. Following rescue by low-temperature, chemical correctors, thapsigargin or overexpression of GRASP55, ΔF508CFTR diffusion was more rapid than that of wild-type CFTR because of reduced interactions with PDZ domain-containing scaffold proteins. Knock-down of the plasma membrane quality control proteins CHIP and Hsc70 partially restored ΔF508CFTR–scaffold association and reduced ΔF508CFTR mobility. In experiments that quantitatively compared CFTR cell surface diffusion and endocytosis kinetics there was an association between reduced scaffold binding and CFTR internalization. As such, our surface diffusion measurements in live cells indicate defective scaffold interactions of rescued ΔF508CFTR at the cell surface and suggest that restoration of ΔF508CFTR–scaffold interactions may be therapeutically beneficial. Further studies to investigate the role of CFTR–scaffold interactions in surface stability have used chimeras of CFTR and scaffold protein domains. This approach has indicated that bypassing CFTR–PDZ interactions by directly linking CFTR molecules to the actin cytoskeleton or ezrin stabilizes CFTR at the cell surface and focuses attention on modulation of CFTR–scaffold interactions as a mechanism to stabilize CFTR at the cell surface. In summary, live cell fluorescence microscopy provides novel insight into CFTR cell biology; in the case of ΔF508CFTR correction, these studies have established that modulation of scaffold interactions may be of therapeutic benefit.

S6.3 Potentiating and correcting double effect of snake venom Phospholipase A2 on ΔF508-CFTR

Grazyna Faure[1], Naziha Bakouh[2], Norbert Odolczyk[3], Haijin Xu[1,4], Gabrielle Planelles[2], Piotr Zielenkiewicz[3], Gergely L. Lukacs[4], Mario Ollero[2,5], Aleksander Edelman[2]

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Cystic fibrosis is caused by mutations in the gene coding for Cystic fibrosis transmembrane conductance regulator (CFTR), a protein with Cl^- channel function. The most frequent mutation, ΔF508-CFTR, leads to abnormal Cl^- transport across epthelia. The aim of this study was to investigate the interaction of human and snake venom phospholipases A2 group IIA (PLA2-IIA, important regulators of lipid signaling) with wild type- and ΔF508-CFTR, and their potential regulation of CFTR Cl^- channel activity. We present new and unexpected data showing that both PLA2-IIA bind to the nucleotide binding domain 1 (NBD1)- and ΔF508NBD1-CFTR. Based on these observations, we postulate that intracellular binding of PLA2-IIA regulates CFTR and/or ΔF508-CFTR activity. To verify this hypothesis, we performed two-electrode voltage clamp experiments in Xenopus laevis oocytes in which CFTR or ΔF508-CFTR were expressed and in which we injected snake or human PLA2-IIA. The results show that human and snake PLA2-IIA increase CFTR Cl^- current activity, and snake PLA2-IIA (the basic CB subunit of crotoxin) increases CFTR- Cl^- channel function and addresses ΔF508-CFTR to the plasma membrane. Molecular docking analysis of snake PLA2-IIA interactions with NBD1 provides a hypothetical 3D model of the binding interface between the two proteins.
In conclusion, we describe a novel function of sPLA2-IIA as a regulator of CFTR activity, demonstrating a clear-cut link between CFTR-Cl^- channel transport and PLA2 lipid signaling pathways. The unexpected, potentiating and correcting double effect of snake venom PLA2 on mutated CFTR provides an original perspective to investigate the pharmacotherapy of cystic fibrosis.

S6.4 - Interaction between Keratin 8 and Cystic Fibrosis Transmembrane Regulator (CFTR): Structural Studies

Anna Kupniewska[1], Arkadiusz Bonna[2], Tomasz Fraczyk[2], Sara Bitam[1], Iwona Pranke[1], Krzysztof Tarnowski[2], Ariel Roldan[3], Gergely Lukacs[3], Michal Dadlez[2], Aleksander Edelman[1]

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Introduction: Recently, we have shown that interaction between CFTR with a deletion of phenyloalanine 508 (F508del - CFTR) might be responsible for the inefficient trafficking of mutated CFTR to the plasma membrane contributing to the cystic fibrosis (CF) disease. Using functional assay, it was shown that disruption of this interaction allows for addressing the F508del - CFTR to the plasma membrane where it can exert its function (Colas J. et al., Hum Mol Gen 2011). Surface plasmon resonance indicated much stronger interaction between keratin 8 and domain of CFTR F508del - NBD1 than with native NBD1.
Aim: Because the site of the interaction between keratin 8 and mutated CFTR might constitute a new target for pharmacotherapy of CF the aim of this study was to determine it on structural level. For this aim we have applied;
Material and methods: Recombinant protein NBD1 WT/ F508del (Lukacs G., McGill University, Montreal), K8 peptide chemical synthesis, hydrogen - deuterium exchange reaction coupled with mass spectrometry (HDex - MS)
Results: After HDex - MS performed on full keratin 8 protein were able to choose fragments of this protein with a highest exposition for hydrogen - deuterium exchange as regions especially prompted to interact with other molecules. We have synthesized the head domain of keratin 8 which exhibited the highest HDex rate dividing it for 3 overlapped peptides. One of them gave a strong signal in HDex - MS experiments suggesting close interaction with mutated and wild type NBD1. We obtained differential HDex pattern between mutated and wild - type NBD1 with and without keratin 8 peptide. The differential site of the interaction with K8 peptide appeared to expand on a linker region between two subdomains of NBD1 in the proximity of deletion phenyloalanine 508. Previously it was shown that the same linking fragment gains much more flexibility leading to more conformation freedom of NBD1 subdomains upon mutation (F508del) (Wieczorek G. et al., J. of Cystic Fibrosis, 2008). This leads to the exposition of hydrophobic residues preferentially buried in the native NBD1 and creation of 2 cavities on the surface mutated NBD1 according to the in silico modelisation. Moreover, to these cavities 12 of small molecules from National Cancer Institute Library (NCIL) were successfully docked and for 4 of them the functional assay exhibited correcting effect of F508 - CFTR and proximity ligation DNA assay revealed ability to disrupt with keratin 8 interaction.
Conclusion: Taking into account above findings we propose the interruption of pathogenic interaction with keratin 8 as highly probable mechanism of action of small corrector [NCIL ID: 407882].

S6.5 - A Functional CFTR Assay Using Primary Cystic Fibrosis Intestinal Organoids

Florijn Dekkers[1,2,3], C.L. Wiegerinck[3], H.R. de Jonge[4], I Bronsveld[5], H.M. Janssens[5], Karin M de Winter-de Groot[1], A.M. Brandsma[1,3], N. W. M. de Jong[1,3], M.J.C. Bijvelds[1,3], B. J. Scholte[6], E.E.S. Nieuwenhuis[7], S. van den Brink[8], H Clevers[8], C.K. van der Ent[1], S. Middendorp[2,7], Jeffrey Beekman[1,2,3]

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Introduction: We have recently established conditions allowing long-term expansion of epithelial organoids from intestine, recapitulating essential features of the in vivo tissue architecture. Here, we apply this technology to study primary intestinal organoids of people with cystic fibrosis.
Methods: Crypts are isolated from rectal biopsies and expanded in growth factor complemented matrigel. Forskolin induced swelling is monitored by live confocal microscopy and unbiased image analysis.
Results: Forskolin induces rapid swelling of organoids derived from healthy controls or wild-type mice, but is strongly reduced in organoids of cystic fibrosis subjects or F508del-Cftr mice and absent in *Cftr*-deficient organoids. This is phenocopied by CFTR-specific inhibitors. Forskolin-induced swelling of *in vitro* expanded healthy control and cystic fibrosis organoids corresponds quantitatively with forskolin-induced anion currents in *ex vivo* freshly excised rectal biopsies, and indicates limited residual function in F508del homozygous subjects. Function of F508del-CFTR is restored upon incubation at low temperature, as well as by CFTR-restoring compounds. We found that CFTR-restoring correctors synergize in restoring CFTR F508del function, and that the efficacy of CFTR-restoring drugs is different in organoids from different patients.
Conclusions: This relatively simple and robust assay will facilitate diagnosis, functional studies, drug development and personalized medicine approaches in cystic fibrosis.

S6.6 - Signaling Detected at ENac N-Termini Controls ENac Proteolysis

Martina Gentzsch[1], Yan Dang[1], Hong He[1], Jack Stutts[1]

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Background: ENaC (epithelial Na^+ channel) is rate limiting for Na^+ absorption by airway epithelia and hyperactivity of ENaC contributes to underhydration of cystic fibrosis airways. Limited proteolysis of ENaC extracellular domains stimulates ENaC open probability and we found that ENaC from CF airways is more cleaved than ENaC from normal airways. Based on reports that ubiquitination affects proteolysis of ENaC extracellular domains, we examined the contribution of lysine clusters in the initial N-termini of β- and γ-ENaC to the proteolytic regulation of ENaC.

Methods and Results: We injected Xenopus oocytes with wild type ENaC subunits or mutant subunits, in which lysine clusters were changed to glutamine or arginine, and used ENaC current (I_{Na}) and biochemistry to assess ENaC proteolytic regulation. Compared to WT ENaC, $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC had decreased basal I_{Na} but I_{Na} in both groups was sharply stimulated by exogenous trypsin. When co-expressed with matriptase, WT ENaC exhibited raised I_{Na} that could not be further stimulated by exogenous protease. In contrast, $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC maintained a low I_{Na} that was stimulated by exposure to trypsin, chymotrypsin or elastase. At baseline, mutant ENaC was expressed equally well at the surface, compared to WT ENaC, but cleavage fragments were sharply reduced. Based on these results, we reasoned that K to Q mutagenesis of ENaC N-termini protected mutant channels from matriptase. Indeed, close examination revealed that mutant channels were activated more slowly by exogenous protease (Time constant of 20 µg/ml trypsin activation: WT = 30 +/- 2 sec; $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC =122 +/- 15 seconds). Most of this effect was attributed to the N-terminus of γ-ENaC. We also discovered that mutagenesis of the γ-ENaC K-cluster to arginines had little effect on ENaC proteolytic regulation, suggesting that loss of net basic charge in the N-terminus of γ-ENaC was a major factor in slowing ENaC proteolytic activation. Co-expressed ubiquitin diminished I_{Na} and slowed acute stimulation by exogenous proteases, whereas co-expressed deubiqutinating enzyme 3 increased basal I_{Na} and sped its activation by exogenous proteases. These effects were absent when ENaC lacking N-terminal lysine cluster was studied. Although basal I_{Na} of ENaC co-expressed with matriptase could not be further stimulated by exogenous protease, oocytes expressing ENaC, matriptase and CFTR produced I_{Na} that was stimulated by trypsin. We found the time constant of acute stimulation of I_{Na} by exogenous proteases was increased 2-4 fold when CFTR was expressed.

Conclusion: We conclude that the basic residues of the initial N-terminus of γ-ENaC mediate interactions that promote susceptibility of ENaC extracellular domains to cleavage in an allosteric manner. Thus, cytosolic signaling events or conditions that foster or diminish these interactions, including ubiquitination and changes in ion activity may regulate ENaC proteolysis.

Saturday 23 March – 08:45-10:30

SYMPOSIUM 7
Mucus and CF
Chairs: Steve Ballard (US) / Gerd Döring (DE)

S7.1 - Airway mucin organisation and maturation

Caroline Ridley, Richard Collins, Karine Rousseau and David J Thornton

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Airways mucus defends the respiratory tract from pathogenic and environmental challenges. In CF, accumulation of mucus with abnormal transport properties is an important pathologic feature, which results in adherence of mucus plaques to the airway epithelium, blocking small airways and providing an environment within which bacteria can flourish. This leads to chronic inflammation and a cycle of airway damage and mucus accumulation, key aspects of morbidity and mortality. The development of effective reagents targeted at reducing mucus accumulation and adherence in CF is an unmet clinical need.

Polymeric mucins are major determinants of the properties of airway mucus, which is comprised of a mixture of MUC5AC and MUC5B glycoproteins (1). Their amounts, size, chemical nature and macromolecular organisation are likely to profoundly effect mucus properties. However, our current understanding of these aspects in CF is incomplete and requires further investigation for a better understanding of mucus barrier function. The current thinking is that mucins change their macromolecular form (or mature) from a cross-linked form in the secretory granule to a linear, expanded form upon release into mucus; this transition being essential for the formation of 'normal' mucus (2). The mechanism that controls this transition is not known. Reduced bicarbonate secretion, as a result of a defective CFTR, may impact upon efficient mucin maturation (3), however the precise role bicarbonate plays is not clear. After secretion bicarbonate is suggested to sequester Ca2+ away from the mucin glycans facilitating efficient mucin-expansion (4). Alternatively, bicarbonate maybe important in maintaining the 'correct' pH for a protease(s) to 'process' the mucins and facilitate their efficient release from the granule and subsequent extracellular expansion. Recent studies by Hansson and colleagues have shed further light on how the intestinal mucin MUC2 matures once released from the secretory granule. Using recombinant MUC2, they showed that the N-terminus of MUC2 forms trimers, which form ring like structures at the low pH and high Ca2+ environment inside the secretory granule. Post-secretion MUC2 expands due to the extracellular increase in pH and decrease in Ca2+ concentration (5). Importantly, whether this happens for airways mucins is yet unknown.

We have investigated (i) the effect of Ca2+ and pH on the structure and homotypic interactions of recombinant MUCB N-terminal domain (NT-MUC5B) and, (ii) attempted to identify potential protease cleavages that may accompany airways mucin maturation. To elucidate the role of Ca2+ and pH in the homotypic interactions of NT-MUC5B we have used sedimentation analyses and multi angle laser light scattering. This has shown that NT-MUC5B forms dimers that in the presence of Ca2+ can form pH-dependent multimers. To gain further insight into the structure of the assemblies of NT-MUC5B we are performing 3D-transmission electron microscopy. To identify potential cleavage sites that accompany MUC5AC maturation we enriched in freshly secreted mucins by using agonist-stimulated stimulation of mucin-producing cells in culture. Using a non-biased LC-mass spectrometry analysis we compared tryptic peptides generated from freshly secreted mucins with mature mucins obtained from the same cultures. Some peptides were found more often in the mature mucins; potentially these could arise from processing of the MUC5AC polypeptide. We do not yet know how this might relate to mucin maturation.

1.Kirkham et al., (2002) Biochem J 361, 537-546.
2. Kesimer et al., (2010) Am J Physiol Lung Cell Mol Physiol 298, L15-L22.
3. Quinton, (2008) Lancet 372, 415-417.
4. Chen et al., (2010) Am J Physiol Lung Cell Mol Physiol 299, L542-549.
5. Ambort et al., (2012) PNAS, 109, 5645-5650.

This work was supported by the Cystic Fibrosis Foundation Therapeutics, Inc. (Mucociliary Clearance Consortium) and the MRC.

S7.2 – Bicarbonate, a functional CFTR channel, and enzymatic activities are required for proper mucin secretion and link Cystic Fibrosis to its mucus phenotype

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Department of Medical Biochemistry, University of Gothenburg, Box 440, 405 30 Gothenburg, SWEDEN

Cystic Fibrosis (CF) is caused by a non-functional chloride and bicarbonate ion channel (CFTR), but the link to the phenomenon of stagnant mucus is not well understood. Part of the reason for this has been the poor understanding of how the mucins are stored and released. Recent understanding have changed this and will be discussed together with a comparision of different mucins. Mice lacking functional CFTR (CftrΔ508) have no lung phenotype, but show similar ileal problems as humans. The ileal mucosa in CF mice have a mucus that was adherent to the epithelium, was denser, and was less penetrable than that of wild-type mice. The release require endogenous protease activities. The properties of the ileal mucus of CF mice were normalized by secretion into a high concentration sodium bicarbonate buffer (about 100 mM) or hypertonic NaCl solution. In addition, bicarbonate added to already formed CF mucus almost completely restored the mucus properties. This information from the small intestine described here will have be discussed in relation to lung CF mucus.

S7.3 – Genesis of Thick Mucus in Cystic Fibrosis Airways

Stephen T. Ballard

Department of Physiology, College of Medicine, University of South Alabama, Mobile, Alabama, USA

A prominent feature of cystic fibrosis (CF) lung disease is the production of an abnormally thick airway mucus. To better understand the mechanism of this pathogenic process, we measured fluid and biomolecule secretion from intact bronchi harvested from the lungs of normal domestic swine as well as from human CF and non-CF lungs removed from patients undergoing transplantation. Excised airways were cannulated and bathed in physiologic salt solution. Bronchi were treated with various combinations of secretagogues and ion transport inhibitors to produce a range of mucous liquid secretion rates. Secreted mucous liquid was collected from the airway lumena, weighed to determine the wet weight and volume, then evaporated to dryness to determine the dry weight. Airways from all three groups (pig, non-CF human and CF human) secreted mucus with very high percent solids (>10% solids) at the lowest rates of mucous liquid secretion whereas a much lower percent solids mucus was produced at the highest mucous liquid secretion rates. The relationship between percent solids and mucous liquid secretion rate in all three groups was curvilinear due to differing linear slopes between solids mass and water mass secretion rate as functions of mucous liquid secretion rate. Model equations derived from these data indicate that normal pig, non-CF human, and CF human bronchi secrete mucus with 2%, 4%, and 8% solids, respectively, at maximum mucous liquid secretion rates. When biomolecular mass was estimated from the total non-volatile solids mass by subtracting the contribution of inorganic salts mass, the slope of the biomolecular mass secretion rate to the total mucous liquid secretion rate was found to be 3.8-fold greater in the CF bronchi than the non-CF bronchi. In pig bronchi, there was no correlation between the biomolecular mass secretion rate and the total mucous liquid secretion rate. These results indicate that the elevated percent non-volatile solids present in CF mucus are largely the consequence of a substantially higher biomolecule secretion rate in CF airways compared to non-CF human airways. We speculate that this higher biomolecular mass secretion rate in CF is attributable to the well-documented mucous and goblet cell hyperplasia that occurs in CF airways.

S7.4 – Modification of the Salivary Secretion Assay in F508del Mice - Salivary Chloride Quantification

Karoline Droebner[1], Qian Mao[1], Peter Sandner[1,2]

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In 2004 Best and Quinton[1] established the salivary secretion assay in mice for the *in vivo* characterization of new drugs against Cystic Fibrosis (CF). However, limited *in vivo* data are available and the predictive value of this assay for treatment effects in CF patients is not fully established.Therefore, we revisited the salivary secretion assay and systematically investigated the salivary secretion rates in different CF mouse models, namely in CFTR knockout mice (CFTR ^{tm1Unc}) as well as in the F508del CFTR mice of different origin (CFTR^{tm1Kth} and CFTR^{tm1Eur}). In addition, we quantified salivary chloride content in these mice since in CF-patients, increased sweat chloride is an established diagnostic tool.

The β-adrenergic salivary secretion response in the presence of Atropine was significantly reduced compared to WT mice. Salivary secretion rates of CF CFTR^{tm1Kth} and CF CFTR^{tm1Eur} mice were reduced 13-fold and 10-fold respectively when compared to WT mice. Salivary rates of CF CFTR^{tm1Unc} mice were 30-fold reduced. Saliva was collected after Pilocarpine stimulation and chloride levels were quantified using a colometric Chloride determination assay. As sweat chloride content in CF-patients, saliva chloride was significantly elevated in CF mouse models. The chloride concentrations were 2.1-fold higher in CF CFTR^{tm1Unc}, 1.8-fold in CF CFTR^{tm1Kth} and 3.3-fold in CF CFTR^{tm1Eur} when compared to WT mice.

Salivary secretion rates and salivary chloride content in CF-mice reflect sweat secretion and sweat chloride content in CF patients. Determination of salivary secretion rates, extended by quantification of salivary chloride content in different CF-mice renders the salivary secretion assay a powerful tool for validation of new CF-treatments.

[1] Best JA, Quinton PM. Salivary secretion assay for drug efficacy for cystic fibrosis in mice. Exp Physiol.2005; 90(2):189-193.

S7.5 – Treatment with VX-809 and VX-770 in Rectal CF Organoids Expressing Uncommon CFTR Genotypes

Evelien Kruisselbrink[1,2,3], Florijn Dekkers[1,2,3], Karin M de Winter-de Groot[1], H.M. Janssens[4], C.K. van der Ent[1], Jeffrey Beekman[1,2,3]

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Background: The potentiator VX-770 (Ivacaftor, Kalydeco) has recently been registered as a drug for cystic fibrosis (CF) patients with a CFTR-G551D mutation. Furthermore, VX-770 treatment in combination with the corrector VX-809 is now being assessed in a phase II clinical trial for patients harboring CFTR-F508del.
Aims: To demonstrate that these CFTR-targeting drugs may also be used to treat patients with CFTR alleles that have not been included in these initial clinical trials.
Methods: We recently developed a quantitative assay to measure CFTR function in primary rectal organoids. This assay can differentiate between patients containing 'mild' and 'severe' CFTR mutations and can detect CFTR restoration by VX-809 or VX-770.
Results: We measured CFTR activity at basal level or upon CFTR restoration by VX-809 and VX-770 in organoids expressing various CFTR mutations, including the most common mutant F508del, and the less common mutants A455E, R117H, (TG)13(T)5 and L997F. We observed a dose-dependent relation between CFTR function and forskolin treatment, which differed between organoids expressing distinct CFTR alleles. Furthermore, VX-770 treatment restored CFTR function in all CF organoids, while VX-809 treatment was effective for specific mutations.
Conclusions: CFTR genotype-specific responses to VX-809 and VX-770 treatment can be detected using organoid-based CFTR measurements. This assay may identify patients who may benefit from CFTR-targeting drugs.

7.6 - SLC26A9-mediated Cl^- Secretion Is Induced In Airway Inflammation and Prevents Mucus Obstruction in Mice

Julia Duerr[1], Brigitte Riederer[2], Pinelopi Anagnostopoulou[1], Sven Michel[3], Aristea Binia[3], Raman Agrawal[1], Xuemei Liu[2], Katrin Kalitzki[2], Fang Xiao[2], Mingmin Chen[2], Jolanthe Schatterny[1], Dorothee Hartmann[4], Thomas Thum[4], Michael Kabesch[3], Manoocher Soleimani[5], Ursula Seidler[2], Marcus A Mall[1,6]

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Recent studies demonstrated that airway inflammation produces a pro-secretory airway epithelial ion transport phenotype characterized by upregulation of Cl^- secretion and inhibition of Na^+ absorption in mouse airways, and have identified active modulation of airway ion transport as an additional epithelial response in the complex in vivo pathogenesis of asthma (Anagnostopoulou P. et al. Eur J Respir 2010; 36:1436-1447). However, the molecular mechanism underlying Cl^- secretion and its relevance in asthma pathophysiology remain unknown. Recently, the solute carrier family 26, member 9 (SLC26A9) Cl^- channel has been shown to contribute to constitutive and cAMP-dependent Cl^- secretion in human bronchial epithelial (HBE) cells, where it has been suggested to interact functionally with CFTR in vitro (Bertrand CA *et al*. *J Gen Physiol*. 2009;133:421-438, Avella M. *et al*. *J Cell Physiol*. 2011;226:212-223). Based on its functional properties in transduced cells and expression pattern in human and mouse airways (Lohi H *et al*. *J Biol Chem*. 2002;277:14246-14254, Chang MH *et al*. *J Membr Biol*. 2009; 228(3):125-140), we hypothesized that SLC26A9 may function as an alternative Cl^- channel that may contribute to ASL homeostasis in health and inflammatory airway disease. To determine the role of the SLC26A9 Cl^- channel in airway inflammation, we compared transepithelial ion transport in freshly excised bronchial tissues, lung morphology, and airway mucus content in wild-type versus *Slc26a9*-deficient (*Slc26a9*$^{-/-}$) mice under physiological conditions and after intratracheal instillation of IL-13 to model Th2-mediated airway inflammation. Further, in a large human study population of 658 healthy children and 661 children with asthma, we tested whether polymorphisms in the *SLC26A9* gene are associated with asthma and determined the functional relevance of a select SNP (rs2282430) in the 3′ UTR of *SLC26A9* in luciferase gene reporter assays.

We found that IL-13 treatment increased Cl^- secretion in the airways of wild-type but not *Slc26a9*$^{-/-}$ mice. While IL-13-induced mucus overproduction was similar in both strains, treated *Slc26a9*$^{-/-}$ mice exhibited airway mucus obstruction, which did not occur in wild-type controls. Moreover, in silico analyses predicted that the A allele of rs2282430 (SNP) strengthens binding of *hsa-miR-632* to the *SLC26A9* 3′ UTR causing reduced protein expression in vitro providing initial evidence that *SLC26A9* may be involved in the pathogenesis of obstructive lung disease in humans.

Our data demonstrate that the SLC26A9 Cl^- channel is activated in airway inflammation and suggest that SLC26A9-mediated Cl^- secretion is essential for preventing airway obstruction in inflammatory airway disease. These results suggest SLC26A9 as a potential disease modifier and novel therapeutic target in airway diseases caused by increased mucus secretion and deficient hydration of airway surfaces, such as CF.

Saturday 23 March – 11:00-12:30

SPECIAL GROUP DISCUSSION II
Correctors / Potentiators / New Treatments

Moderators: Christine Bear (CA) / John Hanrahan (CA)

This Discussion will cover three topics, with short introduction talks that are intended to promote active general discussion by the audience. The first topic will focus on the search for new correctors using new methodologies stemming from recent progress in our understanding CFTR folding and its dependence on both NBD1 stability and domain interactions. This will be followed by a discussion of potentiators and the mechanism of action VX770, focusing on the role of nucleotides and phosphorylation. We will conclude with a discussion of novel in-vitro and/or in-vivo assays of CFTR modulators.

Saturday 23 March – 11:00-12:30

WORKSHOP - II
Imaging – Microscopy
Moderators: Peter Haggie (US) / Rob Tarran (US)

Saturday 23 March – 14:00-15:45

SYMPOSIUM 8
CFTR Structure and Function
Chairs: Oscar Moran (IT) / Robert Ford (UK)

S8.1 - Chimeric constructs endow the human CFTR Cl^- channel with the sub-conductance state behaviour of murine CFTR

Zhiwei Cai[1],Isabelle Callebaut[2],David Lea-Smith[3],Barbara J. Stevenson[3],Ann Doherty[3],Heather Davidson[3],David J. Porteous[3],Jean-Paul Mornon[2], Pierre Lehn[4],A. Christopher Boyd[3] and David N. Sheppard[1]

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The single-channel properties of murine CFTR exhibit two important differences from human CFTR. First, murine CFTR has a reduced single-channel conductance (human, 8.29 ± 0.18 pS; murine, 5.76 ± 0.16 pS; [1]). Second, the gating pattern of human CFTR is characterised by bursts of channel openings interrupted by brief flickery closures, separated by longer closures between bursts; transitions to sub-conductance states are rare for human CFTR. By contrast, murine CFTR resides for prolonged periods in a sub-conductance state and only sojourns briefly to the full open-state. As a result, the open probability of the full open state of murine CFTR is greatly reduced, whereas that of the sub-conductance state is elevated compared to human CFTR [2].

To begin to identify the protein regions responsible for the differences in single-channel behaviour between human and murine CFTR, we constructed human-murine CFTR (hmCFTR) chimeras with sequences from nucleotide-binding domain 1 (NBD1), NBD2 or the regulatory domain (RD) of human CFTR replaced by the equivalent regions of murine CFTR. Transfer of both NBDs of murine CFTR endowed human CFTR with dramatically prolonged channel openings similar to those of the sub-conductance state of murine CFTR [3]. However, transfer of both NBDs failed to endow human CFTR with a sub-conductance state like that of murine CFTR [3].

To investigate whether the membrane-spanning domains (MSDs) define the sub-conductance state of murine CFTR, we constructed the hmCFTR chimeras hmTM1-6, hmTM7-12 and hmTM1-6:TM7-12 containing MSD1, MSD2 and MSD1+2 of murine CFTR and studied their single-channel behaviour in excised inside-out membrane patches. All hmCFTR chimeras exhibited a hybrid gating pattern intermediate between that of human and murine CFTR. Reminiscent of murine CFTR, channel openings were very flickery with open probability decreasing in the rank order: human CFTR > hmTM7-12 > hmTM1-6 > hmTM1-6:TM7-12. The single-channel current amplitude of the full open state of hmCFTR chimeras was smaller than that of human CFTR, decreasing in the rank order: human CFTR > hmTM7-12 > hmTM1-6 ≥ hmTM1-6:TM7-12. Like human CFTR, hmTM7-12 opened predominantly to the full open state; transitions to sub-conductance states were unusual. However, hmTM1-6 resided in multiple sub-conductance states, which were visualised in heavily filtered single-channel recordings and current amplitude histograms. Of note, like murine CFTR, hmTM1-6:TM7-12 resided in a tiny sub-conductance state and only sojourned infrequently to the full open-state. Consistent with hmTM1-6:TM7-12 possessing human NBDs, this construct resided in the sub-conductance state for much shorter periods than murine CFTR. Taken together, our data suggest that amino acid sequences from both MSDs specify the sub-conductance state of murine CFTR and determine intraburst gating.

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S8.2 - Insight into the structure, function and energetics of CFTR and its domains gained from atomic-level molecular simulations

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Cystic Fibrosis (CF) is a lethal, incurable genetic disease caused by mutations to the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) chloride channel. The most common CF causing mutation, occurring in >90% of CF patients, is the deletion of F508 from the first Nucleotide Binding Domain (F508del-NBD1). This mutation leads to a thermally unstable domain and a misfolded, non-functioning CFTR.

Developing an effective treatment for CF by targeting the underlying defect is highly important to the CF community. Rational drug discovery could be greatly assisted by the high resolution structural, functional and energetic characterization of full length CFTR and its domains and by a thorough understanding of the effects brought about by "perturbations" to the native structure introduced by mutations and by the binding of CFTR modulators. Due to the limited solubility and in vitro stability of most of the CFTR domains these studies require the design of new constructs. Such design and characterization efforts could be greatly assisted by atomic-level molecular simulations.

In this work we describe a series of computational studies demonstrating the ability of molecular simulations to direct design efforts of constructs of CFTR and its domains and to probe the structural/energetic consequences of mutations. In particular, these simulations highlight the importance of analyzing the dynamics of CFTR domains (as opposed to studying their static crystal structures) in order to gain insight into their thermal stabilities and the mode of action of both deleterious and stabilizing mutations. In particular, dynamic profiles (expressed in terms of residue fluctuations and termed RMSF profiles) of wt and F508del-NBD1 are shown to be higher for the mutant protein in accord with its lower thermal stability. This correlation persists upon introducing the V510D and 3M (Teem) stabilizing mutations. A comparative analysis of RMSF profiles is revealing in terms of the mechanism by which F508del destabilizes NBD1, renders it aggregation prone, reduces its binding affinity to ATP and impairs its interaction with ICL4 and also in terms of how these effects are counteracted by the V510D and Teem mutations. Moreover, RMSF profiles reveal a complex allosteric network extending over the entire protein that couples regions which are both proximate to and distant from the sites of mutations.

RMSF profiles are also shown to be sensitive to domain conformations and consequently could be used in the design of CFTR domains with alternative conformations (e.g., a dimer-compatible NBD2). Newly designed, better behaved constructs could be used for high throughput screening of CFTR modulators and as stepping-stone for the crystallization of the full length protein.

Finally, we present a new CFTR model optimized under constraints derived from electron microscopy data.

S8.3 - P-glycoprotein, CFTR, and domain unfolding/stability studies: what can we learn about membrane protein structural responses to ligand binding and interdomain cooperativity?

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The cause of disease in most CF patients is an in-frame deletion of phenylalanine (F508del) located in the cystic fibrosis transmembrane conductance regulator (CFTR). This mutation results in the misfolding and degradation of the chloride ion channel before it reaches the plasma membrane. F508del is located in the first nucleotide binding domain, NBD1, and we have shown in studies on the isolated domain that F508del-NBD1 is less stable, both thermodynamically and kinetically, substantially destabilizing the native state and accelerating the formation of an aggregation-prone, unfolding intermediate [1,2]. The greater propensity for F508del-NBD1 to unfold and aggregate is consistent with the instability of cellular F508del-CFTR. These studies exemplify the utility of differential scanning calorimetry (DSC) and of biophysical studies on CFTR domains to shed light on the physical properties of CFTR. DSC is a unique technique that directly measures the heat (enthalpy) of unfolding; it can provide all unfolding thermodynamic parameters without the assumption of an unfolding pathway, unlike all other methods. In fact, the unfolding pathway can be obtained from mathematical fitting of the DSC heat capacity profile [1]. We have built on these previous studies and show here how DSC can also contribute to our understanding of the structural adaptation of CFTR to its solution environment, and to the binding of small molecule modulators. Inferences are drawn from studies on domain constructs and P-glycoprotein, another member of the ABC transporter superfamily and a close structural homolog of CFTR. In particular, anionic and zwitterionic detergents are shown to significantly destabilize NBD1 and P-glycoprotein; addition of lipids mitigate these effects and increase the structural cooperativity of P-glycoprotein. DSC has also contributed to our understanding of the mode of action of CFTR modulator compounds and details will be provided.

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S8.4 - Generation and Characterization of The G551D Mutation in CFTR Expressed in *S. cerevisiae*

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G551D is the second most frequent cystic fibrosis disease-causing mutation and is the target mutation for the new CFTR potentiator VX-770 (Kalydeco). Recently this drug has been approved in the UK for patients with this mutation who are >6 yrs old. Study of CFTR with the G551D mutation is desirable in order to understand the mode of action of VX-770 and where it binds. The mutation is also useful as a negative control in assays of CFTR ion flux. Lastly, the mutation may lock the CFTR protein in a closed state, which might be advantageous for biophysical and structural studies and may enhance its properties for crystallization.
We have generated the G551D mutation in human CFTR expressed in the yeast *S. cerevisiae*. The construct also has a C-terminal GFP tag allowing the cellular location and its expression levels to be monitored. Expression in yeast is also enhanced by the addition of small solutes to the induction media, and we have found that G551D CFTR expresses at levels similar to wt CFTR and with minimal proteolytic degradation. We will present data on the generation of the construct in the human wt CFTR background and on its cellular localization in yeast. We will also present data on the physical properties of the protein (solubility; monodispersity by FSEC; thermal stability by Coumarin maleimide binding) and the effect that VX-770 binding has on these properties.

S8.5 - Purification and Biophysical Characterization of Full-length CFTR Expressed in *Saccharomyces cerevisiae*

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Cystic fibrosis is the most common genetic disorder in the Caucasian population and over 1000 different deleterious mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) have been associated with the disease. There is great medical interest to develop drugs targeted at this ion channel, especially correctors that restore the folding of the CFTR protein with the most common mutation (F508deletion). However, characterisation of full-length wild-type and the F508del CFTR protein has been severely limited by the lack of sufficient quantities of purified protein. This limitation has recently been addressed by the Cystic Fibrosis Foundation (USA) which has formed an international consortium - the CFTR 3D Structure Consortium - to generate methods for CFTR production and crystallization. Reagents such as the murine wt CFTR - expressing yeast generated by our laboratory are now freely available via this consortium.
Here, we describe methodology to overexpress and purify milligram quantities of several full-length CFTR orthologs including F508del CFTR using our *Saccharomyces cerevisiae* (yeast) expression system. CFTR orthologs were solubilised from yeast microsomes using the detergent dodecylmaltoside (DDM) and purified in the same detergent by nickel affinity and size exclusion chromatography to yield 1-2 mg of CFTR protein per 15 litre fermenter culture. CFTR can also be solubilised and purified in the harsher detergent lyso-phosphatidyl glycerol (LPG), but displays no activity in this detergent. A green fluorescent protein (GFP) tag fused to the CFTR C-terminus allows rapid detection of the protein throughout the purification procedure. Purified CFTR was analysed by dynamic light scattering - size exclusion chromatography, mass spectrometry and electron microscopy to assess protein homogeneity. The thermal stability of purified CFTR was probed using tryptophan fluorescence and Coumarin maleimide dye binding assays, and an unfolding transition between 40-50°C was observed for the DDM-purified material. No clear transition was detected for the LPG-purified protein. Mass spectrometry of the DDM-purified CFTR revealed potential CFTR-interacting proteins that appear to remain associated with a small fraction of the CFTR protein molecules during the purification process. Comparisons with the mass spectrometry data for LPG-purified CFTR were consistent with the above interpretation, that LPG at least partially denatures proteins including CFTR. Structural characterization of the DDM- and LPG-purified CFTR material has been carried out by single-particle electron microscopy, showing a monomeric and highly monodisperse LPG-purified CFTR whilst a dimeric and less homogeneous population of particles was observed for the DDM-purified material. Preliminary electron crystallographic analysis of two-dimensional crystals of the chicken CFTR ortholog will be presented. The protein crystallizes in the same two-dimensional plane group and with roughly the same lattice parameters as the previously-reported human CFTR material expressed in BHK cells.

S8.6 - ICL1 Facilitates the Conformational Maturation of CFTR Through Interdomain Interfaces with NBD1 and ICL3

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CFTR is a plasma membrane phosphoglycoprotein, belonging to the ATP-Binding Cassette (ABC) transporter superfamily. CFTR functions as an ATP- and PKA-dependent chloride channel, regulating chloride flux across apical membranes of polarized epithelial cells. Mutations in CFTR cause Cystic Fibrosis; the major mutation, F508del, is located in the first nucleotide binding domain (NBD1). There are multiple conformational defects caused by F508del including disruption of the interaction between NBD1 and the fourth intracellular loop (ICL4) (Loo *et al.*, 2008; Serohijos *et al.*, 2008). Current small molecule 'correctors' lead to partial rescue of the processing defect with some functional (but inadequate) expression at the cell surface, and although the mechanism of action is poorly understood, the NBD1:ICL4 interface is likely repaired to some extent. Further progress in understanding the molecular defects caused by F508del and the development of more effective correctors is limited by the lack of understanding regarding the interdomain interactions necessary for assembly of the full-length protein.

Functionally important interdomain interfaces include: NBD1:ICL4, NBD1:2, ICL3:Regulatory-domain, and membrane spanning domain (MSD) 1:2. Identification of other functionally relevant interfaces is desired. Upon inspection of homology models of CFTR, previously unexplored interfaces (ICL1:2, ICL1:3, ICL1:NBD1) were predicted to play important roles in biosynthesis and gating (Serohijos *et al.*, 2008; Mornon *et al.*, 2009; Dalton *et al.*, 2012). Further analysis identified a cluster of hydrophobic residues in the ICLs that may be important. These residues include: Ile^{177} (ICL1), Val^{181} (ICL1), Leu^{259} (ICL2), Val^{260} (ICL2), Leu^{973} (ICL3) and Val^{1056} (ICL4). Individually mutating Ile^{177} and Leu^{259}, two residues which may form the closest interface, did not affect maturation, but the double substitution of $Ile^{177}Ala/Leu^{259}Ala$ decreased the rate of channel activation by 40%, presumably by altering the ICL1:2 interface, as measured by cell-based iodide efflux assays. Substitution of the Leu^{973} (ICL3) with disease-causing Pro, but not disease-causing Phe or His, affected processing, suggesting that a conserved residue is required at this position for proper assembly and maintenance of helical structure. Interestingly, disruption of the putative ICL1:3 interface by double Ala-substitution of Ile^{177} (ICL1) and Leu^{973} (ICL3) created a mutant protein that was EndoglycosidaseH-sensitive, suggesting that it is synthesized and core-, but not complex-glycosylated at steady-state; therefore this interface is important for maturation.

Subsequently, the putative ICL1:NBD1 interface was disrupted using mutagenesis, and resulted in misprocessing of CFTR; mutations include S169R (ICL1), R170G (ICL1) and W401G (NBD1). This suggests that CFTR models are accurate in this region, and may further support the idea that ICL1 serves as an intramolecular scaffold, allowing for the initial coordinated assembly of NBD1 and possibly MSD2 (via ICL3) during CFTR biosynthesis. By understanding how ICLs transmit signals between the MSDs and NBDs via interdomain interfaces, gating mechanisms will become more apparent, and could allow for the design of therapeutics to treat Cystic Fibrosis. Elucidation of such molecular events would contribute not only to our understanding of CFTR activity, but also to our understanding of channels and transporters in general.

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Saturday 23 March – 16:15-18:00

SYMPOSIUM 9
CFTR and COPD / Smoking
Chairs: Hugo De Jonge (NL) / Gerry McElvaney (IE)

S9.1 - Cigarette Smoke Exposure and the Failure of Airway Surface Liquid Homeostasis

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Chronic obstructive pulmonary disease (COPD) affects over 60 million people world-wide and includes both emphysema and chronic bronchitis (CB). One of the primary causes of COPD is cigarette smoke exposure, which is known to induce trigger a protease/anti-protease imbalance, inflammation and airway remodeling leading to severe lung damage. Whilst cigarette smoke initiates many changes genomically, little is known about the direct effects that cigarette smoke may exert at the protein level. Cigarette smoke exposure has previously been shown to inhibit CFTR, which may contribute to mucus dehydration and a failure of mucus clearance seen in COPD/CB. We therefore tested the hypothesis that cigarette smoke could exert direct effects on the epithelia which led to altered trafficking of the CFTR protein which would impinge upon airways hydration. In vivo and in vitro studies demonstrated that cigarette smoke rapidly abrogated CFTR activity, which correlated with mucus dehydration and a failure of mucus clearance in vivo and lead to airway surface liquid dehydration in vitro. Further studies revealed that cigarette smoke exposure induced rapid internalization of apical membrane CFTR. Cigarette smoke -internalized CFTR did not colocalize with lysosomal proteins. Instead, the bulk of CFTR shifted to a detergent resistant fraction of the cell and co-localized with the intermediate filament vimentin, suggesting that cigarette smoke induced CFTR's movement into an aggresome-like, peri-nuclear compartment. Recent studies in our lab have focused on the upstream signaling events that trigger CFTR internalization. These studies suggest that altered CFTR trafficking occurs in response to specific cellular events. However, whether this is a protective or pathological response to cigarette smoke exposure remains to be determined. In conclusion, we propose that cigarette smoke exposure rapidly inhibits CFTR function by internalizing CFTR, leading to dehydration of airway surfaces. This airway dehydration is predicted to promote mucus stasis and a failure of mucus clearance, leaving smokers at risk of developing airway infections and CB. Furthermore, our data suggest that CB may share a similar etiology as CF, namely the lack of CFTR. In CF, this loss is genetic and can lead to a complete absence of functional CFTR and rapid onset on lung disease. In contrast, in CB, the lack of CFTR is due to the environment (e.g. cigarette smoke exposure) and is more moderate with approximately 50% of CFTR being lost. In the case of CB, onset of the disease is relatively slower. Our data also suggest that strategies aimed at rehydrating CB mucus may provide novel treatments for this disease.

S9.2 - Acquired CFTR Dysfunction in Chronic Obstructive Pulmonary Disease

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University of Alabama at Birmingham

Cystic fibrosis transmembrane conductance regulator (CFTR) plays an essential role in the function of epithelial organs, including the lungs, pancreas, vas deferens, and intestine. Like CF, COPD is characterized by small airway mucus obstruction and is associated with mucus stasis, bacterial infection, airways obstruction, and accelerated loss of lung function. Smoking is also associated with non-respiratory disorders including pancreatitis, male infertility, and cachexia, features characteristic of CF and suggestive of an etiological role for CFTR. Recent data from our laboratory and a number of others indicate that cigarette smoke exhibits deleterious effects on airway epithelial function in vitro and in vivo, including the reduction of CFTR activity and a pronounced decrement in mucociliary transport (MCT) caused by cigarette smoke in vitro. We have also shown that over 40% of patients with COPD have reduced CFTR activity, as detected in the upper airways, lower airways and sweat glands. Exposure of mice to cigarette smoke recapitulates CFTR dysfunction observed in smokers, including that observed in the non-respiratory organs, and was confirmed by intestinal current measurements in human smokers. Furthermore, CFTR dysfunction identified in the nose, lung, and sweat gland are independently associated with chronic bronchitis and COPD severity and can persist despite smoking cessation. Acquired CFTR dysfunction can be reversed by the CFTR potentiator ivacaftor (VX-770) in vitro by activating wild-type CFTR, resulting in a robust increase in mucociliary transport. Combined with unprecedented clinical improvement via augmented mucociliary clearance in CF patients with a responsive CFTR mutation treated with ivacaftor, these data indicate that CFTR represents a viable therapeutic target to address mucus stasis in COPD, an important cause of morbidity and independently associated with COPD progression and mortality. Our data suggest that acquired deficits in CFTR activity may also contribute to extra-pulmonary disorders related to CFTR dysfunction observed more commonly in smokers (e.g. pancreatitis and male infertility) which may also represent potential therapeutic opportunities.

S9.3 CFTR modulates onset and severity of chronic obstructive lung disease in ENaC-overexpressing mice

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Factors determining the onset and severity of chronic obstructive lung disease in CF and COPD remain poorly understood. In previous studies, we demonstrated that airway surface dehydration in mice overexpressing the beta-subunit of the epithelial Na+ channel ENaC (beta-ENaC-Tg) on a mixed genetic background causes either neonatal mortality due to severe airway mucus obstruction, or chronic obstructive lung disease with chronic bronchitis and emphysema. This observation suggested that the onset and severity of obstructive lung disease in this model is modulated by the genetic background. To test this hypothesis, we backcrossed beta-ENaC-Tg mice onto two different inbred mouse strains (C57BL/6 and BALB/c) and studied effects of the genetic background on neonatal mortality, airway ion transport, airway mucus obstruction and inflammation. In independent experiments, we further crossed beta-ENaC-Tg mice with CFTR-deficient mice to determine the role of CFTR as a modulator of disease severity in vivo. The results of these studies showed that the C57BL/6 background has no effect on ENaC-mediated Na+ hyperabsorption, but confers increased CFTR-mediated secretion. Higher levels of CFTR-mediated Cl^- secretion were associated with less severe airway mucus obstruction and reuced mortality in neonatal beta-ENaC-Tg mice on the C57BL/6 background compared to beta-ENaC-Tg mice on the BALB/c background. Conversely, genetic deletion of CFTR increased the severity of early airway mucus obstruction and mortality in beta-ENaC-Tg mice. We conclude that a decrease or absence of CFTR function in airway epithelia in vivo aggravates the severity of early airway mucus obstruction and pulmonary mortality in beta-ENaC-Tg mice. These results suggest that genetic or environmental factors (e.g. cigarette smoke) that reduce CFTR function may contribute to the onset and severity of chronic obstructive pulmonary disease and that CFTR may serve as a novel therapeutic target.

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S9.4 - SLC6A14 Enhances Constitutive CFTR-Mediated Cl^- Secretion in Human Non-CF and CF Primary Bronchial Epithelia

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Background: Genome wide association studies for gene modifiers in CF have recently identified *SLC6A14* as modifier gene for meconium ileus (Sun 2012). *SLC6A14* encodes for a Na^+/Cl^- dependent basic and neutral amino acid transporter (stoichiometry of 2Na+1Cl+1 amino acid). Functional expression of SLC6A14 ($B^{0,+}$) as transporter in human airway has been reported (Sloan 2003, Galietta 1998).
Objective: To investigate whether SLC6A14 activation impacts CFTR-mediated Cl^- currents in human airway epithelia.
Method: Primary human bronchial epithelial cells from CF (CFBE) and non-CF subjects (NBE) were obtained from lung transplants (Drs. Keshavjee and Zabner, University of Toronto and Iowa). SLC6A14 and CFTR-mediated ion transport were recorded as the transepithelial potential difference in Ussing chamber studies (ΔV_{te}, ΔR_{te} , calculated ΔI_{eq}). L-arginine (Arg) and L-lysine (Lys) were used as substrates and α-methyltryptophan (αMT, Karunakaran 2008) as inhibitor for SLC6A14; Forskolin (Fsk)/IBMX as well as $CFTR_{inh-172}$ were used to stimulate and inhibit CFTR-mediated conductance respectively.
Results: RT-PCR analysis and electrophysiological measurements demonstrate functional SLC6A14 expression in NBE and CFBE cells. Apical Arg activated SLC6A14 inducing a sustained depolarization in NBE (ΔI_{eq} = -1.6±-1.0 μA/cm^2, n=25). The Arg- induced ΔI_{eq} was competitively inhibited by Lys and α-MT confirming $B^{0,+}$ activity (p< 0.05, n=4). The SLC6A14 induced I_{eq} was not different in CF cells (ΔI_{eq} = -1.5±-2.2 μA/cm^2, p=0.2, n=8) and unaffected by CFTR activation (ΔI_{eq} = -1.4±-0.9 μA/cm^2, p= 0.3, n=25). Fsk/IBMX stimulated CFTR-mediated currents were not altered by activation of SLC6A14 in NBE ($\Delta I_{eq\ cAMP}$ =-2.7±-2.6 μA/cm^2 versus -3.0±-3.0 μA/cm^2 +/- SLC6A14 activation, p=0.41, n=25). In NBE cells, the $CFTR_{inh-172}$ sensitive I_{eq} ($\Delta I_{eq\ CFTRinh-172}$ =6.1±4.7 μA/cm^2) exceeded the Fsk/IBMX inducible I_{eq} (p< 0.0001), likely reflecting large constitutive CFTR activity. Therefore, we used the $CFTR_{inh-172}$ sensitive I_{eq} as read-out of CFTR-mediated I_{eq} , expressed as fraction of the total epithelial anion conductance (% of $\Delta I_{eq\ totalG}$). Following this analysis, SLC6A14 activation caused a significant increase in the $CFTR_{inh-172}$ sensitive I_{eq} (68±19% compared to 52±13% of $\Delta I_{eq\ total\ G}$ +/- SLC6A14, p< 0.0001, n=25). Similarly, use of Lys as SLC6A14 substrate increased the $CFTR_{inh-172}$ sensitive I_{eq} (60±12% versus 47±17% of $\Delta I_{eq\ total\ G}$ +/- SLC6A14, p=0.03, n=7) suggesting that SLC6A14 expression and not Arg influx *per se* is responsible for the effect on CFTR-mediated currents. In CFBE cells, we observed very small $CFTR_{inh-172}$ sensitive I_{eq} ($\Delta I_{eq\ CFTRinh-172}$ = 0.4±0.41 μA/cm^2), which were nevertheless further enhanced upon activation of SLC6A14 with Arg ($\Delta I_{eq\ CFTRinh-172}$ = 0.9±0.7 μA/cm^2, p< 0.05, n=8).
Conclusion: SLC6A14 is functionally expressed in non-CF and CF primary human bronchial epithelia. Activation of SLC6A14 increases constitutive CFTR-mediated I_{eq} measured as $CFTR_{inh-172}$ sensitive current in non-CF. Small constitutive CFTR-mediated I_{eq} were also detected in CF airway cells. These seemed to be enhanced with SLC6A14 activation. Current studies are performed to examine whether Arg-mediated NO- or AMPK-signaling may play a role in the observed interaction between SLC6A14 and constitutive CFTR activity, and/or whether the SLC6A14 effect on CFTR-mediated currents is induced by altering the electrochemical driving force for CFTR (e.g. re-cycling chloride at the apical membrane).

S9.5 - Small Molecule Inhibitors of GSNOR Possess Anti-inflammatory and Bronchodilatory Actions in Mouse Models of Inflammatory Lung Disease and Modulate CFTR Function in *F508del* CFTR Mice

Joan P Blonder[1], Sarah C Mutka[1], Kirsten Look[1], Michael Suniga[1], Nancy L Quinney[2], Xicheng Sun[1], Charles Scoggin[1], Sherif E Gabriel[1]

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S-nitrosoglutathione reductase (GSNO) serves as a stable reservoir for the labile nitric oxide (NO). GSNO has potent anti-inflammatory and bronchodilatory actions, and may also influence the cystic fibrosis transmembrane conductance regulator (CFTR). GSNO is catabolized by GSNO reductase (GSNOR), an enzyme which has been shown to be dysregulated in inflammatory lung disease. Therefore, small molecule inhibitors of GSNOR may provide therapeutic benefit toward inflammatory, airway smooth muscle, and ion channel conductance endpoints in cystic fibrosis (CF) by restoring GSNO levels and function. The efficacy of the GSNOR inhibitors N6022, N91115, and N91169 were tested in both *in vitro* and *in vivo* models. Relaxation of airway smooth muscle was tested using rat tracheal rings maintained at isometric tension in a wire myograph. Anti-inflammatory and bronchodilatory actions were assessed in mouse models of inflammatory lung disease including tobacco smoke-induced COPD and ovalbumin-induced asthma. The potential to modulate CFTR function was explored via intestinal current measurements (ICM) after treatment of *F508del* CFTR mice with GSNOR inhibitors. N6022, N91115, and N91169 selectively and potently inhibit GSNOR enzyme activity with IC_{50} values of 9.8, 17.3, and 25.6 nM and K_i values of 2.5, 6.6, and 14.0 nM, respectively. *In vitro*, GSNOR inhibition caused dose-dependent smooth muscle relaxation in rat tracheal rings contracted with 1 µM methacholine (MCh). In an asthma model, a single IV dose of 0.1 mg/kg N6022 attenuated airway hyperresponsiveness to MCh aerosol challenge, eosinophil infiltration into BALF, and NFκB activity in the lung. In an 11 day smoke COPD model, daily oral administration of either N91115 or N91169 at 0.3 mg/kg decreased BALF neutrophils by 37% and 34%, respectively. Finally, in *F508del* CFTR mice, oral administration of 1 mg/kg N91115 once daily for 7 consecutive days corrected CFTR function as noted by a positive ΔI_{sc} signal in the distal colon mucosa in response to 10 µM forskolin + 100 µM IBMX. N91115 increased the I_{sc} to 14% of the current measured in wild-type mice. Collectively, these findings suggest that GSNOR inhibition offers a therapeutic approach to mitigating the pathophysiology of CF via anti-inflammatory, bronchodilatory, and CFTR modulatory activities.

S9.6 - Functional Consequences of the I507ATC➔ATT Silent Codon Change in ΔF508 CFTR

Zsuzsa Bebok[1], Ahmed Lazrak[2], Lianwu Fu[1], Rafal Bartoszewski[3], Andras Rab[1], Eric Sorscher[4], Sadis Matalon[2], James Collawn[1]

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The significance of silent codon changes (sSNP) in the development and severity of human disorders has recently been confirmed. Based on our previous studies on CFTR and by others on P-glycoprotein and the *Catechol-O-methyltransferase* (*COMT),* it is now evident that sSNPs may affect mRNA and protein conformation or alter the expression levels and function of gene product. The most common disease causing mutation in the *CFTR* gene (ΔF508) is the out-of-frame deletion of three nucleotides (CTT or TCT) that not only leads to the loss of phenylalanine-508, but also introduces a silent codon change for isoleucine-507 (I507-ATC⟶ATT). While the ΔF508 deletion has received major attention pertinent to protein misfolding and endoplasmic reticulum associated degradation (ERAD), the consequences of the I507 silent codon change have not been considered, based on the generally assumed insignificance of synonymous codon variations. Concentrating on the I507-ATC⟶ATT substitution in ΔF508 *CFTR* using computational, biophysical and biochemical assays we previously demonstrated that it alters the secondary structure of the mRNA in the vicinity of the mutation. Changes in mRNA structure lead to altered translation dynamics and affect protein levels. In the present studies, using HEK293 cell clones stably expressing the ΔF508 CFTR variants, we compared the biochemical and functional properties of the I507-ATC and I507-ATT encoded ΔF508 CFTR proteins and demonstrate that the I507-ATC encoded ΔF508 CFTR is less accessible to the ER quality control than the I507-ATT ΔF508 CFTR found in cystic fibrosis patients. Together with the altered translation, this contributes to increased protein levels (Band-B CFTR). While some of I507-ATC ΔF508 CFTR escapes ERAD (~8% vs ~1% of I507-ATT), 27°C correction is still required for sufficient cell surface expression of the I507-ATC ΔF508 CFTR. However, our most significant finding is that the functional properties of the low temperature rescued I507-ATC ΔF508 CFTR differ considerably from the I507-ATT. Based on previous reports indicating the thermal instability of low temperature rescued ΔF508 CFTR, we performed a series of studies to compare the biochemical and functional stabilities of the ΔF508 CFTR variants at the cell surface. Whole-cell patch-clamp recordings revealed sustained, thermally stable cAMP-activated Cl^- transport through I508-ATC ΔF508, and thermally unstable function of I508-ATT ΔF508 CFTR. Interestingly, the cell surface half-lives of the low temperature rescued proteins were similar when the cells were replaced to 37°C. Furthermore, single channel recordings indicated wild-type CFTR-like gating properties of the I507-ATC ΔF508 compared to I507-ATT ΔF508 CFTR (open probability=0.45±0.037 vs. 0.09±0.002, respectively ($p< 0.001$)). These results establish the physiological significance of the I507-ATC⟶ATT silent codon change in ΔF508 CFTR and reveal its contribution to channel dysfunction and consequently, to disease severity. Our studies also provide valuable information regarding the biological roles of codon redundancy and codon usage bias, as they contribute to the regulation of protein expression and function.

Saturday 23 March – 18:15-19:15 **Room Malaga**

Keynote Lecture

Pharmacological Strategies to Rescue Chloride Transport in Cystic Fibrosis

Luis J.V. Galietta, Nicoletta Pedemonte, Emanuela Caci, Elvira Sondo, Loretta Ferrera, Paolo Scudieri, Emanuela Pesce, Olga Zegarra-Moran

U.O.C. Genetica Medica, Istituto Giannina Gaslini, 16147 Genova, ITALY

Cystic fibrosis (CF) is caused by reduced chloride and bicarbonate transport in the epithelial cells of various organs. In the lungs, the basic defect causes chronic bacterial infections and plugging of the airways by highly dense mucus secretions. Our aim is to develop novel pharmacological strategies to correct the basic defect in CF. To this purpose, we are considering two different targets: CFTR, the plasma membrane chloride channel protein that is altered by CF mutations, and the calcium-activated chloride channel (CaCC), which represents an alternative pathway to circumvent the basic defect. Our studies have two specific aims: 1) the identification of drug-like small molecules to correct the molecular defect caused by F508del, the most frequent mutation affecting CF patients; 2) the development of strategies to upregulate expression and/or activity of TMEM16A protein, the major component of the epithelial CaCC.

The F508del mutation causes impaired maturation of the CFTR protein. Indeed, F508del-CFTR is detected as a misfolded protein during its biosynthesis and is rapidly degraded by the ubiquitin-proteasome system. Although a small fraction of the mutant protein reaches the plasma membrane, it is rapidly internalized and degraded. To rescue F508del-CFTR, we have screened chemical libraries with a high-throughput functional assay based on the halide-sensitive yellow fluorescent protein (HS-YFP). This approach has detected several active compounds that are able to improve F508del-CFTR targeting to the plasma membrane. In particular, we have identified small molecules, belonging to the class of aminoarylthiazoles (AATs), that cause a dual effect on F508del-CFTR. They improve protein trafficking but also enhance the intrinsic ion channel activity. However, the AATs tested so far have a modest efficacy as correctors. Therefore, we are developing novel compounds, derived from the AAT scaffold, that by themselves, or in combination with other correctors, could lead to better F508del-CFTR correction.

In parallel, we have used the HS-YFP assay to screen a genome-wide siRNA library (6,650 targets). The purpose is to identify genes whose silencing leads to rescue of F508del-CFTR. The screening has identified a series of proteins, involved in ubiquitination and sumoylation. Such proteins may represent novel drug targets in CF.

We are also studying the TMEM16A protein since it is a major component of epithelial CaCC. Interestingly, we found that TMEM16A expression in the airway epithelium is particularly associated with goblet metaplasia, a condition of mucus hypersecretion that is typical of CF, asthma, and other chronic respiratory diseases. In particular, TMEM16A is highly expressed in goblet cells whereas CFTR is expressed in ciliated cells. Such results suggest that TMEM16A may be particularly required for mucin secretion. The separate localization of TMEM16A and CFTR requires further investigation to
assess whether the former protein is a suitable drug target for CF therapy.

Room: Granada

Posters – CFTR Structure / Function

P. 1

Study of Long-range Regulatory Mechanisms of the *CFTR* Gene

Stéphanie Moisan[1,2], *Claude Férec*[1,2,3]

[1]INSERM U1078, Laboratoire de Génétique Moléculaire et Génétique Epidémiologique, Brest, France, [2]Université de Bretagne Occidentale, Faculté de Médecine et des Sciences de la Santé, Brest, France, [3]CHU Brest, Hôpital Morvan, Laboratoire de Génétique Moléculaire, Brest, France

The cystic fibrosis transmembrane conductance regulator (*CFTR)* gene was identified in 1989, but more than 20 years later, the regulatory mechanisms controlling its complex expression are still not fully understood. Although, more than 1930 mutations have been identified, many cases of cystic fibrosis or *CFTR Related Disorders* remain still of unknown origin.

The promoter binds transcription factors and drives some aspects of *CFTR* gene expression, but it cannot alone account for tissue specific control. This implicates other distal *cis*- or *trans*-acting elements in cell-type-specific regulation of *CFTR* expression.

The aim of our project is to study long-range regulatory mechanisms of the *CFTR* gene.

Interestingly, the majority of the human genome is composed of non coding DNA whose function has not been thoroughly investigated. A significant number of conserved non-coding sequences (CNCs) are found in gene-poor regions, these large intergenic regions must have kept a function throughout evolution.

Thus, our first approach consisted to map potentials regulatory elements located within CNCs, which could interact specifically with the *CFTR* gene by tri-dimentional folding mechanism. These interactions can be detected by *Chromosome Conformation Captures* (3C) [Dekker and al. 2002]. The 3C technology is a high-resolution technique of molecular biology allowing the analysis of chromosome organization in cells by detecting physical interactions between pairs of genomic loci. Thus we can measure the proximity of various chromatin areas of DNA in the *in vivo* nuclear space.

We have already tested many CNCs located within the intergenic regions ASZ1/CFTR, MDFIC/TFEC and TFEC/TES. Unfortunately, no region appears to have a possible interaction with the *CFTR* promoter.

Accordingly, we wanted to save time with a high-throughput adaptation of 3C: the *3C-Carbon Copy* (5C) technology. This approach can be used to analyze millions of chromatin interactions in parallel [Dostie and al. 2006]. Thanks to mobility grant and collaboration with Dr. Dostie, we were able to directly learn the 5C approach in this pioneer laboratory. We are now setting up it in our laboratory.

Thanks to 3C and 3C-derivated analyses, we hope to discover new possible mutations at distance from the gene, which may lead to its dysfunction by modifying the chromatin conformation.

These analyses will be pursued on patients affected by cystic fibrosis or by *CFTR Related Disorders*, in whom either a single mutation or none was found in the *CFTR* gene coding sequence.

In the future, we would like to set up the 4C technology [Göndor and al. 2008]. The Circular Chromosome Conformation Capture (4C) is a 3C-derived technique that is a high-throughput approach for performing a genome-wide screen of previously unknown interacting partners with a genomic point of interest, “the bait”. Here the bait will be the *CFTR* promoter.

These regions, which could interact specifically with the *CFTR* gene by tri-dimensional folding mechanism, could be a target of small therapeutic molecules.

Finally we could imagine that identified endogenous enhancers may be incorporated into therapeutic vectors to restore a stable expression of *CFTR*.

Generation and Characterization of The G551D Mutation in CFTR Expressed in *S. cerevisiae*

Xin Meng[1], *Tracy Rimington*[1], *Natasha Cant*[1], *Naomi Pollock*[1], *Robert C Ford*[1]

[1]University of Manchester, Faculty of Life Sciences, Manchester, United Kingdom

G551D is the second most frequent cystic fibrosis disease-causing mutation and is the target mutation for the new CFTR potentiator VX-770 (Kalydeco). Recently this drug has been approved in the UK for patients with this mutation who are >6 yrs old. Study of CFTR with the G551D mutation is desirable in order to understand the mode of action of VX-770 and where it binds. The mutation is also useful as a negative control in assays of CFTR ion flux. Lastly, the mutation may lock the CFTR protein in a closed state, which might be advantageous for biophysical and structural studies and may enhance its properties for crystallization.

We have generated the G551D mutation in human CFTR expressed in the yeast *S. cerevisiae*. The construct also has a C-terminal GFP tag allowing the cellular location and its expression levels to be monitored. Expression in yeast is also enhanced by the addition of small solutes to the induction media, and we have found that G551D CFTR expresses at levels similar to wt CFTR and with minimal proteolytic degradation. We will present data on the generation of the construct in the human wt CFTR background and on its cellular localization in yeast. We will also present data on the physical properties of the protein (solubility; monodispersity by FSEC; thermal stability by Coumarin maleimide binding) and the effect that VX-770 binding has on these properties.

Purification and Biophysical Characterization of Full-length CFTR Expressed in *Saccharomyces cerevisiae*

Natasha Cant[1], *Naomi L Pollock*[1], *Tracy Rimington*[1], *Robert C Ford*[1]

[1]University of Manchester, Manchester, United Kingdom

Cystic fibrosis is the most common genetic disorder in the Caucasian population and over 1000 different deleterious mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) have been associated with the disease. There is great medical interest to develop drugs targeted at this ion channel, especially correctors that restore the folding of the CFTR protein with the most common mutation (F508deletion). However, characterisation of full-length wild-type and the F508del CFTR protein has been severely limited by the lack of sufficient quantities of purified protein. This limitation has recently been addressed by the Cystic Fibrosis Foundation (USA) which has formed an international consortium - the CFTR 3D Structure Consortium - to generate methods for CFTR production and crystallization. Reagents such as the murine wt CFTR - expressing yeast generated by our laboratory are now freely available via this consortium.
Here, we describe methodology to overexpress and purify milligram quantities of several full-length CFTR orthologs including F508del CFTR using our *Saccharomyces cerevisiae* (yeast) expression system. CFTR orthologs were solubilised from yeast microsomes using the detergent dodecylmaltoside (DDM) and purified in the same detergent by nickel affinity and size exclusion chromatography to yield 1-2 mg of CFTR protein per 15 litre fermenter culture. CFTR can also be solubilised and purified in the harsher detergent lyso-phosphatidyl glycerol (LPG), but displays no activity in this detergent. A green fluorescent protein (GFP) tag fused to the CFTR C-terminus allows rapid detection of the protein throughout the purification procedure. Purified CFTR was analysed by dynamic light scattering - size exclusion chromatography, mass spectrometry and electron microscopy to assess protein homogeneity. The thermal stability of purified CFTR was probed using tryptophan fluorescence and Coumarin maleimide dye binding assays, and an unfolding transition between 40-50°C was observed for the DDM-purified material. No clear transition was detected for the LPG-purified protein. Mass spectrometry of the DDM-purified CFTR revealed potential CFTR-interacting proteins that appear to remain associated with a small fraction of the CFTR protein molecules during the purification process. Comparisons with the mass spectrometry data for LPG-purified CFTR were consistent with the above interpretation, that LPG at least partially denatures proteins including CFTR. Structural characterization of the DDM- and LPG-purified CFTR material has been carried out by single-particle electron microscopy, showing a monomeric and highly monodisperse LPG-purified CFTR whilst a dimeric and less homogeneous population of particles was observed for the DDM-purified material. Preliminary electron crystallographic analysis of two-dimensional crystals of the chicken CFTR ortholog will be presented. The protein crystallizes in the same two-dimensional plane group and with roughly the same lattice parameters as the previously-reported human CFTR material expressed in BHK cells.

P.4

The Corrector C18 Modulates the Channel Activity of Purified and Reconstituted CFTR Independent of ATP

Paul D.W. Eckford[1], Mohabir Ramjeesingh[1], Christine E. Bear[1,2,3]

[1]Hospital for Sick Children, Molecular Structure and Function, Toronto, Canada, [2]University of Toronto, Department of Physiology, Toronto, Canada, [3]University of Toronto, Department of Biochemistry, Toronto, Canada

We recently developed a rapid purification and reconstitution method for Wt-CFTR and clinically relevant mutants F508del- and G551D-CFTR. Using this system we developed a halide efflux assay to monitor directly the functional activity of phosphorylated CFTR in a defined lipid membrane environment. The measured activity was dependent on the amount of protein reconstituted, genotype, and phosphorylation status, as well as the presence of ATP. Activity in the assay was sensitive to inhibition by the CFTR-specific inhibitor CFTRinh-172, and potentiated by well-known CFTR potentiators such as VRT-532 and VX-770 (ivacaftor). For VX-770 we noted a similar relative potentiation for Wt-, G551D- and F508del-CFTR. Interestingly, the VX-770 response was both independent and additive with ATP, suggesting that VX-770 binds to all genotypes of CFTR and at a site distinct from the ATP binding pocket to promote significant channel opening even in the absence of ATP. In contrast to potentiators, CFTR correctors are designed to rescue trafficking of mutant CFTR to the cell surface. However it appears that F508del-CFTR has defects in biosynthetic processing and trafficking to the cell surface, functional activity at the cell surface, and resident time at the membrane. An ideal corrector may improve all of these activities.

Since C18 is a highly effective corrector in other assays, it is an analog of VX-809 which is in clinical trials, and it is readily accessible, we probed its direct interaction with CFTR. Direct binding of C18 to CFTR modulates the halide flux activity of the purified and reconstituted protein in our assay, which is independent of membrane trafficking defects. At a concentration of 10 μM, C18 increases CFTR activity in this assay >2-fold for F508del-CFTR over vehicle treated control samples. It produced a similar 2.5-fold increase in Wt-CFTR flux activity.

The G551D mutant has little flux activity due to a defect in the ATP binding-hydrolysis cycle thought to be linked to channel opening and closing. In this clinically-relevant mutant, C18 induced a 3-fold increase in halide flux activity. VX-770 potentiates G551D-CFTR similarly, and in fact VX-770 can potentiate both Wt- and F508del-CFTR in the complete absence of ATP. In light of our previous VX-770 results and our new data on C18 treatment of G551D-CFTR, we were prompted to examine the effect of C18 on the activity of Wt-CFTR in the nominal absence of ATP. We examined a >2-fold increase in the flux activity of this phosphorylated protein in the absence of added nucleotide relative to a control sample in the absence of ATP but in the presence of the DMSO vehicle. These data suggest that like VX-770, C18 can induce channel function independent of ATP-mediated channel opening. There is no significant difference in ATPase activity for purified CFTR protein in the presence of 2 μM C18 or vehicle over a range of ATP concentrations. Taken together, our data suggest that C18 binds directly to CFTR and modulates its channel activity by an ATP-independent mechanism, at a site distinct from the canonical ATP-binding site.

Functional Consequences of the I507ATC➔ATT Silent Codon Change in ΔF508 CFTR

Zsuzsa Bebok[1], Ahmed Lazrak[2], Lianwu Fu[1], Rafal Bartoszewski[3], Andras Rab[1], Eric Sorscher[4], Sadis Matalon[2], James Collawn[1]

[1]UAB, CDIB, Birmingham, United States, [2]UAB, Anesthesiology, Birmingham, United States, [3]Medical University of Gdansk, Department of Biology and Pharmaceutical Botany, Gdansk, Poland, [4]UAB, Medicine, Birmingham, United States

The significance of silent codon changes (sSNP) in the development and severity of human disorders has recently been confirmed. Based on our previous studies on CFTR and by others on P-glycoprotein and the *Catechol-O-methyltransferase* (*COMT),* it is now evident that sSNPs may affect mRNA and protein conformation or alter the expression levels and function of gene product. The most common disease causing mutation in the *CFTR* gene (ΔF508) is the out-of-frame deletion of three nucleotides (CTT or TCT) that not only leads to the loss of phenylalanine-508, but also introduces a silent codon change for isoleucine-507 (I507-ATC⟶ATT). While the ΔF508 deletion has received major attention pertinent to protein misfolding and endoplasmic reticulum associated degradation (ERAD), the consequences of the I507 silent codon change have not been considered, based on the generally assumed insignificance of synonymous codon variations. Concentrating on the I507-ATC⟶ATT substitution in ΔF508 *CFTR* using computational, biophysical and biochemical assays we previously demonstrated that it alters the secondary structure of the mRNA in the vicinity of the mutation. Changes in mRNA structure lead to altered translation dynamics and affect protein levels. In the present studies, using HEK293 cell clones stably expressing the ΔF508 CFTR variants, we compared the biochemical and functional properties of the I507-ATC and I507-ATT encoded ΔF508 CFTR proteins and demonstrate that the I507-ATC encoded ΔF508 CFTR is less accessible to the ER quality control than the I507-ATT ΔF508 CFTR found in cystic fibrosis patients. Together with the altered translation, this contributes to increased protein levels (Band-B CFTR). While some of I507-ATC ΔF508 CFTR escapes ERAD (~8% vs ~1% of I507-ATT), 27°C correction is still required for sufficient cell surface expression of the I507-ATC ΔF508 CFTR. However, our most significant finding is that the functional properties of the low temperature rescued I507-ATC ΔF508 CFTR differ considerably from the I507-ATT. Based on previous reports indicating the thermal instability of low temperature rescued ΔF508 CFTR, we performed a series of studies to compare the biochemical and functional stabilities of the ΔF508 CFTR variants at the cell surface. Whole-cell patch-clamp recordings revealed sustained, thermally stable cAMP-activated Cl^- transport through I508-ATC ΔF508, and thermally unstable function of I508-ATT ΔF508 CFTR. Interestingly, the cell surface half-lives of the low temperature rescued proteins were similar when the cells were replaced to 37°C. Furthermore, single channel recordings indicated wild-type CFTR-like gating properties of the I507-ATC ΔF508 compared to I507-ATT ΔF508 CFTR (open probability=0.45±0.037 vs. 0.09±0.002, respectively ($p< 0.001$)). These results establish the physiological significance of the I507-ATC⟶ATT silent codon change in ΔF508 CFTR and reveal its contribution to channel dysfunction and consequently, to disease severity. Our studies also provide valuable information regarding the biological roles of codon redundancy and codon usage bias, as they contribute to the regulation of protein expression and function.

Thermodynamic Analysis of Temperature-Dependent F508del-CFTR Channel Function

Yiting Wang[1], *Zhiwei Cai*[1], *David N Sheppard*[1]

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Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. The commonest CF mutation F508del produces a misfolded protein with three fundamental defects: defective protein processing and trafficking; aberrant channel gating and thermo-instability. Low-temperature incubation partially rescues the cell surface expression of F508del-CFTR. However, when studied at 37 ^oC in excised inside-out membrane patches, F508del-CFTR channel activity is unstable. Within minutes, there is an irreversible loss of channel activity (termed run-down). Here, we investigate F508del-CFTR run-down by studying the single-channel behaviour of F508del-CFTR in excised inside-out membrane patches at temperatures between 23 and 37 ^oC.

There was no difference in the single-channel current amplitude (i) of the full-open state of wild-type and F508del-CFTR between 23 and 37 ^oC. However, at high temperatures F508del-CFTR opened frequently to a sub-conductance state, which was very rare in wild-type CFTR. Analysis of current amplitude histograms revealed that the amplitude of the sub-conductance state at 33 ^oC was 30% of the full-open state (sub-conductance, -0.21 ± 0.01 pA; full-open, -0.69 ± 0.01 pA, mean ± SEM, n = 7, P < 0.01, t-test), and the probability of F508del-CFTR occupying the sub-conductance state was 0.039 ± 0.07 (n = 7). For the full-open state, open probability (P_o) measurements revealed a marked difference in the effects of temperature on wild-type and F508del-CFTR channel gating. For wild-type, P_o rose exponentially between 23 and 37 ^oC (23 ^oC, P_o = 0.24 ± 0.02; 37 ^oC, P_o = 0.46 ± 0.04; n ≥ 4, p < 0.05). For F508del-CFTR, the relationship between temperature and P_o was bell-shaped, with maximal P_o (0.08 ± 0.01, n > 4) around 30 ^oC.

To understand better the temperature-dependence of F508del-CFTR, we applied the Eyring equation to burst analysis data. The activation enthalpy (ΔH) for both wild-type and F508del-CFTR channel opening (ΔH_o) were higher than those for channel closing (ΔH_c). Despite limitations using the Eyring equation to determine activation entropy (ΔS), for both wild-type and F508del-CFTR higher activation entropy was also required for channel opening (ΔS_o) than channel closing (ΔS_c). Interestingly, similar activation enthalpy and entropy were required to open wild-type and F508del-CFTR channels. However, F508del required higher enthalpy and entropy for channel closing (activation enthalpy ($kJ.mol^{-1}$): wild-type, ΔH_o = 100, ΔH_c = 42; F508del, ΔH_o = 107, ΔH_c = 75; activation entropy ($J.mol\text{-}kelvin^{-1}$): wild-type, ΔS_o = 98, ΔS_c = -93; F508del, ΔS_o = 97, ΔS_c = 14).

In conclusion, wild-type and F508del-CFTR Cl^- channels respond differently to temperature (maximum P_o: wild-type, 37 ^oC; F508del, ~30 ^oC). The opening of F508del-CFTR to a sub-conductance state suggests that the F508del-CFTR pore is unstable. This idea is supported by Eyring analysis, which shows that F508del-CFTR required higher activation enthalpy and entropy for channel closing compared to wild-type CFTR. We interpret our data to suggest that F508del-CFTR has an unstable conformation and the appearance of a sub-conductance state may reflect faulty coupling between the nucleotide-binding domains and the channel pore.

Supported by the CF Trust.

P.7

Mechanistic Studies of CFTR Correctors

André Schmidt[1], Linda Millen[1], Juan L. Mendoza[1], Margaret Fuller[1], Yi Cheng[2], Greg A. Miller[3], W. Christian Wigley[3], Robert J. Bridges[2], Philip J. Thomas[1]

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Cystic Fibrosis (CF) is the most common monogenic, recessive lethal disease in populations of European descent, caused by dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), an anion-channel normally expressed in the apical membrane of some epithelial cells. Defective CFTR leads to multi-organ disease, the most important patho-physiological consequence being impaired mucus clearance from the lung. The most common mutation, found in 90% of CF patients, is a deletion of phenylalanine at position 508, termed F508del-CFTR. This mutation prevents the protein from efficiently folding, and, hence, accumulation in the endoplasmic reticulum prior to degradation. Importantly, the mutant protein is appropriately translated in the physiologically relevant cells. Thus, a possible strategy to treat the disease is to discover small molecules, which correct the impaired maturation of the mutant protein and thereby restore its normal cellular function. A number of small molecules have been identified that partially restore F508del-CFTR Cl^--channel function; some of these are represented in the Cystic Fibrosis Foundation Therapeutics (CFFT) panel. Recent work in our lab (Mendoza *et al.,* Cell 2012), indicates that at least two critical steps are defective in the F508del mutation: 1) decreased stability and folding yield of nucleotide binding domain 1 (NBD1), the domain containing the site of the deletion, and 2) poor assembly of the native multi-domain structure. To determine if CFFT panel small molecules act on these or different steps in CFTR folding, we assessed them in the context of second-site suppressor mutations, which have been demonstrated to counteract each of the F508del defective steps, domain folding and domain assembly. First, we assessed the isolated NBD1: To evaluate domain stability, *in vitro* thermal melts of the purified NBD1 protein were performed. To evaluate domain folding yield a cell-based assay was employed. Second, in combination with step-specific second-site suppressor mutations, we assessed full-length maturation using biochemical methods. Third, we performed assays on these combinations to evaluate their Cl^--channel function. The results suggest that some of the CFFT panel compounds target CFTR directly and act preferentially on one of the biosynthetic steps, but not the other, consistent with their limited efficacy. Importantly, a combination of assembly step specific compounds identified from the CFFT panel, and a compound which acts by a different mechanism of action, is able to affect efficient rescue of F508del-CFTR maturation and function. Understanding of the mechanism of action of these small molecules provides a rational path to effective combinations and to genotype specific treatment of other CFTR folding mutations.

We thank CFFT for providing us with the compounds for this study (Kirsten Hencken, khencken@cff.org or by phone (301) 841-2609).

Mendoza *et al.* (2012): Requirements for efficient correction of ΔF508-CFTR revealed by analyses of evolved sequences. *Cell* **148**(1-2), 164-74.

ICL1 Facilitates the Conformational Maturation of CFTR Through Interdomain Interfaces with NBD1 and ICL3

Steven Molinski[1,2], *Ling Jun Huan*[2], *Christine Bear*[1,2]
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CFTR is a plasma membrane phosphoglycoprotein, belonging to the ATP-Binding Cassette (ABC) transporter superfamily. CFTR functions as an ATP- and PKA-dependent chloride channel, regulating chloride flux across apical membranes of polarized epithelial cells. Mutations in CFTR cause Cystic Fibrosis; the major mutation, F508del, is located in the first nucleotide binding domain (NBD1). There are multiple conformational defects caused by F508del including disruption of the interaction between NBD1 and the fourth intracellular loop (ICL4) (Loo *et al.*, 2008; Serohijos *et al.*, 2008). Current small molecule 'correctors' lead to partial rescue of the processing defect with some functional (but inadequate) expression at the cell surface, and although the mechanism of action is poorly understood, the NBD1:ICL4 interface is likely repaired to some extent. Further progress in understanding the molecular defects caused by F508del and the development of more effective correctors is limited by the lack of understanding regarding the interdomain interactions necessary for assembly of the full-length protein.

Functionally important interdomain interfaces include: NBD1:ICL4, NBD1:2, ICL3:Regulatory-domain, and membrane spanning domain (MSD) 1:2. Identification of other functionally relevant interfaces is desired. Upon inspection of homology models of CFTR, previously unexplored interfaces (ICL1:2, ICL1:3, ICL1:NBD1) were predicted to play important roles in biosynthesis and gating (Serohijos *et al.*, 2008; Mornon *et al.*, 2009; Dalton *et al.*, 2012). Further analysis identified a cluster of hydrophobic residues in the ICLs that may be important. These residues include: Ile^{177} (ICL1), Val^{181} (ICL1), Leu^{259} (ICL2), Val^{260} (ICL2), Leu^{973} (ICL3) and Val^{1056} (ICL4). Individually mutating Ile^{177} and Leu^{259}, two residues which may form the closest interface, did not affect maturation, but the double substitution of $Ile^{177}Ala/Leu^{259}Ala$ decreased the rate of channel activation by 40%, presumably by altering the ICL1:2 interface, as measured by cell-based iodide efflux assays. Substitution of the Leu^{973} (ICL3) with disease-causing Pro, but not disease-causing Phe or His, affected processing, suggesting that a conserved residue is required at this position for proper assembly and maintenance of helical structure. Interestingly, disruption of the putative ICL1:3 interface by double Ala-substitution of Ile^{177} (ICL1) and Leu^{973} (ICL3) created a mutant protein that was EndoglycosidaseH-sensitive, suggesting that it is synthesized and core-, but not complex-glycosylated at steady-state; therefore this interface is important for maturation.

Subsequently, the putative ICL1:NBD1 interface was disrupted using mutagenesis, and resulted in misprocessing of CFTR; mutations include S169R (ICL1), R170G (ICL1) and W401G (NBD1). This suggests that CFTR models are accurate in this region, and may further support the idea that ICL1 serves as an intramolecular scaffold, allowing for the initial coordinated assembly of NBD1 and possibly MSD2 (via ICL3) during CFTR biosynthesis. By understanding how ICLs transmit signals between the MSDs and NBDs via interdomain interfaces, gating mechanisms will become more apparent, and could allow for the design of therapeutics to treat Cystic Fibrosis. Elucidation of such molecular events would contribute not only to our understanding of CFTR activity, but also to our understanding of channels and transporters in general.

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A Gene Optimisation Strategy to Enhance Human CFTR Expression in Yeast Cells

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Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene produce defective CFTR protein which results in the disease cystic fibrosis (CF). With over 70,000 patients worldwide, CF is one of the most prevalent life-threatening genetic diseases in Caucasian populations. Research into CF has lead to a greater understanding of the disease and recent advances in medical treatments have increased life expectancy with over half of CF patients today expected to live into their 40s. Despite this progress, there is still a lack of information pertaining to the structure and functionality of the CFTR protein.

Expression of CFTR, which a membrane protein, is notoriously difficult and obtaining high yields in heterologous systems has previously proven problematic. Using a gene optimisation approach, in which rare codons are substituted for those more frequently occurring in the host system, we have expressed human CFTR (hCFTR) in the budding yeast *Saccharomyces cerevisiae* with some promising results. The recombinant proteins expressed are fused to C-terminal GFP tags which allow the proteins to be tracked throughout expression and also later purification stages using SDS-PAGE analysis viewed under fluorescence. GFP also allows us to study protein localisation using fluorescence microscopy. In addition to wild-type GFP, we also used a yeast-enhanced GFP tag (yEGFP) which too, is codon-optimised to the yeast genome. To further enhance protein expression, a range of compounds were tested which would potentially act as chemical chaperones to aid protein folding and trafficking to the plasma membrane and therefore increase protein yields following expression.

Expression levels of codon-optimised hCFTR were improved compared to wild-type hCFTR as analysed by SDS-PAGE although a major degradation band was present in the codon-optimised hCFTR protein which was absent from the wild-type protein. The use of chemical chaperones had a notable effect on protein expression levels, increasing it by >10 fold in some cases. Fluorescent microscopy showed expression of the codon-optimised hCFTR at or near the plasma membrane. This was confirmed by co-staining of the membrane with a fluorescent probe and further analysis using fluorescence microscopy. Using the same strategy, several other codon-optimised CFTR orthologues and mutated hCFTR proteins have also been expressed in *Saccharomyces cerevisiae*. The codon-optimised ΔF508-hCFTR, along with the codon-optimised hCFTR, will be used to design high-throughput drug screening assays using purified protein reconstituted into lipid vesicles. Preliminary experiments using fluorescent indicators which can be quenched by chloride ions have shown that this assay should be feasible and amenable to scaling up to a high-throughput platform.

Our experiments to date have shown that difficult to express membrane proteins, like CFTR, can be expressed to high levels in heterologous expression systems using a gene optimisation strategy. The use of chemical chaperones and optimising construct features are also important to enhance expression and obtain high yields of protein. We currently employ these techniques to express large quantities of protein for structural and functional analysis with the ultimate aim of designing a high-throughput drug screening assay to identify drugs and small molecules which have the potential to treat CF patients.

Interaction between Keratin 8 and Cystic Fibrosis Transmembrane Regulator (CFTR): Structural Studies

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Introduction: Recently, we have shown that interaction between CFTR with a deletion of phenyloalanine 508 (F508del - CFTR) might be responsible for the inefficient trafficking of mutated CFTR to the plasma membrane contributing to the cystic fibrosis (CF) disease. Using functional assay, it was shown that disruption of this interaction allows for addressing the F508del - CFTR to the plasma membrane where it can exert its function (Colas J. et al., Hum Mol Gen 2011). Surface plasmon resonance indicated much stronger interaction between keratin 8 and domain of CFTR F508del - NBD1 than with native NBD1.
Aim: Because the site of the interaction between keratin 8 and mutated CFTR might constitute a new target for pharmacotherapy of CF the aim of this study was to determine it on structural level. For this aim we have applied;
Material and methods: Recombinant protein NBD1 WT/ F508del (Lukacs G., McGill University, Montreal), K8 peptide chemical synthesis, hydrogen - deuterium exchange reaction coupled with mass spectrometry (HDex - MS)
Results: After HDex - MS performed on full keratin 8 protein were able to choose fragments of this protein with a highest exposition for hydrogen - deuterium exchange as regions especially prompted to interact with other molecules. We have synthesized the head domain of keratin 8 which exhibited the highest HDex rate dividing it for 3 overlapped peptides. One of them gave a strong signal in HDex - MS experiments suggesting close interaction with mutated and wild type NBD1. We obtained differential HDex pattern between mutated and wild - type NBD1 with and without keratin 8 peptide. The differential site of the interaction with K8 peptide appeared to expand on a linker region between two subdomains of NBD1 in the proximity of deletion phenyloalanine 508. Previously it was shown that the same linking fragment gains much more flexibility leading to more conformation freedom of NBD1 subdomains upon mutation (F508del) (Wieczorek G. et al., J. of Cystic Fibrosis, 2008). This leads to the exposition of hydrophobic residues preferentially buried in the native NBD1 and creation of 2 cavities on the surface mutated NBD1 according to the in silico modelisation. Moreover, to these cavities 12 of small molecules from National Cancer Institute Library (NCIL) were successfully docked and for 4 of them the functional assay exhibited correcting effect of F508 - CFTR and proximity ligation DNA assay revealed ability to disrupt with keratin 8 interaction.
Conclusion: Taking into account above findings we propose the interruption of pathogenic interaction with keratin 8 as highly probable mechanism of action of small corrector [NCIL ID: 407882].

Room: Granada

Posters – CFTR Biogenesis, Quality Control and Trafficking

P. 11

Revertants, Low Temperature and Correctors Provide Clues to Mechanism of F508del-CFTR Rescue by VX-809 and Suggest Multiple Agents for Full Correction

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The most common disease-causing mutation in the CFTR gene, F508del, causes Cystic Fibrosis through impairment of protein from exiting the endoplasmic reticulum due to misfolding. The investigational drug VX-809 is a small-molecule that rescues F508del-CFTR localization which recently went into clinical trial but with still unknown mechanism of action (MoA). Herein, we assessed if VX-809 is additive or synergistic with genetic revertants of F508del-CFTR, other correctors (VRT-325 and Corr-4a) and low temperature to attempt determining its MoA.

We explored and integrated those various agents in combined treatments, showing how they add to each other, to identify their complementary MoA and the full scope for F508del-CFTR correction.

Results from biochemical, functional and modelling approaches pointed to major insights into its MoA point to major insights into its MoA:

1) VX-809 is additive to both VRT-325 and Corr-4a, suggesting that each compound operates by a different MoA.

2) VX-809 is additive to low temperature rescue of the mutant almost to wt-CFTR levels.

3) VX-809, VRT-325 and Corr-4a show variable additive effects with the genetic revertants tested (4RK, G550E and R1070W) thus providing clues for their possible action being exerted at specific protein binding pockets: VX-809 at the NBD1:TM2 interface (and VRT-325 at NBD1:NBD2) or acting unspecificallly (Corr-4a).

4) VX-809 does not rescue the diacidic-code traffic mutant ($D^{565}D^{567}$/AA) in contrast to low temperature which seems to act at trafficking surveillance checkpoints.

Besides suggesting a MoA for VX-809, our data also indicate the scope for further synergistic F508del-CFTR correction by other compounds at distinct conformational sites/cellular checkpoints.

Altogether, these data provide a plausible explanation for the limited success of VX-809 in clinical trials and suggest that combination therapies may thus be required to achieve full F508del-CFTR correction, so as to reach the functional CFTR threshold necessary to avoid CF. These findings should be exploited for therapeutic strategies of Cystic Fibrosis.

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The Iminosugar Isolab Corrects the Defective Trafficking of F508del-CFTR by Prevention of Hsp90/CFTR Interaction

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Cystic fibrosis (CF) is an autosomal and recessive disease caused by mutations in Cystic Fibrosis Transmembrane conductance Regulator gene (*CFTR*). Approximately 90% of CF patients have the F508del mutation in one or both *CFTR* alleles. This mutation leads to the retention of F508del-CFTR protein in the endoplasmic reticulum (ER), to abnormal gating of CFTR channel and endocytosis. One of the major therapeutic strategy in Cystic Fibrosis aims at developing correctors of CFTR channels. In previous study we identified an iminosugar, IsoLAB, as a new corrector of the F508del-CFTR defective trafficking independently of the ER-α-glucosidase inhibition (Best et al., 2010). In the present work we studied the mechanism of action of IsoLAB.

Firstly, we investigated the effect of IsoLAB on CF tracheal human epithelial cells (CF-KM4) in competition with several inhibitors of the biosynthetic pathway and of the ER quality machinery using the iodide efflux technique. We observed a lack of correction by IsoLAB in presence of brefeldin A, a vesicular ER/Golgi-intermediate compartment traffic inhibitor. This result indicates that IsoLAB-corrected F508del-CFTR follows a conventional trafficking pathway. We observed that thapsigargin, tunicamycin and miglustat, known to alter the F508del-CFTR/calnexin interaction, potentiate the F508del-CFTR rescue induced by IsoLAB. We also found that a treatment with MG132, a proteasome inhibitor, potentiates the IsoLAB effect. Altogether, these results suggest that calnexin and/or proteasome are not the molecular targets of IsoLAB.

On the contrary, when cells were incubated at low-temperature, a procedure known to rescue F508del-CFTR proteins via the inhibition of heat shock proteins (Hsp), we did not observe potentiation of the correction by IsoLAB, whereas it is the case with other correctors. This result suggests a potential effect of IsoLAB on Hsp proteins. To test this hypothesis we conducted additional experiments. While 4-PBA, an Hsp70 inhibitor, slightly potentiated the effect of IsoLAB, we observed no potentiation when IsoLAB was associated in co-treatment with geldanamycin or ansamycin, two Hsp90 inhibitors. Thus, we focused on the Hsp90-dependent pathway and used a Duolink “Proximity ligation Assay” to detect and quantify Hsp90/CFTR interaction. We observed that transfected CF-KM4 cells with Hsp90 siRNA (1) decreased the Hsp90/CFTR interaction and (2) corrected the abnormal trafficking of F508del-CFTR. Similar results were obtained after a pharmacological treatment by geldanamycin or ansamycin. Finally, IsoLAB decreased by 90% the Hsp90/CFTR interaction resulting in the restoration of a functional F508del-CFTR to the plasma membrane.

In conclusion, we propose that the F508del-CFTR corrector IsoLAB act as an Hsp90-dependent pathway inhibitor. Further experiments will be conducted to strengthen this hypothesis and to determine whether IsoLab is a direct inhibitor of Hsp90.

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Palmitate Accumulates within Phosphatidylcholine of Cystic Fibrosis Human Bronchial Epithelial Cells: Impact on F508del-CFTR Trafficking

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The most common mutation in cystic fibrosis (CF), *F508del-CFTR* (CF transmembrane conductance regulator) results in the retention of the protein in the endoplasmic reticulum (ER), which could be corrected by pharmacological agents. Lipid homeostasis is also altered in CF. In fact, few studies showed lower levels of unsaturated fatty acids (UFA) in CF cells and plasma (Anderson *et al.*, 2008; Kuo *et al.*, 1962; Freedman et *al.*, 2004). This defect also appears to impact the overall fatty acyl content of phospholipids, with a decrease of five phosphatidylcholine (PC) UFA-containing species in the plasma of CF patients (Guerrera *et al.*, 2009).
In the present study, lipid analyses by HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry) show that palmitate, a saturated fatty acid (SFA), accumulates within PC of bronchial epithelial cells from CF patient lungs, as compared to non-CF patients. In a parallel study on a simple eukaryotic model, *Saccharomyces cerevisiae*, we demonstrated that SFA accumulation within PC, by altering membrane fluidity, impacts several important cellular processes such as protein folding in the ER and vesicular budding from the Golgi apparatus. These observations led us to postulate that palmitate accumulation observed *in vivo* in CF patient cells could alter F508del-CFTR trafficking correction by pharmacological means.
Surprisingly, contrary to human bronchial epithelial cells, this accumulation was not observed in a CF bronchial epithelial cell line, CFBE41o$^-$. Thus, CFBE41o$^-$ cells were incubated with exogenous palmitate to mimic what is seen *in vivo* in CF patient cells. In this context, we are presently investigating the possible effects of palmitate accumulation within PC in CFBE41o$^-$ cells on F508del-CFTR trafficking correction by pharmacological agents. Preliminary data concerning these aspects will be presented.

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Exchange Proteins Directly Activated by Cyclic AMP: Novel CFTR Interactors?

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CFTR is stimulated when cyclic AMP levels near the plasma membrane increase enough for protein kinase A (PKA) to be activated, triggering CFTR phosphorylation and channel opening. However, PKA is not the sole cyclic AMP sensor in the cell: the family of exchange proteins directly activated by cyclic AMP (Epacs) also responds to direct binding of this second messenger. Epacs are guanine nucleotide exchange factors for small GTPases of the Rap family, which are involved in the control of cell-cell and cell-matrix adhesion, cytoskeleton rearrangements and cell polarization, processes which in cystic fibrosis are dysregulated. Moreover, Epacs are reported to localize to the plasma membrane by the same scaffolding proteins that bind CFTR and PKA, namely ezrin-radixin-moesin (ERM) proteins. This suggests that activation of both CFTR and Epac might be spatially and temporally coincident. However, little is known concerning the involvement of Epacs in CFTR biology. Our aim was thus to elucidate the impact of Epacs in CFTR biogenesis, processing and trafficking.
In this report, we show that both Epacs and their effector Rap1 are expressed in the A549 expressing CFTR under a Tet-ON promoter and CFBE lung epithelial cell lines, as well as in primary cultures of human bronchial epithelial cells. We also show by co-immunoprecipitation that Epac physically interacts with CFTR in A549 cells, supporting a functional interaction between the two proteins. Lastly, preliminary data suggest a decrease in CFTR steady-state levels when A549 cells are treated with 8-pCPT-2'-O-Me-cAMP-AM (a membrane-permeable Epac-selective cyclic AMP analogue that does not activate PKA). Taken together, our data support the hypothesis that Epacs may have a negative impact on CFTR membrane traffic.

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Rescue of ΔF508 CFTR by Transcomplementation and Corrector, C18

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ΔF508 CFTR is recognized as a misfolded protein by the ER quality control mechanism and targeted for degradation by the proteasome. Transcomplementation of ΔF508 CFTR by truncated forms of CFTR have been observed to overcome defective processing and allow mature ΔF508 CFTR to reach the cell surface. Here we study the combined roles of a truncated form of CFTR, Δ27-264 CFTR, which is missing the first 4 transmembrane segments of CFTR but contains the first 27 amino acids of the n-terminus and the corrector C18 (a molecule related to VX-809) as a way of elucidating the mechanism of ΔF508 CFTR rescue. Δ27-264 CFTR increases the amount of the mature C band of both wt and ΔF508 CFTR through transcomplementation. Immunoprecipitation studies show that Δ27-264 binds to DF508-CFTR suggesting a bimolecular interaction.

We found that both C4 and C18 increase the amount of mature C band of ΔF508 CFTR. We also observed that corrector C4 can increase the steady state protein level of Δ27-264 CFTR whereas C18 has no effect.

In cells cotransfected with ΔF508 CFTR and Δ27-264 CFTR and treated with increasing doses of C4 , there was a significant increase in the mature C band of ΔF508 CFTR compared to the cells cotransfected with ΔF508 and Δ27-264 CFTR in the absence of correctors or cells transfected with ΔF508 CFTR alone. Thus, there is an additive effect of C4 and transcomplementation. In sharp contrast, in cells cotransfected with ΔF508 CFTR and Δ27-264 CFTR and treated with increasing doses of C18, there was no significant increase in the mature C band of ΔF508 CFTR compared to the cells cotransfected with ΔF508 and Δ27-264 CFTR in the absence of correctors. Indeed we found that C18 inhibited transcomplementation and altered the binding of Δ27-264 to ΔF508 CFTR.

In conclusion: rescue of ΔF508 CFTR occurs when cells are treated with the small molecule correctors 4A or C18. The additive effect of C4 on Δ27-264 CFTR and transcomplementation suggests that transcomplementation and C4 rescue ΔF508 through different mechanisms. Because Δ27-264 CFTR is missing the first 4 transmembrane segments of CFTR, the observation that C18 has no effect on Δ27-264 CFTR and inhibits transcomplementation suggests that the mechanism of action of C18 may be through interactions with the initial portions of TMD1.

Room: Granada

Posters – Epithelial Transport

P. 16

Modulation of Cl^- Secretion by Bile Acids in Calu-3 Airway Epithelial Cells

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Bile acids are often present in the lower airways of people with CF, probably resulting from the aspiration of gastroesophageal refluxate, but the effects of bile acids on airway epithelium have not yet been investigated (1,2). In the colon, bile acids have been reported to acutely stimulate or chronically inhibit epithelial Cl^- secretion (3).

We investigated the effects of the unconjugated bile acid, deoxycholic acid (DCA, 25 µM) and the conjugated bile acid tauroDCA (TDCA, 25 µM) on basal transport and carbachol (100 µM) or forskolin (10 µM)-induced ion transport in Calu-3 airway epithelial cells grown in an air-liquid interface and mounted in Ussing chambers. Electrogenic transpeithelial ion transport was measured as short-circuit current (I_{sc}). Data given as Mean ± S.E.M. Statistics were generated using the Student's paired t-test or one-way Anova analysis, where $p \leq 0.05$ is considered significant.

We found that acute (5 min) basolateral TDCA treatment of Calu-3 cells stimulated basal I_{sc} by 39 ± 7% ($p = 0.0001$, $n = 13$) but had no effect on the I_{sc} responses induced by carbachol or forskolin treatment. In contrast, acute apical treatment (30 min) of Calu-3 cells with DCA had no effect on basal I_{sc} or on forskolin-stimulated I_{sc} but did attenuate the I_{sc} responses to carbachol by 43 ± 8% ($p = 0.001$, $n = 7$). The I_{sc} responses to bile acids were abolished in Cl^- -free Krebs solution ($n = 4$) indicating that bile acids modulate Cl^- secretion in Calu-3 cells. Prolonged (24hr) apical treatment with DCA significantly increased the I_{sc} responses to carbachol by 55 ± 21% ($p = 0.038$, $n = 6$) whereas 24hr TDCA treatment of Calu-3 had no effect on I_{sc} responses to carbachol or forskolin. In Ussing chambers where Na^+/K^+ATPase generated currents were measured, acute treatment with TDCA increased Na^+/K^+pump activity by 13 ± 3%, while pre-treatment with 100 µM ouabain eliminated this effect ($p = 0.0005$, $n = 9$). Calcium imaging of Calu-3 cells, grown on glass, revealed that acute treatment with TDCA resulted in a rapid 88 ± 12% increase in calcium mobilization ($p = 0.0002$, $n = 8$), whereas DCA had no effect. The Ca^{2+} mobilization response to TDCA was abolished in Ca^{2+} -free buffer indicating that TDCA induced Ca^{2+} influx into the cell.

In summary, these studies demonstrate a temporal dependence and sidedness of bile acid modulation of airway epithelial Cl^- secretion. Further investigations are required to determine the role of Ca^{2+} signalling pathways and ion channels contributing to TDCA and DCA effects in regulating airway epithelial Cl^- secretion.

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Role of TRPV Channels in Calcium Homeostasis in CF Cells

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Cystic Fibrosis (CF) is caused by mutation of the CF transmembrane conductance regulator (CFTR) gene that leads to defective airway epithelial chloride transport. Previously, we demonstrated that abnormal calcium homeostasis is a hallmark of CF airway epithelial cells and associates the transient receptor potential canonical channel 6 (TRPC6). The functional coupling between CFTR and TRPC6 has been recently proposed. Furthermore, swelling-activated Ca^{2+} entry through Transient Receptor Potential Vanilloid channel (TRPV4) is defective in CF airway epithelia (Arniges et al., 2004). Therefore, TRP channels turn out to be involved in calcium homeostasis regulation of airway epithelial cells. Our objective is to study TRPV channels family and their possible role in calcium homeostasis regulation in CF cells.
First, we identified by Western blot, the different isoforms of TRPV channels in 16HBE14o- cells, CFBE41o- cells, and in freshly isolated airway epithelial cells from CF and non CF patients. Second, the activity of TRPV isoform identified in our models was measured to evaluate their possible implication in the regulation of calcium homeostasis in CF cells.
Our preliminary results showed that TRPV1, 3, 4, 5, and 6 are present in 16HBE14o- and CFBE41o- cells. We also confirmed that TRPV4 activity is decreased in CFBE41o- cells and for the first time this decrease is also observed in freshly isolated human epithelial airway cells from CF patients. In contrast, no difference of TRPV1 activity has been observed between 16HBE14o- and CFBE41o- cells, and between freshly isolated human airway epithelial cells from CF and non CF patients. Surprisingly, TRPV5 expression seems to be 1.5 time higher in CFBE41o- cells compared to 16HBE14o- cells.
Further experiments will examine the consequence of F508del-CFTR pharmacological correction in CF cells on TRPV channels function.

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Non-canonical Translation Start Sites in the TMEM16a Chloride Channel

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TMEM16A, a protein localized in the plasma membrane of different types of cells, has a voltage- and calcium-dependent chloride channel activity. Because of its expression in airway epithelial cells, TMEM16A may represent a potential drug target to correct the basic defect in cystic fibrosis. A better understanding of the structure-function relationship of TMEM16A protein may help to devise strategies to upregulate TMEM16A function. Our specific aim was to clarify the role of the TMEM16A N-terminus. In a previous study, we found that removal of the 116 amino acids was without functional consequences (Ferrera et al., Biochim Biophys Acta 1808: 2214-2223, 2011). Therefore, we concluded that this region is dispensable for TMEM16A processing and channel activity. However, those experiments were done by replacing the first ATG with a nonsense codon (M1X mutation) to force the translation from the second ATG localized at position 117. We have now removed the first 116 codons from the TMEM16A coding sequence. Surprisingly, expression of the resulting mutant, delta(1-116), resulted in complete loss of activity. We hypothesized that, in the mutant M1X, translation may start at a position before the second ATG, using a non-canonical start codon. Therefore, we placed an HA-epitope at position 89 in the M1X mutant. We found, by western blot analysis, that the HA-epitope can be detected, thus demonstrating that translation starts from an upstream non-ATG codon. We truncated the N-terminus of TMEM16A at different sites while keeping the HA-epitope. We found that gradual shortening of TMEM16A caused in parallel a decrease in TMEM16A synthesis and function. Our results indicate that the N-terminus of TMEM16A protein is actually important for its activity. Future experiments will have to address the role of this domain in TMEM16A structure, function, and interaction with other proteins.

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FACE Modulates Transepithelial Water Resorption in the Alveolus

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Tight regulation of the alveolar fluid layer is essential for lung function. Impaired water transport across lung epithelia results in severe health problems ranging from impairment of mucociliary clearance to edema. Yet, the mechanisms and the role of alveolar epithelial cells for maintaining alveolar fluid homeostasis are still controversial. We have recently described that exocytosis of lamellar bodies (LBs) in primary alveolar type II (ATII) epithelial cells results in "fusion-activated cation-entry" (FACE) via $P2X_4$ receptors on LBs. Fusion of LBs with the apical plasma membrane (PM) and subsequent cation entry via $P2X_4$ receptors offers a new potential route for apical to basolateral water movement in the alveolus.

Initially, we looked at the I_{sc} of ATII cell monolayers, and the role for agonist induced activation of $P2X_4$ following LB fusion. While both ATP and UTP can stimulate fusion of LBs with the PM, only ATP can activate FACE. While stimulation with both resulted in an increase in I_{sc} and fusion, an additional increase in I_{sc}, indicating FACE, was only seen when an initial stimulation via UTP was followed by ATP. Over-expressing dominant-negative $P2X_4$ abrogated this effect by ~ 50%, whereas potentiating $P2X_4$ lead to ~ 80% increase in I_{sc}. Using fluorescently labeled dextran loaded apically on ATII cell monolayers grown at air-liquid interphase, we were able to directly establish a role for $P2X_4$ receptors in water transport from the alveolar surface fluid (ASF) across the alveolar epithelium. Fluid transport was seen only in the event of LB fusions that were followed by FACE. Again, monolayers transfected with dominant-negative $P2X_4$ reduced the water resportion from the ASF.

Finally, the link between these two studies of cation movement via the $P2X_4$ receptor and water transport in the whole lung was studied in *in situ* experiments looking at changes in lung compliance upon activation of FACE at time of LB fusion. Results from the in situ experiments confirmed the idea that FACE directly couples surfactant secretion and transepithelial water transport in the lung.

Pharmacological Sensitivity of the TMEM16A Chloride Chanel

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The TMEM16A chloride channel is a potential drug target in cystic fibrosis. Its stimulation by direct pharmacological activators could circumvent the defective anion transport caused by CFTR mutations. On the other hand, selective TMEM16A inhibitors are important as research tools to define the contribution of this protein to epithelial ion transport.
It has been recently reported that TMEM16A has a little role in the human airway epithelium (Namkung et al., J Biol Chem 286(3):2365-2374, 2011). In this study, a selective TMEM16A inhibitor, named T16inh-A01, showed a little effect on calcium-activated chloride secretion in human bronchial epithelial cells. In contrast, a less specific inhibitor, CaCCinh-A01 was much more effective.
Our aim was to define the contribution of TMEM16A to ion transport in human airway epithelial cells, to study the mechanism of action of existing pharmacological modulators, and to identify novel activators and inhibitors of the TMEM16A protein.
We studied function and expression of TMEM16A in primary bronchial epithelial cells and in epithelial cell lines (CFPAC-1 and CFBE41o-). Functional studies, done by patch-clamp, short-circuit current recordings, and iodide flux assays, demonstrated that T16inh-A01, in contrast to CaCCinh-A01, is a partial inhibitor of calcium-dependent anion transport. Even at maximal concentrations, inhibition was less than 40%. In contrast, anion transport could be strongly reduced when cells were transfected with siRNA against TMEM16A but not against a related protein, TMEM16F. We also tested T16inh-A01 on FRT cells with stable transfection of TMEM16A. In short-circuit current recordings, the compound strongly inhibited TMEM16A activity. In contrast, in whole-cell patch-clamp experiments, T16inh-A01 had almost no effect. These results suggest for this compound an indirect mechanism of action that is influenced by experimental conditions. We also studied CaCCinh-A01. This compound was more generally effective in various cells and different experimental conditions and appears to be more useful as a probe to inhibit calcium-activated chloride channels. We monitored the intracellular calcium concentration in cells treated with and without CaCCinh-A01. We found that this compound causes a partial inhibition of calcium release from intracellular stores. This effect was much stronger when using niflumic acid, a classical inhibitor of calcium-activated chloride channels. These results indicate that alteration of intracellular calcium mobilization may be an important side effect of some chloride channel inhibitors, an undesired characteristic for a specific compound.
Finally, using a high-throughput functional assay, we screened a library of compounds with various biological activities. We found compounds that inhibited or activated TMEM16A-dependent activity. These compounds are now under study.

This work was supported by Telethon-Italy (GGP10026) and by the Italian Foundation for Cystic Fibrosis (FFC #2/2012).

SLC6A14 Enhances Constitutive CFTR-Mediated Cl^- Secretion in Human Non-CF and CF Primary Bronchial Epithelia

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Background: Genome wide association studies for gene modifiers in CF have recently identified *SLC6A14* as modifier gene for meconium ileus (Sun 2012). *SLC6A14* encodes for a Na^+/Cl^- dependent basic and neutral amino acid transporter (stoichiometry of 2Na+1Cl+1 amino acid). Functional expression of SLC6A14 ($B^{0,+}$) as transporter in human airway has been reported (Sloan 2003, Galietta 1998).
Objective: To investigate whether SLC6A14 activation impacts CFTR-mediated Cl^- currents in human airway epithelia.
Method: Primary human bronchial epithelial cells from CF (CFBE) and non-CF subjects (NBE) were obtained from lung transplants (Drs. Keshavjee and Zabner, University of Toronto and Iowa). SLC6A14 and CFTR-mediated ion transport were recorded as the transepithelial potential difference in Ussing chamber studies (ΔV_{te}, ΔR_{te} , calculated ΔI_{eq}). L-arginine (Arg) and L-lysine (Lys) were used as substrates and α-methyltryptophan (αMT, Karunakaran 2008) as inhibitor for SLC6A14; Forskolin (Fsk)/IBMX as well as $CFTR_{inh\text{-}172}$ were used to stimulate and inhibit CFTR-mediated conductance respectively.
Results: RT-PCR analysis and electrophysiological measurements demonstrate functional SLC6A14 expression in NBE and CFBE cells. Apical Arg activated SLC6A14 inducing a sustained depolarization in NBE (ΔI_{eq} = -1.6±-1.0 μA/cm^2, n=25). The Arg- induced ΔI_{eq} was competitively inhibited by Lys and α-MT confirming $B^{0,+}$ activity ($p< 0.05$, n=4). The SLC6A14 induced I_{eq} was not different in CF cells (ΔI_{eq} = -1.5±-2.2 μA/cm^2, p=0.2, n=8) and unaffected by CFTR activation (ΔI_{eq} = -1.4±-0.9 μA/cm^2, p= 0.3, n=25). Fsk/IBMX stimulated CFTR-mediated currents were not altered by activation of SLC6A14 in NBE ($\Delta I_{eq\ cAMP}$ =-2.7±-2.6 μA/cm^2 versus -3.0±-3.0 μA/cm^2 +/- SLC6A14 activation, p=0.41, n=25). In NBE cells, the $CFTR_{inh\text{-}172}$ sensitive I_{eq} ($\Delta I_{eq\ CFTRinh\text{-}172}$ =6.1±4.7 μA/cm^2) exceeded the Fsk/IBMX inducible I_{eq} ($p< 0.0001$), likely reflecting large constitutive CFTR activity. Therefore, we used the $CFTR_{inh\text{-}172}$ sensitive I_{eq} as read-out of CFTR-mediated I_{eq} , expressed as fraction of the total epithelial anion conductance (% of $\Delta I_{eq\ totalG}$). Following this analysis, SLC6A14 activation caused a significant increase in the $CFTR_{inh\text{-}172}$ sensitive I_{eq} (68±19% compared to 52±13% of $\Delta I_{eq\ total\ G}$ +/- SLC6A14, $p< 0.0001$, n=25). Similarly, use of Lys as SLC6A14 substrate increased the $CFTR_{inh\text{-}172}$ sensitive I_{eq} (60±12% versus 47±17% of $\Delta I_{eq\ total\ G}$ +/- SLC6A14, p=0.03, n=7) suggesting that SLC6A14 expression and not Arg influx *per se* is responsible for the effect on CFTR-mediated currents. In CFBE cells, we observed very small $CFTR_{inh\text{-}172}$ sensitive I_{eq} ($\Delta I_{eq\ CFTRinh\text{-}172}$ = 0.4±0.41 μA/cm^2), which were nevertheless further enhanced upon activation of SLC6A14 with Arg ($\Delta I_{eq\ CFTRinh\text{-}172}$ = 0.9±0.7 μA/cm^2, $p< 0.05$, n=8).
Conclusion: SLC6A14 is functionally expressed in non-CF and CF primary human bronchial epithelia. Activation of SLC6A14 increases constitutive CFTR-mediated I_{eq} measured as $CFTR_{inh\text{-}172}$ sensitive current in non-CF. Small constitutive CFTR-mediated I_{eq} were also detected in CF airway cells. These seemed to be enhanced with SLC6A14 activation. Current studies are performed to examine whether Arg-mediated NO- or AMPK-signaling may play a role in the observed interaction between SLC6A14 and constitutive CFTR activity, and/or whether the SLC6A14 effect on CFTR-mediated currents is induced by altering the electrochemical driving force for CFTR (e.g. re-cycling chloride at the apical membrane).

Signaling Detected at ENac N-Termini Controls ENac Proteolysis

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Background: ENaC (epithelial Na^+ channel) is rate limiting for Na^+ absorption by airway epithelia and hyperactivity of ENaC contributes to underhydration of cystic fibrosis airways. Limited proteolysis of ENaC extracellular domains stimulates ENaC open probability and we found that ENaC from CF airways is more cleaved than ENaC from normal airways. Based on reports that ubiquitination affects proteolysis of ENaC extracellular domains, we examined the contribution of lysine clusters in the initial N-termini of β- and γ-ENaC to the proteolytic regulation of ENaC.

Methods and Results: We injected Xenopus oocytes with wild type ENaC subunits or mutant subunits, in which lysine clusters were changed to glutamine or arginine, and used ENaC current (I_{Na}) and biochemistry to assess ENaC proteolytic regulation. Compared to WT ENaC, $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC had decreased basal I_{Na} but I_{Na} in both groups was sharply stimulated by exogenous trypsin. When co-expressed with matriptase, WT ENaC exhibited raised I_{Na} that could not be further stimulated by exogenous protease. In contrast, $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC maintained a low I_{Na} that was stimulated by exposure to trypsin, chymotrypsin or elastase. At baseline, mutant ENaC was expressed equally well at the surface, compared to WT ENaC, but cleavage fragments were sharply reduced. Based on these results, we reasoned that K to Q mutagenesis of ENaC N-termini protected mutant channels from matriptase. Indeed, close examination revealed that mutant channels were activated more slowly by exogenous protease (Time constant of 20 µg/ml trypsin activation: WT = 30 +/- 2 sec; $\alpha_{WT},\beta_{5Q},\gamma_{6Q}$ ENaC =122 +/- 15 seconds). Most of this effect was attributed to the N-terminus of γ-ENaC. We also discovered that mutagenesis of the γ-ENaC K-cluster to arginines had little effect on ENaC proteolytic regulation, suggesting that loss of net basic charge in the N-terminus of γ-ENaC was a major factor in slowing ENaC proteolytic activation. Co-expressed ubiquitin diminished I_{Na} and slowed acute stimulation by exogenous proteases, whereas co-expressed deubiqutinating enzyme 3 increased basal I_{Na} and sped its activation by exogenous proteases. These effects were absent when ENaC lacking N-terminal lysine cluster was studied. Although basal I_{Na} of ENaC co-expressed with matriptase could not be further stimulated by exogenous protease, oocytes expressing ENaC, matriptase and CFTR produced I_{Na} that was stimulated by trypsin. We found the time constant of acute stimulation of I_{Na} by exogenous proteases was increased 2-4 fold when CFTR was expressed.

Conclusion: We conclude that the basic residues of the initial N-terminus of γ-ENaC mediate interactions that promote susceptibility of ENaC extracellular domains to cleavage in an allosteric manner. Thus, cytosolic signaling events or conditions that foster or diminish these interactions, including ubiquitination and changes in ion activity may regulate ENaC proteolysis.

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Modulation of Epithelial Repair and Autophagy by Female Hormones in Normal and Cystic Fibrosis Human Epithelial Bronchial Cells

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Background: Cystic fibrosis (CF) is the most frequent recessive disease that can impact directly on an individual's longevity among Caucasians. In the lungs, the perturbation of Cl^- efflux leads to an abnormal thick mucus that cannot be removed by mucociliary clearance. The trapped mucus favours bacterial colonisation, promoting chronic infection and inflammation resulting in lung destruction. It has been proven that CF affects women more seriously than men. Indeed, female CF patients have shorter life expectancies than males [1] and frequency of lung exacerbations in CF females correlates with fluctuations of the concentration in estrogen (E2) in plasma during the menstrual cycle [2]. Epithelial repair plays a key role in CF because of the numerous lesions in the CF airways epithelia. This wound healing process involves different mechanisms such as cell migration, proliferation, apoptosis and autophagy. Autophagy is a process involving the degradation of a cell´s own components through the lysosomal machinery, via the ATG (AuTophagy related Gene) pathway. It has been shown recently that there is a lack of this process in CF [3]. In this context, the aim of this study was to test the effect of female hormones (17β-estradiol, E2, and Progesterone, P4) on epithelial repair and autophagy in CF and non-CF human bronchial epithelial cells

Methods: The effect of female hormones (E2 10 nM, P4 10 nM) on epithelial repair of a non-CF (NuLi-1) and a CF (CuFi-1) cell lines was determined by wound healing assay. Cells were grown on millicell transparent inserts and pictures taken every 3 hours. The modulation of autophagy by these female hormones was studied by immunoblotting and immunofluorescence using LC3B-II, p62 and Beclin-1 as autophagy markers.

Results: We show that, contrary to what has been published previously [5] there is no difference in the rate of wound repair after injury between CF and non-CF cells. This result can be explained by the conditions in which cells were grown i.e. our cells were differentiated on inserts. Performing wound healing assay, we show that female hormones did not affect the rate of wound healing in CF cells but E2 only can decrease epithelial repair in non-CF cells. As previously shown by Luciani *et al.*, there is less LC3B-II in CF than in non-CF cells, strongly suggesting a lack in autophagy in CF airway cells. E2 decreased the LC3B-II and increased p62 expression levels indicating that E2 is able to decrease autophagy in non-CF cells only. In confocal microscopy experiments, E2 seemed to inhibit non-CF and CF cell migration by modulating actin reorganization and decreasing the number of pseudopodes.

Conclusion: These results indicate that E2 can decrease lung repair after injury and autophagy in non-CF bronchial epithelial cells.

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References:

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[3] Luciani *et al*., Autophagy. 2011;7(1):104-6

Planar Cell Polarity Protein Network, Which Controls Ciliogenesis and Cilia Function, Is Altered in Human Cystic Fibrosis Bronchial Epithelial Cells

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Mucociliary clearance (MCC), abnormal in CF, is physiologically regulated by epithelial ion transport together with epithelial cilia movement and mechanical phenomenon such as cough and sneezing. Recently, new insights on ciliogenesis and cilia function have emerged. Planar Cell Polarity (PCP) is a tightly controlled protein network which drives the orientation of specialized structures (such as fly wings, mammal furs or fish scales) within the plane of the epithelial tissue. PCP has been described in mammal lung epithelial cells as a crucial mechanism controlling ciliogenesis and cilia function. Cilia structure and function have been studies in CF. Although the majority of these studies showed no structural abnormality and a normal cilia beat frequency (CBF), it has also been showed that ciliary disorientation, rather than ultrastructural abnormalities or slow CBF, may occur secondary to lung inflammation and result in delayed MCC. We hypothesized that CF HBEs may display abnormalities in PCP network which could further impair coordinated cilia function in the plan of the epithelium.

We demonstrated that HBEs expressed several PCP genes. Among them, expression of CELSR3 (Cadherin EGF LAG seven-pass G-type receptor 3) and Vangl-1 (Van-Gogh like 1) was down-regulated in CF (F508del/F508del) HBEs as compared to non-CF cells. In contrast, Fz3 (Frizzled 3), Fz6, Pk1 (Prickle 1) and Vangl-2 were upregulated in CF cells. Very low levels of CELSR3 protein were found in CF cells; moreover we observed that CELSR3 does not undergo autoproteolytic cleavage (in the endoplasmic reticulum) nor N-glycosylation (in the Golgi apparatus) as it normally does in normal HBEs.

These results suggest that expression and processing of PCP proteins is abnormal in CF-HBEs and may altered coordinated function of cilia within the bronchial epithelium in Cystic Fibrosis.

SLC26A9-mediated Cl^- Secretion Is Induced In Airway Inflammation and Prevents Mucus Obstruction in Mice

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Recent studies demonstrated that airway inflammation produces a pro-secretory airway epithelial ion transport phenotype characterized by upregulation of Cl^- secretion and inhibition of Na^+ absorption in mouse airways, and have identified active modulation of airway ion transport as an additional epithelial response in the complex in vivo pathogenesis of asthma (Anagnostopoulou P. et al. Eur J Respir 2010; 36:1436-1447). However, the molecular mechanism underlying Cl^- secretion and its relevance in asthma pathophysiology remain unknown. Recently, the solute carrier family 26, member 9 (SLC26A9) Cl^- channel has been shown to contribute to constitutive and cAMP-dependent Cl^- secretion in human bronchial epithelial (HBE) cells, where it has been suggested to interact functionally with CFTR in vitro (Bertrand CA *et al*. *J Gen Physiol*. 2009;133:421-438, Avella M. *et al*. *J Cell Physiol*. 2011;226:212-223). Based on its functional properties in transduced cells and expression pattern in human and mouse airways (Lohi H *et al*. *J Biol Chem*. 2002;277:14246-14254, Chang MH *et al*. *J Membr Biol*. 2009; 228(3):125-140), we hypothesized that SLC26A9 may function as an alternative Cl^- channel that may contribute to ASL homeostasis in health and inflammatory airway disease. To determine the role of the SLC26A9 Cl^- channel in airway inflammation, we compared transepithelial ion transport in freshly excised bronchial tissues, lung morphology, and airway mucus content in wild-type versus *Slc26a9*-deficient (*Slc26a9*$^{-/-}$) mice under physiological conditions and after intratracheal instillation of IL-13 to model Th2-mediated airway inflammation. Further, in a large human study population of 658 healthy children and 661 children with asthma, we tested whether polymorphisms in the *SLC26A9* gene are associated with asthma and determined the functional relevance of a select SNP (rs2282430) in the 3′ UTR of *SLC26A9* in luciferase gene reporter assays.

We found that IL-13 treatment increased Cl^- secretion in the airways of wild-type but not *Slc26a9*$^{-/-}$ mice. While IL-13-induced mucus overproduction was similar in both strains, treated *Slc26a9*$^{-/-}$ mice exhibited airway mucus obstruction, which did not occur in wild-type controls. Moreover, in silico analyses predicted that the A allele of rs2282430 (SNP) strengthens binding of *hsa-miR-632* to the *SLC26A9* 3′ UTR causing reduced protein expression in vitro providing initial evidence that *SLC26A9* may be involved in the pathogenesis of obstructive lung disease in humans.

Our data demonstrate that the SLC26A9 Cl^- channel is activated in airway inflammation and suggest that SLC26A9-mediated Cl^- secretion is essential for preventing airway obstruction in inflammatory airway disease. These results suggest SLC26A9 as a potential disease modifier and novel therapeutic target in airway diseases caused by increased mucus secretion and deficient hydration of airway surfaces, such as CF.

An LC-MS Lipidomics Study in Lung Lavage Samples from Infant CF Patients. Prospects for Individualised Therapy of Cystic Fibrosis Using Integrated Clinical and Preclinical Research Platform (AREST-CF)

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Since the identification of the CFTR gene in 1989 it has become apparent that the large variation in pathology among CF patients is due to a combination of environmental and genetic factors. This raised the question whether identification of these factors would lead to new and individualised therapeutic options.
The combined CF clinics Erasmus MC Rotterdam (Prof Tiddens et al) and Utrecht Medical Center (Prof Van der Ent et al) are now engaged in an intensive longitudinal screening protocol, pioneered by the Australian CF community (AREST-CF), in which all Dutch CF patients identified by perinatal screening (heelprick/genetics), are followed by regular checkups including CT scans, bronchoscopy and collection of lavage fluid (BALF). This material provides a growing base of genetic, clinical, microbiological and biochemical material, which offers valuable new opportunities for translational research and personalised medicine.
We and others observed abnormalities in the metabolism of bioactive lipids in the lungs of CFTR mutant mice. These molecules are involved in the resolution of inflammation and tissue remodelling. The AREST-CF platform offers a unique opportunity to establish lipid biomarkers in druggable enzymatic pathways, and to perform intervention studies in infant and juvenile CF patients.
Using a state of the art LC-MS platform (Rob Vreeken et al, Leiden metabolomics center) we have established a method to analyse a panel of oxylipids, polar lipids and fatty acids in BALF from CF infants (1-3 years). This technique was applied to selected samples obtained from the Australian AREST-CF consortium (Prof Steve Stick et al). Ten CF infants with a low-moderate bronchiectasis score, an equal number of CF patients without bronchiectasis, and non-CF patients with chronic inflammation were analysed. Cluster analysis shows that we can distinguish CF from non-CF patients, and CF Patients with high bronchectasis score from CF patients without bronchiectasis. We are presently investigating the opportunities for an novel intervention study based on these data.
This new approach to monitor the development of CF pathology in individual patients from birth (AREST-CF) offers valuable new opportunities for translational research. Correlating biomarker studies with progression of pathology allows us to identify new therapeutic targets, monitor the effect of therapeutic intervention and offer treatment tailored to the individual patient.

TMEM16A/ANO1 Expression in Human Bronchial Epithelia

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TMEM16A protein, also known as anoctamin-1, is an important component of calcium-activated chloride channels (CaCCs). TMEM16A expression and function in human bronchial epithelia is strongly upregulated by IL-4 and IL-13, Th-2 cytokines that induce mucus hypersecretion and goblet cell hyperplasia. We have recently reported that TMEM16A hyperexpression induced by IL-4 occurs mostly in goblet cells whereas CFTR is expressed in ciliated cells (Scudieri et al., J Physiol 590: 6141-6155, 2012). Since cystic fibrosis (CF) is also characterized by mucus hypersecretion, we studied TMEM16A expression in freshly excised CF bronchi and in cultured bronchial epithelia under CF-like conditions. Histological sections of CF bronchi, obtained at the time of lung transplant, were studied by immunofluorescence. We found no evidence of TMEM16A hyperexpression in the surface epithelium despite the abundant presence of goblet cells (detected by MUC5AC staining). We also investigated TMEM16A expression in cultured bronchial epithelia incubated for 24 hours with the supernatant of P. aeruginosa cultures or with single bacterial components. Under this condition, we found little increase in TMEM16A expression and function. The most effective stimulus, pyocyanin (60 µM), increased the percentage of TMEM16A-expressing cells and calcium-dependent chloride secretion by only two-fold. In contrast, incubation with IL-4 (10 ng/ml) increased TMEM16A cells and chloride secretion by more than ten-fold. Our results in cultured cells indicate that strong TMEM16A hyperexpression in goblet cells is a phenomenon specifically evoked by Th-2 cytokines and not by conditions mimicking CF airway infection. These results appear to be consistent with the relatively low expression of TMEM16A in CF airways in vivo. Pharmacological stimulation of TMEM16A expression in vivo could be a possible approach to enhance anion transport in CF airways.

Room: Granada

Posters – Inflammation and Bacterial Killing in CF

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Vardenafil Restores Pro/Anti-Inflammatory Balance in Mouse CF Macrophages

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Lung inflammation is a key feature of CF pathology. We have previously shown that, even in basal conditions, $CFTR^{tm1Eur}$ (homozygous F508del-CFTR) mice display an increased number of alveolar and peritoneal macrophages compared to their WT littermates. Promising anti-inflammatory properties have been reported for Vardenafil, a phosphodiesterase type 5 inhibitor (iPDE5). We have recently demonstrated that this iPDE5 reduces LPS-induced inflammatory responses in CF mice (Lubamba et al., 2012). We hypothesized that: 1) the activity of macrophages is altered in CF and 2) macrophages represent target effector cells of the anti-inflammatory effect of vardenafil.

Macrophages were isolated and purified from lung homogenates and peritoneal lavages from CF and WT mice. To test the hypothesis that the activity of macrophages is altered in CF, LPS-induced inflammatory responses were first evaluated. Then macrophage differentiation in pro- (M1) and anti-inflammatory (M2) effectors was studied after polarization with LPS and IFN-γ or IL-4 and IL-13, respectively. Pro- (TNF-α, IL-1β and CCL-2) and anti-inflammatory (FIZZ-1 and mYm1) mediators were quantified by ELISA (in culture supernatants) or by quantitative RT-PCR (in cultured macrophages). In each condition, the effect of vardenafil (50µM) was evaluated.

CF lung and peritoneal macrophages displayed an exaggerated pro-inflammatory response to LPS and vardenafil treatment reduced it. M1/M2 polarization was altered in CF macrophages. In lung and peritoneal macrophages, M1 response was at least 4-fold larger in CF than in WT cells. In contrast, the M2 response was reduced in CF macrophages. These results confirm a deregulation of the pro/anti-inflammatory balance in CF macrophages. Vardenafil restores the balance by reducing the expression of pro-inflammatory mediators (particularly TNF-α and IL-1β) in lung and peritoneal macrophages. Moreover, vardenafil corrects the overproduction of FIZZ-1 in CF macrophages.

Our results show that macrophages play a critical role in inflammatory responses in CF. Differentiation of macrophages from different body compartments is oriented towards a pro-inflammatory profile associated with a decreased anti-inflammatory status. The immunomodulatory effect of vardenafil could thus be beneficial in CF pharmacotherapy.

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Lipoxin A_4 Delays the Invasion of Cystic Fibrosis Bronchial Epithelial Cells by the Pathogen *Pseudomonas aeruginosa*

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Cystic fibrosis (CF) caused by the mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene results in a decreased Cl^- secretion and hyperabsorption of Na^+ in the airways leading to dehydration of the Airway Surface Liquid (ASL) layer. The reduction in ASL height impairs mucocillary clearance and favours lung infection and inflammation. The eicosanoid lipoxin A_4 (LXA_4) described as a signal for the resolution of inflammation, is decreased in the lungs of patients with CF (*Karp et al, 2004*). We have previously shown that LXA_4 stimulates Cl^- secretion *(Bonnans et al, 2003)*, increases ASL height in Human Bronchial Epithelial (HBE) and Cystic Fibrosis Bronchial Epithelial (CFBE) cells (*Verriere et al, 2012*) and stimulates ZO-1 expression and transepithelial electrical resistance (TER) in Human Airway Epithelial Cells (*Urbach et al, 2008*). We hypothesize that decreased levels of LXA_4 in CF airways could favour the invasion of HBE and CFBE cells by microorganisms. CF lungs are chronically infected by *P. aeruginosa* by adolescence and this leads into the progressive lung destruction. Using a gentamyicin invasion assay and confocal microscopy we investigated the role of LXA_4 on the epithelial integrity of HBE and CFBE when infected with *P. aeruginosa*. LXA_4 (1nM) alone did not affect *P. aeruginosa* growth but prevented the invasion of HBE and HCFBE cells by *P. aeruginosa* within the first 4 hours after inoculation. These results were confirmed by visualisation using confocal microscopy. We report a novel role for LXA_4 in delaying the invasion of CFBE by a microorganism, which may lead to a new therapeutic route for CF patients.

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Regulation of Corticosteroid Binding Globulin (CBG) in the inflammatory context of cystic fibrosis

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Background: Cystic Fibrosis (CF) is characterised by chronic lung inflammation. In CF, glucocorticoids (GC) are a widely used therapeutic tool. However, their efficiency, and the benefit/risk ratio are still discussed. In plasma, 90% of GC is bound to the chaperone protein CBG which regulates its bio-disponibility. CBG is mainly produced by the liver. Recent works enlightened the fact that, more than a simple carrier protein, CBG could also address GC specifically to the inflammation site, thereby modulating the response to GC in an inflammatory context.
Objectives: Study the expression and regulation of CBG in the liver and assess its pulmonary expression in the inflammatory context of CF.
Methods: Hepatic *levels of CBG*: Biopsies from healthy donors, cirrhotic CF and non CF patients: measure of the transcripts levels of CBG and interleukin-6.Hepatocarcinoma derived cell-lines Hep3B and HepG2: regulation of CBG expression.
Lung levels of CBG: Lung biopsies; expression of CBG; Bronchial epithelial cell lines; regulation of CBG expression.
Results: We show an increase in CBG expression (i) in the liver and lung of CF patients; (ii) in the hepatic and lung cell lines in an inflammatory context. GC has no effects on CBG expression in hepatic cell lines, but increases CBG levels in the lung cell lines.
Discussion: We show stimulation of the expression of CBG in the inflammatory context of CF. Comparative results from hepatic and lung cell lines enlighten a different regulation of CBG expression. Overall, increase in CBG expression in CF could mean initially a decrease in GC bio-disponibility but, ultimately, an enhanced corticosteroid half life and possible prolonged effects.

Classically Activated Macrophages Promote Inflammation in Response to *Pseudomonas aeruginosa* but Do Not Provide an Effective Barrier against Bacterial Growth

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Pseudomonas aeruginosa (PA) is an opportunistic pathogen that causes severe respiratory infections in susceptible individuals (e.g. cystic fibrosis (CF) patients). Th1-dominated responses are considered protective against PA infection. CF patients have been proposed to have a Th2-biased immune response suggesting that lack of a Th1 response could contribute to the establishment of chronic infection. We have compared IFN-g production by CF and healthy peripheral blood mononuclear cells (PBMCs) in response to several stimuli. In agreement with these original observations we have detected a significant reduction in IFN-g production by CF PBMCs in response to the mitogen phytohaemagglutinin (PHA) and the superantigen Staphylococcal enterotoxin B (SEB) and established a positive correlation between IFN-g production and lung function measured as FEV_1.

Because classically activated macrophages (M1) are considered key effector cells in Th1 responses we investigated how a Th-1-dominated immune response would affect the interaction of PA with macrophages. Our results show that classical activation in the presence and absence of the pro-inflammatory cytokine GM-CSF did not enhance bacterial clearance or macrophage survival significantly and these were not affected by bacteria opsonisation. On the other hand, IFN-g shifted the cytokine response in response to PA towards increased inflammation. In particular, it caused a dramatic up-regulation of MCP-1 and TNF-a expression and down-regulation of IL-10 expression by PA-infected macrophages. Comparison of TNF-a vs IL-10 and IL-6 vs IL-10 ratios revealed the following hierarchy: M-CSF < M-CSF + IFN-g ≤ GM-CSF < GM-CSF + IFN-g. This shift would result in the recruitment and activation of a variety of host cells including antigen-presenting cells, neutrophils, and lymphocytes. These results indicate that rather than exerting a major microbicidal function, macrophages, even when classically activated, act as regulators of the inflammatory response to PA.

Non-lysosomal Beta-glucosidase 2 (GBA2) as a Target of the Anti-Inflammatory Effect of Miglustat

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Sphingolipids (SLs) are complex membrane lipids with emerging importance for many respiratory disorders including CF (Yang and Uhling, 2011). In particular, SL ceramide plays an important role in the infection by *P.aeruginosa* and it has been suggested that the pathophysiology associated with CF could be at least partly corrected by normalizing the pulmonary ceramide levels. Miglustat, a well known inhibitor of SL metabolism produces an anti-inflammatory effect in vitro and in vivo and reduces the ceramide expression induced by *P.aeruginosa* (Dechecchi, 2011). In this study we addressed the biochemical pathways that are impacted by Miglustat, focusing our attention on non-lysosomal beta glucosidase 2 (GBA2) which hydrolyzes GlcCer, generating free glucose and ceramide and is very sensitive to Miglustat (Walden, 2007). To this aim, we treated IB3-1 and CuFi-1 cells with different doses of Genz 529468 (MZ-21), a very potent inhibitor of GBA2 (Shen, 2004) and we found a significant reduction of IL-8 mRNA expression in response to *P.aeruginosa* (IC_{50}= 7 nM). Based on these results, we used specific siRNA to transiently reduce the expression of GBA2 in CuFi-1 cells before *P.aeruginosa* infection. In these conditions GBA2 expression was significantly reduced by about 60% and, in parallel we observed a significant decrease of IL-8 by about 50% both as mRNA and protein release. These results were confirmed in primary epithelial bronchial cells from CF patients. We also measured the total cell activity of GBA2 in CuFi-1 cells and we found that *P.aeruginosa* increases GBA2 activity by about 30%. Previous assays excluded the presence of GBA2 activity in *P.aeruginosa* inocolum. Very importantly, the treatment with Miglustat decreases the activity of GBA2 at about one third also after the infection with *P.aeruginosa* in CuFi-1 cells. Moreover, Liquid Chromatography-Mass Spectrometry (LC-MS) was done to understand the modulation of different ceramides in CuFi-1 cells treated with Miglustat or Genz 529468 (MZ-21), before infection with *P.aeruginosa*. The results showed that infection with *P.aeruginosa* up-modulates biologically active ceramide species and, interestingly both Miglustat and Genz 529468 (MZ-21) selectively reduce specific ceramide species (i.e.C22). These findings demonstrate that GBA2 is a target of the anti-inflammatory effect of miglustat and provide novel insights on the involvement of GBA2 in the signaling cascade activated by *P.aeruginosa* in bronchial epithelial cells

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Abnormal CFTR Affects the IL-17 Pathway in Cystic Fibrosis

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Cystic fibrosis (CF) is a debilitating, life-limiting disease caused by the loss / impairment of a cellular chloride channel known as CFTR. It is the most common hereditary disorder in Caucasian populations. CF patients are extremely susceptible to chronic respiratory infections caused by extracellular bacteria and fungi, but the reasons for this susceptibility are still unknown. As respiratory infections are the primary cause of morbidity and mortality in CF patients, understanding their establishment and discovering novel therapeutic targets for their prevention / control are essential.

Our data demonstrate that peripheral blood mononuclear cells (PBMCs) from CF patients produced significantly less interleukin 17A (IL-17A) than healthy control PBMCs upon in vitro challenge with a range of stimuli. A similar impairment in IL-17A occurred in healthy PBMCs upon pharmacological inhibition of CFTR. Interestingly, interferon g (IFN-g) - another cytokine whose expression has been found to be impaired in CF patients was not affected by CFTR inhibition in healthy PBMCs, suggesting that CFTR function is intrinsically linked to the induction of an IL-17 response.

IL-17 is a pro-inflammatory cytokine important in granulopoiesis and neutrophil chemotaxis and activation. It has been postulated as a therapeutic target in CF because of both its importance in protection against extracellular bacterial and fungal infections and also its potential role in modulating the host immune response which may result in pulmonary destruction. As with other autoimmune and inflammatory diseases such as Crohn's, psoriasis, multiple sclerosis, and rheumatoid arthritis, overproduction of IL-17 has been hypothesised to drive the chronic pulmonary inflammation seen in CF. This hypothesis is based on the comparison of IL-17A and IL-17F levels in the sputum / bronchoalveolar lavage (BAL) / bronchial biopsies of CF patients vs. control subjects. These studies, as in the case of our investigation using CF PBMCs, can't distinguish between primary defects caused by CFTR deficiency and immune adaptation to the chronic inflammation experienced by CF patients. Our results using CFTR inhibitors [GlyH-101 and $CFTR_{inh172}$] are the first to indicate that CFTR itself could play a role in the induction of IL-17-mediated inflammation. In contrast, our data also suggest that the reduction in IFN-g production observed in CF patients, not seen in the presence of CFTR inhibitors, is likely due to an adaptation to recurrent infection. It is essential to understand the precise role of the CFTR / IL-17 axis in CF respiratory disease given the crucial role that IL-17 plays in mediating immunity against extracellular pathogens at mucosal surfaces, and its potent effects on neutrophils. This knowledge can be used to direct the development of therapeutic approaches aimed at eradicating chronic respiratory infections whilst controlling inflammation-mediated pulmonary damage.

Neutrophil Elastase-mediated Increase in Airway Temperature during Inflammation

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Objectives: *P. aeruginosa* is the dominant pathogen in chronic lung infections in CF The reason(s) for this selection is unclear.
Methods: In CF patients and healthy controls we determined the exhaled airwaytemperature using PLET and the temperature in the airway lumen and in mucus plugs using a probe inserted into a flexible bronchoscope.We determined the enthalpy of the binding reaction between purified human neutrophil elastase (NE) and its endogenous inhibitor α_1-PI using isothermal titration calorimetry. In infected wildtype and isogenic $NE^{-/-}$ mice, we determined the pouch airspace temperature 4 days after *P. aeruginosa* challenge.We cultured bacterial pathogens at 30°C and 38°C for 96 h anaerobically *in vitro*, and measured their density. We compared the transcriptome of *P. aeruginosa* after anaerobic growth at 30°C and 38°C and sequenced the microbiota in 8 paired early and late CF sputum specimens.
Results: PLET temperatures in 56 CF patients were inversely correlated to lung function,suggesting that bronchial obstruction increases airway temperature perhaps due to obstruction. A significantly higher mean temperature of 37.98±0.80°C was measured within mucus plugs in the anterior upper lobe segmental bronchi of 5 CF patients as compared to the airway lumenal temperatures (36.62±0.91°C) ($p< 0.05$) which were similar to that of 3 healthy individuals (36.2±0.66°C). When a 150 µM α_1-PI solution was titrated into a dilute (15 µM) NE solution, the initial injection peaks revealed an exothermic reaction with a binding enthalpy of -18.5±1.3 kcal/mol. Temperatures in pouch airspaces of infected WT mice were significantly higher compared to controls, implicating an exothermic NE:α1-PI complex formation as a relevant mechanism for the local temperature rise. After 96 h at 38°C, the densities of *S. epidermidis, S. aureus, S. maltophilia, P. putida, B. cenocepacia* and *M. abscessus* were significantly lower (or remained constant) vs. 30°C while only *P. aeruginosa* grew at 38°C. At 39°C this difference became even more pronounced. In contrast, *P. aeruginosa* grew at both elevated temperatures. Growth of *P. aeruginosa* was not dependent on the production of alginate because a mucoid *P. aeruginosa mucA*$^{-/-}$deletion mutant grew similarly well as the non-mucoid parent strain and the isogenic non-mucoid variant *P. aeruginosa mucA*$^{-/-}$*/algT*$^{-/-}$. Furthermore, loss of the Type 6 secretion systems (T6SS) did not affect growth. Collectively the data show that from the investigated bacterial strains, only *P. aeruginosa* is capable to grow at elevated temperature. We identified 858 differentially expressed genes at 38°C vs 30°C. Virulence genes, including components of the T3SS and components of the LasR regulated quorum sensing system were significantly up-regulated at 38°C compared to 30°C, suggesting that *P. aeruginosa* particularly activates its protective armory at higher environmental temperatures. In addition, a Mg^{2+} transporter known to enhance the thermotolerance by stabilizing proteins or protein-nucleic acid interactions was highly up-regulated at 38°C.The mean *P. aeruginosa* relative abundance was 0.191 in early sputum samples and 0.592 in late samples.
Conclusion: NE mediates a temperature increase in mucus plugs of CF patients which favours the selection of *P.aeruginosa.*

Residence in Biofilms allows *Burkholderia cepacia* Complex (Bcc) Bacteria to Evade Neutrophil Anti-microbial Activities

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The primary morbidity of Cystic fibrosis (CF) -the generation of unusually viscous pulmonary mucous- greatly diminishes mucociliary clearance from the lung. This engenders an environment well-suited to colonisation by microorganisms. However, despite the continuous recruitment of neutrophils into the airway lumen, many of these microorganisms persist.

Bacteria of the *Burkholderia cepacia* complex (Bcc) are such persistors. Though less prevalent among CF patients than *Pseudomonas aeruginosa*, Bcc members are disproportionally associated with patient mortality owing to a unique morbidity they may induce, termed '*cepacia* syndrome', which is characterised by an acute-onset deterioration of lung function with associated septicaemia. Treatment is a challenge as most members of Bcc are multi-drug-resistant and display an ability to form biofilms, further reducing their antibiotic susceptibility.

Therefore, we examined the role of biofilm formation in protecting Bcc bacteria from neutrophil anti-microbial activity. The nature of the physical biofilm-neutrophil interaction was visualised using confocal laser-scanning microscopy (CLSM). In addition, the impact of neutrophils, or components thereof, on biofilm development was investigated using microtiter plate assays of biofilm density. We also investigated the effect of culture in the presence of Bcc biofilm on neutrophil anti-microbial activity and on the fate of the neutrophil.

CLSM confirmed that differentiated, neutrophil-like HL60 cells (dHL60s) remained at the exterior surface of established *B. multivorans* LMG 13010 biofilms (72 h) up to 2 hours after inoculation, impeding phagocytosis. This was despite limited migration and the appearance of pseudopodia.

The CF-associated species LMG 13010, *B. cenocepacia* K56-2 and *B. dolosa* LMG 18941 were grown as static biofilms for 4, 24 or 48 hours, at which point dHL60 cells were added. Following 24 and 48 hours co-incubation, biofilm biomass was determined using crystal violet.

Culture in the presence of dHL60 cells lead to reduced initial formation of biofilm with respect to untreated biofilms, however, during 72 h maturation, greater biofilm biomass was formed by LMG 13010 and LMG 18941 in the presence of dHL60s ($p < 0.01$).

As neutrophils undergo apoptosis and disintegrate shortly after recruitment to the lung, this experiment was repeated with whole-cell lysates of dHL60 cells in order to investigate the effect of neutrophil components on biofilm development. Again, biofilm density increased after incubation with lysed dHL60s for LMG 13010 and LMG 18941($p < 0.001$).

These results suggest that Bcc members produce denser biofilms when incubated with dHL60 cells, either through interaction with viable neutrophils or components thereof, or incorporation of exogenous molecules such as DNA or actin, and that this impedes phagocytosis.

Furthermore, dHL60s secreted disproportionately low amounts of IL-8 when exposed to biofilms relative to planktonic bacteria ($p < 0.001$) and extracellularly degranulated less myeloperoxidase enzyme when added to more mature biofilms (48-72 h).

Hence, biofilms facilitate Bcc species' persistence in the CF lung by masking them from detection and acting as a barrier, and the presence of neutrophils reinforces that biofilm. Therefore, strategies to improve neutrophil efficacy in clearing colonising Bcc must focus on overcoming biofilm-mediated resistance.

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Potential Role of Expression of E3 Ubiquitin Ligases in Regulating the Inflammatory Phenotype of Cystic Fibrosis

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Cystic Fibrosis (CF) disease is characterized by an aggressive inflammatory response and chronic infection in the airway. One important marker of CF lung disease, the pleiotropic cytokine TGF-beta[1,2], is negatively regulated by E3 ubiquitin ligases, which have been found to be dysregulated in previous studies of F508del-CFTR-related gene expression[3].

To understand the role of E3 ubiquitin ligases in CF, we studied the effects of: 1) F508del mutation and 2) exposure to TGF-beta and TNF-alpha cytokines on mRNA and protein expression of the E3 ubiquitin ligases SMURF1, SMURF2 and NEDD4L in polarized CF bronchial epithelial cell models.

Using real-time quantitative PCR, we demonstrated that the F508del mutation is not sufficient to induce significant differential expression of E3 ubiquitin ligases. However, the F508del-CFTR genotype altered the responsiveness of E3 ubiquitin ligases to both inflammatory cytokines. Our results showed that both TGF-beta and TNF-alpha increased the expression of *SMURF2* mRNA in F508del-CFTR CFBE cells, suggesting an up-regulation of this E3 ubiquitin ligase under inflammatory status. In addition, this increased expression was consistent with an observed decrease in *SMAD2* and *SMAD3* mRNA expression.

These results suggest that increased expression of E3 ubiquitin ligases in CF under inflammation could partly be responsible for the increased pro-inflammatory mediators that characterize CF disease, via an inhibition of the Smad-dependent anti-inflammatory effects of TGF-beta.

Our preliminary data strengthen the potential implications of differential expression of enzymes involved in ubiquitination on modulation of inflammatory responses in CF.

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Anti-Inflammatory Effect of FDA Approved Drugs in CF Cells

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Pulmonary disease is the main cause of morbidity and mortality in cystic fibrosis (CF) patients due to exacerbated inflammation. Loss of CFTR activity is associated with dysregulation of transcription factors implicated in the inflammatory pathway such as NF-κB, AP-1 and PPARγ and the abnormal activation of NF-κB leads to increased Interleukin-8 (IL-8) secretion.
Our aim is to test FDA approved drugs having yet undefined anti-inflammatory effect in CF airway epithelial cells.
We first tested the effect of three molecules codified ATB-3A, FLJ-1 and JR-01 and compared them to ibuprofen, on HeLa cells overexpressing wild-type or F508del-CFTR and Calu-3 cells. We performed luciferase reporter gene assays in order to measure i) IL-8 promoter activity, and ii) the activity of synthetic promoters containing either NF-κB or AP-1 or PPARγ responsive elements. We then assess IL-8 secretion by immunodetection with Immulite®. Finally, the effect of these molecules was studied on CFTR expression, and chloride channel activity by western blot and the fluorescent chloride indicator N(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) respectively.
We observe a significant inhibition of IL-8 secretion in airway epithelial cells after ATB-3A treatment, which was associated to a reduced IL-8 promoter activity in the different cell lines. At the molecular level, we showed an inhibition of NF-κB and AP-1 transcriptional activity, whereas PPARγ activity was enhanced. We then obtained similar results with FLJ-1 and JR-01 on NF-κB and AP-1 activity in addition to a huge response to FLJ-01 on PPARγ activity. Treatment with ibuprofen or ATB-3A induces a significant increase of mature form of CFTR in wt-CFTR HeLa cells. In the case of ATB-3A, this enhancement was also associated with a higher chloride channel activity in wt-CFTR HeLa cells. An increase of CFTR activity was also observed in human primary nasal epithelial cells as assessed by short-circuit measurement. These data suggest that ATB-3A could also act as a CFTR potentiator.
Altogether, these results are consistent with a global anti-inflammatory effect of these drugs relevant in the CF context. Further experiments are ongoing on CF and non CF hNEC cultured at the air-liquid interface and CF mouse model.

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Involvement of Enzymes of the Protein Disulphide Isomerase Family in the Interaction of *Burkholderia cenocepacia* with Epithelial Cells

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As a consequence of CFTR mutations, the airway surface liquid (ASL) of CF patients is characterized by a marked decrease in reduced glutathione (GSH) and by the inability to modulate extracellular GSH concentration in response to oxidative stimuli or to bacterial infections. To investigate whether such defect in GSH export may favor lung colonization by opportunistic pathogens, we have recently analyzed the effects of extracellular GSH on the ability of *Burkholderia cenocepacia* to interact with epithelial respiratory cells. We have observed that extracellular GSH can drastically reduce *B. cenocepacia* ability to adhere and invade epithelial cells (either stable cell lines or primary cells from the lung of a transplanted CF patient). This effect is correlated to a drastic reduction of the expression of the proinflammatory cytokines IL-8, TNF-α and IL-1β and to a GSH-dependent increase in the number of free thiols on the surface of epithelial cells. These results suggested that GSH-mediated modifications of the oxidoreductive status of membrane proteins containing labile disulfide bonds can be useful to contrast *B. cenocepacia* infections.

Quite unexpectedly, we have more recently found that a similar reduction of bacterial invasion may be obtained by treating cells with the powerful thiol oxidant DTNB. The comparable effects of agents able either to reduce disulphide bonds or to oxidize free thiols prompted us to investigate the possibility that high levels of extracellular GSH interfere with the activity of enzymes catalyzing the formation and breakage of disulfide bonds. Mechanisms of this kind are known to be used by HIV or by *Chlamydia* to penetrate within host cells. In support to this view, we have found that bacitracin, an inhibitor of enzymes of the protein disulphide isomerase (PDI) family, decreases *B. cenocepacia* adhesion and invasion to levels comparable to those obtained with GSH or DTNB. Notably, although bacitracin has antibiotic activity against Gram-positive bacteria, it has no effects on *B. cenocepacia* growth. A reduction of *B. cenocepacia* invasivity was also obtained also with other PDI inhibitors. Taken together, our observations suggest that low levels of extracellular GSH may promote *B. cenocepacia* lung infections in CF by creating conditions favorable for the activity of membrane-associated enzymes of the PDI family.

On the one hand our study suggests that therapies aimed at restoring normal levels of GSH in the ASL might be beneficial to control lung damage in CF patients colonized by *B. cenocepacia*. On the other hand, the observation that disulphide exchanging enzymes contribute to *B. cenocepacia* invasivity supports the hypothesis that drugs targeting DPI could compensate for the lack of GSH in the epithelial lining fluids of CF patients. This possibility would be important to overcome a major limitation of the discontinuous supply of GSH through aerosol, i.e. its rapid tendency to oxidize.

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Characterization of the Effect of *Burkholderia cenocepacia* Exoproteome in Human Cystic Fibrosis Lung Epithelial Cell Models

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Bacterial infections of the airways of cystic fibrosis (CF) patients are one of the major causes of morbidity and mortality in CF disease. *Burkholderia cenocepacia* is an opportunistic bacterial species characterized by long-term colonization causing diminished lung function and in some instances accompanied by "cepacia syndrome". These bacteria develop a high-level resistance to the best known clinically used antibiotics, causing serious complications in CF treatment. Airway epithelial cell responses to the *B. cenocepacia* exoproteome are poorly characterized. Our objective was therefore to study the effect of addition of *B. cenocepacia* IST4113 strain[1] supernatant on 1) Cystic Fibrosis transmembrane conductance regulator (CFTR) protein expression, 2) epithelial cell integrity and finally 3) inflammatory signalling pathways in polarized human CF lung epithelial cell models expressing F508del-CFTR and infected with *B. cenocepacia* supernatant. CFTR protein analysis by Western blot demonstrated an interference of factors secreted by *B. cenocepacia* IST4113 strain on both WT- and F508del-CFTR expression, showing an apparent decline of protein levels, without, however, any effect on *CFTR* mRNA expression, as measured by real-time quantitative PCR (qPCR). This suggested an interference of an unknown component of the *B. cenocepacia* exoproteome on post-translational processing of the CFTR protein. In addition, our data demonstrated undisturbed organization of tight junction-associated cytoplasmic protein ZO-1 and E-cadherin, as well as, differentiation markers such as cytokeratin 18 and 14 following exposure to *B. cenocepacia* supernatant, suggesting the absence of a secreted factor capable of disrupting epithelial cell integrity. Analysis by qPCR also demonstrated that this bacterial supernatant causes down-regulation of *SMURF1* and *NEDD4L* gene expression, which are negative regulators of the TGF-beta signalling pathway, in F508del-CFTR CFBE cells. This study suggests that infection with the *B. cenocepacia* IST4113 strain may interfere with expression of F508del-CFTR. Understanding the mechanism behind these alterations will be crucial for understanding the impact of bacterial infection on the CF airway and in the design of improved therapeutic strategies. *The authors thank Professor Dr. Isabel Sá Correia (IST, Lisboa, PT) for the B. cenocepacia supernatants.*

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Electrophysiology Measurement of the Functional Properties of CFTR in Human Monocytes

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CFTR is a protein widely distributed in a variety of cell types. We have recently shown that human monocytes express CFTR and we developed a single-cell fluorescence imaging assay to evaluate CFTR activity in isolated cells. In the present work, we characterized the electrophysiological properties of CFTR, by patch clamp technique, in human monocytes from healthy and CF subjects and related it to single cell fluorescence imaging. cAMP-dependent chloride currents were detected only in cells from healthy subjects with a concordance between the two methods. These data further support previous observations from our group showing that monocytes could be useful for diagnosing CF and possibly to assess the effects of drugs active on the basic defect.

Modification of the Salivary Secretion Assay in F508del Mice - Salivary Chloride Quantification

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In 2004 Best and Quinton[1] established the salivary secretion assay in mice for the *in vivo* characterization of new drugs against Cystic Fibrosis (CF). However, limited *in vivo* data are available and the predictive value of this assay for treatment effects in CF patients is not fully established.Therefore, we revisited the salivary secretion assay and systematically investigated the salivary secretion rates in different CF mouse models, namely in CFTR knockout mice (CFTR ^{tm1Unc}) as well as in the F508del CFTR mice of different origin (CFTR^{tm1Kth} and CFTR^{tm1Eur}). In addition, we quantified salivary chloride content in these mice since in CF-patients, increased sweat chloride is an established diagnostic tool.

The β-adrenergic salivary secretion response in the presence of Atropine was significantly reduced compared to WT mice. Salivary secretion rates of CF CFTR^{tm1Kth} and CF CFTR^{tm1Eur} mice were reduced 13-fold and 10-fold respectively when compared to WT mice. Salivary rates of CF CFTR^{tm1Unc} mice were 30-fold reduced. Saliva was collected after Pilocarpine stimulation and chloride levels were quantified using a colometric Chloride determination assay. As sweat chloride content in CF-patients, saliva chloride was significantly elevated in CF mouse models. The chloride concentrations were 2.1-fold higher in CF CFTR^{tm1Unc}, 1.8-fold in CF CFTR^{tm1Kth} and 3.3-fold in CF CFTR^{tm1Eur} when compared to WT mice.

Salivary secretion rates and salivary chloride content in CF-mice reflect sweat secretion and sweat chloride content in CF patients. Determination of salivary secretion rates, extended by quantification of salivary chloride content in different CF-mice renders the salivary secretion assay a powerful tool for validation of new CF-treatments.

[1] Best JA, Quinton PM. Salivary secretion assay for drug efficacy for cystic fibrosis in mice. Exp Physiol.2005; 90(2):189-193.

Role of KCNN4 Potassium Channel in Neutrophil Chemotactic Response

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Arrival of neutrophils to the sites of inflammation or infection is one fundamental mechanism that sustains an adequate innate immune response. Diseases that are characterized by impaired neutrophils migration coincide with repeated and often lethal infections. On the other hand an overamplified response of neutrophils can lead to severe host tissue damage. The latter scenario includes patients affected by cystic fibrosis, a disease distinguished by severe destruction of lung parenchyma due to an aggressive neutrophil response during infections. The KCNN4 channel has been described participating in the mechanism of migration of several cells, but their presence in neutrophils has not yet been described. We aim to explore if KCNN4 is present in neutrophils and by means of genetic and pharmacological silencing to test if the channel participates in neutrophil migration.

RT-PCR analysis demonstrated the presence of mRNA for KCNN4 in human and mouse neutrophils. Human cells responded with a 3-fold increase in fMLP-induced migration, an effect that was blocked when the cells were incubated with the KCNN4 specific inhibitor TRAM-34. Same results were obtained in neutrophils isolated from wild type mice (WT). But when the cells tested were isolated from a *Kcnn4*$^{-/-}$ animal, fMLP was unable to induce chemotaxis. Nasal inoculation of LPS (*Pseudomonas aeruginosa*) into mice demonstrated that the percentage of neutrophils arriving to the lung was lowered in the *Kcnn4*$^{-/-}$ animals when compared to WT. At 8hr post-LPS neutrophils in broncoalveolar lavage (BAL) was 58 ± 14% and 24 ± 13% in WT and *Kcnn4*$^{-/-}$ respectively. 20hr post-LPS the values obtained were 80 ± 4% and 55 ± 14% in WT and *Kcnn4*$^{-/-}$ respectively. Control animals of both genotypes inoculated with saline gave values between 3 to 10% of neutrophils in BAL. Histological analysis of lung samples from WT animals showed a notorious neutrophil infiltration that was not observed in the *Kcnn4*$^{-/-}$ mice. Literature reports that when mice with CF are challenged with nasal LPS they have a greater percentage of neutrophils in BAL than their WT littermates. Preliminary results on animals carrying the F508 CFTR deletion showed 82 ± 2% of neutrophils in BAL 8hr post-LPS, and when these animals were breed into the *Kcnn4*$^{-/-}$ background and challenged, the value obtained was lowered to 41 ± 5%.

Taken together these data suggests that KCNN4 is participating in the migration of neutrophils. Experiments in the whole animal demonstrated that the impairment in migration due to KCNN4 silencing is not exclusively observed for fMLP stimulation, is also occurring in a more pathophysiological context including a mouse model for CF disease.

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Polymorphisms of Macrophage Migration Inhibitory Factor Impact on Severity of Cystic Fibrosis

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Macrophage migration Inhibitory Factor (MIF) is a pro-inflammatory molecule able to sustain exaggerated Gram negative inflammatory responses by inducing Toll-like receptor-4 expression. A MIF regulatory role in CF was suggested by evidences that the 5-CATT polymorphism repeat (MIF5), with lowest promoter activity, was associated with a milder disease phenotype in CF patients with different CFTR genotypes (Barry J et al., Am J Respir Crit Care Med, 2005).
Allele frequencies were determined in 134 non-CF and 189 CF patients homozygous for F508del-CFTR mutation with the goal to assess association between CATT polymorphism and disease severity. DNA was amplified using the forward and reverse primers as described (Baugh JA et al., Genes & Immunity, 2002) and PCR results were analyzed using GenescanView 1.2 software.
In this multicenter study, key clinical features of F508del homozygous CF patients were recorded. Patients with MIF5 displayed concordant trends to better nutritional status, lower diabetes incidence and slower decrease of FEV1 in comparison with those carrying other polymorphic alleles. Quartile analysis based on Kulich's normalized FEV1 showed that MIF5 is associated to a better lung function expressed as FEV1 ($p = 0.03$). Kaplan-Meier analysis showed a delay in first measurement of FEV1 < 60% ($p = 0.03$) in MIF5 patients.
This work provides compelling support for a regulatory role of MIF in the severity of CF disease highlighting the relevant roles of macrophages in CF.

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Identification of a Novel 5' Alternative *CFTR* Mrna Isoform in a Patient with Nasal Polyposis and *CFTR* Mutations

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Cystic Fibrosis (CF) may be revealed by nasal polyposis (NP) starting early in life. We performed *CFTR* DNA and mRNA analyses in the family of a 12y-old boy presenting with isolated NP and a normal sweat test. Routine DNA analysis only showed the heterozygous c.2551C>T (p.Arg851*) mutation in the child and the father. mRNA analysis showed a significant increase of about 30% of total *CFTR* mRNA in the patient and the mother, which was associated with the heterozygous c.-2954G>A variant in the distant promoter region, as demonstrated by *in vitro* luciferase assays. 5'RACE analysis showed the presence of a novel transcript, in which the canonical exon 1 was replaced by an alternative exon that we called 1a-L (1a-Long). This case report could represent the first description of a CFTR pathology associated with an increase of *CFTR* mRNA amount and the presence of a novel and probably non functional transcript.

Haplotype Patterns of the Cystic Fibrosis Transmembrane Conductance Regulator Gene (CFTR) as Potential Screening Tool for Cystic Fibrosis (CF) in the Sultanate of Oman

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Background: Prevalence of cystic fibrosis in the Sultanate of Oman is unknown. However, an established mutational panel at the second largest populated area revealed that the mutations S549R and delF508 cover 89.2% of all CF cases. The mutation S549R is the predominant mutation in the patient cohort with an allele frequency of 0.75. CF and other mutations are inherited with specific haplotype patterns. The identification of haplotypes of unrelated CF patients and volunteers will provide information about frequencies of disease associated haplotypes in the normal population, support risk assessment and might be considered as a potential screening tool for CF.

Objectives:

(1) Identification of allele frequencies of the polymorphism M470V (M/V) at exon 10 and the dimorphic 4-bp GATT structure with either 6 or 7 repeats (6/7) at intron 6.

(2) Defining haplotypes for the CF cohort and the Omani unaffected volunteer population

(3) Evaluation of the association of CF causing mutations and specific haplotypes

(4) Assessing the allele frequencies of disease associated haplotype(s) in the control population and risk stratification.

Methods: Informed consent was taken from 14 unrelated CF patients and 204 unrelated volunteers from Oman Medical College in Sohar. DNA was isolated from EDTA buffered blood. A questionnaire was used to document the origin over three generations of the volunteers in the Sultanate of Oman. CF mutations in the patient cohort were screened by DGGE and SSCP, covering all CF exons and intron borders. Detected mutations were confirmed by sequencing. The M/V and 6/7 polymorphism was analysed by restriction enzyme using HphI and PAGE shift assays, respectively.

Results:

(1) The M and V polymorphism occurs in the unaffected population with allele frequencies of 0.607 and 0.392, respectively. In addition, the 6 and 7 polymorphisms were detected in the unaffected population with allele frequencies of 0.177 and 0.822, respectively.

(2) The haplotype V6 was not observed at all. Therefore, three haplotypes (M6, M7 and V7) out of four possible are characteristic for the Omani population.

(3) The mutations S549R and delF508 are completely associated with M6.

(4) The disease associated haplotype M6 occurs only with an allele frequency of 0.174 in the control population. In addition, the CF associated diplotype M6M6 of the mutations S549R and delF508 has the lowest frequency of 0.023 in the control population.

Conclusions: The complete association of the mutation S549R and delF508 to M6 suggests that a carrier risk for these mutations has a higher probability for individuals with a M6 haplotype. Furthermore, the absence of the V6 haplotype and the low frequency of M6M6 in the control population suggest that the 6 polymorphism in a homozygote state appears to be highly predictive for CF and might serve as an initial screening method for CF in the Sultanate of Oman.

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Targeting cGMP Pathway with Vardenafil to Correct F508del-CFTR Function and Localization in the Gastrointestinal Tract

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Vardenafil, a cGMP-dependent phosphodiesterase type 5 inhibitor, normalizes defective CFTR-mediated chloride secretion across the nasal mucosa of mice homozygous for the F508del mutation (CF). This work aimed at 1) studying the potential of vardenafil to rescue CFTR function across the rectal mucosa, representative of the gastrointestinal (GI) tract; 2) validating immunohistostaining assays to quantify CFTR expression in mouse colon tissue and to evaluate correction of mislocalized F508del-CFTR by treatment with vardenafil.

CFTR function was assessed by quantifying potential difference across the rectal mucosa. Sodium hyperabsorption (40.2 ± 4.0 mV *vs* 20 ± 1.8 mV; $p< 0.001$; mean ± SEM) and reduced chloride transport (-4.2 ± 0.5 mV *vs* -9.4 ± 0.9 mV; $p=0.002$) were typically found in CF compared to wild-type (WT) mice. Vardenafil, applied as a single intraperitoneal dose (0.14 mg/kg) completely restored chloride transport (-9.3 ± 1.2 mV) in CF. Immunohistostaining studies showed reduced CFTR expression at cell membrane in CF mouse colon preparations: in untreated conditions, the ratio of CFTR-specific fluorescence (RF) of the cell membrane and that of the rest of the cell was lower in CF (1.4 ± 0.1) than in WT (1.9 ± 0.1; $p < 0.001$). The RF was increased in vardenafil-treated (2.2 ± 0.1) compared to untreated CF colon tissues ($p < 0.0001$). Our findings pointed out the intestinal mucosa as a valuable target tissue to study CFTR function and localization and to evaluate efficacy of therapeutic strategies in CF. Vardenafil restores ion transport abnormalities across the GI epithelium acting as a corrector of the cell mislocalization of F508del-CFTR. This study provides compelling support for targeting cGMP signaling pathway in CF pharmacotherapy.

S-nitrosoglutathione Reductase Inhibitors Modulate *F508-del* CFTR Trafficking and Chloride Secretion *In Vitro*

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S-nitrosoglutathione (GSNO) is an endogenous nitric oxide (NO) donor that serves as a depot for NO in the body and plays an integral role in communicating NO mediated signaling functions. GSNO levels are regulated by activity of GSNO reductase (GSNOR), the primary enzyme responsible for catabolizing GSNO. In the lung, GSNO and NO-based signaling can regulate processes such as the inflammatory response and smooth muscle function. Furthermore, GSNO has previously been shown to be reduced in lungs from Cystic Fibrosis (CF) patients and has been proposed to modulate CFTR both by cGMP-mediated activation as well as possibly enhancing *F508-del* CFTR trafficking to the apical membrane. However, pharmaceutical development of GSNO is difficult due to chemical instability and lack of direct cellular uptake. N30 Pharmaceuticals has developed potent small molecule inhibitors of GSNOR. In contrast to GSNO, GSNOR inhibitors are stable, have a high bioavailability and are highly specific, with low nanomolar IC_{50} values for the target enzyme.
Using GSNOR inhibitors, we now demonstrate stimulation of chloride secretion in *F508del-CFTR* human airway epithelial cells grown under air-liquid interface conditions. This modulation of *F508del-CFTR* function is observed with GSNOR inhibitors alone and in combination with known CFTR correctors (e.g. C3, C4, C18, and others). A 24 hour incubation with N91115, an orally bioavailable compound, generates a forskolin-mediated ΔIsc of ~ 3 μA/cm^2, whereas co-incubation of N91115 and a CFTR corrector yields a ΔIsc of up to 15 μA/cm^2. Furthermore, GSNOR inhibitor treatment alone and in combination also increases the level of cell surface *F508del-CFTR* protein up to 4-fold as compared to vehicle treated cells. These data suggest that GSNOR inhibitors can modulate CFTR function. Together with other benefits of restoring GSNO levels in the lung such as bronchodilation, anti-inflammatory effects, and enhanced ciliary function, these inhibitors may be beneficial in the treatment of *F508del*-CF patients.

CFTR Modulators VX770, VX809 and PTC124 Correct Chloride Transport in Rectal Biopsies from CF Patients

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Objectives: CFTR modulators VX770 (ivacaftor), VX809 and PTC124 have been investigated in clinical phase trials. Prediction of clinical effects at a preclinical stage is complicated by the lack of optimal models. Aim of this study was to evaluate the preclinical ex vivo effect of CFTR modulators on human rectal CFTR function, to provide a basis for long-term correlations with clinical in vivo effects.
Methods: Rectal suction biopsies (n=256) from 11 PI-CF patients (6 F508del-homozygous, 5 with nonsense-mutation), 8 PS-CF patients (Class IV-V mutation) and 13 healthy controls were freshly procured for Intestinal Current Measurement (ICM). Transepithelial short-circuit current was measured according to the ECFS ICM SOP, before and after ex vivo incubation for 16 hours (37°C, 95% O2, 5% CO2) with VX809 (10-30µM), VX770 (10-30µM), PTC124 (10µM), Gentamicin 400µg/ml, combinations or DMSO 0.1%.
Results: F508del-CFTR function was partially corrected by ex vivo treatment with VX809 to a level up to 25% of non-CF control (DMSO). PTC124 enhanced CFTR function in CF rectal biopsies with at least one nonsense-mutation to 14.4% of non-CF control, whereas gentamicin showed no effect. In PS-CF with class IV-V mutations, VX770 normalised CFTR function from residual to levels of non-CF.
Conclusion: These data indicate the high potential of ICM for preclinical CFTR modulator testing in native ex vivo human tissues. The technique might be helpful for mutation-specific individual prediction of clinical in vivo drug effects and early identification of responders and non-responders of CFTR modulating therapies.

This study was supported by Mukoviszidose e.V., Germany.

Identification of G Protein-Coupled Receptor Activators that in Combination with CFTR Restoring Drugs Induce Fluid Secretion

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Background: Cystic Fibrosis is caused by mutations in the CFTR gene. This leads to a defective CFTR protein and a decrease in CFTR function. CFTR is activated by cyclic AMP (cAMP),which is one of the main seconds messengers in cells and is produced after G protein-coupled receptors (GPCR) signaling. We hypothesize that GPCR agonist can increase the efficacy of CFTR restoring drug in restoring fluid secretion in a primary cell model.
Aim: Identify novel agonists of CFTR-mediated fluid secretion in CF epithelium.
Methods: 60 Modulators of GPCR signalling were added to intestinal organoids of heatlhy control and CF and their ability to induce fluid secretion was monitored for 60 minutes using microscopy. CFTR-F508del cultures were pre-treated for 24 hours with VX-809.
Results: Four compounds were identified to induce fluid secretion in HC organoids, however only 3 compounds were able to induce fluid secretion in VX-809 treated CFTR-F508del organoids. Dopamine induced fluid secretion in HC, but failed to induce fluid secretion in CF.
Conclusions: Specific GPCR agonists induce fluid secretion in CF organoids upon rescue of CFTR by chemical compounds. This supports the use of GPCR modulators as combination treatment for CF patients treated with CFTR-restoring drugs.
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Potentiator Discovery for the Defective CFTR dF508 and G551D Mutants

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CFTR dF508 mutant channels are defective in folding and trafficking yielding low channel numbers at the cell surface, whereas G551D mutant channels reach the cell surface but are impaired in gating. Stable CHO-K1 cell lines were utilized to identify novel potentiators to enhance channel activities of both mutants. Potentiators were identified using a membrane potential (MP) assay based on an anionic bis-oxonol derivative. CFTR mediated efflux of chloride and thereby cell depolarization and increased fluorescence signals. As CFTR is activated by phosphorylation via the cAMP-dependent protein kinase A, the mutant channels were stimulated with the adenylyl cyclase activator forskolin for potentiator finding. To enable an assay for the dF508 mutant, cells were cultivated at 27°C for three days before the assay, which causes temperature correction and hence partial restoring of channel levels at the cell surface. Potent and efficacious potentiators were found active for either CFTR mutant, as well as many enhancing the activity of both mutants. The latter indicates that despite structural alterations caused by these disease mutations, pharmacological binding sites are maintained. Potentiators were confirmed by directly measuring chloride flux using electrophysiology readouts. Assay data and pharmacological data for potentiators will be presented.

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rAAV2/5 Mediated Perinatal Gene Therapy for Cystic Fibrosis

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Aims: Gene therapy offers a generic mutation-independent treatment option for cystic fibrosis (CF). Perinatal gene therapy (fetal or neonatal) has several advantages: the immune system is considered immature possibly leading to tolerance and treatment can be started before the pathological phenotype occurs. In the context of CF, sticky mucus lines the airways preventing efficient gene transfer. Recombinant adeno-associated vector rAAV2/5 efficiently targets the airways. However, the packaging capacity of rAAV is limited and therefore a truncated *CFTR* cDNA is required. In this study we aim to develop and validate rAAV2/5 mediated perinatal gene therapy for CF in a mouse model.

Methods: rAAV2/5 encoding firefly luciferase or β-galactosidase were co-administered by intra-amniotic injection to mouse fetuses (fetal) or by intranasal instillation to neonatal pups (neonatal). Reporter gene expression was monitored by bioluminescence imaging (BLI) and X-gal staining. Immune response was assessed by a transduction inhibition assay, ELISA and ELISPOT. Expression of truncated CFTR was analyzed by western blot (WB) and immunocytochemistry (ICC). The functionality of the construct was validated by an $^{125}I^-$ efflux assay.

Results: Both BLI and X-gal staining revealed that rAAV2/5 efficiently targets the respiratory epithelium of upper and lower airways of both fetal and neonatal mice. Although efficient, transduction was transient (less than 5% positive cells 3 months post injection), necessitating a second vector dose. Readministration was successful in both groups. After readministration to the fetal group the BLI signal increased 6-fold in the lungs and 10-fold in the nose compared to non-readministered controls, whereas in the neonatal group, the signal increased 5-fold in the lungs and even decreased in the nose. After the first rAAV administration, neutralizing Ab (NAB) in serum were 4.5-fold lower in the fetal than in the neonatal group. However, after a second vector administration, NAB increased in both groups.

Next, we tested a truncated CFTR missing a portion of the regulatory domain (CFTRΔR, Ostedgaard, 2002) in cell culture for future use in a CF mouse model. The truncated CFTR showed an expression profile similar to wild-type CFTR with membrane localization in ICC and the presence of both band B and C in WB. In an $^{125}I^-$ efflux assay, the CFTRΔR expressed in HeLa cells displayed anion channel activity upon stimulation with forskolin and genistein.

Conclusions: Our data suggest that readministration of rAAV2/5 is feasible after perinatal vector delivery in a mouse model. Ongoing experiments will determine if repeated readministration is limited and whether immune tolerance induced by perinatal administration mediates this effective readministration. The final goal is to translate the optimized perinatal gene therapy model to rAAV2/5 mediated delivery of CFTRΔR in a CF mouse model correcting the disease phenotype.

Small Molecule Inhibitors of GSNOR Possess Anti-inflammatory and Bronchodilatory Actions in Mouse Models of Inflammatory Lung Disease and Modulate CFTR Function in *F508del* CFTR Mice

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S-nitrosoglutathione reductase (GSNO) serves as a stable reservoir for the labile nitric oxide (NO). GSNO has potent anti-inflammatory and bronchodilatory actions, and may also influence the cystic fibrosis transmembrane conductance regulator (CFTR). GSNO is catabolized by GSNO reductase (GSNOR), an enzyme which has been shown to be dysregulated in inflammatory lung disease. Therefore, small molecule inhibitors of GSNOR may provide therapeutic benefit toward inflammatory, airway smooth muscle, and ion channel conductance endpoints in cystic fibrosis (CF) by restoring GSNO levels and function. The efficacy of the GSNOR inhibitors N6022, N91115, and N91169 were tested in both *in vitro* and *in vivo* models. Relaxation of airway smooth muscle was tested using rat tracheal rings maintained at isometric tension in a wire myograph. Anti-inflammatory and bronchodilatory actions were assessed in mouse models of inflammatory lung disease including tobacco smoke-induced COPD and ovalbumin-induced asthma. The potential to modulate CFTR function was explored via intestinal current measurements (ICM) after treatment of *F508del* CFTR mice with GSNOR inhibitors. N6022, N91115, and N91169 selectively and potently inhibit GSNOR enzyme activity with IC_{50} values of 9.8, 17.3, and 25.6 nM and K_i values of 2.5, 6.6, and 14.0 nM, respectively. *In vitro*, GSNOR inhibition caused dose-dependent smooth muscle relaxation in rat tracheal rings contracted with 1 µM methacholine (MCh). In an asthma model, a single IV dose of 0.1 mg/kg N6022 attenuated airway hyperresponsiveness to MCh aerosol challenge, eosinophil infiltration into BALF, and NFκB activity in the lung. In an 11 day smoke COPD model, daily oral administration of either N91115 or N91169 at 0.3 mg/kg decreased BALF neutrophils by 37% and 34%, respectively. Finally, in *F508del* CFTR mice, oral administration of 1 mg/kg N91115 once daily for 7 consecutive days corrected CFTR function as noted by a positive ΔI_{sc} signal in the distal colon mucosa in response to 10 µM forskolin + 100 µM IBMX. N91115 increased the I_{sc} to 14% of the current measured in wild-type mice. Collectively, these findings suggest that GSNOR inhibition offers a therapeutic approach to mitigating the pathophysiology of CF via anti-inflammatory, bronchodilatory, and CFTR modulatory activities.

Comparative Study of F508del-CFTR Rescue in Response to Combination of VX809, SAHA, Corr4a and the Iminosugars Isolab and Miglustat

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Cystic fibrosis (CF) is an autosomal and recessive disease due to mutations introduced in the gene encoding for a chloride ion channel CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). Among the most common mutations, around 90% of people with CF have at least one copy of the F508del mutation and over 70% are homozygous for this mutation. The deletion of phenylalanine at position 508 leads to a mistrafficking of the CFTR protein resulting from its retention in the endoplasmic reticulum (ER) to abnormal gating of CFTR channel and endocytosis. In previous studies, several molecules were identified individually as correctors of the F508del-CFTR defective trafficking: VX809, SAHA, Corr4a, and Isolab, Miglustat. The aim of the present work was to determine the best combination of these molecules, leading to an optimal pharmacological and functional rescue in cells expressing F508del-CFTR.

To that end, F508del-CFTR activity was recorded using the patch-clamp technique in whole-cell configuration in response to a cocktail of activators (forskolin + genistein) ± the CFTR inhibitor ($CFTR_{inh}$-172) in the human epithelial carcinoma HeLa cell line stably transfected with F508del-CFTR encoding plasmid and incubated at 37°C with the test correctors. Preliminary cellular toxicity experiments showed cytotoxic effects only for Corr4a and SAHA at concentrations used previously to rescue F508del-CFTR activity (10 µM).

We incubated HeLa cells with one, two, three and four correctors and recorded the forskolin+genistein dependent F508del-CFTR chloride current in each experimental condition using the patch-clamp technique. First we confirmed that all correctors individually restored the F508del- CFTR activity in CF cells. But because the level of rescue with Corr4a was very modest we focused our study only on VX809 (10 µM), SAHA (1 µM) and the two iminosugars (100 µM). The magnitude of the rescue of F508del-CFTR activity was dependent on the combination of correctors and on the duration of incubation. The combination of two correctors always gave better correction of F508del-CFTR activity than with one corrector. Including VX809 in the cocktail always produced better correction. On the contrary the presence of SAHA only show modest synergy when added with either iminosugars or VX809. The most favorable combinations that restored the activity of F508del-CFTR to a level similar to or above that of the wt-CFTR were: VX809 + IsoLab; VX809 + Miglustat + SAHA; VX809 + Miglustat + IsoLab; VX809 + IsoLab + SAHA; VX809 + Miglustat + IsoLab + SAHA. Focusing on the VX809 + IsoLab cocktail, we observed that the level of rescue reached after 4h of incubation with these compounds was similar to the one obtained in optimal conditions of combination for each molecule (i.e. 24h for VX809 + 4h for IsoLab).

Our study opens new perspectives on the development of a pharmacological additive to rescue F508del-CFTR and suggests that a combination of correctors should lead to a more complete rescue of F508del-CFTR activity.

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Modulation of Annexin A5 Expression by Gonadotropin-Releasing Hormone (Gnrh) in Human Bronchial Epithelial Cell Lines: Consequences on CFTR Protein

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Background: Changes in CFTR's partners are implicated in the pathophysiology of cystic fibrosis with an unknown extend. Nevertheless, we previously showed that annexin A5 (anxA5) binds to both normal (wt-CFTR) and F508del-CFTR. In transfected epithelial cells, anxA5 overexpression increased CFTR's levels in plasma membranes and enhanced CFTR-dependent Cl^- secretion in wt- and F508del-CFTR expressing cells.
Objectives: One therapeutic approach would be to find a drug to modulate anxA5 expression and subsequently to improve CFTR-dependent Cl^- secretion in F508del-CFTR cells. Because GnRH is known to stimulate anxA5 expression in the gonadotropes, it is a potential candidate. At present, GnRH analogs represent a successful therapeutic option to restore fertility or to suppress the pituitary-gonadal axis for treatment of hormone-related cancers. Interestingly, the presence of GnRHR has been demonstrated in a wide variety of extra-pituitary tissues, indicating that GnRH analogs may also act directly via tissue GnRHRs. The ubiquitous nature of GnRHR expression may lead to other clinical applications for GnRH analogs
Materials and methods: Using RT-PCR and western blotting, expression of GnRHR was studied in human bronchial epithelial cell lines expressing either wt-CFTR or F508del-CFTR (16HBE14o-, CFBE41o-, CFBE41o-/wt, CFBE41o-/F508del). Cells were further treated with native GnRH (1 nM) and anxA5 expression was assessed by western blotting. Effect of GnRH treatment on CFTR-dependent Cl^- secretion is now under investigation by iodide effluxes experiments. Its impact on CFTR cell surface expression was studied by membrane biotinylation techniques. Finally, RT-PCR, western blotting and immunohistochemistry studies of GnRHR are now performed in human airways and primary cultures of human bronchial and nasal epithelial cells to check potential clinical use of the hormone.
Results: Beside the GnRHR expression in the different epithelial cell lines, we showed that anxA5 expression is increased after 1h incubation with GnRH. A significant iodide efflux peak was measured in CFBE41o- cells after treatment (1h) with 1nM GnRH and was maximal with 10nM GnRH (EC_{50}, 0.8nM). Interestingly, a significant iodide efflux peak was also measured in 16HBE14o- cells. Biotinylation experiments after GnRH exposure revealed an increased surface expression of CFTR in wt-CFTR expressing cells. Preliminary RT-PCR and western blotting experiments show that GnRHR is present in human bronchial and nasal epithelial cells.
Conclusion-Perspectives: In addition to anxA5 overexpression, GnRH treatment enhances CFTR-dependent Cl^- secretion in both wt- and F508del-CFTR expressing cells. AnxA5 is a Ca^{2+}-dependant phospholipid binding protein, moreover, studies showed that anxA5 binds to both the wt-CFTR and the F508del-CFTR in a Ca^{2+}-dependent manner, so, it could be of interest to study the impact of GnRH treatment on intracellular Ca^{2+} mobilisation. Finally, because GnRH analogs are available in different forms (nasal sprays or aerosols), one perspective of this project is to study the effects of such molecules on human bronchial and nasal epithelial cells.

Effects of New Correctors on Function and Maturation of F508del-CFTR

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The most frequent mutation in the *CFTR* gene is the deletion of a single amino acid (F508). This mutation leads to a abnormally folded and unstable protein, F508del-CFTR, and results in abnormal transepithelial ion transport including defective cAMP mediated (CFTR)-Cl^- secretion. We have recently postulated that an abnormal interaction between keratin 8 (K8) and F508del-CFTR prevents mutated protein to be delivered to the plasma membrane (Colas *et al.,* Hum Gen Mut 2012), and that this interaction site is a target for pharmacotherapy for F508delCFTR. Using dynamic modelling and molecular docking approaches, we have identified 4 molecules which should bind the abnormal folded NBD1 domain of F508del-CFTR protein, including the interaction site K8/CFTR-NBD1 and restore the mutated protein to the plasma membrane.
The aim of the study was twofold. First, to evaluate the efficacy of the most active compounds by evaluating the correction of the Cl^- transport defect in different cells, and in homozygous F508del mice models. Second, to investigate the mechanisms underlying the action of correctors *i.e.* maturation of F508del-CFTR and effect on interaction K8-F508delCFTR.
Methods: CFTR function was studied by (i) patch clamp technique in whole cell configuration in Hela cells expressing F508del-CFTR (ii) short-circuit current (Isc) in primary bronchial CF cells in culture (primary hcf), (iii) nasal potential difference in F508del/F508del mice. Maturation of CFTR was studied by western blotting in Hela cells expressing F508del-CFTR. Interaction K8-CFTR was studied by proximity ligation DNA assay in Hela cells expressing F508del-CFTR and primary hcf.
Results showed that treatment of the cells with the 2 most active compounds (1µM during 24h) restore the CFTR-Cl^- currents in F508delCFTR HeLa cells, with efficiency similar to VX-809 corrector (whole cell patch clamp recordings). In parallel experiments maturation of F508delCFTR (band C) was observed. The same treatment of primary hcf from 2 homozygous F508del patients with the compound expected to bind to K8/F508del-NBD1 interaction site partially corrected functional defect of F508del-CFTR as measured by Isc approach. In F508del/F508del mice, two nasal instillation (two days apart) of this corrector (10µM) induced CFTR-dependent changes of nasal potential difference without changingsodium transport. Proximity ligation experiments in F508delCFTR HeLa cells showed that treatment with the 2 most active correctors significantly diminished the number of interaction K8-F508delCFTR.
In conclusion, we have identified new correctors which interrupt interaction K8-F508delCFTR. Those correctors restore Cl^- transport in F508del/F508del mice and F508del/F508del primary human CF cells. This compound should be further tested since it may be useful for treatment of F508del/F508del patients.

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A Functional CFTR Assay Using Primary Cystic Fibrosis Intestinal Organoids

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Introduction: We have recently established conditions allowing long-term expansion of epithelial organoids from intestine, recapitulating essential features of the in vivo tissue architecture. Here, we apply this technology to study primary intestinal organoids of people with cystic fibrosis.
Methods: Crypts are isolated from rectal biopsies and expanded in growth factor complemented matrigel. Forskolin induced swelling is monitored by live confocal microscopy and unbiased image analysis.
Results: Forskolin induces rapid swelling of organoids derived from healthy controls or wild-type mice, but is strongly reduced in organoids of cystic fibrosis subjects or F508del-Cftr mice and absent in *Cftr*-deficient organoids. This is phenocopied by CFTR-specific inhibitors. Forskolin-induced swelling of *in vitro* expanded healthy control and cystic fibrosis organoids corresponds quantitatively with forskolin-induced anion currents in *ex vivo* freshly excised rectal biopsies, and indicates limited residual function in F508del homozygous subjects. Function of F508del-CFTR is restored upon incubation at low temperature, as well as by CFTR-restoring compounds. We found that CFTR-restoring correctors synergize in restoring CFTR F508del function, and that the efficacy of CFTR-restoring drugs is different in organoids from different patients.
Conclusions: This relatively simple and robust assay will facilitate diagnosis, functional studies, drug development and personalized medicine approaches in cystic fibrosis.

Treatment with VX-809 and VX-770 in Rectal CF Organoids Expressing Uncommon CFTR Genotypes

Evelien Kruisselbrink[1,2,3], *Florijn Dekkers*[1,2,3], *Karin M de Winter-de Groot*[1], *H.M. Janssens*[4], *C.K. van der Ent*[1], *Jeffrey Beekman*[1,2,3]

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Background: The potentiator VX-770 (Ivacaftor, Kalydeco) has recently been registered as a drug for cystic fibrosis (CF) patients with a CFTR-G551D mutation. Furthermore, VX-770 treatment in combination with the corrector VX-809 is now being assessed in a phase II clinical trial for patients harboring CFTR-F508del.
Aims: To demonstrate that these CFTR-targeting drugs may also be used to treat patients with CFTR alleles that have not been included in these initial clinical trials.
Methods: We recently developed a quantitative assay to measure CFTR function in primary rectal organoids. This assay can differentiate between patients containing 'mild' and 'severe' CFTR mutations and can detect CFTR restoration by VX-809 or VX-770.
Results: We measured CFTR activity at basal level or upon CFTR restoration by VX-809 and VX-770 in organoids expressing various CFTR mutations, including the most common mutant F508del, and the less common mutants A455E, R117H, (TG)13(T)5 and L997F. We observed a dose-dependent relation between CFTR function and forskolin treatment, which differed between organoids expressing distinct CFTR alleles. Furthermore, VX-770 treatment restored CFTR function in all CF organoids, while VX-809 treatment was effective for specific mutations.
Conclusions: CFTR genotype-specific responses to VX-809 and VX-770 treatment can be detected using organoid-based CFTR measurements. This assay may identify patients who may benefit from CFTR-targeting drugs.

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Posters – Others

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Correction of Bone Defect in Cystic Fibrosis: Studies from F508del Cftr Human and Mouse Models

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In patients with cystic fibrosis (CF), bone fragility is recognized as a severe complication of the disease and is a real problem of management of patients. Vertebral and rib fractures are particularly harmful to the respiratory function in CF decreasing their ventilatory capacity and promoting lung infections. Whether the role of F508del mutation in the dysfunction of bone metabolism remains to be elucidated, its involvement in this dysfunction has been demonstrated in KO-CFTR and F508del-CFTR mouse models. We have showed that young and adult F508del *Cftr*^{tm1Eur} mice, in two sexes, have a severe osteopenia characterized by a decrease in bone mass and bone formation accompanied with reduced level of serum insulin-like Growth Factor 1 (IGF-1) (Le Henaff et al., 2012). Miglustat (N-butyldeoxynojyrimicin, NB-DNJ, Zavesca®), an orally approved drug by the US Food and Drug Administration and European Union for type I Gaucher disease and for children and adults with Niemann-Pick type C disease, was reported to normalize sodium and CFTR-dependent chloride transport in human F508del CFTR lung cells and in nasal mucosa of F508del mice.

Here, we investigated the bone mass and microarchitecture of F508del mice relative to wild type (WT) littermates after oral administration of 120 mg/kg/day miglustat for 28 days using *in vivo* micro-CT, bone mass and dynamic parameters of bone formation. Levels of two serum growth factors, insulin-like growth factor 1 (IGF-1) and 17b-estradiol (E_2), were also determined. Once-day treatment with miglustat over 4 weeks normalized trabecular bone volume and improved bone microarchitecture in the lumbar spine of F508del mice. This increase in bone volume and structure was associated with enhanced new bone formation and significantly higher serum levels of E_2, but without changes in IGF-1 levels in miglustat-treated F508del mice. Interestingly, in the miglustat-treated F508del mice, the body weight attained at the end of the treatment were 10 % higher compared to untreated F508del mice. The miglustat-treated F508del mice had no diarrhea at any stage of the study.

Human F508-CFTR osteoblasts (cells that form bone) were cultured from breastbone fragments of a 20-year-old CF male homozygous for the F508del-CFTR mutation undergoing lung transplantation. Compared to normal human osteoblasts, we found a lower CFTR-dependent chloride conductance in F508del-CFTR osteoblasts. The exposure of F508del-CFTR osteoblasts to 10 nM E_2 for 48 hours restored a CFTR-dependent chloride current. F508del-CFTR osteoblasts produced a lower amount of osteoprotegerin (OPG, a key regulator of bone turnover) compared to normal osteoblasts. Interestingly, 10 nM E_2 for a 48 hour-period markedly increased OPG secretion in F508del-CFTR osteoblasts.

The implication of our work is to propose, in the short term, the first treatment to fight against the CF bone disease and, thus allowing for longevity and a better quality of life for patients with cystic fibrosis.

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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) 186-8T/C Allele in a Patient with Cystic Fibrosis (CF) Diagnosis

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186-8T/C mutation (c.54-8T/C) in intron 1 of the CFTR gene has never been reported to our knowledge. Ferec et al. previously reported (NL#50 Cystic Fibrosis Mutation Database, www.genet.sickkids.on.ca) a possible exon 2 splicing in the case of the 186-13C->G variant detected in trans with F508del in a child 13 years old, with nasal polyposis as the main symptom, pancreas sufficient.
186-8T/C CFTR mutation was identified in a 38 years old Italian woman who referred to our Center for evaluation because she was treated as CF since 2010 abroad in Europe following diagnosis based on repeated sweat tests Cl 60 and 74 mEq/L (normal values < 50 mEq/L, at the reference clinic) and clinical history consistent with CF (chronic sinusitis, recurrent bronchitis, gallstones); no mutations were identified by first level genetic analysis; small interstitial-alveolar infiltration were evident in CT scan; deviation of nasal septum was reported.
The variant 186-8T/C was identified by sequencing in Italy. Afterwards the patient referred to our center for assessing the relevance of these findings. Nasal potential difference (NPD) measurements were therefore performed with results in the normal range. Moreover the sweat test was performed twice in a period of 3 months according to the Gibson and Cook method obtaining Cl^- 35 and 57 mEq/L. Lung function was normal; lung infection was absent according to several sputum cultures and previous bronco alveolar lavage (BAL). We considered possible differential diagnosis: ciliary dyscinesia was excluded in a specialized center.
We hypothesized that the genetic variant 186-8T/C could affect splicing and therefore CFTR mRNA from blood cells was analyzed by RT-PCR using primers designed to this aim. Amplification products in the patient were the same as expected and detected in a healthy donor.
Moreover CFTR function was tested by single cell fluorescence analysis (Sorio et al. 2011) in leukocytes having responses to stimuli by cAMP analogue and forskolin overlapping with those obtained in healthy donors. We previously defined as CF index the outcome of this assay which was positive in all healty subjects and negative in all CF patients. In this case CF index was positive (mean: +43±12; the assay was performed three times). In this patient sweat test and genetic tests were inconclusive and required a CFTR functional test performed according to a standardized method (NPD). Moreover we evaluated a possible exon skipping and CFTR function in leukocytes. All results obtained at our Center were not consistent with CF diagnosis. Our innovative approach in leukocytes could be of interest for diagnosis because it is more simple for patients and for investigators than currently available and standardized approaches. Validation in a large group is in progress and more simplified and feasible functional assays in leukocytes are under investigation in order to provide an additional tool for investigating genetic variants of uncertain clinical relevance as well as the effects of drugs targeting the CF basic defect.

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Comparative Study for the Evaluation of a New Technology for Cystic Fibrosis Screening

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Introduction: Cystic fibrosis (CF) is one of the most frequently diagnosed autosomal-recessive diseases in the Caucasian population. Screening for Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene mutations, including poly T, is strongly recommended in infertile couples planning a pregnancy by assisted reproductive technology (ART). This study evaluated the performance of the new Nanochip CF70 kit (Savyon Diagnostic, Israel), a microarray assay, and compared it with the Innolipa kits (Innogenetics, Belgium).
Methods: From January to July 2012 we analyzed 392 blood samples with Innolipa and Nanochip technologies that identify respectively 70 and 56 CFTR mutations. Both tests include the most common Italian mutations and the poly-T screening. Discordant results were analyzed with the Devyser CFTR Core Kit (Devyser, AB, Sweden) based on PCR allele specific technology, MLPA (MRC Holland), Direct Sequencing (DS) on the 3730 DNA Analyzer (AppliedBiosystems), and Sequenom's MassArray system (Diatechpharmacogenetics, Italy).
Results: Innolipa and NanoChip were concordant for 371/392 samples although some samples were repeated several times on Nanochip because of no call and low signal results. 21/392 (0.5%) discordant results were tested with the aforementioned technologies: DS confirmed Innolipa results in 18/21 samples and Nanochip results in 1/21, while Devyser and Sequenom did not recognize some mutations not included in their panels. DS was essential for the identification of two different homozygous deletions; although they were not present in Innolipa panels, in 2/21 samples Innolipa indicated a mutation with the warning no interpretation possible.
Conclusions: In this study the Innolipa assay confirmed its reliability and Nanochip showed that it could become competitive with slight changes to the software.

Spatial cAMP/PKA Compartmentalization and Activity in Primary Airway Cells

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The F508del CFTR mutation is associated with a miss-folded protein and consequent abolition of chloride secretion, which underlies the impairment of lung function. Recent research has shown that treatment with small molecule 'correctors' facilitates some rescue of F508delCFTR and delivery to the apical surface, by promoting the correct folding of F508del CFTR. However, despite cell surface localization of rescued F508del CFTR, it is often observed that stimulators of cAMP- and Protein Kinase A (PKA)-dependent CFTR activity fail to activate or have a reduced activation of F508del CFTR that may be due to the unavailability of cAMP/PKA signalling.

We have found that in secondary airway cells expressing wt CFTR cAMP is, indeed, compartmentalised in that cAMP-raising stimuli generate an increase in cAMP levels that is higher in the sub-plasma membrane compartment than in the cytosol while in secondary CF cells expressing F508del mutation, the subcortical compartmentalization of cAMP is altered and cAMP levels, as well as PKA phosphorylation activity, are significantly increased in the bulk cytosol at the expense of the sub-plasma membrane compartment (Monterisi S. et al. 2012). These findings confirm the importance of a correctly organised intracellular milieu for the appropriate functioning of CFTR. Specifically, they point to the requirement for a sufficiently high concentration of cAMP in the sub-plasma membrane space to achieve effective activation of local PKA and consequent efficient regulation of CFTR activity. This may be relevant for the selection of pharmacological compounds aiming at re-establishing CFTR function since some of these only slightly restore cAMP-mediated chloride secretion despite their ability to rescue F508del CFTR apical expression. This refractoriness of rescued F508del CFTR to activation by cAMP/PKA may be even more severe in primary airway cell monolayers, suggesting that these processes need to be validated in primary cells before clinical trials begin.

Here, we found that in primary cells derived from the bronchi of CF patients homozygous for F508CFTR deletion (CF primary cells: CFPC), the actin cytoskeleton is disorganized as previously shown in secondary cells. Moreover, using real-time FRET reporters of cAMP levels cloned in adenoviral vectors containing the cDNA for the two probes measuring cytosolic and membrane cAMP (H30 and mpH30, respectively), we found that, in contrast to what observed in primary cells expressing wt CFTR (WTPC), CFPC cells exhibited a significant accumulation of cAMP in the bulk cytosol at the expense of the sub-plasma membrane compartment. These data suggest that, indeed, the disorganized cytoskeleton found in CF cells results in a loss of sub-apical cAMP and a subsequent reduction in CFTR activity.

Monterisi S**,** Favia M, Guerra L, Cardone RA, Marzulli D, Reshkin SJ, Casavola V, Zaccolo M. CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity. J Cell Sci. 125:1106-17, 2012.

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