2009
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

Tavira, Portugal

Chairpersons

Michael Gray, Mitchell Drumm
and
Margarida D. Amaral

15-19 April 2009

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Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to welcome you to the Sixth European Cystic Fibrosis Conference entirely dedicated to Basic Science which this year takes place in the Algarve region of Portugal. This year we are delighted to welcome Mike Gray as the conference Chairperson who will be supported by Mitchell Drumm and Margarida Amaral as co-chairs. I would like to especially acknowledge the enormous contribution of Margarida Amaral in establishing these conferences on the international stage. Thanks to her vision and hard work the ECFS Basic Science Conference has a high reputation for disussion of high quality science and it provides a platform to stimulate new ideas and collaborations in CF research aimed at improving treatment.

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 proud to provide you with a opportunity 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. Your active participation contributes to a productive conference and we thank you for coming to discuss your work.

I extend a very warm welcome to an exciting conference

Stuart Elborn
President
European Cystic Fibrosis Society

Welcome from the Conference Chairpersons

It is a great pleasure to welcome you to the 2009-ECFS Basic Science Conference: “New Frontiers in the Basic Science of Cystic Fibrosis”. Our aim in organizing this meeting is to help foster and initiate new research towards a better understanding of the pathogenesis of CF. We believe that ultimately this will lead to new and improved therapies for CF being developed which will have a real and lasting impact on treating this disease.

This year we are back in the Algarve Region of Portugal, in the lovely setting of Tavira, which some past attendees will remember fondly! The organization of this 2009 meeting represents something of a break from past conferences as our co-chair and initiator of these meetings, Margarida Amaral, has ‘stepped down’ from the helm, but has kindly agreed to continue sharing her valuable experience and support. However, we hope you will find that the key characteristic of these conferences which has made them so special and popular with researchers worldwide has been maintained.

We would also like to extend a special welcome and thanks to those groups on both sides of the Atlantic who have supported this conference. Without this support this meeting would not be possible. So we welcome you all to Tavira and look forward to an exciting conference with plenty of high quality open discussion in the spirit of traditional Portuguese hospitality!

Michael Gray
Newcastle University, UK

Mitchell Drumm
Case Western Reserve University, US

Margarida D. Amaral
University of Lisboa, Portugal

CONFERENCE PROGRAMME

2009 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

Algarve (Portugal), 15-19 April 2009

Programme
(webpage: http://www.ecfs.eu/tavira2009)

Chairpersons: Mike Gray (Newcastle, UK) & Mitch Drumm (Cleveland, OH, US)
Margarida Amaral (Lisboa, Portugal)

Wednesday, 15 April 2009 (Day 1)

13:00-18:00	Registration, Light Meal Set-up of Posters – Room: Alcacer Quibir
18:00-18:30	**Official opening of the meeting –** Room: Infante Santo **Stuart Elborn, ECFS President** **Conference Chairpersons**
18:30-19:30	**Opening keynote lecture** Using Novel Post-Genomic Tools for CF Therapeutic Development in the Network Age - M. D. Amaral (PT)
19:30-20:30	**Welcome Reception**
20:30-21:30	*Dinner*

Thursday, 16 April 2009 (Day 2)	
07:30-09:00	*Breakfast*
09:00-10:30	**Symposium 1 – New insights into CFTR structure and function** **Chairs:** C. Bear (CA) / O. Moran (IT)
09:00-09:20	Direct Interaction of Small Molecules with NBD1-CFTR – P. Thomas (US)
09:20-09:40	Modeling of the whole CFTR 3D structure and its conformational transitions – I. Callebaut (FR)
09:40-10:00	Switching Open the CFTR Channel by the Signature Sequence – TC Hwang (US)
10:00-10:10	Structural changes upon phosphorylation of CFTR: detection by electron microscopy and single particle analysis – J. Birtley (UK)
10:10-10:20	Characterizing compounds affecting ΔF508 CFTR folding and conformation – F. Peters (NL)
10:20-10:30	The Impact of the Regulatory Region and Regulatory Insertion in NBD1 on Processing of wt- and F508del-CFTR - A. Da Paula (PT)
10:30-11:00	*Coffee break* & **Poster viewing –** Room: Alcacer Quibir
11:00-12:30	**Symposium 2 – ER quality control and trafficking of WT- and mutant CFTR** **Chairs:** M. Amaral (PT) / A. Mehta (UK)
11:00-11:20	CFTR and SLC26A9: functional and trafficking interactions in airway cells – R. Frizzell (US)
11:20-11:40	Proteostasis in Cystic Fibrosis – W. Balch (US)
11:40-12:00	Manipulation of folding and conformation of CFTR and its NBD1 domain – I. Braakman (NL)
12:00-12:10	Evaluation of CFTR expression in delF508 affected lower airway epithelium compared to wild-type controls from the same patient, shows no difference in extent of apical localisation but demonstrates reduced signal intensity – L. Borthwick (UK)
12:10-12:20	Biochemical Analyses of Potential CK2-sites in CFTR – C. Farinha (PT)
12:20-12:30	sHsps target DF508 CFTR for degradation via a SUMO-dependent, ubiquitin-independent pathway – R. Frizzell (US)
12:30-14:30	*Lunch*
14:30-16:00	**Symposium 3 – Regulation of Epithelial Ion Transport** **Chairs:** H. Danahay (UK) / R. Tarran (US)
14:30-14:50	TMEM16A, a novel type of epithelial chloride channel – L. Galietta (IT)
14:50-15:10	Defective Ca^{2+} dependent chloride secretion in TMEM16A -/- pups – K. Kunzelmann (DE)
15:10-15:30	Regulation of amiloride-sensitive Na+ transport in human H441 airway epithelial cells – D. Baines (UK)
15:30-15:40	Different Levels of F508del-CFTR Rescuing by Correctors in BHK and in Human Bronchial Epithelial cells – M. Sousa (PT)
15:40-15:50	Metformin increases the risk for Pancreatitis in Diabetes patients bearing the CFTR variant S573C – F. Romeiras (DE)
15:50-16:00	Respiratory Syncytial Virus Infection of Ciliated Cells Increases Airway Surface Liquid Height in Normal Airway Epithelium but not in Cystic Fibrosis Airway Epithelium – E. Worthington (US)
16:00-16:30	*Coffee Break* & **Poster Viewing –** Room: Alcacer Quibir

16:30-18:00 **Special Group Discussion I –** Room: Dom Sebastio
Mucus: What controls hydration?

Moderators: J. Pearson (UK) / R. Boucher (US)

Special Group Discussion II – Room: Infante Santo
Calcium Signalling: implications for CF?

Moderators: C. Ribeiro (US) / M. Chanson (CH)

20:00-21:30 *Dinner*

21:30-23:30 Evening posters – Room: Alcacer Quibir

Friday, 17 April 2009 (Day 3)

07:30-09:00 *Breakfast*

09:00-10:30 **Symposium 4 – Lung Development and Regeneration**
Chairs: M. Gray (UK) / C. Ribeiro (US)

09:00-09:25 Airway Epithelial Regeneration – C. Coraux (FR)

09:25-09:50 The CFTR -/- pig: implications for CF pathogenesis – D. Meyerholz (US)

09:50-10:15 Progressive Nasal and Inflammatory Lung Disease in a Mouse model of Cystic Fibrosis – Bob Scholte (NL)

10:15-10:30 Connexin 26 is implied in the regulation of airway epithelium repair – S. Crespin (CH)

10:30-11:00 *Coffee Break* & **Poster Viewing –** Room: Alcacer Quibir

11:00-12:30 **Symposium 5 – Lung Physiology and Pathophysiology**
Chairs: D. Baines (UK) / JP Clancy (US)

11:00-11:20 The effect of estrogens on airways calcium signalling and the implications for lung disease in CF females – R. Tarran (US)

11:20-11:40 Airway surface liquid volume regulation determines airways phenotype in Liddle's versus βENaC-overexpressing mice – M. Mall (DE)

11:40-12:00 The design of inhaled ENaC blockers for the treatment of lung disease in cystic fibrosis – H. Danahay (UK)

12:00-12:10 Human lung explant tissue as a new ex vivo model for preclinical optimisation of CFTR pharmacotherapy – N. Derichs (DE)

12:10-12:20 Ion transport characteristics of human nasal epithelial cells from wild type and cystic fibrosis donors – H. Danahay (UK)

12:20-12:30 Regulation of ENaC in Human Bronchial Epithelial Cell Cultures by protein, sPLUNC1 – B. Rollins (US)

12:30-14:00	*Lunch*
14:00-18:00	**Free Afternoon**
18:00-19:30	**Special Group Discussion III –** Room: Dom Sebastio **Modifier genes – what have we learnt?** **Moderators:** G. Cabrini (IT) / M. Drumm (US) **Special Group Discussion IV –** Room: Infante Santo **Pharmacology- how do correctors and potentiators work?** **Moderators:** D. N. Sheppard (UK) / O. Moran (IT)
20:00-21:30	*Dinner*
21:30-23:30	Evening posters – Room: Alcacer Quibir
Saturday, 18 April 2009 (Day 4)	
07:30-09:00	Breakfast
09:00-10:30	**Symposium 6 – Genes and the environment** **Chair:** M. Drumm (US) / B. Scholte (NL)
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09:20-09:40	ENaC in CF and CF-like disease – H. Cuppens (BE)
09:40-10:00	Quantifying the contribution of genetic and non-genetic factors to variation in CF – G. Cutting (US)
10:00-10:15	Toll-like Receptor 5 as Modifier Gene in CF Lung Disease – C.J. Blohmke (CA)
10:15-10:30	Effects on mRNA Processing of CFTR Splicing Mutations assessed by Novel Mini-genes – A. Ramalho (PT)
10:30-11:00	*Coffee Break* & **Poster Viewing –** Room: Alcacer Quibir
11:00-12:30	**Symposium 7 – Inflammation in CF** **Chair:** T. Bonfield (US) / G. Cabrini (IT)
11:00-11:20	Accumulation of NKT cells in cystic fibrosis – G. Döring (DE)
11:20-11:40	Identification of components of innate defence in the airway surface fluid – O. Zegarra-Moran (IT)
11:40-12:00	CFTR as an inflammation suppressor: the role of protein kinase CK2 – A. Mehta (UK)
12:00-12:15	Glucocorticoids fail to inhibit inflammatory process in CF human bronchial epithelial cell lines – C. Rebeyrol (FR)
12:15-12:30	Pro-inflammatory Toll-like receptor 2 expression in CF epithelium – B. Schock (UK)
12:30-14:30	*Lunch*

14:30-16:00	**Symposium 8 – Bacterial killing in CF airways** **Chair:** S. Elborn (UK) / G. Döring (DE)
14:30-14:50	CFTR genotype determines the functional responses of murine alveolar macrophages – D. Nelson (US)
14:50-15:10	How CFTR modulates survival of *P. aeruginosa* in the lung – G. Pier (US)
15:10-15:30	CFTR and Myeloid Cell Immunity – T. Bonfield (US)
15:30-15:40	Ceramide is a potential biomarker of epithelial injury which is raised in the airway of people with cystic fibrosis – M. Brodlie (UK)
15:40-15:50	Proteomic analysis of proteins released from Pseudomonas aeruginosa clinical and laboratory strains: effects of azithromycin – G. Bergamini (IT)
15:50-16:00	Decreased Levels of Secretory Leucoprotease Inhibitor in the Pseudomonas-infected Cystic Fibrosis Lung are due to Neutrophil Elastase Degradation – S. Weldon (UK)
16:00-16:30	*Coffee Break* & **Poster Viewing –** Room: Alcacer Quibir
16:30-18:30	**Special Symposium Translating Science into Clinical Practice** **Chairs:** M. Amaral (PT) / D.N. Sheppard (UK)
16:30-16:50	Mechanisms of mucus over-production in CF Mice – L. Touqui (FR)
16:50-17:10	CFTR correctors and potentiators: pre-clinical tests in CF mice – H. De Jonge (NL)
17:10-17:30	Sweat Gland Potential Difference. Possible Role as an Outcome Measure of CFTR Function in Clinical Trials? – P. Durie (CA)
17:30-17:50	ICM as a biomarker for CFTR restorative strategies. – JP Clancy (US)
17:50-18:10	From disease mechanism to medicine: a drug discovery perspective – C. Poll (UK)
18:10-18:30	Opportunities and Challenges of Translational Research in Cystic Fibrosis – E. Olson (US)
20:00	*Dinner / social event*

Sunday, 19 April 2009 (Day 5)

08:00-09:30 Breakfast

09:30-11:00 Symposium 9 – Strategies to correct ion transport defects
Chairs: E. Olson (US) / K. Kunzelmann (DE)

09:30-09:50 Chloride Channel Activator Therapies for CF, and CF Lung Disease Mechanisms – A. Verkman (US)

09:50-10:10 A pharmacological strategy towards a therapeutic hope: Miglustat – C. Norez (FR)

10:10-10:30 Functional rescue of deltaF508-CFTR by peptides designed to mimic sorting motifs – C. Bear (CA)

10:30-10:40 Biophysical Characterization of TMEM16A, a Membrane Protein with Calcium-dependent Chloride Channel Activity – L. Ferrera (IT)

10:40-10:50 Inhibition of renal CFTR by insulin-sensitizing agents: Implications for a central role for CFTR in renal-based pathologies of fluid balance – B.L. Blazer-Yost (US)

10:50-11:00 Transcriptional profiles and PARP-1 inhibitors as correctors of DF508 trafficking – S. Anjos (CA)

11:10-12:00 Closing lecture
Mucus: What controls hydration? – R. Boucher (US)

12:00-12:10 Closing remarks
Conference Chairs

12:10 End of the Meeting – Departure

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AWARD WINNERS

Novartis Young Fellows Travel Award:

Christopher Blohmke, CA
Sophie Crespin, CH
Dorothée Raveau, FR
Erin Wothington, US

Student Helper Award:

Diana Faria, DE
Simao Luz, PT
Anabela Ramalho, PT
Brett Rollins, US
Elvira Sondo, IT
Marisa Sousa, PT

UK CF Trust Young Investigator Travel Awards:

James Birtley
Lee Borthwick
Malcolm Brodlie
Paul Buchanan
Sinead Weldon
Mark Williams

15 April – 18:00-19:00 **Room: Infante Santo**

Opening Lecture

Using Novel Post-Genomic Tools for CF Therapeutic Development in the Network Age

Margarida D. Amaral (PT)

Department of Chemistry and Biochemistry, Faculty of Sciences of the University of Lisboa, Portugal
Centre of Human Genetics - National Institute of Health, Lisboa, Portugal

As several model genomes have been sequenced, post-genomic approaches like transcriptomics and proteomics, aimed at identifying gene products differentially expressed in association with a given pathology, have held promise of both understanding the pathways that are putatively associated with the respective disease and as a fast track to therapy.

Our first aim was to apply these post-genomic approaches to Cystic Fibrosis (CF) so as to identify biological markers (mRNAs and proteins) showing robust differential expression in human CF-vs-normal epithelial cell lines and native tissues. We have used GSEA (Gene Expression Enrichment Analysis) to compare the gene expression profiles of three independent CF-related microarray datasets, two of which we produced in our lab. Our findings confirmed a tendency for the top/bottom 1% of each gene list to be located within the top/bottom half of the other two gene sets.

The most representative of our CF-related microarray datasets compares gene expression in individual nasal epithelial cell populations from F508del-CFTR homozygous CF patients and healthy controls. Bioinformatic data mining and robust statistical analysis revealed a network of regulated genes in CF with "a central hub" at estrogen receptor alpha (ESR1), which was found to be upregulated in CF samples. Although the mechanism link is yet to be solved, this is significant as female CF patients experience an exacerbation in CF symptoms during cyclical peaks in systemic estrogen, and several hypotheses have been put forward linking estrogen signalling and CFTR function.

Notwithstanding, this approach also evidences that new high-content strategies and tools are critically needed to distinguish genes and proteins with mere secondary pathological association from those that are primarily responsible for the basic cellular defect(s) in disease. Accordingly, elucidation of protein function is the next post-genomic challenge towards the understanding of biological processes in CF. Strategies and tools distinguishing genes and proteins with mere pathologic association from those primarily responsible for the basic cellular defect(s) in CF pathophysiology in a global manner are also underway and will be presented.

Thursday 16 April – 09:00-10:30 **Room: Infante Santo**

SYMPOSIUM 1
New Insights into CFTR structure and function
Chairs: C. Bear (CA) & O. Moran (IT)

S1.1 Direct Interaction of Small Molecules with NBD1-CFTR

Andre Schmidt, John Richardson, Emmanuel N. Caspa and Philip J. Thomas

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A number of small molecules have been described which modulate CFTR maturation and function. These compounds can be classified according to their effect on CFTR: small molecules that modulate CFTR channel function are termed potentiators or inhibitors, whereas small molecules that rescue mutant CFTR maturation efficiency are termed correctors. One possible mechanism by which these compounds may affect CFTR, is by direct interaction. We have developed assays to test whether a given compound is able to directly interact with the critical NBD1-CFTR or predicted sites on the full length CFTR. NBD1-CFTR contains the most common CF-causing maturation mutation, the deletion of Phenylalanine at position 508, a mutation that destabilizes the isolated domain: Known NBD1-CFTR ligands (such as ATP) stabilize the domain. We undertook the task of screening the panel of CFFT compounds and other known CFTR modulators. Under mild denaturant conditions, a number of the tested compounds were able to protect the native conformation of NBD1-CFTR by directly interacting with this subdomain of CFTR. Specific, predicted, interdomain binding sites shared with NBD1-CFTR were tested utilizing mutants designed to fill these sites in full length CFTR. Current efforts are focused on determining the high resolution structures of co-complexes of NBD1-CFTR and modulators determined to bind specifically.

S1.2 – Modeling of the whole CFTR 3D Structure and its conformational Transistions

Jean-Paul Mornon[a], Pierre Lehn[b], Isabelle Callebaut[a]

1 IMPMC, UMR7590, CNRS, Universités Pierre et Marie Curie-Paris 6 et Denis Diderot-Paris 7, Paris, France
2 U613, INSERM, Université de Bretagne Occidentale, Brest, France

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The high resolution, experimental 3D structure of Sav1866 from *Staphylococcus aureus* has provided invaluable insights into the structural basis underlying the function of ATP-Binding Cassette (ABC) exporters (1). It has encouraged the homology modeling of eukaryotic ABC exporters, in particular those involved in multi-drug resistance and in severe human diseases, such as Cystic Fibrosis (CF).
We have recently modeled the structure of the membrane-spanning domains (MSD1/MSD2) and nucleotide-binding domains (NBD1/NBD2) of human CFTR, in an ATP-bound, outward-facing conformation (2), using the complete Sav1866 structure as template. This modeling was based on the use of sensitive methods allowing accurate alignments in the MSDs at high level of sequence divergence. The obtained model of the CFTR MSD1:NBD1:MSD2:NBD2 assembly highlighted at the atomic level the crucial roles of some amino acids within the MSDs (for substrate translocation) and at the interface between the MSDs and NBDs (coupling interfaces).
Here, we have pursued this approach, by modeling the inward-facing conformation of human CFTR, based on the more recent corrected structures of the bacterial ABC lipid flippase MsbA (3).
This modeling provides a framework to understand the probable conformational transition between the inward- and outward-facing conformations. This transition involves a large conformational rearrangement within the MSDs and an evolution of NBDs from a close conformation, in which the P-loops face each other, towards a tight interface, with the nucleotide being sandwiched between the P-loop and the signature sequence. Noteworthy is that despite this important reorganization, the coupling interfaces between MSDs and NBDs are largely maintained, supporting their crucial role as constant pivots and highlighting the importance of F508 in this context.
In addition, we also constructed a two-segment model of the regulatory R domain, with the first segment corresponding to a globular domain located at the bottom of the NBD heterodimer and the second one having an extended configuration. This model of the R domain as well as our model of the whole MSDs:NBDs:R assembly are supported by the recent 3D reconstruction of CFTR from negatively stained electron microscopy images (4).

Supported by Vaincre La Mucoviscidose.

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(2) Atomic model of human cystic fibrosis transmembrane conductance regulator: membrane-spanning domains and coupling interfaces. Mornon JP, Lehn P, Callebaut I. Cell Mol Life Sci. 2008 65:2594-2612.
(3) Flexibility in the ABC transporter MsbA: Alternating access with a twist. Ward A, Reyes CL, Yu J, Roth CB, Chang G. Proc Natl Acad Sci U S A. 2007 104:19005-19010.
(4) Three-dimensional reconstruction of human cystic fibrosis transmembrane conductance regulator chloride channel revealed an ellipsoidal structure with orifices beneath the putative transmembrane domain. Mio K, Ogura T, Mio M, Shimizu H, Hwang TC, Sato C, Sohma Y. J Biol Chem. 2008 283:30300-30310.

S1.3 – Switching Open the CFTR Channel by the Signature Sequence

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Opening and closing (gating) of the protein kinase A-phosphorylated CFTR are controlled by ATP binding/hydrolysis at its nucleotide binding domains (NBDs). It is believed that opening of CFTR by ATP is coupled to the formation of NBD dimer in a head-to-tail configuration with two ATP molecules sandwiched at the dimer interface. Each ATP binding pocket is composed of the Walker A and B motifs of one NBD and the signature sequence of the partner NBD. G551D, the third most common disease-associated mutation located at the signature sequence of NBD1, completely loses its responsiveness to ATP. We fortuitously found that cadmium (Cd^{2+}), a soft metal ion with distinctive affinity for cysteine, can open G551D-CFTR at micromolar concentrations in the absence of ATP. A smaller but similar effect was seen with zinc (Zn^{2+}). This effect of Cd^{2+} or Zn^{2+} is not seen with wild-type CFTR. Pretreatment of the G551D channel with thiol-specific reagents abolished the effect of Cd^{2+}, suggesting an involvement of endogenous cysteine(s). Supporting the idea that a multi-valent coordination is responsible for the effect of Cd^{2+}, G551C-CFTR channels, which remain responsive to ATP, can be better gated by Cd^{2+} with a higher apparent affinity for Cd^{2+} than G551C. The mutants G551C, L548C, and S549C, all in the signature sequence of CFTR's NBD1, show robust response to Cd^{2+}. On the other hand, negligible effects of Cd^{2+} were seen with T547C, Q552C, and R553C, indicating that a specific region of the canonical LSGGQ sequence is involved in transmitting the signal of Cd^{2+} binding to the gate. In searching for the endogenous cysteine(s) responsible for the effect of Cd^{2+}, we first converted all cysteines in NBD2 to serines under the G551D background. Surprisingly, Cd^{2+} remains an effective ligand for this construct, suggesting that NBD dimerization is not involved in Cd^{2+}-dependent gating of G551D-CFTR. Since the same region involved in Cd^{2+}-mediated gate opening interacts with the gamma-phosphate of ATP in numerous crystal structures of NBD dimers, we propose that the signature sequence serves as a switch that transduces the signal of ligand (ATP or Cd^{2+}) binding to the channel gate. Consistent with this idea, we found that magnesium pyrophosphate, which preserves the beta-gamma phosphates of ATP, by itself can open CFTR abide with a much lower apparent affinity than MgATP. Taken together, these results suggest that the signature sequence may serve as a drug target for developing CFTR potentiators

P5

S1.4 - Structural changes upon phosphorylation of CFTR: detection by electron microscopy and single particle analysis

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Mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) can lead to loss of channel activity, mis-folding and truncation of the protein, and often give rise to the disease cystic fibrosis in humans. Little is known about the structure of the whole CFTR protein, how phosphorylation primes the protein and how ATP initiates the opening of the channel. Moreover there is only partial understanding of how the most common human CFTR mutation (the deletion of phenylalanine 508) affects the structure and activity. On the other hand, there is increasing structural information about isolated domains of the protein, such as the first nucleotide-binding domain (NBD1), and the regulatory (R) domain. There is also structural data for bacterial ATP-binding cassette proteins such as Sav1866 and MsbA, which are distantly related to CFTR. These have allowed the generation of molecular homology models of the CFTR protein.

The scene is therefore set for low resolution structural studies of the whole CFTR protein which should be able to visualise large scale conformational rearrangements. A better understanding of the dynamic rearrangements in CFTR should allow insights into the effects of the various mutations that give rise to cystic fibrosis. Moreover the work may lead to the development or optimisation of drugs to treat the patients. Low resolution structural data has been obtained so far from 2D crystals of the whole CFTR protein, although the constraints of a rigid crystalline lattice did not allow (so far) any information on the 3D structure of the phosphorylated protein. Here we describe low resolution structural studies of non-crystalline CFTR protein using electron cryomicroscopy and single particle analysis, yielding a maximal resolution of ~1/14$Å^{-1}$. We have studied the structure of the channel both before and after phosphorylation by the addition of ATP and protein kinase A, providing the first insight into the global conformational rearrangements that must take place for channel opening to occur. Even at the relatively modest resolution obtained, there are significant changes in the configuration of the protein domains. The nucleotide binding domains move closer together after phosphorylation and ATP addition, as expected from studies of isolated nucleotide-binding domains. However this movement is accompanied by a ratcheting motion within the transmembrane domains. These data can be interpreted in terms of a general model for the mechanism of action of the family of ATP-binding cassette proteins, of which CFTR is an unusual member.

P3

S1.5 - Characterizing compounds affecting ΔF508 CFTR folding and conformation

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The F508 deletion in CFTR changes the protein´s conformation, as a result of which it can no longer travel to the plasma membrane but is retained in the endoplasmic reticulum (ER). In search of new drugs to rescue the ΔF508 phenotype many compound screens have been done and promising candidates have been identified, but their molecular mechanisms of action are still unknown.

We developed an *in vitro* assay to follow conformational changes in CFTR and more specifically, in the F508-containing NBD1 domain. We subject *in vitro* translated radiolabeled NBD1 to limited proteolysis to examine conformational differences between wt and ΔF508 NBD1.

We clearly detect changes in conformation and are optimizing this assay further for examination of compound effects. To find out whether the screened compounds can affect NBD1 folding directly or indirectly we will add the drug at various stages of the limited proteolysis assay. Different compounds have been tested, including corrector 4a, which did not affect NBD1 folding.

We concluded that these compounds do not rescue ΔF508 CFTR by directly correcting NBD1, but must either affect CFTR domain assembly or act by changing the cell, for instance by activating a crucial chaperone

P6

S1.6 - The Impact of the Regulatory Region and Regulatory Insertion in NBD1 on Processing of wt- and F508del-CFTR

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The high-resolution structures of murine and human wt- and F508del-NBD1 of CFTR have provided invaluable information about the structure of NBD1 [1,2]. The reported data showed that these structures are very similar to those of other ABC transporter NBDs except for two segments [2]: the regulatory insertion, RI (Glu403-Leu435), a ~30-residue insert between the first two β-strands of the NBD1 β-subdomain, and the regulatory extension, RE (Ser654- Gly673), at the C-terminus of NBD1, where it extends ~20 residues longer than canonical ABC domains forming a helix packing against NBD1 at the NBD1-NBD2 interface. Both these segments have been suggested to be conformationally dynamic (undergoing ~180^{o} reorientations), namely upon phosphorylation (Ser422, Ser659, Ser660, Ser670) possibly to permit formation of the NBD1/NBD2 ATP-sandwich for channel gating [2]. He hypothesized that the dynamic flexibility of these regions may also result in exposition of hydrophobic surfaces contributing to the low folding efficiency of both wt- and F508del-CFTR.
Our goal here is to elucidate the possible role of RE and RI on the *in vivo* folding of CFTR. by biochemically and functionally characterizing wt- and F508del-CFTR lacking RI or RE.
To this end, wt- and F508del-CFTR-cDNA-pNUT constructs lacking RE and RI were produced by *in vitro* mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA) and used to generate stable BHK cell lines. Steady-state levels of immature and processed forms of these CFTR variants were assessed by Western blot analysis with the 596 antibody (CFF) and efficiency of processing was determined by metabolic pulse-chase followed by CFTR immunoprecipitation (IP). Functional assessment of CFTR activity was performed by the iodide efflux assay with an ion-selective electrode (ThermoElectron Corporation, Waltham, MA)
Preliminary results show wt-CFTR lacking either RE or RI, is efficiently processed, although the RI appears to show a lower steady-state level of maturation. These data are indicative that both these variants acquire a native (folded) conformation *in vivo*, consistently with a previous study [4]. Additional biochemical analyses to determine the maturation efficiency and functional characterization of these CFTR variants as well as of F508del-CFTR lacking RE or RI are underway.

Work supported by pluriannual funding of CIGMH (FCT, Portugal). AC DaPaula is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT, Portugal).

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[2] Lewis H *et al* (2005) *JBiolChem* **280**:1346-53.
[3] Serohijos AW *et al* (2008) *PNAS USA* **105**:3256-61.
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Thursday 16 April – 11:00-12:30 Room: Infante Santo

SYMPOSIUM 2 – ER Quality Control and Trafficking of WT- and Mutant CFTR
Chairs: M.D. Amaral (PT) & A. Mehta (UK)

S2.1 - CFTR and SCL26A9: functional and trafficking interactions in airway cells

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Airway surface liquid (ASL) composition and volume are tightly regulated to maintain healthy lung function. Electrolyte transport across the surface epithelium, together with accompanying osmotic water flow, establishes the volume and composition of the ASL, and this in turn determines the efficiency of mucociliary clearance. Members of the SLC26 family of transporters interact with CFTR physically and functionally. Muallem and co-workers have proposed that their reciprocal regulation provides for tissue specific, CFTR regulated SLC26 transporter function (1). Human bronchial epithelial cells (HBE) exhibit constitutive anion secretion that is absent in CF HBE. The identity of this conductance is unknown, but SLC26A9 is found in human airway (2), and it exhibits chloride channel behavior during heterologous expression (1). The possibility that SLC26A9 is a CFTR-regulated chloride channel raises several significant questions: Does SLC26A9 contribute to basal and/or cAMP/PKA-stimulated anion currents in airway epithelia, is it sensitive to CFTR inhibitors, and does its activity depend on functional CFTR? We used HEK cells and differentiated, primary HBE epithelia to address these questions. We sought differences in the properties of SLC26A9 and CFTR expressed in HEK 293 cells as a fingerprint to identify their contribution to HBE apical anion conductance. HEK cells expressing SLC26A9 displayed a constitutive chloride current that was inhibited by the CFTR blocker GlyH-101 (71 ± 4%, 50 μM). This conductance exhibited a near-linear current-voltage (I/V) relation under control conditions and during block, while GlyH-101 blocked CFTR exhibited a strong inward-rectified (IR) I/V relation. We then tested polarized HBE endogenously expressing either WT or ΔF508 CFTR for similar activity. After electrical isolation of the apical membrane using basolateral α-toxin permeabilization, WT CFTR monolayers showed constitutive chloride currents that were inhibited by 50 μM GlyH-101 (68 ± 6%) while maintaining a near-linear I/V relation. In the absence of blocker, addition of forskolin stimulated a current increase having a linear I/V, and GlyH-101 blocked 69 ± 7% of this current while shifting the I/V relation IR, consistent with CFTR activation. HEK cells co-expressing SLC26A9 and WT CFTR displayed similar properties, as well as forskolin stimulated currents that exceeded the sum of those in cells separately expressing SLC26A9 or WT CFTR. Their I/V relation during GlyH-101 block was moderately IR, indicating that both channels contributed to the stimulated current. HBE from CF patients expressed SLC26A9 mRNA, but no constitutive chloride currents. Likewise, HEK co-expressing SLC26A9 with ΔF508 CFTR failed to exhibit SLC26A9 currents. WT CFTR co-immunoprecipitated with SLC26A9; bands B and C were present in the IP at a ratio similar to that of the input. The co-expression of each channel was stabilized by expression of the other. We conclude that SLC26A9 functions as an anion conductance in the apical membranes of HBE, it contributes to transepithelial chloride currents under basal and cAMP/PKA stimulated conditions, its activity in HBE requires functional CFTR, and physically, these channels are closely associated, beginning with their biosynthesis in the ER.

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2. Lohi et al. 2002. *J. Biol. Chem.* 277:14246-14254.

S2.2 - Proteostasis in Cystic Fibrosis

William E Balch[1], David Hutt[2], David Herman[3], Atanas Koulov[2], Pual Lapointe[2], Bingwen Lu[4], Ana Rodriquez[5], Scott Becker[5], Sabrina Noel[6], Martina Gentzsch[7], Joseph Pilewski[8], John Riordan[7], John Yates IIIrd[4], Gerard Manning[5], Ray Frizzell[6], Joel Gottesfeld[9]

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Protein folding for export through the exocytic pathway of the eukaryotic cell is sensitive to the local composition of membrane trafficking components and requires the assistance of multiple chaperones that define the folding buffer of the cell- the chaperome. Different cell types exploit the variable composition of the chaperome to maintain protein folding homeostasis or proteostasis (Ann. Rev. Biochem. 2009, In press) - an adaptive, cell-type specific biological environment that works the kinetic and thermodynamic properties of the protein fold to achieve functionality. Perturbation of this relationship occurs in misfolding diseases such as cystic fibrosis (CF), where a Phe508 deletion in the NBD1 domain of the multi-membrane spanning chloride channel cystic fibrosis transmembrane conductance regulator (ΔF508-CFTR*) prevents its delivery to the cell surface. The Phe508 deletion results in an imbalance between the energetic properties of the variant protein fold and the local proteostasis environment, leading to disruption of coupling of the nascent protein to the COPII endoplasmic reticulum (ER) export machinery and targeting of ΔF508-CFTR* to ER-associated degradation (ERAD). It is now apparent that protein misfolding in disease and in aging can be altered by use of pharmacological chaperones to stabilize the fold, and/or by manipulation of pathways that favor metabolic stability through use of proteostasis regulators. Our analysis of newly identified mechanisms that efficiently correct CF point to a multi-target solution to normalize transport and restore channel function at the cell surface. Our results suggest the existence of a tunable range of proteostatic environments that can be used favor correction of disease phenotypes in the clinic.

S2.3 - Manipulation of folding and conformation of CFTR and its NBD1 domain

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The ΔF508 mutation in its first nucleotide binding domain (NBD1) leads to misfolding of CFTR and its degradation by the proteasome. To study its folding process we in vitro translate CFTR in the presence of digitonin-permeabilized cells as a source of ER membrane and subject the protein to mild protease treatment. The resulting protease-resistant fragments represent folded (sub)domains of CFTR, the identity of which we determine using C-terminally truncated CFTR constructs and epitope-specific antibodies. The influence of cellular factors we examine by comparing a range of proteins: from purified, in vitro translated, and transfected NBD1, to in vitro translated and transfected full-length CFTR.
Using these assays and reagents we established the influence of various factors on folding and conformation of CFTR, including temperature, chaperones, and intragenic suppressor mutations. A selection of these will be presented.

S2.4 - Evaluation of CFTR expression in delF508 affected lower airway epithelium compared to wild-type controls from the same patient, shows no difference in extent of apical localisation but demonstrates reduced signal intensity

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The expression of CFTR in respiratory epithelia of delF508 homozygous CF patients remains controversial. Reports in the literature support conflicting hypotheses of either a reduction in the fraction of cells expressing apical CFTR or a reduction only in the quantity of CFTR expressed apically. These studies are further confounded by changes during tissue processing and/or the use of nasal as opposed to lower airway epithelia. In this study, these issues were addressed by examining CFTR localisation and signal intensity in the lower airways of patients who have undergone lung transplantation for advanced CF. Using this approach, both wild-type and delF508 CFTR epithelia can be freshly harvested simultaneously from the same individual and immediately fixed.

12 lung transplant recipients with CF consented to the study bronchoscopy. Epithelia were harvested by bronchial brushing above (cells with delF508 mutation) and below (wild-type) the airway anastomosis joining the native bronchus and the transplanted lung. Cells were immediately fixed in paraformaldehyde and stained with MATG1061 CFTR monoclonal antibody (RD-Biotech). The % of ciliated cells expressing CFTR as a distinct apical band and the intensity of apical CFTR staining was examined by confocal microscopy and compared in CF and wild-type cells from each recipient by student's paired t-test. All results expressed as mean (±SEM).

There was no significant difference in the % of cells expressing apical CFTR, above 71(±2.51)% vs below the airway anastomosis 68(±2.55)%, ($p=0.2106$, $n=12$). Eight of the 12 recipients were delF508 homozygous and 4 delF508 heterozygous. In the delF508 homozygous recipients, no significant difference in the % of cells expressing apical CFTR was observed (above 71(±3.24)% vs below 67(±3.16)%, ($p=0.1427$, $n=8$)). However, there was a significant difference in both the average pixel intensity (above 69(±10.04) vs below 146(±22.92), $p=0.0300$, $n=5$) and the total pixel intensity (above 3729 (±805) vs below 9173 (±2465), ($p=0.0419$, $n=5$) of the apical band. No difference in the number of cells counted or number of pixels quantified ($p>0.05$) was observed.

These results suggest the delF508 mutation leads to a reduced apical expression of the mutant protein in ciliated bronchial epithelial cells compared to wild-type cells from the same patient. There was no evidence that the percentage of cells with apical-localised CFTR was different in wild-type and CF cells from the lower respiratory tract. We speculate that CFTR potentiators may have a role in therapy for delF508 homozygous individuals without the need for enhanced trafficking.

Funded by CF Trust UK

P8

S2.5 - Biochemical Analyses of Potential CK2-sites in CFTR

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Despite great efforts to elucidate the mechanism and the molecular factors involved in CFTR biogenesis, trafficking and function, many of these processes are not fully understood. Protein kinases and phosphatases are known for long to regulate CFTR function. However, the role of phosphorylation in CFTR biogenesis and trafficking remains less explored. Recently, it was shown that recombinant casein kinase 2 (CK2) phosphorylates in vitro CFTR NBD1 at the residue serine 422 [1]. In addition, CFTR possesses several CK2 consensus phosphorylation sites, namely S511 (very close to F508) and T1471 at the C-terminus. Our aim here was to determine how mutation of these putative CK2 P-sites affects the CFTR biogenesis, turnover and processing.

We have produced CFTR mutants in which the consensus residues S422, S511 and T1471 were substituted by either a neutral (alanine, A) or an acidic residue (aspartic acid, D) in both wt and F508del-CFTR backgrounds by site-directed mutagenesis and used these constructs to generate novel stable BHK cell lines. Pulse-chase experiments by CFTR immunoprecipitation and Western blot, as well as functional assessment by iodide efflux assay, were performed in these cells. Quantification of bands B (immature form) and C (mature form) of CFTR in pulse-chase shows that substitution of S511 does not affect the turnover or processing of either wt- or F508del-CFTR. However, substitution of threonine at position T1471 to D (but not to A) completely impairs the processing of wt-CFTR without affecting F508del-CFTR and increases the turnover of F508del-CFTR. Interestingly, T1471A although not interfering in the production of mature protein, abolishes CFTR function. Moreover, treatment of cells with 20 mM TBB (tetrabromobenzotriazole, a specific inhibitor of CK2) for 90 min significantly reduces the processing efficiency of wt-CFTR.

Altogether, our data suggest a putative stabilizing role for CK2 upon wt-CFTR in these cells, which can be mediated by CFTR residue T1471. So far, our data do not support a role for residue 511 on the functional interaction of CFTR and CK2. Experiments are underway to determine whether this effect is also dependent on the S422 putative CK2 consensus P-site.

Work supported by POCTI/PTDC/BIA-BCM/67058/2006 grant, EU grant FP6-LSH-2005-037365 (TargetScreen2) and pluriannual funding of CIGMH (FCT, Portugal).

[1] Pagano M *et al* (2008) *Biochemistry* **47**, 7925.

S2.6 - sHsps target DF508 CFTR for degradation via a SUMO-dependent, ubiquitin-independent pathway

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Key mediators of CFTR folding and degradation are molecular chaperones, which help protein substrates fold, but can target them for degradation if folding efficiency is compromised. A family of chaperones termed small heat shock proteins (sHsps) modulates CFTR expression levels in yeast and mammalian cells (1). Relative to WT CFTR, sHsps such as Hsp27 selectively bind and degrade DF508 CFTR when co-expressed in HEK cells, and the effect on DF508 steady-state levels is reduced by the proteasome inhibitor, MG132. The physiological relevance of Hsp27 action was assessed by knockdown using a shRNA construct. Both wt and DF508-CFTR protein levels were augmented 2-3 fold. Thus, sHsps increase CFTR's accessibility to proteasome-mediated degradation pathways and are able to distinguish between the wt and DF508-CFTR proteins. Hsp27 has been reported (2) to interact with Ubc9, the SUMO (small ubiquitin-like modifier) conjugating enzyme. We confirmed this interaction by co-immunoprecipitation and identified Ubc9 and Senp1 (SUMO specific protease 1) expression in airway cells. As for Hsp27, over-expression of Ubc9 selectively decreased steady-state levels of DF508 CFTR, while over-expression of Senp1 increased wt as well as DF508-CFTR levels. Pulse-chase experiments indicated that Ubc9 co-expression selectively degraded DF508 CFTR, similar to the action of sHsps. Inhibition of the SUMO pathway by knock-down of the SUMO E1 enzyme (SAE1) increased wt and DF508-CFTR protein levels 2-3 fold, an effect observed also in CFBE-DF508 airway cells. Moreover, decreased levels of SAE1 blocked the ability of Hsp27 to promote DF508 CFTR degradation. In vitro and in vivo studies indicated that CFTR, and particularly NBD1, is sumoylated, and that Hsp27 facilitates this modification. Additionally, interference with the sumoylation pathway elicited a decline in SUMO-modified DF508-CFTR. Although expression of a (lysine-less) ubiquitin mutant that cannot form poly-ubiquitin chains increased DF508-CFTR levels, it did not interfere with the ability of Hsp27/SUMO pathway components to promote DF508 degradation. These findings indicate that Hsp27 selects DF508 CFTR for ERAD by facilitating its sumoylation, leading to its proteasomal degradation via a SUMO-dependent, but ubiquitin-independent, pathway.

[Supported by the NIH (DK68196 & DK72506) and the Cystic Fibrosis Fdn]
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Thursday 16 April – 14:30-16:00 **Room: Infante Santo**

SYMPOSIUM 3 – Regulation of Epithelial Ion Transport
Chairs: H. Danahay (UK) & R. Tarran (US)

S3.1 - TMEM16A, A Novel Type of Epithlial Chloride Channel

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Calcium-dependent chloride channels (CaCCs) are plasma membrane proteins that play an important role in various physiological processes. In epithelial cells, CaCCs mediate chloride secretion with a mechanism independent of CFTR. Stimulation of CaCCs in cystic fibrosis (CF) airway epithelial cells may be a strategy to correct the defective chloride transport and therefore to restore mucociliary clearance. However, to develop such a strategy, it is important to define the molecular identity and mechanism of regulation of the other chloride channels expressed in epithelial cells. We have recently identified TMEM16A as a membrane protein associated with CaCC activity (Caputo et al., Science 322: 590-594, 2008). TMEM16A is part of a protein family containing other 9 members (TMEM16B - TMEM16K).

It is uncertain whether TMEM16A forms the entire CaCC channel or other proteins are also involved in CaCC activity. We have studied CFPAC-1 cells, which have a high expression of TMEM16A and high levels of calcium-dependent chloride transport. Silencing of TMEM16A expression by siRNA caused a strong inhibition of CaCC activity as measured by three different functional assays. In contrast, silencing of the other TMEM16 proteins did not cause a significant inhibition. Similarly, siRNA molecules against bestrophins were unable to inhibit CaCC activity. Comparable results were also obtained in bronchial epithelial cells (primary cultures and immortalized cells).

Heterologous expression of TMEM16A in different cell types generated chloride currents that were highly similar to those previously described for CaCCs in epithelial and non-epithelial cells. Indeed, TMEM16A-dependent currents showed activation and inactivation at positive and negative membrane potentials, respectively, and were highly sensitive to NPPB and niflumic acid inhibition. Furthermore, channel activation occurred at cytosolic calcium concentrations in the 200 - 600 nM range.

Our results suggest that the activity of native CaCC is strongly dependent on TMEM16A expression with little contribution from the other TMEM16 proteins and from bestrophins. However, the involvement of other proteins that are still to be identified cannot be ruled out.

S3.2 - Defective Ca^{2+} dependent chloride secretion in TMEM16A -/- pups

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A novel family of putative Ca^{2+} dependent chloride channels, the TMEM16 proteins, has been identified recently. TMEM16A and its closest paralog TMEM16B produce Ca^{2+} activated Cl^- currents when expressed in salamander oocytes and HEK293 cells. These current reflect properties of native CaCCs, such as time and voltage dependence, anion selectivity and pharmacological properties. The family of TMEM16 proteins comprises 10 different proteins which exist as different splice variants. Thus TMEM16 proteins may produce a variety of Ca^{2+} activated Cl^- channels with slightly different biophysical properties in epithelial and non-epithelial cells. *In situ* hybridization and immunohistochemistry detected TMEM16A in epithelial acinar cells from various glands such as pancreas, salivary and mammary gland. Less clear is the expression of TMEM16A in airways and intestine. Results from cultured airway epithelial cells in which expression of endogenous TMEM16A was knocked down by siRNA, suggest a role of TMEM16A for calcium-dependent chloride secretion in these tissues. However as Tmem16a expression in the epithelium of trachea and lungs changes remarkably during development, the functional role of TMEM16A in the postnatal lung is not clear. As reported recently knockout of TMEM16A in mice leads to death within one month after birth, most like due to severe tracheomalacia with gaps in the tracheal cartilage rings along the entire length of the trachea. Thus Tmem16a was identified as a novel regulator of epithelial and smooth muscle cell organization in murine development. Using this model, we examined ion transport in various epithelia tissues such as trachea, colon, pancreatic and submandibular acinar cells. TMEM16A-/- pups typically died within the first 3 to 4 days after birth, which required examination of the tissues at an early postnatal state. The preliminary results demonstrate impaired Ca^{2+} dependent Cl^- secretion in different epithelial tissues of TMEM16A-/- pups.

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S3.3 - Regulation of amiloride-sensitive Na^+ transport in human H441 airway epithelial cells

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Transepithelial transport of Na^+ across the lung epithelium via amiloride-sensitive Na^+ channels regulates fluid volume in the lung lumen. Cell-attached patch clamp recordings identified two distinct constitutively active cation channels in the apical membrane of human H441 airway epithelial cell monolayers that were likely to contribute to Na^+ conductance (G_{Na+}): a 5 pS highly Na^+ selective ENaC–like channel (HSC) and a 18 pS non-selective cation channel (NSC). Substituting NaCl with NMDG-Cl in the patch pipette solution shifted the reversal potentials of HSC and NSC respectively from +23 mV to –38 mV and 0mV to –35 mV. 1μM amiloride inhibited HSC activity in cell attached patches and 56% of short circuit current (I_{sc}) across monolayers, whereas 10μM amiloride partially reduced NSC activity and inhibited 30% of $I_{sc.}$ Constructing a concentration response curve showed that the IC_{50} amiloride for control monolayers was 0.7μM.

Activators of AMP-activated protein kinase (AMPK), the adenosine monophosphate mimetic AICAR and the biguanide metformin (2mM) decreased amiloride-sensitive I_{sc} and G_{Na+} by ~50 and 30% respectively. This effect was reversed by the AMPK inhibitor Compound C. AICAR inhibited HSC and NSC activity in cell attached patches, significantly decreased the component of I_{sc} inhibited by 1μM amiloride and increased the IC_{50} amiloride to 9μM indicating that there was a reduction in the amiloride-sensitivity of HSCs and/or the contribution of HSCs to Na^+ transport across the monolayer. Exposure to physiological factors that effect cellular energy status such as hypoxia (3% O_2, 5% CO_2) or glucose deprivation (< 0.4 mM) increased AMPK activity and inhibited I_{sc} to 70% of control. Both treatments predominantly inhibited the component of I_{sc} inhibited by 1μM amiloride. Activation of AMPK with AICAR was not associated with changes in the apical abundance of the α, β or γ epithelial Na+ channel (ENaC) subunits. Immunoprecipitation of phosphatidylinositol 4,5-bisphosphate (PIP_2), present in the membrane of lung epithelial cells, revealed that it was associated with α, β and γ ENaC proteins in control monolayers. However, treatment with AICAR inhibited the interaction of PIP_2 with β and γ ENaC proteins.

In conclusion, these data indicate that activation of AMPK in H441 cell monolayers is associated with inhibition of HSCs and a change in amiloride-sensitivity of monolayers by a mechanism that, at least in part, reduces channel open probability by compromising the interaction of ENaC proteins with PIP_2.

Supported by the BBSRC and St George's University of London.

S3.4 – Different Levels of F508del-CFTR Rescuing by Correctors in BHK and in Human Bronchial Epithelial cells

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Recent high-throughput compound screens identified several novel small-molecules that partially rescue the trafficking defect of F508del-CFTR both in recombinant and human bronchial epithelial cells. These include potentiator VRT-532 and correctors VRT-325, VRT-640, Genzyme compound 48, KM11060 (sildenafil analogue), compounds 4a, 3a, 4c and 2b [1,2,3,4].

Our aim here is two-fold: (i) to determine their efficacy to rescue F508del-CFTR in HBE cells and in polarized primary human airway epithelial cells (AEC); and (ii) to investigate the mechanism by which these correctors rescue cell-surface expression and restore function of F508del-CFTR.

Pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation in BHK cells, show that F508del-CFTR is fully processed, albeit at low levels by VRT-325 6.7μM/24h (~6.0%); VRT-532 20μM/48h (~6.5%); VRT-640 6μM/24h (~4.5%) and C4 10μM/accute (~2.0%). Similar experiments with F508del-revertants [3] show that VRT-325 (but not VRT-532, VRT-640 nor C4) increases the efficiency of maturation of F508del-4RK-CFTR, whereas F508del-G550E-CFTR remains unaffected, suggesting an effect directly on the folding of F508del-CFTR.

Preliminary data obtained by CFTR immunoprecipitation with [^{35}S]methionine labbeling in CFBE41o– cells [5] and western-blot show that F508del-CFTR is fully processed, although at different levels, after a 24h incubation with potentiator VRT-532 and correctors VRT-325, VRT-640 and compound 4a. Currently micro-Ussing chamber experiments are underway to measure CFTR-mediated Cl^- currents in polarized F508del-CFBE41o cells after treatment with the corrector compounds, VRT-532, VRT-325, VRT-640, Genzyme compound 48, KM11060 (sildenafil analogue), compounds 4a, 3a, 4c and 2b, as described [6].

Overall, our data show that the correctors tested here are able to promote F508del-CFTR maturation and restore its function in BHK and CFBE41o– cell lines. Also, correctors tested here appear to rescue F508del-CFTR by distinct mechanisms in BHK cells. Future work will be done in order to assess compound efficacy in polarized human primary AEC cells by Ussing chamber measurements [6].

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S3.5 - Metformin increases the risk for Pancreatitis in Diabetes patients bearing the CFTR variant S573C

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP and protein kinase A (PKA) regulated Cl^- channel in the apical membrane of epithelial cells, and plays an important role in the HCO_3^- secretion by pancreatic duct cells [1]. It has been shown that the metabolically regulated and adenosine monophosphate stimulated kinase (AMPK), colocalizes with CFTR and contributes to the attenuation of its function. [2]. Here we studied the effect of metformin upon CFTR. This biguanidine compound is used in the clinical treatment of type II diabetes, and is described to activate AMPK through the inhibition of the Complex I of the Respiratory Chain [3]. Metformin increases the risks of lactic acidosis and patients using this compound tend to develop pancreatitis secondary to metformin poisoning, or at therapeutic metformin doses in the case of renal failure [4]. Patients with pancreatitis have an increased incidence of genotypic alterations in the *CFTR* gene, including the non-disease causing variant, S573C [5]. We used double electrode voltage clamp (DEVC) to study whole cell conductances activated by 3-Isobutyl-1-methylxanthine (IBMX 1 mM) and forskolin (FSK 2 mM) in *Xenopus laevis* oocytes expressing wt CFTR or S573C CFTR. We are currently studying the effect of extracellular pH on CFTR function. Preliminary data show that acid extracellular pH 5.5 inhibits S573C by 75% but wt-CFTR only by 18% inhibition. We also found that whole cell Cl^- conductances due to sub-maximal activation of S573C-CFTR were inhibited by 25%, upon incubation with metformin (500mM), which had no effects on wt-CFTR. These data suggest that the presence of the *CFTR* gene variant S573C in non-CF diabetes patients renders them more susceptible to the development of pancreatitis, particularly when treated with metformin.

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S3.6 - Respiratory Syncytial Virus Infection of Ciliated Cells Increases Airway Surface Liquid Height in Normal Airway Epithelium but not in Cystic Fibrosis Airway Epithelium

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Respiratory virus infection is a major cause of exacerbation of cystic fibrosis (CF) lung disease and CF patients suffer more severe consequences of virus infection. Our goals are to understand the impact of respiratory viruses on the pathogenesis of CF lung disease. To study the effects of virus infection on the human CF ciliated airway epithelium we used an *in vitro* model of human ciliated airway epithelium (HAE) and respiratory syncytial virus (RSV) as a virus that commonly infects CF patients. Using non-CF HAE, we show that RSV infects only ciliated cells and causes an acute increase in airway surface liquid height (ASL) height as measured by Texas red-dextran staining of ASL followed by confocal microscopy. This effect was maximum at ~3 days post-inoculation (pi) in non-CF HAE (ctrl,7.71±0.5;RSV,14.8±0.9µm, n=4). Parallel experiments with CF HAE revealed that RSV infection of ciliated cells failed to induce an increase in ASL (CF-ctrl,5.9±0.1;CF-RSV,5.9±0.1µm; n=4). These data suggest that non-CF HAE, but not CF HAE, respond to RSV infection by stimulation of fluid secretion into the airway lumen and is likely mediated by epithelial cell ion transport mechanisms. To determine whether the increase in ASL height after RSV was due to active Cl^- secretion, we used bumetanide (100µM) which significantly inhibited RSV-induced ASL height (ctrl,7.3±0.6; RSV,14.4±1.3; RSV+bumetanide,9±0.6; ctrl+bumetanide,5.6±0.1µm; n=4). To determine the specific contribution of CFTR-mediated Cl^- secretion to this response we used CFTR172 that also significantly inhibited RSV-induced ASL height (ctrl,6.6±0.3;RSV,11.4±1;RSV+CFTR172, 8 ±0.3; ctrl+CFTR172, 6.8±0.5µm; n=4). Since adenosine triphosphate (ATP) and adenosine (ADO) have been previously shown to regulate Cl^- secretion and ASL height in non-CF HAE, we determined the ASL concentration of ATP and ADO 3 days after RSV infection in non-CF HAE and found that both were increased by RSV infection (ATP, ctrl,16±5; RSV,26±3nM; ADO, ctrl,151±25; RSV,245±15nM, n=3) suggesting that the increased ASL after RSV infection was due to the release of nucleotides known to regulate ion transport in airway epithelium.

We conclude that RSV infection results in a CFTR-dependent increase in ASL height which is mediated by increased nucleotide levels in the ASL and that this cell response to infection is defective in CF airway epithelial cells. We speculate that increased fluid secretion is a host defense mechanism that attempts to clear virus from the airways and this clearance is diminished in CF airways possibly resulting in prolonged RSV infection of the CF airways.

Supported by the CFF and NIH.

Thursday 15 April – 16:30-18:00 Room: Dom Sebastio

SPECIAL GROUP DISCUSSION I
Mucus: What Controls Hydration?
Moderators: J. Pearson (UK) & R. Boucher (US)

The aim of the workshop is to advance our understanding of how mucus is hydrated and what controls that hydration in normal and C. F. lungs.

In particular the following questions need to be addressed:

1) Are mucus gels stable or can they be diluted out i.e. can they be dissolved by increasing levels of hydration?
2) Can mucus rheology and clearance rates from the lungs be altered by increasing hydration/altering ionic strength e.g. hypertonic saline?
3) Mucus expansion at and after secretion does it involve ion exchange/substitution or is it enzymatic?
4) Which mucin gene products are important in the bilayer on the lung surface?
5) Is sputum a good model of what is happening in the lungs?
6) What is the relationship between cilia beat frequency and mucus rheology?
7) Consider how the level of hydration may be modulated therapeutically.

Thursday 10 April – 16:30-18:00 Room: Infante Santo

SPECIAL GROUP DISCUSSION II
Calcium Signalling: implications for CF
Moderators: C. Ribeiro (US) & M. Chanson (CH)

A hyperinflammatory state has been postulated to play a role in the pathogenesis of CF airways disease. Many reports have also suggested that intracellular Ca^{2+} ($Ca^{2+}i$) signals are elevated in CF airway epithelia. Because rises in $Ca^{2+}i$ signals regulate many cellular functions relevant to CF, particularly $Ca^{2+}i$-mediated inflammatory responses, alterations in $Ca^{2+}i$ signals can play a role in hyperinflammatory responses in CF. A polemic issue has been whether the pro-inflammatory state and the alterations in $Ca^{2+}i$ signals are linked to mutated CFTR or whether they are an acquired epithelial response to the chronic infectious and inflammatory milieu of CF airways.

This Discussion Group Session will address the following issues relevant to this field:

1) Is there a difference in Ca^{2+}_i mobilization between CF and non-CF cells in response to pro-inflammatory or infectious stimuli, and GPCR activation? Can altered Ca^{2+} homeostasis explain the pro-inflammatory phenotype of CF cells?
2) Does ER retention of misfolded F508del cause ER stress or ER expansion? Are these responses associated with the pro-inflammatory CF phenotype?
3) Is there a correlation between ER Ca^{2+} store depletion and/or refilling and abnormal processes of CF cells?
4) What is the link between altered Ca^{2+} homeostasis in CF and abnormal CFTR processing?

The aim of this session is to promote a solid discussion of these important issues to foster future research on the role of Ca^{2+} signaling in CF.

Friday 17 April – 09:00-10:30 Room: Infante Santo

SYMPOSIUM 4 – Lung Development and Regeneration
Chairs: M. Gray (UK) & C. Ribeiro (US)

S4.1 – Airway Epithelial Regeneration

CORAUX Christelle, ROUX Jacqueline, JOLLY Thomas, BIREMBAUT Philippe

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In chronic inflammatory and infectious respiratory diseases such as cystic fibrosis (CF), the airway epithelium is frequently injured and remodelled, resulting in alteration of its defence functions. In CF, the most important remodelling is observed in the small non cartilaginous airways. In these patients, the epithelium covering these bronchioles switches from a small mono / pseudostratified structure composed of ciliated cells, secretory Clara cells, and basal cells with a few number of goblet cells, to a high pseudostratified epithelium exhibiting a bronchial like structure with some Clara cells and goblet cells hyperplasia (Hubeau et al., Clin Exp Immunol, 2001).
In order to determine the cellular and molecular mechanisms of the human bronchiolar airway epithelial regeneration and remodelling, we have examined the sequence of events leading to the restoration of the bronchial epithelium in both *in vivo* and *in vitro* models. When seeded on denuded rat tracheas engrafted in nude mice, human bronchiolar epithelial cells dedifferentiate and adhere on basement membrane, then proliferate to form a transient squamous metaplasia, followed by their differentiation in a remodelled epithelium similar to that observed in CF patients, and finally reconstitute a mature small mono / pseudostratified functional bronchiolar epithelium. *In vitro*, when seeded on type IV collagen-coated porous membranes and cultured at the air-liquid interface, bronchiolar epithelial cells adhere to the membranes, then proliferate and finally differentiate to reconstitute a mature functional bronchiolar epithelium. In light of the sequence of events observed *in vivo* and *in vitro*, it appears that the inflammatory environment present in the xenografts and absent in the *in vitro* cultures could be responsible of the remodelling of the bronchiolar epithelium. The molecular mechanisms of the bronchiolar epithelium regeneration and remodelling remain to be determined

S4.2 - The CFTR-/- pig: implications for CF pathogenesis

Meyerholz DK[1], Stoltz DA[2], Welsh MJ[2,3,4]

Departments of Pathology[1], Internal Medicine[2] and Molecular Physiology and Biophysics[3]; and Howard Hughes Medical Institute (HHMI)[4], Roy J. and Lucille A. Carver College of Medicine; University of Iowa, Iowa City, Iowa, 52242

Cystic fibrosis (CF) is an autosomal recessive disease culminating in respiratory failure for most patients (~95%), thus the study of lung disease development is of critical importance. For the contemporary CF investigator, the ability to study early pediatric lung disease development has been limited due to improved medical therapies and lack of relevant animal models. Recently, we described the development of CFTR-/- pigs that are born with lesions mimicking severe CF disease including meconium ileus, exocrine pancreas destruction, microgallbladder, and focal biliary cirrhosis. In contrast to abdominal organs, neonatal CFTR-/- pig lungs did not have quantitative evidence of submucosal gland hyperplasia, mucinous metaplasia, cellular inflammation, or bronchiectasis, lesions commonly associated with CF. A review of early literature suggests our findings in the CFTR-/- pig is consistent with CF infants. These CFTR-/- pigs require surgerical intervention to correct the meconium ileus. As these pigs grow, preliminary data suggests progressive destruction of the exocrine pancreas, mild advancement of the liver phenotype and development of intermittent respiratory disease. As we continue to document the maturation of CFTR-/- pigs, it will be interesting to define the predisposing to causative factors that precipitate CF disease.

S4.3 – Progressive Nasal and Inflammatory Lung Disease in a Mouse model of Cystic Fibrosis

Bob J Scholte[1], Ruvalic Buijs-Offerman[1], Jamil Aarbiou[1], Harm Tiddens[2], Martina Wilke[3]

Erasmus MC Rotterdam, [1]Department of Cell Biology, [2]Sophia children hospital ped. Pulmonol., [3]Biochemistry

The lung is in continuous repair. This process can be accelerated and biased by pathological situations involving recurrent inflammation and tissue damage. The ensuing tissue damage is repaired during recovery by a process that recapitulates developmental patterns.
CF patients suffer from chronic inflammatory lung disease, opportunistic pathogen infections, characterised by frequent exacerbations. Typical of CF lung disease is the progressive tissue remodelling that can be observed in longitudinal CT scans, showing air trapping and extensive airway stenosis. This process, which is observed early in CF disease, is an extreme example of the irreversible nature of tissue remodelling in chronic inflammatory lung disease. The elucidation of the molecular pathways involved in this derailment of the normal repair processes, and the role of CFTR deficiency in this, may lead to new therapeutic approaches in CF.
CF mutant mice have a lung phenotype that shows interesting parallels with CF lung disease in humans. Although reports vary due to variations in genetic background and experimental conditions, there is consensus that unchallenged CF mutant mice tend to suffer from chronic lung inflammation, and show enhanced and sustained sensitivity to bacterial infection and lipo-polysaccharides. Recent work in different labs shows that mutant lung macrophages play a central role in mouse CF lung disease, displaying a more pro-inflammatory character in terms of cytokine secretion. Whether this is also the case in human CF lung disease, and whether other CFTR expressing blood cell lineages are involved in CF inflammatory disease remains to be seen. The effect of CFTR deficiency on cytokine secretion in epithelial cells has also been documented by several authors. However the molecular mechanism involved and the relevance to CF lung disease is still under study.
In order to understand the complex interactions between epithelium and the underlying connective tissue we study the transcriptional and proteomic responses in a mouse model of airway injury. Our data show that the regenerating lung produces increased amounts of extracellular matrix mRNA's, apparently driven by a marked induction of EGFR related growth factors. We propose that this process plays a prominent role in fibrotic processes associated with recurrent infection.
Abnormalities of the airways in CF mice are not limited to inflammation. Bonora et al have shown previously that CF KO and F508del mice have incompletely developed tracheal rings. We will show that also the development of the nasal cavity is abnormal in F508del (C57bl/6) mice. These observations demonstrate that CFTR deficiency can also interfere with cartilage and bone formation, depending on the genetic context. The aetiology and clinical relevance of these observations remain to be elucidated.
In a model of the most frequent CF mutation F508del ($Cftr^{tm1eur}$) that we have made and characterised we observe in unchallenged animals lung inflammation, abnormal pro-inflammatory macrophage acivity (Leal et al, Brussels), abnormal bioactive lipid levels (DHA/ceramide, Danutha Radzioch, McGill) and enhanced mucus production (Lhousseine Touqui. Paris). This suggests that CFTR deficiency activates several aspects of the inflammatory cascade, even in the absence of pathogens. This points towards novel therapeutic options in CF lung disease..

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S4.4 - Connexin 26 is implied in the regulation of airway epithelium repair

Sophie Crespin[1], Marc Bacchetta[1], Isabelle Scerri[1], Tecla Dudez[1], Marc Chanson[1]

[1]Geneva University Hospitals and Medical Faculty, Geneva, Switzerland

Aim: Functional integrity of the airway epithelium is altered in cystic fibrosis (CF). Epithelial integrity depends on the expression and assembly of specific proteins into specialized junctional structures. Gap junctions, made of connexins (Cx) hexamer, play crucial roles in these interactions by contributing to the ability of cells to share signaling factors directly between adjacent cells. The pattern of Cx expression in Human Airway Epithelial Cell (HAEC) cultures is associated with the differentiation state. Thus, Cx26 is specifically expressed during proliferation phase and its expression decreases to undetectable level with HAEC differentiation to a polarized airway epithelium. We questioned if a specific pattern of Cx was associated with the epithelium repair following injury.
Methods: A model of HAEC repair was established by wounding mechanically the cultures. The cultures were followed for 24h before fixation, immunostaining and confocal analysis. To confirm the role of gap junctions in the epithelium repair, different blockers were used. The coupling between adjacent cells was evaluated by microinjection of Lucifer yellow.
Results: In our model of HAEC repair, Cx26 was transiently re-expressed at the wound area and in basal cells behind the wound. The re-expression of Cx26 was associated with enhanced spreading of the gap junction tracer Lucifer Yellow. In normal HAEC, Cx26 detection was concomitant with the cell ability to proliferate, as evaluated by Ki-67 detection, to close the gap following injury. The same phenomenon was seen in HAEC from CF patients in higher proportion. Interestingly, the amount of Cx26, the duration of its expression and the number of Ki-67-positive cells were amplified, suggesting a hyperproliferative state of the CF airway epithelium. Moreover, the use of gap junction blockers delayed the epithelial repair and reduced HAEC proliferation.
Conclusions: These results suggest that gap junctions, and more specifically Cx26, plays a role in airway epithelium wound repair and that understanding of the underlying mechanisms may lead to identify new targets for controlling CF HAEC proliferation and differentiation.

Supported by "Vaincre la mucoviscidose" and FNRS

Friday 17 April – 11:00-12:30 Room: Infante Santo

SYMPOSIUM 5 – Lung Physiology and Pathophysiology
Chairs: D. Baines (UK) & JP Clancy (US)

S5.1 - The effect of estrogens on airways calcium signalling and the implications for lung disease in CF females

Robert Tarran

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Gender differences have been described in many types of lung disease. For example, cystic fibrosis (CF) females exhibit an accelerated decline in lung function and reduced survival rates compared to CF males, although the mechanisms underlying such a gender disadvantage are unknown. We hypothesized that estrogens could adversely affect airway epithelial function. To test this hypothesis, we examined how estrogens could affect airway epithelial cell signaling and ion transport. Normal airways regulate airway surface liquid (ASL) volume homeostasis and mucus transport through both cAMP and Ca^{2+} dependent regulation of ion and water transport. In cystic fibrosis (CF) airways, a genetic lack of cAMP-regulated CFTR activity leads to diminished Cl^- and water secretion and mucus plugging that serves as the focus for infections. Whilst CF airways lack the CFTR Cl^- channel, they can regulate airway hydration and mucus transport by Cl^- secretion through a second, Ca^{2+}-activated Cl^- channel (CaCC) which is active in CF airways due to mechanical (i.e. breathing-induced) stimulation of ATP release and activation of the $P2Y_2$ pathway which raises intracellular Ca^{2+} to stimulate Cl^- secretion, suggesting that CF airways retain a limited capability to regulate airways hydration which is entirely dependent upon Ca^{2+} homeostasis. ATP-stimulated $P2Y_2$ receptors raise intracellular IP_3 which stimulates Ca^{2+} release from the endoplasmic reticulum. This is thought to trigger stromal interacting molecule 1 (STIM1) to relocate along microtubules towards the plasma membrane where it activates Ca^{2+} influx through the Ca^{2+} channel Orai1 and together, these increases in intracellular Ca^{2+} stimulate CaCC. We have demonstrated that UTP/Ca^{2+}-activated Cl^- secretion via CaCC declines by 50% in CF female subjects when estrogen is elevated around the time of ovulation, which is likely a consequence of impaired Ca^{2+} entry due to elevated estrogen levels. Estrogen also inhibited Ca^{2+} signaling and ASL volume homeostasis in non-CF and CF airway epithelia and in an estrogen receptor null cell line which was transfected with estrogen receptor alpha. Thus, we propose that peak levels of estrogen impair Ca^{2+} signaling and downstream Cl^- secretion leading to mucus dehydration in CF females leaving them more at risk of mucus stasis and infections than either CF males or non-CF females with functional CFTR. How estrogen receptor alpha can interact with the Ca^{2+} pathway, and whether this interaction involves STIM1/Orai1 remains to be determined

S5.2 – Airway surface liquid volume regulation determines airways phenotype in Liddle's versus βENaC-overexpressing mice

Marcus A. Mall[1,2], Brian Button[4], Bjarki Johannesson[1,2], Zhe Zhou[1], Alessandra Livraghi[4], Ray A. Caldwell[4], Susanne C. Schubert[1], Carsten Schultz[2,3], Wanda K. O'Neal[4], Sylvain Pradervand[5], Edith Hummler[5], Bernard C. Rossier[5], Barbara R. Grubb[4], Richard C. Boucher[4]

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Studies in cystic fibrosis (CF) patients and mice overexpressing the epithelial Na^+ channel β-subunit (βENaC-Tg) suggest that raised airway Na^+ transport and airway surface liquid (ASL) depletion are central to the pathogenesis of CF lung disease. However, patients or mice with Liddle's gain-of-function βENaC mutations exhibit hypertension but no lung disease. To investigate this paradox, we compared the airway phenotype (nasal vs. tracheal) of Liddle's with Cftr-deficient, βENaC-Tg, and double mutant mice. In mouse nasal epithelium, the region that functionally mimics human airways, high levels of Cftr expression inhibited Liddle's ENaC hyperfunction. Conversely, in mouse trachea, low levels of Cftr failed to suppress Liddle's ENaC hyperfunction. Indeed, Na^+ transport measured in Ussing chambers ('flooded' conditions) was raised comparably in Liddle's vs. βENaC-Tg mice. Because this Na^+ transport measurement did not correlate with lung disease, measurements in tracheal cultures under physiologic 'thin film' conditions and *in vivo* were performed, which demonstrated that regulation of ASL volume and ENaC-mediated Na^+ absorption were intact in Liddle's, but defective in βENaC-Tg mice. We conclude that the capacity to regulate Na^+ transport and ASL volume, not absolute Na^+ transport rates in Ussing chambers, describes the key physiologic function protecting airways from dehydration-induced lung disease.

S5.3 - The design of inhaled ENaC blockers for the treatment of lung disease in cystic fibrosis

Henry Danahay[1] & Stephen P Collingwood[2]

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There are now compelling data to support the hypothesis that CF lung disease results from a failure of innate host defence mechanisms as a consequence of airway mucosal dehydration. The regulation of airway surface liquid (ASL) volume through the coordinated activity of ion channels and transporters in the airway epithelium is crucial to the effectiveness of mucus clearance by both mucociliary and cough clearance mechanisms. In the simplest pathophysiological paradigm for CF the absence or reduction of functional CFTR at the apical membrane of ciliated airway epithelial cells results in an imbalance of CFTR-mediated Cl^- secretion and ENaC (epithelial sodium channel)-mediated Na^+ absorption resulting in a net loss of fluid from airway surfaces. Approaches to normalize this imbalance and restore fluid to the airway mucosa are predicted to improve mucus clearance and thereby positively impact upon lung health in CF.

One approach to restore hydration to the airway mucosa is to attenuate ENaC function, thereby blocking Na^+ and fluid reabsorption. Early studies failed to demonstrate a robust clinical benefit of inhaled amiloride, a low potency ENaC blocker, in the CF lung. It has been proposed that the lack of clinical efficacy observed with amiloride was as a consequence of its low potency, short duration of action in the airway and the dose limiting side effect of hyperkalaemia. Novel ENaC blockers that demonstrate a long duration of action in the airway and that are safe, may therefore represent a novel therapeutic opportunity for the treatment of CF lung disease. An approach taken by Novartis, has been to design ENaC blockers specifically for inhaled dosing by dry powder delivery. Drug candidates will be described that exemplify the design strategy adopted to achieve both a potent and sustained activity in the lung together with a reduced potential to induce the mechanism-based side effect of hyperkalaemia, in pre-clinical models.

S5.4 - Human lung explant tissue as a new ex vivo model for preclinical optimisation of CFTR pharmacotherapy

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In cystic fibrosis (CF) the CF transmembrane conductance regulator (CFTR) basic defect is currently targeted by multiple approaches to restore CFTR chloride (Cl^-) channel activity using correcting and potentiating pharmacotherapeutic compounds. These drugs are evaluated and pharmacologically optimised *in vitro* in studies on cell lines, animal models and tissue cultures. However, CFTR modulators might have a different efficacy in native human CF epithelia. Availability of native human CF tissues is relatively limited, and size and condition are important quality markers. Hence, aim of this study was to establish lung explant epithelia from CF and disease control patients receiving lung transplantation as a new *ex vivo* model for preclinical optimisation of CFTR pharmacotherapy.

We included native human bronchial tissue sheets from 21 lung explants (CF, COPD, pulmonary fibrosis) and donor bronchi obtained during lung transplantation at the Hannover transplant center in this study so far. Multiple (n=8) tissue pieces per subject were prepared immediately after surgery, transepithelial short-circuit current (I_{sc}) measurements in perfused Mini Ussing chambers were registrated and structure control was obtained by histopathology.

Basal tissue bioelectric properties and I_{sc} responses to inhibition of the epithelial sodium channel (ENaC) and stimulation of CFTR and alternative Cl^- secretion in CF and (disease) control bronchi showed reproducible results so far. The performed preparation technique resulted in tissue segments with intact respiratory epithelia including submucosal glands.

The results of this pilot study confirm the feasibility of *ex vivo* Cl^- secretion measurements in native human lung explant tissue as an additional resource of human CFTR relevant tissue. More data about variability, optimal evaluation protocols and possible limitations of the method will be obtained, before most promising CFTR correctors/potentiators with potency in the nanomolar range can be included in the analysis. Our strategy can be an essential step in the translation of small molecule compounds into future clinical trials aiming to rescue the CFTR basic defect.

Supported by a financial grant from Mukoviszidose e.V., Bonn, the German Cystic Fibrosis Association, and an EuroCareCF training grant

S5.5 - Ion transport characteristics of human nasal epithelial cells from wild type and cystic fibrosis donors

Hazel C Atherton-Watson[1], Francine De Courcey[2], Thomas Riley[3], Grzegorz Skibinski[2], Madeleine Ennis[2], J Stuart Elborn[2], Henry Danahay[1]

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

In cystic fibrosis (CF), patients with class IV and V mutations in CFTR generally have a mild phenotype. Patients with an R117H allele, generally have more severe allele such as F508del or G551D. The aims of this study were to develop a culture method that would enable the routine investigation of the ion transport characteristics of primary human nasal epithelial cells derived from CF donors.
Nasal epithelial cells (NEC) were extracted from wild type and cystic fibrosis volunteers by nasal brushing. The cells were expanded and frozen for future use. These cells were then further expanded and passaged onto collagen coated Snapwell inserts (Costar) using a modification of a previously described method (Danahay et al 2002). Once confluent, the cells were cultured at an apical air interface for 14-21 days, at which point, the ion transport phenotype was assessed.
Each of the wild type donors studied, developed an amiloride-sensitive short-circuit current (I_{SC}). These cells also developed a $CFTR_{inh}172$-sensitive I_{SC} upon the addition of forskolin. Similarly, each of the CF donors NEC demonstrated an amiloride-sensitive current, irrespective of genotype. F508del/R117H and R117H/G551D NEC did exhibit a forskolin-induced I_{SC} which was sensitive to $CFTR_{inh}172$, but that was smaller than that observed with the wild type donors. The F508del/F508del NEC failed to elicit a forskolin-induced response, however, the cells demonstrated a UTP-induced I_{SC}. Following a 20 hour incubation at 27°C, the F508del/F508del NEC demonstrated a forskolin-induced current which could be further potentiated by genistein.
This study has illustrated a cell culture method suitable for the routine culture of primary human nasal epithelial cells. The cells display an ion transport phenotype consistent with the native epithelium, and that is dependent upon CFTR genotype. Furthermore, F508del/F508del cells can be temperature corrected consistent with similar observations made in engineered cell systems. This culture method will allow characterisation of *in vitro* ion transport phenotype of different CF genotypes.

Danahay et al. 282 (2): 226. (2002) AJP Lung

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S5.6 - Regulation of ENaC in Human Bronchial Epithelial Cell Cultures by protein, sPLUNC1

Brett Rollins[1], Agustin Caballero[1], Jack Stutts[1], Robert Tarran[1]

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One significant pathophysiological mechanism underlying Cystic Fibrosis (CF) is the hyperabsorption of sodium through ENaC. Further understanding of this misregulation could help develop novel therapeutic targets for treating CF. Channel Activating Proteases (CAPs) have been shown to increase ENaC activity but it is not clear whether CAP activity is physiologically regulated, or whether this regulation is defective in CF airways.

We have previously shown that normal primary human bronchial epithelial cultures (HBECs) exhibit a trypsin-sensitive transepithelial voltage (Vt) when airway surface liquie (ASL) has accumulated for 48 hours. Conversely, freshly washed normal and CF HBECs, as well as CF HBECs with accumulated ASLs, remained trypsin-insensitive indicating that ENaC is maximally activated in these cultures. This implies that the CAP inhibitor is soluble and accumulates in the ASL. Additionally, accumulation of this inhibitor is unable to inhibit ENaC conductance in CF cultures. (Tarran et al., 2006).

We then searched for soluble CAP inhibitors with trypsin-coated beads, which were added to the ASL. Albumin-coated beads were used as a control for non-specific binding. Using mass spectrometry, we identified sPLUNC1. This protein is secreted, *in vivo* and *in vitro,* in the ASL although its function is unknown.

To understand of the role of sPLUNC1 in airway physiology, we examined the capacity for ASL regulation in HBECs treated with either anti-sPLUNC1 or anti-luciferase shRNA as a control. qPCR verified a >90% knockdown efficiency, which resulted in significant ASL volume depletion in the sPLUNC1 knockdown HBECs as compared to the controls (ctrl, 9.0 ±1.8 uM; anti-sPLUNC1, 5 ±0.9uM; n=5). This was accompanied by a persistent trypsin-sensitive Vt in sPLUNC1-defecient cultures, indicating that they could no longer regulate ENaC. Our next aim was to examine the effect of sPLUNC1 on ENaC current. To do this, xenopus oocytes were injected with sPLUNC1 and α,β,γ ENaC subunits. sPLUNC1 was detected in the media of these oocyte by Western blot and showed a 43% (± 4%, n=40) inhibition of ENaC current when compared to ENaC injected oocytes alone. Western blot analysis revealed that this reduction in current was accompanied by a reduction in cleavage of α, β and γ ENaC subunits suggesting that SPLUNC1 prevents cleavage of ENaC by serine proteases.

These findings indicate an important role for sPLUNC1 in airway physiology as it regulates ENaC current and consequently, ASL volume. Understanding the mechanism of sPLUNC1 may provide insight into novel therapies for regulating hyperactive ENaC in CF.

Friday 17 April – 18:00-19:30 **Room: Dom Sebastio**

SPECIAL GROUP DISCUSSION – III
Modifier Genes- what have we learnt?
Moderators: G. Cabrini (IT) & M. Drumm (US)

Geneticists exploit naturally occurring variation to identify genes that contribute to, or cause, a phenotype. With advances in genotyping technology, the speed, scope and affordability of genotype/phenotype studies has increased dramatically in recent years and thus the number of genetic association reports has risen accordingly. For monogenic diseases such as cystic fibrosis, this technology has been applied to the search for genes that modify some aspect of the CF phenotype. This special group discussion will review studies that have examined relationships between clinically relevant and important phenotypes to CF, such as pulmonary function, inflammatory state of the airways, resistance of the lungs to infection by bacteria, growth indices, just to name a few. Other topics for discussion will include gender differences in genotype/phenotype associations and gene x environment interactions. These genetic approaches are designed to identify genes involved in a process, but they are not designed to elucidate mechanism. Thus, some discussion will be devoted to how specific variants may be giving rise to the observed phenotypic associations.

The goals of the session will be:
1. Describe the concepts of genotype/phenoytpe associations for the non-geneticist to make the discussions accessible to the entire research community.
2. Point out the power of these studies and the caveats inherent to them.
3. Critically evaluate the studies presented to understand the nature of the associations.
4. Describe how the genes implicated would influence the pathophysiology of CF and discuss the biologic plausibility.

Friday 17 April – 18:00-19:30 **Room: Infante Santo**

SPECIAL GROUP DISCUSSION – IV
Pharmacology - how do correctors and potentiators work?
Moderators: D.N. Sheppard (UK) & O. Moran (IT)

Current therapies for CF target the consequences of CFTR dysfunction rather than the defects at the root of the disease. In the search for new drugs to treat CF, high-throughput screening (HTS) has been used to identify small molecules that rescue the cell surface expression of CF mutants (termed CFTR correctors) and correct the defective gating of mutant Cl- channels (termed CFTR potentiators). Excitingly, clinical trials of CFTR correctors and potentiators have now begun and early results are promising. But, how do small molecules that target defects at the root of CF work? To address this pressing issue, this Special Group Discussion will consider the following questions:

1. At what steps during the synthesis, maturation and trafficking of CF mutants do CFTR correctors act? Do they promote folding, facilitate the interaction of CF mutants with chaperones and other components of the cell trafficking machinery or alter quality control mechanisms?
2. What are the consequences for CFTR function of delivering mutant CFTR to the cell surface? Will potentiation of CFTR activity still be required?
3. Do CFTR correctors affect the stability of mutant CFTR at the cell surface?
4. Despite the identification of many CFTR potentiators, structure-activity relationships are poorly defined. Is there a common pharmacophore for CFTR potentiators or will structure-activity relationships be specific to different classes of small molecules?
5. There is evidence to suggest that small molecules interact with a number of sites on CFTR to modulate channel function. However, the binding sites of small molecules have not been conclusively identified. Where do CFTR potentiators dock with CFTR and which site achieves the best potentiation of CFTR function?
6. Many CFTR potentiators also inhibit the CFTR Cl- channel at elevated concentrations. Is it possible to develop CFTR potentiators without this untoward effect?
7. Will CFTR correctors and potentiators treat the consequences of CFTR dysfunction including increased mucus viscosity, inflammation and bacterial infection?
8. How much correction or potentiation of CFTR can be achieved with small molecules? Is there a ceiling? Or is a pharmacological “cure” for CF feasible.

We invite you to discuss these questions at this SGD.

Work in OM’s laboratory is supported by the Italian Cystic Fibrosis Research Foundation (Prog. FFC #2/2008),with contribution of Mille bambini a Via Margutta, Blunotte, Lega Italiana FC - Toscana.
DNS’s group is supported by the CF Trust and EPSRC.

Saturday 18 April – 09:00-10:30 **Room: Infante Santo**

SYMPOSIUM 6 – Genes and the Environment
Chairs: M. Drumm (US) & B. Scholte (NL)

S6.1 - The CFTR active chromatin hub

Christopher J Ott, Neil P Blackledge, Jenny L Kerschner, Shih-Hsing Leir, Ann Harris

Children's Memorial Research Centre/ Northwestern University
Chicago, Il, USA

S6.2 - ENaC in CF and CF-like disease

Abul Kalam Azad,[1] Robert Rauh,[2] François Vermeulen,[3] Martine Jaspers,[1] Judit Korbmacher,[2] Kris De Boeck,[3] Jean-Jacques Cassiman,[1] Christoph Korbmacher[2] and Harry Cuppens[1]

[1]Center for Human Genetics, Katholieke Universiteit Leuven, Belgium; [2]Institut für Zelluläre und Molekulare Physiologie, Universität Erlangen-Nürnberg, Germany; [3]Department of Pediatrics, UZ Gasthuisberg, Belgium

Not in all patients with CF or CF-like disease, a mutation can be found on both CFTR genes. Moreover, CF disease severity can be very variable, irrespective of the CFTR genotype because of the action of modifier genes and environmental factors.

Apart from the defective chloride transport, sodium absorption is increased in CF patients. Sodium transport is mediated by the amiloride sensitive epithelial sodium channel (ENaC), which is build up of three subunits, SCNN1A, SCNN1B and SCNN1G.

We investigated if mutations in the ENaC subunits might be involved in unexplained CF(-like) disease (76 patients) and/or modulate CF lung disease severity (683 CF patients homozygous for F508del originating from different regions from Europe; 156 Danish, 121 Czech, 104 Belgian, 101 British, 101 Italian, 100 German).

In a small fraction of patients with unexplained CF(-like) disease, disease could be potentially explained by an ENaC mutation by a Mendelian mechanism, such as p.V114I and p.F61L in *SCNN1A*.

More importantly, a more than 3-fold increase in incidence of several rare ENaC polymorphisms having a MAF<2.5% (c.45+5G>C, c.4614C>T, p.P33P, p.F61L, p.L180L-R181W, p.V114I, p.W493R in *SCNN1A*; p.S82C, c.904-5T>C, p.I515I, p.G589S, p.D629D in *SCNN1B*, and p.E197K in *SCNN1G*) was found in this patient group (30% versus 9% in controls; $p<0.0001$), indicating an involvement of ENaC in some patients by a polygenetic mechanism.

With regard to SNPs having a MAF>2.5%, homozygosity for the A allele at the c.1176+14A>G locus in *SCNN1G* was found at a 3-fold incidence in patients (11.8%) versus controls (4.1%) ($p<0.002$), with an odds ratio of 3.2 (95% CI: 1.4–6.9. This association was also found to a lesser extent for other loci that were in strong linkage disequilibrium with c.1176+14A>G.

From a genetic point of view, all these findings suggest an involvement or susceptibility of ENaC mutations in disease in some of these patients.

Some of these SNPs were also found to associate with lung disease severity in F508del homozygous CF patients.

Of special interest was the p.W493R-*SCNN1A* polymorphism, which was also found to result in a 4-fold more active ENaC channel when heterologously expressed in *Xenopus laevis* oocytes, which could be confirmed in *in vivo* nasal potential difference measurements. It was found that this polymorphism increases ENaC channel open probability and reduces Na^+ self inhibition. The p.W493R-*SCNN1A* favors constitutive activation of ENaC by reducing the inhibitory effect of extracellular sodium and by reducing the pool of near-silent channels.

About 1 in 975 individuals in the general population will be heterozygous for the hyperactive p.W493R-*SCNN1A* mutation and a *CFTR* gene that results in very low amounts (0-10%) functional CFTR. These ENaC/CFTR genotypes may play a hitherto unrecognized role in lung diseases.

S6.3 – Quantifying the contribution of genetic and non-genetic factors to variation in CF

Garry R. Cutting, M.D. on behalf of the U.S. CF Twin and Sibling Study

S6.4 - Toll-like Receptor 5 as Modifier Gene in CF Lung Disease

Christoph J Blohmke[1], Rachel E Victor[1], Aaron Hirschfeld[1], Julie Park[2], Denise Daley[2], Dorota Stefanowicz[2], Andrew J Sandford[2], Stuart E Turvey[1]

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Introduction: There is growing evidence that polymorphic variants in genes other than *CFTR* play an important role in determining severity of CF lung disease. We recently demonstrated that the innate immune receptor TLR5 mediates much of the damaging inflammatory response generated by CF airway cells following exposure to *P. aeruginosa*. Furthermore, it has been suggested that TLR5 is involved in bacterial clearance from the airways in humans and mice. Together, this evidence identifies *TLR5* as a biologically plausible candidate gene that may modify CF lung disease.
Objective: To confirm the pro-inflammatory phenotype of CF airway epithelial cells and to determine if polymorphisms in the *TLR5* gene modify the severity of pulmonary disease in patients with CF.
Methods: CF and non-CF cell lines were stimulated with *P. aeruginosa* wt and a strain lacking flagellin (Δ*fli*C). We genotyped one functional non-synonymous SNP in the *TLR5* gene (*TLR5 R392X*, rs5744168) in healthy individuals and stimulated PBMCs from carriers and non carriers with purified flagellin to asses the impact of the SNP on TLR5 signaling capacity. TLR5 genotypes of 2,441 CF patients were correlated with severity of CF lung disease. Outcome variables measured were a) lung function (i.e. cross-sectional measurement of FEV1 %predicted) and b) the annual decline of lung function (i.e. longitudinal measurement of FEV1 %predicted).
Results and Conclusions: CF epithelial cell lines recognize *P. aeruginosa* through the flagellin-TLR5 interaction and produce an exaggerated pro-inflammatory cytokine response. The *TLR5 R392X* SNP was functionally active, significantly decreasing TLR5 responsiveness of PBMCs up to 75% ($p < 0.0001$). Our data identify *TLR5* as an excellent biologically plausible candidate gene that may modify CF lung disease. As a follow up to this study, the *TLR5 R*392X SNP has been genotyped in 2,441 CF patients and ongoing statistical analysis of the impact of this SNP on lung function will examine whether genetic variation in *TLR5* modifies CF lung disease.

S6.5 - Effects on mRNA Processing of CFTR Splicing Mutations assessed by Novel Mini-genes

Anabela Santo Ramalho[1,2], Margarida D Amaral[1,2]

[1]National Institute of Health Dr. Ricardo Jorge, Department of Genetics, Lisboa, Portugal, [2]Faculty of Sciences, University of Lisboa, Department of Chemistry and Biochemistry, Lisboa, Portugal

Despite that more than 1,600 variants of the CFTR gene have been identified [1], the functional effects at the level of CFTR expression and/or function remain largely unknown for most of them. Moreover, the major deleterious effect of some "missense" mutations is actually at the level of mRNA processing rather than a coding defect.

Our goal here was to create adequate cellular models to study the effects of splicing or PTC mutations found in CF patients, so as to allow characterizing the effects of CF mutations at the RNA level and also to enable further analysis of the respective protein products.

As most CFTR introns are longer than 2 kb and hence difficult to introduce into the complete CFTR cDNA (4.7 Kb). Our strategy was to produce CFTR mini-genes including the complete CFTR coding sequence plus one or two introns (IVS). We, thus introduced by mutagenesis the full length sequence of the smallest CFTR intron (IVS22 -600bp) or a shorter version (artificial) of IVS19 (art-IVS19-442bp), containing the original splicing sites into the CFTR-cDNA pNUT construct. We also created a CFTR mini-gene containing both introns. Stable BHK cells expressing these CFTR mini-genes were produced.

Results from RT-PCR analysis confirmed that correct splicing occurs for transcripts from all three mini-genes and Western blot (WB) shows that they all generate fully-processed CFTR protein. The p.I1234V (c.3832A>G) CFTR mutation was inserted into the art-IVS19 mini-gene and RT-PCR analysis shows that an alternative transcript lacking the last 18 nucleotides of IVS19 is produced, consistently with results in native tissues from a patient with this mutation. The resulting protein is mostly detected in its core-glycosylated form but reduced function as Cl^- channel could be detected. When this mutation was introduced into a simple CFTR cDNA construct, the resulting I1234V-CFTR exhibited function as a Cl^- channel undistinguishable from that of wt-CFTR. By using this mini-gene and spicies homology studies we discovered a second-site mutation (c.3836G>A) that reverts the alternative splicing of c3832A>G and hence, also restoring normal protein production.

In conclusion, the mini-genes described here are useful tools to characterize the defects of CFTR splicing mutations and possibly also to test therapeutic approaches aimed at correcting CFTR such defects.

[1] The CFTR mutation database, 2009, http://www.genet.sickkids.on.ca/cftr/

ASR is supported by postdoctoral fellowship SFRH/BPD/20622/2004 (FCT, Portugal) and BioFiG.

Saturday 18 April – 11:00-12:30 **Room: Infante Santo**

SYMPOSIUM 7 – Inflammation in CF
Chairs: T. Bonfield (US) & G. Cabrini (IT)

S7.1 - Accumulation of NKT cells in cystic fibrosis

Nanna Siegmann[1], Thilo Biedermann[2], Mischo Kursar[3], Uta Griesenbach[4], Erich Gulbins[5], Gerd Döring[1]

Institute of Medical Microbiology and Hygiene[1], Department of Dermatology[2], University of Tübingen, Tübingen, Max Planck Institute for Infection Biology[3], Berlin, Germany, Imperial College[4], London , UK, Department of Molecular Biology and Center for Medical Biology[5], University of Essen-Duisburg, Germany.

S7.2 – Identification of Components of Innate Defence in the Airway Surface

Olga Zegarra-Moran, Nicoletta Pedemonte, Emanuela Caci, Elvira Sondo, Antonella Caputo, Luis J.V. Galietta.

Laboratory of mOlecular Genetics, Istituto Giannina Gaslini
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The airways are continuously challenged by material arriving with breathed air, such as pollen, ash, dust, mold spores, bacteria, and viruses, that may damage the lungs or produce lung infections. The first line of defence is given by mucociliary clearance, the effectiveness of which depends on the volume and composition of the airway surface fluid (ASF), that is maintained through a precise balance between ion/water secretion and absorption. This is obtained by exerting a tight control on the activity of ion channels and transporters localized on the apical and basolateral membranes of epithelial cells. The ASF contains also other molecules that defend the airways from microbial colonization.

We are interested in identifying some of the components of the innate immunity present in the ASF and in understanding the mechanisms involved. To this aim, we have used proinflammatory cytokines (i.e. interleukin-4, interferon-gamma, tumor necrosis factor alpha, interleukin 1-beta) as tools to mimick inflammatory conditions and modulate epithelial components. This approach has previously permitted other authors and us to show that inflammatory conditions modify the ion transport properties of the airway epithelial cells by changing the expression and activity of ion channels like CFTR, the epithelial sodium channel and the Ca^{2+}-dependent Cl^- channel, TMEM16A. The consequent changes in the volume and composition of ASF secretions might favour the fluidification of mucous secretion and, in consequence, the mucociliary clearance.

We have used proinflammatory cytokines in a proteomics study to identify the proteins secreted by the airway epithelium under physiological and pathological conditions. This approach has led to the identification of several proteins secreted in the ASF, including immune-related proteins, enzymes, protease inhibitors, and a metalloproteinase, the secretion of some of which resulted modulated by proinflammatory cytokines.

Incubation of airway epithelia cells with interleukin-4 has also permitted us to identify an apically-located electroneutral transport system mediated by pendrin (SLC26A4) and involved in transepithelial SCN^- transport. SCN^-, permeable also through CFTR and TMEM16A, is an anion involved in innate defense because the oxidized product, hypothiocyanite, has antimicrobial activity.

All these results underscore the active role that the epithelium has in the ASF barrier function.

Supported by a grant from Italian Cystic Fibrosis Research Foundation

S7.3 – CFTR as an inflammation suppressor: the role of protein kinase CK2

Hunter MJ, Land SC, Winter A, Cassidy D, Treharne KJ and Mehta A

Division of Medical Sciences Centre for Cardiovascular and Lung Biology University of Dundee

In CF, links between disrupted ion transport and inflammation are not understood but both ensue after F508 deletion in NBD1. This presentation links these entities via F508 and the pluripotent protein kinase CK2. Last year, we reported that wild-type CFTR (wtCFTR) expression significantly depressed basal NFκB activation (HeLa cells by ~50%). Now, Vij *et al* confirm wtCFTR as an inflammation suppressor (PLoS one, 4, e4664;2009). Thus, independent laboratories agree on a new CF concept.

***Our results**:* When H441 epithelial cells (basally wtCFTR-null) are incrementally-transfected with wtCFTR (0-400ng cDNA), basal-inflammation (NFκB promoter activity) is dose-dependently suppressed (~50%,$p<0.05$;$n=5$). Conversely, after suppressing pre-existing wtCFTR in Calu-3 cells, NFκB-inflammation is enhanced (~5-fold,$p<0.05$;$n>5$). Interestingly, Vij *et al* report that inflammation suppression does not occur after co-expression of F508delCFTR+wtCFTR and some 'F508delCFTR-correctors' also fail to suppress F508delCF-inflammation (Talebian et al Cell Physiol Biochem 23:199-204;2009). The combined data suggest a pro-inflammatory role for F508delCFTR dominating over our proposed basal, counter-balancing and inflammation-suppressive role for its wild type cousin.

Could protein kinase CK2 link ion transport to inflammation? CF research is dominated by kinases controlling CFTR function. What about the reverse? Could F508 in CFTR control a kinase? Pagano *et al* (Biochemistry 47:7925-36;2008) find that the F508-residue, in the context of certain adjacent amino acids, controls the function of a CFTR-regulated protein kinase, CK2, such that F508-deletion allosterically enhances kinase activity several fold. CK2 phosphorylates/controls NFκB, thus potentially bypassing canonical activation pathways. Could wtCFTR normally act as a suppressor of NFκB-inflammation using CK2 as an (F508-regulated) amplifier? Preliminary data show when wtCFTR is present, pharmacological CK2 inhibition significantly elevates NFκB-mediated inflammation. This regulatory inhibition via CK2 is consistent with our *in vivo* work expressing the same F508-peptide region of NBD1 where we show that F508-dependent manipulation of our proposed CK2-NBD1 control-interface disrupts effector proteins within the epithelial NADPH-oxidase complex required for bacterial killing by lung epithelial cells (Cohen et al, Dev Dyn 238:386-93;2009). Since CK2 also controls sodium channels (Bachhuber et al J Biol Chem 283:13225-32;2008; Brechet et al J Cell Biol 183:1101-14;200;2008), as well as CFTR channel function (submitted), we believe protein kinase CK2 is a latent player in CF pathology.

Patient Implications: CF inflammation might not be a homogeneous entity i.e. 'inflammation' without wtCFTR (*eg* G542X; failed suppression) might be different from 'inflammation' induced by F508delCFTR (dysregulated CK2). Since CK2 controls CFTR and sodium channels, our invocation of CK2 in CF pathogenesis might co-locate inflammation and ion transport into a single model.

Question: By proposing that CK2 requires F508 for control, could the multi-system nature of F508del CF arise from a mishapen internal NBD1 surface near the missing F508 thereby mis-controlling an essential, multi-functional, pluripotent kinase?

Speculation: CK2 is an oncogene controlling >300 genes/proteins *in vivo* regulating inflammation/apoptosis/proliferation (and now, ion transport). We propose that a latent allosteric role for residues near F508 exists thereby creating one of the R's in CFTR.

Support: KD-calu-3 cells: Alan Cuthbert, Cambridge. **MJH**: CF Trust-PJ538. **KJT/DC**: Wellcome Trust (075237); **AW** (Chest Research-Education Fund); **Cohen *et al***: The Russell Trust/Brady Foundation.

S7.4 – Glucocorticoids fail to inhibit inflammatory process in CF human bronchial epithelial cell lines

Carine Rebeyrol[1], Loic Guillot[1], Vinciane Saint-Criq[1], David Ray[2], Annick Clement[1], Olivier Tabary[1], Philippe Le Rouzic[1]

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Background: Lung dysfunction is the main cause of mortality in CF patients. Infection combined with inflammation lead to progressive destruction of respiratory epithelium. Glucocorticoids (GC) are powerful anti-inflammatory molecules commonly used to treat inflammation but with controversial efficiency among studies. Recent clinical trials showed that inhaled and oral corticosteroids have no significant effect on lung function or markers of inflammation. These results were confirmed in *in vitro* studies using cell lines and primary cell cultures from CF patients.
In order to characterise the molecular basis of such dysregulation, our project focuses on the key steps in GC activation pathway in bronchial epithelial cells.

Methods: CF and non CF human bronchial epithelial cell lines were incubated with IL-1β or TNF-α (10 ng/ml) at 4h, 8h, and 16h with or without dexamethasone (dex, 1 µM). Rates of secreted IL-8 were assessed by ELISA 16h after treatment. To evaluate GR transrepression and transactivation at 4h and 8h, we transfected cells using plasmids containing NF-κB, AP-1 or TAT3 promoter coupled to luciferase.

Results: In presence of IL-1β, basal secretion of IL-8 was restored by dex at 16h in non CF cells, whereas CF cells barely respond (only 30% of inhibition). Other results showed there is no effect of dex on NF-κB activation at 8h in CF cells.

Conclusions: This study shows a lack of GC efficiency to decrease IL-8 secretion via NF-κB pathway in CF bronchial epithelial cells.

Identify the origin of the GC resistance in CF will allow to adapt the anti-inflammatory treatments of patients.

Supported by the french Cystic Fibrosis association "Vaincre La Mucoviscidose"

S7.5 – Pro-inflammatory Toll-like receptor 2 expression in CF epithelium

C Kelly, PJ Buchanan, A Bingham, SJ Elborn and BC Schock

Centre of Infection and Immunity, Queen's University Belfast, UK

Background: Increased and prolonged *Pseudomonas aeruginosa* (PA)-induced signalling via activation of Toll-Like Receptor 2 (TLR2) contributes to chronic pulmonary inflammation in Cystic Fibrosis (CF). Internalisation of the receptor complex leads to activation of NF-kB and AP-1 pathways and subsequent pro-inflammatory response (eg. IL-8). Prolonged intracellular signalling may occur when a receptor remains perinuclear, suggesting that endosome formation and subsequent lysosomal degradation may be important for signal termination.

Hypothesis: A defect in the elimination of the TLR2-PA-signalling-complex in CF epithelium raises intracellular TLR2 leading to prolonged pro-inflammatory signalling.

Methods: We used the well established cell lines HBE (human bronchial epithelial cells, non-CF) and CFBE (CF bronchial epithelial cells, F508D) Using CF (F508Δ) and non-CF epithelial cell lines (CFBE, HBE). The kinetics of TLR2 expression upon LPS (50 ug/ml) stimulation is determined by FACS analyses. The endo-lysosomal degradation is followed using immunohistochemistry and antibodies to the early endosome (EEA-1) and the lysosome (LAMP-1).

Results: We show increased TLR2 expression (by FACS). In CFBE cells TLR2 protein expression was higher but not significantly different from that in HBE cells. However, intracellular expression was significantly higher in CFBE (1h, 8h and 12 h of PA-LPS exposure ($p<0.05$ for 8 and 12h). The high expression of TLR2 coincided with increased secretion of IL-8 (24 h, $p<0.05$) in CFBE compared to HBE cells. Investigating endo-lysosomal degradation (immunocytochemistry) showed prolonged expression of the early endosome (EEA1) and failure to form lysosomes (LAMP1) in CFBE cells suggesting decreased processing of endocytosed material and sorting into the lysosomal degradation pathway in CFBE cells. Furthermore we investigated the ubiquitiniation enzyme A20 in CF epithelium and show that A20 protein expression is significantly decreased in CF epithelium which may contribute to the prolonged signalling in CF epithelium.

Conclusion: We conclude that a defect in the elimination of the TLR2-PA-signalling-complex raises intracellular TLR2 leading to prolonged pro-inflammatory signalling.
The importance of the formation of the endosome/lysosome and mechanisms involved in regulation of TLR2 signalling may represent new therapeutic targets to combat chronic PA inflammation in CF airways.

Symposium 8 – Bacterial Killing in the CF Airways
Chairs: S. Elborn (UK) & G. Döring (DE)

S8.1 -.CFTR genotype determines the functional responses of murine alveolar macrophages

L.V. Deriy[1], G. Zhang[1], E.A. Gomez[1], A.J. Gallan[1], J.A. Hopson[1], D. Beacham[2], V. Bindokas[1], and D.J. Nelson[1]

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Alveolar macrophages and neutrophils play major roles in host defense against microbial infections in the lung. In order to perform this function, they must ingest and destroy pathogens, generally in phagosomes, as well as secrete a number of products that signal other immune cells to respond. Recently, we demonstrated that murine alveolar macrophages (AMs) but not neutrophils employ the CFTR (Cystic Fibrosis Transmembrane conductance Regulator) Cl^- channel as a major shunt mechanism in lysosomal acidification. Lysosomes and phagosomes in murine *cftr*$^{-/-}$ AMs failed to acidify and the cells were deficient in bacterial killing compared to wild-type controls. In this study, we investigate the role of CFTR genotype plays in a common set of phagocyte core functions namely, organellar acidification, granule secretion, and microbicidal activity. We establish the differential dependence of lysosomal acidification on CFTR expression in murine AMs, to that of peripheral blood monocytes and peritoneal macrophages where lysosomal acidification is CFTR independent. Utilizing primary AMs obtained from normal, CFTR-deficient as well as mutant mice, we show that there is a tight correlation between levels of lysosomal acidification, bacterial killing and agonist-induced secretory responses and CFTR genotype. Mice lacking CFTR expression or expressing the ΔF508 mutation showed a significant decrease in organellar acidification, bacterial killing and GTP-γ-S induced secretion as assayed using high resolution membrane capacitance techniques. Organellar acidification and bacterial killing for the G511D AMs showed intermediate levels of both organellar acidification and bacterial killing and a significant decrease in the agonist-induced secretory response over that observed in wild type cells. Taken together these data are consistent with a model in which the functional response of AMs is differentially dependent upon CFTR genotype.

S8.2 - How CFTR modulates survival of *P. aeruginosa* in the lung

Gerald B. Pier

Channing Laboratory,
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The extreme hypersusceptibility of CF patients to chronic *Pseudomonas aeruginosa* infection implicates the cystic fibrosis transmembrane conductance regulator (CFTR) as a key factor in host resistance to this pathogen. No other genetic disease is associated so closely with a single pathogen able to cause the vast majority of the morbidity and life-shortening mortality as *P. aeruginosa* does in individuals around the world with CF. While a panoply of host physiologic conditions emanating from loss of functional CFTR contribute to susceptibility to *P. aeruginosa* infection, a key factor is the loss of CFTR as a major epithelial cell receptor for *P. aeruginosa*. Binding of this bacterium to CFTR via the conserved outer core oligosaccharide of the bacterial lipopolysaccharide initiates wild-type (WT) host resistance that quickly eliminates this pathogen from the lung. In individuals with WT-CFTR a rapid, coordinated inflammatory response is initiated when *P. aeruginosa* binds to CFTR, including release of interleukin-1 (IL-1) within 2-10 minutes, activation of nuclear translocation of NF-κB in 5-15 min, transcription of NF-κB dependent genes and secretion of proteins needed to recruit PMN into the airways within 3-6 hrs, with and ultimate resolution of this inflammatory responses in 4-24 hr by apoptosis of the cells binding to *P. aeruginosa*. Lack of this response in CF results in continued *P. aeruginosa* presence in the airways, sticking of bacteria to mucins, entry into mucus plugs that cannot be cleared quickly and eventual formation of a microcolony with subsequent elaboration of alginate, leading to the initiation and maintenance of chronic infection. Lack of *P. aeruginosa* binding to CF airway epithelial cells is well documented from over 20 years of pathology studies of lungs from patients and confirmed in animal studies using transgenic CF mice, while *P. aeruginosa* binding to epithelial cells in the lungs of mice and monkeys with WT-CFTR has also been documented. Thus, the proposal that airway epithelial cells with WT-CFTR binds *P. aeruginosa* while in CF there is no binding to these cells is entirely consistent observations in patients and animals. Additionally, PMN recruitment appears to be key, even when responding to low infectious doses (<50 cfu into the lung) as neutropenic mice cannot resist infection with very low challenges doses of *P. aeruginosa*. Overall it appears CFTR is the key host factor orchestrating human innate immunity to *P. aeruginosa* infection and its functional loss in CF leading to >80% infection rates in most populations indicates other host anti-bacterial factors do not compensate for loss of CFTR's function. The ultimate consequence is that almost all CF patients with severe gene mutations are unable to counter the progressive infection with this organism that destroys the lungs over time.

S8.3 - CFTR and Myeloid Cell Immunity

Craig A. Hodges, Mitchell L. Drumm, Mary F. Koloze, Tracey L. Bonfield

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Cystic fibrosis (CF) patients have mutations in the gene encoding the chloride transport protein CFTR which when defective in the airway epithelium results in significant pulmonary morbidity and eventual mortality. The morbidities are associated with inefficient resolution of pulmonary infection with *Pseudomonas aeruginosa* and the ensuing inflammatory response. The airways are uniquely exposed to a continuous mix of inhaled stimuli. A delicate balance between constitutive immune activation and tolerance of inhaled agents is crucial for host homeostasis. A central cell maintaining this balance is the alveolar macrophage, which functions as a key initiator of innate immune responses and is necessary to perpetuate adaptive responses. The status of macrophage activation, cytokine production, phagocytosis, and antigen presentation, orchestrates the intensity and duration of the immune response including recruitment of neutrophils. How the unique CF lung milieu or direct C*ftr* expression impacts macrophage and neutrophil activity in response to infection is unknown. To define the role of *Cftr* expression on macrophage/neutrophil activity and function, animals were generated which were *Cftr* deficient in only myeloid cells. This was achieved by expressing cre recombinase from the lysozyme promoter in a mouse line carrying a floxed *Cftr* allele in which exon 10 is removed in the presence of cre recombinase. We demonstrated that mice homozygous for the floxed *Cftr* gene and positive for lysozyme-cre promoter lost exon 10 of *Cftr* in peritoneal macrophages and elicited neutrophils but not splenic T-cells validating the specificity of the knockout. Bronchoalveolar lavage (BAL) was done on the myeloid *Cftr* knockout mice and showed elevated numbers of cells $3.4\pm0.3 \times 10^4$ compared to controls $1.7\pm0.2 \times 10^4$ (n=6, p=0.003). Pathogen-recognition receptors toll-receptor 4 (TLR-4) and TLR-2 bind to pathogens and induce a series of molecular and physiological changes in macrophage and neutrophils enhancing innate bactericidal activity. To determine if the absence of functional *Cftr* had an impact on TLR gene or protein expression, macrophages and neutrophils were evaluated for TLR protein and gene expression by flow cytometry and real-time PCR respectively. BAL cell pellets from the myeloid-*Cftr* knockout had -4.1±0.4 fold less TLR-4 mRNA (n= 6, $p<0.05$) and -2.9±0.5 fold less TLR-2 mRNA (n=3, $p<0.05$) relative to controls. Using F480 as a surface marker for macrophages, BAL cells were evaluated for cell-specific presence of TLR-4 and TLR-2. Consistent with the gene expression in the BAL pellets, TLR-4 and TLR-2 surface expression was decreased on macrophages (34±5% TLR-4 and 66±4% TLR-2). These studies suggest that *Cftr* gene expression and CF lung environment impact immune cell function in CF, potentially through regulating the expression of TLRs.

S8.4 - Ceramide is a potential biomarker of epithelial injury which is raised in the airway of people with cystic fibrosis

Malcolm Brodlie[1,2,3], Michael C Mckean[3], Gail Johnson[2], Andrew J Fisher[1,2], Paul A Corris[1,2], James Lordan[1,2], Chris Ward[1]

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Background:
Over 95% of mortality and morbidity in cystic fibrosis (CF) is associated with lung disease. CF results from alterations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene but the exact pathogenesis of CF lung disease remains poorly understood. The sphingolipid ceramide is an essential constituent of plasma membranes and regulates many physiological cellular responses, including apoptosis and cell survival.[1] It has recently been shown that CFTR-deficient mice accumulate ceramide in respiratory epithelial cells due to alkalinisation of intracellular vesicles. Ceramide accumulation resulted in constitutive age-dependent pulmonary inflammation, death of respiratory epithelial cells and susceptibility to *Pseudomonas aeruginosa* infection.[2] Furthermore, similar accumulation was demonstrated in respiratory epithelial cells and a small number of sections of airway from people with CF.[2] Ceramide has also been identified as a central mediator in the development of emphysema by inducing oxidative stress and apoptosis of alveolar endothelial and epithelial cells.[3]

Aim:
To compare the distribution of ceramide in the respiratory epithelium of unused lung donors and patients with CF, emphysema and pulmonary hypertension (PH).

Methods:
Airway from lungs removed at the time of transplantation (8 CF, emphysema and PH respectively) and 8 unused donor lungs were fixed in formalin and embedded in paraffin. Immunohistochemistry was performed on 5µM sections using 2 monoclonal antibodies (Sigma mouse-IgM and Glycobiotech mouse-IgM-enriched) along with appropriate negative controls. Positive staining was evaluated in the epithelium in 5 high-power fields/patient using image analysis software and expressed in terms of percentage area. Groups were compared using the Mann-Whitney test.

Results:

Antibody	CF	PH	Emphysema	Unused Donor
Sigma	5.2	0.46**	2.2	0.33**$^{\#}$
Glycobiotech	13	3.3**	6.5**	3.4**

[Mean Percentage Positive Staining In Epithelium]

**P< 0.01 compared to CF. $^{\#}$P< 0.05 compared to emphysema.

Conclusions:
Staining for ceramide in the respiratory epithelium is significantly increased in people with CF compared to PH and unused lung donors with both antibodies and to emphysema using the Glycobiotech antibody. There was a clear trend towards higher levels of staining in emphysematous lungs compared to PH or unused donor, although this was only statistically significant for emphysema against unused donor lungs using the Sigma antibody. This data provides further evidence to support the hypothesis that ceramide accumulation occurs in the respiratory epithelium of people with CF.

References:

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S8.5 - Proteomic analysis of proteins released from *Pseudomonas aeruginosa* clinical and laboratory strains: effects of azithromycin

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Patients with cystic fibrosis (CF) are particularly susceptible to chronic *P. aeruginosa* (*Pa*) infection in the airways. Modulation of *Pa* virulence factors was suggested as mechanism for azithromycin (AZM) beneficial effects in CF patients. Our work was aimed to study the regulation of proteins released by *Pa* clinical and laboratory strains after AZM treatment.

Looking for secreted virulence factors active on lung epithelium of CF patients we focused on the induction of pro-inflammatory markers and measured the expression of IL-8 and TNF-a genes in a CF epithelial airway cell line in response to conditioned medium derived from *Pa* strains. *Pa* clinical isolates and PAO1 were grown overnight in aerobic conditions in the presence or absence of AZM. We demonstrated that conditioned medium (CM) from the clinical strain AA2, unlike CM from the laboratory strain PAO1, induced a statistically significant increase of about 3.6 and 2.7 times, respectively of IL-8 and TNF-a mRNA, in CF airway epithelial cells.. This induction was reduced of about 30% when AA2 was grown in the presence of AZM, suggesting that this macrolide reduces *Pa* pathogenicity. In the attempt to gain information on the identity of the molecules released by *Pa* strains before and after treatment with AZM and to identify candidate molecules involved in *Pa* virulence, we applied a recent proteomic approach (2DC-MS/MS called also MudPIT). Two-dimensional capillary chromatography - tandem mass spectrometry (MudPIT) identified polypeptides released by PAO1 and AA2 in the absence and presence of drug. 7 proteases were released from AA2 while only 1 was detected in PAO1. AZM appear to down-regulate their release in AA2 strain. This result has been confirmed by zymography.

Investigating *Pa* secretome is critical for identification of molecules involved in bacterial virulence, lung damage, inflammation and drug resistance. This approach could identify potential targets for pharmacological intervention and provide candidates for cellular pathways leading to better understanding of CF pathogenesis.

Supported by Italian Cystic Fibrosis Research Foundation(FFCgrant#17/2006); Comitato Vicenza-Associazione Veneta Lotta contro la Fibrosi Cistica.

S8.6 - Decreased Levels of Secretory Leucoprotease Inhibitor in the *Pseudomonas*-infected Cystic Fibrosis Lung are due to Neutrophil Elastase Degradation

Sinead Weldon[1], Paul Mc Nally[2], Noel McElvaney[2], Stuart Elborn[1], Rodney Levine[3], Clifford Taggart[1]

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Secretory leucoprotease inhibitor (SLPI) is a neutrophil serine protease inhibitor constitutively expressed at many mucosal surfaces including the lung. Originally identified as a serine protease inhibitor, it is now evident that SLPI also has anti-microbial and anti-inflammatory functions, and therefore plays an important role in host defence. Previous work has shown that some host defence proteins such as SLPI and elafin are susceptible to proteolytic degradation. Furthermore, the integrity of elafin was impaired in the lungs of patients with cystic fibrosis (CF). Consequently, we investigated the status of SLPI in the CF lung. A major factor that contributes to the high mortality rate among CF patients is *Pseudomonas aeruginosa* infection. In this study, we report that *P. aeruginosa*-positive CF BAL, which contains lower SLPI levels compared to *P. aeruginosa*-negative samples and higher neutrophil elastase (NE) activity, was particularly effective at cleaving recombinant human SLPI. Additionally, we found that only NE inhibitors were able to inhibit SLPI cleavage, thereby implicating NE in this process. NE in excess was found to cleave recombinant SLPI at 2 novel sites in the NH_2-terminal extremity (Ser15-Ala16 and Ala16-Glu17) and abrogate the ability of SLPI to bind LPS and NF-kappaB consensus binding sites. In conclusion, this study provides evidence that SLPI is cleaved and inactivated by NE present in CF lung secretions and that *P. aeruginosa* infection promotes this effect. Overall, these novel findings broaden our understanding of the destruction caused by *P. aeruginosa* infection and how it contributes to chronic damage seen in the CF lung.

Saturday 18 April – 16:30-18:30 Room: Infante Santo

SPECIAL SYMPOSIUM – Translating Science into Clincal Practice
Chairs: M.D. Amaral (PT) & D.N. Sheppard (UK)

SS.1 - Mechanisms of mucus over-production in CF mice

Fariel Dif[1], Yong-Zheng Wu[1], Pierre-Régis Burgel[2], Mario Ollero[3], Dominique Leduc[1], Jamil Aarbiou[4], Sonja Tattermusch[1], Florence Borot[3], Ignacio Gacia-Verdugo[1], Clémence Martin[2], Michel Chignard[1], Dominique Israel-Biet[5], Yoshihiro Kita[6], Takao Shimizu[6], Bob J. Scholte[4], Lhousseine Touqui

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Cystic Fibrosis Foundation Therapeutics

Normally, the epithelium of conducting airways is covered with a thin layer of mucus that plays important roles in airway defense against inhaled pathogens by facilitating their eviction via cough and mucociliary clearance to the upper airways. Mucins are large glycoproteins that are secreted in the airway lumens by airway epithelial and submucosal gland cells. Expression of numerous mucin genes have been reported in lung tissues. Among these mucins, the secreted gel-forming mucins MUC5AC and MUC5B have been repeatedly identified as important components of airway mucus in normal subjects. In patients with CF, airway disease is characterized by progressive airway obstruction by viscous mucous secretions. Studies of mucins in CF airways excised at the time of transplantation have shown that: (i) MUC5AC mRNA expression is increased in CF epithelium compared to the epithelium of control subjects, (ii) immunostaining for MUC5AC protein is markedly increased in CF epithelium compared with control subjects, (iii) MUC5AC protein is present in the airway lumens of CF subjects, contributing to airway plugging and (iv) MUC5B is also increased in CF airway epithelium and lumens. Altogether these data suggest that overproduction and secretion of MUC5AC and MUC5B mucins occur in CF airways and contribute to airway obstruction. Many studies have examined the mechanisms involved in the induction of pulmonary mucus hypersecretion and mucin expression in various animal models of asthma and COPD. However, little is known about the processes involved in the induction of mucin expression in CF. Therefore, mechanisms that may link *CFTR* gene mutations to mucus hypersecretion remain unclear. This hypersecretion can be a direct consequence of CFTR alteration or secondary to the exacerbated inflammation that CFTR alteration induces. Cytosolic phospholipase A2α (cPLA2α) may potentially play a critical role in the pathogenesis of CF. cPLA2α hydrolyses membrane phospholipids at the sn-2 position leading to a selective release of arachidonic acid (AA). The latter is further converted by cyclooxygenase (COX) and lipoxygenase (LOX) into prostaglandins (PG) and leukotrienes (LT), respectively. The implication of cPLA2α in the development of lung inflammation has been extensively examined in various animal models of lung inflammatory diseases but its involvement in CF has not been addressed. The present study aim to investigate the role of cPLA2α in mucus hypersecretion in a mouse model of CF (*cftr*$^{-/-}$ and F508del mice) and animal mortality caused by lung infection by a clinical strain of *Pseudomonas aeruginosa* isolated from a CF patient.

SS.2 - CFTR correctors and potentiators: pre-clinical tests in CF mice

Hugo de Jonge, Martina Wilke, Alice Bot, [1]David Sheppard

Dept. of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands;
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Mouse models for cystic fibrosis (CF) provide new opportunities to study disease pathogenesis, to correlate genotype and phenotype, and to evaluate the safety and efficacy of novel CF therapies. We use the Cftr^{tm1Eur} mice homozygous for the F508del mutation in a congenic FVB background as a tool in pre-clinical testing of therapeutic strategies aimed at the correction of the F508del folding, gating and cell surface stability defect. The validity of CF mice as a surrogate for human CF patients depends on the assumption that human and mouse CFTR, despite their limited sequence homology (78%) and difference in open probability (P_o) and channel subconductance state, behave similarly in their response to pharmacological correctors and potentiators.

To verify this assumption, we have studied the responsiveness of mouse F508del-CFTR towards several classes of human F508del CFTR correctors and potentiators in two assay systems: (i) CHO cells stably transfected with mouse F508del-Cftr; (ii) freshly excised ileal mucosa from F508del-*Cftr*^{tm1Eur} mice mounted in Ussing chambers.

All hF508del-CFTR correctors tested sofar, including the Vertex compounds VRT-325 and -640, DH1 (Balch lab), and the Verkman correctors C1 (=1a), C4 (=4a) and C6 (=5c) caused a moderate to strong dose-dependent rescue of mF508-Cftr, as evidenced by a 2-6 fold increase in forskolin+genistein-stimulated ^{125}Iodide efflux following 26h of incubation at 37^{0}C (CHO assay). EC_{50} values for rescue of mouse and human F508del-CFTR were similar. A subset of "fast-acting" correctors (VRT-640; VRT-325) was additionally capable of enhancing the forskolin+genistein-activated short-circuit current across ileal mucosa by 2-3 fold (i.e. up to 50-70% of the response in Cftr+/+ littermates) following 6h of *ex vivo* incubation in William's E Glutamax medium (37^{0}C). The maximal level of pharmacological correction reached was comparable to the effect of low-temperature incubation (26h, 26^{0}C) in both assays. Correction was associated with the appearance of mature band C CFTR protein on Western blots, and immunostainable CFTR in intestinal crypt cells.

In contrast, most hF508del-CFTR potentiators tested over a wide concentration range (0.1-100 μM), including NPPB-AM (Kirk lab) and the CFFT toolbox potentiators P1-P5 (i.e. VRT-532; PG-01; SF-03; UCCF-853; $\Delta F508_{act}$-02) and P10, failed to potentiate forskolin-activated mF508-Cftr, both in low-temperature-rescued CHO cells and native intestinal epithelium. Only three of the CFFT compounds, P6 (genistein), P8 and P9, were able to potentiate mF508del-CFTR in both models, with similar potency and efficacy as hF508del-CFTR (2.5-6 fold enhancement of forskolin-induced ^{125}Iodide efflux).

These results indicate that mouse and human F508del-CFTR (1) respond to low temperature incubation and to small molecule correctors with a similar gain in surface expression and function, supporting the use of CF mouse models for *in vivo* tests of CFTR correctors; (2) respond differently to various classes of CFTR potentiators, in line with their pronounced differences in gating behaviour, and emphasizing the specificity of CFTR-potentiator interaction. Further studies focussing on human/mouse CFTR chimera and aimed to identify the domain(s) accounting for the differential response to the CFTR potentiators are in progress.

Acknowledgement: We wish to thank Robert Bridges, Rosalind Franklin University, and Cystic Fibrosis Foundation Therapeutics for providing us with the CFFT modulator library.

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SS.3 – Sweat Gland Potential Difference. Possible Role as an Outcome Measure of CFTR Function in Clinical Trials?

T Gonska[1], W Ip[1], D Turner[2], W-S Han[4], J Rose[1], P Durie[1*], P Quinton[4,5*]

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* Equal senior authorship

Background: For nearly 50 years Cystic Fibrosis (CF) has depended on measurements of Cl^- concentration in the sweat for diagnosis. While the validity of this test is universally accepted, it requires careful technical training and costly laboratory analytical devices for accurate determinations. We demonstrate a novel concept for a simpler, more economical, real time method for diagnosis that also may be of value to measure the effect of potentiators or correctors that are being developed to ameliorate CF.

Methods: Cholinergic and β-adrenergic agonists were iontophoresed to stimulate sweating. After gently rubbing mineral oil over the skin surface, the electrical potential from stimulated sweat glands (SPD) was measured *in vivo* using a standard electrocardiogram (ECG) electrode applied to the skin surface. SPD and simultaneous sweat chloride concentrations [Cl^-] ere compared in cohorts predicted to express a range of CFTR function as presented by healthy controls (HC), heterozygotes (Hz), pancreatic sufficient (CFPS) and pancreatic insufficient CF patients (CFPI).

Results: The median SPD was hyperpolarized in CF compared to control subjects (-47.4 mV *vs.* -14.5 mV, $p<0.0001$). In distinguishing between control and CF subjects, SPD (Area under Receiver-Operator Curve, AUC = 0.997) was similar to sweat [Cl^-] (AUC = 0.986). Sequential cholinergic/β-adrenergic sweat stimulation dramatically depolarized the SPD in CF ($p<0.001$), but had no effect in control subjects ($p>0.6$) or on sweat [Cl^-] in either group ($p>0.5$) SPD results after cholinergic/β-adrenergic stimulation further distinguished CFPS from CFPI subjects ($p=0.04$).

Interpretation: These data provide evidence that SPD may exploited to diagnose CF as well as to assess expressed CFTR function conveniently, accurately, and economically.

SS.4 – ICM as a biomarker for CFTR restorative strategies.

JP Clancy, MD(1); Sherif Gabriel, PhD(2); Phil Karp(3); Jasna Hocevar-Trnka(4); James Lymp, PhD(4); Melissa Ashlock, MD (5); and Judy Williams, RN(4)

(1) University of Alabama at Birmingham; (2) University of North Carolina at Chapel Hill; (3) University of Iowa; (4) Seattle Children's Hospital; (5) Cystic Fibrosis Foundation Therapeutics; and the CFFT-TDN

Mutations within the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR) lead to Cystic Fibrosis (CF). The most common cause of CF is deletion of phenylalanine from position 508 (ΔF508), causing defects in protein maturation, Cl- channel gating, and plasma membrane residence time. As recently identified study drugs capable of restoring function to ΔF508 CFTR enter clinical trials, biomarkers capable of detecting normal maturation, localization and function are of high priority to confirm biologic activity and accelerate therapeutic development. Currently, at least two biomarkers specific for CFTR activity have been developed, including the nasal potential difference (NPD) and the sweat Cl- test. Both of these tests have been successfully used in multi-center clinical trials, but concerns over sensitivity, and limitations in the assays due to their in vivo nature have lead investigators to consider Intestinal Current Measurements (ICM) of CFTR activity from rectal biopsy samples. This assay is attractive since CFTR is expressed at high levels in the rectum, it is not altered by disease manifestations, and the tissue can be studied ex vivo, providing flexibility in the nature of the techniques to detect/quantify CFTR activity. ICM studies have been examined for > 15 years in European CF care and research sites, and have been used as a standard mechanism to diagnose CF. This experience indicates that the assay is safe and well tolerated by study subjects, that the ICM is sensitive and specific for CFTR function, and that the assay is highly discriminatory between severe CF (with no detectable CFTR activity), milder CF (with residual, low level CFTR activity), and non-CF (normal CFTR function). There are numerous variations in how the ICM assay can be performed, including the nature of tissue acquisition (forceps or suction biopsy), buffers and reagents used, data acquisition (stripchart or digital), recording conditions (voltage clamp with short circuit current vs open circuit), tissue study conditions (gassed with continuous recirculating perfusion vs continuous exchange perfusion without gas), and means of tissue mounting. In an effort to develop this assay as a clinical study biomarker, CFFT has supported the development of ICM techniques and protocols that are suitable for use in multicenter clinical trials of CFTR restorative agents. In this presentation, we will summarize our steps in protocol development, including visits to ICM sites, choice of biopsy methods, hands on training, a priori data inclusion, reagent sequence and equipment.

SS.5 – From disease mechanism to medicine: a drug discovery perspective

Christopher Poll

Respiratory Diseases Area,
Novatis Institutes for Biomedical Research,
Wimblehurst Road,
Horsham, West Sussex, UK

Within the Novartis Institutes for Biomedical Research (NIBR) our goal is to bring medicines rapidly into the clinic by focusing on biological targets which are both tractable and have a strong rationale for addressing unmet medical need. Increasingly the evaluation of new compounds in the clinic is being guided by knowledge of molecular pathways, mechanism of action and human genetics. Therefore, we see increasing our knowledge and understanding of the molecular pathways of disease and more effectively accessing and harnessing genetic and genomic information as key to the future discovery and development of safe and effective medicines.

Respiratory diseases represent a major area of drug discovery research within Novartis and I will briefly introduce the respiratory disease indications we are pursuing and some of the scientific approaches being used to tackle them. Using examples primarily from our program of Cystic Fibrosis drug discovery activities, I will then describe the various steps in the journey from identifying and validating a molecular target to the selection of a molecule to test in proof of concept clinical studies in man. Topics addressed will include the considerations in the establishment of translational *in vitro* and *in vivo* models and the importance of strong scientific interactions between preclinical and clinical scientists throughout the various stages of drug discovery. The contribution of research collaborations with external academic groups to drug discovery activities will also be highlighted.

SS.6 - Opportunities and Challenges of Translational Research in Cystic Fibrosis

Eric Olson

Vertex Pharmaceuticals Incorporated, Cambridge, Massachusetts, USA

Demonstration that defective CFTR function could be partially restored in vitro fueled the concept that a drug discovery approach might be a viable strategy for identifying potential new CF therapies. Efforts by several groups over the last few years have resulted in the identification of many small molecules that are capable of increasing defective CFTR function in vitro. Since few compounds have progressed into clinical development, it is premature to fully understand the strengths and weaknesses of each approach employed in identifying potential new therapies. However, with a few of these compounds now in clinical development, and with early data in CF subjects becoming available, it is instructive to analyze some of the strategies employed in moving these compounds from research into development as well as the scientific rationale and practical issues behind the strategies. During a 9-year collaboration between Vertex Pharmaceuticals and Cystic Fibrosis Foundation Therapeutics (CFFT) two compounds have been discovered, VX-770 (a potentiator) and VX-809 (a corrector), both of which are currently in clinical development. Throughout the course of the collaboration the two organizations wrestled with several important strategic and tactical questions. Highlights of the strategy from the VX-770 program include: focusing on functional assays of chloride ion secretion in only a few cell systems, bypassing the need to demonstrate efficacy in an animal model in selecting a clinical candidate, setting a criterion for the level of efficacy in vitro, and measuring multiple outcomes in the Ph2a clinical study to help understand the relationship between biomarkers of CFTR function and clinical endpoints. In addition to addressing the scientific issues, the two organizations also had to define how to productively work together to ensure the best possible outcome. Several important lessons from the evolving relationship will be highlighted

Sunday 19 April – 09:30-11:00 **Room: Infante Santo**

SYMPOSIUM 9 – Strategies to Correct Ion Transport Defects
Chairs: E. Olson (US) & K. Kunzelmann (DE)

S9.1 - Chloride Channel Activator Therapies for CF, and CF Lung Disease Mechanisms

Alan S. Verkman

University of California, San Francisco, CA

S9.2 - A pharmacological strategy towards a therapeutic hope: Miglustat

Caroline Norez

Institut de Physiologie et Biologie Cellulaires, UMR6187, 40 avenue du recteur Pineau, 86000 Poitiers France

Cystic fibrosis (CF) is a fatal, autosomal and recessive genetic disease that is mainly due to inactivating mutations in the chloride channel CF transmembrane conductance regulator (CFTR). Sodium hyperabsorption by the airways, mediated by the epithelial Na^+ channel (ENaC), profound lung inflammation and dysregulation of the calcium homeostasis are presumably causally related to loss of CFTR-dependent chloride function in CF patients.

One strategy for development of CF therapeutics is the identification of pharmacological agents that correct processing defect of F508del-CFTR and/or stimulate the channel activity of mutated proteins. The protein-repair therapy is based on several observations showing that in the presence of a pharmacological corrector, tailored to the specific F508del genotype, the misfolded protein escapes the ER and targeted to the plasma membrane.

We have identified numerous F508del correctors with a CF drug discovery program using a robotic cell-based assay combined to molecular, biochemical and electrophysiological approaches. Among them, we identified miglustat, an orally bioavailable N-alkylated imino sugar (N-butyldeoxynojirimycin, Zavesca®). We investigated effects of high concentration (100 µM) of miglustat on several CF characteristics and demonstrated after short-term (2-4 h) treatment of CF cells, a partial rescue of the defective F508del-CFTR trafficking and function [1], an improvement of the altered Ca^{2+} homeostasis [2], a down-regulation of ENaC-dependent Na^+ hyperabsorption [3] and an anti-inflammatory effect of miglustat [4]. We suggested that the mechanism by which miglustat corrects the defective F508del-CFTR trafficking is correlated to a disturbance of the ER quality control system in CF cells. In support of that, miglustat is an α1,2-glucosidase inhibitor preventing the interaction between F508del-CFTR and calnexin in the ER [1].

More recently, we explored the concentration- and time-dependence of miglustat-induced correction of ionic transports in the human respiratory CF epithelial cells. The most salient result is the demonstration that a daily treatment for 2 months with low concentration of miglustat (3µM) resulted in progressive, stable, reversible and sustained correction of the F508del-CFTR deficient trafficking [5]. Then by investigating different biological and cellular aspects of cystic fibrosis such as Na^+ hyperabsorption and dysregulation of the Ca^{2+} homeostasis, we were able to show paralleled normalization of these parameters correlated with the restoration of the F508del-CFTR function [5].

In conclusion, we provided the first evidence that a respiratory CF cell can acquire a non-CF like phenotype when chronically treated with low-concentration of a pharmacological drug resulting in progressive, stable, reversible and sustained correction of F508del-CFTR trafficking, down-regulation of sodium hyperabsorption and regulation of the calcium homeostasis. This body of information makes the use of miglustat attractive as a potential pharmacologic therapy for those CF patients who have at least one F508del-CFTR allele. Miglustat, a medicament already prescribed in another orphan disease, is now evaluated in CF patients within a pilot phase 2a clinical trial (http://clinicaltrials.gov/).

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[2] Antigny et al, (2008) *Cell Calcium 43:175-83*
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[5] Norez et al, (2009) *Am. J. Respir. Cell Mol. Biol. In press*

S9.3 – Functional rescue of deltaF508-CFTR by peptides desgned to mimic sorting motifs

Patrick Kim Chiaw[1,4], Ling-Jun Huan[1], Stephane Gagnon[3] , Diane Ly[2], Neil Sweezey[3,5], Daniela Rotin[2,4], Charles M. Deber[1,4], Christine E. Bear[1,4,5].

Molecular Structure and Function[1], Cell Biology[2] and Physiology and Experimental Biology[3] Programmes in the Research Institute, Hospital for Sick Children and the Departments of Biochemistry[4] and Physiology[5], University of Toronto, Toronto, Canada

S9.4 – Biophysical Characterizaton of TMEM16A, a Membrane Protein with Calcium-Dependent Chloride Channel Activity

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Ca^{2+}-activated Cl^- channels (CaCCs) play important roles in various cellular mechanisms, including fluid secretion in epithelia, sensory transduction, and regulation of neuronal and smooth muscle excitability. Molecular identity of this type of channels was controversial. Our group has recently identified TMEM16A as a possible CaCC.

The aim of our present study is to characterize the properties of the Cl^- currents associated with TMEM16A expression and to compare them with those of classical CaCCs described in several previous studies. For this purpose, we have used the patch clamp technique in the whole-cell configuration on FRT cells stable-transfected with the TMEM16A(*abc*) isoform.

To analyse the ion channel selectivity, we substituted Cl^- in the extracellular solution with other anions (I^-, Br^-, SCN^-, and gluconate) and we measured the resulting shift in the reversal potential of membrane currents. Our data indicate that TMEM16A-dependent channels have ion selectivity properties similar to those of native CaCCs (Hartzell, Putzier and Arreola, Annu. Rev. Physiol. 2005. 67:719-58).

We also studied the Ca^{2+}-dependence of TMEM16A channels by changing the cytosolic free Ca^{2+}-concentration in the 0.017 - 1.35 µM range. By plotting the maximal current elicited at + 100 mV versus the cytosolic Ca^{2+} concentration, and fitting the data with a Hill function, we found a K_d = 91.8 nM and a Hill coefficient n_H = 2.32. These values are close to those published previously for CaCCs (K_d = 61 nM, n_H = 2.7; Arreola, Melvin and Begenisish, J. Gen. Physiol. 1996. 108:35-47).

In conclusion, our results confirm that TMEM16A is a membrane protein involved in Ca^{2+}-dependent Cl^- transport. This remark evidences that TMEM16A may represent an important pharmacological target to treat cystic fibrosis in which activation of an alternative Cl^- channel may compensate for the defective CFTR activity

P17

S9.5 – Inhibition of renal CFTR by insulin-sensitizing agents: Implications for a central role for CFTR in renal-based pathologies of fluid balance

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Thiazolidinediones (TZDs) are synthetic ligands for the nuclear peroxisome-proliferator activated receptor gamma (PPARγ). These agents have potent insulin-sensitizing capabilities and are used clinically for treatment of non-insulin dependent Type II diabetes mellitus. However, their use is limited in patients at risk for cardiovascular disease due to fluid retentive side effects. The side effect etiology is unknown, but the nature of presentation suggests modulation of renal salt and water homeostasis. This contention was strengthened by the creation of renal collecting duct specific PPARγ knockout animals that were resistant to the fluid retentive effects of two clinically used TZDs, rosiglitazone and pioglitazone. This renal site of action suggested a TZD effect on the epithelial Na^+ channel (ENaC), a hormone regulated channel involved in fluid-electrolyte balance. However, several anomalies and conflicting data argue against an ENaC-mediated response as the primary target of PPARγ agonist-mediated fluid retention. In several collecting duct cell lines, we have shown that TZDs did not affect ENaC expression or activity. We have used the $mpkCCD_{cl4}$ (mouse principal cells of the kidney cortical collecting duct, clone 4) cell line to show that PPARγ agonists inhibit vasopressin-stimulated Cl^- secretion with agonist dose response relationships that mirror receptor trans-activation profiles. Analyses of the components of the vasopressin-stimulated intracellular signaling pathway indicated no PPARγ agonist-induced changes in basolateral membrane conductances, intracellular cAMP or protein kinase A. The PPARγ agonist-induced decrease in anion secretion is the result of decreased mRNA of the final effector in the pathway, the apically located cystic fibrosis transmembrane regulator (CFTR).

These data showing that CFTR is a target for PPARγ agonists provides new insight into the physiology of PPARγ agonist-induced fluid retention. The data, if substantiated by *in vivo* experiments, indicate that CFTR can play a primary rather than secondary role in renal-mediated fluid balance. In addition, this finding may suggest potential treatment options for diseases in which CFTR activity plays a role. One of the most common of these is polycystic kidney disease (PKD). In PKD, CFTR-mediated Cl^- secretion is known to contribute to the growth and maintenance of both renal and hepatic bile duct cysts. TZD inhibition of CFTR should, theoretically, inhibit cyst growth. Animal studies using an orthologous rat model of PKD are in progress. These studies can provide proof-of-principle for TZD-mediated CFTR inhibition as well as providing the basis for a new class of drug therapy for PKD.

S9.6 – Transcriptional profiling of chemical correctors of CFTRΔF508 trafficking

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[2] McGill Center Bioinformatics, McGill University

We have identified and validated a variety of chemically distinct molecules as CFTR ΔF508 trafficking correctors. Overcoming the challenge of merging hit validation with mechanism of action remains a daunting task. To elucidate proximal and distal pathways involved in correcting CFTR ΔF508 we generated a compendium of corrector profiles in the hopes of identifying novel drugable targets. We have generated a library of transcriptional profiles of the 16 compounds in our pipeline including the established correctors such as VRT-325 and rescue at 28°C, and the potentiator VRT-532. We chose the dual color Agilent platform and our study design involved labeling each treated sample reciprocally with Cy3 or Cy5 along with the reciprocally labeled universal reference RNA, with each treatment including a biological replicate. We have generated distinct signatures 12 hours and 24 hours post-treatment in the HEK293 cell line with the correctors at previously determined corrective concentrations. We also generated signatures at earlier time points, including 1hr and 6 hr post treatment for some of our compounds to obtain a temporal signature for correction. As proof of principle we queried specific genes known to be associated with CFTR folding and trafficking and found they were significantly ($p<0.05$) and differentially expressed, in the expected direction. For instance, the chaperone Hsp70 which associates preferentially with the mutant CFTRΔF508, was significantly down-regulated in some of our compound signatures. We have derived distinct transcriptional signatures in categories promixal to CFTR folding, including ER chaperones, N-Glycan biosynthesis, ERAD, and ER-total and are currently validating these hits, in addition to signatures more distal to CFTR biogenesis. So far, we have identified a novel gene, involved in N-glycan biosynthesis which is significantly downregulated upon treatment with one of our correctors a sodium pump inhibitor, Ouabain. Targeting this gene with siRNA in human epithelial cells expressing CFTRΔF508 restored expression at the cell surface by 2 fold over control, as determined by flow cytometry. We are in the process of validating other hits generated from this data by siRNA and overexpression, real-time PCR and western blotting. Generating such profiles will elucidate novel pathways and mechanisms of CFTR rescue, and drive a new generation of target-driven therapies.

Sunday 19 April – 11:10-12:00 **Room: Infante Santo**

Closing Lecture

Mucus: What controls hydration?

Richard Boucher

CF Center, The University of NC at Chapel Hill, US

The syndrome of CF has been defined clinically by “salty sweat”. The high salt sweat phenotype was crucial in assembling the accurately characterized pedigrees required to clone the *CFTR* gene. The pulmonary phenotype of CF reflects the failure of airways defense against chronic bacterial infection, which eventually results in death in >90% of CF patients. Studies of airway cell cultures, transgenic mice, and CF patients suggest that the initiating event in CF airways disease pathogenesis is reduced airway surface liquid (ASL) volume, i.e., dehydration. Because airway epithelia are very water permeable, the volume of ASL reflects the mass of salt on airway surfaces. The capacity of CF airways to transport ions (salt) is defective due to absence of CFTR function in CF airway epithelia. In contrast to sweat, the CF airway ion transport defects produce too little salt and, hence, water on airway surfaces. CF airways attempt to compensate for their intrinsic salt transport defects via an extracellular purinergic (ATP) signaling system to keep airway surfaces minimally hydrated. However, reliance on a single signaling system renders the CF airway more vulnerable to disease-causing insults, e.g., viruses, than normal airways, which regulate ASL volume by redundant ATP and adenosine signaling pathways. Clinical studies have explored the hypothesis that treating the dehydration of CF airways by adding salt to airway surfaces would be therapeutically beneficial. These studies have been greatly aided by a new understanding of the nature of the periciliary layer (PCL), now viewed as a “grafted polyanionic brush” than a “liquid” layer. This new understanding has led to quantitative predictions relating ASL hydration to mucus clearance based on the interactions of the mucus layer moving over a gel-like PCL brush. Clinical “proof of concept” studies demonstrated that inhaled hypertonic saline osmotically draws water onto airway surfaces, improves mucus clearance and pulmonary function, and reduces clinical exacerbations in CF patients. Based on these data, a spectrum of rehydration therapies are now being developed that may reduce the progression of CF lung disease in patients with established bacterial infection and may prevent the onset of CF lung disease if initiated early in life.

Room: Alcacer Quibir
Poster Session – CFTR Structure and Function

P. 1

Annexin V binds to CFTR and regulates its endocytosis

Diana D Faria[1,2], Toby S Scott-Ward[3], Luisa Alessio[1], Rainer Schreiber[2], Margarida D. Amaral[1,3], Karl Kunzelmann[2]

[1]University of Lisbon, Chemistry and Biochemistry, Lisboa, Portugal, [2]University of Regensburg, Department Of Physiology, Regensburg, Germany, [3]National Institute of Health Dr. Ricardo Jorge, Centre of Human Genetics, Lisboa, Portugal

Cystic fibrosis (CF) is primarily cause by deletion of a phenylalanine residue at position 508 (F508del) in the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR). Altered interactions of F508del CFTR with endoplasmic reticulum quality control proteins promote its proteasomal degradation. However, it is believed that crucial CFTR-interacting proteins (CIPs) important for trafficking and function of CFTR remain unknown. In this study, the NBD1 of CFTR was used as 'bait' in affinity chromatography to capture novel interacting proteins. Analysis of the recovered proteins by high resolution two-dimensional electrophoresis followed by mass spectrometry (Maldi-Toff) identified annexin V as binding partner of CFTR-NBD1.Annexin V belongs to a class of calcium-dependent phospholipids binding proteins, some of which have been implicated in membrane-related events along exocytosis and endocytosis pathways. Annexin V is a protein kinase C (PKC) inhibitor protein, which binds acidic phospholipids. This protein is overexpressed in CF epithelial cells and it was recently shown that annexin V is necessary for CFTR membrane expression and function. We report here that annexin V has a stimulatory effect on CFTR-mediated Cl^- currents in HEK cells taking part in the traffic regulation. Since annexin proteins are well known to control protein trafficking, we examined whether it contributed to CFTR trafficking. When patch clamp experiments were preformed on HEK cells by overexpressing the CFTR mutant Y1424A/I1427A, that disrupts the internalization signal, the effect of annexin V overexpression was no longer observed. The same was also observed by using the endocytosis (dynamin) inhibitor dynasore. This suggests a contribution of annexin V on CFTR endocytosis.
Experiments were also done in Xenopus oocytes. In this overexpressing system annexin V has an inhibitory effect on CFTR currents that is not abolish when CFTR-Y1424A/I1427A is overexpressed. Nevertheless in the presence of dynasore, CFTR was no longer inhibited by annexin V. Also the actin filament disrupter cytochalasin D (CD) reduced activation of CFTR by IBMX/Forskolin. However, in the presence of CD, annexin V had no further inhibitory effects on CFTR, sugesting an actin dependent mechanism.

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Interaction between CFTR and Genistein at different values of intracellular pH

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Cystic fibrosis transmembrane conductance regulator (CFTR) potentiators are compounds with a double effect. At lower concentration they increase the activity of CFTR, whereas at higher concentrations they inhibit it. The binding site for potentiators is hypothesized to be located in the interface of the dimer formed by the two nucleotide binding domains (NBDs), interacting mostly with NBD1 and to a lesser extent with NBD2. This binding site seems to involve at least two protonable residues, Cys491 and His1348. The binding of potentiators to the NBDs probably relies on electrostatic interaction, as electrostatic interactions between oppositely charged amino acids might be involved in the formation and stabilization of the dimer. Here we have analysed the electrostatic contribution to the binding of genistein by modifying intracellular pH. We have analysed the effect of pH on both, the activating and the inhibitory dissociation constants. The study was done on polarized epithelia expressing high levels of wild type CFTR after establishing that modifications in the pH of the basolateral solution, between pH 6 and 8, change the intracellular pH to the same extent. We found that at pH 6 it was necessary a lower concentration of CPTcAMP to activate CFTR. In addition, we found that the maximal CFTR current was significantly lower at this pH value. Regarding genistein binding, we found that at alkaline pH the apparent dissociation constant for the activating site was shifted to higher concentrations. In contrast, we found that the inhibitory apparent dissociation constant was affected by alkaline and acidic pH values to the same extent. In fact, at pH 6 and at pH 8 the affinity of genistein for the inhibitory site was similarly increased. This study suggests that cysteine, possibly Cys491, is involved in the activating binding site. In contrast, an histidine (e.g. His1348) does not seem to be part of it. Besides, our data indicate that the inhibitory site, whose location is still unknown, may contain both, a cysteine and a histidine, the only two amino acids with a pKa in the range of the pH analysed.

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Characterizing compounds affecting ΔF508 CFTR folding and conformation

Florence Peters[1], Elena Ganusova[1], Hanneke Hoelen[1], Mieko Otsu[1], Ineke Braakman[1]

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The F508 deletion in CFTR changes the protein´s conformation, as a result of which it can no longer travel to the plasma membrane but is retained in the endoplasmic reticulum (ER). In search of new drugs to rescue the ΔF508 phenotype many compound screens have been done and promising candidates have been identified, but their molecular mechanisms of action are still unknown.

We developed an *in vitro* assay to follow conformational changes in CFTR and more specifically, in the F508-containing NBD1 domain. We subject *in vitro* translated radiolabeled NBD1 to limited proteolysis to examine conformational differences between wt and ΔF508 NBD1.

We clearly detect changes in conformation and are optimizing this assay further for examination of compound effects. To find out whether the screened compounds can affect NBD1 folding directly or indirectly we will add the drug at various stages of the limited proteolysis assay. Different compounds have been tested, including corrector 4a, which did not affect NBD1 folding.

We concluded that these compounds do not rescue ΔF508 CFTR by directly correcting NBD1, but must either affect CFTR domain assembly or act by changing the cell, for instance by activating a crucial chaperone

Effects on mRNA Processing of CFTR Splicing Mutations assessed by Novel Mini-genes

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Despite that more than 1,600 variants of the CFTR gene have been identified [1], the functional effects at the level of CFTR expression and/or function remain largely unknown for most of them. Moreover, the major deleterious effect of some "missense" mutations is actually at the level of mRNA processing rather than a coding defect.

Our goal here was to create adequate cellular models to study the effects of splicing or PTC mutations found in CF patients, so as to allow characterizing the effects of CF mutations at the RNA level and also to enable further analysis of the respective protein products.

As most CFTR introns are longer than 2 kb and hence difficult to introduce into the complete CFTR cDNA (4.7 Kb). Our strategy was to produce CFTR mini-genes including the complete CFTR coding sequence plus one or two introns (IVS). We, thus introduced by mutagenesis the full length sequence of the smallest CFTR intron (IVS22 -600bp) or a shorter version (artificial) of IVS19 (art-IVS19-442bp), containing the original splicing sites into the CFTR-cDNA pNUT construct. We also created a CFTR mini-gene containing both introns. Stable BHK cells expressing these CFTR mini-genes were produced.

Results from RT-PCR analysis confirmed that correct splicing occurs for transcripts from all three mini-genes and Western blot (WB) shows that they all generate fully-processed CFTR protein. The p.I1234V (c.3832A>G) CFTR mutation was inserted into the art-IVS19 mini-gene and RT-PCR analysis shows that an alternative transcript lacking the last 18 nucleotides of IVS19 is produced, consistently with results in native tissues from a patient with this mutation. The resulting protein is mostly detected in its core-glycosylated form but reduced function as Cl^- channel could be detected. When this mutation was introduced into a simple CFTR cDNA construct, the resulting I1234V-CFTR exhibited function as a Cl^- channel undistinguishable from that of wt-CFTR. By using this mini-gene and spicies homology studies we discovered a second-site mutation (c.3836G>A) that reverts the alternative splicing of c3832A>G and hence, also restoring normal protein production.

In conclusion, the mini-genes described here are useful tools to characterize the defects of CFTR splicing mutations and possibly also to test therapeutic approaches aimed at correcting CFTR such defects.

[1] The CFTR mutation database, 2009, http://www.genet.sickkids.on.ca/cftr/

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P. 5

Structural changes upon phosphorylation of CFTR: detection by electron microscopy and single particle analysis

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Mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) can lead to loss of channel activity, mis-folding and truncation of the protein, and often give rise to the disease cystic fibrosis in humans. Little is known about the structure of the whole CFTR protein, how phosphorylation primes the protein and how ATP initiates the opening of the channel. Moreover there is only partial understanding of how the most common human CFTR mutation (the deletion of phenylalanine 508) affects the structure and activity. On the other hand, there is increasing structural information about isolated domains of the protein, such as the first nucleotide-binding domain (NBD1), and the regulatory (R) domain. There is also structural data for bacterial ATP-binding cassette proteins such as Sav1866 and MsbA, which are distantly related to CFTR. These have allowed the generation of molecular homology models of the CFTR protein.

The scene is therefore set for low resolution structural studies of the whole CFTR protein which should be able to visualise large scale conformational rearrangements. A better understanding of the dynamic rearrangements in CFTR should allow insights into the effects of the various mutations that give rise to cystic fibrosis. Moreover the work may lead to the development or optimisation of drugs to treat the patients. Low resolution structural data has been obtained so far from 2D crystals of the whole CFTR protein, although the constraints of a rigid crystalline lattice did not allow (so far) any information on the 3D structure of the phosphorylated protein. Here we describe low resolution structural studies of non-crystalline CFTR protein using electron cryomicroscopy and single particle analysis, yielding a maximal resolution of ~1/14$Å^{-1}$. We have studied the structure of the channel both before and after phosphorylation by the addition of ATP and protein kinase A, providing the first insight into the global conformational rearrangements that must take place for channel opening to occur. Even at the relatively modest resolution obtained, there are significant changes in the configuration of the protein domains. The nucleotide binding domains move closer together after phosphorylation and ATP addition, as expected from studies of isolated nucleotide-binding domains. However this movement is accompanied by a ratcheting motion within the transmembrane domains. These data can be interpreted in terms of a general model for the mechanism of action of the family of ATP-binding cassette proteins, of which CFTR is an unusual member.

The Impact of the Regulatory Region and Regulatory Insertion in NBD1 on Processing of wt- and F508del-CFTR

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The high-resolution structures of murine and human wt- and F508del-NBD1 of CFTR have provided invaluable information about the structure of NBD1 [1,2]. The reported data showed that these structures are very similar to those of other ABC transporter NBDs except for two segments [2]: the regulatory insertion, RI (Glu403-Leu435), a ~30-residue insert between the first two β-strands of the NBD1 β-subdomain, and the regulatory extension, RE (Ser654- Gly673), at the C-terminus of NBD1, where it extends ~20 residues longer than canonical ABC domains forming a helix packing against NBD1 at the NBD1-NBD2 interface. Both these segments have been suggested to be conformationally dynamic (undergoing ~180^{o} reorientations), namely upon phosphorylation (Ser422, Ser659, Ser660, Ser670) possibly to permit formation of the NBD1/NBD2 ATP-sandwich for channel gating [2]. He hypothesized that the dynamic flexibility of these regions may also result in exposition of hydrophobic surfaces contributing to the low folding efficiency of both wt- and F508del-CFTR.
Our goal here is to elucidate the possible role of RE and RI on the *in vivo* folding of CFTR. by biochemically and functionally characterizing wt- and F508del-CFTR lacking RI or RE.
To this end, wt- and F508del-CFTR-cDNA-pNUT constructs lacking RE and RI were produced by *in vitro* mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA) and used to generate stable BHK cell lines. Steady-state levels of immature and processed forms of these CFTR variants were assessed by Western blot analysis with the 596 antibody (CFF) and efficiency of processing was determined by metabolic pulse-chase followed by CFTR immunoprecipitation (IP). Functional assessment of CFTR activity was performed by the iodide efflux assay with an ion-selective electrode (ThermoElectron Corporation, Waltham, MA)
Preliminary results show wt-CFTR lacking either RE or RI, is efficiently processed, although the RI appears to show a lower steady-state level of maturation. These data are indicative that both these variants acquire a native (folded) conformation *in vivo*, consistently with a previous study [4]. Additional biochemical analyses to determine the maturation efficiency and functional characterization of these CFTR variants as well as of F508del-CFTR lacking RE or RI are underway.

Work supported by pluriannual funding of CIGMH (FCT, Portugal). AC DaPaula is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT, Portugal).

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P. 7

Implication of calnexin in the F508del-CFTR correction by miglustat

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The most common mutation in cystic fibrosis (CF), F508del, results in CFTR (CF transmembrane conductance regulator) protein that is retained in the endoplasmic reticulum (ER). Previously, we have shown that miglustat corrects the defective trafficking of F508del-CFTR and hypothesized that by inhibiting the interaction of F508del-CFTR with calnexin, a lectin implicated in the ERQC (ER quality control), miglustat prevents the retention and the degradation of F508del-CFTR (Norez et al., 2006). However, others contest the role of calnexin in the F508del-CFTR retention (Okiyoneda et al., 2008). The purpose of the study was i) to determine the effect of small interfering RNA (siRNA) calnexin treatment on endogenous F508del-CFTR trafficking, ii) to compare these results with a miglustat induced-correction, iii) to understand whether calnexin is implicated in the F508del-CFTR trafficking and in its correction induced by miglustat.

The human CF tracheal cell line CF-KM4 was transfected with a siRNA calnexin (0.5µg/mL, 72h), a siRNA control (0.5µg/mL) or was treated by miglustat (100µM, 2h). Then, the level of calnexin expression was tested by biochemical technique and consequences on CFTR and ENaC activities were assessed using single-cell fluorescence imaging. The results were compared with those obtained on untreated- and reverted- (CF-KM4 stably transfected with the CFTR wild type) CF-KM4 cells. We showed that decreasing calnexin expression (~ 75%) restores F508del-CFTR activity at the plasma membrane in correlation with a decrease (66%) of ENaC activity. Moreover, we found a 1.5 fold higher level of correction induced by miglustat than with siRNA calnexin : this level corresponds to the level of CFTR and ENaC activities measured in reverted CF-KM4.

In conclusion, this work is in favor of a role of calnexin in the F508del-CFTR retention and confirms calnexin as a valuable CF therapeutic target. Nevertheless, our results also suggest that inhibition of calnexin/F508del-CFTR interaction is probably not solely sufficient to fully explain the effects of miglustat raising the hypothesis that another molecular target for this drug exists.

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Biochemical Analyses of Potential CK2-sites in CFTR

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Despite great efforts to elucidate the mechanism and the molecular factors involved in CFTR biogenesis, trafficking and function, many of these processes are not fully understood. Protein kinases and phosphatases are known for long to regulate CFTR function. However, the role of phosphorylation in CFTR biogenesis and trafficking remains less explored. Recently, it was shown that recombinant casein kinase 2 (CK2) phosphorylates in vitro CFTR NBD1 at the residue serine 422 [1]. In addition, CFTR possesses several CK2 consensus phosphorylation sites, namely S511 (very close to F508) and T1471 at the C-terminus. Our aim here was to determine how mutation of these putative CK2 P-sites affects the CFTR biogenesis, turnover and processing.

We have produced CFTR mutants in which the consensus residues S422, S511 and T1471 were substituted by either a neutral (alanine, A) or an acidic residue (aspartic acid, D) in both wt and F508del-CFTR backgrounds by site-directed mutagenesis and used these constructs to generate novel stable BHK cell lines. Pulse-chase experiments by CFTR immunoprecipitation and Western blot, as well as functional assessment by iodide efflux assay, were performed in these cells. Quantification of bands B (immature form) and C (mature form) of CFTR in pulse-chase shows that substitution of S511 does not affect the turnover or processing of either wt- or F508del-CFTR. However, substitution of threonine at position T1471 to D (but not to A) completely impairs the processing of wt-CFTR without affecting F508del-CFTR and increases the turnover of F508del-CFTR. Interestingly, T1471A although not interfering in the production of mature protein, abolishes CFTR function. Moreover, treatment of cells with 20 mM TBB (tetrabromobenzotriazole, a specific inhibitor of CK2) for 90 min significantly reduces the processing efficiency of wt-CFTR.

Altogether, our data suggest a putative stabilizing role for CK2 upon wt-CFTR in these cells, which can be mediated by CFTR residue T1471. So far, our data do not support a role for residue 511 on the functional interaction of CFTR and CK2. Experiments are underway to determine whether this effect is also dependent on the S422 putative CK2 consensus P-site.

Work supported by POCTI/PTDC/BIA-BCM/67058/2006 grant, EU grant FP6-LSH-2005-037365 (TargetScreen2) and pluriannual funding of CIGMH (FCT, Portugal).

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A Role for Spleen Tyrosine Kinase (SYK) in CFTR Processing and Trafficking?

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Regulation of CFTR early and late intracellular trafficking as well as channel activation is the result of a complex network of CFTR interacting proteins (CiPs) namely, molecular chaperones, glycosidases, the basal trafficking machinery (Rab GTPases, SNAREs and PDZ-domain- proteins) and other factors among which molecular switches (like protein kinases and phosphatases). Among CiPs yet to be identified and/or characterized are those affecting CFTR biogenesis/traffic by phosphorylation and dephosphorylation. Spleen Tyrosine Kinase (SYK) is a non-receptor tyrosine kinase, described to have a role in signal transduction and signalling in haematopoietic cells, where the consensus for protein phosphorylation includes a tyrosine followed two negative residues. In CFTR, this consensus appears only once at residue 512 (i.e., very close to F508, the residue which is deleted in most CF patients).
Our aim goal here was to identify the putative role of Spleen Tyrosine Kinase (SYK) upon CFTR biogenesis and trafficking.
To this end, we produced cell lines stably expressing CFTR mutants, where Y512 was substituted by either a neutral residue (alanine, A, or phenylalanine, F, to mimic the bulky side group of tyrosine) or by an acidic residue (aspartic acid, D) in both the wt- and F508del-CFTR backgrounds. Pulse-chase experiments followed by CFTR immunoprecipitation and Western blot, were performed using these cells lines. Quantification of bands B (immature form) and C (mature form) of CFTR show that the substitution of Y512 by either A or D decreases both the steady-state levels and the efficiency of processing of CFTR. Moreover,, in vitro phosphorylation assays, using immunoprecipitated SYK and purified CFTR NBD1, show that in vitro SYK not only undergoes autophosphorylation but also phosphorylates CFTR NBD1, being this effect abolished when a dead kinase mutant of SYK is used in the same assay. Finally, RT-PCR analysis of epithelial respiratory cells (Calu-3 and virally transduced wt-or F508del-CFBE) show that SYK is endogenously expressed in these cells, arguing for the possible physiological significance of these findings.
Altogether, our data suggest a putative positive role for SYK on wt-CFTR stability and processing and that this effect seems to be mediated by the CFTR consensus site for SYK at Y512. At present, indirect effects (i.e, independent of direct CFTR phosphorylation by SYK) cannot be completely put aside.

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Large-Scale Western-Blots to Assess Effects of siRNAs on CFTR Trafficking.

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CFTR biogenesis starts with the co-translational folding and insertion into the endoplasmic reticulum (ER) membrane, followed by core-glycosylation and exit to the Golgi. CFTR glycan moieties are then processed along the Golgi, generating the mature membrane-resident form. This process is known as processing or maturation and a simple Western blot (WB) analysis distinguishes the core-glycosylated immature ER-specific form of CFTR (band B) from its fully-glycosylated mature post-ER form (band C). This biochemical assay is thus used to monitor CFTR exit from the ER. Moreover, as it provides quantitative information on steady-state levels, it can be used to assess the impact on CFTR of chemicals or molecules (over or underexpression of a given gene).
Our goal was to optimize WB to establish a robust, simple and sensitive method to analyse CFTR expressed in cell lines (epithelial and non-epithelial) grown on 96-well plates which can be used as a secondary test after high-content siRNA screen for proteins involved in CFTR trafficking.
Different cell lines were tested here: Calu-3, CFBE [1] and BHK stably expressing either wt- or F508del-CFTR. WB was performed as previously [2] but using benzonase to shear DNA. In each lane of a 7% (w/v) SDS-PAGE gel, contents of 1 to 6 wells from a 96-well plate were applied (about 7.5 mg to 45 mg protein). After electrophoresis and electroblotting, the membrane was blocked and incubated with the 596 anti-CFTR antibody (CFF) at 1:1000, followed by incubation with secondary antibody. Detection was done using West Pico System (Pierce).
First, we determined the minimal amount of cells needed to detect CFTR by WB and we found that ~4 x 10^4 cells (number present in one well) is enough to detect both bands B and C of CFTR in the above cells lines. We then determined the sensitivity of this assay to effects caused by siRNA transfection by using as control siRNAs against CFTR (Silencer Select, Ambion, ref. s2945, s2947). We found a significant decrease (~65 and ~85% respectively) in the amounts of band B and C for wt-CFTR, after 48h of transfection with 1.2 pmol of siRNA.
We conclude that this large-scale WB assay is robust to test the effect of different siRNAs against genes possibly affecting the trafficking of wt- and F508del-CFTR.

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Work supported by EU grant FP6-LSH-2005-037365 (TargetScreen2)

P. 11

Quantitative Transcript Analysis in Nasal Epithelial Samples of Patients Bearing the 2789+5G>A Mutation and Controls: Validating Assays

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Real-time PCR has proven to be a useful method to quantify gene expression in samples with small number of cells. We have applied this technology to validate the relative quantification of CFTR RNA from nasal brushings in a Taqman assay (ABI 7300).Normalization is an absolute prerequisite for the accurate measurement of gene expression and the selection of a suitable set of endogenous genes or gene is a critical step. Four endogenous genes have been evaluated (HPRT1, B2M, GUSB, PMCA4) in this study. First, four-point standard curves (1, 1/5, 1/25, 1/125) were performed for CFTR and endogenous genes from the cell line HEK293 over expressing CFTR and nasal epithelial (NE) samples to determine and compare PCR efficiencies. Second, four different software programs (NormFinder, qBase, geNorm and SPSS) were used to evaluate expression stability in NE samples.The 2789+5G>A is a splicing mutation located in intron 14b which determines the loss of exon 14b and the alteration of the reading frame, leading to a low level of normal transcripts (1). Blood and NE samples from adult individuals were grouped into: negative controls (healthy or lung disease no CFTR associated individuals, wt/wt, n=23); positive controls (CF patients F508del/F508del, n=7); CF patients compound heterozygotes for the 2789+5G>A mutation (n=9); CF carriers, 2789+5G>A/wt (n=9).A comprehensive analysis of CFTR gene was performed in all DNA samples.One costume Taqman assay was designed, the probe detecting the14a-14b boundary of the CFTR gene and one inventoried Taqman assays used for each endogenous gene (ABI). Stability of endogenous genes and quantitative transcript analysis was performed from two independent 500ng RT and each sample was analyzed in triplicate. Acceptable quality of RNA samples was limited to RIN above 5.2 (2100 Bioanalyzer, Agilent Technologies).GUSB and PMCA4, were validated as suitable for normalization. A pool from negative controls has been considered as 100% in the expression analysis. The geometrical mean expression and standard deviation for the different groups were as follows: wt/wt 59.80 ± 0.37%; F508del/F508del 41.60 ± 0.19%; CF carriers 21.10 ± 0.07% and CF patients 14.70 ± 0.07% meaning a fold change reduction of 1.67, 2.40, 4.74 and 6.80, respectively. Statistical significant differences ($p < 0.05$) were found between each control group (wt/wt, F508del/F508del) and patients / carriers. Differences were not statistically significant between positive and negative controls neither between carriers and patients.(1) Highsmith et al. Hum Mutat 1997,9:332- 38

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Evaluation of CFTR Expression in delF508 Affected Lower Airway Epithelium Compared to Wild-Type Controls from the Same Patient, Shows no Difference in Extent of Apical Localisation but Demonstrates Reduced Signal Intensity

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The expression of CFTR in respiratory epithelia of delF508 homozygous CF patients remains controversial. Reports in the literature support conflicting hypotheses of either a reduction in the fraction of cells expressing apical CFTR or a reduction only in the quantity of CFTR expressed apically. These studies are further confounded by changes during tissue processing and/or the use of nasal as opposed to lower airway epithelia. In this study, these issues were addressed by examining CFTR localisation and signal intensity in the lower airways of patients who have undergone lung transplantation for advanced CF. Using this approach, both wild-type and delF508 CFTR epithelia can be freshly harvested simultaneously from the same individual and immediately fixed.

12 lung transplant recipients with CF consented to the study bronchoscopy. Epithelia were harvested by bronchial brushing above (cells with delF508 mutation) and below (wild-type) the airway anastomosis joining the native bronchus and the transplanted lung. Cells were immediately fixed in paraformaldehyde and stained with MATG1061 CFTR monoclonal antibody (RD-Biotech). The % of ciliated cells expressing CFTR as a distinct apical band and the intensity of apical CFTR staining was examined by confocal microscopy and compared in CF and wild-type cells from each recipient by student's paired t-test. All results expressed as mean (±SEM).

There was no significant difference in the % of cells expressing apical CFTR, above 71 (±2.51)% vs below the airway anastomosis 68(±2.55)%, (p=0.2106, n=12). Eight of the 12 recipients were delF508 homozygous and 4 delF508 heterozygous. In the delF508 homozygous recipients, no significant difference in the % of cells expressing apical CFTR was observed (above 71(±3.24)% vs below 67(±3.16)%, (p=0.1427, n=8)). However, there was a significant difference in both the average pixel intensity (above 69(±10.04) vs below 146(±22.92), p=0.0300, n=5) and the total pixel intensity (above 3729 (±805) vs below 9173 (±2465), (p=0.0419, n=5) of the apical band. No difference in the number of cells counted or number of pixels quantified (p>0.05) was observed.

These results suggest the delF508 mutation leads to a reduced apical expression of the mutant protein in ciliated bronchial epithelial cells compared to wild-type cells from the same patient. There was no evidence that the percentage of cells with apical-localised CFTR was different in wild-type and CF cells from the lower respiratory tract. We speculate that CFTR potentiators may have a role in therapy for delF508 homozygous individuals without the need for enhanced trafficking.

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sHsps Target DF508 CFTR for Degradation via a SUMO-Dependent, Ubiquitin-Independent Pathway

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Key mediators of CFTR folding and degradation are molecular chaperones, which help protein substrates fold, but can target them for degradation if folding efficiency is compromised. A family of chaperones termed small heat shock proteins (sHsps) modulates CFTR expression levels in yeast and mammalian cells (1). Relative to WT CFTR, sHsps such as Hsp27 selectively bind and degrade DF508 CFTR when co-expressed in HEK cells, and the effect on DF508 steady-state levels is reduced by the proteasome inhibitor, MG1 32. The physiological relevance of Hsp27 action was assessed by knockdown using a shRNA construct. Both wt and DF508-CFTR protein levels were augmented 2-3 fold. Thus, sHsps increase CFTR's accessibility to proteasome-mediated degradation pathways and are able to distinguish between the wt and DF508-CFTR proteins. Hsp27 has been reported (2) to interact with Ubc9, the SUMO (small ubiquitin-like modifier) conjugating enzyme. We confirmed this interaction by co-immunoprecipitation and identified Ubc9 and Senp1 (SUMO specific protease 1) expression in airway cells. As for Hsp27, over-expression of Ubc9 selectively decreased steady-state levels of DF508 CFTR, while over-expression of Senp1 increased wt as well as DF508-CFTR levels. Pulse-chase experiments indicated that Ubc9 co-expression selectively degraded DF508 CFTR, similar to the action of sHsps. Inhibition of the SUMO pathway by knock-down of the SUMO E1 enzyme (SAE1) increased wt and DF508-CFTR protein levels 2-3 fold, an effect observed also in CFBEDF508 airway cells. Moreover, decreased levels of SAE1 blocked the ability of Hsp27 to promote DF508 CFTR degradation. In vitro and in vivo studies indicated that CFTR, and particularly NBD1, is sumoylated, and that Hsp27 facilitates this modification. Additionally, interference with the sumoylation pathway elicited a decline in SUMO-modified DF508-CFTR. Although expression of a (lysine-less) ubiquitin mutant that cannot form poly-ubiquitin chains increased DF508-CFTR levels, it did not interfere with the ability of Hsp27/SUMO pathway components to promote DF508 degradation. These findings indicate that Hsp27 selects DF508 CFTR for ERAD by facilitating its sumoylation, leading to its proteasomal degradation via a SUMO-dependent, but ubiquitin-independent, pathway.

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Room: Alcacer Quibir
Poster Session – Epithelial Transport

P. 14

Metformin Increases the Risk for Pancreatitis in Diabetes Patients Bearing the CFTR Variant S573C

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP and protein kinase A (PKA) regulated Cl^- channel in the apical membrane of epithelial cells, and plays an important role in the HCO_3^- secretion by pancreatic duct cells [1]. It has been shown that the metabolically regulated and adenosine monophosphate stimulated kinase (AMPK), colocalizes with CFTR and contributes to the attenuation of its function. [2]. Here we studied the effect of metformin upon CFTR. This biguanidine compound is used in the clinical treatment of type II diabetes, and is described to activate AMPK through the inhibition of the Complex I of the Respiratory Chain [3]. Metformin increases the risks of lactic acidosis and patients using this compound tend to develop pancreatitis secondary to metformin poisoning, or at therapeutic metformin doses in the case of renal failure [4]. Patients with pancreatitis have an increased incidence of genotypic alterations in the *CFTR* gene, including the non-disease causing variant, S573C [5]. We used double electrode voltage clamp (DEVC) to study whole cell conductances activated by 3-Isobutyl-1-methylxanthine (IBMX 1 mM) and forskolin (FSK 2 mM) in *Xenopus laevis* oocytes expressing wt CFTR or S573C CFTR. We are currently studying the effect of extracellular pH on CFTR function. Preliminary data show that acid extracellular pH 5.5 inhibits S573C by 75% but wt-CFTR only by 18% inhibition. We also found that whole cell Cl^- conductances due to sub-maximal activation of S573C-CFTR were inhibited by 25%, upon incubation with metformin (500mM), which had no effects on wt-CFTR. These data suggest that the presence of the *CFTR* gene variant S573C in non-CF diabetes patients renders them more susceptible to the development of pancreatitis, particularly when treated with metformin.

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Biophysical Characterization of TMEM16A, a Membrane Protein with Calcium-Dependent Chloride Channel Activity

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Ca^{2+}-activated Cl^- channels (CaCCs) play important roles in various cellular mechanisms, including fluid secretion in epithelia, sensory transduction, and regulation of neuronal and smooth muscle excitability. Molecular identity of this type of channels was controversial. Our group has recently identified TMEM1 6A as a possible CaCC.

The aim of our present study is to characterize the properties of the Cl^- currents associated with TMEM16A expression and to compare them with those of classical CaCCs described in several previous studies. For this purpose, we have used the patch clamp technique in the whole-cell configuration on FRT cells stable-transfected with the TMEM1 6A(*abc*) isoform.

To analyse the ion channel selectivity, we substituted Cl^- in the extracellular solution with other anions (I^-, Br^-, SCN^-, and gluconate) and we measured the resulting shift in the reversal potential of membrane currents. Our data indicate that TMEM16A-dependent channels have ion selectivity properties similar to those of native CaCCs (Hartzell, Putzier and Arreola, Annu. Rev. Physiol. 2005. 67:719-58).

We also studied the Ca^{2+}-dependence of TMEM16A channels by changing the cytosolic free Ca^{2+}-concentration in the 0.017 - 1.35 µM range. By plotting the maximal current elicited at + 100 mV versus the cytosolic Ca^{2+} concentration, and fitting the data with a Hill function, we found a K_d = 91.8 nM and a Hill coefficient n_H = 2.32. These values are close to those published previously for CaCCs (K_d = 61 nM, n_H = 2.7; Arreola, Melvin and Begenisish, J. Gen. Physiol. 1996. 108:35-47).

In conclusion, our results confirm that TMEM16A is a membrane protein involved in Ca^{2+}-dependent Cl^- transport. This remark evidences that TMEM16A may represent an important pharmacological target to treat cystic fibrosis in which activation of an alternative Cl^- channel may compensate for the defective CFTR activity.

Ion Transport Characteristics of Human Nasal Epithelial Cells from Wild Type and Cystic Fibrosis Donors

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In cystic fibrosis (CF), patients with class IV and V mutations in CFTR generally have a mild phenotype. Patients with an R1 1 7H allele, generally have more severe allele such as F508del or G551 D. The aims of this study were to develop a culture method that would enable the routine investigation of the ion transport characteristics of primary human nasal epithelial cells derived from CF donors.

Nasal epithelial cells (NEC) were extracted from wild type and cystic fibrosis volunteers by nasal brushing. The cells were expanded and frozen for future use. These cells were then further expanded and passaged onto collagen coated Snapwell inserts (Costar) using a modification of a previously described method (Danahay et al 2002). Once confluent, the cells were cultured at an apical air interface for 14-21 days, at which point, the ion transport phenotype was assessed.

Each of the wild type donors studied, developed an amiloride-sensitive short-circuit current (I_{SC}). These cells also developed a CFTRinh172-sensitive I_{SC} upon the addition of forskolin. Similarly, each of the CF donors NEC demonstrated an amiloride-sensitive current, irrespective of genotype. F508del/R1 17H and R1 17H/G551 D NEC did exhibit a forskolin-induced I_{SC} which was sensitive to $CFTR_{inh}172$, but that was smaller than that observed with the wild type donors. The F508del/F508del NEC failed to elicit a forskolin-induced response, however, the cells demonstrated a UTP-induced I_{SC}. Following a 20 hour incubation at 27°C, the F508del/F508del NEC demonstrated a forskolin-induced current which could be further potentiated by genistein.

This study has illustrated a cell culture method suitable for the routine culture of primary human nasal epithelial cells. The cells display an ion transport phenotype consistent with the native epithelium, and that is dependent upon CFTR genotype. Furthermore, F508del/F508del cells can be temperature corrected consistent with similar observations made in engineered cell systems. This culture method will allow characterisation of *in vitro* ion transport phenotype of different CF genotypes.

Danahay et al. 282 (2): 226. (2002) AJP Lung

Inhibition of Renal CFTR by Insulin-Sensitizing Agents: Implications for a Central Role for CFTR in Renal-Based Pathologies of Fluid Balance

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Thiazolidinediones (TZDs) are synthetic ligands for the nuclear peroxisome-proliferator activated receptor gamma (PPARy). These agents have potent insulin-sensitizing capabilities and are used clinically for treatment of non-insulin dependent Type II diabetes mellitus. However, their use is limited in patients at risk for cardiovascular disease due to fluid retentive side effects. The side effect etiology is unknown, but the nature of presentation suggests modulation of renal salt and water homeostasis. This contention was strengthened by the creation of renal collecting duct specific PPARy knockout animals that were resistant to the fluid retentive effects of two clinically used TZDs, rosiglitazone and pioglitazone. This renal site of action suggested a TZD effect on the epithelial Na^+ channel (ENaC), a hormone regulated channel involved in fluid-electrolyte balance. However, several anomalies and conflicting data argue against an ENaC-mediated response as the primary target of PPARy agonist-mediated fluid retention. In several collecting duct cell lines, we have shown that TZDs did not affect ENaC expression or activity.

We have used the $_{mpkCCDcl4}$ (mouse principal cells of the kidney cortical collecting duct, clone 4) cell line to show that PPARy agonists inhibit vasopressin-stimulated Cl^- secretion with agonist dose response relationships that mirror receptor trans-activation profiles. Analyses of the components of the vasopressin-stimulated intracellular signaling pathway indicated no PPARy agonist-induced changes in basolateral membrane conductances, intracellular cAMP or protein kinase A. The PPARy agonist-induced decrease in anion secretion is the result of decreased mRNA of the final effector in the pathway, the apically located cystic fibrosis transmembrane regulator (CFTR).

These data showing that CFTR is a target for PPARy agonists provides new insight into the physiology of PPARy agonist-induced fluid retention. The data, if substantiated by *in vivo* experiments, indicate that CFTR can play a primary rather than secondary role in renal-mediated fluid balance. In addition, this finding may suggest potential treatment options for diseases in which CFTR activity plays a role. One of the most common of these is polycystic kidney disease (PKD). In PKD, CFTR-mediated Cl^- secretion is known to contribute to the growth and maintenance of both renal and hepatic bile duct cysts. TZD inhibition of CFTR should, theoretically, inhibit cyst growth. Animal studies using an orthologous rat model of PKD are in progress. These studies can provide proof-of-principle for TZD-mediated CFTR inhibition as well as providing the basis for a new class of drug therapy for PKD.

Cleavage of αENaC is Associated with Increased Sensitivity of Human H441 Airway Epithelial Monolayers to Amiloride

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We reported two Na^+ permeable cation channels in the apical membrane of polarised human airway H441 epithelial monolayers contributing to amiloride-sensitive transepithelial short-circuit current (I_{SC}) [1]; a 5pS, Na^+ selective ENaC-like channel (HSC) inhibited by 1µM amiloride and an 18pS non-selective cation channel (NSC) inhibited with ≥ 10µM amiloride. Extracellular proteases, activated by airway fluid dilution of protease inhibitors, have been shown to cleave ENaC proteins and activate amiloride-sensitive Na^+ transport [2].

Our aim was to determine whether extracellular protease activity altered the 2 components which contribute to amiloride-sensitive I_{SC} across H441 monolayers. H441 monolayers were cultured at AI for 7 days, inserted into Ussing chambers and the sensitivity of the monolayers to amiloride was determined by generating a concentration response curve. Amiloride-sensitive I_{SC} inhibited by 1 µM was attributed to inhibition of HSC whilst that inhibited by 10µM attributed to NSC. We appreciate higher concentrations of amiloride are non-specific.

Airway fluid volume expansion in culture (5-100µl) upregulated transepithelial Na^+ transport in Ussing style electrophysiological studies. This was associated with proteolysis of the αENaC subunit. Using α subunit specific antibodies, full length 90kDa & cleaved 65kDa (N-terminal) or 20kDa (C-terminal) proteins were detected. The appearance of cleavage products correlated with airway fluid volume expansion and upregulation of amiloride-sensitive I_{SC}. The protease inhibitor aprotinin (30µM) abolished this effect. The effective applied airway fluid volume to upregulate I_{SC} & induce 50% proteolysis was calculated to be ~ 7µl. In control monolayers, I_{SC} was 15.3±4.7 µA.cm^{-2}. 1µM amiloride inhibited 68.5% of I_{SC}, whilst 10µM amiloride inhibited a further 31.5% of I_{SC}. The EC_{50} for amiloride was 0.9±0.3µM (n = 10). After apical application of trypsin (0.5mM) I_{SC} was 18.9±3.4 µA.cm^{-2} and EC_{50} for amiloride was 0.5±0.2 mM (both P=0.1 & n=8). The protease inhibitor, aprotinin (30µM), reduced I_{SC} to 4.76±1.84 µA.cm^{-2}. 1 µM amiloride inhibited 32.9% of I_{SC} whilst 10µM amiloride inhibited a further 47.8 % of I_{SC}. In addition, aprotinin increased the EC_{50} value to 8.7±2.3µM (both P< 0.05; n=8).

The data indicate that apical fluid induces protease cleaveage of αENaC and increases the activity of channels contributing to I_{SC}. The shift in dose response curve indicates a change in either the amiloride sensitivity of cleaved channels or the channel populations (HSC/NSC) that contribute to I_{SC} across airway epithelial cells.

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Connexin 26 is Implied in the Regulation of Airway Epithelium Repair

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Aim: Functional integrity of the airway epithelium is altered in cystic fibrosis (CF). Epithelial integrity depends on the expression and assembly of specific proteins into specialized junctional structures. Gap junctions, made of connexins (Cx) hexamer, play crucial roles in these interactions by contributing to the ability of cells to share signaling factors directly between adjacent cells. The pattern of Cx expression in Human Airway Epithelial Cell (HAEC) cultures is associated with the differentiation state. Thus, Cx26 is specifically expressed during proliferation phase and its expression decreases to undetectable level with HAEC differentiation to a polarized airway epithelium. We questioned if a specific pattern of Cx was associated with the epithelium repair following injury.
Methods: A model of HAEC repair was established by wounding mechanically the cultures. The cultures were followed for 24h before fixation, immunostaining and confocal analysis. To confirm the role of gap junctions in the epithelium repair, different blockers were used. The coupling between adjacent cells was evaluated by microinjection of Lucifer yellow.
Results: In our model of HAEC repair, Cx26 was transiently re-expressed at the wound area and in basal cells behind the wound. The re-expression of Cx26 was associated with enhanced spreading of the gap junction tracer Lucifer Yellow. In normal HAEC, Cx26 detection was concomitant with the cell ability to proliferate, as evaluated by Ki-67 detection, to close the gap following injury. The same phenomenon was seen in HAEC from CF patients in higher proportion. Interestingly, the amount of Cx26, the duration of its expression and the number of Ki-67-positive cells were amplified, suggesting a hyperproliferative state of the CF airway epithelium. Moreover, the use of gap junction blockers delayed the epithelial repair and reduced HAEC proliferation.
Conclusions: These results suggest that gap junctions, and more specifically Cx26, plays a role in airway epithelium wound repair and that understanding of the underlying mechanisms may lead to identify new targets for controlling CF HAEC proliferation and differentiation.

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Influence of Genetic Background on Tracheal Abnormalities in Different Cystic Fibrosis Mice

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We have previously demonstrated that two mouse models for cystic fibrosis (CF) on a mixed genetic background exhibit structural abnormalities of the trachea characterized by disrupted or incomplete cartilage rings, tracheal constriction and subsequent altered respiratory airflow (*Bonvin et al., J. Physiol. 586, pp 323 1- 3243, 2008).* In the present study, we aimed at investigating whether such CF lung phenotype would be modified in CF mice on a congenic background. We, thus, examined 1) the tracheal morphology in adult and newborn *Cftr* (CF transmembrane conductance regulator) knockout (*Cftr$^{-/-}$)* mice congenic on C57Bl/6 compared to that of adult and newborn *Cftr$^{-/-}$* mice on a mixed genetic background (C57Bl/6 and 129sv) and to their respective controls (*Cftr$^{+/+}$),* 2) the pattern of breathing in adult *Cftr$^{-/-}$* and *Cftr$^{+/+}$* congenic mice by using whole-body plethysmography.

Our data show that adult *Cftr$^{-/-}$* congenic mice exhibit severe cartilaginous ring abnormalities, with a larger number of incomplete or disrupted cartilage rings (5 to 13) than in *Cftr$^{-/-}$* mice on a mixed background (1 to 7), inducing a tracheal architecture weakness. Similarly, we detected in newborn *Cftr$^{-/-}$* congenic mice numerous abnormal rings (5 to 11) with large frontal interruptions of the cartilage, in particular on the first five rings below the larynx. In both control mice, a few adult and newborn animals had never more than one abnormal ring. This altered tracheal structure was associated in most adult and newborn *Cftr$^{-/-}$* congenic mice to marked upper tracheal stenosis. Moreover, the breathing pattern of adult *Cftr$^{-/-}$* congenic mice was significantly altered compared to control animals with a reduced breathing rate due to a lengthening of inspiratory and expiratory times.

We conclude that 1) adult and newborn *Cftr$^{-/-}$* mice congenic on C57Bl/6 exhibit tracheal abnormalities which are more severe than in *Cftr$^{-/-}$* mice on a mixed genetic background, 2) the marked alteration in tracheal architecture is associated to an abnormal respiratory airflow, 3) such structural and functional abnormalities of the respiratory system in *Cftr$^{-/-}$* congenic mice, which are already present at birth, may contribute to their poor survival rate before weaning regardless of intestinal obstruction, and put forward the important role of genetic background on the development of CF lung phenotype.

The Role of TLR Expression and CFTR Channel Activity on the Inflammatory Response to LPS from *Pseudomonas Aeruginosa* and *Staphylococcus Aureus* Peptidoglycan

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Cystic Fibrosis (CF) infection by gram-neg and -pos bacteria such as *Pseudomonas aeruginosa(PA) and Staphylococcus aureus*(*SA)* causes increased Interleukin (IL)-8 release and chronic inflammation. This immune response is elicited through Toll-like Receptors 2 and 4. During infection PA undergoes genetic changes for instance in the Lipid A component of the Lipopolysaccharide (LPS).
Aims: (1) To compare the inflammatory response of *PA* LPS isolates from CF patients differing in disease severity and *SA* peptidoglycan (PGN) in NON-CF (HTE) and CF (CFTE) tracheal epithelial cells; (2) ascertain the role of the CFTR and TLR expression in the inflammatory response.
CFTE and HTE cells were stimulated with LPS (Sigma: *PA,* PAO1), PGN (*SA)* or *PA* clinical isolates with different Lipid A structure: SE4 (Infant CF), PAK8 (Mild CF), SE22 (Severe CF), Bronch5 (non-CF, bronchiectasis), in the presence or absence of CFTR-inh1 72 (CFTR Cl^-channel inhibitor). IL-8 release (ELISA) and TLR expression (Flow Cytometry) were measured.
LPS isolates (ng/ml) are more potent than Sigma LPS (ug/ml).
Sigma LPS and *PA* isolates SE22 and PAK8 showed significant dose dependent IL-8 release in CFTE (all $p< 0.002$, ANOVA). SE4 and Bronch5 showed no significant response.
IL-8 release differed significantly between both cell lines (100ng/ml): SE22 ($p< 0.01$) and PAK8 ($p< 0.001$) induced a higher response in CFTE compared to HTE. Comparisons between *PA* (PAK8, Se22) and *SA* (all 100ng/ml) showed that *PA* LPS produced a higher inflammatory response than *SA* PGN in CFTE (all $p< 0.02$).
HTE cells treated with CFTR-inh172 showed significantly higher basal IL-8 ($P< 0.05$) compared to HTE DMSO control, but significantly lower than CFTE inhibitor treated cells ($P< 0.05$).
Analyses of TLR expression showed a significant basal increase in both TLR-2 ($P< 0.02$) and 4 ($P< 0.03$) in CFTE cells compared to HTE.
The inflammatory response to *PA* is dependent on cell type and changes in the Lipid A structure over time. These changes correlate with increased pro-inflammatory response, which is greater than that of SA, which may explain the PA dominance over SA. Increased TLR-2 and 4 expression in CFTE cells suggest a mechanism for the heightened response in CF. While inhibiting CFTR Cl^-channel function in HTE's increases basal IL-8 release, it is still significantly lower compare to CFTE, suggesting that CFTR Cl-channel function alone is not responsible for the increase in IL-8 in CFTE cells.

Respiratory Syncytial Virus Infection of Ciliated Cells Increases Airway Surface Liquid Height in Normal Airway Epithelium but not in Cystic Fibrosis Airway Epithelium

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Respiratory virus infection is a major cause of exacerbation of cystic fibrosis (CF) lung disease and CF patients suffer more severe consequences of virus infection. Our goals are to understand the impact of respiratory viruses on the pathogenesis of CF lung disease. To study the effects of virus infection on the human CF ciliated airway epithelium we used an *in vitro* model of human ciliated airway epithelium (HAE) and respiratory syncytial virus (RSV) as a virus that commonly infects CF patients. Using non-CF HAE, we show that RSV infects only ciliated cells and causes an acute increase in airway surface liquid height (ASL) height as measured by Texas red-dextran staining of ASL followed by confocal microscopy. This effect was maximum at ~3 days post-inoculation (pi) in non-CF HAE (ctrl,7.71±0.5;RSV,14.8±0.9µm, n=4). Parallel experiments with CF HAE revealed that RSV infection of ciliated cells failed to induce an increase in ASL (CF-ctrl,5.9±0.1;CF-RSV,5.9±0.1µm; n=4). These data suggest that non-CF HAE, but not CF HAE, respond to RSV infection by stimulation of fluid secretion into the airway lumen and is likely mediated by epithelial cell ion transport mechanisms. To determine whether the increase in ASL height after RSV was due to active Cl^- secretion, we used bumetanide (100µM) which significantly inhibited RSV-induced ASL height (ctrl,7.3±0.6; RSV,14.4±1.3; RSV+bumetanide,9±0.6; ctrl+bumetanide,5.6±0.1µm; n=4). To determine the specific contribution of CFTR-mediated Cl^- secretion to this response we used CFTR172 that also significantly inhibited RSV-induced ASL height (ctrl,6.6±0.3;RSV,11.4±1;RSV+CFTR172, 8 ±0.3; ctrl+CFTR172, 6.8±0.5µm; n=4). Since adenosine triphosphate (ATP) and adenosine (ADO) have been previously shown to regulate Cl^- secretion and ASL height in non-CF HAE, we determined the ASL concentration of ATP and ADO 3 days after RSV infection in non-CF HAE and found that both were increased by RSV infection (ATP, ctrl,16±5; RSV,26±3nM; ADO, ctrl,151±25; RSV,245±15nM, n=3) suggesting that the increased ASL after RSV infection was due to the release of nucleotides known to regulate ion transport in airway epithelium.

We conclude that RSV infection results in a CFTR-dependent increase in ASL height which is mediated by increased nucleotide levels in the ASL and that this cell response to infection is defective in CF airway epithelial cells. We speculate that increased fluid secretion is a host defense mechanism that attempts to clear virus from the airways and this clearance is diminished in CF airways possibly resulting in prolonged RSV infection of the CF airways.

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Regulation of ENaC in Human Bronchial Epithelial Cell Cultures by Protein, sPLUNC1

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One significant pathophysiological mechanism underlying Cystic Fibrosis (CF) is the hyperabsorption of sodium through ENaC. Further understanding of this misregulation could help develop novel therapeutic targets for treating CF. Channel Activating Proteases (CAPs) have been shown to increase ENaC activity but it is not clear whether CAP activity is physiologically regulated, or whether this regulation is defective in CF airways.

We have previously shown that normal primary human bronchial epithelial cultures (HBECs) exhibit a trypsin-sensitive transepithelial voltage (Vt) when airway surface liquie (ASL) has accumulated for 48 hours. Conversely, freshly washed normal and CF HBECs, as well as CF HBECs with accumulated ASLs, remained trypsin-insensitive indicating that ENaC is maximally activated in these cultures. This implies that the CAP inhibitor is soluble and accumulates in the ASL. Additionally, accumulation of this inhibitor is unable to inhibit ENaC conductance in CF cultures. (Tarran et al., 2006).

We then searched for soluble CAP inhibitors with trypsin-coated beads, which were added to the ASL. Albumin-coated beads were used as a control for non-specific binding. Using mass spectrometry, we identified sPLUNC1. This protein is secreted, *in vivo* and *in vitro,* in the ASL although its function is unknown. To understand of the role of sPLUNC1 in airway physiology, we examined the capacity for ASL regulation in HBECs treated with either anti-sPLUNC1 or anti-luciferase shRNA as a control. qPCR verified a >90% knockdown efficiency, which resulted in significant ASL volume depletion in the sPLUNC1 knockdown HBECs as compared to the controls (ctrl, 9.0 ±1.8 uM; anti-sPLUNC1, 5 ±0.9uM; n=5). This was accompanied by a persistent trypsin-sensitive Vt in sPLUNC1-defecient cultures, indicating that they could no longer regulate ENaC. Our next aim was to examine the effect of sPLUNC1 on ENaC current. To do this, xenopus oocytes were injected with sPLUNC1 and a,â,y ENaC subunits. sPLUNC1 was detected in the media of these oocyte by Western blot and showed a 43% (± 4%, n=40) inhibition of ENaC current when compared to ENaC injected oocytes alone. Western blot analysis revealed that this reduction in current was accompanied by a reduction in cleavage of a, â and y ENaC subunits suggesting that SPLUNC1 prevents cleavage of ENaC by serine proteases.

These findings indicate an important role for sPLUNC1 in airway physiology as it regulates ENaC current and consequently, ASL volume. Understanding the mechanism of sPLUNC1 may provide insight into novel therapies for regulating hyperactive ENaC in CF.

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Toll-Like Receptor 5 as Modifier Gene in CF Lung Disease

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Introduction: There is growing evidence that polymorphic variants in genes other than *CFTR* play an important role in determining severity of CF lung disease. We recently demonstrated that the innate immune receptor TLR5 mediates much of the damaging inflammatory response generated by CF airway cells following exposure to *P. aeruginosa.* Furthermore, it has been suggested that TLR5 is involved in bacterial clearance from the airways in humans and mice. Together, this evidence identifies *TLR5* as a biologically plausible candidate gene that may modify CF lung disease.
Objective: To confirm the pro-inflammatory phenotype of CF airway epithelial cells and to determine if polymorphisms in the *TLR5* gene modify the severity of pulmonary disease in patients with CF.
Methods: CF and non-CF cell lines were stimulated with *P. aeruginosa* wt and a strain lacking flagellin (Δ*fli*C). We genotyped one functional non-synonymous SNP in the *TLR5* gene (*TLR5 R392X,* rs5744168) in healthy individuals and stimulated PBMCs from carriers and non carriers with purified flagellin to asses the impact of the SNP on TLR5 signaling capacity. TLR5 genotypes of 2,441 CF patients were correlated with severity of CF lung disease. Outcome variables measured were a) lung function (i.e. cross-sectional measurement of FEV1 %predicted) and b) the annual decline of lung function (i.e. longitudinal measurement of FEV1 % predicted).
Results and Conclusions: CF epithelial cell lines recognize *P. aeruginosa* through the flagellin-TLR5 interaction and produce an exaggerated pro-inflammatory cytokine response. The *TLR5 R392X* SNP was functionally active, significantly decreasing TLR5 responsiveness of PBMCs up to 75% ($p < 0.0001$). Our data identify *TLR5* as an excellent biologically plausible candidate gene that may modify CF lung disease. As a follow up to this study, the *TLR5 R*392X SNP has been genotyped in 2,441 CF patients and ongoing statistical analysis of the impact of this SNP on lung function will examine whether genetic variation in *TLR5* modifies CF lung disease.

Azithromycin Fails to Reduce Inflammation in Human Cystic Fibrosis Bronchial Epithelial cells

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Objective: The aim of this study was to explore the effects of macrolides: azithromycin (AZM), clarithromycin (CAM) and erythromycin (EM) on molecular mechanisms involved during inflammation in bronchial epithelial cystic fibrosis (CF) cells.
Methods: Human CF bronchial epithelial (IB3-1) and CFTR-corrected bronchial epithelial cell lines (S9) were pre-treated 30 minutes with the macrolide (10 µg/ml) and TNF-α (10 ng/ml) was added for additional 16h of culture. To confirm results, we have treated with the same protocol human bronchial gland cells (HBG) isolated from non-CF patients with or without the CFTR inhibitor Inh-172 (10 µM, 72h).
IL-8 concentrations were evaluated in culture supernatants by ELISA and NF-κB pathway was investigated by a p65-luciferase plasmid at 4 and 8h of treatment.
Results: CAM and EM were not able to modulate TNF-α induced IL-8 secretion. Interestingly AZM reduced TNF-α induced IL-8 secretion in non-CF cells (S9 and HBG cells) but not in CF cells (IB3-1 in HBG cells treated with the CFTR inhibitor). Next, we have investigated molecular mechanisms after 4 and 8h of treatment. We have demonstrated that AZM significantly decreased TN F-α induced NF-κB transcriptionnal activity in S9 but not in IB3-1 cell line.
Conclusion: Our study demonstrates that azithromycin is not able to decrease inflammation in CF bronchial epithelial cells and suggests that CFTR chloride function may be involved in normal cells response to macrolides.

We thank Pfizer for their generous gift of azithromycin

Glucocorticoids Fail to Inhibit Inflammatory Process in CF Human Bronchial Epithelial Cell Lines

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Background: Lung dysfunction is the main cause of mortality in CF patients. Infection combined with inflammation lead to progressive destruction of respiratory epithelium. Glucocorticoids (GC) are powerful anti-inflammatory molecules commonly used to treat inflammation but with controversial efficiency among studies. Recent clinical trials showed that inhaled and oral corticosteroids have no significant effect on lung function or markers of inflammation. These results were confirmed in *in vitro* studies using cell lines and primary cell cultures from CF patients.
In order to characterise the molecular basis of such dysregulation, our project focuses on the key steps in GC activation pathway in bronchial epithelial cells.
Methods: CF and non CF human bronchial epithelial cell lines were incubated with IL-1β or TNF-α (10 ng/ml) at 4h, 8h, and 16h with or without dexamethasone (dex, 1 µM). Rates of secreted IL-8 were assessed by ELISA 16h after treatment. To evaluate GR transrepression and transactivation at 4h and 8h, we transfected cells using plasmids containing NF-κB, AP-1 or TAT3 promoter coupled to luciferase.
Results: In presence of IL-1β, basal secretion of IL-8 was restored by dex at 16h in non CF cells, whereas CF cells barely respond (only 30% of inhibition). Other results showed there is no effect of dex on NF-κB activation at 8h in CF cells.
Conclusions: This study shows a lack of GC efficiency to decrease IL-8 secretion via NF-κB pathway in CF bronchial epithelial cells.
Identify the origin of the GC resistance in CF will allow to adapt the anti-inflammatory treatments of patients.

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Proteomic Analysis of Proteins Released from *Pseudomonas Aeruginosa* Clinical and Laboratory Strains: Effects of Azithromycin

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Patients with cystic fibrosis (CF) are particularly susceptible to chronic *P. aeruginosa* (*Pa)* infection in the airways. Modulation of *Pa* virulence factors was suggested as mechanism for azithromycin (AZM) beneficial effects in CF patients. Our work was aimed to study the regulation of proteins released by *Pa* clinical and laboratory strains after AZM treatment.
Looking for secreted virulence factors active on lung epithelium of CF patients we focused on the induction of pro-inflammatory markers and measured the expression of IL-8 and TNF-a genes in a CF epithelial airway cell line in response to conditioned medium derived from *Pa* strains. *Pa* clinical isolates and PAO1 were grown overnight in aerobic conditions in the presence or absence of AZM. We demonstrated that conditioned medium (CM) from the clinical strain AA2, unlike CM from the laboratory strain PAO1, induced a statistically significant increase of about 3.6 and 2.7 times, respectively of IL-8 and TNF-a mRNA, in CF airway epithelial cells.. This induction was reduced of about 30% when AA2 was grown in the presence of AZM, suggesting that this macrolide reduces *Pa* pathogenicity. In the attempt to gain information on the identity of the molecules released by *Pa* strains before and after treatment with AZM and to identify candidate molecules involved in *Pa* virulence, we applied a recent proteomic approach (2DC-MS/MS called also MudPIT). Two-dimensional capillary chromatography - tandem mass spectrometry (MudPIT) identified polypeptides released by PAO1 and AA2 in the absence and presence of drug. 7 proteases were released from AA2 while only 1 was detected in PAO1. AZM appear to down-regulate their release in AA2 strain. This result has been confirmed by zymography.
Investigating *Pa* secretome is critical for identification of molecules involved in bacterial virulence, lung damage, inflammation and drug resistance. This approach could identify potential targets for pharmacological intervention and provide candidates for cellular pathways leading to better understanding of CF pathogenesis.

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Ceramide is a potential biomarker of epithelial injury which is raised in the airway of people with cystic fibrosis

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Background:
Over 95% of mortality and morbidity in cystic fibrosis (CF) is associated with lung disease. CF results from alterations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene but the exact pathogenesis of CF lung disease remains poorly understood. The sphingolipid ceramide is an essential constituent of plasma membranes and regulates many physiological cellular responses, including apoptosis and cell survival.[1] It has recently been shown that CFTR-deficient mice accumulate ceramide in respiratory epithelial cells due to alkalinisation of intracellular vesicles. Ceramide accumulation resulted in constitutive age-dependent pulmonary inflammation, death of respiratory epithelial cells and susceptibility to *Pseudomonas aeruginosa* infection.[2] Furthermore, similar accumulation was demonstrated in respiratory epithelial cells and a small number of sections of airway from people with CF.[2] Ceramide has also been identified as a central mediator in the development of emphysema by inducing oxidative stress and apoptosis of alveolar endothelial and epithelial cells.[3]

Aim:
To compare the distribution of ceramide in the respiratory epithelium of unused lung donors and patients with CF, emphysema and pulmonary hypertension (PH).

Methods:
Airway from lungs removed at the time of transplantation (8 CF, emphysema and PH respectively) and 8 unused donor lungs were fixed in formalin and embedded in paraffin. Immunohistochemistry was performed on 5µM sections using 2 monoclonal antibodies (Sigma mouse-IgM and Glycobiotech mouse-IgM-enriched) along with appropriate negative controls. Positive staining was evaluated in the epithelium in 5 high-power fields/patient using image analysis software and expressed in terms of percentage area. Groups were compared using the Mann-Whitney test.

Results:

Antibody	CF	PH	Emphysema	Unused Donor
Sigma	5.2	0.46**	2.2	0.33**$^{\#}$
Glycobiotech	13	3.3**	6.5**	3.4**

[Mean Percentage Positive Staining In Epithelium]

**P< 0.01 compared to CF. $^{\#}$P< 0.05 compared to emphysema.

Conclusions:
Staining for ceramide in the respiratory epithelium is significantly increased in people with CF compared to PH and unused lung donors with both antibodies and to emphysema using the Glycobiotech antibody. There was a clear trend towards higher levels of staining in emphysematous lungs compared to PH or unused donor, although this was only statistically significant for emphysema against unused donor lungs using the Sigma antibody. This data provides further evidence to support the hypothesis that ceramide accumulation occurs in the respiratory epithelium of people with CF.

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The Effect of the Fluoroquinolone Antibiotic Ciprofloxacin in a Rat Model of Respiratory Infection with *Pseudomonas Aeruginosa*

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Morbidity and mortality in cystic fibrosis (CF) are primarily caused by progressive lung damage associated with chronic bacterial infection, primarily *Pseudomonas aeruginosa* (*P.a.).* Excessive inflammatory response to chronic bacterial infection of the airways, dominated by neutrophils and consequent release of oxidants and proteases, is thought to play a central role in this tissue destruction. Ciprofloxacin, one of the antibiotics used in the treatment of CF, is effective against *P.a.* and may have some immunomodulatory effects (reviewed by Dalhoff & Shalit, 2003). The objectives of our study were to evaluate if ciprofloxacin could attenuate infection and modulate inflammatory responses in a refined rat model of respiratory infection with *P.a..* Under isofluorane anaesthesia, (day zero), male Sprague Dawley rats (250-300g; n=16/group) were inoculated with agar beads containing 10^5 colony forming units (cfu) *P.a.* PAO1 strain (M. Vasil, University of Colorado) via intra-tracheal dosing. A sham-treated group was given sterile beads. On day one, rats were dosed with ciprofloxacin (50 mg/kg s.c.) or saline vehicle and once again eight hours later. Animals were culled on day two. Cfu counts were performed on lung tissues; total and differential cell counts and ELISAs (to measure cytokines) were performed on broncheoalveolar lavage fluid (BALF). Data are expressed as mean ± standard error of the mean and analysed using a Kruskal-Wallis test followed by a Dunn's post test. In sham animals, lung infection was absent compared to the infected vehicle-treated group which had a significant lung infection (5.57 ± 0.16 log cfu). Ciprofloxacin treatment significantly attenuated infection (1.65 ± 0.15 log cfu, $P< 0.01$). BALF from vehicle-treated infected rats had a significantly greater number of total cells present versus sham treated rats ($5.03 \pm 0.92 \times 10^6$ and $1.59 \pm 0.1\ 8 \times 10^6$ cells respectively, $P< 0.001$), reflected by a significant increase in neutrophils ($2.67 \pm 0.70 \times 10^6$ and $0.21 \pm 0.05 \times 10^6$ cells, $P< 0.001$). This was not significantly altered by ciprofloxacin. BALF levels of CINC-3 were significantly elevated in infected vehicle-treated animals (36.32 ± 9.54 pg/ml, $P< 0.05$) compared to sham (undetectable). There were no significant changes in the levels of IL-1β in infected vehicle-treated animals compared to sham (105.60 ± 30.50 pg/ml versus 21.54 ± 14.37 pg/ml). IL-1β and CINC-3 levels were not affected by ciprofloxacin. IL-6 and TNFα were undetectable.

In conclusion, whilst P.a. infection was significantly attenuated by ciprofloxacin, this compound had no significant effect on the inflammatory parameters measured two days post infection in this model.

Inflammatory Responses of CF and Non-CF Cultured Nasal Epithelial Cells Obtained from Nasal Brushings

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Primary epithelial cell culture models obtained from cystic fibrosis (CF) patients are rare. Sources currently used from nasal polyps or lung explants have limitations. We have successfully developed a method to culture nasal epithelial cells (NECs) from non-CF and CF subjects.

NECs from F508del homozygous (n=12), R1 17H heterozygous (n=8) and non-CF (n=1 1) subjects were obtained by brushing the medial wall and inferior turbinate of the nose using a bronchial cytology brush; two brushings were taken from each nostril and the combined cells were resuspended in airway epithelial cell growth medium for culture and expansion. Cultured cells stained positive for cytokeratin 18. In these experiments we mimicked the insult of bacterial invasion in the respiratory tract by causing an inflammatory response. To accomplish this cells were cultured in 24-well plates and were stimulated with a cytomix containing 10 ng/ml TNF-α, 5 µg/ml LPS (from *P. aeruginosa)* and 5 ng/ml interleukin (IL)-1β over 2, 6 and 24 hours. Culture supernatants were then analysed for both spontaneous and stimulated release of IL-6, IL-8, IL-10 and IFN-γ using the BioPlex suspension array system (Luminex technology).

Cytomix elicited significant release ($P < 0.05$) of IL-6, IL-8 and IFN-γ above spontaneous levels from all three of the subject populations (between a 3- and 20-fold increase across the populations dependant on time and cytokine measured). Whilst there was an increase in stimulated compared to spontaneous cytokine release, contrary to expectation there was no difference between the populations at any time point across any of the cytokines analysed (e.g. 24 h stimulated IL-8 secretion was as follows: F508del homozygous: 16340 ± 3308 pg/ml; R117H heterozygotes: 19610 ± 4537 pg/ml and non-CF controls: 29510 ± 6549 pg/ml. IL-10 was below the limit of detection in all populations at all timepoints.

We have shown that there is no difference in the inflammatory response between patients with class II or IV mutations compared to responses from healthy controls. In conclusion, inflammatory responses cannot therefore be explained entirely by genetic background.

Effect of Cigarette Smoke Extract on IL-8 Release from Primary Nasal Epithelial Cells in CF and Healthy Volunteers

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Cigarette smoke extract (CSE) decreases the expression of the CFTR. Furthermore, CSE has profound effects on epithelial cells but different results are reported with cell lines and primary cells. This study aimed to compare the effects of CSE on spontaneous and induced IL-8 release from primary nasal epithelial cells isolated from CF patients possessing two different classes of CFTR mutation (F508del homozygous; R1 17H heterozygote) and healthy volunteers. Nasal epithelial cells were obtained via nasal brushings of the inferior turbinate of CF patients; F508del homozygote (n=5), R1 17H heterozygote (n=7) and healthy volunteers (n=6). The cells were expanded in culture and used at passage 3. Monolayer cultures were pre-exposed for 4h in the presence or absence of 5% CSE. The cells were then stimulated with LPS from *P. aeruginosa* (LPS-PA 50 or 100 µg/ml); Cytomix 1 (TNF-α 10 ng/ml, IL-1β 5 ng/ml, LPS-PA 5 µg/ml) or Cytomix 2 (TNF-α 10 ng/ml, IL-1β 10 ng/ml, IFN-γ 1000 U/ml) for 24 h. Culture supernatants were harvested and IL-8 release measured using ELISA (R & D systems). LPS (100 µg/ml) induced IL-8 release in control cells was increased by CSE (1188±292 vs. 1774±501.6, $p< 0.05$). In cells from F508del homozygous patients CSE increased cytomix 2 and LPS 50 µg/ml induced IL-8 secretion. In the R1 17H heterozygotes only the spontaneous IL-8 secretion was significantly increased by CSE exposure. Comparing the responses from the various genetic backgrounds of the subjects; the only significant differences between the groups were with cytomix 2 where the releases followed R1 17H< F508del< control (8157±1746, vs 16031±3729 vs 21296±2576). R1 17H cells were also significantly less responsive to cytomix 2 compared to F508del cells in the presence of CSE.In contrast to our data using cell lines, where CSE consistently decreased IL-8 secretion from the CF cell line (CFBE41o-), preincubation of primary cells with CSE only caused sporadic increases in IL-8 release [1]. Cytomix 2 induced significantly different levels of IL-8 secretion depending on the genetic background of the cells. As yet, we have no explanation for these data but further studies will investigate the action of IFN-γ alone on primary cells.
Williams MTS et al. Ped Pulmon 2008;287:246

Decreased Levels of Secretory Leucoprotease Inhibitor in the *Pseudomonas*-Infected Cystic Fibrosis Lung are due to Neutrophil Elastase Degradation

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Secretory leucoprotease inhibitor (SLPI) is a neutrophil serine protease inhibitor constitutively expressed at many mucosal surfaces including the lung. Originally identified as a serine protease inhibitor, it is now evident that SLPI also has anti-microbial and anti-inflammatory functions, and therefore plays an important role in host defence. Previous work has shown that some host defence proteins such as SLPI and elafin are susceptible to proteolytic degradation. Furthermore, the integrity of elafin was impaired in the lungs of patients with cystic fibrosis (CF). Consequently, we investigated the status of SLPI in the CF lung. A major factor that contributes to the high mortality rate among CF patients is *Pseudomonas aeruginosa* infection. In this study, we report that *P. aeruginosa*-positive CF BAL, which contains lower SLPI levels compared to *P. aeruginosa* -negative samples and higher neutrophil elastase (NE) activity, was particularly effective at cleaving recombinant human SLPI. Additionally, we found that only NE inhibitors were able to inhibit SLPI cleavage, thereby implicating NE in this process. NE in excess was found to cleave recombinant SLPI at 2 novel sites in the NH_2-terminal extremity (Ser15-Ala16 and Ala16-Glu17) and abrogate the ability of SLPI to bind LPS and NF-kappaB consensus binding sites. In conclusion, this study provides evidence that SLPI is cleaved and inactivated by NE present in CF lung secretions and that *P. aeruginosa* infection promotes this effect. Overall, these novel findings broaden our understanding of the destruction caused by *P. aeruginosa* infection and how it contributes to chronic damage seen in the CF lung.

Pro-Inflammatory Toll-Like Receptor 2 Expression in CF Epithelium

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Background: Increased and prolonged *Pseudomonas aeruginosa* (PA)-induced signalling via activation of Toll-Like Receptor 2 (TLR2) contributes to chronic pulmonary inflammation in Cystic Fibrosis (CF). Internalisation of the receptor complex leads to activation of NF-kB and AP-1 pathways and subsequent pro-inflammatory response (eg. IL-8). Prolonged intracellular signalling may occur when a receptor remains perinuclear, suggesting that endosome formation and subsequent lysosomal degradation may be important for signal termination.

Hypothesis: A defect in the elimination of the TLR2-PA-signalling-complex in CF epithelium raises intracellular TLR2 leading to prolonged pro-inflammatory signalling.

Methods: We used the well established cell lines HBE (human bronchial epithelial cells, non-CF) and CFBE (CF bronchial epithelial cells, F508D) Using CF (F508Δ) and non-CF epithelial cell lines (CFBE, HBE). The kinetics of TLR2 expression upon LPS (50 ug/ml) stimulation is determined by FACS analyses. The endo-lysosomal degradation is followed using immunohistochemistry and antibodies to the early endosome (EEA-1) and the lysosome (LAMP-1).

Results: We show increased TLR2 expression (by FACS). In CFBE cells TLR2 protein expression was higher but not significantly different from that in HBE cells. However, intracellular expression was significantly higher in CFBE (1 h, 8h and 12 h of PA-LPS exposure ($p < 0.05$ for 8 and 1 2h). The high expression of TLR2 coincided with increased secretion of IL-8 (24 h, $p < 0.05$) in CFBE compared to HBE cells. Investigating endo-lysosomal degradation (immunocytochemistry) showed prolonged expression of the early endosome (EEA1) and failure to form lysosomes (LAMP1) in CFBE cells suggesting decreased processing of endocytosed material and sorting into the lysosomal degradation pathway in CFBE cells. Furthermore we investigated the ubiquitiniation enzyme A20 in CF epithelium and show that A20 protein expression is significantly decreased in CF epithelium which may contribute to the prolonged signalling in CF epithelium.

Conclusion: We conclude that a defect in the elimination of the TLR2-PA-signalling-complex raises intracellular TLR2 leading to prolonged pro-inflammatory signalling.
The importance of the formation of the endosome/lysosome and mechanisms involved in regulation of TLR2 signalling may represent new therapeutic targets to combat chronic PA inflammation in CF airways.

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Evaluation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) function in Human Monocytes

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The functional evaluation of cystic fibrosis transmembrane conductance regulator (CFTR) is critical for assessing new therapies and define diagnosis of atypical cases in cystic fibrosis (CF). Currently available protocols are cumbersome and practicable only for selected subjects in few centers. As it is known that leukocytes express detectable levels of CFTR we investigated the possibility to assess CFTR activity in these cells obtained from a 5 cc venous blood sampling.
We measured higher levels of CFTR expression in monocytes and lymphocytes than in polymorphonuclear cells by immune-precipitation and flow cytometric assays. To determine CFTR activity in monocytes isolated from non-CF donors (non-CF), heterozygous subjects (HTZ) and CF patients we measured cell membrane depolarization, by single-cell fluorescence imaging, using the potential-sensitive probe bis-(1 ,3- diethylthiobarbituric acid) trimethine oxonol ($DiSBAC_2$). We found that, upon stimulation, only non-CF and, to a lesser extent, HTZ monocytes showed an increase in fluorescence signal while monocytes from CF patients failed to respond. In comparison, we measured Nasal Potential Difference in selected subjects obtaining overlapping results.
Our findings promote the evaluation of CFTR activity in monocytes by optical techniques as an useful tool to assess CFTR activity for basic and translational research, including drug development and testing as well as diagnostic applications.

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Human Lung Explant Tissue as a new Ex Vivo Model for Preclinical Optimisation of CFTR Pharmacotherapy

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In cystic fibrosis (CF) the CF transmembrane conductance regulator (CFTR) basic defect is currently targeted by multiple approaches to restore CFTR chloride (Cl^-) channel activity using correcting and potentiating pharmacotherapeutic compounds. These drugs are evaluated and pharmacologically optimised *in vitro* in studies on cell lines, animal models and tissue cultures. However, CFTR modulators might have a different efficacy in native human CF epithelia. Availability of native human CF tissues is relatively limited, and size and condition are important quality markers. Hence, aim of this study was to establish lung explant epithelia from CF and disease control patients receiving lung transplantation as a new *ex vivo* model for preclinical optimisation of CFTR pharmacotherapy.
We included native human bronchial tissue sheets from 21 lung explants (CF, COPD, pulmonary fibrosis) and donor bronchi obtained during lung transplantation at the Hannover transplant center in this study so far. Multiple (n=8) tissue pieces per subject were prepared immediately after surgery, transepithelial short-circuit current $_{(Isc)}$ measurements in perfused Mini Ussing chambers were registrated and structure control was obtained by histopathology.Basal tissue bioelectric properties and I_{sc} responses to inhibition of the epithelial sodium channel (ENaC) and stimulation of CFTR and alternative Cl^- secretion in CF and (disease) control bronchi showed reproducible results so far. The performed preparation technique resulted in tissue segments with intact respiratory epithelia including submucosal glands.
The results of this pilot study confirm the feasibility of *ex vivo* Cl^- secretion measurements in native human lung explant tissue as an additional resource of human CFTR relevant tissue. More data about variability, optimal evaluation protocols and possible limitations of the method will be obtained, before most promising CFTR correctors/potentiators with potency in the nanomolar range can be included in the analysis. Our strategy can be an essential step in the translation of small molecule compounds into future clinical trials aiming to rescue the CFTR basic defect.

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Dual Activity of Aminoarylthiazoles on Trafficking and Gating Defects Caused by CF Mutations

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There is an increasing interest in developing small molecules able to correct the basic defect caused by cystic fibrosis (CF) mutations. Many types of CF mutations impair the function of CFTR protein by altering the protein targeting to the plasma membrane and/or by causing an alteration in the channel gating. Pharmacotherapy of CFTR needs to be tailored to the different classes of CF mutations. Typically, class 3 mutations, which cause a channel gating defect, require potentiators, i.e. molecules that enhance the response to the cAMP signal by increasing open channel probability. Conversely, class 2 mutations, essentially F508del, are sensitive to correctors, small molecules that improve the targeting of the mutant protein to the plasma membrane.

In a previous study (Pedemonte et al., *J. Clin. Invest.* 115: 2564-2571, 2005), we found that aminoarylthiazoles act as correctors of F508del mistrafficking. However, these compounds also had the interesting ability to improve F508del activity after long-term incubation, a mechanism that seems different from that of classical CFTR potentiators.

We have now tested aminoarylthiazoles on class 3 mutations. CFTR activity was evaluated in transfected FRT and A549 cells with the halide-sensitive yellow fluorescent protein and (for FRT cells only) by measuring transepithelial chloride currents. Cells were stimulated with forskolin (20 μM), to fully enhance intracellular cAMP, and with genistein (50 - 200 μM), to correct the gating defect. Interestingly, we found that long-term incubation with aminoarylthiazoles strongly enhanced the current elicited by forskolin, $I_{(F)}$, relative to that elicited by forskolin plus genistein, $I_{(FG)}$. This effect, observed in G551 D, G1349D, and D1 152H cells, persisted even after removing the compound before the functional assay. In G551 D cells, 24 hour treatment with aminoarylthiazoles enhanced $I_{(F/FG)}$ from less than 0.05 to 0.5, a highly significant effect given that $I_{(F/FG)}$ for wild type CFTR is about 0.8. As also reported previously, aminoarylthiazoles did not act acutely on mutant CFTR in intact cells, as expected for a classical potentiator. Experiments are in progress to clarify the mechanism of action of aminoarylthiazoles. It is possible that they induce post-translational modifications and/or long-term changes in folding that promote dual effects on CFTR trafficking and gating.

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Identification and Analysis of ΔF508 Correctors

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The ΔF508 mutation stops the maturation of the CFTR protein and causes its early degradation by the ubiquitin/proteasome system. Small molecules called correctors may rescue ΔF508-CFTR from the endoplasmic reticulum and enhance its targeting to the plasma membrane. However, the efficacy of most correctors identified so far is low when they are tested in native airway epithelial cells.

We have performed a screening of a small molecule library using a fluorescence-based assay on CFBE41 o- bronchial epithelial cells. A small set of molecules, including corr-194 and corr-207, was found to enhance the activity of ΔF508-CFTR after 24 hours of incubation. Such putative correctors were compared to known correctors previously identified, particularly corr-4a (Pedemonte et al., *J. Clin. Invest.* 115: 2564-2571, 2005), and to low temperature treatment (27 °C). In CFBE41 o- cells, responsiveness of ΔF508 to rescue treatments follows the order: low temperature > corr-194 > corr-207 > corr-4a. Interestingly, we found that some combination of correctors caused a synergic effect. In particular, correction by low temperature was enhanced by corr-4a and by corr-207 but not by corr-194. At the protein level, we found that most treatments increased the intensity of the immature form, band B, of CFTR protein. In particular, corr-194 was the most effective, by causing a > 10-fold increase in band B with a relatively small effect on the mature form, band C. Our results suggest that the various ΔF508 correctors act on different targets, probably associated with different steps of the quality control process in the endoplasmic reticulum. Identification of such targets and rational design of correcting maneuvers may help to maximize ΔF508 rescue.

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Different Levels of F508del-CFTR Rescuing by Correctors in BHK and in Human Bronchial Epithelial cells

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Recent high-throughput compound screens identified several novel small-molecules that partially rescue the trafficking defect of F508del-CFTR both in recombinant and human bronchial epithelial cells. These include potentiator VRT-532 and correctors VRT-325, VRT-640, Genzyme compound 48, KM11060 (sildenafil analogue), compounds 4a, 3a, 4c and 2b [1,2,3,4].

Our aim here is two-fold: (i) to determine their efficacy to rescue F508del-CFTR in HBE cells and in polarized primary human airway epithelial cells (AEC); and (ii) to investigate the mechanism by which these correctors rescue cell-surface expression and restore function of F508del-CFTR.

Pulse-chase experiments with [^{35}S]methionine followed by CFTR immunoprecipitation in BHK cells, show that F508del-CFTR is fully processed, albeit at low levels by VRT-325 6.7μM/24h (~6.0%); VRT-532 20μM/48h (~6.5%); VRT-640 6μM/24h (~4.5%) and C4 10μM/accute (~2.0%). Similar experiments with F508del-revertants [3] show that VRT-325 (but not VRT-532, VRT-640 nor C4) increases the efficiency of maturation of F508del-4RK-CFTR, whereas F508del-G550E-CFTR remains unaffected, suggesting an effect directly on the folding of F508del-CFTR.

Preliminary data obtained by CFTR immunoprecipitation with [^{35}S]methionine labbeling in CFBE41o– cells [5] and western-blot show that F508del-CFTR is fully processed, although at different levels, after a 24h incubation with potentiator VRT-532 and correctors VRT-325, VRT-640 and compound 4a. Currently micro-Ussing chamber experiments are underway to measure CFTR-mediated Cl^- currents in polarized F508del-CFBE41o cells after treatment with the corrector compounds, VRT-532, VRT-325, VRT-640, Genzyme compound 48, KM11060 (sildenafil analogue), compounds 4a, 3a, 4c and 2b, as described [6].

Overall, our data show that the correctors tested here are able to promote F508del-CFTR maturation and restore its function in BHK and CFBE41o– cell lines. Also, correctors tested here appear to rescue F508del-CFTR by distinct mechanisms in BHK cells. Future work will be done in order to assess compound efficacy in polarized human primary AEC cells by Ussing chamber measurements [6].

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Transcriptional profiling of chemical correctors of CFTRΔF508 trafficking

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We have identified and validated a variety of chemically distinct molecules as CFTR ΔF508 trafficking correctors. Overcoming the challenge of merging hit validation with mechanism of action remains a daunting task. To elucidate proximal and distal pathways involved in correcting CFTR ΔF508 we generated a compendium of corrector profiles in the hopes of identifying novel drugable targets. We have generated a library of transcriptional profiles of the 16 compounds in our pipeline including the established correctors such as VRT-325 and rescue at 28°C, and the potentiator VRT-532. We chose the dual color Agilent platform and our study design involved labeling each treated sample reciprocally with Cy3 or Cy5 along with the reciprocally labeled universal reference RNA, with each treatment including a biological replicate. We have generated distinct signatures 12 hours and 24 hours post-treatment in the HEK293 cell line with the correctors at previously determined corrective concentrations. We also generated signatures at earlier time points, including 1hr and 6 hr post treatment for some of our compounds to obtain a temporal signature for correction. As proof of principle we queried specific genes known to be associated with CFTR folding and trafficking and found they were significantly ($p<0.05$) and differentially expressed, in the expected direction. For instance, the chaperone Hsp70 which associates preferentially with the mutant CFTRΔF508, was significantly down-regulated in some of our compound signatures. We have derived distinct transcriptional signatures in categories promixal to CFTR folding, including ER chaperones, N-Glycan biosynthesis, ERAD, and ER-total and are currently validating these hits, in addition to signatures more distal to CFTR biogenesis. So far, we have identified a novel gene, involved in N-glycan biosynthesis which is significantly downregulated upon treatment with one of our correctors a sodium pump inhibitor, Ouabain. Targeting this gene with siRNA in human epithelial cells expressing CFTRΔF508 restored expression at the cell surface by 2 fold over control, as determined by flow cytometry. We are in the process of validating other hits generated from this data by siRNA and overexpression, real-time PCR and western blotting. Generating such profiles will elucidate novel pathways and mechanisms of CFTR rescue, and drive a new generation of target-driven therapies.

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Role of CLCA Proteins in Mucus Homeostasis in Cystic Fibrosis and Other Diseases with Secretory Dysfunctions

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CLCA proteins are thought to play a role as modulators of mucus homeostasis in diseases with secretory dysfunctions including Cystic Fibrosis (CF) by contributing to different aspects of processing of the mucus. In contrast to the fully secreted CLCA proteins of goblet cells, the CLCA proteins in non-goblet cell epithelial cells are anchored to the cell membrane via a transmembrane domain in their carboxy-terminal cleavage product. This part of the protein might therefore be the key to distinguish between different functions of CLCA family members. Here we present immunohistochemical and biochemical characteristics of the murine mCLCA3 and mCLCA6 proteins with a focus on their membrane association and their cellular distribution patterns in the body. Computational predictions of possible transmembrane domains of mCLCA3 and mCLCA6 were performed and tested experimentally using polyclonal rabbit antibodies, including immunoblotting, deglycosylation analyses, acid release, immunohistochemistry, immune electron microscopy and co-localization with the cystic fibrosis transmembrane conductance regulator (CFTR) protein via confocal laser scanning analyses. Furthermore, the results of the immunolocalization were corroborated on the mRNA level with laser capture microdissection (LCM) followed by RT-qPCR. In contrast to the fully secreted murine mCLCA3 protein, mCLCA6 possesses a transmembrane domain within the carboxy-terminal cleavage product. Unlike mCLCA3, which is expressed in mucin granules of intestinal goblet cells in various organs, mCLCA6 is exclusively located at the apical plasma membrane of non-goblet cell enterocytes in both the small and large intestine. In the large intestine, mCLCA6 co-localizes with the CFTR protein. Furthermore, both mCLCA3 and mCLCA6 appear to be upregulated in the intestine of the CF mouse models cftr tm1 Cam and cftr TgH(neoim)1 Hgu. Both mCLCA3 and mCLCA6 are thus expressed in adjacent but distinct intestinal cellular microenvironments. Co-localization of mCLCA6 with the CFTR protein and increased expression in CF mouse models suggest that murine CLCA proteins might play a role in modulating the CF phenotype. The functional significance and structural details of this co-localization have to be determined in the future.

Primary Bronchial Epithelial Cell Cultures from the Explanted Lungs of People with Cystic Fibrosis - A Valuable Resource for Translational Research

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Introduction: Mortality and morbidity in cystic fibrosis (CF) are largely due to lung disease. Despite improvements in survival the exact pathogenesis of CF lung disease remains poorly understood. Ultimately, it results in progressive bronchiectasis and premature death. It follows that experimental models are vital for use in CF research.
Studies involving animal models and immortalised cell lines have contributed significantly to our current knowledge of CF lung disease. However, there are inherent limitations to both approaches, including poor replication of lung pathology and failure to reflect *in vivo* findings. The opportunity to work on primary tissue from patients with CF is rare.
Aim: The aim of this work was to establish an *ex vivo* culture system for primary bronchial epithelial cells (PBECs) from the lungs of people with CF removed at the time of transplantation.
Methods: Pieces of segmental bronchus were removed immediately after explantation and treated with patient-specific antimicrobials and mucolytics to achieve disinfection. PBECs were harvested and submerged cultures established before transfer to an air-liquid interface (ALI). Cultures were characterised morphologically and histologically using light and scanning electron microscopy. Mucus production at ALI was assessed by enzyme-linked immunosorbent assay and amylase-periodic acid Schiff staining.
Results: PBECs have been successfully cultured from 14 of 21 patients attempted. Mucus production and tight junction formation has been demonstrated at ALI. The PBECs remained viable after cryopreservation. PBEC cultures failed from 2 patients due to immediate overgrowth with *Burkholderia cepacia* complex and in 5 patients initially successful cultures overgrew with *Pseudomonas aeruginosa* once antimicrobials were withdrawn.
Conclusions: PBEC culture is possible from lungs removed at the time of transplantation from people with CF. Tailored antimicrobial strategies are practicable and yield a favourable success rate. This technique represents a valuable resource that provides a cellular model to elucidate the pathogenic mechanisms in CF lung disease and to investigate potential therapeutic targets.