2011
European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Conference Programme & Abstract Book

Tirrenia-Pisa, Italy

Chairpersons

Christine Bear, Harry Cuppens and Luis Galietta

30 March - 02 April 2011

CONTENTS

CONFERENCE SPONSORSHIP & SUPPORT

The ECFS thanks the following for their support

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Welcome from the ECFS President

Dear Friends and Colleagues,

It is a great pleasure to welcome you to the 8th European Cystic Fibrosis Conference entirely dedicated to Basic Science which, this year, takes place in Tirrenia, Italy. This year we are delighted to welcome Prof. Harry Cuppens as the conference Chairperson who will be supported by Prof. Luis Galietta and Prof. Christine Bear as co-chairpersons.

Basic scientific discovery is critical to our understanding of CF, and already we can see new therapies coming through from the hard work of this endeavour. The ECFS is committed to providing you with a platform to discuss your ideas with your colleagues during this important conference which will comprise a series of symposia with invited European and International guest speakers. It is a high priority for us to support the work of scientists in CF and we are proud that this conference is now established as a key annual event in the ECFS. Your active participation will contribute to a productive exchange of information and I hope productive collaborations.

I extend a very warm welcome to an exciting conference.

Stuart Elborn
President
European Cystic Fibrosis Society

Conference Chairpersons' Welcome

We are very happy to welcome you to the 8th ECFS Basic Science CF conference 2011, taking place in the town of Tirrenia in Italy. Located at the centre of the Tuscan coast, Tirrenia has a coastal stretch of about 10km long. It is completely immersed in nature and at a short distance from the two important cities of Pisa and Livorno, and not too far from Florence.

With this conference, you can expect to hear about recent unpublished results and engage in discussions of data and ideas in an informal and interactive environment. A key characteristic of the conference is the high attendance of young participants, both PhD students and post-docs, who contribute enormously to the success of this annual event with their high enthusiasm. Here you will have excellent opportunities to discuss your data and interact in a great atmosphere with the best of European and International experts in this field.

This year's programme includes a number of symposia with international experts covering topical aspects of basic research in cystic fibrosis together with invited talks from submitted abstracts; there will also be two key-note lectures as well as four informal and interactive special interest group discussions. For two evenings we will finish the day with poster sessions and a chance to win the best poster prize! This combination has been extremely successful in the past and the conference offers a forum for informal brain-storming-type discussions.

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

Christine Bear
University of Toronto
Canada

Harry Cuppens
University of Leuven
Belgium

Luis Galietta
University of Genoa
Italy

2011 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

Italy, 30 March-2 April 2011

Programme

Chairpersons: C. Bear (Toronto, Canada), Harry Cuppens (Leuven, Belgium), Luis Galietta (Genoa, Italy)

Wednesday, 30 March 2011 (Day 1)	
	Registration, Light Meal
14:00-17:30	Set-up of Posters – Room: SALONE BRUSCHI
17:30-18:00	**Official opening of the meeting by the Conference Chairpersons** – Room: SALA FERRETTI
18:00-19:00	**Opening Keynote Lecture** Cellular Mechanisms Modulating the Response of Patients Carrying Nonsense Mutations to Read-through Treatments – B. Kerem (IL)
19:00-20:00	**Welcome Reception**
20:00-21:30	*Dinner*

Thursday, 31 March 2011 (Day 2)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 1 – CFTR Structure: Indications for Modulators** **Chairs: I. Braakman (NL) / P. Thomas (US)**
08:45-09:10	*Pichia pastoris* as an expression toolbox for mammalian ABC transporters suitable for structure determination- A successful strategy for P-glycoprotein with promise for structural studies of CFTR – I. Urbatsch (US)
09:10-09:35	Mis-steps in Mutant CFTR Folding – P. Thomas (US)
09:35-10:00	Sequential Domain Folding in Wild-Type and F508del-CFTR – I. Braakman (NL)
10:00-10:15	Abstract 1: Structural Features of the Nucleotide Binding Domains of the CFTR in Solution – L. Galeno (IT)
10:15-10:30	Abstract 2: Assessment of the Direct Interaction of Potentiator Molecules on CFTR in Functional Assays for Purified and Reconstituted Protein – P. Eckford (CA)
10:30-11:00	*Coffee break* & **Poster viewing** – Room: SALONE BRUSCHI
11:00-12:45	**Symposium 2 – Mutant CFTR: Search for Targets and Modulators** **Chairs: C. Bear (CA) / J. Hanrahan (CA)**
11:00-11:25	Early Folding Events of CFTR NBD1 Detected by FRET– W. Skach (US)
11:25-11:50	Challenges in CF Drug Development – J. Hanrahan (CA)
11:50-12:15	Autophagy Inhibition as Link between Deffective CFTR and Inflammation in Cystic Fibrosis Airways – L. Maiuri (IT)
12:15-12:30	Abstract 4: Cytosolic pH Effect on CFTR Activity and on Binding of Different CFTR Activators – R. Melani (IT)
12:30-12:45	Abstract 3: Model of the cAMP Activation of Chloride Transport by CFTR Channel and the Mechanism of Potentiators – O. Moran (IT)
12:45-14:30	*Lunch*
14:30-16:15	**Symposium 3 – Alternative Targets for CF Basic Defect** **Chairs: H. De Jonge (NL)/ K. Kunzelmann (DE)**
14:30-14:55	Physiological Relevance of Anoctamins – K. Kunzelmann (DE)
14:55-15:20	Regulation of ENaC by Soluble Signals in Airway Surface Liquid – J. Stutts (US)
15:20-15:45	Compensatory Chloride Channels in CF - H. De Jonge (NL)
15:45-15:55	Abstract 19: The Anti-Inflammatory Mediator, Lipoxin A4, Increases ASL Height in Normal and Cystic Fibrosis Bronchial Epithelium – V. Urbach (IE)
15:55-16:05	Abstract 18: Native Calcium-Activated Chloride Channels and their Association with TMEM16A Protein Expression – P. Scudieri (IT)
16:05-16:15	Abstract 40: Molecular Determinants Underlying the Stimulatory Action of the CFTR Potentiator CBIQ on the Ca^{2+} -Activated K^{+} Channel KCa3.1 – R. Sauvé (CA)

16:15-16:45	*Coffee Break* & **Poster Viewing** – Room: SALONE BRUSCHI
16:45-18:15	Room: SALA FERRETTI **Special Group Discussion-I – CFTR Structure as a Therapeutic Target** **Moderators: C. Bear (CA)/ W. Skach (US)** Room: SALA NATALI **Special Group Discussion-II – ENaC and/or Alternative Chloride Channels as Relevant Targets for CF Treatment** **Moderators: H. Cuppens (BE)/ R. Frizzell (US)**
20:30-21:30	*Dinner*
21:30-23:00	Evening Poster Session – Posters with Even Numbers – Room: SALONE BRUSCHI

Friday, 1 April 2011 (Day 3)

07:30-08:45	*Breakfast*
08:45-10:30	**Symposium 4** – **Understanding Epithelial Ion Transport** **Chairs: R. Frizzell (US) / M. Gray (UK)**
08:45-09:10	Orchestration of Epithelial Bicarbonate Secretion in Human Airway Serous Cells– M. Gray (UK)
09:10-09:35	Estrogen and Lipoxin Regulation of Ion Transport and Airway Surface Liquid Dynamics in Normal and Cystic Fibrosis Bronchial Epithelium – B. Harvey (IE)
09:35-10:00	Co-dependent Ion Channels: CFTR and SLC26A9 – R. Frizzell (US)
10:00-10:15	Abstract 17: Molecular Structure, Packing and Release of MUC2 with Relevance to Cystic Fibrosis – H. Nilsson (SE)
10:15-10:30	Abstract 13: CFTR Regulation in Human Airway Epithelial Cells Requires Integrity of the Actin Cytoskeleton and Compartmentalized cAMP and PKA Activity – S. Monterisi (IT)
10:30-11:00	*Coffee break* & **Poster viewing** – Room: SALONE BRUSCHI
11:00-12:45	**Symposium 5 – Inflammatory Mechanisms in CF as Therapeutic Targets** **Chairs: B. Scholte (NL) / G. Cabrini (IT)**
11:00-11:25	Modulating Chemotactic Signaling: Novel Molecular Targets – G. Cabrini (IT)
11:25-11:50	Inflammation, Bioactive Lipids and Mucus Production in CF Mutant Mice - B. Scholte (NL)
11:50-12:15	Links between CFTR-Cl^- Channel Function and Eicosanoid Inflammation Pathway in Cystic Fiborosis – A. Edelman (FR)
12:15-12:25	Abstract 26: Characterization of Altered Inflammatory Signalling Pathways in CFTR-Defective Cells – F. Dekkers (NL)
12:25-12:35	Abstract 24: Inhibitors of Glucosylceramide Synthase (GluCerT) Reduce the Transcription of IL-8 Induced by *P.aeruginosa* in CF Bronchial Cells – MC Dechecchi (IT)
12:35-12:45	Abstract 32: COMMD1: An Anti-Inflammatory Protein in the Context of Cystic Fibrosis? – A. Aissat (FR)
12:45-14:00	*Lunch*

14:00-18:00	**Free Afternoon, Pisa guided tour**
18:00-19:35	**Symposium 6 – Host Pathogen Interactions in Cystic Fibrosis** **Chairs: M. Surette (CA) / M. Knowles (US)**
18:00-18:25	The Cystic Fibrosis Airway Microbiome: Polymicrobial Interactions and Overlooked Pathogens – M. Surette (CA)
18:25-18:50	The Basic Link between Lack of CFTR, Mucin Secretion and the Sticky Mucus in CF and Relation to Bacterial Overgrowth- G. C. Hansson (SE)
18:50-19:05	Abstract 35 : Effects of Azythromycin on the Regulation of Metalloproteases Released by *Pseudomonas aeruginosa* Clinical and Laboratory Strain – G. Bergamini (IT)
19:05-19:20	Abstract 29: Investigation of Putative Virulence Factors of an Emerging Cystic Fibrosis Pathogen, *Pandoraea* – L. Fabunmi (IE)
19:20-19:35	Abstract 27: ICAM-1 Expression in Host Cells Following *Burkholderia cepacia* Complex (Bcc) Infection – R. Pilkington (IE)
20:00-21:30	*Dinner*
21:30-23:30	Evening Poster Session - Posters with Odd Numbers – Room: SALONE BRUSCHI

Saturday, 2 April 2011 (Day 4)

07:30-08:45	Breakfast
08:45-10:30	**Symposium 7 – Lessons from Animal Models** **Chair: J. Engelhardt (US) / J. Wine (US)**
08:45-09:10	Fishing for New Regulators of CFTR and Fluid Secretion – M. Bagnat (US)
09:10-09:35	Lung Disease and Diabetes in CFTR-knockout Ferrets – J. Engelhardt (US)
09:35-10:00	The F508del Mutation Causes CFTR Misprocessing and Cystic Fibrosis-like Disease in Pigs – L. Ostedgaard (US)
10:00-10:15	Abstract 20: Tracheal Smooth Muscle Abnormalities in the CF Mouse – H. Wallace (UK)
10:15-10:30	Abstract 36: Host Response to *Pseudomonas aeruginosa* Adaptation During Airway Chronic Infection – A. Bragonzi (IT)
10:30-11:00	*Coffee break* & **Poster viewing** – Room: SALONE BRUSCHI
11:00-13:00	**Symposium 8 – Insights in the CF Modulating Gene Products** **Chair: H. Cuppens (BE) / M. Amaral (PT)**
11:00-11:25	CFTR Traffic: Friends and Foes – M. Amaral (PT)
11:25-11:50	CF Lung Disease Modifier at the Chromosome 11p13 Locus: Update on the Potential Mechanism of Genetic Variation – M. Knowles (US)
11:50-12:15	Variation in the *MSRA* Gene Decreases Risk of Neonatal Intestinal Obstruction in Cystic Fibrosis -P. Durie (CA)/ G. Cutting (US)/ M. Drumm (US)
12:15-12:40	First Results of the Genome Wide French CF Modifier Study – H. Corvol (FR)
12:40-12:50	Abstract 12: Trafficking Of CFTR Is Unconventionally Regulated By Key Components Of The COPII Complex – M. Cutrona (IT)
12:50-13:00	Abstract 10: Proteomic Identification of Calumenin as a G551D - CFTR Associated Protein – P. Trouvé (FR)
13:00-14:30	*Lunch*

14:30-16:00	Room: SALA FERRETTI **Special group discussion-III – CFTR Post Translational Modifications** **Moderators: A. Mehta (UK)/ J. Hanrahan (CA)**) Room: SALA NATALI **Special group discussion-IV – CF Animal Models** **Moderators: L. Galietta (IT)/ J. Engelhardt (US)**
16:00-16:30	*Coffee Break* & **Poster Viewing** – Room: SALONE BRUSCHI
16:30-18:15	**Symposium 9 – Clinical Trials and Therapeutic Strategies** **Chairs: L. Galietta (IT) / M. Wilschanski (IL)**
16:30-16:55	*Ex vivo* Surrogate Assays to Assess Efficacy of Ion Channel-Targeted CF Drugs – N. Derichs (DE)
16:55-17:20	Rescue of Mutant CFTR: Current Shortcomings and Future Strategies in Drug Discovery - M. Mense (US)
17:20-17:30	Abstract 42: Innovative Strategies for the Suppression of Fluid Hyperabsorption and the Recovery of Airways Hydration in Cystic Fibrosis – A. Gianotti (IT)
17:30-17:40	Abstract 44: Restoration of Chloride Efflux by Azithromycin in CF Human Bronchial Epithelial Cells – M. Ruffin (FR)
17:40-17:50	Abstract 46: Differential pattern of microRNAs expression in Cystic Fibrosis and Normal Human Bronchial Epithelial cells – S. Noel (BE)
17:50-18:15	Small Molecules in Treatment of CF - M. Wilschanski (IL)
18:15-18:30	*Break*
18:30-19:30	**Closing Lecture** Submucosal Gland Secretion in Four Species with CF – J. Wine (US)
20:30	*Dinner / social event*

Sunday, 3 April 2011 – Delegates depart

POSTER TITLES & AUTHORS

P.1 **Structural Features of the Nucleotide Binding Domains of the CFTR in Solution**
Lauretta Galeno, Oscar Moran

P.2 **Assessment of the Direct Interaction of Potentiator Molecules on CFTR in Functional Assays for Purified and Reconstituted Protein**
Paul D.W. Eckford, Canhui Li, Mohabir Ramjeesingh, Ling Jun Huan, Christine E. Bear

P.3 **Model of the cAMP Activation of Chloride Transport by CFTR Channel and the Mechanism of Potentiators**
Oscar Moran

P.4 **Cytosolic pH Effect on CFTR Activity and on Binding of Different CFTR Activators**
Raffaella Melani, Valeria Tomati, Ambra Gianotti, Luis J.V. Galietta, Olga Zegarra-Moran

P.5 **Structural Biology of ABC Transporters of Medical and Pharmacological Interest**
Ateeq Alzahrani

P.6 **Expression and Purification of Full-Length CFTR from *Saccharomyces cerevisiae***
Natasha Cant, Liam O'Ryan, Tracy Rimington, Robert Ford

P.7 **An Improved Fluorescent Sensor to Measure CFTR Activity**
Lodewijk A.W. Vijftigschild, Jeffrey M. Beekman, Cornelis K. van der Ent

P.8 **CFTR Specific Effects of CPT-cAMP and Forskolin on Cell Metabolism**
Matthias Zwick, Manuel Hellstern, Anna Seelig

P.9 **Direct visualization of CFTR conformation by atomic force microscopy imaging**
Carlotta Marasini, Massimo Vassalli

P.10 **Proteomic Identification of Calumenin as a G551D - CFTR Associated Protein**
Pascal Trouvé, Ling Teng, Nathalie Benz, Mathieu Kerbiriou, Mehdi Taiya, Olivier Mignen, Claude Ferec

P.11 **Cdc42 Involvement in CFTR Post-Golgi Trafficking in Epithelial Airway Cells**
Romain Clément, Fleur Fresquet, Alain Kitzis, Vincent Thoreau

P.12 **Trafficking Of CFTR Is Unconventionally Regulated By Key Components Of The COPII Complex**
Meritxell B. Cutrona, Seetharaman Parashuraman, Oliviano Martella, Ramanath N Hegde, Anastasia Egorova, Alberto Luini

P.13 **CFTR Regulation in Human Airway Epithelial Cells Requires Integrity of the Actin Cytoskeleton and Compartmentalized cAMP and PKA Activity**
Stefania Monterisi, Maria Favia, Rosa A Cardone, Lorenzo Guerra, Stephan Reshkin, Valeria Casavola, Manuela Zaccolo

P.14 **Analysis of CFTR Expression in Nasal Epithelial Cells by Flow Cytometry**
Marit A. van Meegen, Suzanne W.J. Terheggen, Cornelis K van der Ent, Jeffrey M. Beekman

P.15 **Non-Genomic Estrogen Regulation of Airway Surface Liquid Height in Normal and Cystic Fibrosis Bronchial Epithelia**
Vinciane Saint-Criq, John A. Katzenellenbogen, Brian J. Harvey

P.16 **Glucocorticoids Differentially Modulate the CFTR-Channel**
Mandy Laube, Ulrich H. Thome

P.17 **Molecular Structure, Packing and Release of MUC2 with Relevance to Cystic Fibrosis**
Harriet Nilsson, Daniel Ambort, Gunnar C Hansson, Hans Hebert

P.18 **Native Calcium-Activated Chloride Channels and their Association with TMEM16A Protein Expression**
Paolo Scudieri, Loretta Ferrera, Elvira Sondo, Nicoletta Pedemonte, Emanuela Caci, Ifeoma Ubby, Franco Pagani, Luis J.V. Galietta

P.19 **The Anti-Inflammatory Mediator, Lipoxin A4, Increases ASL Height in Normal and Cystic Fibrosis Bronchial Epithelium**
Valerie Urbach, Valia Verriere, Mazen Mazen Al-Alawi, Gerry Higgins, Raphael Chiron, Richard Costello, Brian J Harvey

P.20 **Tracheal Smooth Muscle Abnormalities in the CF Mouse**
Helen L. Wallace, Marilyn G Connell, Susan Wray, Kevin Southern, Theodor Burdyga

P.21 **Decreased Apical Expression of CFTR by Pseudomonas Aeruginosa Infection in Respiratory Cells: Role of NHERF1 Phoshorylation**
Anna Tamanini, Rosa Rubino, Moira Paroni, Lorenzo Guerra, Maria Favia, Maria Cristina Dechecchi, Valentino Bezzerri, Alessandra Bragonzi, Giulio Cabrini, Valeria Casavola, Stephan J Reshkin

P.22 **Translational Medicine - From Bedside to Basic Science. On the Clinical Impact of Oxidative Stress in Cystic Fibrosis**
Peter O. Schiotz, Joergen Froekjaer, Lars P Tolbod

P.23 **Expression of Interferon Developmental Regulator 1 (IFRD1) and Histone-Deacetylase (HDAC1-2) in CFTR-Deficient Airway Cells**
Elise Blanchard, Monique Bonora, Annick Clement, Jacky Jacquot

P.24 **Inhibitors of Glucosylceramide Synthase (GluCerT) Reduce the Transcription of IL-8 Induced by *P.aeruginosa* in CF Bronchial Cells**
Maria Cristina Dechecchi, Valentino Bezzerri, Elena Nicolis, Anna Tamanini, Maela Tebon, Valentina Lovato, Seng H Cheng, Giulio Cabrini

P.25 **CFTR Is Important for Phagocytosis and Killing of Pseudomonas *aeruginosa* by Human Monocytes but not Neutrophils**
Pauline B. van de Weert - van Leeuwen, Jennifer Speirs, Marit van Meegen, Lodewijk A.W. Vijftigschild, Hubertus G.M. Arets, Cornelis K. van der Ent

P.26 **Characterization of Altered Inflammatory Signalling Pathways in CFTR-Defective Cells**
Florijn Dekkers, Jeffrey Beekman

P.27 **ICAM-1 Expression in Host Cells Following *Burkholderia cepacia* Complex (Bcc) Infection**
Ruth Pilkington, Siobhan Mcclean, Maire Callaghan

P.28 **Lack of RAB7 Expression and Activation Promotes Toll-Like Receptor Recycling in CF Epithelial Cells**
Bettina C. Schock, Catriona Kelly, Paul J Buchanan, J. Stuart Elborn, Madeleine Ennis

P.29 **Investigation of Putative Virulence Factors of an Emerging Cystic Fibrosis Pathogen, *Pandoraea***
Lydia Fabunmi, Siobhan McClean, Emma Caraher

P.30 **Exploiting Antimicrobial Mechanisms of Human Macrophages**
Ramona Marrapodi, Paola Del Porto, Noemi Cifani, Serena Quattrucci, Fiorentina Ascenzioni

P.31 ***Pseudomonas aeruginosa* Increase Gap Junction Channels in Calu-3 Cells by a TLR5-Dependent Mechanism**
Davide Losa, Thilo Köhler, Tecla Dudez, Christian Van Delden, Marc Chanson

P.32 **COMMD1: An Anti-Inflammatory Protein in the Context of Cystic Fibrosis?**
Abdel Aissat, Alix de Becdelievre, Loic Drevillon, Alexandre Hinzpeter, Agathe Tarze, Pascale Fanen

P.34 **Three-Dimensional Modelling of Native Mucins Using Electron Tomography**
Guy A. Hughes, David J. Thornton, Robert C. Ford

P.35 **Effects Of Azythromycin on the Regulation of Metalloproteases Released by *Pseudomonas aeruginosa* Clinical and Laboratory Strain**
Gabriella Bergamini, Baroukh M. Assael, Claudio Sorio, Paola Melotti

P.36 **Host Response to *Pseudomonas aeruginosa* Adaptation During Airway Chronic Infection**
Alessandra Bragonzi, Cristina Cigana, Nicola Ivan Lorè, Leo Eberl, Irene Bianconi,

P.37 **A European Meta-Analysis of Ancestral Haplotype 8.1 and Lung Disease Severity in Cystic Fibrosis**
Julie Beucher, Pierre-Yves Boelle, Celine Charlier, Pierre-François Busson, Annick Clement, Felix Ratjen, Hartmut Grasemann, Judith Laki, Colin Palmer, Stuart Elborn, Anil Mehta, Harriet Corvol

P.38 **Implication of the ESE Transcription Factors (Epithelium-Specific Ets-Like Factors) in *CFTR* Gene Expression**
Alexandre Hinzpeter, Alix de Becdelievre, Natacha Martin, Bruno Costes, Pascale Fanen

P.39 **Regulation of Ion Transporters and Airway Surface Dynamics by Lipoxin in Cystic Fibrosis Bronchial Epithelium**
Valerie Urbach, Mazen Al-Alawi, Valia Verriere, Richard Costello, Brian Harvey

P.40 **Molecular Determinants Underlying the Stimulatory Action of the CFTR Potentiator CBIQ on the Ca^{2+}-Activated K^+ Channel KCa3.1**
Rémy Sauvé, Line Garneau, Hélène Klein, Patricia Morales-Espinosa, Manuel Simoes, Emmanuelle Brochiero

P.41 **New Approaches of Cell Therapy for Cystic Fibrosis: *In Vitro* Differentiation of Human Amniotic Mesenchymal Stem Cells in Airway Epithelium and Correction of the CF Phenotype**
Valentina Paracchini, Annalucia Carbone, Stefano Castellani, Silvia Mazzucchelli, Federico Colombo, Manuela Seia, Carla Colombo, Massimo Conese

P.42 **Innovative Strategies for the Suppression of Fluid Hyperabsorption and the Recovery of Airways Hydration in Cystic Fibrosis**
Ambra Gianotti, Raffaella Melani, Emanuela Caci, Luis J.V. Galietta, Olga Zegarra-Moran

P.43 **Shift U1 snRNAs Targeted to an Intronic Splicing Silencer Correct Aberrant CFTR Exon 12 Skipping**
Eugenio Fernandez Alanis, Andrea Dal Mas, Franco Pagani

P.44 **Restoration of Chloride Efflux by Azithromycin in CF Human Bronchial Epithelial Cells**
Manon Ruffin, Vinciane Saint-Criq, Carine Rebeyrol, Loïc Guillot, Annick Clement, Olivier Tabary

P.45 **Targeting the "Sick" Conformation of F508del-CFTR Protein**
Norbert Odolczyk, Grzegorz Wieczorek, Danielle Tondelier, Caroline Norez, Janine Fritsch, Nathalie Servel, Patricia Melin-Heschel, Frederic Becq, Aleksander Edelman, Piotr Zielenkiewicz

P.46 **Differential pattern of microRNAs expression in Cystic Fibrosis and Normal Human Bronchial Epithelial cells**
Sabrina Noel, J. Latoche, P. Harvey, J. Pilewski, R.A. Frizzell

P.47 **Defective CFTR Expression and Function are Detectable in Blood Monocytes: Development of a New Blood Test for Cystic Fibrosis**
Claudio Sorio, Mario Buffelli, Chiara Angiari, Jan Johansson, Genny Verzè, Michele Ettorre, Laura Viviani, Baroukh Maurice Assael, Paola Melotti

AWARD WINNERS

Young Fellows Travel Award:

Alexandre Hinzpeter, FR
Ramona Marrapodi, IT

Student Helper Award:

Abdel Aissat, FR
Ambra Gianotti, IT
Stefania Monterisi, IT
Liam O'Ryan, UK
Vinciane Saint-Criq, IE
Kendra Tosoni, IT

Free Registration Young Researchers:
Supported by FFC

Eugenio Fernandez Alanis, IT
Lauretta Galeno, IT
Nicola Ivan Lorè, IT
Davide Losa, CH
Carlotta Marasini, IT

UK CF Trust Young Investigator Travel Award:

Natasha Cant, UK
Guy Hughes, UK
Tracy Rimington, UK
Helen Wallace, UK

SIFC Young Investigator Bursary

Valentina Paracchini, IT
Paolo Scudieri, IT

30 March – 18:00-19:00 **Room: Sala Ferretti**

Opening Keynote Lecture

Cellular Mechanisms Modulating the Response of Patients Carrying Nonsense Mutations to Read-through Treatments

Yifat S. Oren[1], Tamar Geiger[2], Matthias Mann[2] and Batsheva Kerem[1]

[1]Genetics Department, Life Sciences Institute, Hebrew University, Jerusalem, Israel, [2]Proteomics and Signal Transduction department, Max-Planck-Institute for Biochemistry, Martinsried, Germany.

Premature termination codons (PTCs) account for 13% of the mutations causing CF. However, in certain populations the incident of PTCs is much higher. CFTR PTCs are associated with a severe form of the disease. Hence, a great effort is made to develop therapeutic approaches for PTCs aiming to promote translational readthrough of the PTC in order to generate full length functional proteins. This possibility was first studied with the antibiotic gentamicin and more recently with ataluren (PTC124®). Both compounds promote readthrough of PTCs. Importantly; phase III clinical trials with ataluren are currently being performed in ~30 CF centers around the world. The previous readthrough studies were successful, however a variable response to the treatment was found among patients. Aiming to understand the molecular basis for this variability we have found that the CFTR transcript level, which serves as templates to the readthrough process, is a limiting factor of the response. We have further shown that the surveillance mechanism Nonsense Mediated mRNA Decay (NMD), which senses and degrades transcripts carrying PTCs, regulates the level of W1282X transcripts and by this can modulate the response to readthrough treatments.

The CFTR protein is a chloride channel located in the apical membrane of exocrine epithelial cells. The newly synthesized CFTR proteins are folded into their normal structure in the endothelial reticulum (ER). An imbalance between ER protein folding capacity and the level of proteins that cannot be folded correctly activates the unfolded protein response (UPR) that senses the stress condition and alters the transcriptional and translational cellular programs in order to resolve the protein-folding defect.

Since inefficient NMD leads to translation of truncated proteins that are not correctly folded, we hypothesize that inefficient NMD leads to accumulation of truncated proteins in the ER that activates the UPR, and that the UPR further inhibits the NMD (which is translational dependent). Hence, in addition to NMD, the UPR can also regulate the level of CFTR transcripts and proteins carrying PTCs.

In this lecture I will present results and discuss our working hypothesis that both the NMD and UPR mechanisms and the interplay between them plays an important role in modulating the response to readthrough treatment and CFTR function. Significance: our study is expected to shed a new light on the molecular basis underlying differences in disease severity, level of the CFTR proteins and variability in the response to readthrough treatments among patients carrying PTCs. Our results will enable a new screening tool for identifying patients with the potential to respond to readthrough treatments, by pre-analyzing their NMD and/or UPR efficiencies.

Thursday 31 March – 08:45-10:30 **Room: Sala Ferretti**

SYMPOSIUM 1
CFTR Structure: Indications for Modulators
Chairs: I. Braakmann (NL) / P. Thomas (US)

S1.1 *Pichia pastoris* as an expression toolbox for mammalian ABC transporters suitable for structure determination - A successful strategy for P-glycoprotein with promise for structural studies of CFTR.

Ina L. Urbatsch

Department of Cell Biology and Biochemistry and Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA.

Structural and biochemical studies of membrane proteins, especially mammalian proteins, remain hampered by inefficient production of pure protein. We have explored a codon optimization strategy based on highly expressed *Pichia pastoris* genes to enhance co-translational folding and production of P-glycoprotein (Pgp), an ABC transporter involved in multidrug resistance of cancers. Yeast expression levels of codon-optimized "Opti-Pgp" and yield of the purified protein from *P. pastoris* (~150 mg per kg cells) were about three-fold higher than for wild-type protein. Opti-Pgp conveyed full *in vivo* drug resistance against a variety of anticancer and fungicidal drugs. ATP hydrolysis by purified Opti-Pgp was enhanced by 1.2-fold over wild-type Pgp, likely due to higher purity of Opti-Pgp preparations. Analysis of purified wild-type and Opti-Pgp by CD, DSC and limited proteolysis suggested similar secondary and ternary structure. Addition of *E. coli* lipid increased the thermal stability of both proteins, and significantly increased the total unfolding enthalpy ΔH_{cal}. The increase in folded state may account for the increase in drug-stimulated ATPase activity seen with lipids. In conclusion, the significantly higher yields of protein in the native folded state, higher purity and improved function establish the value of our gene optimization approach, and provide a basis to improve production of CFTR for biophysical and biochemical studies.

S1.2 – Mis-steps in Mutant CFTR Folding

Juan Mendoza, Anna Patrick, Andre Schmidt, Andrey Karamyshev, Phil Thomas

UT Southwestern Medical Center at Dallas, Texas, USA

Cystic fibrosis (CF) is caused by a loss-of-function of the cystic fibrosis transmembrane conductance regulator (CFTR) channel. Many mutations that cause CF, including the most common disease allele F508del, interfere with CFTR function because the mutant protein does not efficiently fold into the native channel structure. Significantly, when these mutant proteins are induced to fold, in experimental systems, some CFTR function is recovered, suggesting an avenue for therapeutic development. Exploiting this opportunity requires detailed knowledge of the basic processes of CFTR folding and the relative contributions of the steps altered by the disease-causing mutations. Models of CFTR place the critical F508 residue on the surface of one of the two nucleotide binding domains (NBD1) at a predicted interface with the intracellular loop (ICL4) in the second of two transmembrane domains (TMD2). A variety of biophysical, biochemical, and cell biological studies demonstrate that CFTR folds in a hierarchical manner, with folding of the domains occurring first, during translation and, later, the partially folded domains associating to form the final, functional CFTR structure. Consistent with the location of F508 in the structural models, its deletion interferes with both the folding of NBD1 and with subsequent steps of domain-domain association. The double mutant analyses provides insight into the individual steps in folding and the impact of the F508del mutant and reveal obstacles to and suggest strategies for improving the folding efficiency of the mutant CFTR protein.

Supported by NIH-NIDDK, NIH-NIDCR, Reata Pharmaceuticals, CFF

S1.3 – Sequential Domain Folding in wild-type and F508del-CFTR

Ineke Braakman, Hanneke Hoelen, Bertrand Kleizen, Floor Peters, Mieko Otsu

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

The F508del mutation in its first nucleotide binding domain (NBD1) leads to misfolding of CFTR and its degradation by the proteasome. To study the folding process of mutant and wild-type CFTR and its domains we use 3 different techniques: i) expression of the protein in intact cells and radiolabeling with 35S-methionine and cysteine, followed by a chase in unlabeled medium (1-4); ii) in vitro translation in the presence of digitonin-permeabilized cells as a source of ER membrane (1, 2 ,4); iii) purified NBD1 domain (3). To analyze folding and conformation we subject the protein to mild protease treatment, either in a time course or in a concentration range (1,2). 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.

We found before that CFTR's domains fold already during synthesis and acquire their intrinsic protease resistance co-translationally (1). F508del-NBD1 attains its primary folding defect during synthesis as well (2). Subsequent domain assembly and changes in associating protein complexes complete the maturation process. We examine changes in the domains over time and the influence of various factors, including temperature, chaperones, and intragenic suppressor mutations.

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S1.4 Structural Features of the Nucleotide Binding Domains of the CFTR in Solution

Lauretta Galeno[1], Oscar Moran[1]

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Nucleotide binding domains (NBD1 and NBD2) of the cystic fibrosis transmembrane conductance regulator (CFTR), the defective protein in cystic fibrosis, are responsible for controlling the gating of the chloride channel, and are the putative binding site for several candidate drugs for the treatment of the disease. We have elaborated a succefull protocol to prepare recombinant NBD1 and NBD2, obtaining high purity (>98%) of samples and high of refolding yield. His-tagged proteins are produced as inclusion bodies in *E.* coli, purified by affinity and gel filtration chromatography, and refolded by three steps dialysis. After controling the refolding by fluorescence spectroscopy and circular dichroism, we studied the structural features of recombinant NBD1, NBD2 and an equimolar NBD1/NBD2 mixture in solution by small-angle X-ray scattering. The sole NBD1 or NBD2 in solution have a globular shape. Addition of ATP induce the formation of NBD1 hodimers, while no significant changes in the NBD2 occur. Converselly, the solution containing an equimolar mixture of NBD1/NBD2 spontaneously forms dimers, that become tighter in the presence of ATP. To our knowledge, this is the first direct observation of a conformational change of the NBD1/NBD2 interaction by ATP, that could be related with the CFTR chloride channel. This data may be useful to understand the physiopathology of the cystic fibrosis.

Supported by the Fondazione Ricerca Fibrosi Cistica (grant #2/2008), Mille bambini a Via Margutta - onlus, Blunotte and Lega Italiana FC - Associazione Toscana Onlus

S1.5 - Assessment of the Direct Interaction of Potentiator Molecules on CFTR in Functional Assays for Purified and Reconstituted Protein

Paul D.W. Eckford[1], Canhui Li[1], Mohabir Ramjeesingh[1], Ling Jun Huan[1], Christine E. Bear[1,2,3]

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Small molecule therapies are being developed to restore CFTR activity *in vivo*, and these treatments hold the promise of pharmacological correction of the basic defect in Cystic Fibrosis. However, our understanding of the *direct* interaction between mutant CFTR protein and these therapeutics remains limited. Single channel conductance experiments provide valuable insights but caution must be used in interpretation of these results as they are based on individual channels that may or may not faithfully recapitulate the properties and function of the whole CFTR population. Our recent design of a rapid purification and reconstitution protocol for CFTR has enabled the development of new functional assays to study the interaction of small molecules with purified protein in an unambiguous system. These tools are allowing us to study directly the channel and ATPase activities of wildtype and mutant CFTR proteins and the effects of small molecules on these activities nearly simultaneously in a purified and defined *in vitro* system. Our flux assay measures iodide release from reconstituted proteoliposomes of defined lipid and highly purified wildtype or mutant CFTR protein at concentrations as low as 1 µg/assay. This assay is highly sensitive to the quantity of protein reconstituted, the phosphorylation status of the protein, and the presence of ATP. Flux activities as high as 20 nmols/µg CFTR/s are measured. The specific CFTR inhibitor, CFTRinh-172, significantly reduces the flux activity in this system, and reduces the ATPase activity of CFTR as well. Both the Vmax and Km for ATP are reduced in CFTRinh-172 ATPase inhibition experiments, suggesting a mixed inhibition mechanism. The clinically relevant G551D-CFTR mutation results in an ATPase defective protein that has dramatically lowered flux activities in our assay compared to wildtype protein. Small molecule potentiators such as VRT-532 are known to affect the apparent affinity of CFTR for ATP in ATPase experiments and in our flux-based assay we detect a dramatic increase in the flux rate to wildtype levels upon incubation of reconstituted G551D protein with VRT-532 and other small molecules in the low µM range. The major mutant, F508del-CFTR, shows limited activity in our flux assay that is also greatly enhanced in the presence of VRT-532. We are currently investigating the requirements of mutant CFTR for phosphorylation and hydrolysable nucleotide in the presence of such potentiator molecules to better define how the molecules may interact with and increase the activity of both G551D and F508del-CFTR. We believe our assays will have great utility in the elucidation of the direct interaction of CFTR with potential small molecule therapeutics and their effects on the channel and enzymatic activities of the protein, and allow clarification of the molecular mechanism of action underlying CFTR function. This work is supported by the Canadian Cystic Fibrosis Foundation (CCFF) and the Canadian Institutes of Health Research (CIHR). P.D.W.E. was supported by a fellowship from the CCFF. We gratefully acknowledge the support of the CFFT and Dr. Robert Bridges, Rosalind Franklin University, USA, for providing modulator compounds.

Thursday 31 March – 11:00-12:45

SYMPOSIUM 2
Mutant CFTR: Search for Targets and Modulators
Chairs: C. Bear (CA) / J. Hanrahan (CA)

S2.1 – Early Folding Events of CFTR NBD1 Detected by Fret

William R. Skach, Amardeep Khushoo, Zhongying Yang, Yoshihiro Matsumura, Soo Jung Kim,

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CFTR biogenesis requires the coordinated folding and assembly of five peptide domains in three cellular compartments. F508del, disrupts one or more steps along this pathway and as a result, prevents plasma membrane expression by targeting newly synthesized CFTR to ER associated degradation. Phe508 is located in the first nucleotide-binding domain (NBD1) of CFTR where it is required for normal NBD1 folding and where it facilitates the coupling of ATP hydrolysis to transmembrane helix movement during channel gating, Unfortunately, knowledge of how F508del affects CFTR folding is limited. A major obstacle in understanding the F508del defect is that NBD1 folding begins during synthesis as the nascent polypeptide emerges from the ribosome in a complex cellular environment that is poorly amenable to traditional biophysical approaches. To overcome this, used fluorescence resonance energy transfer (FRET) to study cotranslational folding of the CFTR NBD1 domain. Out strategy is based on the principle that the distance between incorporated donor and acceptor fluorophores should decrease, and hence the FRET between them should increase, as the nascent chain transitions from an extended to a folded conformation. Donor (CFP) and acceptor (6-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino) hexonate) fluorescent probes were incorporated into nascent polypeptides synthesized in vitro from truncated mRNA templates. This approach generate uniform cohorts of nascent chains that remain tethered to the ribosome and thus reflect biogenesis intermediates at a defined stage of synthesis. Results demonstrate that NBD1 utilizes a multistep folding pathway that proceeds vectorially at the nascent chain exits the ribosome. Folding is initiated by rapid, cotranslational compaction of a small N-terminal subdomain (residues 389-~490) that forms a minimal ATP-binding site. This peptide region contains the 3-stranded ABC beta subdomain (S1-S3), the regulatory insertion (RI), Walker A motif (Helix-1), and two beta-strands from F1-type ATPase core (S4 & S6). Real-time folding kinetics following synchronized ribosome release revealed that N-terminal subdomain folding is rapid (<1 min) and occurs coincident with synthesis of the downstream alpha-subdomain. Moreover, N-terminal folding is facilitated by ATP binding. Because Phe508 is located in the alpha-helical subdomain (residue 501-564) it has not yet emerged from the ribosome when the N-terminal subdomain folds. Consistent with this F508del, has no observable effect on N-terminus folding efficiency. Studies are underway to examine the mutation's affect on alpha-subdomain folding and formation of the beta-sheet core. Interestingly, deletion of the regulatory insertion (residues 404-435) appeared to increase N-teminus folding efficiency based on increased FRET for all truncations tested. These findings suggest a revised classification for NBD subdomain organization, localize the F508del defect to a late stage of the NBD1 folding pathway, and provide an example in which a ubiquitious cellular ligand, ATP, promotes vectorial-cotranslational domain folding. (Supported by NIH and CFFTI)

S2.2 - Challenges in CF Drug Development

John W. Hanrahan[1] and David Y. Thomas[2]

[1]Department of Physiology, [2]Department of Biochemistry, [1-2]McGill University, Montreal, Quebec Canada

The development of small molecules as therapeutics for cystic fibrosis poses special challenges. Most other channels, receptors, and enzymes that have become successful drug targets have well characterized binding sites for agonists and substrates that enabled screens based on a relatively simple mechanism of action. By contrast, CFTR folding and quality control are complex, and the mechanisms leading to ER retention of ΔF508 CFTR are not well understood. There is limited structural information, and the most useful structure when developing a corrector might well be that of a folding intermediate rather than the mature protein. Moreover it is not yet clear whether CFTR is the ideal target, since rescue of the mutant could be achieved by direct binding or by interaction with other proteins that influence the folding environment. Faced with such uncertainties, we have developed cell-based, high throughput screening methods and secondary validation assays to search for CFTR correctors. In parallel, domain-based biophysical screening and systems biology approaches have been used to identify potential chemical chaperones and alternative targets, respecitively. This presentation will describe these approaches, the lessons we have learned, and prospects for the future.

S2.3 - Autophagy Inhibition as Link between Deffective CFTR and Inflammation in Cystic Fibrosis Airways.

Luigi Maiuri

European Institute for Research in Cystic Fibrosis, Laboratory for Experimental Treatment of Cystic Fibrosis, San Raffaele Scientific Institute, Milan, Italy

Autophagy is a cytoprotective mechanism for the degradation of misfolded/ polyubiquitinated proteins and damaged organelles via lysosome-mediated self digestion. Autophagy is important in clearing protein aggregates following overload of polyubiquitinated proteins. Aggregation occurs when the load of ubiquitinated proteins exceeds the capacity of proteasome degradation, as a cytoprotective mechanism to delay degradation. The accumulation of "unwanted"/misfolded protein aggregates has been described in several human pathologies, such as neurodegenerative diseases, myopathies, and cancer.
Oxidative stress, inflammation and aggresome accumulation characterize CF airways. Increased levels of ROS and Tissue Transglutaminase (TG2) drive cross-linking and secondary decrease of beclin 1 protein, a key regulator of autophagy, and result in accumulation of the LC3-binding protein p62, which induces proteasome overload and regulates aggresome formation. Beclin 1 accumulation in aggresomes leads to sequestration of phosphatidyl-inositol-3-kinase (PI3K) complex III, thus inhibiting autophagosome formation. Restoration of beclin 1 by either beclin 1 overexpression, cystamine, or antioxidant molecules rescues Endoplasmic Reticulum localization of beclin 1 interactome and autophagy in CF epithelia and reverts airway inflammation *in vitro*, in *ex vivo* cultured nasal biopsies of CF patients, and *in vivo* in $Cftr^{F508del}$ homozygous mice. These observations highlight CF as an autophagy-related disease and support a pathogenic model that linking CFTR defect to autophagy deficiency is responsible for the accumulation of proteins aggregates and lung inflammation.
They also suggest that the restoration of beclin 1 and autophagy may be a novel approach to treat CF and may pave the way for the development of a new class of drugs that by enhancing beclin 1 could be effective treatments for CF patients.

S2.4 - Cytosolic pH Effect on CFTR Activity and on Binding of Different CFTR Activators

Raffaella Melani[1], Valeria Tomati[1], Ambra Gianotti[1], Luis J.V. Galietta[1], Olga Zegarra-Moran[1]

[1]Istituto G. Gaslini, Genova, Italy

Potentiators are molecules that increase the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). At higher concentrations, some potentiators can also inhibit it. After comparing theoretical and functional affinities of several of these compounds on a molecular model of the nucleotide binding domains (NBDs) of CFTR, we proposed that the activating binding site may be located at the interface of a dimer formed by the NBDs. In the model, the binding site seems to involve cysteines and histidines protonable residues. We have hypothesized that if potentiators binding involves titratable residues forming salt bridges, then modifications of cytosolic pH (pHi) would alter the binding constant.
To test this hypothesis, we have analyzed the effect of pHi on CFTR activation and on the binding of potentiators genistein, UCCF-029 and felodipine. The study has been done measuring CFTR-mediated apical Cl^- currents on Fisher Rat Thyroid epithelia in Ussing chamber. The basolateral membrane was permeabilised with amphotericin B and pH was changed in the basolateral solution.
The effect of pHi on CFTR activation was analysed first. We found that pHi does modify CFTR maximum current, Im, and the half-activation concentration, Kd (Im = 127.7, 185.5, and 231.8 μA/cm^2; Kd = 32.7, 56.6 and 71.9 μm at pHi 6, 7.35, and 8, respectively).
The role of pHi on potentiators binding was characterized using genistein, the classical CFTR activator. We found that the genistein apparent dissociation constant for activation (Ka) increased at alkaline pHi, near cysteine pK (Ka = 1.83, 1.81 and 4.99 μm at pHi 6, 7.35, and 8, respectively), suggesting the involvement of cysteines in the binding site. Acidic pHi does not affect genistein binding.
Mutations of cysteine residues predicted to be within (Cys491) or outside (Cys1344) the potentiator-binding site indicated Cys491 as the responsible for the sensitivity of potentiator binding to alkaline pHi, since only the mutation C491A, but not C1344A, abolished the effect of pH 8 on genistein binding. This effect was confirmed also for another potentiator, UCCF-029.
In contrast, we found that the apparent dissociation constant for activation with felodipine increased at alkaline pHi for C491A as for WT-CFTR suggesting that the dihydropyridines bind to a different site in CFTR.
In conclusion our results confirm and extend previous observations about the modulatory effect of pHi on CFTR activity. Our results suggest that binding of at least some potentiators to CFTR depends on electrostatic interactions with amino acid residues of NBD1 including Cys491.

Supported by Italian Cystic Fibrosis Foundation grants FFC#2/2008 with the contribution of Mille bambini a Via Margutta onlus, Blunotte, and Lega Italiana FC - Associazione Toscana, and FFC#7/2009 with the contribution of Work in progress communication SRL and Brooks Brothers Retail Brand Alliance Europe SRL

S2.5 - Model of the cAMP Activation of Chloride Transport by CFTR Channel and the Mechanism of Potentiators

Oscar Moran

CNR, Istituto di Biofisica, Genova, Italy

The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral membrane protein that forms an ATP-gated anion channel activated by a cAMP-dependent phosphorylation. CFTR is responsible for the chloride and bicarbonate permeability on the apical membrane of most epithelia. Mutations of the gene coding for CFTR produce cystic fibrosis (CF), an autosomic recessive lethal disease. In an attempt to increase the ion transport in defective CFTR, several molecules, called potentiators, have been identified. These molecules have been proposed to be potentially useful for CF treatment on patients with class 3 and 4 mutations, and probably also for patients carrying the most common mutation, dF508, a class 2 mutations that, after been pharmacologically rescued, may present a reduced ion transport. Most research to find more effective potentiators have been done by "brute force" methods, like high throughput screening, or by studying the properties of analogues of already known putative potentiators. A more "intelligent" design of drugs has not been tried because of the lack of informations about the precise target for drugs, either in terms of a well identified binding site based on direct structural data, or by the insufficient knowledge on the precise step on the activation and gating pathways that determine the CFTR function. I have attempted to construct a model of the pathways that lead to the activation of CFTR upon an increase of intracellular cAMP. The interest to describe the system as a function of cAMP concentration is because this parameter is currently used when CFTR physiology or pharmacology is studied on cell monolayers, applying either a permeable cAMP analogue, or activating the adenylate cyclase by forskolin. I have designed the model putting together data describing several sections of the complete pathway. The model is described in steady-state conditions, as most transitions on these pathways may occur with relaxation times (between 10 μs and 10 s) faster than the time course of the anion transport with physiological meaning (>1 min), rendering irrelevant the kinetics of these events. The model of activation and gating of CFTR presented is capable to reproduce the characteristics of a potentiator. The model yields a potentiator mechanism of action that is in contrast to that proposed previously. This novel proposal, that needs, indeed, a series of experimental tests to support the model, may represent an excellent framework to plan functional experiments for the design and develop of CFTR potentiator drugs.

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Thursday 31 March – 14:30-16:15

SYMPOSIUM 3
Alternative Targets for CF Basic Defect
Chairs: H. De Jonge (NL) / K. Kunzelmann (DE)

S3.1 – Physiological Relevance of Anoctamins

Yuemin Tian, Patthara Kongsuphol, Jiraporn Ousingsawat, Ralph Witzgall, Rainer Schreiber, Karl Kunzelmann

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Ca^{2+} activated Cl^- channels of the anoctamin family (anoctamin 1, Ano1, TMEM16A) show biophysical and pharmacological properties that are typical for endogenous Ca^{2+} dependent Cl^- channels. TMEM16-proteins are expressed abundantly and an increasing number of reports demonstrate their physiological importance in epithelial as well as non-epithelial cells. These channels have also other exciting properties as they are activated by cell swelling and may therefore control cell volume, proliferation and apoptosis. TMEM16A null mice exhibit severe defects in epithelial transport along with tracheomalacia and death within one month after birth. Despite its outstanding physiological significance, the mechanisms for activation of TMEM16A remain obscure. TMEM16A is activated upon increase in intracellular Ca^{2+}, but it is unclear whether Ca^{2+} binds directly to the channel or whether additional components are required. We found recently that TMEM16A is strictly membrane localized and requires cytoskeletal interactions to be fully activated. Despite the need for cytosolic ATP for full activation, phosphorylation does not seem to be required. In contrast, the Ca^{2+} binding protein calmodulin appears indispensable and interacts physically with TMEM16A. Openers of small and intermediate conductance Ca^{2+} activated potassium channels like 1-EBIO, DCEBIO or riluzole known to interact with calmoduline, also activated TMEM16A. These results reinforce the use of these compounds for activation of electrolyte secretion in diseases such as cystic fibrosis.

S3.2 - **Regulation of ENaC by Soluble Signals in Airway Surface Liquid**

M. Jackson Stutts, Martina Gentzsch, Robert Tarran.

Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC USA

Human bronchial epithelia (HBE) employ both Na+ absorption and Cl- secretion to keep the airway mucosal surface properly hydrated for mucociliary clearance. These processes are controlled by signals embedded in airway surface liquid (ASL). When ASL height is abruptly increased, Na+ absorption is activated. As ASL height is restored, Na+ absorption slows in normal epithelia, until matched by Cl- secretion. Cystic fibrosis epithelia have impaired Cl- secretion, but also fail to slow Na+ absorption. We wish to identify the signals and conditions in ASL that maintain homeostatic control of ASL depth, and to understand why these signals are ineffective in CF. We found that Na+ absorption was stimulated by acute volume challenge. Excess aprotinin prevented ~70% of the increased Na+ absorption in CF HBE cultures, but had almost no effect in normal HBE cultures. This difference is consistent with a greater extent of ENaC proteolysis and activation that we and others have observed in CF HBE. In contrast, UTP was equi-effective in NL and CF, inhibiting up to 80% of volume induced Na+ absorption. To explore the molecular basis of homeostatic control of ASL, we identified potential soluble regulators of ENaC. We studied SPLUNC1 (Short palate, lung, and nasal epithelium clone 1), a protein secreted into ASL. Interestingly, SPLUNC1 co-immunoprecipitates with ENaC, decreases its proteolytic activation, and decreases surface expression. In ASL height studies, NL HBE cultures with knocked down SPLUNC1 expression lost the ability to restrain Na+ absorption. These results implicate SPLUNC1 as an important soluble factor in ASL that restrains Na+ absorption. Why this control is not exerted in CF HBE cultures as ASL volume decreases is under investigation. Thus, we have identified two classes of soluble mediators contributing to steady state control of Na+ absorption through restraint of ENaC. In addition, our results make it clear that the effectiveness of slowing ENaC proteolytic stimulation depends on the presence and function of CFTR. These results support multiple approaches for restoring ASL homeostasis in CF airway epithelia, including replacing CFTR functions such as bicarbonate conduction and control of ENaC proteolysis, and by direct block of ENaC.

S3.3 - Compensatory chloride channels in CF

Hugo de Jonge[1], Alice Bot[1], Marjan Wouthuyzen-Bakker[2], Jeng-Haur Chen[3], Sarah Ernst[3], Michael Welsh[3], Henkjan Verkade[2]

[1]Erasmus University Medical Center, Dept. of Gastroenterology & Hepatology, Rotterdam, The Netherlands, [2]University Medical Center Groningen, Dept. of Pediatrics, Groningen, The Netherlands, [3]University of Iowa, Dept. of Internal Medicine, Iowa City, U.S.A.

Aside CFTR corrector therapy, pharmacological stimulation of surrogate, non-CFTR chloride channels could be used as a therapeutic strategy to prevent or mitigate the CF phenotype. The ideal pseudo-CFTR channel should (i) localize to the apical membrane of CF-relevant epithelia; (ii) respond to intracellular cAMP, cGMP or Ca^{2+} signals; (iii) respond directly or indirectly to pharmacological stimuli; (iv) function properly in the absence of CFTR; (v) display a CFTR-like tissue distribution; (vi) compensate for multiple other CF abnormalities, including defective bicarbonate secretion.

Many molecular candidates became disqualified because of their intracellular or basolateral localization (ClC-2 [1]; bestrophins [2]), their dependence on functional CFTR (SLC26-A9 [3]), or their downregulation in CF (Best2 [4]; ClC-2 in the intestine). However a few candidates remained, including several members of the TMEM16/anoctamin family of Ca^{2+}-activated Cl^- channels (CaCCs), in particular TMEM16a [5]. The dominance of CaCCs in mouse airways is often used to explain why CFTR-/- mice do not develop human-like CF lung disease, and CFTR-/- and TMEM16A-/- mice in part share the same phenotype (e.g. tracheomalacia).

Interestingly, the recent phenotypic analysis of CF pig models revealed that liver and gallbladder disease including mucus plugging and prenatal microgallbladder formation is seen in 100% of the CF piglets [6]. The same phenotype occurs only in a minority of CF patients and is absent in CF mouse models. To find out whether these species differences in disease severity correspond with a different expression and activity of CaCCs, we compared the expression of TMEM16A by Q-PCR and immunostaining, and the activity of CaCC-mediated Cl^- secretion in freshly excised gallbladders from CFTR+/+, CFTR-/- and F508del/F508del CF pigs *versus* mice. Carbachol-activated, Ca^{2+}-linked Cl^- secretion measured in Ussing chambers appeared highly active in gallbladders from Cftr+/+ mice (116 $\pm$ 33 µAmp/cm^2) but was low in pig gallbladders (15$\pm$12 µAmp/cm^2). CaCC-mediated anion secretion was slightly upregulated in CF mice, resulting in wild-type levels of total (i.e. CFTR- *plus* CaCC-mediated) anion secretion. In contrast, forskolin/cAMP-activated anion secretion was similar in WT mice and pigs (85$\pm$14 and 70$\pm$9 µAmp/cm^2 respectively) and reduced by ~50% in F508del/F508del CF pigs and by ~70-80% in CFTR-/- pigs and mice. Consequently the residual total anion secretory capacity in CF pigs was 4 to 5-fold lower in comparison with CF mice. Q-PCR measurements of the TMEM16A transcript level did not show major differences between mouse and pig gall bladders, but immunostaining of TMEM16A in the apical membrane was much stronger in pigs as compared to mice, suggesting species differences in TMEM16A mRNA or protein stability.

In conclusion, the much stronger dependence of pig gallbladder on CFTR for performing Cl^- and HCO_3^- secretion may explain why a modest reduction of CFTR-mediated anion secretion in F508del/F508del CF pigs (~50%) as compared to WT pigs results in severe gallbladder pathology, not discernible from CFTR-/- pigs [7]. However final proof for a protective role of CaCCs/TMEM16A against gall bladder disease in CF awaits future pathophysiological studies in crossings of Cftr-/- and conditional TMEM16A-/- mice, or the transgenic expression of TMEM16A in CF pigs.

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[2] Kunzelmann K et al 2011 Biol Chem 392: 125-134
[3] Bertrand CA et al 2009 J Gen Physiol 33: 421-438
[4] Yu K et al 2010 J Clin Invest 120: 1722-1735
[5] Galietta LJV 2009 Biophys J 97 : 3047-3053
[6] Meyerholz DK et al 2010 Am J Pathol 176: 1377-1389
[7] Ostedgaard LS et al, manuscript submitted

S3.4 - The Anti-Inflammatory Mediator, Lipoxin A4, Increases ASL Height in Normal and Cystic Fibrosis Bronchial Epithelium

Valerie Urbach[1,2,3], Valia Verriere[2], Mazen Al-Alawi[2], Gerry Higgins[1], Raphael Chiron[3], Richard Costello[2], Brian J Harvey[2]

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Cystic fibrosis (CF) affects many organs but the progressive lung destruction is the main cause of morbidity and mortality. Mutations of the CFTR gene result in defective Cl^- secretion and Na^+ hyperabsorption. This contributes to a reduction of the airway surface liquid layer (ASL) height and impairs mucociliary clearance, thus promoting bacterial colonization and chronical inflammation. Identification of agents that promote hydration of the ASL is likely to be of therapeutic benefit to patients with CF.

Lipoxins are bioactive lipids derived from omega-6 polyunsaturated fatty acids. The lipoxin A4(LXA_4) is produced at inflammatory sites from the interaction of lipoxygenase activities of several cell types including neutrophils, platelets and epithelium. This lipid mediator is one member of the newly identify molecules playing a role in ending/resolving the inflammatory process by modulating neutrophilic inflammation, clearing apoptotic PMN and inhibiting pro-inflammatory cytokines production. The levels of LXA_4 have been reported to be decreased in the airways of patients with CF.

We have discovered a pro-secretory effect of the endogenous LXA_4 in human bronchial epithelium. Human bronchus epithelial (HBE) cell primary cultures and CF(CuFi-1) and non-CF(NuLi-1) bronchial epithelial cell lines were grown under an air-liquid interface into well-differentiated epithelia. LXA_4 (1nM) treatment significantly increased ASL height in non-CF and CF HBE. This effect was sustained over 24 hours in the CF HBE and was inhibited by Boc-2, the antagonist of the ALX/FPR2 receptor that we found to be expressed in the apical membrane of HBE. We investigated the contribution of Na^+ absorption (via ENaC) and of Cl^- secretion in the ASL height regulation by LXA_4. LXA_4 pre-treatment reduced the amiloride-sensitive short-circuit current in CuFi-1 epithelium indicating that LXA_4 inhibits ENaC activity. LXA_4 and amiloride produced additive stimulating effects on the ASL height. LXA_4 increased the whole-cell currents of non-CF and CF HBE and this effect was inhibited by BAPTA-AM (chelator of intracellular Ca^{2+}) and NPPB (non selective inhibitor of Ca^{2+}- activated Cl^- channels) but not by the CFTRinh-172 (CFTR inhibitor). Bumetanide abolished the ASL height increase induced by LXA_4. We tested the possible role of an ATP release in mediating the effect of LXA_4 on ASL height. LXA_4 stimulated an apical ATP release. Hexokinase (ATP hydrolysis) and reactive-blue-2 (P2Y purinoreceptor antagonist) had no effect on baseline ASL height and both abolished the LXA_4 effect on ASL height. LXA_4 stimulated an intracellular Ca^{2+} increase in non-CF and CF HBE and this effect was inhibited by reactive-blue-2.

Taken together, our results provide evidence for a novel effect of LXA_4 involving the FPR2 receptor in the apical membrane, luminal ATP secretion and P2Y receptor activation leading to an intracellular Ca^{2+} increase, inhibition of Na^+ absorption and stimulation of Cl^- secretion in CF and non-CF epithelia to finally increase ASL height. These novel pro-resolving effects of LXA_4 reveal a cross-talk between an endogenous anti-inflammatory mediator decrease and the ion transport defect in CF and open up a new therapeutic avenue in the treatment of CF.

S3.5 - Native Calcium-Activated Chloride Channels and their Association with TMEM16A Protein Expression

Loretta Ferrera[1], Paolo Scudieri[1], Elvira Sondo[1], Nicoletta Pedemonte[1], Emanuela Caci[1], Ifeoma Ubby[2], Franco Pagani[2], Luis J.V. Galietta[1]

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Expression of TMEM16A protein in heterologous expression systems is associated with the activity of calcium-activated chloride channels, CaCCs (Ferrera et al., Physiology 25: 357-363, 2010). However, it is not clear whether TMEM16A is the only protein responsible for CaCC activity in epithelial cells. We compared the properties of native CaCCs recorded with whole-cell patch-clamp recordings in the pancreatic cell line CFPAC-1 with those of the chloride currents arising from stable TMEM16A transfection in FRT cells.

Analysis of TMEM16A mRNA in CFPAC-1 cells revealed the prevalent expression of the (ac) isoform which corresponds to a protein of 960 amino acids lacking segment b. Segment b, coded by exon 6b, is a 22 amino acid long region that has been reported previously to affect apparent calcium affinity of TMEM16A-associated chloride currents. CaCC currents in CFPAC-1 cells have time-dependent activation at positive membrane potentials and apparent calcium affinity similar to those of FRT cells expressing the TMEM16A(ac) isoform. The half effective intracellular calcium concentration was 140 and 160 nM for CFPAC-1 and FRT cells, respectively. The kinetics of activation and deactivation of the currents, following depolarization and hyperpolarization of membrane potential, were also comparable. Furthermore, removal of ATP from the intracellular (pipette) solution abolished the CaCC currents in both cell types. This finding suggests that a phosphorylation step is required to allow the activity of the channels. We hypothesized that calcium/calmodulin-dependent kinase (CaMKII) is involved in this process. However, the CaMKII inhibitor KN-93 did not change the size and properties of CaCC currents in FRT cells. We also tested the contribution of other TMEM16 proteins to CaCC activity in CFPAC-1 cells using a functional assay based on the halide-sensitive yellow fluorescent protein. Before the assay, CFPAC-1 cells were transfected with siRNA against TMEM16 proteins (from A to K). We found that only silencing of TMEM16A caused a significant inhibition of CaCC currents.

Our findings confirm that TMEM16A protein expression in null cells is sufficient to generate chloride currents with properties and regulation similar to those of native CaCCs. However, the contribution of other types of proteins to CaCC function cannot be excluded. The study of TMEM16A/CaCC function and regulation is important to assess its relevance as a therapeutic target in cystic fibrosis.

S3.6 - Molecular Determinants Underlying the Stimulatory Action of the CFTR Potentiator CBIQ on the Ca^{2+}-Activated K^+ Channel KCa3.1

Rémy Sauvé[1], Line Garneau[1], Hélène Klein[1], Patricia Morales-Espinosa[2], Manuel Simoes[3], Emmanuelle Brochiero[4]

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Several strategies have been implemented to correct ion transport defects in cystic fibrosis (CF) through a direct action on CFTR activity and/or expression. Other approaches propose to bypass CFTR function and use, as therapeutic targets, channels other than CFTR known to be important for fluid and electrolytes transepithelial transport. There is now increasing evidence that the calcium-activated K^+ channel KCa3.1 expressed both at the apical and basolateral membranes of several epithelia is important to maintain a favorable electrochemical gradient for Cl- secretion, while being a key regulator of the epithelial cell Ca^{2+} signaling process by facilitating Ca^{2+} influx. A research aimed at identifying structural parameters by which CFTR potentiators can simultaneously activate both CFTR and KCa3.1 thus offers new perspectives to the correction of ion transport defects in CF epithelia. A study was undertaken where computer modeling, site directed mutagenesis and single channel patch clamp recordings were used to characterize the molecular determinants underlying the stimulatory action of CBIQ, a KCa3.1 potentiator also documented to activate CFTR. Experiments carried out with the constitutively active A279G-KCa3.1 mutant first confirmed that Ca^{2+} is absolutely required to the action of CBIQ. Single channel recordings next indicated that the fluctuation pattern of KCa3.1 is characterized by bursts of channel openings separated by Ca^{2+} -sensitive inter-burst silent periods, with the main effect of CBIQ being to drastically decrease the inter-burst silent periods while slightly increasing burst duration. These observations provided evidence for an action of CBIQ on the Ca^{2+} -dependence of the KCa3.1 activation process. Studies have already established that the Ca^{2+} -sensitivity of KCa3.1 is conferred by the Ca^{2+} -binding protein calmodulin (CaM) constitutively bound to a domain of the channel intracellular C-terminus. A structural model of the KCa3.1/CaM complex was generated through homology modeling, and the resulting 3D structure showed that the domains in KCa3.1 C-terminus extending from K312 to T329 and L361 to S372 were respectively involved in the constitutive binding of the CaM-C-lobe and the Ca^{2+} -dependent binding of the CaM N-lobe to KCa3.1. Binding of the CaM C-lobe and N-lobe to their respective segment on two adjacent KCa3.1 monomers resulted in turn in the formation of a dimeric structure stabilized in part by interactions between the residues E363 and R352 located at the interface of the dimeric complex. Substituted Cysteine Accessibility experiments confirmed that modulating the interactions between E363 and R352 affects the channel activity, with an increase in interactions mimicking the action of KCa3.1 potentiators such as CBIQ. The importance of these interactions to the stimulatory action of CBIQ was confirmed in patch clamp experiments where the mutation E363A was found to markedly reduce the potency of CBIQ to activate KCa3.1. These observations support docking predictions based on the Multiple Copy Simulation Search Method (CMSS) where the residues R352, E363 and L356 appeared to form a cavity suitable for CBIQ binding. Our results suggest that CBIQ activates KCa3.1 by interacting with residues in the KCa3.1 C-terminus involved in stabilizing the dimeric structure formed by the Ca^{2+} -dependent binding of CaM to KCa3.1.

Thursday 31 March – 16:45-18:15 **Room: Sala Ferretti**

SPECIAL GROUP DISCUSSION I
CFTR Structure as a Therapeutic Target
Moderators: C. Bear (CA) / W. Skach (US)

Thursday 31 March – 16:45-18:15 **Room: Sala Natali**

SPECIAL GROUP DISCUSSION II
EnaC and/or Alternative Chloride Channels as Relevant Targets for CF Treatment
Moderators: H. Cuppens (BE) / R. Frizzell (US)

Friday 01 April – 08:45-10:30 **Room: Sala Ferretti**

SYMPOSIUM 4
Understanding Epithelial Ion Transport
Chairs: R. Frizzell (US) / M. Gray (UK)

S4.1 – Orchestration of Epithelial Bicarbonate Secretion in Human Airway Serous Cells

Michael Gray

Epithelial Research Group, Institute for Cell & Molecular Biosciences, Newcastle University Medical School, Newcastle upon Tyne, NE24HH, UK

Despite the growing awareness of the importance of HCO_3^- in CF pathophysiology, our understanding of the mechanism of HCO_3^- secretion in CF-affected epithelial tissues is still not fully clear, particularly in the lung. HCO_3^- is an important component of epithelial secretions and, via its buffering role, controls the pH of the luminal microenvironment. Aberrant HCO_3^- secretion is likely to contribute to CF lung pathogenesis as efficient secretion and solubilisation of secreted macromolecules (such as mucus) is a pH-dependent process. Furthermore, abnormal pH/[HCO_3^-] may negatively impact mucus properties (hydration and viscosity), bacterial killing and ciliary beat frequency, that collectively would predispose the lungs to mucus blockage, bacterial infection and disease.

In the pancreas, small intestine and salivary glands there is good evidence that CFTR regulates HCO_3^- secretion in at least two ways: (1) directly by conducting HCO_3^- ions and (2) indirectly through regulation of members of the SLC26A family of apically-located anion exchangers. Loss of CFTR could therefore lead to aberrant HCO_3^- secretion via reduced Cl^-/HCO_3^- exchange activity, as well as through reduced HCO_3^- efflux through CFTR. We have been investigating HCO_3^- transport in the Calu-3 cell line, which are used as a model of serous cells of human submucosal glands. These cells have been shown to secrete HCO_3^- through a CFTR-dependent mechanism, but it is unknown whether SLC26A transporters play any role in this process. This talk will describe our recent molecular, biochemical and functional experiments to determine whether polarised cultures of Calu-3 cells express functional Cl^-/HCO_3^- exchangers and whether these exchangers have a role in transepithelial HCO_3^- secretion.

Supported by the BBSRC and Novartis Institutes for Biomedical Research (UK)

S4.2 - Estrogen And Lipoxin Regulation Of Ion Transport And Airway Surface Liquid Dynamics In Normal And Cystic Fibrosis Bronchial Epithelium

Brian J. Harvey[1], Mazen Al-Alawai [1,2], Valia Verriere[1], Vinciane Saint-Criq[1], Olive McCabe[1], John A. Katzenellenbogen[3], Raphaël Chiron[4], Richard W. Costello[2], Valerie Urbach [5, 6]

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Cystic fibrosis (CF) is one of the most common recessive illnesses which impacts directly on an individual's longevity. There are significant differences in the progression of CF in male and female patients termed the "CF gender gap". Lung function among female CF patients deteriorates more rapidly than in male patients and lung exacerbation is reported to vary in CF females throughout the estrous cycle. This points to an endocrine involvement in the regulation of ion transport processes that impact on CF severity. Estrogen exerts a sex-specific and estrous cycle dependent anti-secretory effect in epithelia whereas the endogenous lipoxin LXA_4 produces an opposite pro-secretory response. Recent work from our laboratory has identified the molecular targets for the differential effects of estrogen and lipoxin on airway surface liquid dynamics in normal and CF bronchial epithelium.

We have investigated the role of 17β-estradiol (E2) and the endogenous anti-inflammatory lipoxin LXA_4 in modulating Cl^- secretion and Na^+ absorption, airway surface liquid height (ASLh) and ciliary beat frequency (CBF) in CF and non-CF bronchial epithelia.

Human bronchus epithelial primary cell cultures (HBE) and bronchial epithelial cell lines CF (CuFi-1) and non-CF (NuLi-1) were grown under an air-surface liquid interface into well-differentiated epithelia. ASLh and CBF were measured using confocal fluorescence microscopy and ion transport using patch-clamp and short-circuit current techniques.

Estrogen exerted rapid, sustained anti-secretory and pro-absorptive responses in normal and CF bronchial epithelial cells. E2 as low as 0.1nM reduced the ASL height in normal unstimulated and forskolin-stimulated NuLi cells. The E2-induced reduction in ASLh occurred as a result of inhibition of bumetanide-sensitive Cl^- secretion and an increased amiloride-sensitive ENaC dependent Na^+ absorption. E2 also decreased the already low ASL height in CF HBE and CuFi epithelia and caused a reduction in the ciliary beat frequency in CF HBE. The E2 effects on CBF and ASL height were mimicked by the nuclear-excluded estrogen dendrimer conjugate EDC. E2 (1nM) increased PKCδ activity four-fold and phosphorylated the KCNQ1 channel within 30 mins to inhibit basolateral membrane K^+ currents. Both E2 and the KCNQ1-specific inhibitor chromanol 293B suppressed the cAMP-dependent K^+ conductance in NuLi cells, so demonstrating that KCNQ1 is a component of the K^+ recycling process essential for Cl^- secretion. Thus E2 causes a reduction in ASL height via a novel membrane-associated ERα which initially does not require a genotropic response but instead involves a non-genomic PKCδ /PKA signalling pathway.

Lipoxins are powerful anti-inflammatory agents and synthetic analogues are being investigated for therapeutic potential in a variety of inflammatory diseases. We have discovered a pro-secretory effect of the endogenous lipoxin LXA_4 in human bronchial epithelium. In contrast to the anti-secretory effects of estrogen, LXA_4 (1nM) increased ASLh by 47% and 103 % in normal and CF epithelia respectively ($P<0.001$, $n=18$). The stimulatory effect of LXA_4 on ASLh was sustained over 24 hours in the CF epithelia and was inhibited by the following pre-treatments: bumetanide, Boc-2 (LXA_4 receptor antagonist), reactive blue (P2Y receptor antagonist) and extracellular hexokinase (ATP hydrolysis). LXA_4 also stimulated CBF, intracellular Ca^{2+} mobilization, ATP release, Cl^- secretion and inhibited Na^+ absorption in the CF epithelia. The effect of lipoxin to enhance airway surface liquid dynamics by inhibiting Na^+ absorption and stimulating Cl^- secretion opens up a new therapeutic avenue to promote mucociliary clearance in cystic fibrosis airways.

S4.3 – CFTR and SLC26A9: Co-dependent Ion Channels

Carol A. Bertrand, Yong Liao, Joseph M. Pilewski and Raymond A. Frizzell

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Contributions to the volume and composition of airway surface liquid (ASL) come from both constitutive and stimulated anion secretion, with CFTR once considered the source of both secretory components. This conclusion stemmed from the absence of both components in CF airway, and the actions of CFTR inhibitors on constitutive anion secretion and ASL pH regulation (1). Observations that ASL pH is more acidic in CF (2), and evidence indicating that the CFTR inhibitors, CFI172 and GlyH-101, block the ability of the unstimulated epithelium to adjust surface liquid pH suggest that the loss of both the constitutive and the regulated, CFTR-dependent, secretory components contribute to the airway pathology observed in CF. Nevertheless, we have demonstrated that constitutive anion secretion in primary cultures of Human Bronchial Epithelia (HBE) from non-CF donors bears the pharmacological fingerprint of SLC26A9, an SLC26A family member expressed in surface airway epithelium (3). SLC26A9 exhibits chloride channel properties, and it is inhibited by CFTR channel blockers. In the HEK 293 cell expression system, SLC26A9 interacts with CFTR to enhance both constitutive and cAMP/PKA-stimulated chloride currents, reflecting their functional interaction. Importantly, SLC26A9 activity is absent in HBE derived from F508del CF donors, and its currents are suppressed when it is co-expressed with F508del CFTR in HEK 293 cells. The mechanism by which F508del CFTR suppresses SLC26A9 function appears result from a direct interaction between these channels. For example, co-immunoprecipitation studies in HEK 293 cells reveal that SLC26A9 interacts not only with the mature (band C) form of WT CFTR, but also with the immature (band B) form of both WT and F508del CFTR. This finding suggests that their interaction occurs at an early stage of CFTR biogenesis, and that the functional impact of F508del on SLC26A9 currents may be due to ER associated degradation of the former. In support of this idea, HEK cells co-expressing the gating mutant, G551D CFTR, display SLC26A9 currents at the plasma membrane; however, they are not enhanced by cAMP/PKA stimulation. Yet, these SLC26A9 currents may explain results obtained in patients with the G551D mutation and a second severe mutation, which show a milder clinical phenotype compared with F508del homozygous patients (4). Further support for physical and functional interactions between these channels is provided by the results of SLC26A9 knockdown experiments using adenoviral based shRNA expression in differentiated HBE, which show significant decreases in both constitutive and cAMP/PKA stimulated anion secretion. Accordingly, the presence of physiological levels of SCL26A9 currents at the plasma membrane appears to require both the presence of CFTR at this site and its regulated channel activity. [Supported by the NIH and CFF]

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S4.4 - Molecular Structure, Packing and Release of MUC2 with Relevance to Cystic Fibrosis

Harriet Nilsson[1], Daniel Ambort[2], Gunnar C Hansson[2], Hans Hebert[1,3]

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In CF extremely viscous fluid is built up in fluid transporting tissues likely connected to impaired release and expansion of mucins. O-glycosylated mucins make up the core of the mucus gel, which cover all mucosal surface of the body and play a central role in protection and hydration. MUC2 is present in small and large intestine, where it is stored as a multimer in secretory granules of goblet cells at high [Ca^{2+}] and low pH. It is the MUC2 N-terminus that controls this packing. The extracellular milieu have to trigger the unpacking, a not yet fully understood process. **The aim** is to elucidate organization and structure of MUC2 when it is packed in and secreted.

The N-terminal part of MUC2 was expressed in CHO-K1 cells. The secreted trimerized recombinant mucin was purified from culture medium by anion exchange chromatography and analyzed by transmission electron microscopy (TEM). The pH in the buffers was varied in the range from 5.2 to 8 to mimic conditions of secretory pathway and extracellular environment by adding HAc (pH 5.2), MES (pH 6.2) or Tris (pH 7.4 and pH 8) with or without calcium. Samples were adsorbed onto carbon coated EM grids and negative stained. Processing of micrographs was performed using EMAN1 software.

When pH was low at pH 6.2 and calcium present, rings with an outer and inner diameter of 25-30 and 20-25 nm respectively were observed. Without calcium rings were assembled at pH 5.2 and 6.2, but vanished with increasing pH. 2D refinements of the projections showed rotational 5- and 6- folded symmetry. When the MUC2 N-terminal rings at low pH with calcium were crosslinked and purified by density ultracentrifugation, assemblies of laterally concatenated rings were obtained in the high density fractions. The formations of these rings are probably vital for proper packing and release of full length MUC2.

Harriet N. and Daniel A. have contributed equally to this work.

S4.5 - CFTR Regulation in Human Airway Epithelial Cells Requires Integrity of the Actin Cytoskeleton and Compartmentalized cAMP and PKA Activity

Stefania Monterisi[1], Maria Favia[1], Rosa A Cardone[1], Lorenzo Guerra[1], Stephan Reshkin[1], Valeria Casavola[1], Manuela Zaccolo[2]

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ΔF508CFTR rescued to the apical membrane exhibits regulatory defects suggesting that the intracellular milieu may affect its ability to respond to cAMP regulation. We recently reported that NHERF1 overexpression in CFBE41o- (CFBE) cells rescues ΔF508CFTR functional expression, by promoting F-actin organization and formation of the NHERF1-ezrin-actin complex (1).
The hypothesis that the rescue of ΔF508CFTR activity induced by NHERF1 overexpression may involve recruitment of PKA to the membrane region was supported by immunocytochemical analysis of PKA localization demonstrating that PKA localization shifted from the cytosol to the sub-cortical region in CFBE cells stably overexpressing NHERF1 (CFBE/sNHERF1). Importantly, when we analyzed PKA activity by a FRET-based reporter targeted to the plasma membrane (2), we found that PKA-mediated phosphorylation at the plasma membrane was lower in CFBE cells than that found in CFBE/sNHERF1 cells. By contrast, using an untargeted, cytosolic version of the PKA activity FRET reporter (2), we found that PKA-dependent phosphorylation was significantly higher in the cytosol of CFBE than in CFBE/sNHERF1 cells. To assess if the higher PKA activity detected in CFBE cytosol could be due to a higher concentration of cAMP in this compartment, we measured cAMP changes in cells expressing FRET-based reporters for cAMP levels either untargeted (3) or targeted to the plasma membrane (4). In keeping with the observed difference in PKA activity, CFBE cells showed a significantly larger cAMP response in the bulk cytosol and a lower cAMP accumulation in the sub-plasma membrane compartment with respect to CFBE/sNHERF1.
A possible explanation for the observed differences in the cAMP compartmentalization in the two cell lines is that the restoration of cortical actin cytoskeletal organization induced by NHERF1 overexpression in CFBE cells (1) may contribute to the accumulation of cAMP in the sub-plasma membrane compartment by acting as a physical barrier to cAMP diffusion. In support of this hypothesis, we found that F-actin depolymerization induced by Latrunculin B, had no effect in CFBE cells while significantly increasing both cAMP accumulation and PKA activity in the cytosol of CFBE/sNHERF1 cells at the expense of their levels and activity in sub-cortical region.
Altogether these findings suggest that the organized sub-cortical cytoskeleton constitutes an efficient barrier to cAMP diffusion promoting compartmentalized cAMP/PKA signals.

This work was supported by the Italian Cystic Fibrosis Research Foundation (grant FFC#1/2009) with the contribution of the "Delegazione FFC della Valdadige"; "Nicola Petruzzi e Del FFC di Molfetta"; "Gli amici di Thomas con la Delegazione FFC di Vibo Valentia"; "Delegazione FFC "La Bottega delle Donne" di Montebelluna Treviso".

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Friday 01 April – 11:00-12:45

SYMPOSIUM 5
Inflammatory Mechanisms in CF as Therapeutic Targets
Chairs: B. Scholte (NL) / G. Cabrini (IT)

S5.1 - Modulating Chemotactic Signaling: Novel Molecular Targets

Valentino Bezzerri[1], Pio d'Adamo[2], Alessandro Rimessi[3], Carmen Lanzara[2], Sergio Crovella[2], Elena Nicolis[1], Anna Tamanini[1], Emmanouil Athanasakis[2], Maela Tebon[1], Giulia Bisoffi[1], Mitchell L. Drumm[4], Michael R. Knowles[5], Paolo Pinton[3], Paolo Gasparini[2], Giorgio Berton[6], and Giulio Cabrini[1]

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An excessive neutrophilic inflammation, mainly orchestrated by the release of IL-8 from bronchial epithelial cells and amplified by bacterial infection, leads to progressive tissue destruction in the airways of patients affected by CF. The anti-inflammatory drugs presently utilized have several limitations, indicating the need for identifying novel molecular targets, to open the way to find more effective anti-inflammatory drugs, possibly tailored to the specific pathophysiology of CF lung disease.

CF bronchial epithelial cells play a pivotal role in translating the presence of infectious agents into the release of chemokines. However, the transmembrane signal transduction machinery implicated in the recruitment of leukocytes is characterized by redundancy, as inhibition of one of the multiple receptors recognizing bacterial components, of the different kinases, adapters, phospholipases, etc., could be bypassed by alternative signaling pathways. Thus understanding the real relevance of each single component as a therapeutic target is critical. To start addressing this issue, we preliminarily studied the association of 721 single-nucleotide polymorphisms (nonsynonymous and TagSNPs) from 135 genes of the pro-inflammatory signal transduction machinery in a pilot cohort of F508del homozygous CF patients, grouped as having either severe or mild progression of lung disease, to gain insights on the relative impact on CF lung disease of candidate components of the signaling process. The top ranking association with the progression of lung disease was found for a nonsynonymous SNP of the phospholipase C beta 3 (PLCB3) gene.

Studies on the role of PLCB3 were performed in bronchial epithelial cells exposed to *P.aeruginosa in vitro*. Silencing the expression of PLCB3 reduced significantly the *P.aeruginosa*-dependent transcription and release of IL-8 in IB3-1 and in polarized CuFi-1 cells. This was associated with a parallel reduction of a) the sustained increase of cytosolic calcium, b) the activation of the classical calcium-dependent protein kinase C isoforms alpha and beta, c) the activation of NF-kB, one of the key nuclear transcription factors involved in IL-8 transcription, that were all induced by *P.aeruginosa* infection. We found that PLCB3-mediated pathway is dependent on the release of ATP in the extracellular milieu, upon interaction of *P.aeruginosa* with bronchial epithelial cells. Importantly, we found that the extracellular nucleotides/PLCB3 pathway is not sufficient to activate autonomously the transcription and release of IL-8 but acts by potentiating the Toll-like Receptors' signaling cascade. In this respect, PLCB3 can represent an interesting molecular target to attenuate the excessive recruitment of neutrophils without completely abolishing the inflammatory response.

Supported by the Italian Cystic Fibrosis Research Foundation (grants FFC # 3/2008, FFC # 8/2009, FFC # 13/2009, FFC # 18/2009, FFC # 12/2010 to P.G., G.C. and P.P.), the Italian Association for Cancer Research, Telethon (GGP09128), University of Ferrara, the Italian Ministry of Education, University and Research and the Italian Ministry of Health (to P.P.), NIH grants HL68890 and DK27651, and American Cystic Fibrosis Foundation grants DRUMM00A0, KNOWLES00A0 and RDPR026 (to M.L.D. and M.R.K.). V.B. is fellow of the Italian Cystic Fibrosis Research Foundation and E.N. of the "Azienda Ospedaliera Universitaria Integrata di Verona".

S5.2 – Inflammation, Bioactive Lipids and Mucus Production in CF Mutant Mice

Bob J Scholte[1], Martina Wilke[2], Ruvalic Buijs-Offerman[1]

Erasmus MC, Rotterdam, [1]Cell biology department, [2]Clinical genetics.

Recent developments show that the molecular signalling pathways designed for resolution of inflammation and tissue repair do not function properly in CFTR deficient tissues, also in the absence of pathogens. This offers new opportunities to define and test therapeutic targets. In this presentation we will focus on the results obtained in mouse models, in particular the Cftr^{tm1eur} F508 mouse model developed in our laboratory.

Lung inflammation was reported in various unchallenged CF mutant mouse strains, which appears to be strain-, age- sex- and environment dependent. Further, enhanced responses to LPS and bacterial challenge are reported be several authors (reviewed in Wilke et al, J Cystic fibrosis 2011). This can be at least in part attributed to abnormal behaviour of lung macrophages/monocytes with respect bacterial killing (Nelson et al) and secretion of cytokines upon challenge (Leal et al , Egan et al). The macrolide antibiotic Azithromycin reduces IL1B secretion by monocytes *in vitro* and inflammation in vivo in the Cftr^{tm1eur} F508 model (Leal et al).

In a model of acute airway injury and repair in Cftr^{tm1eur} F508 mice we observed a higher expression of IL6 and EGFR agonists in CF mutant animals one week after injury. These two signalling factors synergise in wound repair, and may play a role in abnormal airway remodelling in CF. Further, a mRNA marker of mucus producing cells Clca3/Gob5 is expressed more than tenfold higher in mutant mice after injury. This suggests an abnormal tendency of CF epithelial progenitor cells to differentiate towards a mucus secreting phenotype after injury.

In addition to abnormal cytokine signalling, a growing body of evidence shows abnormal lipid metabolism in CF mutant mice. Several of these lipids regulate inflammation, mucus production and wound healing, and therefore play an important role in CF pathology. Production of the endogenous PPARg agonist 15-keto-PGE2 was reduced in CFKO mice, and lung hyper inflammation could be corrected by the synthetic PPARg agonist rosiglitazone (Harmon et al 2010). In Cftr^{tm1eur} F508 mutant mice we previously observed a reduction of a parallel prostaglandin synthetic pathway (Cbr2), resulting in a fourfold reduction of PGF2alpha in lung lavage after LPS stimulation. Furthermore, in CFKO mice the increased arachidonic acid (AA)/docosahexaenoic acid (DHA) ratio and related inflammatory phenotype could be corrected by the retinoic acid analogue fenritinide (Radzioch et al). Abnormalities in ceramide metabolism and sensitivity to pseudomonas infections in partially CFTR deficient mice could be corrected by an inhibitor of the acid sphingomyelinase (Gulbins et al). Hyperactivity of the cPLA2 cytoplasmic phospho-lipase, a key enzyme in arachidonic acid metabolism, was observed in lung extracts of CFKO and Cftr^{tm1eur} F508 mice. This correlates with enhanced mucus production and secretion in CF mutant mice, and can be reduced by a cPLA2 inhibitor *in vivo* (Touqui et al 2010).

How CFTR deficiency causes these phenotypic abnormalities at the molecular level is still under investigation. However, the data strongly suggest that experimental therapeutics targeting inflammatory pathways and signalling lipids could be beneficial for CF patients.

S5.3 - Links between CFTR-Cl^- channel function and eicosanoid inflammation pathway in cystic fibrosis

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In cystic fibrosis (CF), abnormal transepithelial ion secretion due to dysfunctional CFTR is accompanied by chronic infection/inflammation leading to lung disease, and ultimately to respiratory insufficiency and death. A number of studies try to investigate the molecular and functional links that might exist between abnormal CFTR and inflammation. In recent years, we have initiated a global proteomic approach, pointing at the importance of the lipid mediator pathway in CF. First, by comparing protein expression in tissues from KO-CFTR and WT-CFTR mice by 2D-gel and MS analysis, we have observed that expression of annexin-1(ANXA-1) an antinflammatory protein, is significantly decreased in colon, lung and pancreas of KO mice (1). In the same tissues, expression of cytosolic phospholipase A2, a proinflammatory protein, is increased. The same analysis performed on primary cultures of nasal epithelial cells confirmed the decreased expression of ANXA-1 in F508del/F508del cells. In another study, we have observed that ANXA,cPLA2, S100A10 and CFTR form a protein macrocomplex in Calu-3 cells (*2*). We also observed a rapid and partial redistribution of all four proteins in detergent resistant membranes (DRM) induced by a pro-inflammatory cytokine, TNF-α. This was concomitant with increased lL-8 synthesis and cPLA2α activation, ultimately resulting in eicosanoid (PGE2 and LTB4) overproduction. To test if dysfunction of CFTR/Cl^- channel is linked to a proinflammatory response, we tested the effect of short exposure of cells to CFTR inhibitors Inh172 and Gly-101. Both inhibitors induced a rapid increase in eicosanoid production. These results show that (i) CFTR may form a complex with cPLA2α and ANXA1 *via* interaction with S100A10, (ii) CFTR inhibition and DRM disruption induce eicosanoid synthesis, and (iii) suggest that the putative cPLA2/ANXA1/p11/CFTR complex may participate in the modulation of TNF-α-induced eicosanoid production, underlining the importance of membrane composition and CFTR function in the regulation of inflammation mediator synthesis. Finally, in a more recent series of experiments we have observed that a secretory phospholipase may correct the trafficking of F508delCFTR in X. oocytes. All together, our studies reveal the existence of a functional link between CFTR/F508del-CFTR-Cl^- channel and the pathway leading to the synthesis and release of lipid mediators of inflammation.

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2. F. Borot, D. L. Vieu, G. Faure, J. Fritsch, J. Colas, S. Moriceau, M. Baudouin-Legros, F. Brouillard, J. Ayala-Sanmartin, L. Touqui, M. Chanson, A. Edelman, M. Ollero, Eicosanoid release is increased by membrane destabilization and CFTR inhibition in Calu-3 cells. *PLoS One* **4**, e7116 (2009).

S5.4 - Characterization of Altered Inflammatory Signalling Pathways in CFTR-Defective Cells

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and characterized by recurrent and eventually chronic pulmonary infections with *P. aeruginosa*. Many *in vitro* and *in vivo* studies suggest that this increased susceptibility to infection may partly result from cellular intrinsic alterations in inflammatory signaling pathways, as CF cells and tissues develop proinflammatory characteristics even in the absence of detectable infection. Normalization of this enhanced inflammatory state may be of great importance to reduce the CF disease severity.
We here studied CFTR-dependent alterations in expression and activation of inflammatory signaling pathways by analyzing total cell lysates of human bronchial epithelial cells expressing either wild type CFTR (HBEs) or ΔF508 CFTR (CFBEs) under sterile conditions or upon P. *aeruginosa* stimulation using Western Blot analysis.
We observed decreased expression of the NF-κB inhibitor IκB in CFBEs compared to HBEs under sterile conditions, suggesting increased activation of NF-κB that leads to proinflammatory cytokine production. IκB levels decreased to a greater extend in CFBEs compared to HBEs upon bacterial stimulation. Interestingly, we detected for the first time increased basal levels of phospho-STAT5 and phospho-STAT3 in CFBEs compared to HBEs and bacterial stimulation increased phospho-STAT3 and phospho-STAT5 levels in CFBEs but not in HBEs. Besides, preliminary data suggested increased levels of phospho-ERK, decreased levels of phospho-PKB and equal levels of phospho-p38 in CFBEs compared to HBEs under sterile conditions.
Taken together, these data suggest CFTR-dependent alterations in activation of several inflammatory signaling routes that may be important for the enhanced inflammation observed in CF. We are now further characterizing these pathways and extending these data to primary CFTR-defective cells, including CF lymphocytes, monocytes and nasal epithelial cells. These data may help to better understand the proinflammatory events observed in CF and may lead to identification of novel drug targets to correct CF-related inflammation.

S5.5 - Inhibitors of Glucosylceramide Synthase (GluCerT) Reduce the Transcription of IL-8 Induced by *P.aeruginosa* in CF Bronchial Cells

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CF individuals respond to chronic bacterial lung infections with an exaggerated immune response which destroys the lungs, leading to respiratory insufficiency. The chemokine IL-8, abundantly expressed at sites of chronic inflammation, seems to play a major role in driving the formation of a neutrophil-rich exudate into the lung of CF patients. Therefore, reduction of the exaggerated production of IL-8 is a key therapeutic target in CF. Consensus is growing on sphingolipids (SLs) as novel targets for the treatment of pulmonary disorders, including CF, since modulation of cellular ceramide reduces lung inflammation. We previously demonstrated that the imino sugar miglustat, an inhibitor of the synthesis of glycoshingolipids (GSLs), used for treating type I Gaucher disease, reduces the expression of IL-8 gene (Dechecchi, 2008), induced by *P.aeruginosa* infection in human bronchial epithelial cells. Miglustat inhibits the first step in GSL synthesis which is the transfer of glucose to ceramide by the GluCerT (Platt, 1994). In addition, the galactose analogue N-butyldeoxygalactonojirimycin (NB-DGJ), also inhibitor of GluCerT, produces an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). Therefore we extended the investigation to the effect of the pharmacological modulation of GluCerT on the inflammatory response to *P.aeruginosa*, by inhibiting its function with a different, structurally unrelated compound, Genz-123346 (Zhao, 2009). CF bronchial epithelial IB3-1 cells were treated with the inhibitor, at doses ranging from 1 nM to 50 microM for 24 hours, before infection with *P.aeruginosa*. In these experimental conditions no toxicity was observed. Genz-123346 significantly reduces the expression of IL-8 mRNA by about 50%, starting from 100 nM. Interestingly, no effect on the inflammatory response was found when the cells were treated with the compound, 4 hours before infection. Therefore Genz-123346 exerts an inhibitory effect very similar to that previously observed with miglustat and NB-DGJ. The parallel effects obtained with Genz-123346 and the structurally unrelated compounds, the imino sugars miglustat and NB-DGJ, suggest that they could be mediated by the inhibition of the activity of GluCerT, one of the common molecular targets of these compounds, although their effect on additional molecular targets can not be excluded. Our results further strengthen the hypothesis that the pharmacological modulation of SL metabolism, that can intercept the ceramide metabolic pathway at many levels, may be an effective approach for the treatment of CF lung inflammation.

Supported by: Italian Cystic Fibrosis Research Foundation (#16/2010) with the contribution of "*Assistgroup*", "*Latteria Montello SpA*" and "*Delegazione FFC di Imola*".

S5.6 - COMMD1: An Anti-Inflammatory Protein in the Context of Cystic Fibrosis?

Abdel Aissat[1,2], Alix de Becdelievre[1,2,3], Loic Drevillon[1,3], Alexandre Hinzpeter[1,2], Agathe Tarze[1,2], Pascale Fanen[1,2,3]

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Cystic fibrosis (CF) is a severe disease caused by mutations in the *CFTR* gene, which encodes an epithelial anion channel. Morbidity is mainly due to the lung disease, characterized by a chronic neutrophilic inflammation. Deregulation of inflammatory pathway is observed in the airways of CF patients, as evidenced by an increased NF-κB response. Consequently, pro-inflammatory cytokines such as IL-8 are increased.
We have previously identified COMMD1 as a new CFTR partner and shown that CFTR was protected from ubiquitination by COMMD1, which sustains CFTR expression at the plasma membrane. COMMD1 is a protein associated with multiple cellular pathways, including sodium uptake through interaction with ENaC (epithelial sodium channel) and NF-κB signalling.
We studied the influence of COMMD1 overexpression/extinction on NF-κB pathway in CF and non-CF bronchial epithelial cells (IB3-1 and S9 respectively) as well as in stably transfected F508del- or wild-type-CFTR HeLa cells. To decipher the underlying mechanisms, we have first performed a set of luciferase assays in order to test NF-κB transcriptional activity in the presence or absence of each protein. TNFα or p65/p50 stimulation produced a higher increase of NF-κB activity in CF versus non CF cells. Overexpression of COMMD1 in the same experimental conditions decreased NF-κB activity by half in both cell lines. As IL-8 promoter contains NF-κB responsive elements, we performed such experiments with the wild-type and mutant IL-8 promoter constructs.
Our results show for the first time the anti-inflammatory properties of COMMD1 in bronchial cells. Thus, increasing COMMD1 expression may provide an approach to simultaneously inhibit ENaC absorption, enhance CFTR trafficking and inhibit NF-κB pathway, three major issues in cystic fibrosis.

This work was supported by public grants from Institut National de la Santé et de la Recherche Médicale (INSERM) and Chancellerie des Universités de Paris, and the French Association "Vaincre la Mucoviscidose".

Friday 01 April – 18:00-19:35

SYMPOSIUM 6
Host Pathogen Interactions in Cystic Fibrosis
Chairs: M. Surette (CA) / M. Knowles (US)

S6.1 - The Cystic Fibrosis Airway Microbiome: Polymicrobial Interactions and Overlooked Pathogens

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The microbiome of the respiratory tract, including the nasopharyngeal and oropharyngeal microbiota, is a dynamic community of microorganisms as diverse as the gastrointestinal microbiome. Cystic Fibrosis (CF) is a disease in which complex dynamic microbial communities colonize the airways of patients. The CF airway microbiome (the microbiota present in the lower airways of these patients) is comprised of chronic opportunistic pathogens (such as *Pseudomonas aeruginosa*) and a variety of organisms derived mostly from the microbiota of the upper respiratory tract. Traditionally, only a small number of bacterial pathogens have been associated with airway disease in CF, including *Pseudomonas aeruginosa, Staphylococcus aureus* and the *Burkholderia cepacia* complex. Surprisingly, the population dynamics of these microbes do not readily account for the occurrence of most pulmonary exacerbations: in many patients the level of these principal pathogens is the same before, at the onset, during and at the resolution of pulmonary exacerbations. Can a more comprehensive view of the CF airway microbiome inform us about disease? We hypothesized that at least some pulmonary exacerbations arise from 1) pathogens overlooked by conventional CF microbiology, and 2) the interaction of multiple species (polymicrobial infections). Our findings have validated these hypotheses. We observe that the *Streptococcus milleri* group (SMG) contributes to almost half the pulmonary exacerbations and hospitalizations in our adult CF patient population. The SMG are not detected by standard clinical microbiology and consequently easily overlooked in CF. These results have lead directly to changes in treatment and antibiotic therapy directed towards management of SMG has improved outcome for these patients. To address the second hypothesis, we have used simple infection models to examine the interaction of 40 oropharyngeal isolates individually with *Pseudomonas aeruginosa* in pair wise infections. This study revealed that a large proportion of the organisms in CF airways have the ability to be somewhat pathogenic on their own. Most surprising was that about 1/3 of the organisms examined in this model were able to enhance the pathogenicity of the principal CF pathogen *P. aeruginosa* in mixed infections while being avirulent on their own. These **synergens** (avirulent organisms that enhance the virulence of a pathogen) may be a common feature of polymicrobial infections. Making sense of the disease process and host response in chronic polymicrobial infections like CF is often complicated by incomplete microbiology. However, *patient specific* CF airway microbiome profiling may be used guide complementary therapies to current treatments.

S6.2 - The basic link between lack of CFTR, mucin secretion and the sticky mucus in CF and relation to bacterial overgrowth

Gunnar C. Hansson, Jenny K. Gustafsson, Daniel Ambort, Anna Ermund, Harriet E. Nilsson, Kaisa Thorell, Hans Hebert, Henrik Sjövall, and Malin E.V. Johansson

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Programme note: Abstract details are not authorized for inclusion

S6.3 Effects of Azythromycin on the Regulation of Metalloproteases Released by *Pseudomonas aeruginosa* Clinical and Laboratory Strain

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Modulation of *Pseudomonas aeruginosa* virulence factors was suggested as mechanism for azithromycin (AZM) beneficial effects in CF patients particularly susceptible to chronic *Pseudomonas aeruginosa* (Pa) infection in the airways. Our work was aimed to study the regulation of metalloproteases (MMPs), known for their strong proteolytic activities in cells and tissues, released by Pa strains after AZM treatment.
The functional assay such as zymography revealed that the members of the metalloprotease family of enzymes were poorly expressed by the laboratory strain PAO1 and clinical strain AA43 (collected 7.5 years after Pa acquisition). The AA2 clinical strain collected at the onset of chronic colonization (0.5 year) appeared to express a much larger set of proteases and decreased their expression after exposure to AZM. Western blot analysis for alkaline metalloprotease (AprA), one of the metalloproteases detected in the conditioned medium (CM) from PAO1 and clinical strains in our previous study, demonstrated a diminished release of this enzyme in CM from AA2 strain cultured in presence of AZM.
Given that higher expression of MMPs was detected in AA2 strain, in comparison with AA43, we have evaluated MMPs activity and AprA expression in the clinical isolates derived from different CF patients featuring sporadic or chronic colonization. Pa strains from about 400 CF patients followed at the Cystic Fibrosis Center of Verona were available for this study.
We have evaluated MMP activity in CM derived from 49 isolates defined as chronic and 44 classified as sporadic. In 32 of the sporadic strains we detected MMP activity (73%) while this was true only for 17 of 49 (34%) among the chronic strains ($p < 0.0001$). We then evaluated whether MMP activity was associated to AprA expression in a subset of these strains. This association was not detected in all the strains positive for MMP activity indicating the presence of other MMPs as the major source of proteolytic activity in selected strains.
We then evaluated the effect of AZM in the same series of Pa strains isolated from CF patients. Within this context we have observed a decreased MMP activity in CM derived from 22 defined as sporadic isolates of 27 strains analized (81%), cultured in presence of AZM. We also studied whether MMP activity was affected by AZM treatment in CM from other 16 isolates classified as chronic. AZM had effect only on 5 chronic strains (31%).
Finally, the correlation reported among MMP activity/expression and specific clinical conditions suggest that MMPs might play a role in the clinical manifestations of Pa infection supporting a link among MMP expression/activity and Pa virulence in CF patients. Future evaluation of MMP activity in a larger series of clinical isolates may provide insights on the correlation between this parameter and lung function in patients colonized by Pa strains. Moreover the analysis of CM derived from the Pa clinical isolates grown in absence and in presence of AZM is provided and can contribute to a better explaining the variable response to Pa infection and sensitivity to AZM known to occur in CF patients.

S6.4 Investigation of Putative Virulence Factors of an Emerging Cystic Fibrosis Pathogen, *Pandoraea*

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Chronic bacteria infection and inflammation of the lung are the main cause of morbidity and mortality in patients with Cystic Fibrosis (CF). Typical CF pathogens include *Pseudomonas aeruginosa, Staphylococcus aureus* and *Burkholderia cepacia* which have all been studied extensively, however very little is known about the clinical impact of the new genus, *Pandoraea*. *Pandoraea* species are Gram negative, glucose non-fermenting rods that are capable of causing severe lung infection and in some instances death of CF patients. Little is published to date about the virulent characteristics of *Pandoraea*. This study examined a number of virulence factors that might enhance the pathogenesis of *Pandoraea* in the CF lung.

Previously, we have shown that *Pandoraea* isolates were capable of inducing the production of pro-inflammatory cytokines, IL-6, IL-8 and TNF-α in two human bronchial epithelial cell lines; HBE and CFBE cells (which are CFTR $^{+/+}$ and CFTR $^{-/-}$) with heat treatment having a significant impact on these cytokines production. In this study, the stimulation of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) in a phorbol myristate acetate (PMA) differentiated macrophage cell line; U937 in response to *Pandoraea isolates* (bacterial cell, cell free and heat treated cells) was examined. All the *Pandoraea* isolates triggered the release of these cytokines in U937 cells which demonstrated that macrophages secrete cytokines very effectively in response to exogenous stimulation. This can serve as a key in understanding the importance of macrophage in bacteria infection.

A range of *Pandoraea* isolates were also screened for other potential virulence factors, specifically proteases, lipase, siderophore production and haemolytic activity. In this study, *Pandoraea* isolates were screened for proteolytic activity and the results demonstrated that they expressed serine proteases as their dominant protease. All the *Pandoraea* isolates examined produced high levels of lipase activity and siderophore production thus indicating these could be potential virulence factors for *Pandoraea*. However the results obtained for haemolytic activity demonstrated that only four of seven isolates screened were haemolytic suggesting that haemolytic activity may not be an important virulence for some of the *Pandoraea* isolates. Further investigation of the involvement of these factors in CF pathogenesis is warranted as this will provide us with a comprehensive understanding of the bioactions of *Pandoraea*, allowing for the development of targeted and more effective therapies to treat patients.

This project is funded under HEA-PRTL1 Cycle 4 and the EU Regional Fund.

S6.5 - ICAM-1 Expression in Host Cells Following *Burkholderia cepacia* Complex (Bcc) Infection

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Burkholderia cepacia complex (Bcc) is a group of Gram-negative opportunistic pathogens that colonise the lungs of patients with cystic fibrosis (CF). These pathogens are extremely difficult to eradicate and their mechanisms of pathogenesis are poorly understood. To date, 17 species of Bcc have been identified. The two most virulent are *B. multivorans* and *B. cenocepacia*. Currently, *B. multivorans* is the most commonly identified new acquisition in both Europe and North America. Bcc are inherently resistant to antimicrobial agents, promote a potent inflammatory response in the lung and are capable of disrupting epithelial integrity which results in penetration through lung tissue causing septicaemia. This study aims to establish if Bcc infection is associated with an increase in the expression of the neutrophil ligand intercellular adhesion molecule (ICAM-1). The expression levels of ICAM-1 in response to both *B. multivorans* and *B. cenocepacia* infection in epithelial and endothelial cell lines were determined.

ICAM-1, which is expressed on multiple cell types throughout the body, plays an important role in the migration of leukocytes to the site of infection. ICAM-1 is a major ligand for leukocyte function associated with lymphocyte function associated antigen-1 (LFA-1) and macrophage-1 (Mac-1).

ICAM-1 expression was investigated in both epithelial and endothelial cells in response to either Bcc or LPS exposure using confocal microscopy. ICAM-1 expression levels were compared on CFTR positive (16HBE14o-), CFTR negative (CFBE41o-) lung epithelial cells and umbilical cord endothelial cells (Huvec-cs). The cells were co-cultured with a *B. multivorans* strain LMG13010 and a *B. cenocepacia* strain J2315 for 48 hours and the effect on ICAM-1 determined. ICAM-1 expression was elevated in response to *B. multivorans* LMG13010 and *B. cenocepacia* BC7 in both CFBE41o- and 16HBE14o- cells. There was no significant difference in ICAM-1 levels in CFTR positive and CFTR negative cells. However, the endothelial cells demonstrated a more rapid response to Bcc infection with ICAM-1 levels at 12 hours comparable to that on epithelial cells after 48 hours bacterial exposure.

In order to determine whether the bacterial endotoxin, lipopolysaccharide (LPS) was a contributor to the ICAM-1 response, epithelial and endothelial cells were exposed to purified *B. multivorans* LMG13010 LPS. A significant up-regulation of ICAM-1 expression was observed in the presence of LPS compared to control cells and was comparable to levels induced by intact bacterial cells. As with bacterial exposure, there was no significant difference between ICAM levels in CFTR positive and CFTR negative cells. Furthermore, endothelial cells were sensitive to 10 times less LPS than the epithelial cells, indicating their potential to respond rapidly to the initial stages of Bcc colonization by facilitating an influx of immune cells to the lung.

This host response whilst initially beneficial, is ultimately deleterious to the host in infections which are persistent and associated with limited bacterial clearance by these cells. Therapeutic targets aimed at regulating this response will have the potential to limit the lung damage caused by the trafficking of immune cells to the CF lung.

SYMPOSIUM 7
Lessons from Animal Models
Chairs: J. Engelhardt (US) / J. Wine (US)

S7.1 - Fishing for new regulators of CFTR and fluid secretion

Michel Bagnat

Department of Cell Biology, Duke University

Transport of chloride through the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) channel is a key step in regulating fluid secretion in vertebrates. Loss of CFTR function leads to cystic fibrosis, a disease that affects the lungs, pancreas, liver, intestine and vas deferens. Conversely, un-controlled activation of the channel leads to increased fluid secretion and plays a major role in several diseases and conditions including secretory diarrheas and Polycystic Kidney Disease. Understanding how CFTR activity is regulated in vivo has been limited by the lack of a genetic model. Recently, using forward genetics in zebrafish we identified a mutation in the cse1l gene that leads to the sudden and dramatic expansion of the gut tube. We showed that this phenotype results from a rapid accumulation of fluid due to the un-controlled activation of the CFTR channel. Analyses in zebrafish embryos and mammalian cells indicate that Cse1l is a negative regulator of CFTR-dependent fluid secretion. This work prompted us to carry out a new zebrafish screen to identify more regulators of CFTR and fluid secretion. We have thus far identified 25 mutations casing excessive fluid accumulation in the gut (12), ear (2) and kidney (11). In addition, we are also using the zebrafish model to study the role of CFTR and fluid secretion in organ development and function.
These approaches provide a new genetic model system for studying the regulation of the CFTR channel and fluid secretion.

S7.2 – Lung disease and Diabetes in CFTR-knockout ferrets

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S7.3 – The *ΔF508* Mutation Causes CFTR Misprocessing and Cystic Fibrosis-Like Disease in Pigs

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In vitro expression CFTR-ΔF508, the most common CF-associated mutation, results in a protein that is misprocessed. The *in vivo* consequences of this mutation in humans remain uncertain. To better understand the effects of the Δ*F508* mutation *in vivo*, we produced *CFTR*$^{\Delta F508/\Delta F508}$ pigs. Our biochemical, immunocytochemical and electrophysiological data on CFTR-ΔF508 in newborn pigs paralleled our *in vitro* findings. CFTR-ΔF508 protein was partially processed in both the airway and the intestine of newborn *CFTR*$^{\Delta F508/\Delta F508}$ pigs. In addition, we isolated less CFTR-ΔF508 than wild-type protein and detected lower levels in both the airway and the intestine by immunocytochemistry. These data suggest that studies of recombinant CFTR-ΔF508 misprocessing predict *in vivo* behavior, which validates its use in biochemical and drug discovery experiments. We also found that *CFTR*$^{\Delta F508/\Delta F508}$ airway epithelia retain a small residual CFTR conductance, with maximal stimulation producing ~6% of wild-type function. Interestingly, cAMP agonists were less potent at stimulating current in *CFTR*Δ*F508*/Δ*F508* epithelia, suggesting that quantitative tests of maximal anion current may overestimate transport under physiological conditions. Despite residual CFTR function, older *CFTR*Δ*F508*/Δ*F508* pigs developed lung disease strikingly similar to human CF. These results suggest that this limited CFTR activity is insufficient to prevent lung or gastrointestinal disease in CF pigs. These findings help elucidate the molecular pathogenesis of the common CF mutation and will guide strategies for developing new therapeutics.

S7.4 – Tracheal Smooth Muscle Abnormalities in the CF Mouse

Helen L Wallace[1], Marilyn G Connell[2], Susan Wray[1], Kevin Southern[2], Theodor Burdyga[1]

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Introduction: Recent data point towards changes in tracheal cartilage structure and altered airway smooth muscle (ASM) exacerbating respiratory dysfunction in cystic fibrosis (CF). To date, no clear role of altered contraction has been established and the possible mechanisms involved remain unclear. We performed functional smooth muscle studies and morphological analysis to determine the extent and implications of ASM abnormalities in the CF mouse trachea.

Methods: Smooth muscle function was measured using tracheal rings isolated from 6 week old heterozygous (+/-) and homozygous (-/-) *cftr*^{tm1Cam} MF1/129 mice. Contractile responses to the agonist carbachol (CCh 50µm) in Ca free solution were measured and normalised to a 40 second application of 120 mM high K^+ solution (100%). The role of Rho-kinase in contraction was determined by measuring changes in force after incubating rings with Rho-kinase inhibitor H-1152 for 15 minutes. For morphological analysis, whole tracheas were fixed in 4% paraformaldehyde, embedded in 5% gelatine and snap frozen. 7µm cryosections were stained with haematoxylin and eosin and labelled with anti-actin, α-smooth muscle-Cy3 monoclonal antibody. Sections were observed and photographed under a fluorescent microscope.

Results: The amplitude of contraction in response to CCh was significantly decreased in CF mice compared to controls (89.6±5% (*cftr* -/-) n=6, 118.5±5% (*cftr* +/-) n=6, p< 0.01). The duration of contraction after K^+ depolarisation was significantly decreased in CF mice after incubation with H-1152 compared to controls (30.2±6.3s shorter in *cftr -/-,*10.7± 5.4s, (*cftr* +/-) p< 0.01). Cross sectional areas of trachea were 25.2% smaller (p< 0.01) and less circular than controls (0.73±0.02 (*cftr* -/-) 0.84±0.008 (*cftr* +/-) p< 0.01). Trachea lumens tended to be smaller when normalised to cross sectional area (41.3%±3.6 (*cftr* -/-), 49.5±1.6 (*cftr* +/-)) and the area of epithelium lining the lumen was increased by 10% in CF tracheas, but these changes were not significant. Smooth muscle area was reduced in CF tracheas; however when normalised to whole trachea area, there was no significant difference between genotypes (2.88±0.18% (*cftr* -/-),3.25±0.3% (*cftr* +/-).

Conclusion: These data indicate a loss in contractile capacity of the mouse CF trachea in Ca free conditions. This suggests a reduction in the release of Ca from the sarcoplasmic reticulum leading to a decrease in force. Data also suggest that the role of Ca dependent activation of Rho kinase in contraction may be elevated in the mouse CF airway. Morphological analysis showed that the reduction in ASM contractility is not due to a reduction in smooth muscle area, but is a consequence of altered ASM cell function.

S7.5 - Host Response to *Pseudomonas aeruginosa* Adaptation During Airway Chronic Infection

Cristina Cigana[1], Nicola Ivan Lorè[1], Leo Eberl[2], Irene Bianconi[1], Alessandra Bragonzi[1]

[1]Scientific Institute San Raffaele, Infections and Cystic Fibrosis, Milano, Italy, [2]University of Zurich, Department of Microbiology, Zurich, Switzerland

Pathogen recognition and induction of immune responses are important for efficient elimination of infection. However, life-threatening chronic infections are maintained by bacterial patho-adaptive variants that employ strategies to evade or modulate these defences. In cystic fibrosis (CF), the host response to *P. aeruginosa* patho-adaptive variants remains to be established.

The most effective strategy for hijacking genes involved in innate immune responses involves steric shielding or modification of exposed molecules. Comparing lipid A and peptidoglycan (PGN) of sequential strains isolated from CF patients, including mucoid and non-mucoid phenotypes isolated during a period of up to 7.5 years, we found for the first time specific structural modifications temporally associated with CF lung infection (Cigana et al, PlosOne 2009). Both bacterial structures (LPS and PGN) and whole cell bacteria of early and late clinical strains had different potencies when activating host innate immunity *in vitro*. TNF-α and IL-8 protein release was significantly decreased in epithelial bronchial cells of CF origin IB3-1 and their wt-like isogenic cells C38 and macrophage-like cells (THP-1) after treatment with late strains in comparison to early strain. Similar results were obtained with *P. aeruginosa* mutants defective in several virulence factors (Bianconi et al, PlosPathogen 2011). *P. aeruginosa* mutants stimulated less the immune response when compared to their wt strain. However, microarray analysis showed that late strains are prone to revise their interaction with host by activating pathways relevant for damage and remodelling process. Increased cytotoxicity and matrix metalloprotease-9 expression were observed after infection with late strains compared to early strains.

Next, we analysed the host response to *P. aeruginosa* in a multihost pathogenesis system including four different models, namely, *Caenorhabditis elegans, Galleria melonella, Drosophila melanogaster* and mouse. *P. aeruginosa* strains at the onset of infection are more lethal than late isolates from the same patient when tested in *C. elegans, Galleria melonella* and *Drosophila melanogaster*. In murine model of acute infection, the early *P. aeruginosa* strain induced higher mortality than late clonal strains. Although attenuated in mortality, *P. aeruginosa* late isolates retained their capacity to persist when embedded in agar beads in models of chronic infection. Histological examination, PAS-staining and Tunnel assay of lung tissue sections showed that early strains induced pronounced leukocytes recruitment indicating strong inflammatory response while late strains increased numbers of mucin-positive goblet cells and apoptotic cells, a typical hallmark of damage in the airway chronic diseases.

Our findings suggest that during long-term infection *P. aeruginosa* revises its interaction with CF host by activating alternative pathways including evasion of the immune response, non-inflammatory cell death and those relevant for tissue damage and remodelling process to ultimately result in chronic disease and decline in lung functions.

Supported by the European Union 7th Framework Programme (project NABATIVI) and Italian Cystic Fibrosis Research Foundation.

Saturday 02 April – 11:00-13:00

Symposium 8
Insights in the CF Modulating Gene Products
Chairs: H. Cuppens (BE) / M. Amaral (PT)

S8.1 – CFTR Traffic: Friends and Foes

Margarida D. Amaral

University of Lisboa, Faculty of Sciences, BioFIG - Centre for Biodiversity, Functional and Integrative Genomics

The information of complete genome sequences and the identification and systematic cloning of human cDNAs provide the challenging opportunity to analyse the complexity of biological processes on a large scale. However, these techniques have limitations, not least of which is their lack of demonstrating a functional involvement of the molecules identified.
Recent advances in automated fluorescence scanning microscopy and image processing allow the application of complete genome knowledge in large-scale screening applications with so far unmatched functional information at the single cell or sub-cellular level. Indeed, in combination with genome-wide/ high-content (HC) small-interference RNA (siRNA) or cDNA over-expression strategies, such microscopy-based applications hold promise to help revealing comprehensively the regulatory networks underlying several cellular processes, such as membrane traffic, in intact cells [1].
The application of cutting-edge microscopy-based screening technology to study traffic and function of two CF-related membrane proteins by siRNA approach, namely CFTR and ENaC will be described. For CFTR, novel constructs (wt- and F508del) were generated, namely bearing both a N-terminus fused fluorescent tag (mCherry) and a Flag epitope tag located at CFTR 4th extracellular loop. These two tags allow determining on each individual cell the fraction of expressed CFTR which is residing in the cell membrane [2]. For ENaC, a functional live-cell assay was selected, based on the activity of ENaC as sodium channel and using the FLIPR membrane potential (FMP) voltage-sensitive fluorescent (blue) dye in combination with the specific ENaC-blocker amiloride [2].
Data will be presented from ongoing work regarding the application of these assays in the context of high-content siRNA screens to identify genes/proteins affecting the traffic of CFTR and function ENaC. In these screens, three high-content libraries of human siRNAs (Ambion) are used, namely: 1) the "kinome" library, targeting 710 different kinase genes; 2) the "secretome" library, targeting 1552 genes involved in the trafficking of a temperature sensitive membrane transport marker, tsO45G; and 3) and the "druggable" targeting different 5940 genes of relatively well known function.

[1] Erfle H et al (2007) Nat Protoc 2: 392-399
[2] Almaça et al (2011) Methods Mol Biol, in press.

S8.2 - CF Lung Disease Modifier at the Chromosome 11p13 Locus: Update on the Potential Mechanism of Genetic Variation

MICHAEL KNOWLES, M.D

REPORTING FOR THE CF GENE MODIFIER CONSORTIUM
CYSTIC FIBROSIS/PULMONARY RESEARCH AND TREATMENT CENTER, THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL, CHAPEL HILL, NC, USA

Programme note: Abstract details are not authorized for inclusion

S8.3 - Variation in the *MSRA* Gene Decreases Risk of Neonatal Intestinal Obstruction in Cystic Fibrosis

Lindsay B. Henderson[1], Vishal K. Doshi[1], Scott M. Blackman[1], Kathleen M. Naughton[1], Rhonda G. Pace[2], Jackob Moskovitz[3], Michael R. Knowles[2], Peter R. Durie[4,5] Mitchell L. Drumm[6] and Garry R. Cutting[1]

[1]Johns Hopkins University, [2]Baltimore; University of North Carolina, Chapel Hill; University of Kansas, [4]Hospital for Sick Kids and [5]University of Toronto, [6]Case Western Reserve University, Cleveland.

Cystic fibrosis (CF) is a monogenic disease with considerable phenotypic variability. About 15% of newborns with CF suffer from an intestinal obstruction called meconium ileus (MI) and studies in twins with CF have shown that modifier genes play a substantial role in the development of this complication. While MI has served as a paradigm for the discovery of modifier genes in Mendelian disorders, replicating identified loci has proven to be a challenge. Here, we performed family-based association analysis in a region of the genome that had previously been linked to MI and found that variation in the *MSRA* gene, represented by a combination of SNPs, or haplotype, was protective against MI. The association was replicated in two independent CF populations. Furthermore, as intestinal obstruction is the primary cause of mortality in CF mice, we generated a murine model to evaluate the biological significance of this gene in the disease context of CF. Survival was dramatically improved in CF mice with *MsrA* null alleles compared to those with wild type *MsrA* ($p=1.3x10^{-4}$) due to decreased rates of fatal intestinal obstruction, consistent with the protective modifier effect observed in humans. The *MSRA* gene encodes an antioxidant that preserves the activity of a variety of proteins, including those that regulate intestinal proteolysis. Modulation of MSRA may play a role in normalizing the protein content of meconium in the developing intestine of the CF fetus, thereby reducing the incidence of obstruction in the newborn period. The identification of protective modifiers of MI such as *MSRA* may offer insight into the development of therapies for this life-threatening complication of CF.

S8.4 - First Results of the Genome Wide French CF Modifier Study

Harriet Corvol[1,2,3], Pierre-Yves Boelle[3,4], Jean-François Vibert[3,4], Safa Saker[5], Diana Zelenika[6], Annick Clement[1,2,3] on the behalf of the French CF Modifier Gene Consortium

[1]Inserm U938, A., CDR St Antoine,[2]Pediatric Pulmonology Department, Hôpital Trousseau, Paris,[3]UPMC-Paris VI,[4]Inserm U707, Biostatistics department, Hôpital St Antoine, Paris,[5]Genethon DNA and cell bank, Evry,[6]Centre National de Genotypage, Evry

Programme note: Abstract details are not authorized for inclusion

S8.5 - Trafficking Of CFTR Is Unconventionally Regulated By Key Components Of The COPII Complex

Meritxell B Cutrona[1,2], Seetharaman Parashuraman[1], Oliviano Martella[2], Ramanath N Hegde[1], Anastasia Egorova[1], Alberto Luini[1]

[1]TIGEM - Telethon Institute of Genetics and Medicine, Naples, Italy, [2]Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

Cystic Fibrosis is a genetic disease caused by mutations in the Cystic Fibrosis transmembrane conductance regulator (CFTR) gene. Most of the disease alleles correspond to a deletion mutation, resulting in the loss of phenylalanine at position 508 of the protein (F508del-CFTR). This leads to the misfolding and eventual intracellular retention of CFTR in the endoplasmic reticulum (ER) since the protein cannot pass the ER quality control (ERQC) to enter the conventional secretory pathway. When leaving the ER, the wild type CFTR (wt-CFTR) can follow an unconventional secretory pathway to reach the plasma membrane bypassing the Golgi Apparatus[1,2,3]. Here, our aim was to map the secretory route of the wt-CFTR protein at both biochemical and morpho-functional level.

To investigate the trafficking route of CFTR we developed an RNAi-based approach for manipulation of specific trafficking steps while investigating the consequences on CFTR progression through the secretory pathway. As a starting point, we focused on early steps of the conventional protein secretion. With the known role of the COPII complex in mediating transfer of protein cargo from the endoplasmic reticulum to the Golgi apparatus[4], and specifically, because of the dependence of CFTR protein on COPII function for leaving the ER[2,5], we selected clue components of this machinery as RNAi targets to breakdown the conventional ER-export function.

We established the conditions to obtain the efficient depletion of the mammalian protein isoforms corresponding to the sar1 and sec23 yeast homologues. Accordingly, we found that Sar1-depletion decreased the number of cells where wt-CFTR could reach the Plasma Membrane (PM), while Sec23-depletion supported PM localisation of CFTR. Indeed, the knockdown of Sec23 promoted arrival of the F508del-CFTR to the PM. The complex glycosylation of the CFTR protein was consistently decreased, suggesting a possible unconventional trafficking under COPII perturbation.

Although, the exact mechanism for both the unconventional trafficking and the non-canonical ER-exiting of CFTR is being currently investigated and characterised at ultrastructural level, we raise a plausible scenario where the Sec23 proteins exert a repressive role of an unknown route for CFTR trafficking that may depend on Sar1 function. Therefore, the manipulation of ER-boundary decisions could be a promising resource for the design of therapeutic strategies amenable to correct the basic defect of F508del-CFTR.

1. Bannykh SI. et al, 2000. Traffic pattern of cystic fibrosis transmembrane regulator through the early exocytic pathway. Traffic 1:852-870.
2. Yoo JS. et al, 2002. Non-conventional trafficking of the cystic fibrosis transmembrane conductance regulator through the early secretory pathway. J Biol Chem. 277: 11401-9.
3. Marie M. et al, 2009. The function of the intermediate compartment in pre-Golgi trafficking involves its stable connection with the centrosome. Mol Biol Cell 20: 4458-70.
4. Kuge O. et al, 1994. Sar1 promotes vesicle budding from the endoplasmic reticulum but not golgi compartments. JCB 125: 51-65

Wang X. et al, 2004. COPII-dependent export of cystic fibrosis transmembrane conductance regulator from the ER uses a di-acidic exit code. J Cell Biol 167:65-74.

S8.6 - Proteomic Identification of Calumenin as a G551D - CFTR Associated Protein

Pascal Trouvé[1,2], Ling Teng[1,2], Nathalie Benz[1,2], Mathieu Kerbiriou[1,2], Mehdi Taiya[2], Olivier Mignen[1,2], Claude Ferec[1,2,3]

[1]Inserm, UMR613, Brest, France, [2]Universite de Bretagne Occidentale, Brest, France, [3]Etablissement Francais du Sang, Brest, France

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. To date, over 1700 mutations have been identified in the CFTR gene. Among these mutations, the CF-causing missense mutation G551D-CFTR (approx. 5% of cases) exhibits normal expression on the cell surface but it is associated with severe disease due to its altered channel activation. The aim of the present study was to identify specific interacting proteins of G551D-CFTR. Membrane proteins from Wt-CFTR and G551D-CFTR expressing cells were extracted and resolved by 2D-gel electrophoresis (2-DE). Mass Spectrometry revealed that calumenin was only present in the proteins extracted from G551D-CFTR expressing cells. Co-immunoprecipitations showed that calumenin is linked to Wt- and to G551D-CFTR. The amount of bound calumenin was higher in G551D-CFTR protein complex than in wt-CFTR complex. Nevertheless, its basal expression was not modified in G551D-CFTR expressing cells when compared to Wt-CFTR. Calumenin associated proteins were resolved by 2-DE and the spots were analyzed by MS. The results indicated that calumenin is bound to chaperons, cytoskeleton proteins and Grp78/Bip which is involved in the Unfolded Protein Response (UPR). Therefore, we showed a calumenin-CFTR interaction and we suggest that calumenin maybe involved in the G551D-CFTR maturation and trafficking pathway. Finally, we suggest that UPR may be triggered independently of the retention of G551D-CFTR in the ER.

Saturday 02 April – 14:30-16:00 **Room: Sala Ferretti**

SPECIAL GROUP DISCUSSION – III
CFTR Post Translational Modifications
Moderators: A. Metha (UK) / J. Hanrahan (CA)

Saturday 02 April – 14:30-16:00 **Room: Sala Natali**

SPECIAL GROUP DISCUSSION – IV
CF Animal Models
Moderators: L. Galietta (IT) / J. Engelhardt (US)

Saturday 2 April – 16:30-18:15

SYMPOSIUM 9
Clinical Trials and Therapeutic Strategies
Chairs: L. Galietta (IT) / M. Wilschanski (IL)

S9.1 - Ex vivo surrogate assays to assess efficacy of ion channel-targeted CF drugs

Nico Derichs

Charité University Berlin, CF Centre, Pediatric Pulmonology
CFTR Biomarker Centre, Berlin/Germany

Modulation of ion transport is an attractive strategy for treatment of CF, which gives promise to a clinical benefit for the patients. Several ion-transport modulators are in pre-clinical development or clinical trials, including CFTR potentiators and correctors, ENaC inhibitors, and activators of alternative chloride channels, such as calcium-activated chloride channels (CaCCs). Of great importance in translational CF research is the development of reliable pre-clinical assays to predict the *in vivo* human efficacy of ion-channel targeted CF drugs.

In this study we develop and optimize several pre-clinical *ex vivo* surrogate assays of airway and intestinal epithelial cell function. The assays include the measurement and modulation of airway surface liquid (ASL) depth and viscosity, the fluorimetric determination of chloride secretion from airway epithelial cells and the use of native rectal biopsies for assessment of the efficacy of ion-channel-targeted CF drugs, including ΔF508-CFTR correctors.

Having good pre-clinical assays to prioritize ion channel-modulating CF drugs is important in streamlining the CF drug pipeline in selecting the very best drug candidates to move forward in clinical trials.

S9.2 - Rescue of Mutant CFTR: Current Shortcomings and Future Strategies in Drug

Martin Mense

Cystic Fibrosis Foundation Therapeutics, Bethesda, Maryland, USA

Functional rescue of mutant CFTR has been a drug discovery goal for at least the past 13 years. To a very large degree the focus has been on synthetic small molecules. To date, this effort led to the discovery of two drug candidates (VX-770 and VX-809) by Vertex Pharmaceuticals and one molecule (Ataluren) by PTC Therapeutics. All are currently in clinical trials. Just recently Vertex reported the very promising data for the potentiator VX-770 from a Phase III pivotal.

The discovery starting point at Vertex and also for a number of other efforts aimed at identifying modulators of mutant CFTR were cell-based assays with a phenotypic, functional read-out. To date these assays largely have relied upon non-human cell lines that heterologously express disease-causing mutations of human CFTR. Before entering clinical development CF modulators are usually expected to show activity in the current gold standard assay, short-circuit current measurements from primary CF human bronchial epithelial (CF hBE) cells. This discovery paradigm has been validated by the success of the potentiator VX-770, and other corrector drug candidates including VX-809 were discovered in the same fashion.

A small but statistically significant decrease in sweat chloride in CF patients homozygous for CFTR F508del has been reported for VX-809, demonstrating proof of concept that the F508del CFTR can be partially rescued by a small molecule in human subjects. However, the low level of clinical activity suggests that VX-809 is not likely to yield the same clinical efficacy as VX-770 in the relatively short time frame of a Phase 2 clinical trial. Prior in vitro studies from Vertex suggest that VX-809 and VX-770 in combination confer improved efficacy in F508del hBE cells; nevertheless, more efficacious corrector molecules remain very desirable.

In recognizing this need CF Foundation Therapeutics (CFFT) conducted a strategic planning process to identify how technological advances, new assays, and knowledge learned in the past decade can be utilized for the discovery of second generation CFTR correctors. Key recommendations from the strategic planning include: (1) functional screening will ideally be conducted in primary CF hBE cells at an early stage because the majority of active compounds identified in other screening cell lines fail to show activity in the CF hBE cells. For engineered screening cells lines a human background is desirable. (2) A preferred screening paradigm should rely on two different assays, a functional screening assay and a mechanistic or biochemical assay. Ideally, the latter assay would be a cell-free assay that increases the likelihood of finding molecules directly interacting with CFTR. (3) Evaluation of multiple concentrations of test compound in the primary screening assays could offer additional information and prevent certain molecules from being overlooked due to dose dependent effects.

The presentation will go over the recommendations and second generation efforts and provide a rationale and several approaches for mechanistic or biochemical assays. Also discussed will be the possibility of reaching the efficacy goal by a combination treatment that includes at least two correctors with different mechanisms of action.

S9.3 Innovative Strategies for the Suppression of Fluid Hyperabsorption and the Recovery of Airways Hydration in Cystic Fibrosis

Ambra Gianotti[1], Raffaella Melani[1], Emanuela Caci[1], Luis J.V. Galietta[1], Olga Zegarra-Moran[1]

[1]Istituto G. Gaslini, Genova, Italy

The most serious problem experienced by CF patients is the airway disease, characterized by increased viscosity of mucus with subsequent infection and colonization of the lungs. These conditions are all consequences of airway dehydration. Na^+ absorption and Cl^- secretion are carefully regulated to keep an appropriate airway surface fluid (ASF) volume (7-10 µm) and ensure mucociliary clearance. In CF, the equilibrium between these two processes is disrupted because of the failure of Cl^- transport across the airway epithelium due to CFTR mutations. In this condition, Na^+ and water absorption became predominant leading to airway dehydration. Reduction of epithelial Na^+ channel (ENaC)-mediated sodium absorption, and Cl^- secretion stimulation may improve this situation. We recently found that short interfering RNA (siRNA) against ENaC reduces transepithelial Na^+ currents and fluid absorption in human bronchial epithelia, representing proof of principle that ENaC knockdown may have functional consequences in CF patients. Yet, knockdown was partial, siRNA doses were too high (with the risk of producing off-target effects), and other fluid absorption pathways were not explored. Now we aim to face all these points. In addition, we aim to establish the relative contribution to Na^+ absorption of ENaC and amiloride-insensitive channels.

To this purpose, we have measured the transepithelial Na^+ transport in polarized preparations of H441 cells and on primary human bronchial epithelial cells (HBEC). In spite of lacking of a significant expression of CFTR, H441 cells exhibit robust amiloride-sensitive currents. Therefore, were considered a good model of the bronchial epithelium. To further support this conclusion we have compared the expression of the three ENaC subunits on both cell types with the qRT-PCR. We found that the relative subunit abundance was similar for H441 and HBEC. On both cells, the α subunit resulted markedly more expressed than β, and γ expression was very low. Next, we have studied the transepithelial Na^+ transport in Ussing chamber. Dose-response relationships to amiloride showed that Na^+ current inhibition had to be fitted with two dissociation constants. The first, near 1 µM, is tipically of classical ENaC. The second, about 50 µM, may represent the so called Amiloride Insensitive Currents (AIC). The relative expression of these currents seem to be modulated by cAMP.

In order to knockdown ENaC, we have tested different concentrations of siRNA on H441 cells to find out the lowest effective dose. Silencing was evaluated measuring the short circuit current blocked by amiloride and the best result was obtained with 20 nM. Next, we have used this dose on HBEC and confirmed the reduction of ENaC-mediated currents. To evaluate the functional effects of ENaC silencing, we have also measured the height of ASF with a confocal microscope using Texas red-dextran to color the liquid phase.

S9.4 - Restoration of Chloride Efflux by Azithromycin in CF Human Bronchial Epithelial Cells

Manon Ruffin[1], Vinciane Saint-Criq[1], Carine Rebeyrol[1], Loïc Guillot[1], Annick Clement[1], Olivier Tabary[1]

[1]Inserm UMR S 938, Paris, France

Background: Cystic fibrosis (CF) is a hereditary disease caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene that encodes a chloride (Cl^-) channel. CF pulmonary pathophysiology is characterised by chronic inflammation and bacterial infections. Azithromycin (AZM), a macrolide antibiotic, has shown promising anti-inflammatory properties in inflammatory pulmonary diseases. Moreover, all clinical studies have presented an improvement of the respiratory condition of CF patients, but the molecular and cellular mechanisms remain unknown. The aim of this study was to investigate, in bronchial epithelial cells, the mechanism by which AZM has beneficial effects in CF patients.

Methodology / Principal findings: We demonstrate that AZM does not have an anti-inflammatory effect on the CF human bronchial epithelial cells nor on CFTR-inhibited primary human bronchial glandular cells. Contrary to what was observed in non-CF cells, our data show no effect of AZM on IL-1β- or TNF-α-induced IL-8 secretion and NF-κB pathway. Activation of the NF-κB pathway was investigated by luciferase assay, western blotting, and by Förster Resonance Energy Transfer imaging, allowing the detection of the interaction between the transcription factor and its inhibitor in live cells (Saint-Criq, Biochemical And Biophysical Research Communications, Submitted).
More, we have also analysed the effects of AZM on Cl^- efflux using a specific Cl^- probe.
The effect of AZM on Cl^- efflux using a specific Cl^- probe have also been analyzed and demonstrate that AZM treatment induced a restoration of Cl^- efflux in CF bronchial epithelial cells (Saint-Criq V., Antimicrob Agents Chemother, In press).

Conclusions / Significance: Taken together, these data suggest that AZM could improve CF patients pulmonary pattern by acting on Cl^- efflux rather than by modulating inflammatory parameters.

S9.5 - Differential pattern of microRNAs expression in Cystic Fibrosis and Normal Human Bronchial Epithelial cells

Sabrina Noel[1,3], J. Latoche[2], P. Harvey[2], J. Pilewski[2], R.A. Frizzell[1]

[1]Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh PA, USA
[2]Division of Pulmonary Allergy and Critical Care medicine, UPMC, Montefiore Hospital, Pittsburgh, PA, USA
[3]Galapagos NV, General de Wittelaan L11A3, 2800 Mechelen, Belgium (current affiliation)

Cystic Fibrosis (CF) is due to mutation in the chloride channel CFTR gene causing impairment of chloride secretion in the apical membrane of epithelial cells. The most common CFTR mutation in CF is the deletion of the phenylalanine at the position 508 of the protein (DF508), leading to the retention of the mutant protein within the ER and its rapid degradation via ERAD pathway. More than 1800 mutations have been identified so far, variably affecting CFTR activity, but none of them have been clearly linked to a certain phenotype. In other words, even if carrying the same genotype, CF patients might face a different development of their disease. Clearly, CF disease also depends on other genetic and/or environmental factors. Lately, a number of studies focused on modifier genes that may determine severity of lung disease, as MBL and TGF-B. Recently, Wright et al. performed whole-genome microarrays assays of nasal cells from non-CF individuals, mild CF and DF508-CFTR homozygous patients and found a total of 652 out of 1187 genes differentially expressed in these three groups (1).
MicroRNAs (miRNAs) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. First described in 1993, miRNAs are non-coding RNA ; instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a premiRNA and finally into a functional miRNA. Mature miRNA molecules are partially complementary to one or more mRNA molecules, and thus down-regulate gene expression by inducing mRNA degradation or repression of protein expression by translation inhibition. Differential expression in miRNAs has been shown to influence disease development in Alzheimer's disease, cancer, heart failure and COPD. We performed microarray analysis of miRNAs expression using primary bronchial epithelial cells from three different donors (DF/DF) and from three non-CF donors. Among the 856 miRNAs identified in the human genome at the time we performed the study, 94 were expressed in HBE cells, with 16 of them differentially expressed between CF and non CF patients:

- miR22, miR29a, miR29c, miR30a, miR30b, miR30c, miR30d, miR30e, miR151-3p, miR151-5p were upregulated in CF-HBEs;
- miR103, miR107, miR146a, miR203, miR224, miR1246 were down-regulated in CF-HBEs.

Real-time PCR experiments partially confirmed microarrays results, finding significant differences in expression of 11 of the 16 miRNAs. Further target analysis of the differentially expressed mi-RNAs is expected to identify new therapeutic targets in CF.

Supported by the Cystic Fibrosis Fundation

1. Wright JM, Merlo CA, Reynolds JB, Zeitlin PL, Garcia JG, Guggino WB, Boyle MP (2006). Respiratory epithelial gene expression in patients with mild and severe cystic fibrosis lung disease. Am J Respir Cell Mol Biol. 35(3):327-36.

S9.6 - Small Molecules in the Treatment of CF

M. Wilschanski

Pediatric Gastroenterology Unit and CF Center, Hadassah University Hospitals, Jerusalem, Israel

Since the discovery of the gene that causes Cystic Fibrosis, our knowledge of how mutations in this gene cause the varied pathophysiological manifestations of this disease has increased substantially.
This knowledge has led to the possibility of new therapeutic approaches aimed at the basic defect. Apart from gene therapy, several novel compounds have recently been discovered using High Throughput Screening which appear promising enough to develop effective drugs to cure the basic defect.
This presentation will summarise our current knowledge of mutation specific therapy and will focus on orally bioavailable potentiators and correctors. There will be special reference to possible treatments for the G551D mutation which is in Class III and particularly suppressors of premature termination codons (Class 1) including preclinical model systems and clinical trials in CF.
There will be discussion on the manipulation of ionic conductances to restore Airway Surface Liquid which may be applicable to all CF mutations.

Saturday 2 April – 18:30-19:30

Closing Lecture

Submucosal Gland Secretion in Four Species with CF

Jeffrey J. Wine

Cystic Fibrosis Research Laboratory
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United States

Airway submucosal glands are prevalent in the nasal cavity and larger airways where they secrete most of the antimicrobial rich mucus that protects the airways. Gland serous cells express CFTR. Because people with CF die mainly from airway infections, and because thick, sticky mucus is a hallmark of CF, we hypothesize that defective mucus secretion from airway glands contributes in two ways to the defective mucosal host defence of CF airways: the mucus is more difficult to clear via mucociliary clearance and cough, and its antimicrobial/antiprotease functions are diminished because of inadequate dispersal (reduced bioavailability) of the >100 mucosal host defence molecules secreted by normal glands. Gland mucus secretion has now been studied from individual airway glands in all species with CF: humans, mice, pigs and ferrets. There are large species and regional differences in gland responses to neurotransmitters. However, all species fail to secrete fluid in response to agents that elevate $[cAMP]_i$; in humans solids secretion continues. All species continue to secrete fluid to agents that elevate Ca^{2+}, but the volume is diminished except in mice tracheal glands, where CFTR is a minor component, and in human glands at transplant, which become enlarged over the course of the disease so that their size compensates for their diminished cellular secretory capacity. However, the mucus from these glands has higher solids and is thicker and more acidic. The absence of glands in lower airways and in tracheas of some species has raised questions about their role in innate defense. However, all mammals have abundant glands in their nasal cavities, which first encounter inspired air during normal breathing. In CF humans and CF pigs the nasal turbinate glands also show defects in fluid secretion. Quinton has proposed that deficient secretion of bicarbonate is the principal reason that CF mucus is abnormal, and available information suggests that this holds also for glands—with much work remaining to be done. A major unanswered question regarding airway glands is the nature of their neural control. They are richly innervated by local neurons in the airway wall that release multiple transmitters including acetylcholine, vasoactive intestinal peptide, Substance P, NO, and other compounds, as well as by collaterals from sensory neurons that release Substance P and stimulate the glands via axon reflex. The available evidence, although very slight, suggests that these neurons are always active *in vivo* and hence that the glands always maintain some level of secretion, with increased secretion produced by provocations. There is no evidence that airway gland cells hyper-secrete mucus as a direct consequence of CFTR mutations—rather the opposite. The abundant, purulent sputum that characterizes chronically infected CF airways appears to result from mucus accumulation, secondary to altered mucus properties and increases in the numbers of mucus-producing cells in both the surface epithelium (goblet cells) and within the glands. (The glands themselves do not increase in number). Although the evidence suggests an important role for glands in airway health, they are only one component of a multi-tiered mucosal defence system.

Room: Salone Bruschi

Posters – CFTR Structure and Function

P. 1

Structural Features of the Nucleotide Binding Domains of the CFTR in Solution

Lauretta Galeno[1], Oscar Moran[1]

[1]National Council of Research, Biophysical Institute, Genova, Italy

Nucleotide binding domains (NBD1 and NBD2) of the cystic fibrosis transmembrane conductance regulator (CFTR), the defective protein in cystic fibrosis, are responsible for controlling the gating of the chloride channel, and are the putative binding site for several candidate drugs for the treatment of the disease. We have elaborated a succefull protocol to prepare recombinant NBD1 and NBD2, obtaining high purity (>98%) of samples and high of refolding yield. His-tagged proteins are produced as inclusion bodies in *E.* coli, purified by affinity and gel filtration chromatography, and refolded by three steps dialysis. After controling the refolding by fluorescence spectroscopy and circular dichroism, we studied the structural features of recombinant NBD1, NBD2 and an equimolar NBD1/NBD2 mixture in solution by small-angle X-ray scattering. The sole NBD1 or NBD2 in solution have a globular shape. Addition of ATP induce the formation of NBD1 hodimers, while no significant changes in the NBD2 occur. Conversely, the solution containing an equimolar mixture of NBD1/NBD2 spontaneously forms dimers, that become tighter in the presence of ATP. To our knowledge, this is the first direct observation of a conformational change of the NBD1/NBD2 interaction by ATP, that could be related with the CFTR chloride channel. This data may be useful to understand the physiopathology of the cystic fibrosis.

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Assessment of the Direct Interaction of Potentiator Molecules on CFTR in Functional Assays for Purified and Reconstituted Protein

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Small molecule therapies are being developed to restore CFTR activity *in vivo*, and these treatments hold the promise of pharmacological correction of the basic defect in Cystic Fibrosis. However, our understanding of the *direct* interaction between mutant CFTR protein and these therapeutics remains limited. Single channel conductance experiments provide valuable insights but caution must be used in interpretation of these results as they are based on individual channels that may or may not faithfully recapitulate the properties and function of the whole CFTR population. Our recent design of a rapid purification and reconstitution protocol for CFTR has enabled the development of new functional assays to study the interaction of small molecules with purified protein in an unambiguous system. These tools are allowing us to study directly the channel and ATPase activities of wildtype and mutant CFTR proteins and the effects of small molecules on these activities nearly simultaneously in a purified and defined *in vitro* system. Our flux assay measures iodide release from reconstituted proteoliposomes of defined lipid and highly purified wildtype or mutant CFTR protein at concentrations as low as 1 µg/assay. This assay is highly sensitive to the quantity of protein reconstituted, the phosphorylation status of the protein, and the presence of ATP. Flux activities as high as 20 nmols/µg CFTR/s are measured. The specific CFTR inhibitor, CFTRinh-172, significantly reduces the flux activity in this system, and reduces the ATPase activity of CFTR as well. Both the Vmax and Km for ATP are reduced in CFTRinh-172 ATPase inhibition experiments, suggesting a mixed inhibition mechanism. The clinically relevant G551D-CFTR mutation results in an ATPase defective protein that has dramatically lowered flux activities in our assay compared to wildtype protein. Small molecule potentiators such as VRT-532 are known to affect the apparent affinity of CFTR for ATP in ATPase experiments and in our flux-based assay we detect a dramatic increase in the flux rate to wildtype levels upon incubation of reconstituted G551D protein with VRT-532 and other small molecules in the low µM range. The major mutant, F508del-CFTR, shows limited activity in our flux assay that is also greatly enhanced in the presence of VRT-532. We are currently investigating the requirements of mutant CFTR for phosphorylation and hydrolysable nucleotide in the presence of such potentiator molecules to better define how the molecules may interact with and increase the activity of both G551D and F508del-CFTR. We believe our assays will have great utility in the elucidation of the direct interaction of CFTR with potential small molecule therapeutics and their effects on the channel and enzymatic activities of the protein, and allow clarification of the molecular mechanism of action underlying CFTR function. This work is supported by the Canadian Cystic Fibrosis Foundation (CCFF) and the Canadian Institutes of Health Research (CIHR). P.D.W.E. was supported by a fellowship from the CCFF. We gratefully acknowledge the support of the CFFT and Dr. Robert Bridges, Rosalind Franklin University, USA, for providing modulator compounds.

P. 3

Model of the cAMP Activation of Chloride Transport by CFTR Channel and the Mechanism of Potentiators

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral membrane protein that forms an ATP-gated anion channel activated by a cAMP-dependent phosphorylation. CFTR is responsible for the chloride and bicarbonate permeability on the apical membrane of most epithelia. Mutations of the gene coding for CFTR produce cystic fibrosis (CF), an autosomic recessive lethal disease. In an attempt to increase the ion transport in defective CFTR, several molecules, called potentiators, have been identified. These molecules have been proposed to be potentially useful for CF treatment on patients with class 3 and 4 mutations, and probably also for patients carrying the most common mutation, dF508, a class 2 mutations that, after been pharmacologically rescued, may present a reduced ion transport. Most research to find more effective potentiators have been done by "brute force" methods, like high throughput screening, or by studying the properties of analogues of already known putative potentiators. A more "intelligent" design of drugs has not been tried because of the lack of informations about the precise target for drugs, either in terms of a well identified binding site based on direct structural data, or by the insufficient knowledge on the precise step on the activation and gating pathways that determine the CFTR function. I have attempted to construct a model of the pathways that lead to the activation of CFTR upon an increase of intracellular cAMP. The interest to describe the system as a function of cAMP concentration is because this parameter is currently used when CFTR physiology or pharmacology is studied on cell monolayers, applying either a permeable cAMP analogue, or activating the adenylate cyclase by forskolin. I have designed the model putting together data describing several sections of the complete pathway. The model is described in steady-state conditions, as most transitions on these pathways may occur with relaxation times (between 10 μs and 10 s) faster than the time course of the anion transport with physiological meaning (>1 min), rendering irrelevant the kinetics of these events. The model of activation and gating of CFTR presented is capable to reproduce the characteristics of a potentiator. The model yields a potentiator mechanism of action that is in contrast to that proposed previously. This novel proposal, that needs, indeed, a series of experimental tests to support the model, may represent an excellent framework to plan functional experiments for the design and develop of CFTR potentiator drugs.

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P. 4

Cytosolic pH Effect on CFTR Activity and on Binding of Different CFTR Activators

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Potentiators are molecules that increase the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). At higher concentrations, some potentiators can also inhibit it. After comparing theoretical and functional affinities of several of these compounds on a molecular model of the nucleotide binding domains (NBDs) of CFTR, we proposed that the activating binding site may be located at the interface of a dimer formed by the NBDs. In the model, the binding site seems to involve cysteines and histidines protonable residues. We have hypothesized that if potentiators binding involves titratable residues forming salt bridges, then modifications of cytosolic pH (pHi) would alter the binding constant.
To test this hypothesis, we have analyzed the effect of pHi on CFTR activation and on the binding of potentiators genistein, UCCF-029 and felodipine. The study has been done measuring CFTR-mediated apical Cl^- currents on Fisher Rat Thyroid epithelia in Ussing chamber. The basolateral membrane was permeabilised with amphotericin B and pH was changed in the basolateral solution.
The effect of pHi on CFTR activation was analysed first. We found that pHi does modify CFTR maximum current, Im, and the half-activation concentration, Kd (Im = 127.7, 185.5, and 231.8 μA/cm^2; Kd = 32.7, 56.6 and 71.9 μm at pHi 6, 7.35, and 8, respectively).
The role of pHi on potentiators binding was characterized using genistein, the classical CFTR activator. We found that the genistein apparent dissociation constant for activation (Ka) increased at alkaline pHi, near cysteine pK (Ka = 1.83, 1.81 and 4.99 μm at pHi 6, 7.35, and 8, respectively), suggesting the involvement of cysteines in the binding site. Acidic pHi does not affect genistein binding.
Mutations of cysteine residues predicted to be within (Cys491) or outside (Cys1344) the potentiator-binding site indicated Cys491 as the responsible for the sensitivity of potentiator binding to alkaline pHi, since only the mutation C491A, but not C1344A, abolished the effect of pH 8 on genistein binding. This effect was confirmed also for another potentiator, UCCF-029.
In contrast, we found that the apparent dissociation constant for activation with felodipine increased at alkaline pHi for C491A as for WT-CFTR suggesting that the dihydropyridines bind to a different site in CFTR.
In conclusion our results confirm and extend previous observations about the modulatory effect of pHi on CFTR activity. Our results suggest that binding of at least some potentiators to CFTR depends on electrostatic interactions with amino acid residues of NBD1 including Cys491.

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P. 5

Structural Biology of ABC Transporters of Medical and Pharmacological Interest

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Cystic fibrosis transmembrane conductance regulator (CFTR) is a transmembrane protein that functions as an ion channel. Mutations in this protein cause Cystic Fibrosis. For this reason, it is important to study the structure and function of CFTR. In this study, constructs of CFTR (C- and N-terminii) were cloned, expressed and purified for structural and functional purposes. The purified C-terminus of CFTR was subjected to crystallization trials using a sitting drop vapour diffusion method. The N-terminus of CFTR was expressed in *E.coli* , but no protein was obtained. This may be a result of inclusion body formation. In order to examine the NHERF - CFTR-Cterminus interaction, NHERF PDZ1 (Na+/H+ Exchanger Regulatory Factor) was cloned into two new plasmid vectors (pET-52b and pET-28a). The aim of this Project is to co-express this construct with pET-24a 6-His tagged CFTR1441-1480 C-terminus and attempt to co-purify them together. Preliminary data using MALDI-TOF mass spectrometry show that the two proteins form a very tight complex, and hint at a possible means of regulation of their interaction via phosphorylation. As for the co-crystallization of the two proteins using sitting drop vapor diffusion method, the proteins were mixed with low proteins concentration (as a result of co-expression them into the same E.coli cells) but no crystals were formed. The second part of this project includes the same steps explained previously with one difference that the proteins were expressed separately to manage the concentration of proteins. Site-directed mutation of the C-terminus of CFTR was performed in order to examine the effect of removing a potentially flexible amino acid (Arginine) on protein crystallization. Full-length CFTR has been employed for reconstitution experiments with lipids.

P. 6

Expression and Purification of Full-Length CFTR from *Saccharomyces cerevisiae*

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The expression and purification of the cystic fibrosis conductance regulator (CFTR) in large quantities remains a major obstacle in its biochemical and structural characterisation. Yeast are commonly used as a heterologous overexpression system for such problematic eukaryotic membrane proteins. We aim to produce milligram quantities of pure full-length CFTR from *Saccharomyces cerevisiae*. The fusion of a green fluorescent protein (GFP) to recombinant CFTR has allowed a small-scale high-throughput expression and purification screen of multiple CFTR orthologues, using methods including in-gel fluorescence and whole-cell confocal microscopy. Large-scale expression has also provided sufficient quantities of CFTR for purification by nickel affinity and size exclusion chromatography. Optimisation of conditions for CFTR expression and purification is currently ongoing.

P. 7

An Improved Fluorescent Sensor to Measure CFTR Activity

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Introduction: CFTR function in vitro can be indicated by measurement of cAMP-mediated ion transport between the extracellular and intracellular compartment. Multiple techniques have been developed based on this general principle including patch-clamp analysis, radioactive iodide or chloride influx or efflux, and intracellular chloride-sensitive dyes such as SPQ or proteins such as YFP. Currently, an iodide-sensitive YFP mutant is used in high throughput screens to identify chemical and biological modulators of CFTR. The big caveat of this sensor is that the YFP signal both depends on the expression level of the YFP molecule and the intracellular iodide concentration. Therefore we created a novel fluorescent sensor to quantify CFTR function by fusion of YFP-H148Q/I152L to an iodide insensitive red fluorescent protein (DsRed) or mKate2.

Aim: Our aim was to develop a more accurate fluorescent sensor of CFTR activity that can be used in flow cytometry or confocal microscopy. CFTR activity measurement by flow cytometry allows the analysis of large numbers of suspension cells in an unbiased fashion. Confocal or fluorescence microscopy allows measurements of of CFTR activity in adherent cells in their native state.

Method: The iodide-sensitive YFP-H148Q/I152L was fused with a flexible linker to DsRed or was coexpressed as independent protein by cloning a cleavable E2A peptide between YFP and DsRed. Another sensor was created by cloning YFP-E2A-mKate2. The genetic sensors were transiently expressed in multiple cell lines stably expressing CFTR-wt or CFTR-dF508, and upon transfer of these cells into a buffer containing or lacking iodide, we measured fluorescent intensities of the individual components of the sensor in time. The rate of change in fluorescent ratio between YFP and DsRed or mKate was studied for the different conditions.

Results: Using our novel ratiometric fluorescent sensors, we measured CFTR activity in multiple cellular backgrounds using flow cytometry as well as confocal microscopy. Cells stably expressing CFTR-wt protein have a higher sensor activity than cells lines expressing the CFTR-dF508 protein. Sensor activity was sensitive to CFTR-inhibition (CFTR-inh172) as indicated by a decrease in reporter activity in cells expressing CFTR-wt or CFTR-dF508. Furthermore, our ratiometric measurements showed a much lower standard deviation than the YFP-data alone. The cleavable E2A peptide allowed better detection of YFP signals per unit of DsRed when compared to the linked YFP-DsRed fusion protein.

Conclusion: Using our novel fluorescent sensor we were able to accurately measure CFTR activity in a flow cytometry and confocal microscopy setting using cell lines transfected with CFTR. We are currently preparing lentiviral stocks to assess the capacity of this sensor to measure CFTR function in primary cultures of human subjects. This novel sensor may be useful in identifying novel regulators of CFTR activity, and may be used to accurately quantify residual CFTR-activity in primary cell cultures of CF patients.

CFTR Specific Effects of CPT-cAMP and Forskolin on Cell Metabolism

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CFTR (ABCC7) is the only ABC protein known that functions as an ion channel. Defective CFTR causes severe diseases like cystic fibrosis and secretory diarrhea. It is known that the channel function is regulated in many ways including phosphorylation by protein kinase A and the presence of ATP. Furthermore there is evidence for ATPase activity similar to other ABC transporters. Nevertheless literature seems somewhat contradictory on the velocity of this ATP hydrolysis and on the influence of protein kinase A on ATPase activity. As membrane proteins can behave differently in purified reconstituted systems and native environments, we wanted to address this problem using micophysiometry[1] applied in our lab to measure the effect of drugs on ATP hydrolysis by ABC transporters in living cells. Microphysiometry was shown to allow monitoring the cellular ATP consumption rate by measuring the extracellular acidification and oxygen consumption rate, respectively, in real time in MDR1 transfected cells[2]. As stimulation of PKA by agents like forskolin or CPT-cAMP seems to be necessary for channel function of CFTR it is a prerequisite for our approach to understand the effects of these agents on cell metabolism. We found that both substances have specific effects on extracellular acidification and oxygen consumption rates of CHO cells stably expressing functional CFTR which cannot be observed in non-transfected cells or cells expressing CFTR-ΔF508. Both molecules seem to have at least two effects in the concentration range (0.1 µM ≤ c(CPT-cAMP) ≤ 400 µM and 0.01 µM ≤ c(forskolin) ≤ 100 µM) investigated. There is not only stimulation of activity as seen in electrophysiology experiments, but also inhibition at higher concentrations. This results in bell-shaped activation profiles resembling those seen for ABC transporter substrates. Furthermore forskolin seems to have an additional CFTR independent inhibitory effect on cell metabolism at concentrations c > 10 µM which is most likely due to inhibition of glucose import. We could also show that these effects cannot be completely suppressed by inhibition of protein kinase A, suggesting that especially forskolin has additional CFTR dependent effects in living cells, that might even include direct interactions with the transmembrane domains of CFTR. Microphysiometry can thus be used to measure CFTR specific effects on cell metabolism and allows monitoring the influence of drugs on CFTR function in a native environment. As we could easily distinguish between CFTR specific effects and general effects, i.e. decreased glucose import, that may indirectly influence CFTR mediated chloride currents and may then lead to misinterpretations of experiments using only ion channel conductance as readout. The present analysis shows that microphysiometry successfully complements electrophysiology as well as ATPase activity measurements in purified systems.

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P. 9

Direct visualization of CFTR conformation by atomic force microscopy imaging

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Cystic Fibrosis (CF) is one of the recessive Mendelian genetic diseases more popular in the world. There is no decisive treatment for this disease and, only recently, an effort to find pharmacological therapies for CF has been carried out. This disease is caused by a mutation in the gene encoding for the CF trans-membrane regulator (CFTR) chloride channel present in epithelial cells. A full knowledge of the CFTR structure and conformational changes associated with the activation process would allow a better understanding of the disease, possibly leading to the identification of suitable drugs. Up to now a detailed molecular structure of the first nucleotide binding domain (NBD1) was determined by x-ray diffraction and an homology model of the NBD1-NBD2 dimer was build and successively extended to the complete CFTR. However, the quaternary organization of the protein is still controversial: X-ray experiments suggest that isolated CFTR is organized as monomer, whereas a dimeric organization is proposed on the basis of electron microscopy reconstructions. In addition, Schillers et al. (2004) used Atomic Force Microscopy (AFM) to image the cytoplasmic side of CFTR on transfected Oocytes, showing the presence of peculiar annular structures, probably associated to CFTR oligomers, but never observing features comparable with the proposed structures for monomers and dimers.
The aim of our work is to study CFTR in its natural environment, the plasma membrane, to gather definite information about the native organization of the channel and to open for future studies on the putative functional role of the supramolecular organization of the channel in the plasma membrane that has not yet been fully addressed.
Atomic Force Microscopy (AFM) imaging was performed on the cytoplasmic side of cellular membranes extracted from fetal rat thyroid (FRT) cells permanently transfected with CFTR. The protocol to obtain flat and clean membranes, crucial to reach the highest resolution in AFM images, was carefully assessed by means of AFM and confocal microscopy. CFTR channels were identified in the plasma membrane using an immunogold labeling approach and the organization of their quaternary structure was studied. Our preliminary results indicate that several CFTR conformations co-exist in the plasma membrane: monomers, dimers and annular rings.

Room: Salone Bruschi

Posters – CFTR Biogenesis, Quality Control and Trafficking

P. 10

Proteomic Identification of Calumenin as a G551D - CFTR Associated Protein

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Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. To date, over 1700 mutations have been identified in the CFTR gene. Among these mutations, the CF-causing missense mutation G551D-CFTR (approx. 5% of cases) exhibits normal expression on the cell surface but it is associated with severe disease due to its altered channel activation. The aim of the present study was to identify specific interacting proteins of G551D-CFTR. Membrane proteins from Wt-CFTR and G551D-CFTR expressing cells were extracted and resolved by 2D-gel electrophoresis (2-DE). Mass Spectrometry revealed that calumenin was only present in the proteins extracted from G551D-CFTR expressing cells. Co-immunoprecipitations showed that calumenin is linked to Wt- and to G551D-CFTR. The amount of bound calumenin was higher in G551D-CFTR protein complex than in wt-CFTR complex. Nevertheless, its basal expression was not modified in G551D-CFTR expressing cells when compared to Wt-CFTR. Calumenin associated proteins were resolved by 2-DE and the spots were analyzed by MS. The results indicated that calumenin is bound to chaperons, cytoskeleton proteins and Grp78/Bip which is involved in the Unfolded Protein Response (UPR). Therefore, we showed a calumenin-CFTR interaction and we suggest that calumenin maybe involved in the G551D-CFTR maturation and trafficking pathway. Finally, we suggest that UPR may be triggered independently of the retention of G551D-CFTR in the ER.

Cdc42 Involvement in CFTR Post-Golgi Trafficking in Epithelial Airway Cells

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Cystic Fibrosis (CF) Transmembrane conductance Regulator (CFTR) functions as a cAMP-activated chloride channel at the apical plasma membrane (PM) of epithelial cells. Regulation of its activity requires the control of its trafficking to and from the PM. Many keyplayers have already been specifically involved in these processes, for several other proteins. In our lab we focus on Cdc42, a Rho small GTPase-subfamilly member acting as a cortical actin cytoskeleton organizer, and analyse its role in CFTR regulation.

In this study, we tested the effects of Cdc42 activity modulation on the CFTR biochemical properties. We choose the CFBE41o- epithelial airway cell model stably expressing wild type (WT)-CFTR. Overexpression of GFP-tagged forms of either WT or mutant (dominant negative T17N or constitutively activated Q61L) versions of the Cdc42 protein was first carried out. Western blot analysis revealed that any Cdc42 elicits a depletion of the total CFTR protein level. Cell surface biotinylation experiments indicated that this is correlated to a decrease of the CFTR amount exposed at the PM. Notably, CFTR internalization is significantly increased by the Q61L mutant. Because overexpressions of small GTPases could mislead to confusing conclusions we performed siRNA-mediated Cdc42 RNA interference. We observed that Cdc42 depletion induces an increase of the total CFTR protein amount whereas the CFTR PM fraction is reproducibly reduced. To clarify these divergent effects of Cdc42 protein level variations, we are now investigating both the post-golgi half-life of CFTR and its internalization and recycling properties.

Since small GTPases trigger their molecular functions through specific effectors, we will test the potential requirement of the Toca-1 and N-WASP Cdc42 effectors in the above mentionned processes. This work should be extended to F508del-CFTR-expressing CFBE41o- cells in temperature-rescuing conditions. It takes place in the understanding of the molecular mechanisms governing the post-golgi CFTR journey which cannot be fully corrected, so far, in CF cells.

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P. 12

Trafficking Of CFTR Is Unconventionally Regulated By Key Components Of The COPII Complex

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Cystic Fibrosis is a genetic disease caused by mutations in the Cystic Fibrosis transmembrane conductance regulator (CFTR) gene. Most of the disease alleles correspond to a deletion mutation, resulting in the loss of phenylalanine at position 508 of the protein (F508del-CFTR). This leads to the misfolding and eventual intracellular retention of CFTR in the endoplasmic reticulum (ER) since the protein cannot pass the ER quality control (ERQC) to enter the conventional secretory pathway. When leaving the ER, the wild type CFTR (wt-CFTR) can follow an unconventional secretory pathway to reach the plasma membrane bypassing the Golgi Apparatus[1,2,3]. Here, our aim was to map the secretory route of the wt-CFTR protein at both biochemical and morpho-functional level.

To investigate the trafficking route of CFTR we developed an RNAi-based approach for manipulation of specific trafficking steps while investigating the consequences on CFTR progression through the secretory pathway. As a starting point, we focused on early steps of the conventional protein secretion. With the known role of the COPII complex in mediating transfer of protein cargo from the endoplasmic reticulum to the Golgi apparatus[4], and specifically, because of the dependence of CFTR protein on COPII function for leaving the ER[2,5], we selected clue components of this machinery as RNAi targets to breakdown the conventional ER-export function.

We established the conditions to obtain the efficient depletion of the mammalian protein isoforms corresponding to the sar1 and sec23 yeast homologues. Accordingly, we found that Sar1-depletion decreased the number of cells where wt-CFTR could reach the Plasma Membrane (PM), while Sec23-depletion supported PM localisation of CFTR. Indeed, the knockdown of Sec23 promoted arrival of the F508del-CFTR to the PM. The complex glycosylation of the CFTR protein was consistently decreased, suggesting a possible unconventional trafficking under COPII perturbation.

Although, the exact mechanism for both the unconventional trafficking and the non-canonical ER-exiting of CFTR is being currently investigated and characterised at ultrastructural level, we raise a plausible scenario where the Sec23 proteins exert a repressive role of an unknown route for CFTR trafficking that may depend on Sar1 function. Therefore, the manipulation of ER-boundary decisions could be a promising resource for the design of therapeutic strategies amenable to correct the basic defect of F508del-CFTR.

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Room: Salone Bruschi

Posters – Epithelial Ion and Fluid Transport

P. 13

CFTR Regulation in Human Airway Epithelial Cells Requires Integrity of the Actin Cytoskeleton and Compartmentalized cAMP and PKA Activity

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ΔF508CFTR rescued to the apical membrane exhibits regulatory defects suggesting that the intracellular milieu may affect its ability to respond to cAMP regulation. We recently reported that NHERF1 overexpression in CFBE41o- (CFBE) cells rescues ΔF508CFTR functional expression, by promoting F-actin organization and formation of the NHERF1-ezrin-actin complex (1).
The hypothesis that the rescue of ΔF508CFTR activity induced by NHERF1 overexpression may involve recruitment of PKA to the membrane region was supported by immunocytochemical analysis of PKA localization demonstrating that PKA localization shifted from the cytosol to the sub-cortical region in CFBE cells stably overexpressing NHERF1 (CFBE/sNHERF1). Importantly, when we analyzed PKA activity by a FRET-based reporter targeted to the plasma membrane (2), we found that PKA-mediated phosphorylation at the plasma membrane was lower in CFBE cells than that found in CFBE/sNHERF1 cells. By contrast, using an untargeted, cytosolic version of the PKA activity FRET reporter (2), we found that PKA-dependent phosphorylation was significantly higher in the cytosol of CFBE than in CFBE/sNHERF1 cells. To assess if the higher PKA activity detected in CFBE cytosol could be due to a higher concentration of cAMP in this compartment, we measured cAMP changes in cells expressing FRET-based reporters for cAMP levels either untargeted (3) or targeted to the plasma membrane (4). In keeping with the observed difference in PKA activity, CFBE cells showed a significantly larger cAMP response in the bulk cytosol and a lower cAMP accumulation in the sub-plasma membrane compartment with respect to CFBE/sNHERF1.
A possible explanation for the observed differences in the cAMP compartmentalization in the two cell lines is that the restoration of cortical actin cytoskeletal organization induced by NHERF1 overexpression in CFBE cells (1) may contribute to the accumulation of cAMP in the sub-plasma membrane compartment by acting as a physical barrier to cAMP diffusion. In support of this hypothesis, we found that F-actin depolymerization induced by Latrunculin B, had no effect in CFBE cells while significantly increasing both cAMP accumulation and PKA activity in the cytosol of CFBE/sNHERF1 cells at the expense of their levels and activity in sub-cortical region.
Altogether these findings suggest that the organized sub-cortical cytoskeleton constitutes an efficient barrier to cAMP diffusion promoting compartmentalized cAMP/PKA signals.

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Analysis of CFTR Expression in Nasal Epithelial Cells by Flow Cytometry

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Background: Cystic fibrosis is the most common lethal hereditary disease amongst Caucasians and caused by loss-of-function mutations of the CFTR gene. CFTR protein is relatively highly expressed in epithelial tissues and is critically important forion transport across epithelial surfaces. Quantification of CFTR protein expression in primary patient material has been proven difficult and thus far limited data is available on CFTR protein expression at single cell level.

Methods: We developed a novel assay to analyze CFTR expression in primary nasal epithelial cells at single cell level using flow cytometry. Nasal cells collected from the inferior turbinate were fixed immediately upon collection and stained for markers to discriminate epithelial cells from non-epithelial cells such as leukocytes by using cytokeratin, E-cadherin and CD45. CFTR- BHK cells were used as an internal standard to quantify CFTR expression in the nasal epithelial cells.

Objective: Validation of a panel of CFTR-directed monoclonal antibodies for flow cytometry and investigation of CFTR expression levels in nasal epithelial cells from healthy controls and CF patients.

Results: Using our novel assay, we assessed CFTR expression in specific cell types isolated from the nasal cavity with a variety of CFTR monoclonal antibodies. Our data indicate that CF patients homozygous for DF508 express significant levels of CFTR protein in nasal epithelial cells.

Conclusion: The analysis of primary nasal epithelial cells of CF patients by flow cytometry is feasible and may be used to monitor pharmacological intervention aimed to restore CFTR expression and to study *in vivo* regulation of CFTR expression levels.

Non-Genomic Estrogen Regulation of Airway Surface Liquid Height in Normal and Cystic Fibrosis Bronchial Epithelia

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Introduction: There are significant differences in the progression of CF in male and female patients: lung function among female patients deteriorates 26% more rapidly than in male patients and on average, male CF patients survive 9 years longer than females. Moreover, measurement of nasal trans-epithelial potential difference in female CF subjects has shown an effect of circulating estrogen on ion transport properties of the epithelium. These observations indicate an endocrine (estrogen) component of the CF gender gap. The surface mucus is a key component of the innate immune system of the respiratory tract and proper hydration of the airway surface layer (ASL) is a prerequisite for efficient mucociliary clearance. Estrogen has been reported to reduce ASL height in female CF bronchial epithelium. We have previously shown an anti-secretory effect of estrogen in epithelia and here investigated the role of estrogen receptors and basolateral K^+ channels (KCNQ1) in mediating estrogen effects on ASL height.

Methods: Confocal fluorescence microscopy was used to investigate the effects of 17β-estradiol (E2, 0.1 to 10 nM) on ASL height in a normal human bronchial epithelial cell line (NuLi-1) and a F508del CF cell line (CuFi-1). To investigate signal transduction via membrane and nuclear estrogen receptors, we tested the effects of free E2 and a nuclear-impeded Estrogen Dendrimer Conjugate (EDC) on ASL height. Chloride secretion was assessed as bumetanide (10 µM) sensitive short-circuit current and KCNQ1 channel activity was determined using the specific inhibitor HMR1556 (1µM).

Results: In control conditions, ASL height was significantly higher in normal compared to CF cells (6.82 ± 0.33 µm versus 5.58 ± 0.14 µm, n=20, $p< 0.001$). Moreover, E2 reduced ASL height in both normal (25% decrease, n=5, $p< 0.05$, ANOVA) and CF (20% decrease, n=5, $p< 0.05$, ANOVA) cell lines after 30 min treatment using concentrations of E2 between 0.1 and 10 nM. Bumetanide or HMR1556 treatment decreased ASL height significantly in both cell lines. E2 had no additive effect on ASL height in the presence of these inhibitors. Treatment with EDC (at 1 nM E2 equivalent concentration) produced a significant reduction in ASL height in both cell lines (4.72 ± 0.25 µm in NuLi-1 and 4.86 ± 0.42 µm in CuFi-1, n=5, $p< 0.05$, ANOVA) whereas the empty dendrimer had no effect.

Conclusion: These results demonstrate an anti-secretory effect of estrogen in both normal and CF bronchial epithelial cell lines which results in a reduction in ASL height. These rapid responses to estrogen target the Cl^- secretory pathway and basolateral KCNQ1 channels. These rapid estrogen responses are non-genomic and are initiated by an estrogen receptor at the plasma membrane or restricted to the cytosol rather than via a genotropic nuclear estrogen receptor signal transduction pathway.

Acknowledgements: This work was supported by a NBIP Ireland Career Enhancement and Mobility Fellowship to V.S-C. co-funded by EU FP7 Marie Curie Actions and the Irish Higher Education Authority. Support from the Higher Education Authority of Ireland PRTLI Cycle 4 (to B.J.H) and the National Institutes of Health (R37 DK015556, to J.A.K.) is acknowledged.

Glucocorticoids Differentially Modulate the CFTR-Channel

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Cystic Fibrosis (CF) is the most common recessive, lethal genetic disease in Caucasians. The hallmark ion transport defects in CF are a diminished or absent Cl^--secretion and Na^+-hyperabsorption. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) are pathognomonic and agents improving the transcription, rescue or channel activity supposedly lessen the pathologic features and may increase life expectancy. Glucocorticoids are known to increase the function of epithelial sodium channels (ENaC), but little information exists about the impact on CFTR. However, experimental evidence also suggests a stimulatory role, supposedly through enhancing the activity of the serum and glucocorticoid-dependent kinase 1 (SGK1). Therefore, we sought to analyze the influence of glucocorticoids on CFTR in different cell types. We used Real Time-PCR to determine the CFTR mRNA-expression level and Ussing-Chamber measurements to analyze the transport activity by measuring short-circuit currents (I_{SC} in $\mu A/cm^2$; Mean ± SEM). In the electrophysiological analysis we used different agonist (forskolin) and antagonists (amiloride, diisothiocyano-stilbenedisulfonic acid (DIDS) and glybenclamide) to distinguish between effects on ENaC, CFTR and other chloride channels. In subbronchial gland cell-derived Calu-3 cells dexamethasone (D: 100 nM) has little influence on CFTR mRNA-expression, but increases the forskolin-induced and glybenclamide-sensitive channel activity significantly (I_{forsk} from 65.61 ± 3.21 to 86.02 ± 4.09, $p< 0.001$ and I_{glyb} from 5.45 ± 0.27 to 9.27 ± 0.76, $p< 0.001$). The basal and DIDS-sensitive I_{SC} are not altered by D. However, in fetal distal lung epithelial (FDLE) cells from rat fetuses, the CFTR mRNA-expression is dramatically reduced by 80 % through D ($p< 0.001$) and the electrophysiological activity diminished accordingly (I_{forsk} from 2.38 ± 0.13 to 0.97 ± 0.03, $p< 0.001$ and I_{glyb} from 0.47 ± 0.02 to 0.25 ± 0.01, $p< 0.001$). The basal and amiloride-sensitive I_{SC} are increased by D and no DIDS-sensitive I_{SC} is detected. SGK1 mRNA-expression is increased by D in both cell types. The results show opposing effects of glucocorticoids on CFTR mRNA-expression and channel activity depending on the analyzed cell type. One difference between these two cell types is the expression of ENaC in FDLE-cells but not Calu3, which is greatly increased by D. It is known that CFTR is a regulator of ENaC activity, but our experiments suggest that ENaC might also regulate CFTR and reduces its activity upon D treatment. Further analysis will investigate this assumption in more detail. However these results may caution against using glucocorticoids in inflammatory CF-associated complications, because any further inhibition of CFTR channels might worsen the clinical outcome.

P. 17

Molecular Structure, Packing and Release of MUC2 with Relevance to Cystic Fibrosis

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In CF extremely viscous fluid is built up in fluid transporting tissues likely connected to impaired release and expansion of mucins. O-glycosylated mucins make up the core of the mucus gel, which cover all mucosal surface of the body and play a central role in protection and hydration. MUC2 is present in small and large intestine, where it is stored as a multimer in secretory granules of goblet cells at high [Ca^{2+}] and low pH. It is the MUC2 N-terminus that controls this packing. The extracellular milieu have to trigger the unpacking, a not yet fully understood process. **The aim** is to elucidate organization and structure of MUC2 when it is packed in and secreted.

The N-terminal part of MUC2 was expressed in CHO-K1 cells. The secreted trimerized recombinant mucin was purified from culture medium by anion exchange chromatography and analyzed by transmission electron microscopy (TEM). The pH in the buffers was varied in the range from 5.2 to 8 to mimic conditions of secretory pathway and extracellular environment by adding HAc (pH 5.2), MES (pH 6.2) or Tris (pH 7.4 and pH 8) with or without calcium. Samples were adsorbed onto carbon coated EM grids and negative stained. Processing of micrographs was performed using EMAN1 software.

When pH was low at pH 6.2 and calcium present, rings with an outer and inner diameter of 25-30 and 20-25 nm respectively were observed. Without calcium rings were assembled at pH 5.2 and 6.2, but vanished with increasing pH. 2D refinements of the projections showed rotational 5- and 6- folded symmetry. When the MUC2 N-terminal rings at low pH with calcium were crosslinked and purified by density ultracentrifugation, assemblies of laterally concatenated rings were obtained in the high density fractions. The formations of these rings are probably vital for proper packing and release of full length MUC2.

Harriet N. and Daniel A. have contributed equally to this work.

P. 18

Native Calcium-Activated Chloride Channels and their Association with TMEM16A Protein Expression

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Expression of TMEM16A protein in heterologous expression systems is associated with the activity of calcium-activated chloride channels, CaCCs (Ferrera et al., Physiology 25: 357-363, 2010). However, it is not clear whether TMEM16A is the only protein responsible for CaCC activity in epithelial cells. We compared the properties of native CaCCs recorded with whole-cell patch-clamp recordings in the pancreatic cell line CFPAC-1 with those of the chloride currents arising from stable TMEM16A transfection in FRT cells.

Analysis of TMEM16A mRNA in CFPAC-1 cells revealed the prevalent expression of the (ac) isoform which corresponds to a protein of 960 amino acids lacking segment b. Segment b, coded by exon 6b, is a 22 amino acid long region that has been reported previously to affect apparent calcium affinity of TMEM16A-associated chloride currents. CaCC currents in CFPAC-1 cells have time-dependent activation at positive membrane potentials and apparent calcium affinity similar to those of FRT cells expressing the TMEM16A(ac) isoform. The half effective intracellular calcium concentration was 140 and 160 nM for CFPAC-1 and FRT cells, respectively. The kinetics of activation and deactivation of the currents, following depolarization and hyperpolarization of membrane potential, were also comparable. Furthermore, removal of ATP from the intracellular (pipette) solution abolished the CaCC currents in both cell types. This finding suggests that a phosphorylation step is required to allow the activity of the channels. We hypothesized that calcium/calmodulin-dependent kinase (CaMKII) is involved in this process. However, the CaMKII inhibitor KN-93 did not change the size and properties of CaCC currents in FRT cells. We also tested the contribution of other TMEM16 proteins to CaCC activity in CFPAC-1 cells using a functional assay based on the halide-sensitive yellow fluorescent protein. Before the assay, CFPAC-1 cells were transfected with siRNA against TMEM16 proteins (from A to K). We found that only silencing of TMEM16A caused a significant inhibition of CaCC currents.

Our findings confirm that TMEM16A protein expression in null cells is sufficient to generate chloride currents with properties and regulation similar to those of native CaCCs. However, the contribution of other types of proteins to CaCC function cannot be excluded. The study of TMEM16A/CaCC function and regulation is important to assess its relevance as a therapeutic target in cystic fibrosis.

The Anti-Inflammatory Mediator, Lipoxin A4, Increases ASL Height in Normal and Cystic Fibrosis Bronchial Epithelium

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Cystic fibrosis (CF) affects many organs but the progressive lung destruction is the main cause of morbidity and mortality. Mutations of the CFTR gene result in defective Cl^- secretion and Na^+ hyperabsorption. This contributes to a reduction of the airway surface liquid layer (ASL) height and impairs mucociliary clearance, thus promoting bacterial colonization and chronical inflammation. Identification of agents that promote hydration of the ASL is likely to be of therapeutic benefit to patients with CF.

Lipoxins are bioactive lipids derived from omega-6 polyunsaturated fatty acids. The lipoxin A4(LXA_4) is produced at inflammatory sites from the interaction of lipoxygenase activities of several cell types including neutrophils, platelets and epithelium. This lipid mediator is one member of the newly identify molecules playing a role in ending/resolving the inflammatory process by modulating neutrophilic inflammation, clearing apoptotic PMN and inhibiting pro-inflammatory cytokines production. The levels of LXA_4 have been reported to be decreased in the airways of patients with CF.

We have discovered a pro-secretory effect of the endogenous LXA_4 in human bronchial epithelium. Human bronchus epithelial (HBE) cell primary cultures and CF(CuFi-1) and non-CF(NuLi-1) bronchial epithelial cell lines were grown under an air-liquid interface into well-differentiated epithelia. LXA_4 (1nM) treatment significantly increased ASL height in non-CF and CF HBE. This effect was sustained over 24 hours in the CF HBE and was inhibited by Boc-2, the antagonist of the ALX/FPR2 receptor that we found to be expressed in the apical membrane of HBE. We investigated the contribution of Na^+ absorption (via ENaC) and of Cl^- secretion in the ASL height regulation by LXA_4. LXA_4 pre-treatment reduced the amiloride-sensitive short-circuit current in CuFi-1 epithelium indicating that LXA_4 inhibits ENaC activity. LXA_4 and amiloride produced additive stimulating effects on the ASL height. LXA_4 increased the whole-cell currents of non-CF and CF HBE and this effect was inhibited by BAPTA-AM (chelator of intracellular Ca^{2+}) and NPPB (non selective inhibitor of Ca^{2+}- activated Cl^- channels) but not by the CFTRinh-172 (CFTR inhibitor). Bumetanide abolished the ASL height increase induced by LXA_4. We tested the possible role of an ATP release in mediating the effect of LXA_4 on ASL height. LXA_4 stimulated an apical ATP release. Hexokinase (ATP hydrolysis) and reactive-blue-2 (P2Y purinoreceptor antagonist) had no effect on baseline ASL height and both abolished the LXA_4 effect on ASL height. LXA_4 stimulated an intracellular Ca^{2+} increase in non-CF and CF HBE and this effect was inhibited by reactive-blue-2.

Taken together, our results provide evidence for a novel effect of LXA_4 involving the FPR2 receptor in the apical membrane, luminal ATP secretion and P2Y receptor activation leading to an intracellular Ca^{2+} increase, inhibition of Na^+ absorption and stimulation of Cl^- secretion in CF and non-CF epithelia to finally increase ASL height. These novel pro-resolving effects of LXA_4 reveal a cross-talk between an endogenous anti-inflammatory mediator decrease and the ion transport defect in CF and open up a new therapeutic avenue in the treatment of CF.

Room: Salone Bruschi

Posters – Lung Physiology and Pathophysiology

P. 20

Tracheal Smooth Muscle Abnormalities in the CF Mouse

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Introduction: Recent data point towards changes in tracheal cartilage structure and altered airway smooth muscle (ASM) exacerbating respiratory dysfunction in cystic fibrosis (CF). To date, no clear role of altered contraction has been established and the possible mechanisms involved remain unclear. We performed functional smooth muscle studies and morphological analysis to determine the extent and implications of ASM abnormalities in the CF mouse trachea.

Methods: Smooth muscle function was measured using tracheal rings isolated from 6 week old heterozygous (+/-) and homozygous (-/-) *cftr*^{tm1Cam} MF1/129 mice. Contractile responses to the agonist carbachol (CCh 50µm) in Ca free solution were measured and normalised to a 40 second application of 120 mM high K^+ solution (100%). The role of Rho-kinase in contraction was determined by measuring changes in force after incubating rings with Rho-kinase inhibitor H-1152 for 15 minutes. For morphological analysis, whole tracheas were fixed in 4% paraformaldehyde, embedded in 5% gelatine and snap frozen. 7µm cryosections were stained with haematoxylin and eosin and labelled with anti-actin, α-smooth muscle-Cy3 monoclonal antibody. Sections were observed and photographed under a fluorescent microscope.

Results: The amplitude of contraction in response to CCh was significantly decreased in CF mice compared to controls (89.6±5% (*cftr* -/-) n=6, 118.5±5% (*cftr* +/-) n=6, $p< 0.01$). The duration of contraction after K^+ depolarisation was significantly decreased in CF mice after incubation with H-1152 compared to controls (30.2±6.3s shorter in *cftr* -/-,10.7± 5.4s, (*cftr* +/-) $p< 0.01$). Cross sectional areas of trachea were 25.2% smaller ($p< 0.01$) and less circular than controls (0.73±0.02 (*cftr* -/-) 0.84±0.008 (*cftr* +/-) $p< 0.01$). Trachea lumens tended to be smaller when normalised to cross sectional area (41.3%±3.6 (*cftr* -/-), 49.5±1.6 (*cftr* +/-)) and the area of epithelium lining the lumen was increased by 10% in CF tracheas, but these changes were not significant. Smooth muscle area was reduced in CF tracheas; however when normalised to whole trachea area, there was no significant difference between genotypes (2.88±0.18% (*cftr* -/-),3.25±0.3% (*cftr* +/-).

Conclusion: These data indicate a loss in contractile capacity of the mouse CF trachea in Ca free conditions. This suggests a reduction in the release of Ca from the sarcoplasmic reticulum leading to a decrease in force. Data also suggest that the role of Ca dependent activation of Rho kinase in contraction may be elevated in the mouse CF airway. Morphological analysis showed that the reduction in ASM contractility is not due to a reduction in smooth muscle area, but is a consequence of altered ASM cell function.

P. 21

Decreased Apical Expression of CFTR by Pseudomonas Aeruginosa Infection in Respiratory Cells: Role of NHERF1 Phoshorylation

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Cystic Fibrosis (CF) is a still life-threatening disease, although therapies have augmented the life span of CF individuals. In these patients the chronic infection and inflammation represents a key pathogenetic event of lung damage and respiratory insufficiency.P. aeruginosa infections of the host airway cell have been shown to decrease apical expression of both wild-type (wt) and F508del CFTR through the inhibition of apical endocytic recycling, thus potentially impairing the efficacy of the potentiator/corrector therapies (Swiatecka-Urban A. *et al*. 2002, Guerra L. *et al*. 2005, Kwon SH. *et al*. 2007)The host cell intracellular molecular mechanism(s) underlying this P. aeruginosa infection-dependent inhibition of CFTR membrane recycling is still unknown. CFTR endocytic recycling is regulated by its interaction with PDZ domain containing proteins and by actin cytoskeleton organization and recent work has shown that the PDZ domain adaptor protein, NHERF1, finely regulates both wt and F508del CFTR membrane recycling through its association with the C-terminal PDZ-binding motif (DTRL) of CFTR (Favia M. *et al*. 2010)In this study, we have investigated the effect of P. aeruginosa on the NHERF1 and CFTR expression, *in vitro* and *in vivo*. CFTR and NHERF1 were analyzed by Western Blot in wt respiratory cells and in murine lung upon exposure of the cells and mice to P. aeruginosa.P. aeruginosa induced a reduction of CFTR expression and increased NHERF1 and its molecular weight in both bronchial epithelial cells and in the lung of the mice. To determine if the increase in NHERF1 molecular weight was caused to its hyper-phosphorylation, the extract from wt respiratory cells was exposed to increasing levels of the enzyme, Alkaline Phosphatase (AP). The results demonstrated that the exposure to P. aeruginosa does indeed hyper-phosphorylate NHERF1 as it was very sensitive to AP but a certain amount was resistant to this enzyme and might represent a different group of phosphorylated residues, which could further be investigated. Our experiments have also shown that the increased expression of mature CFTR driven by the overexpression of NHERF1 does not render it insensitive to the negative effects of P. aeruginosa infection.The molecular identification of the pathological mechanisms responsible for the effect of P. aeruginosa on the phosphorylation of NHERF1 and reduction of CFTR expression could identify novel targets to block this process and also block the P. aeruginosa interference with the efficacy of drugs capable of increasing CFTR apical membrane expression.

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Translational Medicine - From Bedside to Basic Science. On the Clinical Impact of Oxidative Stress in Cystic Fibrosis

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In the normal lung the ventilation/perfusion ratio decreases from top to bottom of the lung. This leaves the upper part of the lung with the highest oxygen koncentration.
(Respiratory Physiology - the essentials. 5 th edition. J. B. West)
With the low level of antioxidants (in particular glutathione) in CF lungs, there is plenty of possibility for inflammatory damage by ROS (reactive oxygen species) - also called "oxidative stress" (W. M. Hudson et al. Treat. Resp. Med. 2004; 3(6): 253-263.)
If we accept that oxidative stress is a major factor in lung distruction - this explains the established finding that CF lung disease starts in the upper part of the lung.
(G. Santis et al. Clinical Radiology; 1991; 44: 20-22).
It also explains the interesting finding that high FEV1 is a risk factor for "rate of decline" and the risk factor with the most significant impact across all ages (M. W. Konstan et al. J. Pediatric; 2007; 151:134-139).
With [^{18}F] fluorodeoxyglycose (FDG) positron emission tomography, a new tool for evaluation of pulmonary inflammation is at hand (D. L. Chen; Am J Crit Care Med, 2006; 173: 1363-1369).
This communikation is a presentation of the first cases of a larger study of patients selected as patients with high rate of decline, based on fall in FEV1 over the last 4 years.
After baseline evaluation of leucocyt + differential count, CRP, C-reactive protein and a PET CT-scan - the patients recieved 600 mg x 3 po of N-acetylcystin and 1 gram x 3 as inhalation via E-flow rapid for 3 weeks.
No astma- or antiinflammatory medication, nor any additional antibiotics were adminestered during the period - after which the PET CT-scan and the other perameters were repeated.
The PET CT-scan was done as a dynamic study after intravenous injection of 18F-FDG, and subsequent analysis of the entire lung, apical and basal segments of intact lung tissue. The quantitative optake of 18F-FDG by the lungs was meassured as the net influx rate constant using Patlak plot analysis.

Results: Characteristically, patients show marked chronic lesions on CT. Lung parenchyma which seems preserved can be discriminated from destructed lung tissue and with PET it is possible to quantify the FDG uptake as a measure of inflammation. From the preliminary results 18F-FDG demonstrates a significant difference in the uptakte between apical and basal segments pointing to a correlation between FDG uptake and the ventilation-perfusion ratio.
This suggests that the analysis is measuring the results of oxidative stress.
The preliminary conclusions are, that PET CT-scan seems to be a valuable tool in the evaluation of CF-lung inflammation, and that high dose antioxidant theraphy seems promising from a clinical and serological point of view, to selected patients.

Room: Salone Bruschi

Posters – Inflammation and Bacterial Killing in CF

P. 23

Expression of Interferon Developmental Regulator 1 (IFRD1) and Histone-Deacetylase (HDAC1-2) in CFTR-Deficient Airway Cells

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Interferon related developmental regulator 1 (IFRD1) was identified as an important co-factor involved in the severity of lung inflammation in patients with cystic fibrosis (CF). IFRD1 acts in a histone-deacetylase (HDAC1, 2, 3)-dependent manner to mediate transcriptional co-repression of NF-κB (p65) transactivation controlling the expression of inflammatory genes. We therefore hypothesize that intrinsic alterations in the expression level of IFRD1 and HDAC1-2, might occur in CFTR-deficient airway cells compared to CFTR-sufficient airway cells.

First, we examined the level of IFRD1 protein in tracheal and lung extracts of adult *Δf508/ Δf508* (n=9) and wild-type (n=8) mice. The IFRD1 protein was detected in tracheal and lung extract, but the level of IFRD1 protein differs widely in samples of both *Δf508/ Δf508* and *wtCFTR* mice.

We next examined the expression level (mRNA and protein) of IFRD1 and two HDACs (1 and 2) in the CF bronchial cell line (CFBE41o- cells with F508del/ F508del mutation) compared to the CFTR-sufficient bronchial cell line (16HBE14o-cells).

We demonstrated a higher expression (a 5.5 fold increase) of IFRD1 mRNA (evaluated by qPCR) in cultured CFBE41o- cells compared to 16HBE14o- cells. By contrast, a 50% decreased level of IFRD1 protein (evaluated by western blot) was measured in CFTR-deficient cells compared to the CFTR-sufficient cells.

Interestingly, after an antioxidant treatment, i.e. with glutathione (GSH) at 2mM for 2hrs, we show a significant increase of IFRD1 protein level in CFTR-deficient airway cells.

The expression level of HDAC1 and HDAC2 (mRNA and protein) did not differ between CFTR-deficient cells and CFTR-sufficient airway cells.

Our data show a low IFRD1 protein level in CFTR-deficient airway cells. To explain this result, it will be interesting to measure the level of phospho-eIF2α, known as a protein stabilizing IFRD1 mRNA, in stress condition. Understanding how loss of CFTR function leads to modification in the role of IFRD1/p65/HDACs complex in exaggerated inflammatory response in CFTR-deficient airway cells is currently under investigation.

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Inhibitors of Glucosylceramide Synthase (GluCerT) Reduce the Transcription of IL-8 Induced by *P.aeruginosa* in CF Bronchial Cells

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CF individuals respond to chronic bacterial lung infections with an exaggerated immune response which destroys the lungs, leading to respiratory insufficiency. The chemokine IL-8, abundantly expressed at sites of chronic inflammation, seems to play a major role in driving the formation of a neutrophil-rich exudate into the lung of CF patients. Therefore, reduction of the exaggerated production of IL-8 is a key therapeutic target in CF. Consensus is growing on sphingolipids (SLs) as novel targets for the treatment of pulmonary disorders, including CF, since modulation of cellular ceramide reduces lung inflammation. We previously demonstrated that the imino sugar miglustat, an inhibitor of the synthesis of glycoshingolipids (GSLs), used for treating type I Gaucher disease, reduces the expression of IL-8 gene (Dechecchi, 2008), induced by *P.aeruginosa* infection in human bronchial epithelial cells. Miglustat inhibits the first step in GSL synthesis which is the transfer of glucose to ceramide by the GluCerT (Platt, 1994). In addition, the galactose analogue N-butyldeoxygalactonojirimycin (NB-DGJ), also inhibitor of GluCerT, produces an anti-inflammatory effect in bronchial epithelial cells (Dechecchi, 2008). Therefore we extended the investigation to the effect of the pharmacological modulation of GluCerT on the inflammatory response to *P.aeruginosa*, by inhibiting its function with a different, structurally unrelated compound, Genz-123346 (Zhao, 2009). CF bronchial epithelial IB3-1 cells were treated with the inhibitor, at doses ranging from 1 nM to 50 microM for 24 hours, before infection with *P.aeruginosa*. In these experimental conditions no toxicity was observed. Genz-123346 significantly reduces the expression of IL-8 mRNA by about 50%, starting from 100 nM. Interestingly, no effect on the inflammatory response was found when the cells were treated with the compound, 4 hours before infection. Therefore Genz-123346 exerts an inhibitory effect very similar to that previously observed with miglustat and NB-DGJ. The parallel effects obtained with Genz-123346 and the structurally unrelated compounds, the imino sugars miglustat and NB-DGJ, suggest that they could be mediated by the inhibition of the activity of GluCerT, one of the common molecular targets of these compounds, although their effect on additional molecular targets can not be excluded. Our results further strengthen the hypothesis that the pharmacological modulation of SL metabolism, that can intercept the ceramide metabolic pathway at many levels, may be an effective approach for the treatment of CF lung inflammation.

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CFTR Is Important for Phagocytosis and Killing of Pseudomonas *aeruginosa* by Human Monocytes but not Neutrophils

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Introduction: The mechanisms underlying increased Pseudomonas *aeruginosa* (P. *aeruginosa*) infection susceptibility in patients with CF are still incompletely understood. We hypothesized that P. *aeruginosa* infection partly results from impaired function of human immune cells due to CFTR deficiency, as has been shown for phagocytosis and killing of P. *aeruginosa* by alveolar macrophages from CFTR-deficient mice. Thus far, human neutrophils have been suggested to functionally express CFTR protein but these reports have been challenged. Human monocytes have shown to express CFTR protein, but functional data in these cells have not been shown.

Aim: The aim of our study was to show CFTR protein expression in peripheral blood leukocyte subsets and to evaluate whether phagocytosis and killing of P. *aeruginosa* by monocytes and neutrophils is regulated by CFTR function.

Method and results: ***CFTR protein expression in peripheral blood leukocytes.***
Peripheral blood leukocyte subsets derived from healthy controls (HC) and patients with CF were stained with various CFTR-specific monoclonal antibodies and analyzed by multicolour flow cytometry and confocal microscopy. We observed CFTR protein expression in peripheral blood mononuclear cells such as lymphocytes and monocytes, but not neutrophils.

Phagocytosis and killing of P. aeruginosa by monocytes and neutrophils.
EGFP labelled P. *aeruginosa* (PAO1 strain) was incubated for 30 minutes at 37°C with (heat inactivated) serum to opsonize bacteria. Next, opsonized P. *aeruginosa* was co-cultured with peripheral blood leukocytes for 30 min at 37°C. Cells were infected with an effector:target ratio of 10. Pharmacological inhibition of CFTR with CFTR inhibitor 172 (25 µM) in peripheral blood leukocytes was performed by pretreatment of cells for 60 min. Phagocytosis was measured by determining the percentage of EGFP-positive cells using flow cytometry. Killing capacity was determined by counting number of viable bacteria intracellularly and in the supernatant. Results showed that dysfunctional CFTR inhibits the capacity of monocytes, but not neutrophils, to phagocytose and kill P. *aeruginosa*.

Conclusion: These data suggest that CFTR protein is expressed by peripheral blood mononuclear cells such as monocytes and lymphocytes, but not neutrophils. Since we observed a CFTR-dependent modulation of P. *aeruginosa* phagocytosis and killing in human monocytes, these data support the hypothesis that functional impairment of CFTR in mononuclear cells of the immune system can contribute to CF disease pathology in humans. Restoration of the CFTR-dependent defect in these cells by therapeutic intervention may therefore be important to enhance bacterial clearance and limit the inflammatory phenotype in CF.

Characterization of Altered Inflammatory Signalling Pathways in CFTR-Defective Cells

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and characterized by recurrent and eventually chronic pulmonary infections with *P. aeruginosa*. Many *in vitro* and *in vivo* studies suggest that this increased susceptibility to infection may partly result from cellular intrinsic alterations in inflammatory signaling pathways, as CF cells and tissues develop proinflammatory characteristics even in the absence of detectable infection. Normalization of this enhanced inflammatory state may be of great importance to reduce the CF disease severity.

We here studied CFTR-dependent alterations in expression and activation of inflammatory signaling pathways by analyzing total cell lysates of human bronchial epithelial cells expressing either wild type CFTR (HBEs) or ΔF508 CFTR (CFBEs) under sterile conditions or upon P. *aeruginosa* stimulation using Western Blot analysis.

We observed decreased expression of the NF-κB inhibitor IκB in CFBEs compared to HBEs under sterile conditions, suggesting increased activation of NF-κB that leads to proinflammatory cytokine production. IκB levels decreased to a greater extend in CFBEs compared to HBEs upon bacterial stimulation. Interestingly, we detected for the first time increased basal levels of phospho-STAT5 and phospho-STAT3 in CFBEs compared to HBEs and bacterial stimulation increased phospho-STAT3 and phospho-STAT5 levels in CFBEs but not in HBEs. Besides, preliminary data suggested increased levels of phospho-ERK, decreased levels of phospho-PKB and equal levels of phospho-p38 in CFBEs compared to HBEs under sterile conditions.

Taken together, these data suggest CFTR-dependent alterations in activation of several inflammatory signaling routes that may be important for the enhanced inflammation observed in CF. We are now further characterizing these pathways and extending these data to primary CFTR-defective cells, including CF lymphocytes, monocytes and nasal epithelial cells. These data may help to better understand the proinflammatory events observed in CF and may lead to identification of novel drug targets to correct CF-related inflammation.

ICAM-1 Expression in Host Cells Following *Burkholderia cepacia* Complex (Bcc) Infection

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Burkholderia cepacia complex (Bcc) is a group of Gram-negative opportunistic pathogens that colonise the lungs of patients with cystic fibrosis (CF). These pathogens are extremely difficult to eradicate and their mechanisms of pathogenesis are poorly understood. To date, 17 species of Bcc have been identified. The two most virulent are *B. multivorans* and *B. cenocepacia*. Currently, *B. multivorans* is the most commonly identified new acquisition in both Europe and North America. Bcc are inherently resistant to antimicrobial agents, promote a potent inflammatory response in the lung and are capable of disrupting epithelial integrity which results in penetration through lung tissue causing septicaemia. This study aims to establish if Bcc infection is associated with an increase in the expression of the neutrophil ligand intercellular adhesion molecule (ICAM-1). The expression levels of ICAM-1 in response to both *B. multivorans* and *B. cenocepacia* infection in epithelial and endothelial cell lines were determined.

ICAM-1, which is expressed on multiple cell types throughout the body, plays an important role in the migration of leukocytes to the site of infection. ICAM-1 is a major ligand for leukocyte function associated with lymphocyte function associated antigen-1 (LFA-1) and macrophage-1 (Mac-1).

ICAM-1 expression was investigated in both epithelial and endothelial cells in response to either Bcc or LPS exposure using confocal microscopy. ICAM-1 expression levels were compared on CFTR positive (16HBE14o-), CFTR negative (CFBE41o-) lung epithelial cells and umbilical cord endothelial cells (Huvec-cs). The cells were co-cultured with a *B. multivorans* strain LMG13010 and a *B. cenocepacia* strain J2315 for 48 hours and the effect on ICAM-1 determined. ICAM-1 expression was elevated in response to *B. multivorans* LMG13010 and *B. cenocepacia* BC7 in both CFBE41o- and 16HBE14o- cells. There was no significant difference in ICAM-1 levels in CFTR positive and CFTR negative cells. However, the endothelial cells demonstrated a more rapid response to Bcc infection with ICAM-1 levels at 12 hours comparable to that on epithelial cells after 48 hours bacterial exposure.

In order to determine whether the bacterial endotoxin, lipopolysaccharide (LPS) was a contributor to the ICAM-1 response, epithelial and endothelial cells were exposed to purified *B. multivorans* LMG13010 LPS. A significant up-regulation of ICAM-1 expression was observed in the presence of LPS compared to control cells and was comparable to levels induced by intact bacterial cells. As with bacterial exposure, there was no significant difference between ICAM levels in CFTR positive and CFTR negative cells. Furthermore, endothelial cells were sensitive to 10 times less LPS than the epithelial cells, indicating their potential to respond rapidly to the initial stages of Bcc colonization by facilitating an influx of immune cells to the lung.

This host response whilst initially beneficial, is ultimately deleterious to the host in infections which are persistent and associated with limited bacterial clearance by these cells. Therapeutic targets aimed at regulating this response will have the potential to limit the lung damage caused by the trafficking of immune cells to the CF lung.

Lack of RAB7 Expression and Activation Promotes Toll-Like Receptor Recycling in CF Epithelial Cells

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Introduction: Prolonged *Pseudomonas aeruginosa* (*PA*)-induced Toll-like Receptor (TLR4) signalling contributes to chronic pulmonary inflammation in Cystic Fibrosis (CF). Internalisation and subsequent endo-lysosomal degradation is important in terminating inflammatory TLR signalling. Our previous work suggested that sustained endosome formation and rapid recycling of TLR4 contribute to prolonged pro-inflammatory responses in the CF epithelium [1]. The guanosine triphosphatase (GTPase) Rab7b plays an important role in directing TLR4 towards the lysosome for degradation. This results in a negative regulation of TLR4 protein levels on the cell membrane [2]. Rab7b may be activated by the nucleoside diphosphate (NDP) kinase Nm23 which phosphorylates GDP in GTP-binding proteins such as Rab7b. However, the CFBE cell line displays a reduction in the phosphorylation and phosphotransferase activities of NM23 [3] which may account for the apparent failure in timely endo-lysosomal degradation in the CF epithelium. Here, we sought to investigate the role of Rab7b and Nm23 in endo-lysosomal processing in CF epithelium.

Methods: Non-CF (16HBE41o-) and CF (CFBE410-) bronchial epithelial cell lines grown in submersion were stimulated with 50µg/ml *PA*-lipopolysaccharide (LPS; Sigma) for 0-24h. Primary nasal epithelial cells (NECs) from CF patients (homozygous F508del) and age-matched controls were fully differentiated at air-liquid interface and stimulated with LPS (*PA*, Sigma) for 24h. Expressions of Rab7b and the small GTPase NM23 (splice variants A and B) were investigated using qPCR (Lightcycler, Roche) and Western Blotting. Statistics were calculated by ANOVA.

Results: Following LPS treatment, CFBE cells displayed persistent protein expression of the late endosomal marker Rab7b at all time points examined, while HBE cells showed peak expression 1h post-stimulation ($p < 0.01$, Kruskal Wallis) which subsequently retuned to basal levels. NECs (24h only) showed no significant change in Rab7b mRNA expression. In HBE following stimulation, mRNA expression of Nm23 increased over time with peak expression at 4-8h. In contrast, in CFBE cells Nm23A mRNA levels diminished over time. In both cell lines Nm23A reached basal levels 24h after stimulation. Nm23B followed a similar pattern. NECs stimulated with LPS for 24h showed no significant change in Rab7b mRNA expression between the CF and non-CF controls. Nm23A mRNA expression increased over time in HBE cells with peak expression 4-8h post-stimulation. In contrast, LPS treatment of CFBE cells caused a time dependent reduction in NM23A mRNA levels up to 8h post-stimulation. In both cell lines Nm23 expression returned basal levels 24h after stimulation. Nm23B followed a similar pattern.

Conclusion: Despite high and persistent protein expression of the late endosomal GTPase RAB7b in CF epithelial cells RAB7b may not be activated because of reduced expression of the diphosphate (NDP) kinase Nm23. Together with the described reduced activities of Nm23 in CFBE cells [3], this may contribute to dysregulated endo-lysosomal degradation and subsequent predominant recycling of TLR in the CF epithelium. Future work will investigate the role of Rab effector proteins.

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1. Kelly *et al.* Pediatric Pulmonol 2010, 2. Wang *et al.* Blood 2007, 3. Treharne *et al.* FEBS Lett. 2009

Investigation of Putative Virulence Factors of an Emerging Cystic Fibrosis Pathogen, *Pandoraea*

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Chronic bacteria infection and inflammation of the lung are the main cause of morbidity and mortality in patients with Cystic Fibrosis (CF). Typical CF pathogens include *Pseudomonas aeruginosa, Staphylococcus aureus* and *Burkholderia cepacia* which have all been studied extensively, however very little is known about the clinical impact of the new genus, *Pandoraea*. *Pandoraea* species are Gram negative, glucose non-fermenting rods that are capable of causing severe lung infection and in some instances death of CF patients. Little is published to date about the virulent characteristics of *Pandoraea*. This study examined a number of virulence factors that might enhance the pathogenesis of *Pandoraea* in the CF lung.

Previously, we have shown that *Pandoraea* isolates were capable of inducing the production of pro-inflammatory cytokines, IL-6, IL-8 and TNF-α in two human bronchial epithelial cell lines; HBE and CFBE cells (which are CFTR $^{+/+}$ and CFTR $^{-/-}$) with heat treatment having a significant impact on these cytokines production. In this study, the stimulation of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) in a phorbol myristate acetate (PMA) differentiated macrophage cell line; U937 in response to *Pandoraea isolates* (bacterial cell, cell free and heat treated cells) was examined. All the *Pandoraea* isolates triggered the release of these cytokines in U937 cells which demonstrated that macrophages secrete cytokines very effectively in response to exogenous stimulation. This can serve as a key in understanding the importance of macrophage in bacteria infection.

A range of *Pandoraea* isolates were also screened for other potential virulence factors, specifically proteases, lipase, siderophore production and haemolytic activity. In this study, *Pandoraea* isolates were screened for proteolytic activity and the results demonstrated that they expressed serine proteases as their dominant protease. All the *Pandoraea* isolates examined produced high levels of lipase activity and siderophore production thus indicating these could be potential virulence factors for *Pandoraea*. However the results obtained for haemolytic activity demonstrated that only four of seven isolates screened were haemolytic suggesting that haemolytic activity may not be an important virulence for some of the *Pandoraea* isolates. Further investigation of the involvement of these factors in CF pathogenesis is warranted as this will provide us with a comprehensive understanding of the bioactions of *Pandoraea*, allowing for the development of targeted and more effective therapies to treat patients.

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Exploiting Antimicrobial Mechanisms of Human Macrophages

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It is well documented that macrophages phagocyte and ten kill intracellular bacteria, thereby contributing to defend many organs from invading bacteria. Macrophages posses several weapons to kill engulfed microorganisms such as reactive oxygen species, and proteins/peptides which become activated following interaction with bacteria and/or release of bacterial substances. The contributions of these mechanisms to bacterial clearance is still not completely clear additionally, different bacterial species have evolved mechanisms to counteract macrophages microbicidal activity. Accordingly to previously published data, we have observed that macrophages from subjects affected by CFTR possess less ability to kill *Pseudomonas aeruginosa* with respect to healthy individuals. It has been claimed that CFTR deficiency alters phagolysosomal acidification which in turn would affect bacterial killing. Additionally, by using murine lung macrophages, it has been recently proposed that CFTR deficiency results in a chronic accumulation of ceramide, failure to stimulate NADPH oxidase activation, inability to release ROS and to kill *Pseudomonas*. In order to explore the mechanisms underlying the bactericidal activity of human macrophages we have analyzed the contribution of the oxidative-dependent mechanisms on the ability of macrophages to kill the engulfed bacteria. For this analysis we have used macrophages differentiated *in vitro* by conditioned medium. First, we have tested the susceptibility of different bacterial strains to oxidative stress in vitro then, macrophages treated or untreated with a specific inhibitors of NADPH-oxidase (DPI) were infected with the *P.aeruginosa* strains. The results obtained revealed a strong correlation between the bacterial sensibility to hydrogen peroxide and their NADPH oxidase-dependent killing by macrophages: the bacteria showing higher resistance to H_2O_2 were also less susceptible to NADPH-dependent killing; on the contrary an H_2O_2-sensitive *P.aeruginosa* strain was rapidly killed. Subsequently we have analyzed the fraction of intracellular bacteria killed via NAPDH-oxidase in macrophages from Cystic Fibrosis patients and healthy donors. Preliminary data suggest that this mechanism is less active in CF macrophages with respect to healthy donors.Overall we present evidence of NADPH oxidase-dependent microbicidal activity against *P.aeruginosa* strains in human macrophages differentiated *in vitro* from peripheral monocytes.

Pseudomonas aeruginosa Increase Gap Junction Channels in Calu-3 Cells by a TLR5-Dependent Mechanism

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The airway surface liquid (ASL) plays a critical role in mucociliary clearance, a mechanism that is compromised in CF patients. We have recently reported (Scheckenbach et al. 2011, Am J Respir Cell Mol Biol 44:74-82) that gap junction channels contribute to CFTR activity and ASL volume in the Calu-3 cell line. This observation prompted us to investigate whether connexins (Cxs), which form gap junctions, are modulated during the airway epithelial response against *Pseudomonas aeruginosa* (*Pa*). To address this question, we infected Calu-3 airway epithelial cells grown on Transwell inserts with the *Pa* laboratory strain PAO1. PAO1 increased Cx43 expression of about 2 fold in Western blots. Cx43 was also up-regulated in Calu-3 cells exposed to heat-killed PAO1, suggesting that this effect was mediated by activation of pathogen recognition receptors (PRRs). To evaluate which PRRs may regulate Cx43, we infected Calu-3 cells with PAO1 mutant strains lacking either pili (*pilA*), flagella (*fliC*) or lipopolysaccharide (LPS) O-side chains (*algC*, *wbpL*). Interestingly, only the *fliC* mutant failed to increase Cx43 expression in Calu-3 cells. PAO1 activated the innate immune response of Calu-3 cells, as evidenced by enhanced interleukin-8 (IL-8) production. However, unlike any other mutant strains, the *fliC* mutant failed to induce IL-8 release, suggesting that innate immune response against *Pa* in Calu-3 cells is mainly triggered by flagellin binding to Toll-like receptor 5 (TLR5). Inhibitors of phosphatidylinositol 3-kinases (PI3K) and extracellular signaling-regulated kinases (ERK) blocked the increase of Cx43 expression induced by PAO1. These results indicate that Cx43 is a target of *Pa*-dependent signaling via TLR5 in airway epithelial Calu-3 cells. Gap junction channels enable the intercellular spread of ions, nucleotides and second messengers for coordinating the tissue activity. Increased Cx43 expression in Calu-3 cells may represent a defense mechanism of the airway epithelium against *Pa* infection.

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P. 32

COMMD1: An Anti-Inflammatory Protein in the Context of Cystic Fibrosis?

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Cystic fibrosis (CF) is a severe disease caused by mutations in the *CFTR* gene, which encodes an epithelial anion channel. Morbidity is mainly due to the lung disease, characterized by a chronic neutrophilic inflammation. Deregulation of inflammatory pathway is observed in the airways of CF patients, as evidenced by an increased NF-κB response. Consequently, pro-inflammatory cytokines such as IL-8 are increased.

We have previously identified COMMD1 as a new CFTR partner and shown that CFTR was protected from ubiquitination by COMMD1, which sustains CFTR expression at the plasma membrane. COMMD1 is a protein associated with multiple cellular pathways, including sodium uptake through interaction with ENaC (epithelial sodium channel) and NF-κB signalling.

We studied the influence of COMMD1 overexpression/extinction on NF-κB pathway in CF and non-CF bronchial epithelial cells (IB3-1 and S9 respectively) as well as in stably transfected F508del- or wild-type-CFTR HeLa cells. To decipher the underlying mechanisms, we have first performed a set of luciferase assays in order to test NF-κB transcriptional activity in the presence or absence of each protein. TNFα or p65/p50 stimulation produced a higher increase of NF-κB activity in CF versus non CF cells. Overexpression of COMMD1 in the same experimental conditions decreased NF-κB activity by half in both cell lines. As IL-8 promoter contains NF-κB responsive elements, we performed such experiments with the wild-type and mutant IL-8 promoter constructs.

Our results show for the first time the anti-inflammatory properties of COMMD1 in bronchial cells. Thus, increasing COMMD1 expression may provide an approach to simultaneously inhibit ENaC absorption, enhance CFTR trafficking and inhibit NF-κB pathway, three major issues in cystic fibrosis.

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Room: Salone Bruschi

Posters – CF Microbiology

P. 34

Three-Dimensional Modelling of Native Mucins Using Electron Tomography

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Although the primary defect in cystic fibrosis is the dysfunctional CFTR protein, the major pathology of the disease is due to aberrant mucus properties which give rise to repeated and prolonged infections of the lungs. Electron tomography was used to generate a three-dimensional model of a mucin network in its native physiological state. This has allowed the examination of intrinsic differences in the properties of healthy and cystic fibrosis phenotype mucus which could potentially lead to a better understanding of how mucus gels behave *in vivo*. Human sublingual and submaxillary mucous secretions were extracted and 90% of the water was removed using 14 kDa MWCO dialysis tubing immersed in Aquacide II followed by centrifugation through a 1 MDa MWCO filter to remove small proteins. The resultant partially dehydrated mucus was then frozen in vitrified ice and imaged using an FEI Polara 300kV FEG transmission electron microscope. Tomograms were generated using IMOD.

Effects Of Azythromycin on the Regulation of Metalloproteases Released by *Pseudomonas aeruginosa* Clinical and Laboratory Strain

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Modulation of *Pseudomonas aeruginosa* virulence factors was suggested as mechanism for azithromycin (AZM) beneficial effects in CF patients particularly susceptible to chronic *Pseudomonas aeruginosa* (Pa) infection in the airways. Our work was aimed to study the regulation of metalloproteases (MMPs), known for their strong proteolytic activities in cells and tissues, released by Pa strains after AZM treatment.
The functional assay such as zymography revealed that the members of the metalloprotease family of enzymes were poorly expressed by the laboratory strain PAO1 and clinical strain AA43 (collected 7.5 years after Pa acquisition). The AA2 clinical strain collected at the onset of chronic colonization (0.5 year) appeared to express a much larger set of proteases and decreased their expression after exposure to AZM. Western blot analysis for alkaline metalloprotease (AprA), one of the metalloproteases detected in the conditioned medium (CM) from PAO1 and clinical strains in our previous study, demonstrated a diminished release of this enzyme in CM from AA2 strain cultured in presence of AZM.
Given that higher expression of MMPs was detected in AA2 strain, in comparison with AA43, we have evaluated MMPs activity and AprA expression in the clinical isolates derived from different CF patients featuring sporadic or chronic colonization. Pa strains from about 400 CF patients followed at the Cystic Fibrosis Center of Verona were available for this study.
We have evaluated MMP activity in CM derived from 49 isolates defined as chronic and 44 classified as sporadic. In 32 of the sporadic strains we detected MMP activity (73%) while this was true only for 17 of 49 (34%) among the chronic strains ($p < 0.0001$). We then evaluated whether MMP activity was associated to AprA expression in a subset of these strains. This association was not detected in all the strains positive for MMP activity indicating the presence of other MMPs as the major source of proteolytic activity in selected strains.
We then evaluated the effect of AZM in the same series of Pa strains isolated from CF patients. Within this context we have observed a decreased MMP activity in CM derived from 22 defined as sporadic isolates of 27 strains analized (81%), cultured in presence of AZM. We also studied whether MMP activity was affected by AZM treatment in CM from other 16 isolates classified as chronic. AZM had effect only on 5 chronic strains (31%).
Finally, the correlation reported among MMP activity/expression and specific clinical conditions suggest that MMPs might play a role in the clinical manifestations of Pa infection supporting a link among MMP expression/activity and Pa virulence in CF patients. Future evaluation of MMP activity in a larger series of clinical isolates may provide insights on the correlation between this parameter and lung function in patients colonized by Pa strains. Moreover the analysis of CM derived from the Pa clinical isolates grown in absence and in presence of AZM is provided and can contribute to a better explaining the variable response to Pa infection and sensitivity to AZM known to occur in CF patients.

Host Response to *Pseudomonas aeruginosa* Adaptation During Airway Chronic Infection

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Pathogen recognition and induction of immune responses are important for efficient elimination of infection. However, life-threatening chronic infections are maintained by bacterial patho-adaptive variants that employ strategies to evade or modulate these defences. In cystic fibrosis (CF), the host response to *P. aeruginosa* patho-adaptive variants remains to be established.

The most effective strategy for hijacking genes involved in innate immune responses involves steric shielding or modification of exposed molecules. Comparing lipid A and peptidoglycan (PGN) of sequential strains isolated from CF patients, including mucoid and non-mucoid phenotypes isolated during a period of up to 7.5 years, we found for the first time specific structural modifications temporally associated with CF lung infection (Cigana et al, PlosOne 2009). Both bacterial structures (LPS and PGN) and whole cell bacteria of early and late clinical strains had different potencies when activating host innate immunity *in vitro*. TNF-α and IL-8 protein release was significantly decreased in epithelial bronchial cells of CF origin IB3-1 and their wt-like isogenic cells C38 and macrophage-like cells (THP-1) after treatment with late strains in comparison to early strain. Similar results were obtained with *P. aeruginosa* mutants defective in several virulence factors (Bianconi et al, PlosPathogen 2011). *P. aeruginosa* mutants stimulated less the immune response when compared to their wt strain. However, microarray analysis showed that late strains are prone to revise their interaction with host by activating pathways relevant for damage and remodelling process. Increased cytotoxicity and matrix metalloprotease-9 expression were observed after infection with late strains compared to early strains.

Next, we analysed the host response to *P. aeruginosa* in a multihost pathogenesis system including four different models, namely, *Caenorhabditis elegans, Galleria melonella, Drosophila melanogaster* and mouse. *P. aeruginosa* strains at the onset of infection are more lethal than late isolates from the same patient when tested in *C. elegans, Galleria melonella* and *Drosophila melanogaster*. In murine model of acute infection, the early *P. aeruginosa* strain induced higher mortality than late clonal strains. Although attenuated in mortality, *P. aeruginosa* late isolates retained their capacity to persist when embedded in agar beads in models of chronic infection. Histological examination, PAS-staining and Tunnel assay of lung tissue sections showed that early strains induced pronounced leukocytes recruitment indicating strong inflammatory response while late strains increased numbers of mucin-positive goblet cells and apoptotic cells, a typical hallmark of damage in the airway chronic diseases.

Our findings suggest that during long-term infection *P. aeruginosa* revises its interaction with CF host by activating alternative pathways including evasion of the immune response, non-inflammatory cell death and those relevant for tissue damage and remodelling process to ultimately result in chronic disease and decline in lung functions.

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Room: Salone Bruschi

Posters – Genes and the Environment

P. 37

A European Meta-Analysis of Ancestral Haplotype 8.1 and Lung Disease Severity in Cystic Fibrosis

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The clinical course of cystic fibrosis (CF) varies considerably among Caucasians carrying the same *CFTR* mutation suggesting that additional genetic modifiers may contribute to this variability. The highly conserved ancestral haplotype 8.1AH contains a cassette of linked genes and is carried by up to 20% of Caucasians, comprising linked gene polymorphisms that play a key role in the inflammatory response: *LTA* +252A/G (Lymphotoxin A), *TNF* -308G/A (Tumor necrosis factor), *HSPA1L* +1267A/G (Heat shock protein A1 Like) and *RAGE* -429T/C (Receptor for Avanced Glycation Endproducts). Moreover, 8.1AH has recently been associated with delayed onset of lung bacterial colonization in CF patients in some cohorts. As airway inflammation is a key component inducing CF lung damage, we investigated whether the 8.1AH represents such modifier in CF patients.

To test whether the 8.1AH modified lung disease CF severity, we analyzed a cohort of 436 European CF patients from France (n=230), UK (n=111) and Germany (n=95). Lung function was evaluated by forced expiratory volume in one second (FEV_1) measurements. Age adjusted FEV_1) differences were calculated in each country, and a random effect meta-analysis was used to pool the results.

Distribution of the 8.1AH carriers was similar: 22% in the French; 28% in the UK and 29% in the German CF patients. We found 8.1AH was significantly associated with lower FEV_1 in all 3 cohorts (meta-analysis: p=0.03). These findings support the concept that the 8.1AH appears to be an important genetic modifier of lung disease in CF. We suggest that examination of multiple linked genes provides an alternative avenue of research into genetic variability in CF outcome.

Implication of the ESE Transcription Factors (Epithelium-Specific Ets-Like Factors) in *CFTR* Gene Expression

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Aim: Despite the important number of mutations, less than 1% have been identified in regulatory regions or in the *CFTR* promoter. These may account in part for the phenotypic variability observed in some patients. In order to identify new cis- and trans- regulatory elements implicated in *CFTR* gene expression, we evaluated members of the ESE transcriptions factors (Epithelium-specific Ets-like factors) as potential regulators of *CFTR* expression.

Methods: Different sizes of the *CFTR* promoter were subcloned upstream of the luciferase reporter gene. These constructs were co-transfected in IB3-1 epithelial cells with either ESE-1, ESE-2 or ESE-3 and luciferase activity measured. Interaction between ESE-1 and the *CFTR* promoter was assessed by chromatine immunoprecipitation. Endogenous *CFTR* expression levels were measured by qRT-PCR after over-expression of the ESE transcription factors in Calu-3 cells (Amaxa technology).

Results: Transcriptional activity of *CFTR* promoter was enhanced after ESE-1 over-expression by a 2 to 6 fold depending of the tested construct while it was weakly affected by either ESE-2 or ESE-3. Site directed mutagenesis of a potential binding site at position -98 prevented this increase. Interaction between ESE-1 and *CFTR* promoter was validated by chromatine immunoprecipitation obtained from Calu-3 cells. Over-expression of ESE-1 in Calu-3 cells doubled endogenous *CFTR* expression in these cells (n=2) while over-expression of ESE-2 or ESE-3 induced a moderate variation of gene expression (n=2). This assay will also be performed in human primary airway epithelial cells (Epithelix).

Discussion and conclusions: The *CFTR* gene appears to be a transcriptional target of the ESE-1 transcription factor, implicating a binding site located in position -98. Identifying sequence variations within this binding site or associated to ESE-1 altered expression levels could explain in part the phenotypic variability for some patients. This study therefore contributes to a better phenotype-genotype understanding.

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Room: Salone Bruschi

Posters – Therapeutic Strategies to Correct Ion Transport Defects

P. 39

Regulation of Ion Transporters and Airway Surface Dynamics by Lipoxin in Cystic Fibrosis Bronchial Epithelium

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We have investigated the role of the endogenous anti-inflammatory lipoxin LXA_4 in modulating Cl^- secretion and Na^+ absorption, airway surface liquid height (ASLh) and ciliary beat frequency (CBF) in CF and non-CF bronchial epithelia.
CF (CuFi-1) and non-CF (NuLi-1) bronchial epithelial cell lines were grown under an air-surface liquid interface into well-differentiated epithelia. ASLh and CBF were measured using confocal fluorescence microscopy and ion transport using patch-clamp and short-circuit current techniques.
LXA_4 (1nM) treatment for 15 minutes, increased ASLh by 47.5±0.5% and 103.0±3.0% in NuLi and CuFi epithelia respectively ($P< 0.001$, n=18). The stimulatory effect of LXA_4 on ASLh was sustained over 24 hours in the CF epithelia and was inhibited by the following pre-treatments: bumetanide, amiloride, Boc-2 (LXA_4 receptor antagonist), reactive blue (P2Y receptor antagonist) and extracellular hexokinase (ATP hydrolysis). LXA_4 stimulated CBF, intracellular Ca^{2+} mobilization, Cl^- secretion and inhibited Na^+ absorption in the CF epithelia. These effects of lipoxin involving the FPR2 receptor, apical ATP release, purinoreceptor activation, inhibition of Na^+ absorption and stimulation of Cl^- secretion to enhance airway surface liquid dynamics open up a new therapeutic avenue to promote mucociliary clearance in cystic fibrosis airways.

Molecular Determinants Underlying the Stimulatory Action of the CFTR Potentiator CBIQ on the Ca^{2+}-Activated K^+ Channel KCa3.1

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Several strategies have been implemented to correct ion transport defects in cystic fibrosis (CF) through a direct action on CFTR activity and/or expression. Other approaches propose to bypass CFTR function and use, as therapeutic targets, channels other than CFTR known to be important for fluid and electrolytes transepithelial transport. There is now increasing evidence that the calcium-activated K^+ channel KCa3.1 expressed both at the apical and basolateral membranes of several epithelia is important to maintain a favorable electrochemical gradient for Cl^- secretion, while being a key regulator of the epithelial cell Ca^{2+} signaling process by facilitating Ca^{2+} influx. A research aimed at identifying structural parameters by which CFTR potentiators can simultaneously activate both CFTR and KCa3.1 thus offers new perspectives to the correction of ion transport defects in CF epithelia. A study was undertaken where computer modeling, site directed mutagenesis and single channel patch clamp recordings were used to characterize the molecular determinants underlying the stimulatory action of CBIQ, a KCa3.1 potentiator also documented to activate CFTR. Experiments carried out with the constitutively active A279G-KCa3.1 mutant first confirmed that Ca^{2+} is absolutely required to the action of CBIQ. Single channel recordings next indicated that the fluctuation pattern of KCa3.1 is characterized by bursts of channel openings separated by Ca^{2+}-sensitive inter-burst silent periods, with the main effect of CBIQ being to drastically decrease the inter-burst silent periods while slightly increasing burst duration. These observations provided evidence for an action of CBIQ on the Ca^{2+}-dependence of the KCa3.1 activation process. Studies have already established that the Ca^{2+}-sensitivity of KCa3.1 is conferred by the Ca^{2+}-binding protein calmodulin (CaM) constitutively bound to a domain of the channel intracellular C-terminus. A structural model of the KCa3.1/CaM complex was generated through homology modeling, and the resulting 3D structure showed that the domains in KCa3.1 C-terminus extending from K312 to T329 and L361 to S372 were respectively involved in the constitutive binding of the CaM-C-lobe and the Ca^{2+}-dependent binding of the CaM N-lobe to KCa3.1. Binding of the CaM C-lobe and N-lobe to their respective segment on two adjacent KCa3.1 monomers resulted in turn in the formation of a dimeric structure stabilized in part by interactions between the residues E363 and R352 located at the interface of the dimeric complex. Substituted Cysteine Accessibility experiments confirmed that modulating the interactions between E363 and R352 affects the channel activity, with an increase in interactions mimicking the action of KCa3.1 potentiators such as CBIQ. The importance of these interactions to the stimulatory action of CBIQ was confirmed in patch clamp experiments where the mutation E363A was found to markedly reduce the potency of CBIQ to activate KCa3.1. These observations support docking predictions based on the Multiple Copy Simulation Search Method (CMSS) where the residues R352, E363 and L356 appeared to form a cavity suitable for CBIQ binding. Our results suggest that CBIQ activates KCa3.1 by interacting with residues in the KCa3.1 C-terminus involved in stabilizing the dimeric structure formed by the Ca^{2+}-dependent binding of CaM to KCa3.1.

New Approaches of Cell Therapy for Cystic Fibrosis: *In Vitro* Differentiation of Human Amniotic Mesenchymal Stem Cells in Airway Epithelium and Correction of the CF Phenotype

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The results of CF gene therapy show that the correction of the respiratory defect is only temporary, therefore novel approach for reconstitution of the airway epithelium and CFTR expression should be explored. Regenerative medicine needs a safe and ethically acceptable stem cell source for the development of new therapeutic strategies. In the present study, we evaluated the possibility of obtaining stem cells from human placenta and their ability to differentiate *in vitro* in airway epithelium for the cure of respiratory disease of CF.

Through mechanical separation and successive enzymatic digestions, amniotic mesenchymal stromal cells (AMSC) were isolated from twelve placentas. At least 33 x 10^6 cells (range: 26- 160 x 10^6) were recovered in each isolation with a viability of 85-90%. Isolated cells readily attached to plastic or basement membrane-coated culture dishes. Karyotype, immunofluorescence and real time PCR analyses of various stem cell markers were performed at different passages. hAMSCs displayed the capacity to differentiate towards mesodermal and endodermal lineages.

In order to evaluate the ability of hAMSCs to differentiate towards airway epithelium, we performed a dose-dependent experiment: hAMSCs stained with the vital dye CM-DiI were mixed with F508del homozygous CFBE41o- cells at different increasing ratios (1:20-1:5) and seeded onto semi-permeable filters. In co-cultures at the ratio of 1:5, 10.6% of the whole population showed both CFTR and CM-DiI positive staining by cytofluorimetric analysis, and this value decreased by reducing hAMSC number. Overall, our data showed that at least 50-80% of hAMSCs has acquired a detectable CFTR expression on the apical membrane above the CFBE background. Confocal microscopy showed that CFTR was highly expressed on the apical membrane of some hMSCs since red labelled cells showed a green staining at membrane level, whereas CFBE41o- monolayers in absence of hMSCs showed a background signal for CFTR expression, consistent with the lack of CFTR transport to the apical membrane in CF cells. Co-culturing induced also a small re-organisation of ZO-1 at the level of tight juntions at the highest ratio. The transepithelial resistance of co-cultures grown onto semi-permeable filters increased during culture above the values given by CFBE41o- cells, suggesting the establishment of tight junctions across the monolayer. Amiloride-dependent fluid absorption decreased when CFBE41o- were co-cultured with hAMSCs respect to CFBE41o- cells alone, above all with higher hAMSC-CFBE ratios, approaching the behaviour of non CF 16HBE14o- cell monolayers.

Overall, our data showed that amniotic cells has stem cell characteristics including self-renewal and multi-epithelial lineage differentiation potential; these cells may contribute to partial correction of the CF phenotype and are very likely an ideal candidate for cell-based therapy for CF. Whether the beneficial effects of placenta-derived cells could be due to differentiation of the transplanted cells themselves or to paracrine actions on the surrounding host tissue in order to reduce inflammation and promote regeneration remains to be fully elucidated. Future studies testing these cells in *in vivo* model, will help in finding a strategy of cell therapy for the cure of respiratory disease of CF, directly transferable to CF patients.

P. 42

Innovative Strategies for the Suppression of Fluid Hyperabsorption and the Recovery of Airways Hydration in Cystic Fibrosis

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The most serious problem experienced by CF patients is the airway disease, characterized by increased viscosity of mucus with subsequent infection and colonization of the lungs. These conditions are all consequences of airway dehydration. Na^+ absorption and Cl^- secretion are carefully regulated to keep an appropriate airway surface fluid (ASF) volume (7-10 µm) and ensure mucociliary clearance. In CF, the equilibrium between these two processes is disrupted because of the failure of Cl^- transport across the airway epithelium due to CFTR mutations. In this condition, Na^+ and water absorption became predominant leading to airway dehydration. Reduction of epithelial Na^+ channel (ENaC)-mediated sodium absorption, and Cl^- secretion stimulation may improve this situation. We recently found that short interfering RNA (siRNA) against ENaC reduces transepithelial Na^+ currents and fluid absorption in human bronchial epithelia, representing proof of principle that ENaC knockdown may have functional consequences in CF patients. Yet, knockdown was partial, siRNA doses were too high (with the risk of producing off-target effects), and other fluid absorption pathways were not explored. Now we aim to face all these points. In addition, we aim to establish the relative contribution to Na^+ absorption of ENaC and amiloride-insensitive channels.

To this purpose, we have measured the transepithelial Na^+ transport in polarized preparations of H441 cells and on primary human bronchial epithelial cells (HBEC). In spite of lacking of a significant expression of CFTR, H441 cells exhibit robust amiloride-sensitive currents. Therefore, were considered a good model of the bronchial epithelium. To further support this conclusion we have compared the expression of the three ENaC subunits on both cell types with the qRT-PCR. We found that the relative subunit abundance was similar for H441 and HBEC. On both cells, the α subunit resulted markedly more expressed than β, and γ expression was very low. Next, we have studied the transepithelial Na^+ transport in Ussing chamber. Dose-response relationships to amiloride showed that Na^+ current inhibition had to be fitted with two dissociation constants. The first, near 1 µM, is tipically of classical ENaC. The second, about 50 µM, may represent the so called Amiloride Insensitive Currents (AIC). The relative expression of these currents seem to be modulated by cAMP.

In order to knockdown ENaC, we have tested different concentrations of siRNA on H441 cells to find out the lowest effective dose. Silencing was evaluated measuring the short circuit current blocked by amiloride and the best result was obtained with 20 nM. Next, we have used this dose on HBEC and confirmed the reduction of ENaC-mediated currents. To evaluate the functional effects of ENaC silencing, we have also measured the height of ASF with a confocal microscope using Texas red-dextran to color the liquid phase.

Shift U1 snRNAs Targeted to an Intronic Splicing Silencer Correct Aberrant CFTR Exon 12 Skipping

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Different mutations, at or near donor splice sites (5´ ss) or within exonic regulatory elements, may cause cystic fibrosis inducing aberrant exon skipping. In the CFTR exon 12 we have studied several mutations either located at the 5'ss consensus or in the exon that induce exon skipping. We also identified a critical splicing regulatory element with inhibitory function (Intronic Splicing Silencer, ISS) located downstream the 5´ss, whose deletion partially activated exon 12 inclusion.
To favour normal over aberrant pre-mRNA splicing we explored the potential therapeutic effect of modified U1 snRNAs. Using engineered U1 snRNAs with modified 5´ ends, complementary either to the 5´ss or to the downstream ISS, we were able to revert CFTR exon 12 skipping induced by several disease-associated mutations. For exonic mutations c.1696G>A (A566T) and c.1731C>T (Y577Y) a single complementary change in position +4, which increases complementarity of U1 snRNA 5' end to exon 12 5'ss, stimulate exon inclusion. However, for mutations lying within donor site sequence (c.1766G>A, +3A>G and +5G>A) we observed rescue of exon 12 inclusion only when introducing an additional compensatory change, specific for each mutation. On the other hand, three shift U1s targeted to the ISS were able to induce exon inclusion (although to variable degrees) in all mutants. Interestingly, a unique shift U1, shift U1+11, corrected exon skipping caused by both exonic and 5'ss mutations. Antisense oligonucleotides or engineered U7 snRNAs complementary to the ISS did not correct exon skipping suggesting that specific U1 snRNA components are important for splicing rescue. This result highlights the potential therapeutic role of shift U1 targeted to ISS downstream the 5'ss in correcting different exon skipping mutations in CFTR. Experiments with splicing competent exon 12 CFTR expression vectors, with flanking intronic regions, are in progress to evaluate the shift U1 rescue at the CFTR protein levels and function.

Restoration of Chloride Efflux by Azithromycin in CF Human Bronchial Epithelial Cells

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Background: Cystic fibrosis (CF) is a hereditary disease caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene that encodes a chloride (Cl^-) channel. CF pulmonary pathophysiology is characterised by chronic inflammation and bacterial infections. Azithromycin (AZM), a macrolide antibiotic, has shown promising anti-inflammatory properties in inflammatory pulmonary diseases. Moreover, all clinical studies have presented an improvement of the respiratory condition of CF patients, but the molecular and cellular mechanisms remain unknown. The aim of this study was to investigate, in bronchial epithelial cells, the mechanism by which AZM has beneficial effects in CF patients.

Methodology / Principal findings: We demonstrate that AZM does not have an anti-inflammatory effect on the CF human bronchial epithelial cells nor on CFTR-inhibited primary human bronchial glandular cells. Contrary to what was observed in non-CF cells, our data show no effect of AZM on IL-1β- or TNF-α-induced IL-8 secretion and NF-κB pathway. Activation of the NF-κB pathway was investigated by luciferase assay, western blotting, and by Förster Resonance Energy Transfer imaging, allowing the detection of the interaction between the transcription factor and its inhibitor in live cells (Saint-Criq, Biochemical And Biophysical Research Communications, Submitted).
More, we have also analysed the effects of AZM on Cl^- efflux using a specific Cl^- probe.
The effect of AZM on Cl^- efflux using a specific Cl^- probe have also been analyzed and demonstrate that AZM treatment induced a restoration of Cl^- efflux in CF bronchial epithelial cells (Saint-Criq V., Antimicrob Agents Chemother, In press).

Conclusions / Significance: Taken together, these data suggest that AZM could improve CF patients pulmonary pattern by acting on Cl^- efflux rather than by modulating inflammatory parameters.

Targeting the "Sick" Conformation of F508del-CFTR Protein

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The most frequent mutation in the *CFTR* gene responsible for Cystic Fibrosis (CF), among the nearly 1800 classified up to date (http://www.genet.sickkids.on.ca/), is a deletion of three nucleotides that causes the lack of phenylalanine 508 in NBD1 (F508del-CFTR). The resulting protein has reduced ability to escape from the endoplasmic reticulum (ER) and undergoes premature degradation in a proteasome-dependent manner. This results in a significant decrease in the population of functional CFTR channels at the apical plasma membrane and has been defined as a trafficking or folding defect. Moreover, a residual amount of F508del-CFTR, which in some conditions reaches the native destination, exhibits another dysfunction associated with its lower activity, which is known as a gating defect.

As it has previously been shown by Molecular Dynamic(MD) simulation (Wieczorek and Zielenkiewicz 2008) the F508del mutation increases the conformational freedom of the NBD1 domain. As a consequence, a much greater average surface of solvent-accessible hydrophobic residues can be observed in F508del than in wild-type NBD1. This substantial exposure of hydrophobic surface could represent a signal for the interaction with housekeeping proteins and result in premature degradation of mutated CFTR. We assumed that such an unwanted situation could be interrupted pharmacologically by binding small molecules around the hydrophobic areas and subsequently inhibit the interactions between F508del-CFTR and proteins involved in the degradation process.

To address this problem we used a structure-based Virtual Screening (VS) approach, to find chemical compounds displaying favorable binding to two pockets around hydrophobic surfaces of F508del-NBD1. We screened the structures of small molecules retrieved from the National Cancer Institute Database using the DOCK 6 program against two binding pockets defined on the surface of the F508del-NBD1 unique conformation. The number of complexes initially obtained were gradually minimized and evaluated by seven scoring functions (D_Score, ChemScore, PMF_score, G_score, HPScore, HMScore and HSScore). Instead of using a consensus scoring protocol, we focused our attention on the top 10 results from each scoring function. A critical visual assessment resulted in the selection of twelve chemical compounds for experimental tests.

Afterward we evaluated the effects of these compounds on three F508del-CFTR parameters: protein processing, cell localization in HeLa cells stably expressing F508del-CFTR, and function in two cell lines (HeLa and CF-KM4). Our results show that at least four of the tested molecules exhibit a correcting activity on F508del-CFTR. Moreover, two of them also correct CFTR function in epithelial cell lines derived from CF patients, while they are not toxic in our culture conditions.

Differential pattern of microRNAs expression in Cystic Fibrosis and Normal Human Bronchial Epithelial cells

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Cystic Fibrosis (CF) is due to mutation in the chloride channel CFTR gene causing impairment of chloride secretion in the apical membrane of epithelial cells. The most common CFTR mutation in CF is the deletion of the phenylalanine at the position 508 of the protein (DF508), leading to the retention of the mutant protein within the ER and its rapid degradation via ERAD pathway. More than 1800 mutations have been identified so far, variably affecting CFTR activity, but none of them have been clearly linked to a certain phenotype. In other words, even if carrying the same genotype, CF patients might face a different development of their disease. Clearly, CF disease also depends on other genetic and/or environmental factors. Lately, a number of studies focused on modifier genes that may determine severity of lung disease, as MBL and TGF-B. Recently, Wright et al. performed whole-genome microarrays assays of nasal cells from non-CF individuals, mild CF and DF508-CFTR homozygous patients and found a total of 652 out of 1187 genes differentially expressed in these three groups (1).
MicroRNAs (miRNAs) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. First described in 1993, miRNAs are non-coding RNA ; instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a premiRNA and finally into a functional miRNA. Mature miRNA molecules are partially complementary to one or more mRNA molecules, and thus down-regulate gene expression by inducing mRNA degradation or repression of protein expression by translation inhibition. Differential expression in miRNAs has been shown to influence disease development in Alzheimer's disease, cancer, heart failure and COPD. We performed microarray analysis of miRNAs expression using primary bronchial epithelial cells from three different donors (DF/DF) and from three non-CF donors. Among the 856 miRNAs identified in the human genome at the time we performed the study, 94 were expressed in HBE cells, with 16 of them differentially expressed between CF and non CF patients:

- miR22, miR29a, miR29c, miR30a, miR30b, miR30c, miR30d, miR30e, miR151-3p, miR151-5p were upregulated in CF-HBEs;
- miR103, miR107, miR146a, miR203, miR224, miR1246 were down-regulated in CF-HBEs.

Real-time PCR experiments partially confirmed microarrays results, finding significant differences in expression of 11 of the 16 miRNAs. Further target analysis of the differentially expressed mi-RNAs is expected to identify new therapeutic targets in CF.

Supported by the Cystic Fibrosis Fundation

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Room: Salone Bruschi

Posters – Others

P. 47

Defective CFTR Expression and Function are Detectable in Blood Monocytes: Development of a New Blood Test for Cystic Fibrosis

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Background: Evaluation of cystic fibrosis transmembrane conductance regulator (CFTR) functional activity to assess new therapies and define diagnosis of cystic fibrosis (CF) is cumbersome. It is known that leukocytes express detectable levels of CFTR but the molecule has not been characterized in these cells.
In this study we aim at setting up and validating a blood test to evaluate CFTR expression and function in leukocytes.

Description: Western blot, PCR and cell membrane depolarization analysis by single-cell fluorescence imaging, using the potential-sensitive $DiSBAC_2(3)$ probe were utilized. Expression of CFTR isoform was detectable by western blot in monocytes. CFTR agonist administration induced membrane depolarization in monocytes isolated from non-CF donors (31 subjects) and, to a lesser extent, obligate CFTR heterozygous carriers (HTZ: 15 subjects), but it failed in monocytes from CF patients (44 subjects).
We developed an index (named “CF-index”) capable to identify CF from both non-CF and HTZ (both $p< 0.001$) and between non-CF and HTZ groups ($p=0.015$). Nasal Potential Difference, measured in selected subjects had concordant results with monocytes assay.

Results and significance: CFTR has been found expressed in human monocytes. We also showed that CFTR can be functionally evaluated in 5 ml of peripheral blood suggesting applications in both basic and translational research: from drug development to the evaluation of functional outcomes in clinical trials.

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