Final Report

HSC R&D Division
Final Report

Award Details

HSC R&D File Reference:	COM/4978/14				
Project Title:	Opportunity-led Research: Chronic Lung Infection				
Start Date:	12.03.14	**End Date:**		**Duration:**	
Name of Award Holder:	Professor Michael Tunney				
Employer of Award Holder:	Queen's University Belfast				
Host Research Organisation:	Queen's University Belfast				
Name of Lead Supervisor: **(only applicable to training awards)**	NA				

Report

Summary of Research for Lay Audience (200 words)

Information which may be used to communicate your research to the public. (involvement of a lay person in writing/reviewing this summary is recommended)

Lung infection is the main cause of disease and death in people with cystic fibrosis (CF). In this project, lungs were collected from people with CF and other respiratory diseases undergoing transplantation and airway tissue and sputum samples from different areas of the lungs examined. Bacteria present in these samples, and their numbers, were determined using sensitive methods which detect even small quantities of these infectious agents.

Our results show that when patients undergo lung transplantation, there are very few different types of bacteria present in their lungs with only bacteria, such as *Psueudomonas aeruginosa*, known to result in poor lung function detected in large numbers. In contrast, a wide range of different types of bacteria were present in healthy control lung tissue. There were also differences in the number of bacteria present in different areas of the lung.

Future work will determine whether these findings are important to the health of people with CF by comparing the relationship between numbers of bacteria detected in a particular tissue sample and damage to that issue. We will also investigate factors that result in one or two bacteria dominating in the airways and how ‘healthy’ bacteria can be maintained in the airways of people with CF.

Description of progress in achieving research objectives (1000 words)

Describe the progress of the research programme referring to: the research objectives (including any deviation from original objectives); the methodologies used; any problems/difficulties encountered and how these were overcome; and the findings to date.

1. Background

Previous studies, including those within our own group have revealed a complex and diverse microbiota in sputum of patients with chronic respiratory disease (1-4). However, the impact that the microbiota has on structural organization and changes of the lung in chronic pulmonary disorders are poorly understood. Furthermore, no studies have determined if the microbiota is different in distinct anatomical regions in the lung and whether such differences, if present, account for the regional variation in structural damage and lung disease present in patients with chronic infection.

In this project, multiple airway tissue and sputum samples from distinct anatomical regions of have been extracted from frozen explanted lungs of patients with different types of chronic respiratory disease: Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD) and multiple sub-types of Chronic Lung Allograft Dysfunction (CLAD). This sampling was carried out by our collaborators at the KU Leuven (Belgium), a world-leading transplant centre. In our laboratory, bacteria present in these samples, and their numbers, has been determined using state-of-the art Illumina next-generation sequencing and Quantitative Polymerase Chain Reaction (qPCR). The data generated from these techniques will be compared with clinical, histological, radiological (High Resolution and micro Computed Tomography) and microarray data generated in collaborating institutions to determine the relationship between microbiota composition, localised structural damage and disease progression.

2. Aims

The aims of the study were to:

- Determine the lower airway microbiota in patients undergoing lung transplantation for chronic respiratory disease and investigate how it relates to localised structural damage and disease progression.
- Compare the microbiota in sputum versus lower airway tissue in chronic respiratory disease.
- Determine the spatial distribution of bacteria within the airways.
- Determine if the microbiota in end-stage respiratory disease is spatially heterogeneous and if so, how this relates to localised structural damage.

3. Methods

3.1 Sample Collection

To date, explanted lungs have been obtained from 62 patients with multiple types of chronic respiratory disease. Furthermore, control tissue cores were obtained from 13 patients who died from non-respiratory related causes. Full details of sample types are provided in Table 1. Transplantation was carried out at the University Hospital of Gasthuisberg, Leuven (Belgium), with subsequent tissue sampling carried out in the Laboratory of Pneumology, KU Leuven (Belgium) according to a previously published protocol (5-7). Tissue cores were stored at -80°C until shipped to Queens University Belfast (QUB).

Table 1: Samples received in Belfast

Disease	CF	COPD	RAS	BOS	EAA	Controls
No. of Samples	127	44	56	44	36	52
No. of Patients	17	11	14	11	9	13

* RAS = Restrictive Allograft Syndrome; BOS = Bronchiolitis Obliterans Syndrome; EAA = Extrinsic Allergic Alveolitis.

3.2 Lung Homogenate Preparation and DNA Enrichment

Prior to processing, descriptive notes were made on each tissue core, documenting size, shape, number of bronchioles visible, presence of blood / sputum plugs within sample, etc. A lung homogenate was prepared by dissecting the tissue into small pieces, followed by two rounds of vortexing (5mins) and homogenization at 6.0 m/s for 40s (Fast-Prep-24, MP Biomedicals, Calfironia, USA) in 2.8mm ceramic bead tubes (Cambio, Cambridge, UK), aided by the addition of 1mL of sterile Quarter Strength Ringer's Solution (QSRS). The resulting mixture was subjected to 10 minutes of ultrasonication (Branson 3510 sonicator, Branson, Danbury, USA) before being filtered through a 70µm cell strainer (Fisher Scientific, Waltham, Massachussets, USA) with the aid of centrifugation at 1RPM for 3 minutes.

Isolation and enrichment of bacterial DNA from lung homogenate was carried out using a QIAamp DNA microbiome kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions. DNA concentration and purity measurements were obtained using an Omega FluoStar multi-plate reader (BMG Labtech, Ortenberg, Germany). The resulting DNA was stored at -80°C until further analysis.

Any sputum plugs identified within tissue cores were isolated, pre-lysed using physical and enzymatic degradation steps and processed for automated bacterial DNA extraction using the MagNA pure system (Roche Diagnostics Ltd., West Sussex, UK.), before quantification and storage at -80°C. Process

controls, consisting of substitution of the sample material with DEPC water were performed with all extractions.

Prior to processing clinical samples, a considerable period of work was undertaken to optimise our in-house protocol for DNA extraction from lung tissue samples. This optimisation work was undertaken using mouse lung tissue and was critical to ensure the success of the project as the samples received from Leuven were low biomass samples containing small amounts of DNA. This work resulted in the introduction of two homogenization steps, additional vortexing procedures and increased sonication time. This improved DNA yield (with the aim of increasing success of PCR amplification) both using mouse lung tissue and subsequently when tested with clinical samples.

3.3 Development of a qPCR Assay for Estimating Total Bacterial Load

Two different detection methodologies (SYBR Green 1 and probe-based qPCR) were tested and optimized to develop this assay targeting the 16S ribosomal RNA gene, a highly conserved region of the bacterial genome and thus a suitable target to quantify as an estimate of total bacterial load. Primer and probe sets were selected from existing literature (8, 9) for each detection method and assays optimised for annealing temperature, primer / probe concentration, reaction volume and cycle length using the Roche LightCycler 480 II qPCR platform against bacterial type strains, with DEPC water controls. Following optimization of both assays, it was decided to move forward with probe-based detection; this was based on achieving comparable Cp (crossing point) values to the SYBR Green 1 assay for samples, but with greater specificity to the gene target of interest, producing a cleaner negative control (Figure 1). The specificity of the chosen primer / probe set for this assay for the 16S rRNA gene was confirmed by testing against 11 different bacterial species.

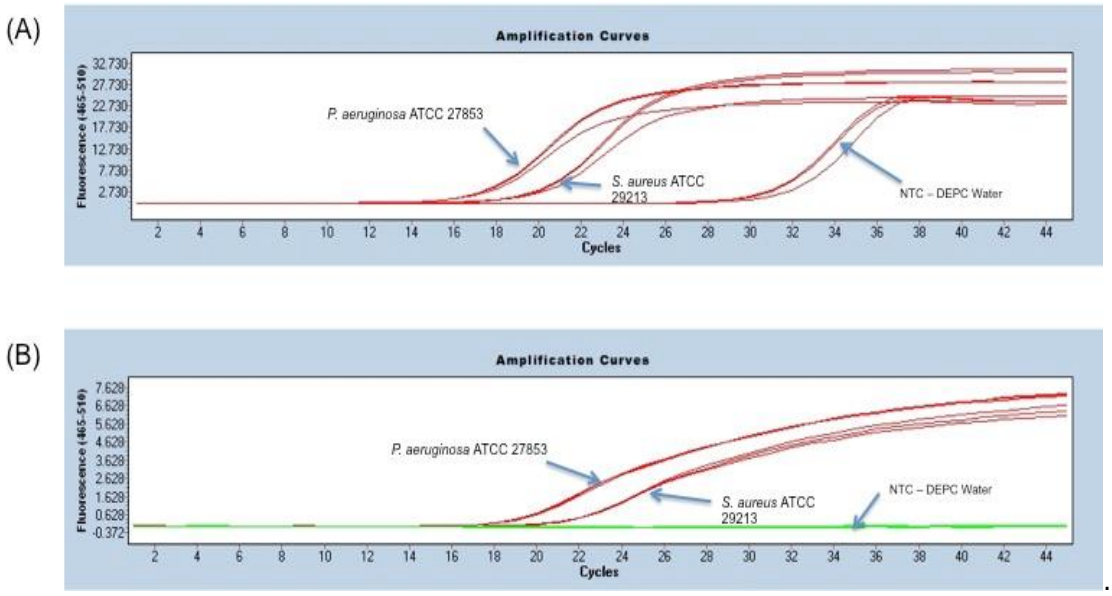


Figure 1: Amplification curves at optimum temperature for each detection method; (A) SYBRGreen 1; (B) Probe-based detection.

Following optimization, all reactions were performed in triplicate alongside positive, negative and non-template controls. The concentration of 16S copies/mL of sample were calculated using the absolute quantification detection method via the LightCycler 480 software (version 1.5; Roche Diagnostics Ltd., West Sussex, UK) using a standard curve prepared from purified 16S amplicons from *P. aeruginosa* type strain ATCC 27853. Results were deemed acceptable provided this standard curve remained within the efficiency range 90-100% as per MIQE guidelines (10). Crossing point (Cp) values were deemed within the limit of detection of the assay if they occurred ≤2cycles from the negative control.

3.4 Illumina MiSeq Microbiota Analysis

Amplicon libraries for paired-end 16SrRNA marker gene community sequencing were prepared using previously published barcoded primers (515F/806R) targeting the V4 variable region of the 16S rRNA gene (11). Due to the low biomass of these samples, our in-house 16S rRNA amplicon library preparation method was optimized to enhance performance. This involved alterations to incubation times and sample:magnetic bead ratios during PCR clean up steps.

Following library preparation, correct amplicon size (approx. 450bp) was confirmed via electrophoresis on a 1.5% agarose gel. Pooled amplicons were purified using the Agencourt AMPure XP magnetic bead system (Beckman Coulter [UK] Ltd., High Wycomb, UK), before quantification of amplicons via the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Loughborough, UK) according to the manufacturer's protocol. Following pooling based on PicoGreen measurements, pooled amplicons were ran on a 1.5% agarose gel and purified via gel extraction using a QIAEX II kit (Qiagen, Hilden, Germany). This final pooled library was then again quantified by PicoGreen. Pooled library sequencing was performed on the Illumina MiSeq system (Illumina UK, Cambridge, UK) using the 500-cycle V2 reagent kit as per the manufacturer's protocol.

Following MiSeq paired-end sequencing, sequences were processed using the QIIME (12) pipeline and aligned using the GreenGenes database. After normalisation based on the sample with the fewest reads and initial quality filtering, sequences were clustered into their representative operational taxonomic units (OTUs) based on 97% sequence similarity. Potential contaminant OTU reads, due to reagent / technical contamination, were removed based on presence in both the negative control and samples.

4. Findings

4.1 Estimation of total bacterial load in CF lungs via 16S qPCR

To date, 58 CF lung tissue cores (n=12 patients) and 11 sputum (n=5 patients) samples excised from cores have been analysed. Furthermore, 3 tissue cores from a single control patient have been analysed. The number of 16S copies per mL was observed to be significantly (p<0.001, Kruskal-Wallis H test, Dunn's test *post-hoc*) higher in sputum compared to tissue cores (Figure 2A) for CF patients. No

difference was observed between either of the two CF groups and the control groups and this is likely due to the small (n=3) number of control tissue cores analysed. Additionally, the number of 16S copies per mL was observed to be significantly higher (p<0.05, Wilcoxon signed-rank test) in sputum compared to tissue for 9 matched CF sputum and tissue samples (collected from n=4 patients, Figure 2B). Eight CF patients in the analysis group had n≥3 tissue cores analysed; in 7 of these patients, significant differences in 16S copies per mL were apparent between different tissue cores. Interestingly, 16S copies per mL were also significantly different between the 3 tissue cores analyzed for the single control patient (p<0.05, Kruskal-Wallis H test, Dunn's test *post-hoc*).

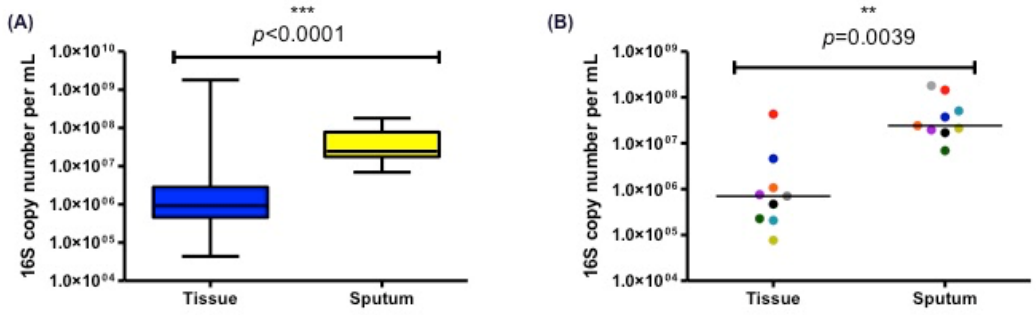


Figure 2: Comparison of number of 16S copies per mL in CF tissue compared to sputum (A) overall and (B) for matched tissue and sputum samples.

4.2 Determination of the microbiota in end-stage CF lungs via 16S Illumina Sequencing

To date, 77 samples have been analysed: 48 CF tissue cores (n=11 patients), 12 CF sputum plugs (n=5 patients), 16 control tissue cores (n=3 patients) and a single control tissue sputum plug (n=1 patient).

From this analysis, the end-stage CF tissue / sputum microbiota was observed to exhibit extremely low bacterial diversity with 10/12 (83%) sputum and 22/48 (46%) tissue samples having 90% or more of sequence reads generated attributable to a single taxa. Generally, this dominance was due to typical CF-related taxa, with *Pseudomonas* most commonly being found. However, *Stenotrophomonas, Achromobacter* and *Staphylococcus* were also detected as dominant pathogens. The typical microbiota observed in these end-stage samples is shown in Figure 3.

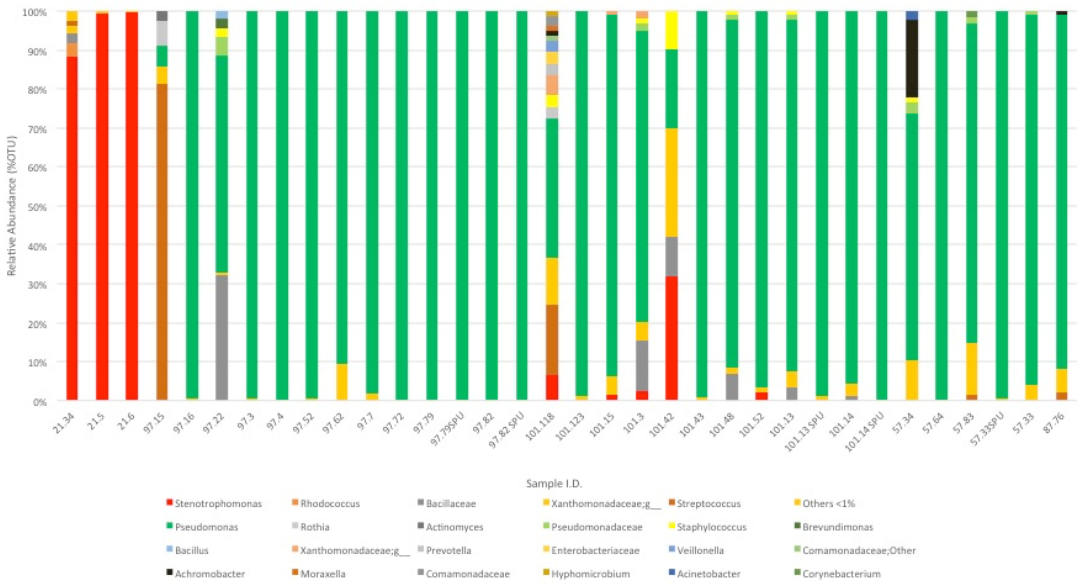
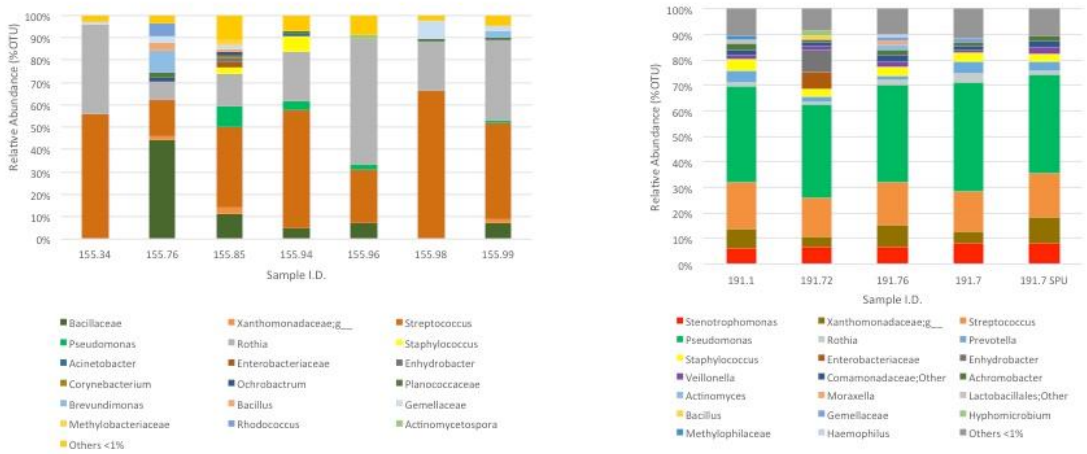


Figure 3: Typical microbiota composition observed in end-stage CF lung tissue samples.

Conversely, the control samples analysed exhibited much greater diversity, without a single dominating taxa. The typical taxa observed dominating samples in the CF cohort (e.g. *Pseudomonas)* were either not found or found in low relative abundances. Interestingly, members of, what can be considered to be a 'healthy' microbiota were more prevalent in these samples, including *Streptococcus, Rothia, Prevotella, Veillonella* and *Staphylococcus* (Figure 4).



(A) (B)

Figure 4: Microbiota composition in control patients (A) 155 and (B) 191.

As can be observed in Figure 3, microbiota composition was generally similar between multiple tissue cores from individual patients; however, some evidence of heterogeneity in bacterial communities was observed. For example, 3 tissue samples have been analysed for patient 43, with 2 of these cores (37

and 92) showing high relative abundances of *Pseudomonas*, contrasting with core 39, which exhibits a high relative abundance of *Stenotrophomonas,* with very little *Pseudomonas* detected (Figure 5). Similarly, for patient 177, where 2 samples have been analysed, one is dominated almost entirely by *Achromobacter,* with the other showing high levels of *Staphylococcus,* with low relative abundance of *Achromobacter* (Figure 5).

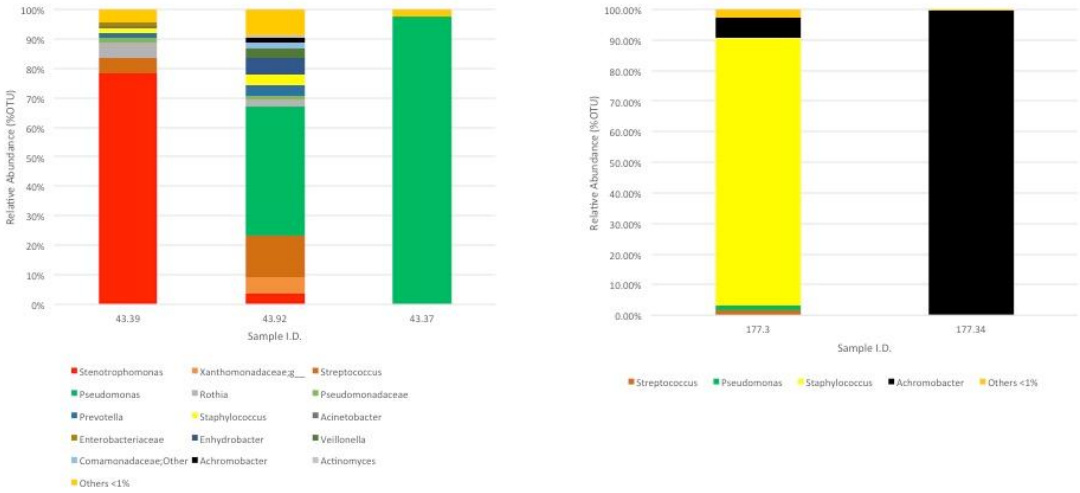


Figure 5: Microbiota composition in patients 43 and 177, highlighting potential regional variation in microbiota composition.

All 12 sputum samples analysed have shown high levels of dominance by *Pseudomonas*. Moreover, even when *Pseudomonas* has not been the dominant taxa detected in the tissue sample from which the sputum plug was isolated, it has still been the dominant species in the sputum plug. For example, the matched sputum and tissue sample (sample 12) from patient 90 exhibit contrasting microbiota profiles, with the sputum sample being dominated by *Pseudomonas,* whereas the tissue shows a much higher relative abundance of *Staphylococcus,* with lower levels of *Pseudomonas* (Figure 6).

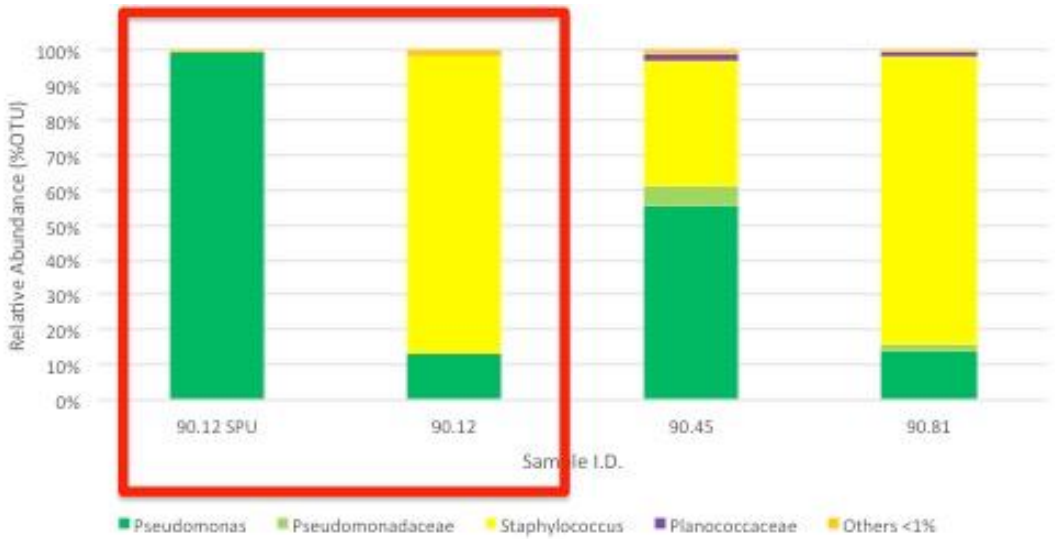


Figure 6: Microbiota profile for patient 90, with highlighted disparity in profiles between sputum and tissue sample 12.

5. Conclusions

Findings to date have illustrated that the end-stage CF lung is characterised by an extremely low diversity microbiota, which is dominated by traditional CF-related bacteria and differs markedly from healthy control lung tissue. Furthermore, these results illustrate that regional variation in bacterial load and microbiota composition may be a feature of the CF lower airway. Future work will determine whether this finding has clinical implications in terms of the relationship to regional variation observed in structural damage and disease progression observed in CF.

In this study, the sputum and tissue microbiota in CF has also been shown to differ; with sputum showing a higher estimated bacterial load and exhibiting lower bacterial diversity, with dominance of *Pseudomonas*. This suggests that *Pseudomonas* may be enriched in the lower airway sputum, where it can predominate and then move into the lower airway tissue, displacing the normal, diverse 'healthy' microbiota.

Future work will involve analysing microbiota composition in samples from patients with other types of chronic respiratory disease (as outlined in Table 1) and then analysing this data alongside the clinical, histological and radiological data collected by our collaborators in KU Leuven.

References

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Description of outputs related to the Award

List the various outputs from the Research Award (HSC R&D Division expects to receive copies of all reports or publications stemming from the research supported by this award)

Publications in peer reviewed journals & other publications/reports e.g. policies/guidelines

Boutin S, Graeber SY, Weitnauer M, Panitz J, Stahl M, Clausznitzer D, Kaderali L, Einarsson G, Tunney MM, Elborn JS, Mall MA, Dalpke AH. Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. PLoS One. 2015 Jan 28;10(1):e0116029. doi: 10.1371/journal.pone.0116029. eCollection 2015

Einarsson, G. G., Ronan, N. J., McIlreavey, L., O'Callaghan, G., Murphy, D. M., Tunney, M. M., Elborn, J., and Plant, B. J. The effect of ivacaftor treatment on airway microbial community dynamics in patients with G551D (the "Celtic" mutation). *Pediatr Pulm*. 2015: 50, 302-303.

Tunney, M. M., McLean, D., Einarsson, G., Elborn, J., Lammertyn, E., Bosch, B., Vermeulen, F., Verleden, S., De Boeck, K., Dupont, L., and Vanaudenaerde, B. (2015) Microbial community composition in explanted cystic fibrosis (CF) lungs. *Pediatr Pulm*. 2015: 50, 306-306.

The role of anaerobic bacteria in the cystic fibrosis airway. Sherrard LJ, Bell SC, Tunney MM. Curr Opin Pulm Med. 2016 Nov;22(6):637-43.

Presentations at local, national & international meetings and conferences

Anaerobes: Bystanders or thriving contributors. 40th European Cystic Fibrosis Conference, Sevilla, June 2017.

Microbiome analysis of the airway microbiome. Novartis Respiratory Research meeting. Novartis Campus, Basel, Swuitzerland, June 2016.

Antimicrobial resistance in the respiratory microbiota of people with Cystic Fibrosis. 16th Irish National Cystic Fibrosis Clinical Meeting, Killarney, February 2016.

Microbial composition of lung biopsy tissue and sputum from CF patients. KU Leuven, Leuven, Belgium, June 2015.

Changes in the Cystic Fibrosis airway microbiota with time and treatment. Mater Misericordiae University Hospital Seminar Series, Dublin, May 2015.

Lung and normal airway microbiota and implications for Cystic Fibrosis. The Society for General Microbiology Annual Meeting (Microbiome in health and disease), Birmingham, April 2015.

Interpreting the CF microbiome and the challenge of emerging pathogens in CF patients with advanced lung disease. 15th Irish National Cystic Fibrosis Clinical Meeting, Killarney, February 2015.

Training opportunities

There have been a number of opportunities for staff and PhD students working on this research programme to undergo training to support both the ongoing research and also their personal development. These included training on molecular methodologies such as next generation sequencing and bioinformatics analysis.

Other awards/grants obtained, or grant applications made

TUNNEY, M.,ELBORN, S. & GILPIN, D.
Project title: Activity of ALX-009, Lactoferrin and Hypothiocyanite against clinical isolates from Cystic Fibrosis (CF) Patients
Funding body: Alaxia SAS
Amount awarded: £22,080, August 2016

SCHOCK, B.,ELBORN, S.,SHIELDS, M. & TUNNEY, M.
Project title:Development of the GUT faecal microbiome in infants with chronic airway disease
Funding body: Northern Ireland Chest Heart and Stroke:
Amount awarded: £56,078, July 2016

ELBORN JS,TUNNEY MM.
Project title: Development of a Cystic Fibrosis Pathogen Array.
Funding body: Randox Laboratories Ltd.
Amount awarded: £64,505, January 2015

ELBORN JS, INGRAM R, GILPIN DF, TUNNEY MM.
Project title: Inhaled antibiotics in Bronchiectasis and Cystic Fibrosis
Funding body: EC Framework 7 projects (Innovative Medicines Initiative)
Amount awarded: €49,881,635, August 2015

IP being disclosed or other commercial outputs (including patent applications filed/granted)

Not applicable.

Indicators of prestige awarded to members of research team or any other personal or institutional benefits gained

Both investigators on the study (Tunney & Elborn) have been invited to present research findings as keynote speakers and to chair symposia at a number of major national and international conferences e.g. European Cystic Fibrosis Conference, North American Cystic Fibrosis Conference, European Society of Clinical Pharmacy, British Thoracic Society Annual Meeting, American Thoracic Society Meeting, Society for Applied Microbiology Conference and the Society for General Microbiology Conference.

Similarly, both investigators have acted as invited assessors for major grant awarding bodies including the UK Medical Research Council, the French National Research Agency, the Netherlands Organisation for Health Research and Development (ZonMw), UK Cystic Fibrosis Trust, Cystic Fibrosis Foundation (USA) and the Australian Cystic Fibrosis Research Trust and have acted as advisory board members for a number of companies.

Moreover, Professor Tunney is currently a member of the European Cystic Fibrosis Society (ECFS) Scientific Committee which is responsible for organising the Annual ECFS Conference and is chair of the Microbiology/Antibiotics Assembly.

Other benefits (e.g. to communities involved in project), examples of knowledge exchange or future plans for this work

Based on the findings of this study and the collaborative links developed with KU Leuven, we are currently working with KU Leuven on a project to determine the effect of the macrolide antibiotic, azithromycin, on the development of Chronic Lung Allograft Dysfunction (CLAD) in lung transplant (LTx) recipients. We are investigating the post-LTx microbiota, the impact of azithromycin on this, and how the microbiota changes with the development of CLAD. We are using methods developed in this study to collaborate with colleagues in Cork to determine the effect of Ivacaftor, a CFTR potentiator, on airway microbial community composition.

Personal and Public Involvement (PPI) (500 words)

Describe the involvement of services users and the public in the research
(Involvement of a PPI representative in writing/reviewing this section is recommended)

Benefits/impacts of PPI to the study

In this study, we have shown that end-stage CF lung is characterised by an extremely low diversity microbiota, which is dominated by traditional CF-related bacteria and differs markedly from healthy control lung tissue. Future work will focus on determining factors that result in this loss of diversity and how a ‘healthy’ airway microbiota may be maintained. This study would not have been possible, without the participation of a large number of patients at the Leuven Transplant Centre who agreed to take part in this clinical study. However, further detailed analysis of the data generated in Belfast and other

Impact *Applicable to Final Reports*

How the research impacts on practice and patient/client care with an indication of the expected time-line to impact and specific examples where possible. (500 words)

The key finding of this study is that end-stage CF lung is characterised by an extremely low diversity microbiota, which is dominated by traditional CF-related bacteria and differs markedly from healthy control lung tissue. Furthermore, we have also shown that regional variation in bacterial load and microbiota composition may be a feature of the CF lower airway. The use of Illumina next-generation sequencing enabled us to study healthy and disease airway microbiomes in much more detail than previously possible. This enabled detection of difficult to culture and non-culturable species, enhancing the ability to identify bacteria in samples with low abundance.

These findings could be of potential clinical significance to people with CF and a range of other lung diseases characterised by chronic infection such as bronchiectasis and COPD. Future work will focus on determining factors that result in loss of diversity with progression of lung disease and how a 'healthy' airway microbiota may be maintained. Work we have undertaken with colleagues in Cork has clearly shown that the use of the CFTR potentiator, Ivacaftor, reverses the loss of airway microbiota diversity in people with CF and this is associated with improved lung function. Moreover, the relative abundance of anaerobic bacteria such as Prevotella increased in patients taking Ivacaftor. Unfortunately, Ivacaftor is only effective in a small percentage of CF patients carrying a specific G551D mutation. We have a large collection of clinical Prevotella isolates and can use these to determine if bacteria from this genus can be added to the airways to create a 'healthy' airway microbiota. This work will take 2-3 years and within the next 5 years we should know if we can manipulate the CF airway microbiota to limit the damage that occurs to the lung and improve patient health.

Future work will also determine if the regional variation in bacterial load and microbiota composition observed in the CF lower airways in this study is related to the regional variation observed in structural damage in the airways and disease progression in CF. If this is the case, we can again determine whether this finding has clinical implications by manipulating the airway microbiota and determining if this prevent structural tissue damage. Such work could be undertaken in a mouse model of chronic lung infection such as that recently developed by our collaborator, Dr Beckie Ingram. We would anticipate a similar timeframe of approximately 5 years to complete such work and transition the findings to people with CF.