Manchester Metropolitan
University

Cystic Fibrosis Lung Microbiome

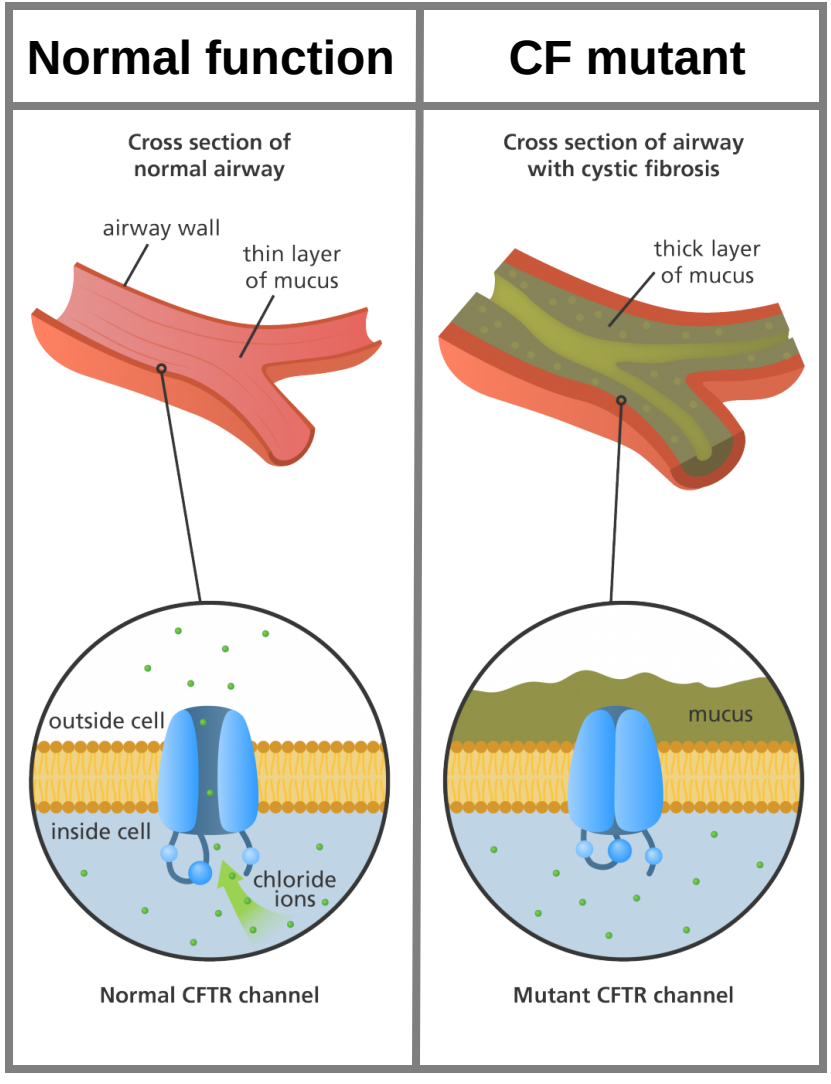
Prof Chris van der Gast

Cystic Fibrosis

Cystic fibrosis (CF) is a common autosomal recessive genetic disorder, affecting approximately 10,000 and 30,000 people in the UK and USA, respectively.

Mutation of the CF Transmembrane conductance Regulator (CFTR) genes.

CFTR genes encode for the CFTR epithelial cell membrane protein and chloride channel



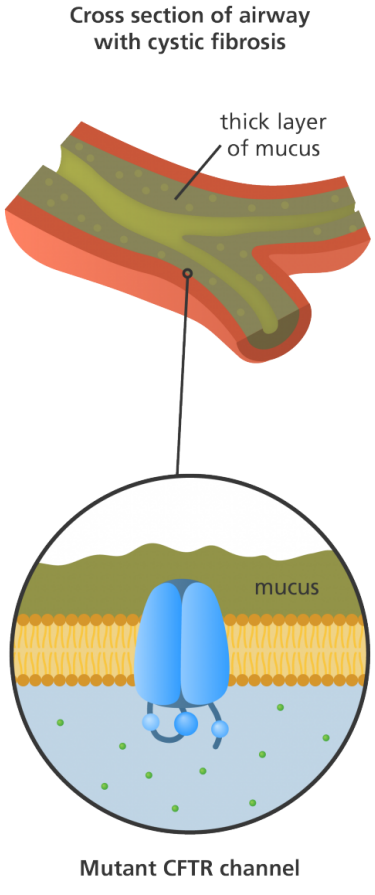
Adapted from www.yourgenome.org/facts/what-is-cystic-fibrosis

Cystic Fibrosis

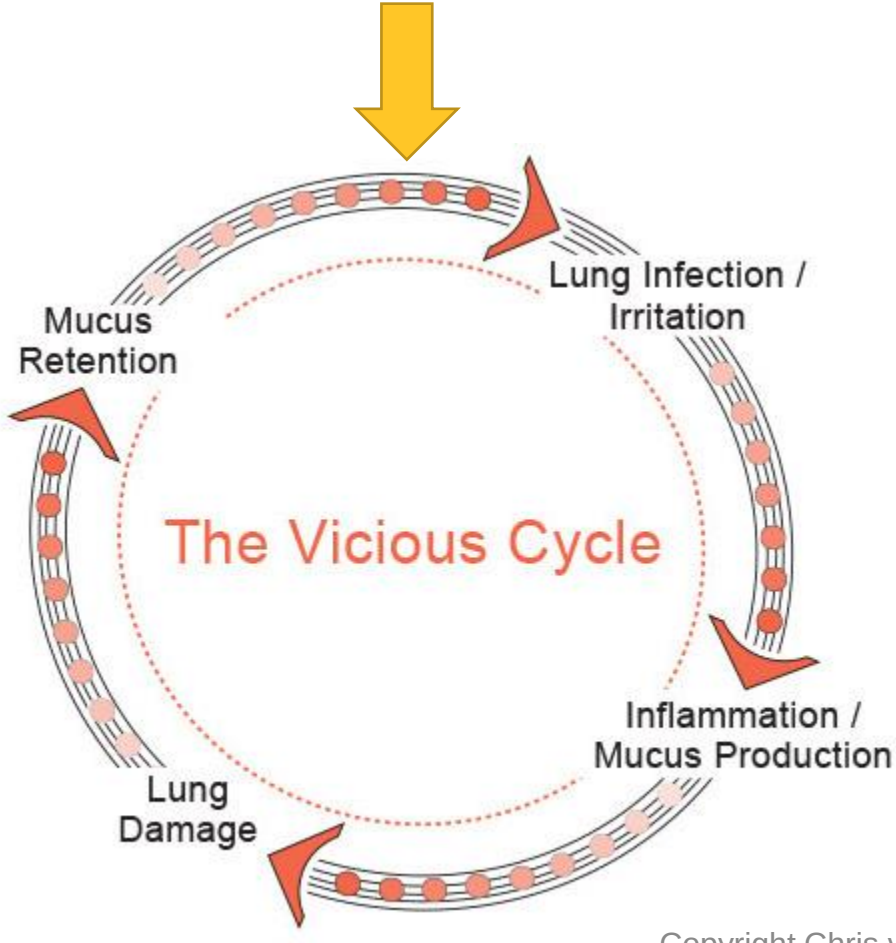
The disorder is multi-systemic, affecting the lungs, gastrointestinal tract, pancreas, reproductive organs, liver, and kidneys.

However, lung disease, as a result of chronic microbial infection and concomitant airway inflammation, is the leading cause of morbidity and mortality in the majority of patients.

CF mutant



Absent or impaired mucociliary clearance leads to a Viscous Cycle of infection and inflammation



Infection pathogenesis and control

Targeted culture-dependent assays remain the gold standard for pathogen detection.

Typically screening for a limited palette of bacterial species, including: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex, *Haemophilus influenzae*, *Stenotrophomonas maltophilia, Acromobacter xylosoxidans*

Infection pathogenesis and control

The traditional ‘one microbe, one disease’ concepts of infection pathogenesis and infection control are not optimal in CF management.

Why?

- Absent or impaired mucociliary clearance - eradication not possible

- Chronic lung infection in CF is polymicrobial - an **infection microbiota** of interacting microbes (interacting with each other and the host), ranging from strict aerobes through to anaerobic bacteria and fungal species.

Manchester Metropolitan
University

How to look at lung microbiota in CF

Targeted amplicon high-throughput sequencing of CF microbiota

Workflow

Manchester Metropolitan
University

Sample handling and preparation

Stabilising respiratory sample

Biobanking (freeze/thawing samples)

Propidium monoazide – viable cells

Sample handling & preparation

Respiratory sample (BAL, sputum, …)

Stabilise at -80°C within 12 hours – if not results in significant change in microbiota composition

JCM
Journals.ASM.org

Time between Collection and Storage Significantly Influences Bacterial Sequence Composition in Sputum Samples from Cystic Fibrosis Respiratory Infections

Leah Cuthbertson,[a,b] Geraint B. Rogers,[b,g] Alan W. Walker,[c] Anna Oliver,[a] Tarana Hafiz,[d] Lucas R. Hoffman,[e,f] Mary P. Carroll,[d] Julian Parkhill,[c] Kenneth D. Bruce,[b] Christopher J. van der Gast[a]

August 2014 Volume 52 Number 8 Journal of Clinical Microbiology p. 3011–3016

Sample handling & preparation

Biobanking – freeze-thawing samples 4 times or more results in significant shifts in the microbiota composition

ELSEVIER

Journal of Cystic Fibrosis 14 (2015) 464 – 467

Journal of Cystic Fibrosis
www.elsevier.com/locate/jcf

Short Communication

Implications of multiple freeze-thawing on respiratory samples for culture-independent analyses ☆

Leah Cuthbertson [a,b], Geraint B. Rogers [c], Alan W. Walker [d,e], Anna Oliver [a], Lucas R. Hoffman [f,g], Mary P. Carroll [h], Julian Parkhill [d], Kenneth D. Bruce [b], Christopher J. van der Gast [a,*]

Sample handling & preparation

Removing bias from dead cells, damaged cells, or extracellular DNA

Propidium Monoazide (PMA)

The ISME Journal (2013) 7, 697–706

www.nature.com/ismej

ORIGINAL ARTICLE

Reducing bias in bacterial community analysis of lower respiratory infections

Geraint B Rogers[1], Leah Cuthbertson[2], Lucas R Hoffman[3,4], Peter AC Wing[5], Christopher Pope[3,4], Danny AP Hooftman[2], Andrew K Lilley[1], Anna Oliver[2], Mary P Carroll[5], Kenneth D Bruce[1] and Christopher J van der Gast[2]

Sample handling & preparation

Removing bias from dead cells, damaged cells, or extracellular DNA

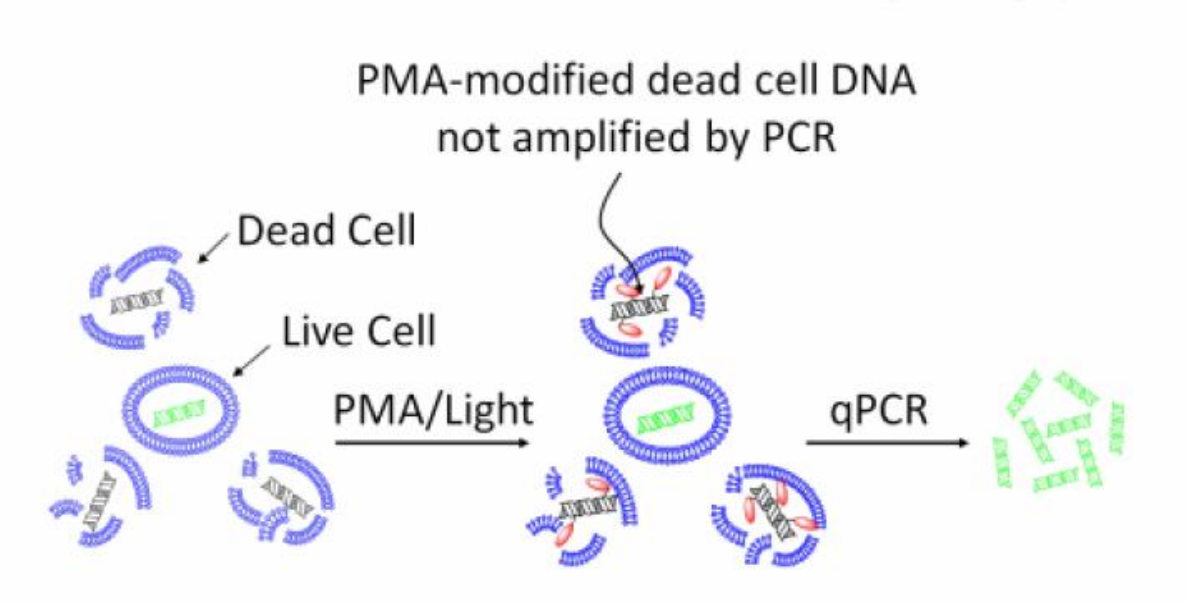


Figure 2. Principle of PMA modification for quantitation of viable bacteria by qPCR. The cell membrane-impermeable PMA dye selectively and covalently modifies DNA from dead bacteria with compromised membranes. Subsequent PCR amplification of PMA-modified DNA templates is inhibited, allowing selective quantitation of viable bacteria.

Sample handling & preparation

Removing bias from dead cells, damaged cells, or extracellular DNA

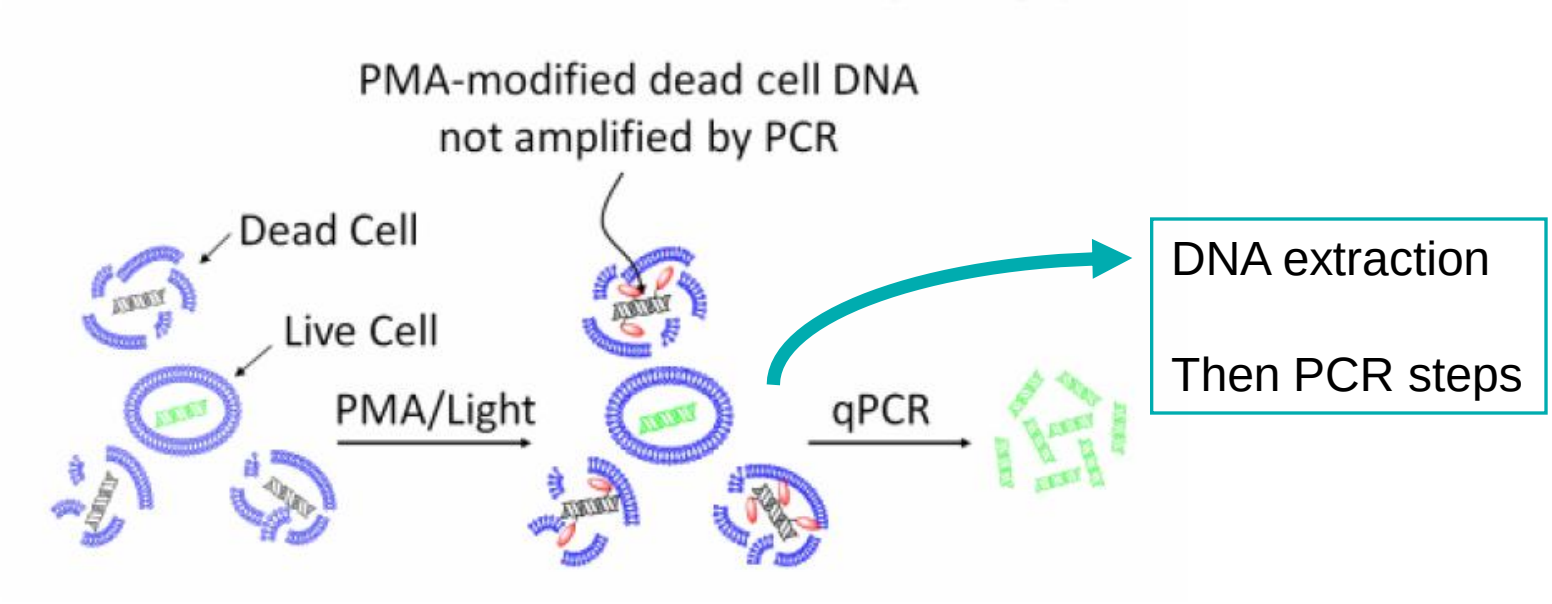


Figure 2. Principle of PMA modification for quantitation of viable bacteria by qPCR. The cell membrane-impermeable PMA dye selectively and covalently modifies DNA from dead bacteria with compromised membranes. Subsequent PCR amplification of PMA-modified DNA templates is inhibited, allowing selective quantitation of viable bacteria.

Manchester Metropolitan
University

DNA extraction

Effective DNA extraction protocols

Be aware of kit contamination

Manchester Metropolitan
University

DNA extraction

Within a community / microbiota, the cells of some bacterial species are difficult to break open due to cell wall structure. e.g. high GC containing Gram +ve bacteria and especially *Mycobacterium* spp.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2000, p. 5488–5491 Vol. 66, No. 12
0099-2240/00/$04.00+0

Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition

ROBERT I. GRIFFITHS,[1,2] ANDREW S. WHITELEY,[1] ANTHONY G. O'DONNELL,[2] AND MARK J. BAILEY[1]*

Protocol combines rigorous chemical lysis with physical disruption via bead beating

Manchester Metropolitan
University

DNA extraction

Commercial high-throughput DNA extraction kits

Kits can have microbial contaminants in reagents – especially a problem in low biomass samples.

Run positive (mock communities) and negative controls

Salter *et al. BMC Biology* 2014, **12**:87
http://www.biomedcentral.com/1741-7007/12/87

RESEARCH ARTICLE Open Access

Reagent and laboratory contamination can critically impact sequence-based microbiome analyses

Susannah J Salter[1*], Michael J Cox[2], Elena M Turek[2], Szymon T Calus[3], William O Cookson[2], Miriam F Moffatt[2], Paul Turner[4,5], Julian Parkhill[1], Nicholas J Loman[3] and Alan W Walker[1,6*]

Manchester Metropolitan
University

PCR & Sequencing

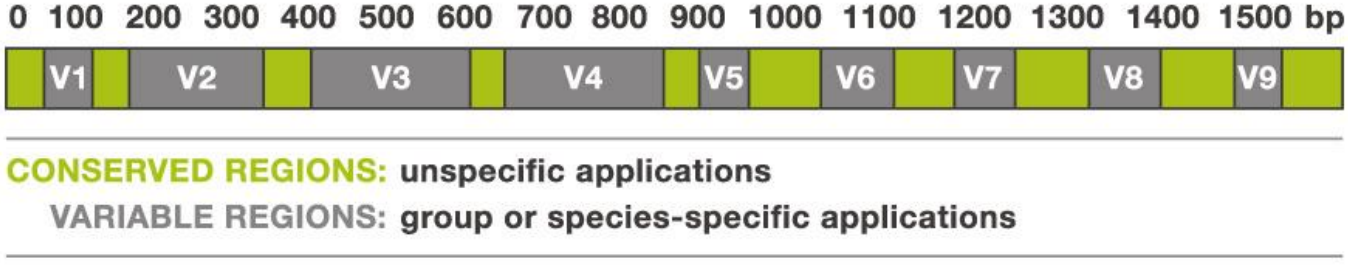
Which amplicon?

Illumina MiSeq

PCR & Sequencing

Which amplicon to target?

16S rRNA gene: bacteria and / or archaea



18S rRNA gene: microbial eukaryotes (protists, amoeba..), fungi, will pick up host (but can use blocking PCR to control for human DNA)

ITS (internal transcribed spacer): for fungi

https://biology.stackexchange.com

Manchester Metropolitan
University

PCR & sequencing

Commercial high-throughput PCR reaction kits

Kits can have microbial contaminants in reagents – especially a problem in low biomass samples.

Run positive (mock communities) and negative controls

Salter *et al. BMC Biology* 2014, **12**:87
http://www.biomedcentral.com/1741-7007/12/87

BMC Biology

RESEARCH ARTICLE Open Access

Reagent and laboratory contamination can critically impact sequence-based microbiome analyses

Susannah J Salter[1*], Michael J Cox[2], Elena M Turek[2], Szymon T Calus[3], William O Cookson[2], Miriam F Moffatt[2], Paul Turner[4,5], Julian Parkhill[1], Nicholas J Loman[3] and Alan W Walker[1,6*]

Manchester Metropolitan
University

PCR & sequencing

When preparing library for sequencing run include positive and negative controls from DNA extraction and PCR steps. Also include blanks for sequencing run.

Salter *et al. BMC Biology* 2014, **12**:87
http://www.biomedcentral.com/1741-7007/12/87

BMC Biology

RESEARCH ARTICLE Open Access

Reagent and laboratory contamination can critically impact sequence-based microbiome analyses

Susannah J Salter[1*], Michael J Cox[2], Elena M Turek[2], Szymon T Calus[3], William O Cookson[2], Miriam F Moffatt[2], Paul Turner[4,5], Julian Parkhill[1], Nicholas J Loman[3] and Alan W Walker[1,6*]

Manchester Metropolitan
University

PCR & sequencing

Illumina MiSeq – reasonably easy to use (compared to previous sequencing platforms)

Possible to multiplex up to 384 samples

We typically run 192 samples with 100,000 average sequence reads per sample – more than adequate of CF microbiota

Illumina.com

Manchester Metropolitan
University

PCR & sequencing

In general, sequencing power and quality is going up and price is going down

Experienced that from using Roche 454 pyrosequencing > Ion Torrent > Illumina MiSeq > …?

Illumina.com

Manchester Metropolitan
University

Bioinformatic pipelines

Converting raw sequence reads into something usable

Operational Taxonomic Units (OTUs) and Exact Sequence Variants (ESVs)

Bioinformatic pipelines

Sequencing platform produces millions of raw sequences – pipelines work to organise those sequences back to corresponding sample, remove poor sequences.

Lots of different pipelines (constantly evolving), e.g.

QIIME or Mothur (Operational Taxonomic Units)

DADA2 on the R platform (Exact Sequence Variants)

Manchester Metropolitan
University

Bioinformatic pipelines

A move from Operational Taxonomic Units (OTUs) to Exact Sequence Variants (ESVs)

OPEN

The ISME Journal (2017) 11, 2639–2643
www.nature.com/ismej

PERSPECTIVE

Exact sequence variants should replace operational taxonomic units in marker-gene data analysis

Benjamin J Callahan[1], Paul J McMurdie[2] and Susan P Holmes[3]
[1]*Department of Population Health and Pathobiology, NC State University, Raleigh NC, USA;* [2]*Whole Biome Inc, San Francisco CA, USA and* [3]*Department of Statistics, Stanford University, Stanford CA, USA*

Manchester Metropolitan
University

Bioinformatic pipelines

A move from Operational Taxonomic Units (OTUs) to Exact Sequence Variants (ESVs)

AMERICAN SOCIETY FOR MICROBIOLOGY | mSphere®

OBSERVATION
Ecological and Evolutionary Science

Check for updates

Broadscale Ecological Patterns Are Robust to Use of Exact Sequence Variants versus Operational Taxonomic Units

Sydney I. Glassman,[a,b] Jennifer B. H. Martiny[a]

July/August 2018 Volume 3 Issue 4 e00148-18

Manchester Metropolitan
University

Multivariate analyses

Getting to point of answering the question you set off at the beginning to answer

Manchester Metropolitan University

Multivariate analyses

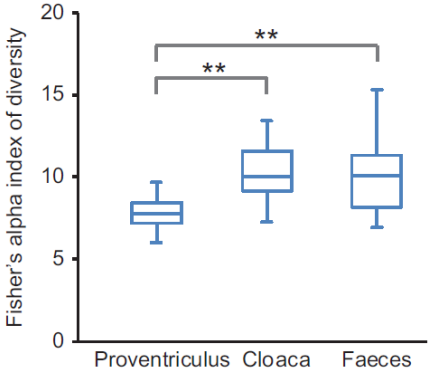
Getting to point of answering the question you set off at the beginning to answer

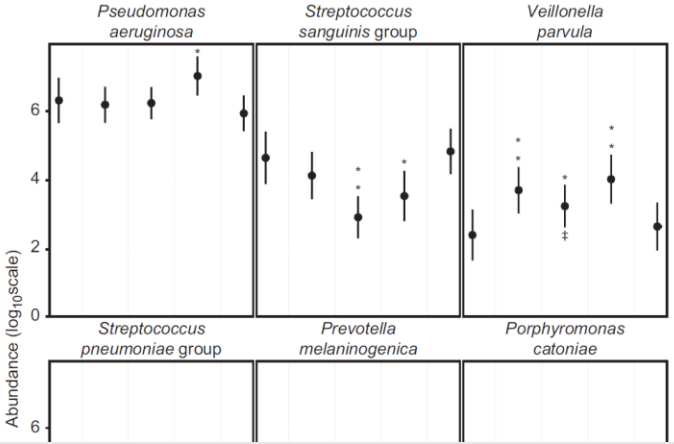
						Sequence reads per sample														
						18705	101789	7221	5777	9148	25698	44726	13573	11042	29248	9561	29993	8255	28801	11547
Phylum	**Class**	**Order**	**Family**	**OTU#**	**Taxa ID**	**MAI0016**	**LAR16**	**MAI0022**	**SHT0042**	**MAI0023**	**BEL0072**	**BEL0058**	**DTM024**	**SHT0063**	**SHT0062**	**DTM015**	**BEL0027**	**MAI0006**	**SHT0074**	**MAI0019**
p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Pseudomonadaceae	1	Pseudomonas aeruginosa 490	23	91641	4778	1068	8083	18021	29833	3888	10914	27041	6880	4225	421	28400	11360
p__Proteobacteria	c__Betaproteobacteria	o__Burkholderiales	f__Burkholderiaceae	2	Burkholderia 1176	31	0	68	32	48	2	6	30	80	66	36	16	8	132	84
p__Firmicutes	c__Bacilli	o__Bacillales	f__Staphylococcaceae	3	Staphylococcus aureus 491	12	8	9	56	700	1	1	8	12	15	76	8746	902	29	42
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Porphyromonadaceae	4	Porphyromonas_855	0	4	144	2624	0	0	678	0	0	14	1	1431	12	0	1
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	5	Prevotella melaninogenica_825	0	733	476	215	184	515	8420	44	2	14	1705	316	79	17	1
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	7	Prevotella_296	0	3	0	1	15	0	0	8768	0	28	0	75	2	0	0
p__Proteobacteria	c__Gammaproteobacteria	o__Pasteurellales	f__Pasteurellaceae	8	Haemophilus influenzae_221	0	0	0	0	0	1	1	0	1	1	0	3	1	1	0
p__Proteobacteria	c__Gammaproteobacteria	o__Xanthomonadales	f__Xanthomonadaceae	9	Stenotrophomonas maltophilia_1052	18511	4	16	13	16	0	0	6	14	33	17	1	6726	59	49
p__Firmicutes	c__Clostridia	o__Clostridiales	f__Veillonellaceae	10	Veillonella dispar_398	0	370	210	53	31	396	2550	139	0	321	301	101	5	0	1
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	12	Streptococcus_995	13	77	1	10	0	220	561	5	0	69	9	1553	7	1	0
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	13	Streptococcus_1274	0	1148	12	58	1	10	1	37	0	0	107	566	6	0	0
p__Proteobacteria	c__Betaproteobacteria	o__Neisseriales	f__Neisseriaceae	15	Neisseria subflava_613	0	0	1	1	1	0	3	0	1	0	0	0	4	0	0
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	17	Streptococcus_1043	0	307	0	20	1	2	226	22	0	0	0	1	4	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__[Paraprevotellaceae]	18	Prevotella_849	0	4	66	120	0	0	0	0	0	0	137	85	1	0	0
p__Actinobacteria	c__Actinobacteria	o__Actinomycetales	f__Micrococcaceae	19	Rothia mucilaginosa_562	45	355	9	66	1	4	124	2	0	0	3	2041	5	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	20	Prevotella_277	0	0	0	60	0	0	0	0	0	39	0	89	0	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	21	Prevotella_853	0	60	48	16	4	0	0	3	0	4	0	58	6	1	1
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	26	Prevotella nanceiensis_802	0	4	0	115	5	0	0	0	0	0	0	13	1	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Porphyromonadaceae	28	Porphyromonas_805	0	0	2	0	0	0	8	0	0	11	0	485	0	0	0
p__Proteobacteria	c__Gammaproteobacteria	o__Pasteurellales	f__Pasteurellaceae	30	Haemophilus parainfluenzae_706	0	0	0	1	0	0	0	2	0	0	12	1	0	1	0
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Carnobacteriaceae	31	Granulicatella_1232	6	29	1	73	0	6	64	1	0	2	5	1	3	0	0
p__Actinobacteria	c__Actinobacteria	o__Actinomycetales	f__Micrococcaceae	32	Rothia dentocariosa_596	6	3023	1	4	0	5	107	2	0	8	0	157	0	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	33	Prevotella pallens_1228	0	18	0	23	1	0	0	1	0	0	0	6	2	0	0
p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	34	Oribacterium_627	0	1	3	0	1	0	0	1	0	0	0	5	0	0	0
p__Proteobacteria	c__Gammaproteobacteria	o__Pasteurellales	f__Pasteurellaceae	35	Haemophilus parainfluenzae_962	0	2	0	0	0	0	0	0	0	0	12	2	0	0	0
p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__[Paraprevotellaceae]	36	Prevotella tannerae_276	0	5	0	0	1	0	0	1	1	5	8	5	7	1	0
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	37	Streptococcus_1049	0	32	1	12	2	3	0	0	0	1	0	0	0	1	0
p__Fusobacteria	c__Fusobacteriia	o__Fusobacteriales	f__Leptotrichiaceae	38	Leptotrichia_188	0	5	0	0	0	0	0	1	0	0	1	0	8	1	0
p__Fusobacteria	c__Fusobacteriia	o__Fusobacteriales	f__Fusobacteriaceae	39	Fusobacterium_946	0	1	0	0	0	3857	2	0	0	0	0	4	0	0	0
p__Actinobacteria	c__Actinobacteria	o__Actinomycetales	f__Actinomycetaceae	41	Actinomyces_310	8	6	12	8	0	0	0	0	0	0	1	1	0	0	0
p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	42	Streptococcus_816	0	10	6	3	0	2	0	1	0	0	0	2	0	0	0

Manchester Metropolitan
University

Multivariate analyses

How microbiota organisation (e.g. diversity, composition, structure) is associated with clinical and host factors…





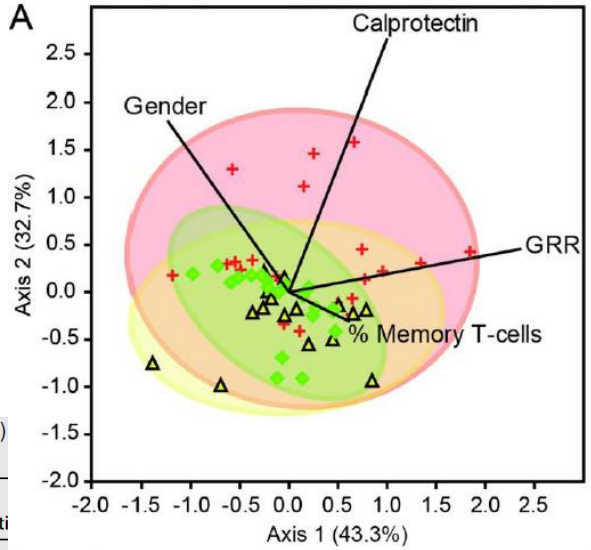


Table 2 Similarity of percentages (SIMPER) analysis of microbial community dissimilarity (Bray–Curtis) between core species groups for (A) and siblings, (B) healthy controls and siblings and (C) CD and healthy cohorts

A Name	Mean abundance in the CD cohort	Mean abundance in the siblings cohort	% Contribution	Cumulati
Faecalibacterium prausnitzii	22.4	24.2	20.7	20.7
Escherichia fergusonii	21.4	9.7	15.9	36.6
Shigella flexneri	13.6	7.2	10.7	47.3
Ruminococcus gnavus	13.1	5.2	8.9	56.2
Bacteroides vulgatus	13.2	7.6	7.8	64.0
Eubacterium rectale	9.8	6.4	6.6	70.6
Oscillospira guilliermondii	0	8.0	5.9	76.5
Escherichia coli	6.5	0	4.5	81.0
Sutterella wadsworthensis	0	6.0	4.5	85.5

Manchester Metropolitan
University

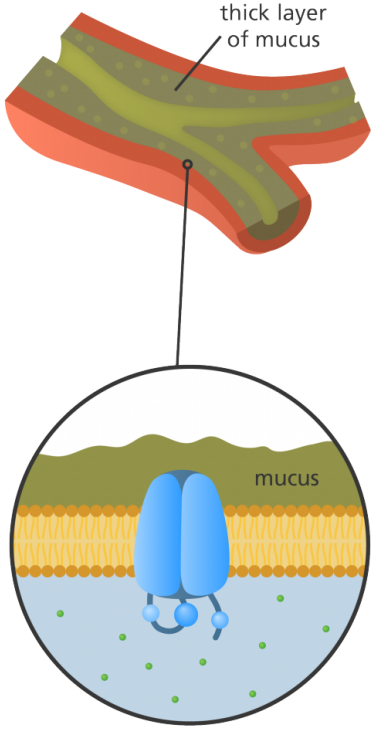
How to look at lung microbiota in CF

Targeted amplicon high-throughput sequencing of CF microbiota

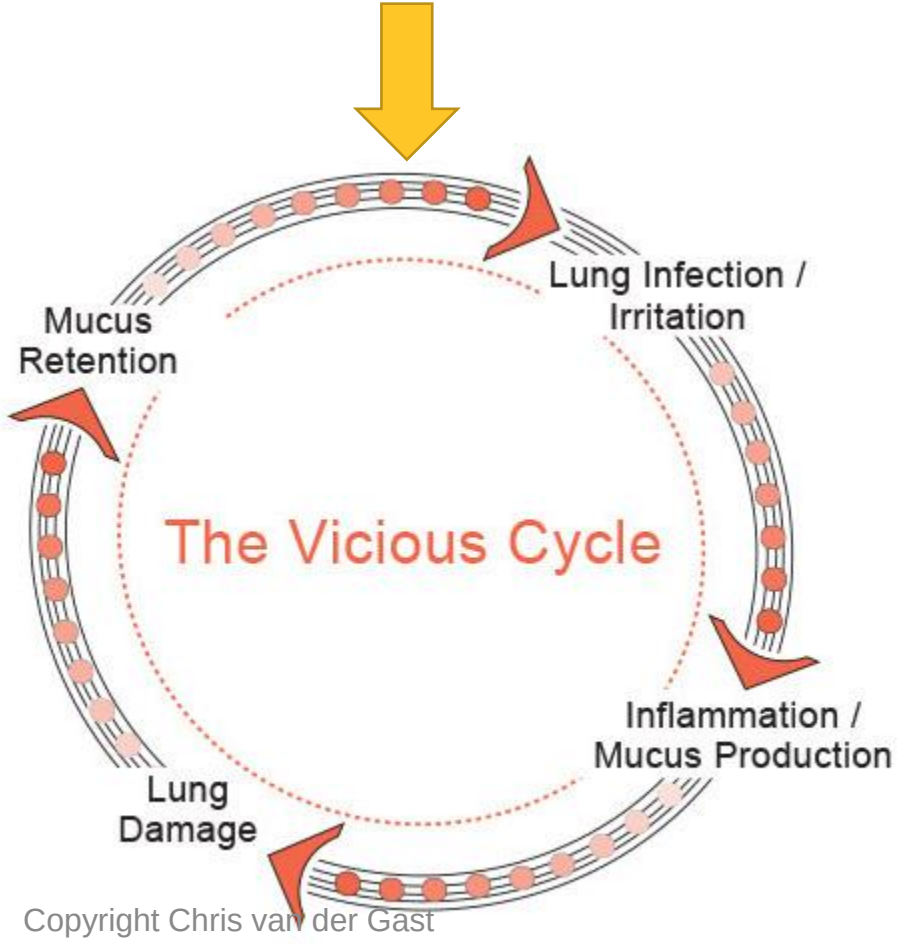
Workflow

CF mutant

Cross section of airway with cystic fibrosis



Absent or impaired mucociliary clearance leads to a Viscous Cycle of infection and inflammation



Manchester Metropolitan
University

C.vanderGast@mmu.ac.uk