Understanding and predicting acute pulmonary exacerbations in adults with cystic fibrosis

Dr Emem-Fong Ukor

Department of Medicine

University of Cambridge

Murray Edwards College

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DECLARATION

This dissertation is the result of my own work, carried out under the supervision of Professor R. Andres Floto, and includes nothing which is the outcome of work done in collaboration, except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted, for any such degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed length of 60,000 words set by the Degree Committee for the Faculties of Clinical Medicine and Veterinary Medicine.

Signed:

(Emem-Fong Ukor)

Date: April 2021

ABSTRACT

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Cystic fibrosis (CF) is the most common, inherited, life-limiting, multi-system disorder among the Caucasian population. Disease arises from mutations in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene. In the lung, defective protein leads to the accumulation of thick viscid mucus, depletion and acidification of the airway surface liquid and impairment of the normal muco-ciliary clearance mechanisms, providing a permissive environment for chronic infection and progressive airway damage.

Pseudomonas aeruginosa is a versatile, Gram-negative opportunistic human pathogen with a predilection for establishing chronic infection in the CF lung. Its extraordinary capacity to cause infections is due to its vast repertoire of secreted and cell-associated virulence determinants, which are subject to a complex regulatory network of intracellular and intercellular signals.

Acute pulmonary exacerbations (APE), are the main cause of morbidity and mortality in CF. Despite their clinical significance, the mechanisms that trigger these events are poorly understood. In this dissertation, I investigate whether home monitoring for changes in patient physiology and symptoms was feasible and could permit advanced detection of an APE. I additionally concentrated on whether temporal fluctuations in the behaviour and structure of established *P. aeruginosa* populations within the CF lung may trigger APEs, and whether such changes could function as a bacterial biomarker(s) and be correlated with home monitored data to facilitate APE diagnosis and prompt initiation of treatment.

First, I conducted a single-centre, pilot study (TeleCF) of 15 adults with CF in order to determine whether daily home monitoring of a single sputum bacterial biomarker (*P. aeruginosa* exotoxin A [PEA]) along with several clinical parameters might provide advanced warning of an APE. Home monitoring was well tolerated and provided high resolution data on physiological and biomarker changes preceding, during and

following antibiotic therapy for an APE. On its own, PEA did not prove an effective biomarker for early detection of APEs in adults with CF, but the study did provide proof of concept for the application of home-monitoring and sputum profiling for bacterial biomarkers to inform further work.

Next, I collected longitudinally sampled sets of 95 isolates per sputum sample from 9 adults with CF before, during and after antibiotic treatment for an APE. Isolate populations were analysed for a series of phenotypic traits associated with *P. aeruginosa* virulence to determine whether changes in phenotype composition were related to exacerbation onset. I also investigated for differences in phenotype composition between isolate populations of the *P. aeruginosa* Liverpool epidemic strain (LES), Manchester epidemic strain (MES) and local non-epidemic strains. I found strong evidence for the uncoupling of the traditional quorum sensing (QS) regulatory hierarchy in CF isolates, with the *rhl* subsystem playing a more dominant role in virulence expression in certain strain types. Importantly, no link was found between APEs and the emergence of a particular sub-population of morphotypic or phenotypic variants.

Finally, I conducted a multicentre UK-based study (SMARTCARE) of 147 adults with CF to assess the acceptability and feasibility of daily monitoring of symptoms and physiology using novel sensor technology and mobile phones. Linked-anonymised data were analysed using machine learning (ML) methods to define the profile of APEs and predict their onset. Survey patient feedback confirmed that home monitoring was easy to do and helped patients track their health over time. Unsupervised machine learning analysis uncovered the typical signal profile of an APE and revealed three distinct classes of APE. We developed an ML predictive classifier that can detect an impending APE on average 11 days earlier than current clinical practice.

This work has contributed greater insights into the day-to-day variation in symptoms and physiology prior to, during and following periods of APE in adults with CF. It has confirmed the important role for home monitoring in CF care delivery and highlighted the power of machine learning methods when applied to high frequency data to advance our understanding of APEs in adults with CF.

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ABBREVIATIONS

$(NH_4)_2SO_4$	ammonium sulphate
3OC12-HSL	*N*-3-oxo-dodecanoyl-L-homoserine lactone
ABC	ATP-binding cassette
AHL	*N*-acyl-homoserine lactone
AI	autoinducer
Alt	alternate
AMP	adenosine monophosphate
ANOVA	Analysis of Variance
APE	acute pulmonary exacerbation
AQ	acyl-quinolone
Azli inh	Aztreonam inhaled
BHL	*N*-butanoyl-*L*-homoserine lactone
BMI	body mass index
bpm	beats per minute
BTS	British Thoracic Society
C4-HSL	*N*-butanoyl-*L*-homoserine lactone
$CaCl_2$	calcium chloride
CAS	chrome azurol S
CCLI	Cambridge Centre for Lung Infection
CF	cystic fibrosis
CFLD	cystic fibrosis-related liver disease
CFQ-R	Cystic Fibrosis Questionnaire-Revised
CFRD	cystic fibrosis-related diabetes
CFRSD	Cystic Fibrosis Respiratory Symptom Diary
CFTR	cystic fibrosis trans-membrane conductance regulator
CIMR	Cambridge Institute for Medical Research
Col inh	Colomycin inhaled
COPD	chronic obstructive pulmonary disease
CRISS	Chronic Respiratory Infection Symptom Score
CRP	C-reactive protein
CTAB	cetyl trimethylammonium bromide

DAPA	diaminopimelic acid
DNA	deoxyribonucleic acid
DNase	dornase alpha
E	exacerbation
eDNA	extracellular deoxyribonucleic acid
eICE	early intervention in cystic fibrosis exacerbation (trial)
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
EPIC	Early Pseudomonas Infection Control (trial)
EPS	extracellular polymeric substances
Fe	iron
$FeCl_3$	iron (III) chloride
$FeSO_4$	iron (II) sulphate
FEV_1	forced expiratory volume in one second
FPH	Frimley Park Hospital
HCl	hydrogen chloride
HDTMA	hexadecyltrimethylammonium bromide
HEE	Health Enterprise East
HHQ	2-heptyl-4-quinolone
HR	heart rate
HRQoL	health-related quality of life
HTS	hypertonic saline
IGT	impaired glucose tolerance
IL-8	interleukin-8
IQR	interquartile range
KCH	King's College Hospital
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KH_2PO_4,	potassium dihydrogen phosphate
LB	luria broth
LES	Liverpool epidemic strain
LTF	lost-to-follow-up
Mero inh	Meropenem inhaled

MES	Manchester epidemic strain
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
ML	machine learning
MLST	multi-locus sequence typing
mRNA	messenger ribonucleic acid
MRSA	methicillin sensitive *Staphyloccocus aureus*
MTA	Materials Transfer Agreement
NA	Not applicable
NaCl	sodium chloride
NE	non-exacerbation
NH_4Cl	ammonium chloride
NHS	National Health Service
NRES	National Research Ethics Service
NTM	non-tuberculous mycobacteria
OdDHL	*N*-3-oxo-dodecanoyl-L-homoserine lactone
PA	*Pseudomonas aeruginosa*
PCA	principal component analysis
PDA	personal digital assistant
PEA	*P. aeruginosa* exotoxin A
PI	pancreatic insufficiency
PIPES	piperazine-N, N'-bis (2-ethansulfonic acid)
PPGAS	proteose-peptone-glucose-ammonium salts
PQS	*Pseudomonas* quinolone signal
QOL	quality of life
QS	quorum sensing
R	recovery
R&D	research and development
RBH	Royal Brompton Hospital
RNA	ribonucleic acid
SBRI	Small Business Research Initiatives
SCV	small colony variant
SD	standard deviation

SpO_2	percentage saturation of arterial haemoglobin by oxygen
SUH	Southampton University Hospital
T1SS	type I secretion system
T2SS	type II secretion system
T4P	type 4 pili
Tob inh	Tobramycin inhaled
w/v	weight per volume

1. INTRODUCTION

1.1 Cystic fibrosis

Cystic fibrosis (CF) is an inherited, life-limiting, multi-systemic disorder which affects over 90 000 individuals worldwide (1). It is recognised as the most common life-limiting genetic condition to affect individuals of northern European descent, with an incidence rate of around 1 in 3000 live births in Europe, North America and Australasia (2,3). However, in the last two decades, epidemiological studies based on data from emerging patient data registries in South America, Africa, the Middle East, Turkey and Asia, have confirmed that the disease is more prevalent than previously thought among populations of non-European descent (4).

In the UK, approximately 10 000 individuals are currently living with CF, with around 200 - 300 new cases diagnosed each year (5). Diagnosis is made with a positive sweat test (sweat chloride > 60 mmol•L^{-1}), DNA mutation analysis and cystic fibrosis-typical electrophysiology (1). In the UK, the majority of cases are identified in the neonatal period, particularly since the implementation of newborn screening (the Guthrie heelprick test) in 2007. However, a small proportion of people living with CF are diagnosed in adulthood. For example, 15 people aged over 16 years were diagnosed with CF in the UK in 2018 (5).

Over the last six decades, significant advances in disease understanding, diagnosis, symptomatic treatments, and the coordination of care through dedicated multi-disciplinary health-care teams have led to substantial improvements in survival (1,6). In the UK, a child born with CF between 2014 and 2018 will have a median predicted life expectancy of 47 years of age (5). This represents a remarkable leap from the 1960s where the median survival was less than 5 years of age (7). With more people with CF surviving into adulthood, the adult CF population in the UK has surpassed the number of paediatric cases and is expected to continue to rise, reflecting the changing demographic of the CF population in many developed countries (8,9). Population forecasts based on European CF Society patient registry data provided by 16 countries, including the UK, estimate the CF adult population to rise by 75% between 2010 and 2025 (10). This growth in the ageing CF population will necessitate an increase in resources and CF service capacity to meet demands and poses a growing

challenge for adult services in many countries (10). Furthermore, the recent arrival of mutation-specific modulator therapies; medications which target the basic cellular defect caused by CF-causing gene mutations, heralds a transformative era in CF care (3,9). One which will likely alter greatly the natural history of this disease for those born with CF today, as well as for a significant subset of the CF population already living with the disease (Figure 1.1).

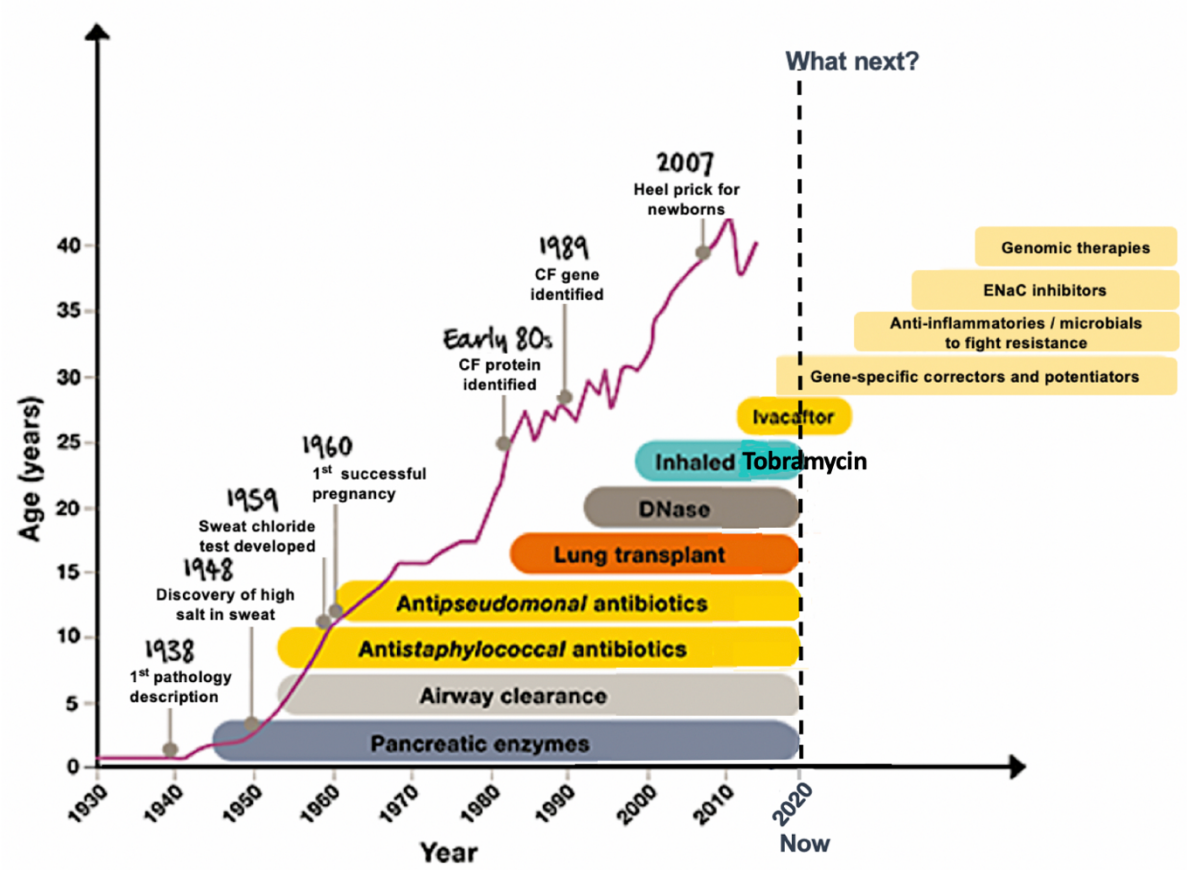


Figure 1.1. Significant advances in CF care and accompanying improvements in median life expectancy. Figure adapted from the Cystic Fibrosis Trust UK website (5). Accessed July 2020.

1.1.1 Genetics

CF is an autosomal recessive monogenetic disorder. A landmark discovery in 1989 confirmed that disease results from possession of a loss-of-function mutation in each allelic copy of the cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene, located on the long arm of chromosome 7 (11–13). This gene encodes for the 1480 amino acid CFTR protein, an ABC-transporter class, cyclic adenosine monophosphate-dependent anion channel located at the plasma membrane of

epithelial cells and other cell types (14).

Over 2 000 genetic variants have been identified in the *CFTR* gene to date (15). The most prevalent of which is the three base-pair deletion of phenylalanine at position 508 (Phe-508del or F508del), found in approximately 80% of people with CF worldwide (9). Approximately 40% of these individuals carry two copies of the F508del allele whilst a similar proportion of individuals carry one F508del allele combined with another loss-of-function mutation (15). Notably, only 360 of the genetic variants are classified as pathogenic in the CFTR2 database and account for the genetic mutations found in more than 96% of all patients with CF of northern European ancestry (15).

CFTR variants may cause impairment in gene translation; protein expression, trafficking, function and stability at the apical cell membrane; or a combination of these abnormalities (6,16). Traditionally, on the basis of their impact on CFTR function and production, these mutations have been grouped into six classes (17). More recently, with the advent of CFTR modulator therapies, an alternate classification has been proposed by Marson *et al.* that splits Class I into Class IA and Class IB, to better accommodate for not only the CFTR defect and clinical disease severity, but also the possibility of precision medicine therapy (Figure 1.2) (17). However, although helpful, this classification system has been criticised as over simplistic, since multiple cellular processes may be affected by the same *CFTR* variant (e.g., *F508del* mutation belongs to Class II but also leads to defects associated with Class III and Class VI mutations) (6,9,18).

Class I mutations lead to an absence of functional CFTR protein at the apical plasma membrane and are caused primarily by the presence of nonsense, frameshift mutations and mRNA splicing mutations (19). Class II mutations (which include F508del) cause abnormal CFTR protein processing and trafficking to the cell surface, with most of the protein degraded in the proteasome (4). Class III mutations lead to normal localisation of CFTR at the plasma membrane but the ion channel's opening time is severely disrupted due to impaired activation of the gating mechanism by cyclic AMP (20). Class IV mutations cause reduced conductance (e.g., flow) of chloride and bicarbonate ions through the CFTR channel, but trafficking to the cell membrane is normal (4). Class V mutations are often splicing mutations and lead to a reduced

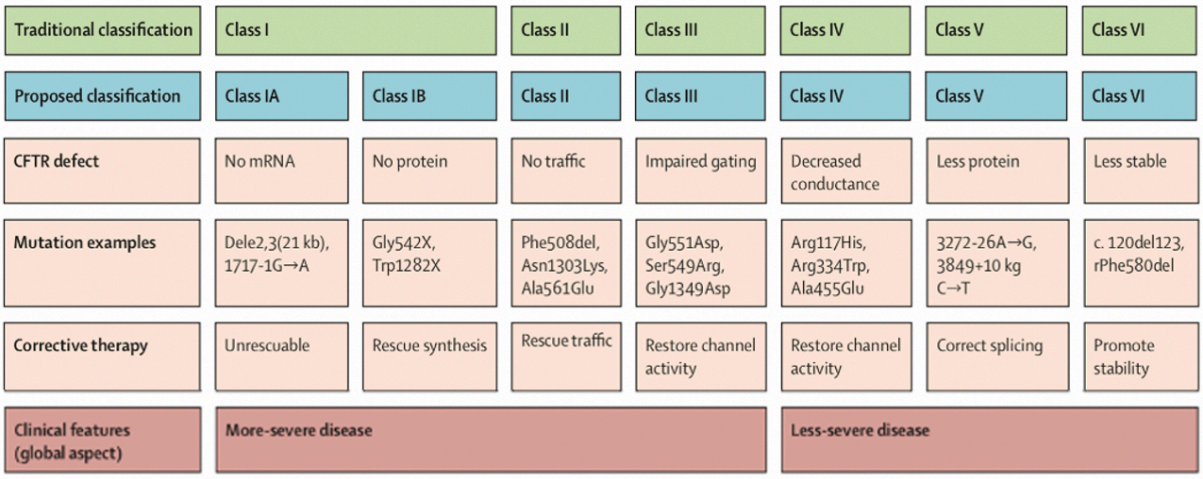


Figure 1.2. *CFTR* mutations and therapeutic strategies in the traditional classification system and the proposed classification by Marson, Bertuzzo and Ribeiro. Figure adapted from Marson *et al.,* 2016 (17).

amount of normal CFTR protein reaching the plasma membrane (21). Class VI mutations lead to conformationally unstable CFTR protein that is prematurely recycled from the plasma membrane and degraded in lysosomes (21).

Class I, II and III mutations are associated with minimal or absent CFTR function and confer a more severe (or classical) disease phenotype (1). Class IA and Class IB mutations share the same functional outcome (e.g., absence of the CFTR protein), but Class IA mutations are not amenable to pharmacotherapy (17). Class IV, V and VI mutations are considered residual function mutations and are associated with milder (typically pancreatic-sufficient) disease phenotypes (1).

1.1.2 Airway pathophysiology

The CFTR protein plays an important and complex role in normal physiology. Its main function is to provide a pathway for the transport of anions (e.g., chloride and bicarbonate) across the apical membrane of epithelial cells (which line multiple organs, including the lung, pancreas, intestines, liver, sweat glands and the vas deferens) (22,23). CFTR also interacts with other ion channels and transporters, such as the epithelium sodium channel (ENaC; also known as the amiloride-sensitive sodium channel), which together regulate the hydration of secretions and mucins in the airway (24). CFTR expression has also been found in non-epithelial cells, including

cells involved in immune function such as platelets, neutrophils, macrophages, and lymphocytes (25–28). CFTR dysfunction in these bone-marrow derived cells is thought to further exacerbate the pathophysiological changes seen in the CF lung (29).

The primary outcome of defective or absent CFTR in epithelial cells, regardless of organ or tissue, is impaired transepithelial chloride and bicarbonate secretion and impaired inhibition of the ENaC (30). This leads to disruption of ion and fluid homeostasis and a lowering in pH at the apical surface (3). With the exception of the sweat gland (where CFTR dysfunction hampers recovery of salt from sweat), abnormal CFTR function leads to dehydration and acidification of mucosal surfaces, resulting in thick, viscous secretions that cause progressive obstruction of luminal compartments and ducts; the hallmark pathological process in CF-associated disease (24,31,32).

In the airway, the epithelial surface (composed mainly of ciliated cells) is covered by an airway surface liquid (ASL) consisting of two layers, a periciliary layer (PCL) and an overlying mucus layer (33). The mucus layer acts as a physical barrier, trapping inhaled pathogens and particles. The periciliary layer, a membrane-tethered mucin gel that is more densely packed than the overlying mucus layer, acts as a lubricant surface to facilitate ciliary beating and promote clearance of mucus out of the airway (34). The hydration status of the ASL is a key determinant of muco-ciliary clearance efficiency, (the first line of defence against aerial pathogens) (35). Regulation of ASL volume is mediated via CFTR, which determines the mass of salt in the ASL, and consequently, the osmotic gradient for fluid secretion into the luminal space (22). The ASL also contains a number of peptide and protein antimicrobials, which are key components of the innate immune response to inhaled microbes (36).

In the CF airway, the net physiological impact of aberrant CFTR chloride/bicarbonate secretion and unopposed ENaC-mediated sodium and water absorption is reduction in the ASL volume and airway surface pH (37,38). Depletion of the ASL leads to hyper-concentration of the mucus layer which in turn causes a rise in the osmotic pressure of the mucus layer. The resultant osmotic gradient drives water out of the PCL, causing compression of the PCL and cilia, and leading to failure of the normal muco-ciliary clearance mechanisms (34,39). Changes in ASL pH, due to impaired cAMP-

dependent HCO_3^- secretion by mutant CFTR and unopposed H^+ secretion by ATP12A, is proposed to lead to diminished activity of many antimicrobials (37,40). Impaired bicarbonate secretion may also play an important role in the formation of dehydrated and hyper-viscous mucus, independent of its impact on airway surface pH (41). Evidence in favour of this hypothesis comes from studies in the CF mouse intestine which have shown that failure of CFTR-mediated bicarbonate secretion leads to abnormally folded mucin molecules, with impaired rheology and reduced transportability, leading to further exacerbation of mucus stasis and plugging (42).

It remains cause for debate whether airway inflammation arises as a direct consequence of CFTR dysfunction, or rather in response to airway infection. Initial observational studies in CF piglets had suggested an indirect causal link between reduced airway surface pH and the development of CF lung disease - via impaired bacterial killing leading to promotion of airway inflammation (37). However, more recently, a study by Shultz *et al.* comparing ASL in children with and without CF found no difference in ASL pH, calling into question the pathogenic role of ASL acidification (43). But, subsequent work by Simonin *et al.* on bronchial epithelial cell cultures (obtained post-mortem from patients with and without CF) showed further evidence for delayed bacterial clearance as a consequence of ASL acidification (40). Discordances in the results of these studies may be attributed to differing methodology and models used, and clearly further work is needed to clarify the differences found.

Of note, emerging evidence from observational studies in infants and preschool children with CF and animal models with CF-like lung disease, suggests that mucous plugging alone (as a primary consequence of CFTR dysfunction), may trigger sterile inflammation and play a critical role in the pathogenesis of early lung disease, independent of the onset of bacterial infection (44,45). However, mechanistic understanding of the link between mucous plugging and sterile inflammation in early CF lung disease is still poor. One proposed mechanistic pathway may be via activation of IL-1 signalling in response to cytokine release from airway epithelial cells that have sustained hypoxic injury in mucus-obstructed airways (46).

Nonetheless, the complex interplay of all these factors (ASL dehydration, hyper-concentration of mucus, diminished bicarbonate secretion and impaired muco-ciliary

clearance) initiates and maintains a permissive environment for recurring polymicrobial infections. Eventually, chronic endobronchial infection (frequently multi-drug resistant), persistent and excessive airway inflammation, muco-obstruction and bronchiectatic lung damage (e.g., irreversible abnormal dilatation of affected segmental and subsegmental bronchi) is established (47). The exaggerated inflammatory response is characterised by raised airway concentrations of neutrophil-derived proteases such as neutrophil elastase, neutrophil extracellular traps, reactive oxygen species such as hydrogen peroxide and pro-inflammatory cytokines such as interleukin-8. Neutrophil-derived proteases further exacerbate the CF basic defect through several functions: degradation of residual CFTR, proteolytic cleavage of inhibitory peptide segments leading to activation of the ENaC (contributing to hyper-reabsorption of sodium), degradation of antimicrobials and anti-proteases, and promotion of mucin secretion and pro-inflammatory signalling - adding to mucus adhesiveness and retention within the airways (16,48–52). Over time, progressive respiratory insufficiency ensues, ultimately culminating in respiratory failure and premature death.

1.1.3 Clinical manifestations of CFTR dysfunction

Clinical disease expression in CF varies widely and correlates poorly with *CFTR* genotype (53). This is best exemplified by individuals homozygous for F508del mutation who display a broad spectrum of disease, with heterogeneity in the number of organ systems involved, and the degree of severity and rate of progression of disease (21). Such variation in disease expression highlights differential tissue CFTR expressivity and sensitivity, as well as the importance of additional influences in determining disease severity beyond *CFTR* genotype; including genetic modifiers, environmental exposures and stochastic factors (54). For example, evidence from twin studies have shown that genetic modifiers play a predominant role in the age of establishment of airway infection with *Pseudomonas aeruginosa (P. aeruginosa)*, the age of onset of diabetes and the development of intestinal obstruction (55–57).

At birth, neonates with CF are identified for further screening on the basis of a heel-prick test which confirms elevated levels of circulating immunoreactive trypsinogen caused by pancreatic ductal blockage, autodigestion, and leakage of digestive enzymes into the systemic circulation (58). Up to 20% of CF neonates may present

with meconium ileus (bowel obstruction) as a consequence of reduced fluid and bicarbonate secretion in the gut, whilst pancreatic exocrine function is absent in more than 90% of cases (58,59). Other organ pathologies typically evolve and persist after birth.

The 'classical' CF phenotype is characterised by progressive lung disease, exocrine pancreatic insufficiency associated with malnutrition and poor growth, hepatobiliary and intestinal manifestations, male infertility resulting from obstructive azoospermia and, as a diagnostic marker, elevated sweat chloride concentration; due to the inability to recover salt from sweat (1,60). However, non-classical presentations have also been defined. These manifestations often involve single-organ disease and are referred to as CFTR-related disorders, in recognition of CFTR dysfunction in individuals who do not meet key diagnostic criteria (e.g., with two *CFTR* mutations, at least one of which is not clearly categorised as a CF-causing mutation), such as an elevated sweat chloride (60). Such disorders include, congenital bilateral absence of the vas deferens, idiopathic chronic and acute recurrent pancreatitis, diffuse bronchiectasis, rhinosinusitis and sclerosing cholangitis (23). Most of these individuals are pancreatic sufficient.

1.1.4 CF lung disease

Pulmonary disease secondary to progressive pulmonary obstruction, chronic endobronchial bacterial infection and endobronchial inflammation accounts for the greatest morbidity and mortality in individuals with CF (61,62). Hence, much of the focus of research in the CF domain is targeted at minimising the respiratory sequelae of CFTR dysfunction.

At birth, the lungs of newly diagnosed infants with CF appear free from infectious pathogens and inflammation (63–65). However, signs of pulmonary involvement are evident from within the first few months of life with bronchoalveolar lavage sampling of the lower airways confirming the presence of raised inflammatory cells in the absence of bacterial infection (64). Infant lung function tests are abnormal by age 3 months, with CT scan evidence of structural lung damage present in one third of children with CF by 3 years of age (66–69). Typically, bronchiectasis develops in the upper lobes but eventually progresses to involve the entire lung (16).

1.1.4.1 Bacterial airway infection

Bacterial infection of the lower airways often occurs shortly after birth in CF infants (70). Initial airway infection episodes are intermittent but over the course of months to years, chronic infection becomes established, highlighting the complex relationship between microbial adaptation and the evolving host. Evidence from microbiologic studies and CF registry data reveal a characteristic set of micro-organisms causing infection of the CF airways with the prevalence of these different pathogens varying in an age-dependent fashion (5,71) (Figure 1.3). Typically, non-typeable *Haemophilus influenzae (H. influenzae)* and *Staphyloccocus aureus (S. aureus)* are common in infancy and early childhood with *P. aeruginosa* predominating from adolescence onwards (72). Early infection with *P. aeruginosa* is usually intermittent. However, over half of individuals with CF will develop chronic pseudomonal infection (which persists indefinitely) by their mid-twenties (5,73). The decreased prevalence in *P. aeruginosa* infection seen after the age of 35 is thought to probably reflect a survivor effect, as the mortality risk is higher in individuals with *P. aeruginosa* infection (74). Over recent years, several emerging opportunistic pathogens have been isolated from CF sputum at increased frequency and have been associated with significant lung infection, such as *Stenotrophomonas maltophilia, Ralstonia, Pandoraea* species, *Achromobacter* species, methicillin-resistant *S. aureus* (MRSA) and nontuberculous mycobacteria (NTM) (75,76). By comparison, the overall prevalence of *Burkholderia cepacia* complex organisms, notorious for its association with nosocomial outbreaks in the mid-1980s and a high mortality rate, has fallen worldwide in response to improved infection control measures (77,78).

1.1.4.2 Treatment strategies

The management of CF pulmonary disease is critical to long-term survival and centres around an arduous daily routine of treatments targeted at preserving lung function, and treating and preventing acute pulmonary exacerbations (79). A comprehensive review of this topic is beyond the scope of this work but is available elsewhere (9,79–81). With the exception of CFTR modulator therapy, maintenance treatments for CF pulmonary disease are tailored toward mitigating the downstream consequences of CFTR dysfunction. As such, they aim to restore ASL and muco-ciliary clearance, reduce excessive airway inflammation and limit infection burden (82).

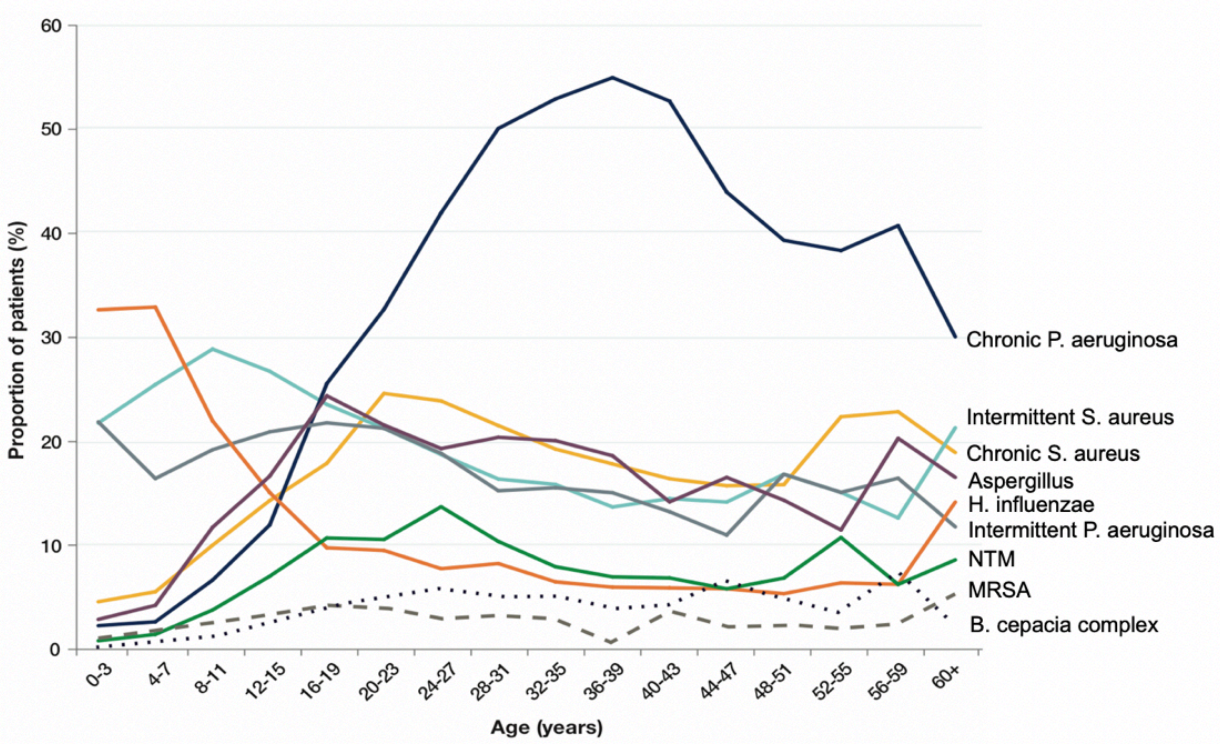


Figure 1.3. Age-specific prevalence of common respiratory pathogens in CF in 2018. NTM: Nontuberculous mycobacteria, MRSA: methicillin resistant *S. aureus*. Figure adapted from the UK Cystic fibrosis Patient Registry, 2018 Annual Data Report (5).

1.1.4.2.1 Targeting the secondary effects of CFTR dysfunction

Maintenance treatments commonly include mucolytics and sputum hydrators, airway clearance therapies, anti-inflammatories and inhaled antibiotics (81). Nebulised agents, such as dornase alpha (DNase) and hypertonic saline, improve the visco-elastic properties of sputum and assist with muco-ciliary clearance (83,84). Both DNase and hypertonic saline have been shown to reduce the rate of decline in lung function, improve quality of life and reduce exacerbations (83,84). Regular, good quality airway clearance is part of recommended care worldwide, despite limited evidence for their long-term benefit (79,80).

Several medications have been investigated as anti-inflammatory agents, including inhaled corticosteroids, systemic steroids, high dose ibuprofen, leukotriene antagonists and macrolide antibiotics (e.g., azithromycin) (85–89). However, results have either been underwhelming, or long-term use limited by concerns over possible adverse effects (85,88,90–92). Azithromycin, is the only agent in wide use and is part of best practice care (79,80). It has been shown to reduce exacerbations and improve

the health status of patients, with the effect greater in patients chronically infected with *P. aeruginosa* (93,94). These benefits are thought to be secondary to its anti-inflammatory and immunomodulatory effects, as well as a possible impact on the virulence factors of *P. aeruginosa* (95). However, the exact mechanism remains poorly defined.

Health outcomes are improved with eradication attempts of initial bacterial infection, particularly in the case of *P. aeruginosa* (96). Once eradication therapy fails and chronic *P. aeruginosa* infection is diagnosed, inhaled anti-pseudomonal antibiotic treatments form part of standard care (79,80). These agents have been shown to improve lung function and reduce pulmonary exacerbation rates, with the best evidence being for inhaled tobramycin (97).

1.1.4.2.2 Targeting the defective CFTR protein

In the last decade, the introduction of CFTR modulator therapy into the treatment armamentarium for CF has been transformative and led to remarkable clinical outcomes for individuals with CF (9). Discovered through high-throughput drug discovery programs, these small molecules are mutation-specific corrective agents which improve epithelial CFTR function and expression (31). However, it is not yet known if they also restore the function of mutant CFTR expressed in phagocytes and other immune cells which regulate airway inflammation (29).

Five main groups have been defined, depending on their effects on mutant CFTR: potentiators, correctors, stabilisers, read-through agents and amplifiers (9). To date, one potentiator compound (which increases mutated CFTR activity at the cell surface) and three corrector agents (which improve altered protein processing and trafficking to the cell surface), have been approved (either as single, dual or triple combination therapy) for clinical use worldwide (98–103). Yet, challenges still remain to ensure equitable access to these costly new treatments for the 90% of individuals with CF who carry genes amenable to CFTR modulator therapy, including those with the most common gene mutation (F508del) (3).

1.1.5 Infection control

Patient-to-patient transmission has been well described for several CF-associated

pathogens, such as certain highly transmissible strains of *P. aeruginosa* and *B. cepacia* complex*,* MRSA and *Mycobacterium abscessus* complex (104–112). Acquisition of all of these organisms is associated with poor clinical outcomes (6). Outbreaks have been traced to inpatient stays and clinic visits to CF centres or social events involving several individuals with CF (109,111–113). Of concern is growing evidence that transmission can occur not only by direct (e.g., patient to patient) but also by indirect (e.g., airborne transmission) routes (105,111,114). Accordingly, infection control guidelines have been developed and widely adopted by CF centres to lessen the risk of cross-infection to individuals with CF, including education, strict cohorting policies for inpatient and clinic settings based on sputum microbiology, hand and cough hygiene, the use of personal protective equipment by staff (e.g., gloves, gowns) and individuals with CF (e.g., masks), equipment and environmental cleaning and disinfection practices (115–117). Although these practices have reduced the prevalence of epidemic *B. cenocopacia* and *P. aeruginosa* infections, many are based on a low level of evidence (1). In addition, implementation has not been without considerable impact on the lives of individuals with CF and strain on existing hospital-based infrastructure (117–121).

With the evolving demographic, clinical characteristics and personal needs of individuals with CF, an important challenge to care providers remains how to evolve accordingly, whilst maintaining health outcomes. Of note, strategies to reduce treatment burden and improve adherence have recently been highlighted as important clinical research priorities in a recent survey of the CF community (122). In the last decade, advances in digital technology and wearable sensors have driven interest in the use of home-based monitoring (reviewed in Home monitoring to detect acute pulmonary exacerbations in CF 1.6.3) as a means to address these priorities, with a potential to reduce routine clinic visits for some individuals with CF, enhance self-monitoring; clinician decision making and peer-to-peer support, whilst mitigating cross-infection concerns.

1.2 Acute pulmonary exacerbations in CF

CF lung disease is clinically characterised by recurrent, periodic worsening in respiratory symptoms and signs (e.g., cough, sputum production and lung function) from an otherwise stable baseline (123). Respiratory deterioration is often associated

with systemic symptoms (e.g., fatigue and low appetite) due to an acute phase inflammatory response (123–125). These episodes, termed acute pulmonary exacerbations (APE), remain one of the most important clinical events experienced by individuals with CF (124). In the short term, depending on the severity of the episode, some individuals with CF may require time off school or work. This can, depending on the frequency, impact adversely on educational and career endeavours (1). Several key studies have underscored the longer-term clinical impact of these events, demonstrating associations with an acceleration in lung function decline, a higher mortality risk, increased health-care costs and a reduction in health-related quality of life (126–131). Moreover, their prevalence has been shown to increase with age and severity of lung disease, further emphasising their role in driving disease morbidity and mortality (132).

Antibiotics, administered either intravenously or orally, are considered a mainstay of treatment (133,134). Nonetheless, 25% of individuals with CF will fail to recover to within 90% of their baseline lung function following an APE, despite aggressive treatment with intravenous antibiotics (135). Of note, factors associated with failure to recover lung function include delays to re-assessment of baseline lung function, and larger falls from baseline FEV_1 before treatment initiation - providing support for closer monitoring to assist earlier detection and prompt treatment of exacerbation events (126).

1.2.1 Pathophysiology of APE

Despite their frequency and clinical significance, a clear understanding of the pathophysiological mechanisms that trigger an APE continues to elude investigators. Current consensus opinion, largely driven by observations of clinical improvement with antimicrobial therapy, is that these events are triggered by a shift in the balance between host defence mechanisms and the predominant chronic airway pathogen(s) (136–139). Yet, strong mechanistic evidence for this is lacking. As the dominant conventional CF pathogen, much investigation has focused on pulmonary exacerbations in individuals chronically infected with *P. aeruginosa*. An early study proposed clonal expansion of the persisting pseudomonal strain from the biofilm reservoir, rather than acquisition of new bacterial strains (140). Studies demonstrating falls in bacterial sputum density following antibiotic treatment for an APE favoured this

hypothesis (136–138,141–143). However, subsequent studies demonstrated no increase in bacterial sputum density within the airway prior to or at the onset of an APE (144–148). Moreover, falls in bacterial density previously noted with antibiotic treatment have since been shown to be transient and not predictive of enhanced clinical outcomes (e.g., improvement in lung function) (147,148).

Respiratory viruses (e.g., rhinovirus, respiratory syncytial virus, parainfluenzae, influenza, adenovirus, coronavirus and coxsackie/echovirus) have been associated with an increased risk of APE (149–151). Rhinovirus infection in addition, has been shown to liberate planktonic *P. aeruginosa* from biofilms *in vitro*, which illicit a stronger inflammatory response than their biofilm dwelling counterparts (152). This has led several authors to hypothesise whether a change in sputum bacterial density may be linked to viral respiratory tract infections. Conflicting results have been reported. Wark *et al.* investigated 17 adults with CF, the majority of whom were chronically infected with *P. aeruginosa* (153). They found a small increase in sputum bacterial density in association with viral-associated APEs. However, in a more recent study by Chin *et al.* of 35 adults with CF and chronic *P. aeruginosa* infection, viral infections were not associated with significant changes in bacterial density at the time of an APE when compared to non-viral-associated APEs (148).

Ambient air pollution has also been linked to APEs in individuals with CF (154,155). In a landmark epidemiological study combining the US CF National Registry to the US Environmental Protection Agency Aerometric Information Retrieval System Goss *et al.* demonstrated that annual average exposure to particulate material was associated with an increased risk of APE (154). Their findings have been supported by more recent work showing a significant association between residential proximity to major roadways, exposure to ambient concentrations of ozone, nitrogen dioxide and particulate matter less than 10 μm in diameter (PM_{10}) and an increased frequency of APEs (156,157).

In the last two decades, the increased use of culture-independent molecular techniques (e.g., 16S ribosomal RNA gene sequencing) has enriched profiling of the CF airway microbiome and revealed the presence of new microbial species co-existing

with conventional, culture-detected CF pathogens (62,158,159). Across several studies common newly detected genera include obligate anaerobes (e.g., *Prevotella*, *Veillonella*, and *Fusobacterium*) and facultative anaerobes (e.g., *Streptococcus*, *Rothia*, *Actinomyces*, *Gemella* and *Granulicatella*) (159,160). These findings have led to investigation of microbial community composition peri-exacerbation to determine whether short-term shifts in the structure of the CF microbiota may drive APEs. However, thus far, the CF microbiota has been found to be relatively stable through periods of exacerbation and antibiotic treatment, providing no new insights into the causal mechanisms for APEs (160–165).

Changes in phenotypic expression of biofilm-associated bacteria may drive APEs. Biofilm-dwelling bacteria employ cell-density-dependent intercellular communication (quorum sensing [QS]) to coordinate expression of several genes, including those associated with virulence (166). Early work exploring microbial factors in APE initiation revealed the presence of *P. aeruginosa* exoproteins in CF sputa only at times of exacerbation in adults chronically infected with *P. aeruginosa* (167). This suggested an increase in bacterial virulence during acute exacerbations. The subsequent discovery of anaerobic biofilm formation in chronic CF lung infection led investigators to speculate whether transient fluctuations in bacterial growth mode, through occasional blooms of pro-inflammatory, planktonic bacterial cells from relatively inert biofilm populations, might trigger worsening in clinical symptoms necessitating antibiotic treatment (168–171). Data from clinical trials have confirmed a reduction in APE risk using macrolides (with neither bacteriostatic nor bactericidal activities against *P. aeruginosa*). Furthermore, *in vitro* studies have suggested that these agents, at sub-inhibitory concentrations, can compromise expression of certain bacterial characteristics, including *P. aeruginosa* QS, motility and exoproducts (93,172,173). Taken together, these findings provide support for the theory that short-term fluctuations in bacterial phenotypic expression (rather than density) may be significant in APE pathogenesis. However, to date, studies investigating this hypothesis have been hindered by either profiling too few phenotypes, low sampling depths, small patient numbers, or few timepoints independent of changes in clinical state, potentially hindering detection of a microbial signal linked to APE initiation (174–184).

1.2.2 Defining a CF exacerbation

To date, no consensus has been reached as to what diagnostic criteria constitutes an APE, although the terminology has been in common use since the mid-1980s (185–187). At present, diagnosis relies heavily on patient-reported changes in baseline symptoms and accompanying declines in spirometric parameters (188). However, individuals with CF invariably experience day-to-day symptom variation.

Several factors have hampered development of an APE definition, not least the poorly understood day-to-day variation in symptoms, the absence of a reliable biomarker and variations in admission and treatment criteria between different CF centres (133,185,189,190). Existing diagnostic criteria have been derived from empirical data and proposed largely to facilitate inclusion criteria for treatment studies or as outcome measures to assess the clinical efficacy of new therapeutic interventions (83,95,132,191–193). For example, most clinical trials employ the definition proposed by Fuch's *et al.* for the rhDNase trials (Table 1.1) (83). These operational definitions have been centred around the clinician's decision to treat for a change in a variable combination of patient-reported symptomology, laboratory tests (especially spirometry) and clinical assessment (186). Common to several of these published definitions are changes in four specific parameters: increased cough, change in purulence or volume of sputum, lung function decline and weight loss (83,132,191,192). Despite their use, the accuracy and precision of these existing criteria are yet to be validated. Nonetheless, given the acknowledged inter-clinician

Table 1.1. Fuch's diagnostic criteria for an acute pulmonary exacerbation (83).

Treatment with IV antibiotics for any 4 of the following 12 signs or symptoms:	
1. Change in sputum	7. Anorexia or weight loss
2. New or increased haemoptysis	8. Sinus pain or tenderness
3. Increased cough	9. Change in sinus discharge
4. Increased dyspnoea	10. Change in chest examination
5. Malaise, fatigue or lethargy	11. ↓ in lung function by ≥ 10% from a previously recorded value
6. Temperature > 38ºC	12. Radiographic changes of chest infection

variability in exacerbation diagnosis and management (194), it is clear that treatment-defined exacerbation definitions lack specificity and therefore have limited use in the development of standardised approaches to care and the provision of meaningful comparative criteria for research purposes.

In spite of their limitations, systematic examination of components of these diagnostic research criteria by several groups has suggested that patient-reported symptoms, rather than clinical examination and laboratory values, were more predictive of a pulmonary exacerbation (132,185,195,196). In all of these studies, increased cough, increased sputum volume or purulence, reduced appetite, weight loss and reduced exercise tolerance were some of the signs and symptoms most predictive of a pulmonary exacerbation (125). However, inter-patient variability in the perception of, and therefore the reporting of, exacerbation-related symptoms and signs is well recognised, not least because early symptoms of APE-onset for adults with CF are often focused on subjective measures (e.g., how they are feeling) (197). Moreover, the symptom descriptors used may vary according to the severity of an individual's underlying CF disease (e.g., "tiredness" for individuals with mild disease versus "fatigue" for those with more advanced disease) (197). Individuals with CF also face a daily onerous burden of treatment and are often reluctant to seek treatment early or attend clinic appointments for declining health, to the detriment of long-term fitness and wellbeing. It is likely, therefore, that decisions to report deteriorations in health lag considerably behind the first physiological signs or symptoms of illness, hindering early intervention and contributing to further lung damage.

Alongside inter-clinician and inter-patient heterogeneity in APE recognition, McCourt *et al.* have highlighted the lack of agreement in perspectives between CF health professionals and adults with CF as to what signs and symptoms are the most important indicators of an APE (123). Indicators rated highly by CF health professionals (e.g., inflammatory markers, respiratory rate, oxygen saturation, chest X-ray changes) were often rated lower by individuals with CF who prioritised subjective measures of an APE (e.g., a change in breathlessness, sputum production, cough and wellbeing) (123). It is worth noting that despite a different hierarchy of importance the indicators common to both groups were a reduction in lung function, increased breathlessness, increased sputum production and increased coughing. These

observations contribute to a growing evidence base that support patient-reported outcome measures (PROMS); standardised sets of questions on health status completed by patients without clinician interpretation of responses (500), as more sensitive to change than clinician-derived measures. Of these, the CFRSD-CRISS (CF Respiratory Symptom Diary/Chronic Respiratory Infection Symptom Score) is the only exacerbation-specific symptom score recently validated for use in people with CF aged ≥ 12 years (501,502). Nonetheless, work by McCourt *et al.* suggests that progression towards a unifying set of criteria that defines an APE may not be feasible, nor indeed most helpful. Rather, it may be more useful to identify a patient-specific combination of symptoms and signs that, when altered for a sustained period of time, constitutes a deviation from that individual's "stable" baseline and could be used to identify an APE. The challenge, however, is in establishing what is typically an undocumented daily baseline, against which occurrence of an APE can be detected. Again, here (in addition to the potential benefits highlighted previously in Infection control 1.5.3), the CF research community is looking at the use of home monitoring of symptoms and lung function as a potential strategy to better understand daily symptom variation and to enable examination of relationships between symptoms and physiological variables that can facilitate earlier detection of APEs in CF (198).

1.2.3 Detecting APEs using home monitoring

Telemedicine (or Telehealth) can be broadly defined as the use of information and communication technologies to deliver medical information, clinical care, education and services over a distance to improve the health of patients and their communities (199,200). Interactions may occur either asynchronously or in real-time (199). Telemedicine services encompass two sub-categories: technology dedicated to communication between health professionals (e.g., teleradiology) and technology used between health professionals and patients (201). Telemonitoring (or home monitoring) falls into the latter category and uses remote patient monitoring technologies, including telephones, smartphones, health-related mobile applications and connected wearable devices (mHealth), with or without audio-visual connections, to transmit patient symptom and physiological data in order to enable diagnosis, follow-up and/or treatment remotely (199,200,202).

The use of home monitoring to detect adverse health trends in individuals with CF is not a new concept. However early methods of home monitoring were reliant on the use of paper diaries to record symptoms and physiology (203–205). One of the earliest such studies was conducted in the mid-1980s by Finkelstein *et al.* who investigated the implementation of a home monitoring program for individuals with CF (203,206). In this study, 122 children and 51 adults with CF were asked to record their daily symptoms and physical measurements (weight, vital capacity, quiet breathing and resting pulse rate) using written diaries collected on a weekly basis over a two-year period. One-hundred and eleven participants completed the study. Diary data was manually collated into a research database within 48 hours of receipt. Despite the potential time and resource burden, the home monitoring program was found to be feasible and acceptable, with 80% compliance among participants. Notably, compliance was promoted through feedback letters, between-visit telephone and written contact and the discussion of home monitoring graphs during clinic visits. Subsequently, these investigators carried out a nonconcurrent cohort study on 50 individuals with CF. Twenty-five participants were selected randomly from the group that used home monitoring and were compared to twenty-five age- and gender-matched participants who had not taken part in home monitoring. The aim was to determine if daily diary recording and self-monitoring alone, in the absence of any therapeutic intervention, resulted in a change in clinical outcomes (physical or psychological health, lung function or growth). Participants were followed for 4 years (207). Importantly, close home monitoring and diary record keeping was not found to have a negative impact on patient health (207).

Since these initial studies there has been rapid advances in telecommunication tools, not least the broad adoption of internet and web-based technologies, improved affordability and access to high-speed, high-bandwidth telecommunication networks and the invention of devices capable of more efficient capture and transmission at a distance of health-related data in digital form (208). Surprisingly, when compared to other chronic diseases such as heart failure, diabetes or COPD (209–211), there have been relatively few telemonitoring studies in CF and evidence for their efficacy has been mixed (198,212–219). Moreover, Cox *et al.* conducted a systematic review in 2012 of the use of telehealth in CF (n = 8) and concluded that despite being feasible and acceptable to individuals with CF, there was an insufficient evidence base from

which to draw firm conclusions about the benefits of telemedicine applications for individuals with CF (217).

To date, prior studies in CF evaluating interventions for the early detection of pulmonary exacerbations have been relatively small, focused on a limited number of home-monitored parameters [such as spirometry (213–216), oxygen saturation (214) and symptoms (213–216)] and used fairly simple statistical cut-offs as triggers to detect exacerbation events (198,215,220,221). Moreover, adherence rates have varied widely across the studies (10-59%), often attributed to either technical difficulties or an increased burden of reporting associated with the home monitoring intervention (200). For example, Bella *et al*. conducted a non-randomised study assessing the use of telehomecare in an adult population at an Italian CF centre (214). Thirty individuals with CF were assigned to record daily symptoms, spirometry and overnight oxygen saturation whereas matched controls received usual care. Symptom data was transmitted via e-mail. Physiological data was collected on a digital multi-channel recorder and transmitted twice weekly via a non-digital, landline, cable modem to a dedicated study server. Monitoring was completed for a minimum duration of seven months. The drop-out rate for the intervention group was high (43%) with participants failing to comply with the minimum requirements for data submission. Investigators proposed that the added burden of the intervention to a participant's usual treatment schedule was a likely explanation for the significant drop-out rate, although qualitative feedback was not sought from participants (214). A 4.5-year analysis of this longitudinal study was recently undertaken by Murgia *et al.* (221). During the follow-up period, participants were contacted if they met intervention criteria for an exacerbation based on a decline in FEV_1 (> 10% compared to the previous value recorded in stable clinical conditions) or nocturnal oxygen saturation (< 90% of the maximum, or a reduction in the mean, or an increase of > 5% in the detection time (T90) spent below an oxygen saturation of 90%). Interestingly, a significantly lower decline in lung function was observed in the group undertaking home monitoring (221).

In another non-randomised study, Sarfaraz *et al.* conducted a feasibility study of once-daily electronic remote monitoring of symptoms and lung function for early identification of pulmonary exacerbations in a cohort of adults with CF (*n* = 51) (213). The remote monitoring system consisted of a mobile-enabled personal digital

assistant (PDA) to record symptoms (cough, sputum, breathlessness and fatigue) and lung function. Monitoring occurred over a 6-month period and data was automatically transmitted from the PDA to a study website. Exacerbations were defined as either device-identified (recorded when the submitted data met any of the following criteria: 1) at least one point increase in three symptom scores over three consecutive days; 2) > 10% decrease in FEV_1 alone over three consecutive days; or 3) at least one point increase in two symptom scores, plus ≥ 10% decreased in FEV_1 over three consecutive days) or patient-defined (symptoms without fulfilment of criteria for a device-identified event). However again, adherence to monitoring was poor with only a third (37%) of participants providing frequent enough measures to form a baseline to explore the natural history of the disease. This was largely due to technical issues encountered when operating the device. Nonetheless, despite these short-comings, the authors reported that 75% of the exacerbations captured had a "prodromal" phase in which one or more symptoms (rather than lung function) worsened in the two weeks prior to identification of the exacerbation (213).

More recently, in an observational cohort study, van Horck *et al*. assessed the use of electronic home monitoring to detect APEs in forty-nine children with CF recruited from three CF centres in the Netherlands (216). In this one-year study, an exacerbation was established in one of two ways: first according to criteria used in the Early Pseudomonas Infection Control (EPIC) trial (222) (Table 1.2) and second, when the clinician determined a course of antibiotics was necessary based on the clinical symptoms expressed. Monitoring involved thrice weekly recording of lung function and symptoms (cough, sputum and breathlessness). Data stored on the home monitor was transmitted to a secure web-based portal weekly. Three participants did not use the monitor (two due to technical issues and one before using the home monitor). Adherence, defined as completion of 70% of the maximum number of requested home measurements over one year, was high (75%). Of note, the authors found an increase in symptoms as early as 4 weeks prior to the diagnosis of an APE being made.

In adults with CF, a recent large, multicentre, randomised controlled trial (the early intervention in cystic fibrosis exacerbation (eICE) trial) was conducted to assess whether electronic home monitoring of lung function and symptoms would facilitate earlier detection of APE and result in a slower decline in lung function over 12 months

compared with usual care (215). Two-hundred and sixty-seven adults with CF took part and the proposed study duration was one year. Participants used home-based spirometers and completed the CFRSD via the Viasys AM2 device (CareFusion, Yorba Linda, CA) to identify exacerbations. The home monitoring system triggered participants to seek clinical review for a potential APE whenever FEV_1 declined greater than 10% from baseline or two or more of eight symptom scores (using the CFRSD)

Table 1.2. EPIC trial definition for a pulmonary exacerbation (222).

Major criteria (One finding alone establishes the presence of an exacerbation)
1. Decrease in FEV_1 of ≥ 10% from best baseline within the past 6 months, unresponsive to beta-2 agonist 2. Oxygen saturation < 90% on room air or ≥ 5% decline from previous baseline 3. New lobar infiltrate(s) or atelectasi(e)s on chest radiograph 4. Haemoptysis (more than streaks on more than one occasion in past week)
Minor criteria (Two findings plus duration criteria, in the absence of major criteria)
1. Increased work of breathing or respiratory rate 2. New or increased adventitial sounds on lung exam 3. Weight loss > 5% of body weight or decrease across 1 major percentile in weight percentile for age in past 6 months 4. Increased cough 5. Decreased exercise tolerance or level of activity 6. Increased chest congestion or change in sputum
Duration (Required with two minor criteria, in the absence of major criteria)
Duration ≥ 5 days or significant symptom severity

worsened from baseline. Despite more protocol-defined exacerbations detected in the home monitoring cohort, the study was stopped prematurely as earlier detection of APEs did not result in a slower decline in lung function. Moreover, adherence with twice weekly data transmission was very low (19%) with participants encountering considerable technical issues with the home monitoring system. This finding (in line with previous home monitoring trials in CF) clearly highlighted the need to streamline the remote monitoring system in order to lower the burden of monitoring to the patient.

The authors also suggest that the study's negative primary outcome may reflect a suboptimal choice in APE threshold identifiers, acknowledging that (in the absence of a consensus definition for an APE) an alternate combination of cut-offs may have led to different results. However, it still remains unclear how best to determine an accurate composite measure of symptoms and signs for early detection of an incipient exacerbation.

1.3 Machine learning as a tool to examine home monitoring data

The CF home monitoring studies reviewed in Section 1.2.3 have shown that with continued innovations in home monitoring technologies, the collection of increasingly larger and more complex, longitudinal datasets is possible (223). These big datasets, however, have revealed the limitations of classical statistical approaches to yield actionable knowledge that can drive progress on identifying and predicting APEs at the individual level (224,225). In general, classical statistical models deal poorly with high-dimensional data due to their underlying assumptions. Therefore, they may fail to uncover complex interactions between variables which may be concealed within the data and which cannot easily be expressed as a mathematical equation (226,227). Consequently, assisted by the availability of faster computation and cloud data storage, there has been a push within the respiratory community to leverage data science techniques (e.g., data mining, machine learning (ML) methods and predictive analytics) to discover useful associations, patterns and trends for prediction and explanation within big data (228–230).

Artificial intelligence is a broad interdisciplinary field which draws on computer science, mathematics and philosophy, among others (231). It involves the use of computer systems to imitate human cognition and intelligent behaviours, such as thinking, reasoning, and learning, in order to solve complex problems in the way an expert might - by careful consideration of the evidence to reach reasoned decisions (227). However, one key advantage over the human expert is that systems driven by artificial intelligence can both observe and rapidly analyse an almost infinite number of inputs (232).

Machine learning is an artificial intelligence technique (232). It broadly refers to an algorithmic framework that can enable new knowledge discovery (e.g., identify hidden

groups or associations between key variables) while facilitating inductive inference and providing a setting to explain and predict particular outcomes from observed data (226,233). Machine learning algorithmic models process data and improve on performing specific tasks autonomously, by pattern recognition and learning from the data they see, rather than through explicit computer programming by a human expert (234,235).

Machine learning algorithms can be divided into two main classes: unsupervised learning and supervised learning (236). Unsupervised learning supports features extraction - algorithms that seek patterns from an unlabelled set of input data, with the goal being to group the data based on their inherent features, rather than on a targeted outcome (235). A large part of the strength of ML comes from unsupervised learning which facilitates the learning of new associations from hidden (or *latent*) aspects of the data, that often have not been considered before (231,237). These hidden aspects often reflect non-linear relationships between many of the input variables. For example, this method has been used to identify novel phenotypes of sepsis (238). In contrast, supervised learning algorithms are trained with a labelled dataset of inputs and a paired known output, with the aim being to learn how to process the inputs in order to reproduce the related output (235). For example, the model may be trained to associate a person's characteristics (e.g., sex, weight, age) to a certain outcome (e.g., onset of hypertension within five years). The fully trained algorithm can then be tested on a given set of inputs, without their associated outcomes. The algorithm's predictions on the testing set can then be compared to their known outcomes to determine the model's predictive performance, and generalisability (239).

The use of ML within the field of respiratory medicine is rapidly expanding (236). Examples include integration of ML for prediction of lung cancer prognosis, risk of hospital admissions with COPD and thoracic imaging analysis to improve diagnostic accuracy (240–243). Machine learning offers a promising solution to the challenge of analysing high-dimensional data collected by home monitoring devices. However, as yet, this powerful analytic technique has not been applied to datasets collected from individuals with CF.

1.4 Detecting APEs in non-CF lung disease using home monitoring

Acute exacerbations in asthma and COPD, as in CF, are significant events with a negative impact on health status, hospitalisations and readmissions, and disease progression (244,245). Home monitoring has also been studied in these conditions, as a means to promote self-management, improve disease control, increase quality of life and prevent hospital admissions (226). In 2013, Nimmon *et al.* conducted a critical review of telehealth interventions for the management of COPD and asthma, using evidence collated from eleven systematic reviews published between 2001 and 2011 (246). The authors concluded that home monitoring showed potential as a patient management approach, supporting early identification of deteriorations in patient condition and symptom control. However, the authors cautioned that evidence for the magnitude of clinical impact was lacking. Reasons given for this included the poor quality of studies, unclear definitions of disease, wide variation in monitoring methods, small sample sizes and short study durations. Moreover, several studies have highlighted the poor performance of conventional algorithms (e.g., threshold values assigned to trigger an alert when a deterioration in patient status occurs) for detecting exacerbations (247–249). These findings have been corroborated in more recent systematic reviews (250,251). The lack of concrete symptoms and signs to distinguish stability from periods of exacerbation in asthma and COPD has made development of early detection systems based on home monitoring data challenging (252).

Recently, data mining and ML approaches have been applied to asthma and COPD telemonitoring datasets to address the task of developing more accurate, clinically reliable, early predictors of acute exacerbations (226). However, a 2016 systematic review of the use of predictive algorithms for exacerbations in home monitoring studies of individuals with COPD and asthma concluded that models with good clinical reliability have yet to be defined and remained an important goal for the future development of telehealth in chronic respiratory conditions (226). Sanchez-Morillo *et al*. identified 20 studies (16 COPD, 4 asthma) which met inclusion criteria (where home telemonitoring was involved, the algorithms used to detect episodes were described and results on the performance of algorithms for the automatic prediction and/or detection of respiratory exacerbations were presented) (226). In the majority of the studies (n = 12) a simplistic strategy was employed for early detection of exacerbations, consisting of a basic decision rule based on assigning threshold values

to the collected parameters. In the remaining studies (*n* = 8), ML techniques were applied. Of note, the authors did highlight that the ML predictive models provided encouraging results whilst, by comparison, the conventional threshold-based algorithms showed poor performance. Many of the underpinning algorithms behind these models still need to be validated in larger samples of patients, for longer periods of times and with well-established protocols. Nonetheless, there is encouraging evidence from this work in individuals with asthma and COPD that identification and prediction of acute exacerbations in respiratory conditions, using home monitored data may be within reach using ML approaches.

1.5 Detecting APEs in CF using bacterial sputum biomarkers

The clinical presentation of APEs in CF is broad, varying from an increase in cough frequency without a change in lung function to significant falls in lung function with accompanying breathlessness and potential respiratory failure (186). In the absence of a consensus diagnostic definition, the need remains for a reliable, clinically relevant biomarker(s) that can objectively reflect the onset of an APE and advance our understanding of the mechanisms that drive APEs and their relationship to airway inflammation and lung function decline (190,253). Moreover, the discovery of such a biomarker(s) would offer the opportunity to initiate therapies promptly, thereby circumventing irreversible loss of lung function.

Ideal biomarkers for APE detection should be able to be measured from samples that can be easily collected from individuals with CF. Sputum (in comparison to blood, urine, bronchoalveolar lavage samples) is a rich, non-invasive and direct source of the by-products of lower airway inflammation and infection (such as inflammatory cells, pro-inflammatory cytokines, mucin, bacteria and bacterial metabolites), and is readily sampled from the majority of adults with CF (254). Moreover, previous studies have confirmed that expectorated sputum provides an accurate measure of infection in the CF airway (255–257). To date, sputum biomarker studies have largely focused on investigating host-derived biomarkers of pulmonary inflammation, mainly for use as outcome measures in clinical trials assessing new therapies or disease progression (258–260). Some of these biomarkers (e.g., calprotectin, neutrophil elastase, IL-8, and myeloperoxidase) have also been investigated to monitor for APEs. However, findings have been inconsistent, with both positive and negative results reported for the same

biomarker (141,261–265). Importantly, certain authors have argued that host-derived inflammatory biomarkers are likely to be limited in their predictive power, as changes in their levels are likely to occur only once upregulation in the inflammatory response has been established during an APE (1,189). Therefore, there remains a need to identify biomarkers that are linked to triggers that upregulate the inflammatory response, in order to more accurately detect APE onset.

In the setting of chronic bacterial infection (usually with *P. aeruginosa*) in the CF airway, alternate approaches may be to measure changes in airway bacteria number or bacterial products in sputum. Mechanistically, it has been postulated that upregulation of the immune response may be triggered by secretion of specific bacterial compounds (associated with virulence behaviour) into the airways, or in response to the presence of bacterial cells in the airways (189,266). However, when bacterial sputum density has been determined through the course of an APE, changes in bacterial load have not been shown to be predictive of an APE (147).

Several substances associated with *P. aeruginosa* virulence (e.g., exotoxins, pyocyanin, pyoverdine and QS signals) have been detectable from CF sputum using various analytical techniques such as immunochemistry, spectrophotometry, mass spectrometry and whole cell reporters (167,267–271). Yet, few studies have been published on microbial-derived biomarkers for APE detection. In the early 1990s Grimwood *et al.* demonstrated an increased concentration of *P. aeruginosa* exoenzymes (Exotoxin A, exotoxin S, elastase, total protease and phospholipase C) in sputum collected during an APE from 17 hospitalised patients compared with 17 stable controls with CF (267). The same authors also demonstrated a fall in exoenzyme concentrations following antibiotic treatment in a separate prospective study of 9 hospitalised patients with CF. Shortly after, Jaffer-Bandjee *et al.* examined secretion of *P. aeruginosa* exoenzymes (elastase, Exotoxin A and alkaline protease) during one or several exacerbation periods (29 in total) in 18 hospitalised patients with CF (167). Three of the patients were also studied during inter-exacerbation periods (when they were admitted for digestive disease). A significant fall in exoprotein levels was observed following antibiotic treatment in the majority of exacerbation episodes with negligible levels detected during the 3 inter-exacerbation periods studied (167). In order for a biomarker to be useful in predicting the onset of an APE, a consistent

change in the level between periods of clinical stability and decline (not just following initiation of treatment) must be demonstrable (189). Evidence from these earlier studies by Grimwood *et al*. and Jaffer-Bandjee *et al*. suggests that a microbial signal may be present prior to APE onset. In addition, combining several biomarkers to augment the significance of association with an APE has been proposed previously, which may be more useful than assessing single markers alone (190). However, this has yet to be confirmed.

Microbial-derived biomarkers in sputum (with particular emphasis on the traditional CF pathogen, *P. aeruginosa*) present a promising means to enrich APE diagnosis and prediction in CF and warrants further investigation. Furthermore, the potential to integrate novel sputum biomarker assays with innovative home monitoring technology offers an exciting opportunity to enhance self-management and clinical decision-making for individuals with CF.

1.6 *Pseudomonas aeruginosa*

P. aeruginosa is a stress-resilient Gram-negative organism. It is an aerobic to facultative anaerobic, rod-shaped bacterium which belongs to the bacterial family *Pseudomonadacea* (272). The organism is widely distributed in terrestrial and aquatic environments (273). *P. aeruginosa* strains possess a large genome (between 5.5 and 7Mbp within the species) which approaches the size and complexity of lower eukaryotes (274–276). The large genome size is primarily the result of a highly conserved (inter-clonal sequence diversity of 0.5 - 0.7%) core genome which encodes many regulatory genes involved in sensing environmental signals, controlling expression of virulence factors, metabolism and resistance mechanisms (277,278). Its genomic diversity results mainly from the presence of a highly variable accessory genome, consisting of integrated islands, transposons, bacteriophages, or IS-elements that are distributed through the core genome at certain loci (regions of genomic plasticity) to form a mosaic-like structure (276,277,279,280). These accessory elements enable *P. aeruginosa* to acquire genetic material from different sources (including other species or genera) by horizontal gene transfer (transformation, conjugation and transduction), nucleotide substitution, insertions of transposons and bacteriophages (277). *P. aeruginosa* prefers to grow in aerobic or microaerobic environments (281). However, it is remarkably versatile, due to a

combination of its large genome, accessory elements and vast array of regulatory systems. These properties make it capable of competitively thriving in diverse ecological niches and maintaining a metabolically flexible lifestyle (276,280).

However, the same properties that confer ecological success have allowed *P. aeruginosa* to excel as an opportunistic human pathogen, with the means to cause various, often overwhelming, acute infections (e.g., pneumonia, bloodstream infections), mostly in the setting of compromised host defences (282). Remarkably, this same pathogen is capable of causing chronic infections that persist in the host over time, such as is seen in the lungs of individuals with CF. This capacity to establish chronic infection results as a consequence of dynamic and complex host-pathogen interactions, where *P. aeruginosa* persists without causing overwhelming injury, and where host defences fail to eradicate the organism (278).

1.6.1 *P. aeruginosa* infection in CF

Of the characteristic set of microbes known to infect the lungs of individuals with CF in an age-dependent fashion (reviewed in section 1.5.1), *P. aeruginosa* remains the most noteworthy, causing chronic airway infection in up to 80% of adults with CF (283). Its persistence in the CF airways is classically associated with poorer survival and an accelerated rate of lung function decline (284–288). Given these major clinical implications, early, aggressive treatment of incident infection (in an attempt at eradication), is an agreed standard of care (79,289). In part this strategy, coupled with improved infection control measures, has seen a gradual decrease in the overall prevalence of *P. aeruginosa* chronic infection in CF over the past two decades (290,291).

Currently, there is no consensus definition to distinguish early, intermittent *P. aeruginosa* infection from chronic infection (292). Whilst there is broad agreement that persistent *P. aeruginosa* growth on repeated airway sampling is characteristic, the proportion/number of positive samples, the sampling interval and the clinical context may differ between CF specialist centres, in accordance with local guidelines (292,293). Where available, serum *Pseudomonas* IgG antibody levels (e.g., strongly positive or a rising trend) may be useful to assist with differentiation between early and chronic infection stages, particularly in the case of inadequate airway sampling (294–

296).

Despite the lack of a universally accepted definition, certain criteria have been applied in the CF clinical and research settings, particularly in paediatric studies (297,298). Arguably, the most well-known of these is the Leeds criteria, which is solely dependent on standard microbiological results (299). These criteria define chronic *P. aeruginosa* infection as > 50% of months with cough swabs/sputum cultures in the preceding 12 months that were positive for *P. aeruginosa* (299). Of note, a recent real-world study by Hoo *et al*. highlighted some important concerns with its use (293). The study compared clinicians' decision with the Leeds criteria in three adult CF centres in the UK. The authors found consistency in decision-making by clinicians across the different centres and that clinicians did not always agree with the Leeds criteria. Furthermore, where disagreement occurred, clinical decision-making was found to be more sensitive in the diagnosis of *P. aeruginosa* chronic infection because clinicians assimilated other relevant information (such as information on strain type, proportion of negative cough swabs *versus* sputum samples) in their decision-making (293,300).

1.6.1.1 Acquisition

A lack of clarity still exists around the aetiology and individual risk factors for first acquisition of *P. aeruginosa* in CF (301). Several paediatric studies have drawn links to seasonal, meteorological and geographical differences in acquisition. A higher incidence rate of first acquisition has been observed in the summer and autumn months and in urban environments, whereas an earlier age of acquisition has been associated with warmer annual ambient temperatures (301–304). Given its ubiquitous presence in the natural world, initial acquisition of *P. aeruginosa* is thought likely to occur from environmental reservoirs (305). This conclusion is supported by genetic fingerprinting studies in children with CF which show that early infecting strains are nonclonal and genotypically resemble those found in the natural environment (306).

Acquisition may also occur via patient-to-patient transmission and several multi-drug-resistant "epidemic" strains have been identified, most prominently in the UK but also in Australia and the US (recently reviewed in detail by Parkins *et al.*) (113). Epidemic strains are clonal strains (genetically identical to a common ancestor) that exist among a local CF population (113). The Liverpool epidemic strain (LES) is the most prevalent

epidemic strain of *P. aeruginosa,* infecting approximately 11% of individuals with CF in the UK (307). It is also notable as the only epidemic clone to be found inter-continentally (North America and Europe) and is unique for very high rates of antibiotic resistance which facilitate its persistence in the CF airway, transmissibility and virulence behaviour (308). Two other transmissible strains are known to circulate in the UK: the Manchester epidemic strain (MES) and the Midlands-1 strain (108,309). The MES was first reported in 2001 in the Manchester Adult CF Clinic (108). This clone, unlike the Midland's-1 strain, is also significant for its high antibiotic resistance and association with increased CF morbidity (e.g., higher exacerbation frequency, admission rates and acute treatment burden) (108,310).

1.6.1.2 Establishment of chronic infection

Upon entering the CF airway, initial infecting *P. aeruginosa* strains encounter a hostile, heterogenous and stressful environment (311). Proposed challenges include osmotic stress due to highly viscid mucus (311), nutritional availability (312,313), other resident microbes (competing for resources or contributing to *P. aeruginosa* mortality) (62), bacteriophages (314), oxidative and nitrosactive stresses due to host immune responses (leading to increased mutation rates due to increased DNA damage) (315,316) and, sublethal concentrations of antibiotics. In this setting, and for reasons not entirely understood, early, recurrent and intermittent infection (which is susceptible to antibiotic treatment) is inevitably superseded by the establishment of intractable chronic infection (305). The transition to chronic infection is accompanied by a fundamental shift in the gene expression profile of *P. aeruginosa* in response to the host environment. These genotypic changes, determined by mutation and selection, enable the organism to adapt and manifest persistent and resistant phenotypes that distinguish chronic isolates from their early-infecting counterparts (which are non-mucoid, free-living, fast growing and relatively antibiotic-susceptible) (321) (Figure 1.4).

Individuals with CF are usually chronically infected with a single strain type of *P. aeruginosa,* although in some instances several unrelated strains can transiently or even permanently, coinfect the same individual (113,179). In a landmark study, Smith *et al.* examined two *P. aeruginosa* strains collected from a chronically infected individual with CF, 7.5 years apart, using whole genome sequencing (179). The

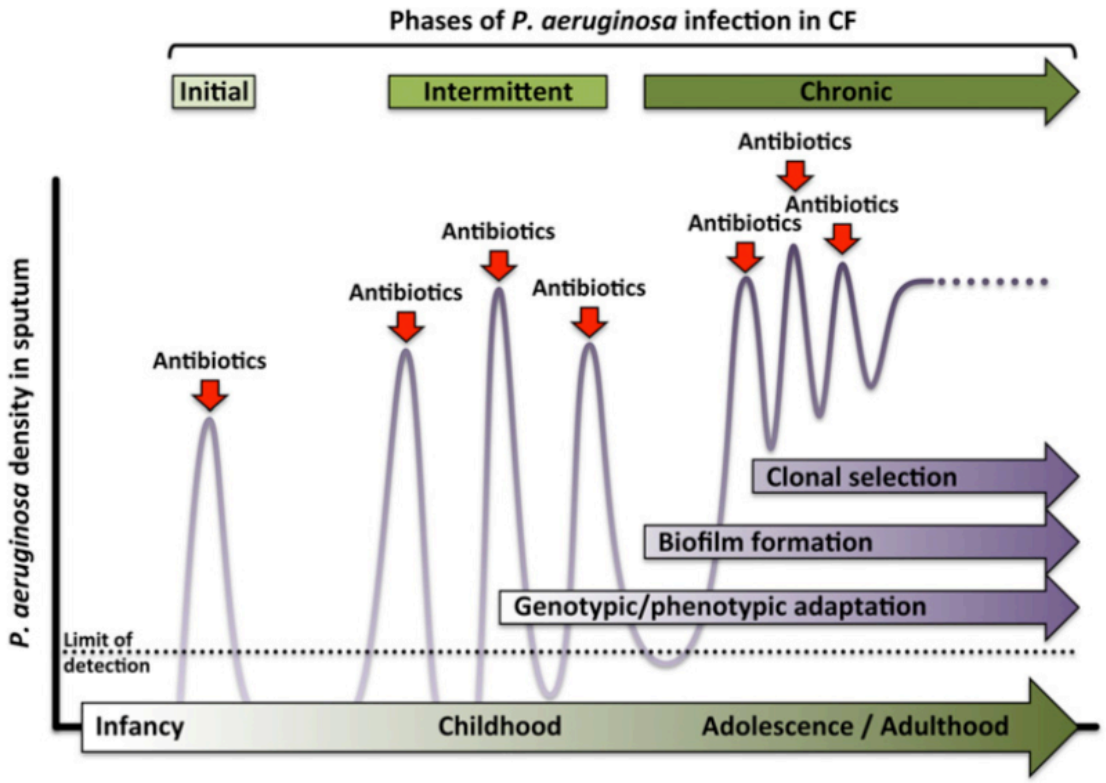


Figure 1.4. The characteristic time course of the development of chronic *P. aeruginosa* infection of the CF lung**.** Adapted with permission from Bouvier *et al.*, 2016 (322).

authors determined that the later isolate had acquired 68 mutations, a higher proportion of which were non-synonymous. Comparable findings were observed when analysing candidate genes from similarly paired isolates from 29 other CF patients. This led the authors to conclude that over the course of chronic *P. aeruginosa* infection there is evolution toward a dominant clone that, through clonal expansion, leads to emergence of populations of subclones characterised by expression of a chronic phenotype that is typically less virulent (e.g., less inflammatory and cytotoxic) and more resistant to antibiotic treatment than the initial infecting strains. Some of these characteristic "CF-evolved" phenotypes (described in further detail below) include conversion to mucoidy, formation of slower-growing small colonies on agar (small colony variants), loss of flagella-dependent motility, biofilm formation, increased auxotrophy, down-regulation of QS and subsequent loss of QS-regulated virulence factors such as secreted proteases and the siderophore pyoverdine (177,179,323,324).

However, it is now well established that clonal populations of *P. aeruginosa* residing

in the CF airway display greater heterogeneity in the expression of virulence-associated determinants than previously suspected (121,271,325–328). Moreover, evidence suggests that this phenotypic diversity is dynamic, with substantial fluctuations seen among clinical isolates within a single sputum sample and between sputum samples collected from the same individual over time (121,271,326,327). However, whether (in the setting of chronic *P. aeruginosa* infection) CF exacerbation onset correlates with the short-term emergence of more virulent clonal sub-populations of *P. aeruginosa* remains to be confirmed.

1.7 *P. aeruginosa* adaptive and virulence-associated phenotypes in chronic infection of the CF lung

1.7.1 Morphology variants

During early intermittent *P. aeruginosa* infection, the non-mucoid (e.g., environmental wild-type) colony morphotype predominates in the bacterial population. However, during the process of adaptation to the stressful CF lung environment mutations in the regulatory gene, *mucA* (which encodes the anti-sigma factor MucA) are commonly acquired (329). These mutations lead to loss of sequestration of the stress-responsive AlgT/U sigma factor to the inner membrane, allowing binding to RNA polymerase and unopposed expression of the alginate biosynthetic genes (329). The consequent overproduction of alginate, a secreted exopolysaccharide, characteristically marks the transition to chronic infection, with the emergence of mucoid colony variants. Alginate overproduction is regulated at two levels: 1) post-translationally, as a response to stress (e.g., antibiotics, iron limitation, host responses) or 2) genetically (from spontaneous chromosomal mutations) (330,331). Alginate contributes to the pathogenesis of *P. aeruginosa* in several ways, including enhancing biofilm formation and providing a protective capsule around the bacterium that impedes host immune responses (e.g., complement-mediated killing and phagocytic killing by macrophages and neutrophils) (74,305) and oxidative damage from oxygen free radicals (332).

Small colony variants (SCVs) are another distinctive colony morphotype associated with chronic *P. aeruginosa* infection in CF (333). These slow-growing isolates are typically described to take more than 48 h to appear on culture plates and exhibit small colonial diameters ranging between 1 and 3 mm (74). SCVs exhibit additional unique

features that favour *P. aeruginosa* pathogenesis, including an enhanced capacity to form biofilms (e.g., auto-aggregation in liquid culture and hyper-adherence to surfaces), a reduction in flagellar motility, upregulation of type III secretion components (which mediates secretion of exotoxins into the host cell) and increased antibiotic resistance (334). SCVs have also been reported to exhibit reversion into fast-growing morphotypes with regained antibiotic susceptibility following several passages in antibiotic-free medium *in vitro* (323). It has therefore been postulated that more resistant SCVs may emerge under the selection pressure of antibiotic therapy. Furthermore, when the selection effect weans, fast-growing revertants may arise and increase bacterial load, ultimately culminating in an exacerbation episode (323). However, prospective studies have yet to explore this hypothesis further.

The molecular mechanisms underlying the SCV morphotype in clinical *P. aeruginosa* isolates are not yet understood. However, SCVs are strongly associated with elevated levels of cyclic-di-GMP (a bacterial second messenger that regulates transition between motile/virulent and sessile/biofilm lifestyles in a wide range of species) (335), as well as increased production of Pel and Psl exopolysaccharides (336). To date, loss-of-function mutations in regulatory genes such as *wspF, yfiR, fleQ* and *rsmA* (which favour increased cyclic-di-GMP production) have been commonly identified in CF-evolved *P. aeruginosa* isolates (335,337–339).

1.7.2 Quorum sensing

Quorum sensing, a bacterial communication system that enables the coordinated expression of multiple genes and microbial social behaviour in a cell density-dependent manner, plays a critical role in the pathogenesis of *P. aeruginosa* (340). Quorum sensing depends on the synthesis, release and groupwide response to a critical threshold of extracellular signalling molecules called autoinducers (AIs) (341). Accumulated AIs activate their cognate receptors to induce or repress the transcriptional expression of numerous target genes in response to environmental stimuli (342,343).

P. aeruginosa possesses three linked canonical QS systems (Las, Rhl and PQS), with a hierarchical network of control mediating integration of multiple cross-signals between the QS signalling pathways (for detailed reviews see Williams and Camara,

and also Lee and Zhang) (282,343,344). These QS systems control the expression of genes mainly involved in virulence factor production, motility, the switch from planktonic to sessile growth mode, biofilm development, antibiotic resistance mechanisms and the adjustment of metabolic pathways for stress responses (343,345) (Figure 1.5).

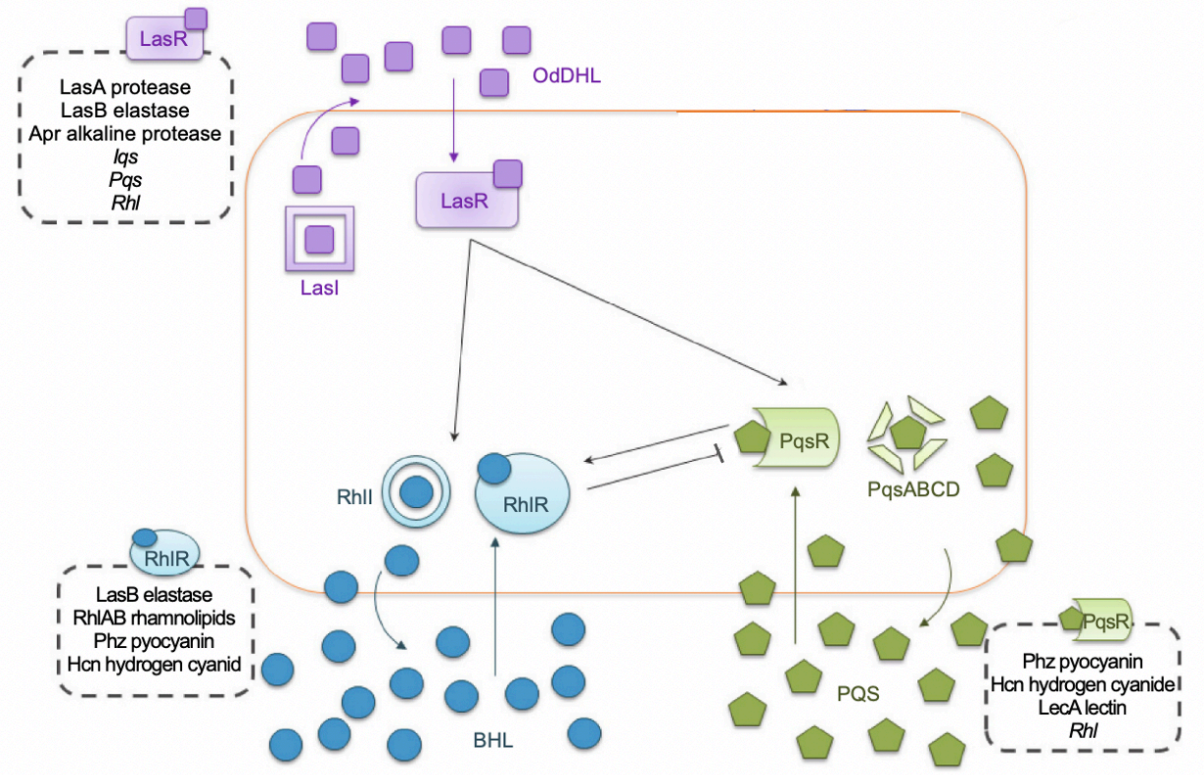


Figure 1.5. Schematic representation of the three QS signalling networks in *P. aeruginosa* and their corresponding regulons. Arrows indicate positive regulation. Perpendicular lines indicate negative regulation**.** Adapted with permission from Lee and Zhang *et al.*, 2015 (343).

The Las system consists of two proteins; LasI and LasR. LasI catalyses synthesis of its specific AI, *N*-acyl-homoserine lactone (AHL) signal molecule *N*-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL, OdDHL), that docks with LasR and in turn binds to the promoter of regulated genes (346,347). The Rhl system has its own specific AI synthase, RhlI, which synthesises *N*-butanoyl-*L*-homoserine lactone (C4-HSL, BHL), which partners with its cognate transcriptional regulator, RhlR (348,349). Microarray data suggest that 6 to 12% of all chromosomal genes of *P. aeruginosa* are regulated by the LasIR and RhlIR QS systems (342).

The two AHL QS systems interact with a third non-AHL QS system, which is mediated by the 2-alkyl-4-quinolone (AQ) AI signalling molecules 2-heptyl-3-hydroxy-4-quinolone [*Pseudomonas* quinolone signal (PQS)] and its biosynthetic precursor 2-heptyl-4quinolone (HHQ) (350). PQS and HHQ bind to PqsR (also called MvfR) to regulate transcription of the *pqsABCDE* operon and expression of the PQS synthesis genes. PqsR is itself regulated by LasR-OdDHL (351–353). The *pqs* operon *pqsABCDE*, together with *pqsH*, codes for HHQ and PQS production. *PqsE,* however, is not required for AQ biosynthesis but is necessary for expression of PQS-dependent virulence determinants, expression of RhlR-activated genes and biofilm formation (354,355). Regulation of the QS network is further complicated by the existence of other regulators such as the global regulators; Vfr and GacA, and the repressors; RsaL, QscR and MvaT and the sigma factors RpoS and RpoN (345).

The Las system regulates several secreted virulence factors including alkaline protease, exotoxin A and LasA, as well as creating a positive feedback loop by inducing lasI. LasIR also induces the Rhl system and RhlR has been shown in turn to upregulate the expression of *lasI* (356). The Rhl system regulates a large regulon of genes, including those encoding virulence factors such as rhamnolipids, elastases, stationary phase sigma factor (*rpoS*), type I and II lectins (*lecA, lecB*), hydrogen cyanide and pyocyanin (357). Some of these genes are also members of the LasR:OdDHL regulon. The PQS system produces multiple small molecules, which are involved in antibiotic resistance, cytochrome inhibition, virulence (e.g., exoenzymes, lectins, siderophores (pyochelin and pyoverdine) and phenazines) (350,358–360), and intercellular communication. The RhlR regulon overlaps with the LasR regulon, for example the promoter of *lasB,* which encodes for elastase, has binding sites for both LasR and RhlR, with LasR showing the strongest *lasB* activation (342). Similarly, many of the genes regulated by PQS are also regulated by the two AHL signals and their cognate receptors (357,361). In addition to controlling virulence, QS also has a role in regulating biofilm formation in *P. aeruginosa* (362).

Much of our understanding of the sophisticated QS systems used by *P. aeruginosa* comes from extensive investigations on the laboratory strain, PAO1 (346,347). In this context, the Las system is often described as the master regulator of the QS hierarchy. However, many QS-dependent virulence factors are mainly activated by RhlR-BHL.

Moreover, *lasR* mutants are commonly isolated from the lungs of CF patients chronically infected with *P. aeruginosa* (177,179,363,364).

1.7.3 *P. aeruginosa* virulence determinants

P. aeruginosa possesses an impressive collection of virulence factors which, along with its genetic and metabolic flexibility, contribute to the bacterium's broad pathogenic potential. Included among these are an array of secreted (e.g., proteases, toxins, pigments, extracellular polysaccharides, siderophores and rhamnolipids) and cell-associated factors (e.g., pili, flagellum and lipopolysaccharide). These virulence factors enable the bacterium to manipulate and evade host defence strategies in order to initiate and establish infection. The specific virulence factors relevant to this body of work, are described in further detail below.

1.7.3.1 Proteases and toxins

Most of the proteases and toxins secreted by *P. aeruginosa* are secreted via the Xcp type II secretion system (T2SS), one of the organism's five (type I, II, III, V, VI) secretory systems (365). *P. aeruginosa* secretes several extracellular proteolytic enzymes which include alkaline protease (AprA), elastase A (LasA/staphylolysin), elastase B, (LasB/pseudolysin/elastase), large exoprotease (LepA), MucD, protease IV (PIV), *P. aeruginosa* aminopeptidase and *Pseudomonas* small protease (366). *P. aeruginosa* proteases are particularly notable for their role in facilitating invasion and necrosis of host tissue (including lung surfactant proteins A and D involved in opsonisation and macrophage function) (367–369). In addition, they are involved in the modulation of host inflammatory responses through their interaction with a diverse range of host molecules (see Hoge *et al.* (367) for a more detailed review), including the destruction of surface receptors on neutrophils leading to impairment of chemotaxis, phagocytosis and the oxidative burst.

Of all the secreted proteases, elastase B is generally the most abundant. It has several properties that distinguish it as one of the major virulence enzymes of *P. aeruginosa*, including its broad substrate specificity, ability to degrade elastin (a major component of connective tissue that is resistant to hydrolysis by most proteases), immunoglobulins and complement proteins, disrupt epithelial tight junctions, and reduce endothelial integrity. Elastase A (also called staphylolysin due to its ability to

cleave the pentaglycine bonds in the peptidoglycan of *S. aureus*) is the second elastase secreted by *P. aeruginosa*. It degrades several glycine-rich proteins (367,374). It exhibits limited intrinsic elastolytic activity whilst significantly enhancing the elastinolytic activity of other proteases, including that of LasB (374).

Alkaline protease, secreted via the type I secretory system (T1SS), degrades host complement proteins and fibronectin (375). It has, in addition, been shown to interfere with flagellin signalling, enabling *P. aeruginosa* to avoid immune detection (376). Alkaline protease exhibits a broad substrate range but is not as potent as elastase B and has no elastolytic activity.

The type II secreted Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections (377). Exotoxin A is responsible for local tissue damage, bacterial invasion and (possibly) immunosuppression via inhibition of host protein synthesis.

Synthesis of the elastases A and B, alkaline protease and exotoxin A is under QS control with LasR-OdDHL regulating expression of *lasB, lasA, aprA and toxA* (378). *LasB* transcription, in addition, is reliant on PQS and activation of the PQS system, with inactivation of PQS signal shown to cause a reduction in elastolytic activity *in vitro* (379). Furthermore, BHL has been shown to act synergistically with PQS to induce *lasB* expression, with greater expression levels observed in the presence of both signalling molecules than with either signal alone (380).

Exoprotein-deficient isolates are commonly isolated from CF patients chronically infected with *P. aeruginosa* (381). These observations are also supported by the frequent identification of *lasR* variants in chronic CF isolates, leading to speculation that these virulence factors play less of a role in chronic infection of the CF airway. However, curiously, there is also evidence indicating that CF-evolved *P. aeruginosa* strains produce higher levels of secreted virulence factors (e.g., elastase, exotoxin A and alkaline protease) at times of exacerbation, suggesting that dynamic fluctuations in virulence factor expression may be important in triggering periods of APE (167,271,325).

1.7.3.2 Flagella- and pilus-mediated motility

P. aeruginosa synthesises a single polar flagellum and several shorter polarly localised Type 4 pili (T4P) (382). These proteinaceous extracellular appendages are readily detectable by the host immune system and therefore are potent activators of the immune response to *P. aeruginosa* infection (383). They play a significant role in *P. aeruginosa* pathogenesis, being essential factors in biofilm formation and a major means of cell motility (382). Flagella-mediated swimming motility is understood to be required for initiation of biofilm formation through facilitation of adhesion to airway mucins (384). Once attached, twitching motility, a form of surface translocation mediated by T4P, promotes further development of biofilm architecture. This occurs through mediation of cell migration and aggregation which enables the formation of microcolonies within the mature biofilm (385,386). *P. aeruginosa* also uses flagella-based swarming motility but this form of motility will not be discussed further here.

In *P. aeruginosa*, flagellar biosynthesis and assembly is under the control of a highly complex, four-tiered, transcriptional regulatory cascade which coordinates the expression of approximately 50 genes (for a detailed review see Dasgupta *et al*.) (387). FleQ is considered the master regulator protein of this cascade. It belongs to the top tier of the pathway and is needed for the expression of all known flagellar genes with the exception of *fliA* (388).

A large number of genes are also involved in T4P biosynthesis and function. Among them are the genes encoding the main structural subunit (*pilA* or pilin), the leader peptidase (*pilD*), other proteins required for pilus assembly and twitching motility and at least three regulatory systems, including the two-component sensor regulator *pilSR* and *algR/fimS* and a complex chemosensory system (*pilGHIJK, chpABCDE*) (389).

In addition, cyclic-di-GMP plays a key role in the regulation of flagellar and T4P biosynthesis, which in turn influence biofilm formation (390). Binding of cyclic-di-GMP to the transcriptional regulator FleQ downregulates flagellar biosynthesis. Consequently, c-di-GMP-bound FleQ converts into a potent transcriptional activator of the exopolysaccharide synthesis genes, favouring biofilm formation (391). Cyclic-di-GMP, in addition, positively regulates T4P assembly at the cell poles when bound to FimX, which again promotes biofilm development (392).

Twitching motility has also been linked to the *las* and *rhl* QS systems in *P. aeruginosa*. Work by Beatson *et al*. demonstrated that although twitch motility is unaffected by mutations in the genes encoding the *las* and *rhl* QS systems, existing mutations in these genes can lead to specific secondary mutations in other significant regulatory genes that affect twitching motility, such as *vfr* and *algR* (389). Furthermore, there is some evidence to suggest that flagellin and alginate production are inversely regulated via the alternative sigma factor AlgT, which upregulates alginate biosynthesis and downregulates flagella-mediated motility (through inhibition of *fleQ*) (388,393).

Loss of flagella- and pilus-based motility is observed almost exclusively in isolates from CF patients chronically infected with *P. aeruginosa* (324,383,394). Repression of motility phenotypes may enable *P. aeruginosa* to evade clearance by phagocytosis *in vivo,* conferring a survival advantage in the CF lung (324,395).

1.7.3.3 Siderophores

Iron is essential for bacterial growth and required for *P. aeruginosa* virulence and survival in the CF lung (396). Iron acquisition is made difficult for the bacterium by the poor solubility of the ferric form (which dominates in aerobic environments) and sequestration of iron intracellularly in heme-containing compounds, or in fluids, by iron-binding proteins such as lactoferrin and transferrin (396). Iron concentrations in the CF lung correlate with the amount of inflammation and tissue damage and vary between 2 and 130 μM. Of note, work by Hunter *et al*. has shown that the relative balance of ferric and ferrous iron alters with the progression of CF lung infection (397). Over time soluble ferrous iron dominates in the increasingly more microaerobic/anaerobic environment of the CF lung. However, and for reasons still unclear, *P. aeruginosa* growth and biofilm formation under anaerobic conditions demands higher concentrations of ferric iron than otherwise expected, underscoring the importance of siderophore-mediated iron acquisition (398).

P. aeruginosa has evolved several strategies (recently reviewed by Cornelis and Dingemans) to aide iron acquisition for growth and pathogenesis (399). The most common of which is the production of low-molecular-mass, iron-chelating molecules termed siderophores (400). Siderophores are secreted by the bacterium, under iron-

limiting conditions, into the extracellular environment. Here they chelate ferric iron and return it to the cell via specific receptor proteins at the bacterial surface. The two major siderophores secreted by *P. aeruginosa* are pyoverdine and pyochelin (which has a lower iron-binding affinity) (401,402). Iron-loaded pyoverdine and pyochelin are taken up by the outer membrane receptors FpvA and FptA, respectively (403). *P. aeruginosa*, in addition, has the ability to utilise siderophores from other pseudomonads as well as other bacterial and fungal species (siderophore piracy) (396,399,404). Although this has yet to be determined, this feature likely confers a selective advantage in the polymicrobial environment of the CF lung.

Pyoverdine synthesis is dependent on the expression of the pyoverdine biosynthetic genes which are activated by the alternative sigma factor, PvdS. Expression of the *PvdS* gene is in turn controlled by the iron-sensing repressor protein Fur (405). Pyochelin upregulates its own biosynthesis and uptake genes by activating the *AraC*/*XylS*-family transcriptional regulator *PchR* (406).

In addition to its iron-scavenging properties, pyoverdine plays a key role in several other important activities that enhance *P. aeruginosa* virulence and pathogenesis. Pyoverdine, upon binding to its outer membrane cognate receptor, is capable of not only regulating its own production (by activating the pyoverdine biosynthetic genes), but also activating the expression of genes that encode two extracellular virulence factors: exotoxin A and PrpL protease (405,407). Pyoverdine has also been demonstrated to be necessary for proper development of non-alginate biofilms *in vitro,* under iron-limiting conditions (408). The exact mechanism, however, is not yet known, although work by Banin *et al*. indicate that a functional iron uptake system is required (408).

Quorum sensing, in addition, likely plays a role in siderophore production through the activity of PQS. Purified PQS, by its ability to chelate and sequester extracellular ferric iron, can activate the expression of genes involved in the regulation (*pvdS*) and biosynthesis of pyochelin (*pvdA)* and pyoverdine (*pvdE*) *in vitro*, suggesting a possible role for PQS in pyoverdine-mediated uptake *in vivo* (359).

Siderophores have been detected in sputum samples from CF individuals chronically infected with *P. aeruginosa* with a previous study reporting a strong correlation between pyoverdine levels and *P. aeruginosa* bacterial load (409). However, the relationship between pyoverdine levels and periods of acute exacerbation is less clear.

1.7.3.4 Rhamnolipids

Rhamnolipids are a class of secondary metabolites secreted by *P. aeruginosa* (410). Previous work indicates that these amphiphilic surface-active compounds play an important role in the spatial differentiation of biofilm architecture, although the exact mechanisms are not fully understood (411). Rhamnolipids are necessary for initial microcolony formation in the early development of biofilms and in the late stages aid structural development that depends on cell migration (412,413). They play a role in alteration of the hydrophobicity of the cell and the detachment and dispersal of cells from the biofilm (especially from the centre of the microcolonies) (414,415). There is also evidence for rhamnolipids participating in the maintenance of open-channel formation within the biofilm (through modulating cell-to-cell and cell-to-surface interactions), which aids nutrient dispersion and metabolic waste removal around the microcolonies (416).

Rhamnolipid production is directly regulated by the *rhl* system which coordinates rhamnolipid synthesis via the *rhlAB*-encoded rhamnosyltransferase. The *rhlAB* operon is transcriptionally and post-transcriptionally regulated by several factors, often linked to the QS system. For example, rhamnolipid synthesis is upregulated at the transcriptional level by the *pqs* system through activation of the *rhlIR* operon. Furthermore, rhamnolipid biosynthesis also seems to be influenced by nutritional and environmental cues (417). Rhamnolipid expression is upregulated under iron-limiting conditions and is associated with increased surface-associated motility (twitching and swarming) and the formation of flat unstructured biofilms (418). However, most of these regulatory mechanisms are not completely understood (419).

Rhamnolipids have been previously detected in sputum from patients chronically infected with *P. aeruginosa*, with the highest concentrations reported to correlate with periods of acute exacerbation (420). They have been shown to have direct effects on host immune cells, including inducing lysis of polymorphonuclear leukocytes and

inhibition of macrophage phagocytic responses (421). They have also been shown to be necessary for *P. aeruginosa* to invade respiratory epithelial cells during acute infection. It is plausible to consider, therefore, whether rhamnolipid-mediated dispersal of *P. aeruginosa* cells from the biofilm may contribute to initiation of these significant clinical events.

1.7.3.5 Biofilm formation

Much of our current insights into the molecular basis for the regulation of biofilm formation have been recently reviewed by Moradali *et al.* and Faure *et al* (278,282). Only summary aspects are outlined here.

CF-evolved *P. aeruginosa* biofilms are highly organised, structured communities composed of aggregations or tight microcolonies of bacteria (predominantly attached to one another inside mucus, and not to the epithelial surface of the lung) surrounded by a matrix of extracellular polymeric substances (EPS) (282,315,422). The ability to phenotypically switch between a free-floating (planktonic) and a sessile (biofilm) lifestyle is a major survival adaptation for *P. aeruginosa* (282). This dynamic transition is mediated through multiple and overlapping regulatory networks which include the RetS/GacS sensor pathway (278). Cellular levels of cyclic-3´5´-diguanylic acid (cyclic di-GMP) play a critical role in the post-transcriptional regulation of this phenotypic switch, with increased levels triggering EPS production while inhibiting flagellar and type IV pilus-mediated motility. Conversely, low levels of cyclic di-GMP promote biofilm dispersal (411).

Formation of biofilm is also intricately linked to QS, although the exact mechanisms by which this occurs are not yet known (390,423). QS-regulated genes encoding secreted exoproducts (e.g., Pel and Psl exopolysaccharides, rhamnolipids and phenazines) have been shown to be critical to biofilm development and maturation, with QS-deficient variants observed to display poorly developed biofilms *in vitro* (168,362,424). The biofilm matrix is a relatively oxygen and nutrient limited environment and functions to protect the bacteria dwelling within from the hostile surrounding environment (e.g., dehydration, reactive oxygen species) (382). However, host responses to *P. aeruginosa* biofilms are complex with biofilms capable of eliciting either less (such as in the case of reduced activation of complement in the absence of

functional flagellar) or more robust immune responses than their planktonic counterparts.

The key components of the *P. aeruginosa* biofilm matrix are the secreted exopolysaccharides; Psl (polysaccharide synthesis locus), Pel (pellicle formation locus) and alginate. Alginate production is not critical for biofilm formation (425). Psl promotes cell surface adherence (the first step of biofilm formation), whereas Pel plays a major role in promoting cell-cell interactions important for self-aggregation (426). Other important functional components of the biofilm include the proteins CdrA and extracellular DNA (eDNA), which reinforce the structural scaffold of the biofilm matrix (390,427).

P. aeruginosa cells in biofilm growth mode exhibit slower growth rates compared to their planktonic counterparts. This may be a consequence of a reduced ability to move to aerobic regions (in the absence of flagellar-mediated motility), where aerobic growth gives the advantage of a faster growth rate (428). The difference in physiology in these two growth modes contributes to differences in cellular metabolism, transcription and expression of protein profiles (429,430). The specific environmental cues that trigger biofilm development along a particular pathway remain unknown (390). Furthermore, there is growing recognition that *P. aeruginosa* lifestyle strategies are more flexible than previously thought. That is to say, some functions typically associated with planktonic/early infection cells are also found within biofilms, and vice versa (431). In line with this thinking, it has been postulated that short-term shifts between planktonic and biofilm growth modes of *P. aeruginosa* biofilm populations within the CF lung may play a part in triggering exacerbation episodes (278). However, evidence for this remains elusive.

1.7.3.6 Auxotrophy

Auxotrophic isolates lack the ability to synthesise one or more specific metabolites necessary for growth and, in the case of *P. aeruginosa*, may fail to grow on minimal media (74,313). Amino acid biosynthesis is metabolically costly (432). In the CF lung high concentrations of free amino acids have been documented (433). It is not yet clear whether these sputum amino acids are of host or microbial origin (434). Nevertheless, in this setting, auxotrophic *P. aeruginosa* variants frequently arise,

suggesting a fitness advantage over their prototrophic counterparts (435).

Methionine, leucine and arginine auxotrophs are most frequently reported among chronic CF isolates of *P. aeruginosa* (435–437). Furthermore, a growth advantage on phenylalanine over parental strains has been reported among *P. aeruginosa* clonal isolates harbouring mutations in the *lasR* gene (184). Related non-synonymous mutations in the biosynthetic pathways for some of these amino acids have been recently identified (438). However, attempts to assign causality for the observed phenotypes have failed due to the complex nature of the regulatory networks controlling metabolism (313).

Auxotrophic isolates have been reported to be less susceptible to antibiotic treatment than their prototrophic counterparts (437). Intriguingly, in this early study by Taylor *et al*., an increased prevalence of auxotrophs in CF sputum collected during periods of APE was reported. However, more recent work by Forthergill *et al*. failed to observe a similar trend (325).

1.8 Aims and objectives

The broad aim of this dissertation is to gain a better understanding of the relationship between changes in microbial factors, patient physiology and symptoms during periods of acute respiratory decline. The interaction between these factors was examined to determine if a change in one or more could predict for APEs in adults with CF. Specifically, this dissertation is focused on addressing the following interrelated questions:

1. Can signals from home monitoring of patient physiology and symptoms be combined with a single sputum biomarker to predict for APE onset?

2. Can high-frequency home monitored data of patient physiology and symptoms be used to better understand the changes preceding APE onset? If so, can home monitoring signals be used to predict APE onset?

3. Do changes in the phenotypic profile of clonal populations of *P. aeruginosa* trigger APEs?

The objectives are:

1. To retrospectively analyse data on home monitored physiology, symptoms and a sputum biomarker (Exotoxin A) for evidence of an early signal of APE onset. This data was collected from 15 adults with CF who took part in the clinical pilot study, TeleCF.

2. To design, implement and analyse the outcomes of a multi-centre, feasibility and acceptability study (SMARTCARE) of home monitoring in an adult CF population, using novel Bluetooth sensors and smart technology.

3. To analyse data collected in two home monitoring clinical studies, TeleCF and SMARTCARE, using ML techniques [as part of a study undertaken at Microsoft Research (Cambridge) and the Laboratory of Molecular Biology (Cambridge)] in order to understand and characterise changes in patient physiology and symptoms leading up to an APE and, if possible, identify a predictive signal for APE.

4. To complete detailed *in vitro* phenotyping of 4408 *P. aeruginosa* clonal isolates for six phenotypic traits associated with virulence in *P. aeruginosa.* These isolates were harvested from longitudinal sputum samples collected before, during and after an APE, from 9 adults with CF (who took part in TeleCF).

5. To characterise the relationships between phenotypic profiles displayed by these co-varying *P. aeruginosa* clonal isolates before, during and after an APE using bioinformatic analyses, in order to determine if a microbial signal is associated with APEs.

2. GENERAL METHODS

The general methods are described in this section. Specific methods are detailed in the relevant chapters.

2.1 Study Population

To investigate changes in symptoms and physiology, as well as changes in phenotypic traits in *P. aeruginosa* clonal isolates within the CF airway, before, during and after periods of APE I studied a pre-defined population of adult CF patients in two clinical studies - TeleCF and SMARTCARE.

Shared inclusion and exclusion criteria between both clinical studies are detailed below. Specific criteria for each study are listed in the relevant chapters.

Individuals met inclusion criteria if they were aged 18 years or older, had a confirmed diagnosis of CF based on genetic testing and/or abnormal sweat chloride levels (cut-off threshold > 60 $mmol \bullet L^{-1}$), and were willing and able to provide daily home monitoring data.

Potential participants were excluded if they were unable to provide written informed consent or were unable to provide regular sputum samples.

2.2 Ethical Considerations

All protocols involving new data acquisition in participants received Royal Papworth Hospital NHS Foundation Trust research approval and favourable ethical opinions from the local ethics committee – REC references 12/EE/0462 (TeleCF) and 14/EE/1244 (SMARTCARE). Ethical approval documents for both studies are included in Appendix 0.

Trials were conducted in keeping with recommendations for good clinical practice including the declaration of Helsinki.

Written informed consent was obtained from all participants. Collection and storage of sputum samples for analysis in future studies was covered by the ethics approval.

2.3 Trial Protocols

Individual trial protocols are detailed in the relevant chapters.

Both home monitoring trials were conducted over a period of six months. The trials were not conducted at the same time of the year and participants were recruited into each trial on a rolling basis. During the trial period I requested that participants collect daily measures for a set of pre-defined clinical symptoms and signs using several devices linked to a home monitoring system.

The pre-defined clinical symptoms and signs I selected for home monitoring were both subjective (participant-reported) and objective (device-detected), and were based on clinical experience, supervisor consultation and a review of relevant literature (reviewed in Section 1.6.2). The monitoring parameters were: 1) Weight (kg), 2) physical activity (step count), 3) lung function (measured as forced expiratory volume in one second [FEV_1]), 4) transcutaneous pulse oximetry (reported as the percentage saturation of arterial haemoglobin by oxygen [SpO_2] and heart rate in beats/minute [bpm]), 5) a 10-point scale of wellness (reported as worst ever (1/10) to best ever (10/10) and 6) a 10-point scale of cough quality [reported as worst ever s (e.g., chronic) (1/10) to best ever (e.g. none) (10/10)]. At the time of conduction of this work, no PROM had been validated for daily use in CF to assess for acute changes in symptoms. Both unvalidated symptom scores were therefore developed specifically for this work, following patient and clinician feedback, and were designed to reflect within-subject acute change in day-to-day perception of cough intensity and general wellbeing from their normal baseline. Similar symptom scores have been used in asthma for the evaluation of daily symptom control (503, 504).

I requested that participants record their daily data in a pre-defined sequence to minimise confounding of resting-state measures (e.g., heart rate) by effort-dependent measures (e.g., lung function). This order differed marginally for each study and is therefore listed in the relevant chapters. Although I encouraged participants to complete data entry for all measures within the same time interval, no specific time of day was set for data collection. I requested that participants perform three lung function attempts, with the best of the three attempts recorded by the monitoring application, in accord with the British Thoracic Society (BTS) criteria (439).

I also requested that participants provide a daily sputum sample (for future biomarker studies) which was stored for collection in a dedicated home freezer with an average temperature between -18 and -20ºC, as indicated by the manufacturer's specifications.

Both trials were non-disruptive to routine clinical practice, therefore data collected during the trial was not routinely reviewed during the trial period and was not used to inform/influence the participant's disease management.

2.4 Sputum collection, processing and storage

Participants were instructed to collect expectorated sputum samples following their usual chest clearance routine in sterile, dated, link-anonymised 50 mL falcons and store them immediately in a dedicated home freezer (average temperature as documented in Trial Protocols 2.3).

Participants were instructed to bring their batched sputum samples in cooler bags with ice packs to the study centre, anytime they attended clinic. These samples were then stored for further analysis at -80ºC.

I undertook all sputum processing at the microbiology research laboratory at Royal Papworth Hospital (Cambridge, UK). Sputum samples were stored long-term in the Royal Papworth Hospital Tissue bank.

I completed Materials Transfer Agreements (MTA) for transfer of any biological research material (e.g., sputum samples) between organisations in accordance with Intellectual Property Law. A template of the MTA form is included in Appendix 1.

2.5 Definition of an acute pulmonary exacerbation

In the absence of a consensus definition (reviewed in Defining a CF exacerbation 1.2.2) I defined an APE pragmatically as the need to initiate either oral or intravenous antibiotic treatment for a deterioration in a participant's respiratory status.

2.6 Statistical analysis

The statistical analyses performed are outlined in the relevant chapters.

3. TeleCF: Investigating the use of home monitoring and single biomarker profiling in CF sputum to predict for acute pulmonary exacerbations

Summary:

Acute pulmonary exacerbations are the main cause of morbidity in patients with cystic fibrosis. Home based monitoring for APE might ensure prompt detection and initiation of therapy which could potentially result in better long-term lung health. We therefore sought to assess whether daily home monitoring of a single sputum bacterial biomarker and clinical parameters might provide advanced warning of an APE.

On a daily basis for a 6 months period, 15 adults with CF were asked to collect sputum samples, undertake home-based telemetric physiological monitoring (of FEV_1, PEF, heart rate, saturations, weight, activity) and complete wellness and cough diaries electronically. Sputum samples were retrospectively analysed for levels of *P. aeruginosa* exotoxin A (PEA) and complete datasets were examined to evaluate which parameters best predicted APE.

Patient compliance with home monitoring was excellent. A number of combinations of physiological parameters may allow early, pre-symptomatic detection of APE. In a subgroup of patients, relative changes in PEA may also predict APE.

In this single centre pilot study, home monitoring was acceptable to patients, provided unprecedented temporal resolution of clinical parameters and allowed analysis of sputum biomarker changes preceding an APE and during antibiotic therapy. PEA did not prove an effective biomarker for early detection of APEs in adults with CF.

Statement of contribution:

I carried out all clinical data collection, data analysis and interpretation. Sputum processing, storage and Exotoxin A enzyme-linked immunosorbent assays (ELISA) were completed by Karen Brown (Floto Lab).

3.1 INTRODUCTION

Early detection and treatment of APEs are a key priority in CF care due to their undisputed significance in disease progression and unfavourable impact on quality of life and survival outcomes (reviewed in Acute pulmonary exacerbations in CF, Section 1.2) ((126–128,130). Triggers for these events are still ill-defined, and therefore reliable diagnostic biomarkers remain elusive (124,188). Yet, it is generally accepted that microbial drivers play a significant role in pathogenesis (reviewed in Pathophysiology of APE, Section 1.2.1) (136–139). Improvement in symptoms and lung function following antibiotic treatment has been observed, despite studies failing to demonstrate significant changes in bacterial density with treatment (133,134,147,148). This observation has led to speculation that changes in factors related to bacterial virulence, rather than number, may be responsible for initiating APEs, and that these compounds may be detectable in sputum prior to the onset of symptoms. (reviewed in Detecting APEs in CF using bacterial sputum biomarkers, Section 1.5).

In the absence of predictive biomarkers for APE onset, clinical decisions around diagnosis and treatment initiation are heavily dependent on patient-reported symptoms associated with, most commonly, a corresponding fall in lung function measured in the clinic (6,188). It is, however, likely that APEs begin sometime before individuals with CF present for assessment and treatment (reviewed in Defining a CF exacerbation, Section 1.2.2). Yet, the ability to more accurately determine the onset of clinical decline remains a challenge, largely as diagnosis is dependent on a change in status from a perceived, and usually unrecorded, baseline. The lack of a means to easily capture day-to-day variations in clinical stability has further hindered our understanding of the nature of APEs and the search for predictive biomarkers.

I hypothesised that if APEs are triggered by a change in pseudomonal virulence behaviour I could anticipate a change in one of the organism's secreted exoenzymes (Exotoxin A), assayed in sputum, prior to initiation of antibiotic treatment for an APE. Similarly, these periods of clinical decline should be associated with objective changes in physiology and symptoms that could be readily captured using daily home monitoring.

I selected Exotoxin A as a candidate sputum biomarker because, in contrast to other exoenzymes secreted by *P. aeruginosa*, levels assayed from isolates sub-cultured from frozen stocks have been shown to remain stable over time when compared to levels derived from their fresh parent strains (505). Furthermore, a commercially available serological assay was accessible for ease and standardisation of screening.

The data analysed in this chapter was generated through the clinical study, TeleCF. The study also generated a foremost sputum biobank for later detailed examination of the dynamic changes in phenotypic composition of populations of *P. aeruginosa* clonal isolates in relation to APEs, to determine if this might prove predictive of APE onset (examined in Chapter 4).

3.2 METHODS

3.2.1 Study design and participants

TeleCF was a single-centre, observational, exploratory pilot study (ClinicalTrials.gov number NCT01877707). National Ethical approval was obtained from the National Research Ethics Service (NRES) Committee of Hertfordshire, UK (REC 12/EE/0462). The study design was peer reviewed by the Cambridge Centre for Lung Infection (CCLI) and the Cambridge Institute for Medical Research (CIMR).

In addition to the inclusion and exclusion criteria previously described in General Methods, Section 2, participants were eligible for enrolment into TeleCF if they fulfilled the following inclusion criteria: to be aged 18 years and over; have had at least two APEs in the 12 months prior to screening; have evidence of chronic airway infection with *P. aeruginosa.* I defined chronic *P. aeruginosa* airway infection as the persistent growth of *P. aeruginosa* in sputum (≥ 2 positive sputum cultures at least one week apart) following anti-pseudomonal antibiotic eradication therapy with a combination of four weeks of nebulised treatment and four to six weeks of oral therapy and (if needed)/or two weeks of intravenous therapy followed by twelve weeks of nebulised treatment, in accordance with local guidelines. We excluded participants if they had

fewer than two APEs within 12 months of screening or had airway co-infection with non-tuberculous mycobacteria.

All participants were recruited from the adult CF centre at Royal Papworth Hospital in England (Cambridge, UK). The study was funded by a competitive Small Business Research Initiatives scheme (SBRI) grant from Health Enterprise East (HEE). Active8rlives (Aseptika Ltd), the software provider for the telemonitoring system, had no role in the study design, analysis or interpretation of the data.

3.2.2 Participant recruitment, screening and enrolment

We identified eligible participants through screening the Centre's clinical database for patients who were chronically infected with *P. aeruginosa.* Individuals who met criteria were contacted by the Centre's research coordinators. Participants who were interested in taking part received patient information letters explaining the study protocol and were followed-up with a telephone call to confirm willingness to enrol. All participants were consented and enrolled into the study between January 2013 and November 2013.

Participants were withdrawn from the study if they withdrew consent or passed away within the first month of the study or if they were unable to record more than one month of telemonitoring data during the course of the study. However, if a participant withdrew consent or passed away more than one month into the study period, I included their demographic and telemonitoring data for comparison with those of the patients who remained in the study.

3.2.3 Home monitoring system and equipment

Telemonitoring was completed with home monitoring devices that were available commercially. We supplied participants with home monitoring devices, a 3G-enabled PC lap-top computer, a portable 3G mobile broadband dongle for ease of data upload, 50ml falcons for sputum collection and a rucksack for transport. The devices used in this study are illustrated in Figure 3.1.

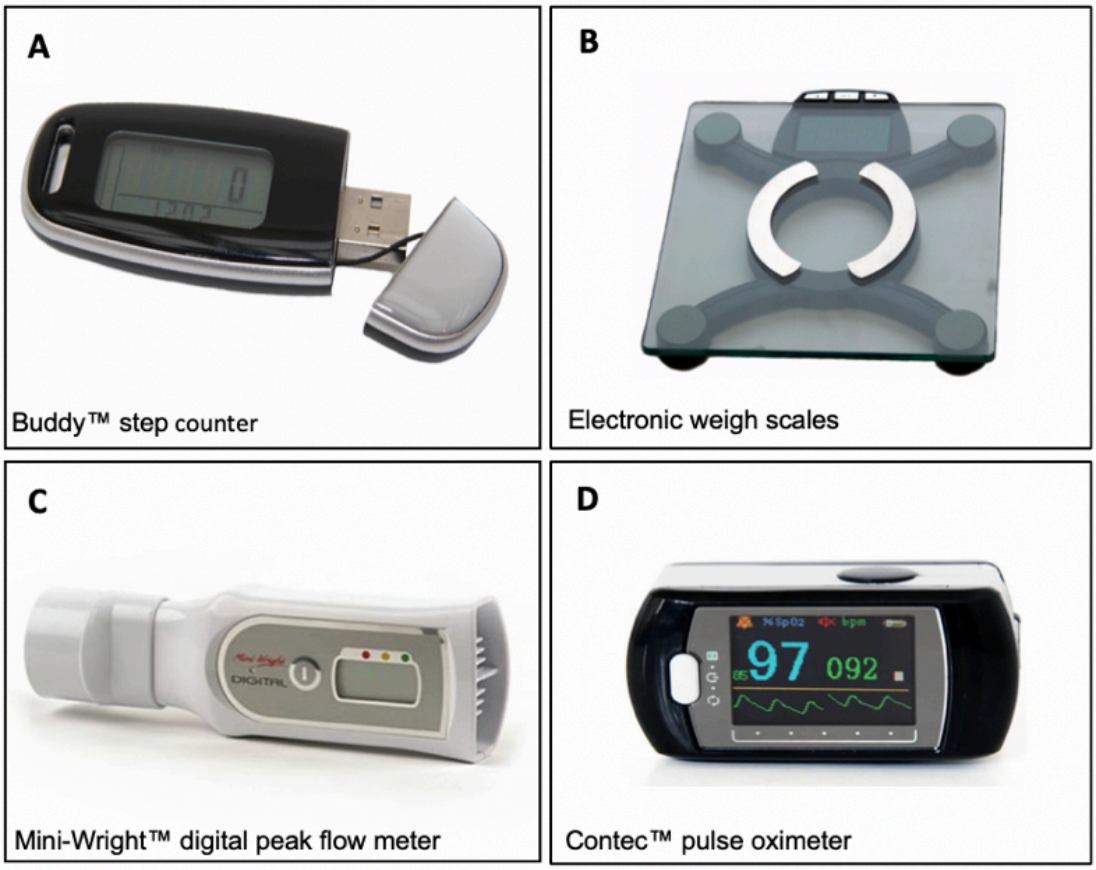


Figure 3.1. Home monitoring devices used in the TeleCF study. These devices were commercially available through Activ8rlives®. (A) Step counter, (B) electronic weigh scales, (C) digital peak flow meter, (D) pulse oximeter.

Study participants received training in use of their study equipment via a structured user training session. We also provided them with a step-by-step instruction manual at study inclusion.

The software platform to upload data from monitoring devices was purchased by Activ8rlives. Data recorded on the home monitoring devices were automatically uploaded to the study PC laptop via a USB port connection. Symptom scores were completed within the Activ8rlives PC application (Figure. 3.2). Data from the telemonitoring system was automatically downloaded to a secure web-based application for review by the research team. Study participants were not blinded to their own datasets. Direct technical support and additional training during the course of the study was provided by Active8rlives (Aseptika Ltd). Data entry was monitored by Active8rlives (Aseptika Ltd). Contact was made with the research team if a

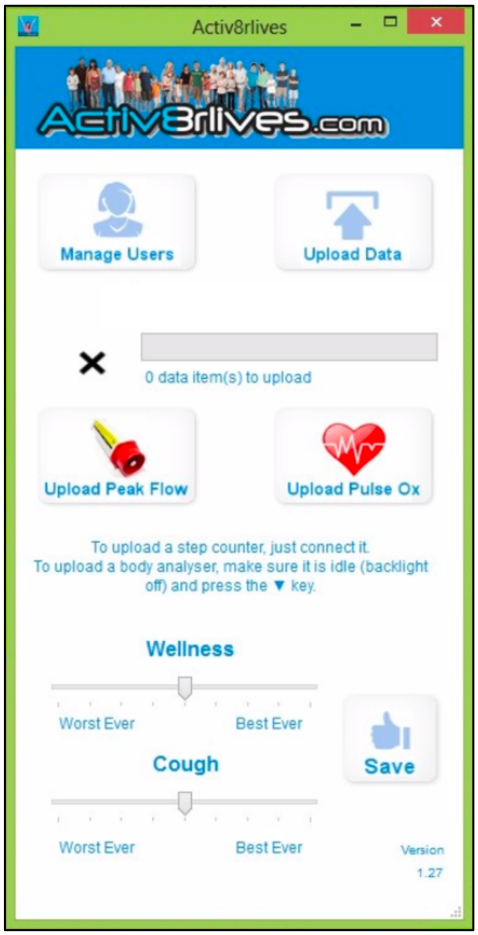


Figure 3.2. Activ8rlives upload touchscreen for the PC application

participant omitted to upload data for several days; following which, the participant would be contacted to encourage participation.

3.2.4 Home monitoring protocol

Participants performed home monitoring and sputum collection as detailed in Trial Protocols, Section 2.3. They were instructed to collect and log their data in the following order: 1) weight (kg), 2) physical activity (step count), 3) lung function (FEV_1), 4) Pulse rate (bpm) and oxygen saturation (%), 5) a 10-point scale of wellness [reported as "worst ever" (1/10) to "best ever" (10/10)] and 6) a 10-point scale of cough quality [reported as "worst ever" (1/10) to "best ever" (10/10)]. Note that the scale terminology used for the 10-point symptom scores was pre-assigned by the app developer.

In order to minimise the added time imposition incurred from daily home monitoring

clear instructions and regular support from the research team were provided to participants to ensure optimal efficiency with home monitoring.

3.2.5 Clinical data

Demographic information was collected at study inclusion by the research nurses. In addition, for each participant, I collected clinical data on the following variables through retrospective review of case notes:

1. Clinical characteristics (CFTR sequencing results; presence of pancreatic insufficiency, CF-related liver disease, CF-related diabetes; macrolide therapy use (azithromycin), inhalation therapy use (antibiotic, hypertonic saline and/or dornase alpha), oral steroid use).
2. Antibiotic requirement during the study period (oral and intravenous).
3. Dates of hospital admission for APEs.
4. Dates of clinic encounters.
5. Positive sputum microbiology during the study period.
6. Hospital-based C-reactive protein measurements during the study period.
7. Hospital-based lung function measurements (FEV_1 in litres and percent of predicted) during the study period.

3.2.6 Compensation

Participants received a £1 per day incentive for completion of daily sputum collection and data upload and were given the option to retain all study equipment at the end of the study.

3.2.7 Clinical samples

Exotoxin A concentrations were measured from daily sputum samples which fell approximately 3 days, 7 days or 14 days before the start of antibiotic treatment for an APE. In most cases, participants had not received antibiotics in the 14 days prior to day -14 (e.g., 28 days prior to the start of the exacerbation). All samples met this criterion, with the exception of: APE 2 for participant K1, APE 2 and 3 for participant K2, APE 2 for participant K3 and APE 3 for participant K4, where, in each case, a course of antibiotics finished 11, 13, 9, 9 and 13 days prior to day -14 respectively.

In addition, Exotoxin A levels were calculated from daily sputum samples which fell from the first day following a course of antibiotic treatment for an APE to approximately -3 days, -7 days and -14 days from the start of antibiotic treatment for the next APE. Of the 39 exacerbation periods captured in this study, sputum samples taken at the required timepoints were only available in 19 of the exacerbation episodes. For 1 of the 39 episodes, samples were not available for analysis of the 3-day and 7-day period leading up to antibiotic treatment for an exacerbation (APE 1 for participant K7).

3.2.8 Exotoxin A detection

ELISA-based extraction of Exotoxin A from stored daily sputum samples was completed by Karen Brown (research scientist, Floto Lab). Reagents used in this study are summarised in Table 3.1.

Table 3.1. List of reagents for *P. aeruginosa* Exotoxin A ELISA

Reagent	Conditions
Solubilisation Solution	4 mM TCEP HCl (1.14 mg/mL) 0.1% Pluronic F127 in 0.5 M guanidine
Anti-PEA-Biotin CJ	1.5 μg/mL, 4ºC
Anti-PEA-HRP CJ	2.0 μg/mL, 4ºC
SureBlue reserve	4ºC
HCl	1 M
1x wash buffer	50 mL 20x wash buffer 950 mL distilled water

Frozen sputum samples were thawed to room temperature and 100 μl aliquots of each sample were mixed with an equal volume of solubilisation solution. Samples were then vortexed and incubated at 37ºC in a shaking incubator at 210 rpm. The samples were vortexed again and 50 μL aliquots, in duplicate, were added to the Streptavidin-coated 96-well assay plate (40 samples per plate). Frozen standard curve solutions were thawed and vortexed. Aliquots of 50 μL of each standard curve solution were then added to the assay plate, in duplicate. Aliquots of 100 μL of 1.5 μg/mL anti-PEA-Biotin CJ were added to each well. The assay plate was then incubated at 37ºC and 1000 rpm in a shaking incubator for 1 hour.

A short wash was performed with distilled water in a plate washer. Excess wash solution was tapped off the plate and 100 μL aliquots of 1.5 μg/mL anti-PEA-Biotin CJ was added to each well. The plate was then re-incubated in the shaking incubator under the same conditions (37ºC ,1000 rpm, 1 hour).

A second short wash was performed and excess wash solution was tapped off the plate. Aliquots of 100μL of SureBlue reserve were added to each well. Following incubation at 37ºC and 1000 rpm in a shaking incubator for 10 minutes, 100 μL of 1 M HCl was added to each well.

Exotoxin A concentration (ng/mL) was quantified by measuring absorbance at 405 nm using a Clariostar (BMG Labtech) plate reader then multiplying the result by 2 (dilution factor).

3.2.9 Statistical analyses

I performed statistical analyses using Microsoft Excel for Mac version 16.27 (Microsoft, Redmond, WA, USA), Prism 6.0 (Graphpad Software Inc, San Diego, CA, USA) for Mac OS X and RStudio version 1.1.456 (RStudio Inc, Boston, MA, USA)

I have expressed descriptive statistics of the baseline characteristics of participants as mean (standard deviation [SD]), median (interquartile range (IQR), minimum and maximum) or mode for numerical variables and as number (percentage) for categorical variables. No attempt was made to substitute missing data.

I compared non-parametric data using unpaired Mann-Whitney signed rank tests for comparison of differences between median Exotoxin A concentrations in sputum taken from participants chronically infected with epidemic versus non-epidemic strains of *P. aeruginosa* and Kruskal-Wallis test for comparison of multiple groups.

I used unpaired Mann-Whitney signed rank tests to compare differences in median Exotoxin A concentrations obtained from sputum samples collected during clinical stability and prior to an APE for each participant. Differences between multiple groups

were assessed using the one-way Analysis of Variance (ANOVA) test for parametric data and the Kruskal-Wallis test for non-parametric data.

Comparisons for differences in variables between periods of non-exacerbation and prior to initiation of antibiotic therapy, across all participants, were assessed using Wilcoxon's matched paired test for non-parametric data and the paired t-test for parametric data. The variables included, lung function, weight, heart rate, oxygen saturations, activity, cough and wellness score and Exotoxin A concentration.

I examined relationships for correlation between continuous and discrete variables using Pearson's correlation coefficient for parametric data and Spearman's Rho for non-parametric data. These variables included lung function, weight, heart rate, oxygen saturations, activity, cough and wellness score and Exotoxin A concentration.

I assessed for agreement between in-clinic and home-based spirometric data using a Bland-Altman analysis (440) of same-day in-home and in-clinic FEV_1 (% predicted).

All statistical tests were two-tailed and a p value of ≤ 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Participants

We screened sixty-five potential participants for inclusion in TeleCF (Figure 3.3). Of these, fourteen were ineligible and five declined to participate. We enrolled sixteen participants, with fifteen participants completing six months of home monitoring. Baseline demographic and clinical characteristics of all fifteen participants are detailed in Table 3.2 and summarised in Table 3.3. The mean age of the cohort was 30 years; more men participated (60%) and almost half of the participants (47%) were homozygous for the F508del mutation. The majority of the study population (87%) had at least moderately impaired lung function with an FEV_1 below 70% of predicted. The nutritional status of the cohort was good, as reflected by the mean BMI of 22. The

majority of participants were taking inhaled antibiotic therapy (87%), with just over half of participants (53%) receiving long-term macrolide therapy with azithromycin. Nearly two thirds of the participants had a co-existing diagnosis of CF-related diabetes. Although all participants were chronically infected with *P. aeruginosa*, just over half (53%) were infected with an epidemic strain, with the majority of that cohort (75%) chronically infected with the Liverpool epidemic strain (LES) and the remainder with the Manchester epidemic strain (MES).

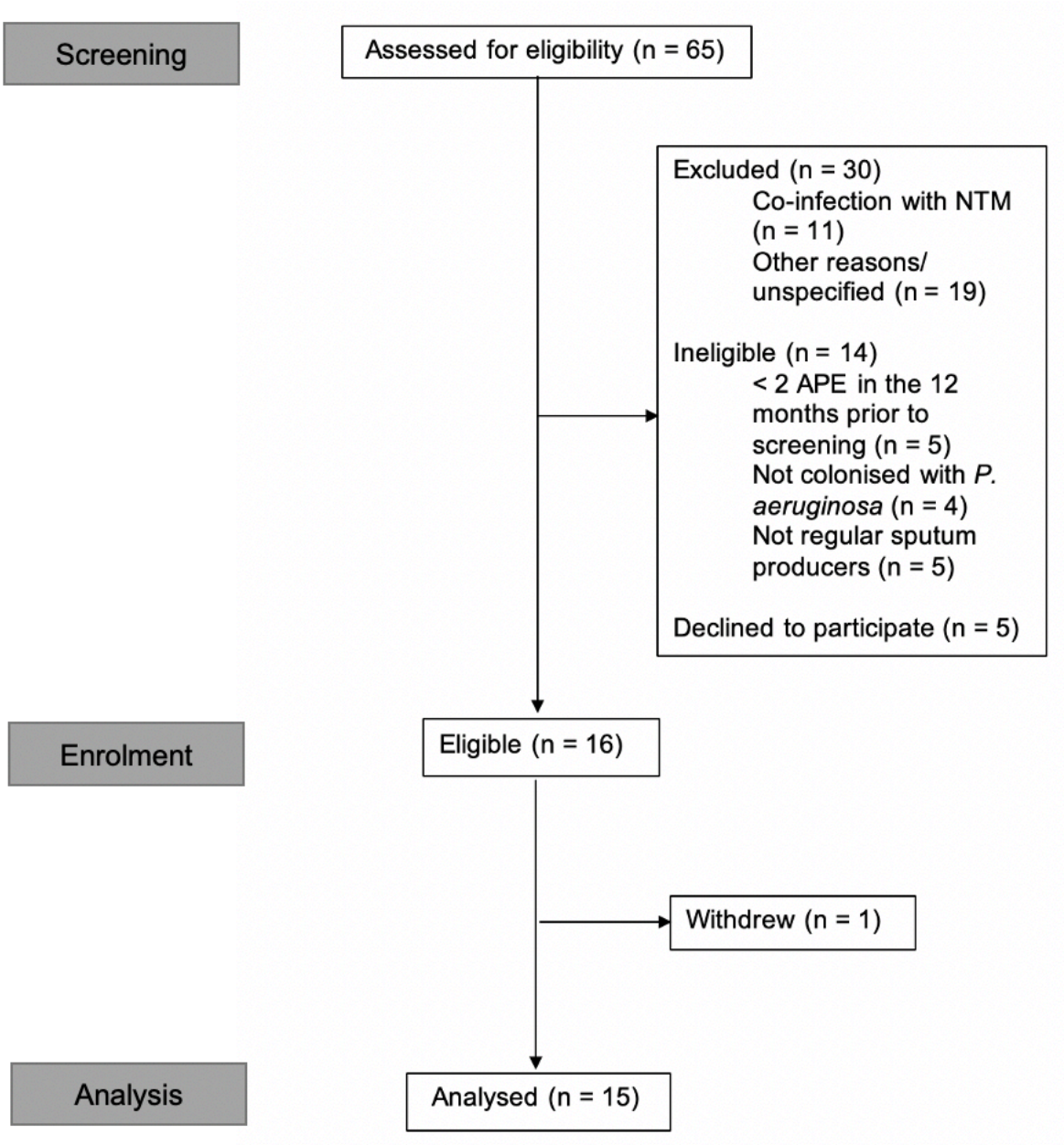


Figure 3.3. Screening, enrolment and follow-up of the TeleCF study participants.

Table 3.2 TeleCF study participant information

Participant	Age	Gender	Genotype I	Genotype II	FEV_1 (% predicted) at study entry	Chronic infection with epidemic strain of *PA*	BMI at study entry	APE episodes during the study period (*n* = 39)	APE episodes analysed here (*n* = 20)	CFRD	CFLD	PI
K1	31	Female	R117H	4326delTC	45.6	MES	29.5	4	2	No	No	No
K2	23	Male	F508del	F508del	45.6	LES	22.4	5	3	Yes	Yes	Yes
K3	32	Female	G85E	Q207X	50.4	No	18.2	4	3	Yes	No	Yes
K4	21	Female	F508del	c.3889dupT	34.7	No	21.1	3	2	No	Yes	Yes
K5	33	Female	F508del	p.Leu1254X	51	LES	23.8	3	1	No	No	Yes
K6	24	Male	F508del	G542X	47.9	No	21.7	3	3	Yes	No	Yes
K7	52	Female	F508del	F508del	72.9	LES	25	2	2	No	No	Yes
K8	28	Male	F508del	F508del	84.7	No	26.8	1	0	Yes	Yes	Yes
K9	36	Male	F508del	c.1766+1A->G	14.1	LES	17	3	1	Yes	No	Yes
K10	38	Male	F508del	F508del	26	No	21	1	0	Yes	Yes	Yes
K11	34	Male	F508del	F508del	32	LES	20	2	1	IGT	No	Yes
K12	35	Male	F508del	621+1G->T	24.2	No	22.9	2	2	Yes	No	Yes
K13	26	Male	F508del	F508del	53.3	No	20.8	1	0	Yes	No	Yes
K14	24	Male	F508del	3752G/A	59.7	MES	19.5	2	0	No	No	Yes
K15	19	Female	F508del	F508del	53.3	LES	19.5	3	0	Yes	No	Yes

FEV_1: forced expiratory volume in 1 second, PA: *P. aeruginosa*, BMI – body mass index, APE: acute pulmonary exacerbation, CFRD: cystic fibrosis-related diabetes, CFLD: cystic fibrosis related liver disease, PI: pancreatic insufficiency, MES: Manchester epidemic strain, LES: Liverpool epidemic strain, IGT: impaired glucose tolerance

Table 3.2 (cont.)

	Long-term medications												Hospital encounters and antibiotic use during the study period			
	Antibiotic therapy								Corticosteroids		Mucolytics					
Participant	Macrolide	Col inh	Tob inh	Azli inh	Tob/Col inh (alt. months)	Tob/Azli inh (alt. months)	Col/Azli inh (alt. months)	Mero inh	Oral	Inhaled	DNase	HTS	Admissions for APE	IP days	Clinic visits	Antibiotic days
K1	Yes	No	No	No	No	Yes	No	No	No	Yes	No	Yes	1	17	9	59
K2	No	No	Yes	No	No	No	No	No	Yes	Yes	No	Yes	1	44	7	80
K3	No	No	No	No	No	No	Yes	No	No	Yes	Yes	No	0	0	15	59
K4	Yes	No	No	No	No	No	No	Yes	No	Yes	No	Yes	0	0	9	36
K5	No	No	No	No	No	Yes	No	No	No	Yes	No	Yes	3	26	5	41
K6	No	No	Yes	No	No	No	No	No	No	Yes	No	No	3	41	3	41
K7	No	No	No	Yes	No	No	No	No	No	Yes	No	No	0	0	8	40
K8	Yes	No	Yes	No	No	No	No	No	No	No	No	No	0	0	4	9
K9	Yes	No	No	No	No	No	No	No	No	Yes	Yes	No	2	36	5	43
K10	Yes	No	No	No	Yes	No	No	No	No	Yes	Yes	Yes	0	0	4	2
K11	No	No	No	No	No	Yes	No	No	Yes	Yes	No	No	4	63	4	58
K12	Yes	No	No	Yes	No	No	No	No	Yes	Yes	No	No	2	20	4	20
K13	No	No	No	No	Yes	No	No	No	No	Yes	No	No	2	19	3	33
K14	Yes	No	No	No	No	No	No	No	Yes	Yes	No	Yes	0	0	6	30
K15	Yes	No	No	No	No	Yes	No	No	No	Yes	No	Yes	3	44	3	44

Col inh: Colomycin inhaled, Tob inh: Tobramycin inhaled, Azli inh: Aztreonam inhaled, Mero inh: Meropenem inhaled, Tob/Col (alt. months): tobramycin/colomycin inhaled on alternating months, Tob/Azli (alt. months): tobramycin/aztreonam inhaled on alternating months, Col/Azli (alt. months): Colomycin/Aztreonam inhaled on alternating months, DNase: Dornase alpha, HTS: hypertonic saline, APE: acute pulmonary exacerbation, IP: inpatient

Table 3.3. Summary of baseline characteristics of participants in the TeleCF study

Characteristic	**TeleCF cohort (*n* = 15)**
Age (yr) at screening – mean ± SD	30 ± 8.1
Female sex - no. (%)	6 (40)
Genotype – no. (%)	
F508del homozygous	7 (47)
F508del heterozygous	6 (40)
Other	2 (13)
Percentage of predicted FEV_1 – mean ± SD	46 ± 5.1
Subgroup – no. (%)	
< 40%	5 (33)
≥ 40 to < 70%	8 (53)
≥ 70 to < 90%	2 (13)
≥ 90%	0 (0)
BMI – mean ± SD	22 ± 3.2
Chronic epidemic *P. aeruginosa* infection – no. (%)	8 (53%)
Number of treatment-defined APE – median (IQR)	3 (2.0-3.0)
Prescribed medications – no. (%)	
Inhaled antibiotic	13 (87)
Inhaled bronchodilator	14 (93)
Inhaled hypertonic saline	7 (47)
Dornase alfa	4 (27)
Azithromycin	8 (53)
Prednisolone	4 (27)
Pancreatic insufficiency – no. (%)	15 (94)
CF-related diabetes – no. (%)	9 (60)
CF-related liver disease – no. (%)	4 (27)

FEV_1: forced expiratory volume in 1 second, BMI: Body mass index, APE: acute pulmonary exacerbation, CF: cystic fibrosis

3.3.2 Hospital encounters for usual clinical care

Current consensus guidelines for CF Standards of Care in the UK recommend clinical review of stable CF patients at least every 2-3 months (441). The frequency of clinic attendance for the study participants was greater than two- to three-monthly (median 5 visits, range 3 – 15 visits) during the 6-month study period, reflecting a less clinically stable cohort. A total of 89 clinic visits were documented during the study period with 50 (56%) of these visits linked to either initiation of oral or intravenous antibiotics for a deterioration in baseline respiratory health, or to follow-up whilst on antibiotic treatment. I did not find a correlation between the severity of baseline lung function (FEV_1 percent of predicted) and frequency of clinic visits (Spearman r = - 0.05, p = 0.82).

Participants received treatment for APEs either in the inpatient or outpatient setting. Twenty-one (51%) of these treatment episodes required admission to hospital for all or part of the treatment episode. The total number of hospital-bed days linked to these admissions was 395.

Participants who were admitted to hospital tended to receive a longer course of treatment with the average hospital stay extending over two weeks (median 17 days, range 0 – 50 days). Although there was a trend toward a weakly negative correlation between severity of baseline lung function (FEV_1 % predicted) and length of hospital admission, this relationship did not meet statistical significance (Spearman r = -0.33, p = 0.19).

3.3.3 Frequency of treatment-defined episodes of APE

The majority of participants (80%) experienced at least two APEs during the study period, with just over half (53%) of the participants experiencing three or more APEs over their time in the study (Figure 3.4).

Of the 39 APE periods experienced by the 15 participants, 2 participants (K14 and K15) did not provide sufficient sputum samples for Exotoxin A detection. Thirty-five of these APE episodes were treated with intravenous antibiotic therapy, whilst the remaining four episodes were treated with oral antibiotics alone. Antibiotic courses were administered for a period of two weeks. In participants with a high APE frequency

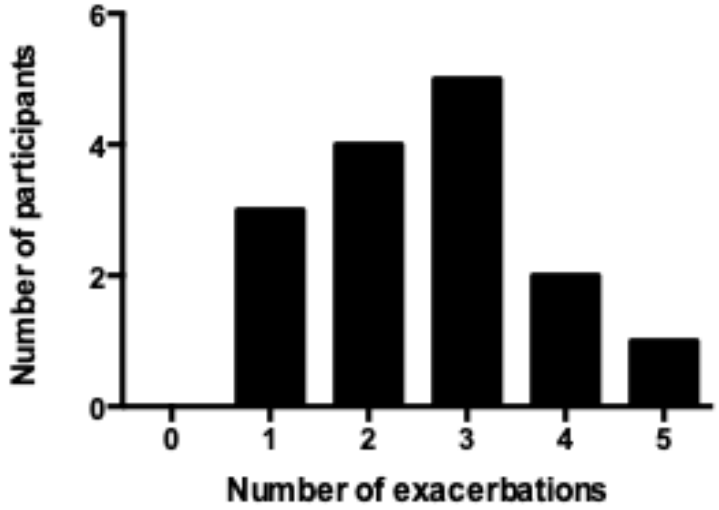


Figure 3.4. Distribution of treatment-defined pulmonary exacerbations during the TeleCF study. The total number of APE during the study period was determined for each participant. An APE was defined based on the initiation of antibiotic therapy (intravenous or oral) for a deterioration in respiratory status. The majority of participants (*n* = 13), experienced 2 or more APEs during the 6-month study period. Only 4 APE episodes were treated with oral antibiotics: two participants with 2 APEs and two participants with 4 and 5 APEs during the study period.

during the study period, I acknowledge the uncertainty in determining whether each treatment-defined episode was reflective of a new APE versus a continuation of the previous. However, a minimum of three to four weeks between treatment courses was typically observed.

3.3.4 Home monitoring data capture and analysis of recordings

Of the 15 participants who completed the TeleCF study, a total of 2698 days of home monitoring was recorded, out of a possible 2700.

The proportion of total study days for which each participant completed a measurement for an individual parameter is summarised in Figure 3.5. Nine participants (60%) completed each home measure daily for at least 80% of the time they were enrolled in the study. The parameter which participants provided the least daily data for was weight (range 4 - 99% of total study days). One participant was unable to provide a daily measure on any of the home monitoring parameters for at least 80% of the time they were enrolled in the study. No lung function data was available from participant K9.

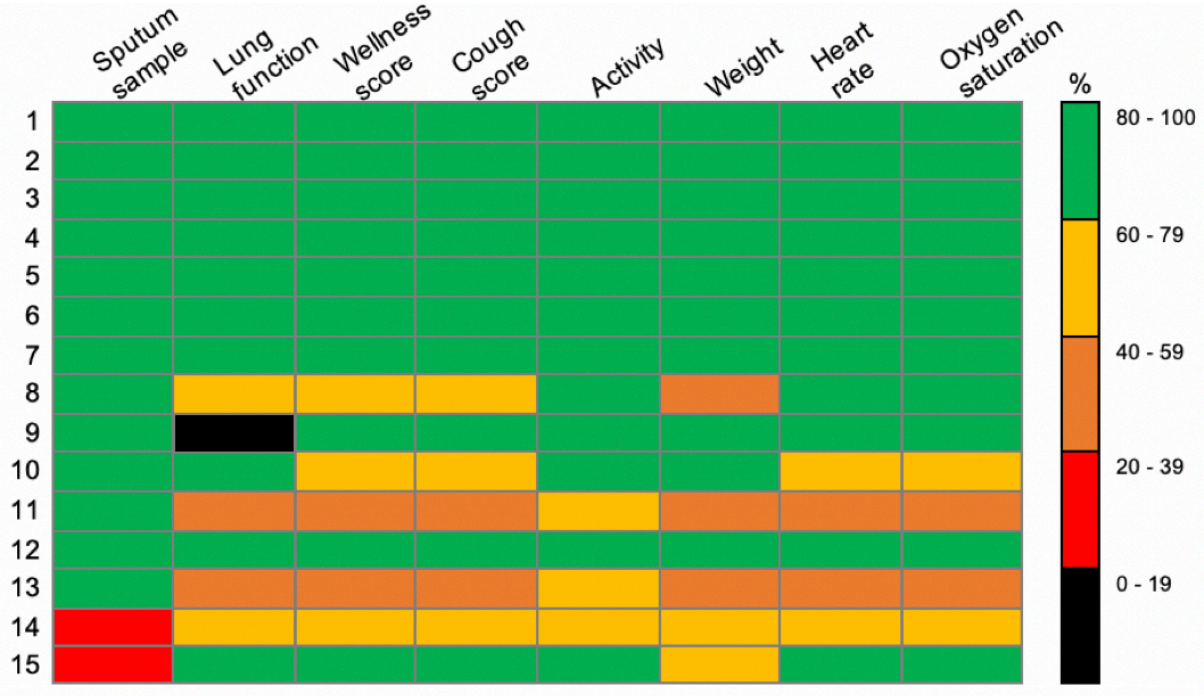


Figure 3.5. Summary of home monitoring frequency for each participant according to home-measured variable. The colour bar indicates the percentage of the total study days for which an individual parameter was completed by the participant.

3.3.4.1 Lung function

The mean lung function for each participant during the study period, measured either on the clinic spirometer or with their home monitoring device, is shown in Figure 3.6. For some participants, I observed large variations between average lung function

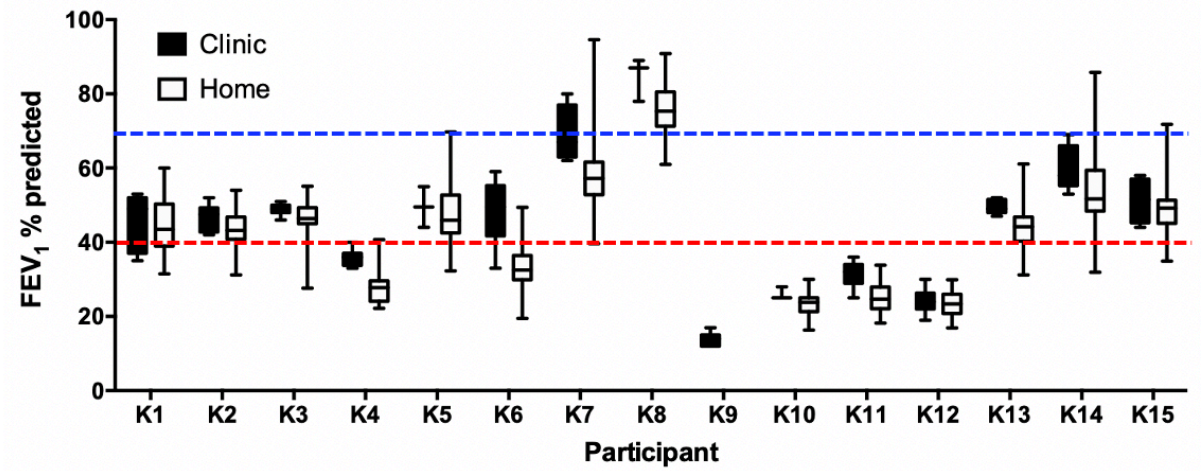


Figure 3.6. Comparison of average lung function [FEV_1 (% predicted)] for each participant over the study period, derived from in-clinic spirometry (black bars) and in-home measures (white bars). The data is presented as mean ± SD. Dashed lines indicate thresholds for severity of lung function impairment. FEV_1 (% predicted) < 40% = severe impairment (red) and FEV_1 (% predicted) ≥ 40 to < 70% predicted = moderately severe impairment (blue). Participant K9 did not record any lung function at home.

measured at home when compared to that measured in the clinic. I therefore compared agreement between lung function recorded at home against lung function measured during clinic attendance on the same day by performing a Bland-Altman analysis. This revealed that home FEV_1 measurements tended to be lower by approximately 5% of predicted and, furthermore, the limits of agreement covered a wide range (-7.5, 18.23). This suggested that the in-home device may either underestimate or overestimate FEV_1 by substantial amounts (Figure 3.7). This may reflect user technique (as lung function is effort-dependent) and/or reflect differences between the two devices.

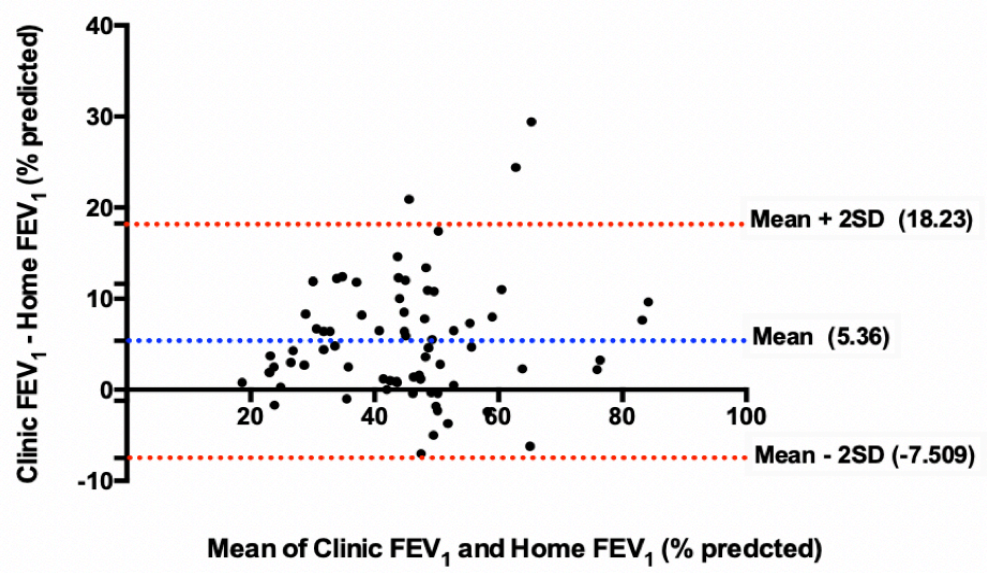


Figure 3.7. Comparison of clinic and home measurements for FEV_1 obtained on the same day on 74 occasions for 15 participants. The difference between clinic and home measurements is on the *y* axis and the average of clinic and home measurements is on the *x* axis. The blue dashed line indicates the mean difference for all data points and the red dashed lines indicate the limits of agreement (upper line: mean +2SD, lower line: mean -2SD).

3.3.4.2 Weight

The average body mass index (BMI) for each participant, derived from their home measurements for weight during the study is shown in Figure 3.8. The majority of participants (73%) maintained a BMI within the healthy weight range (18.5 – 24.9) over the 6-month study period.

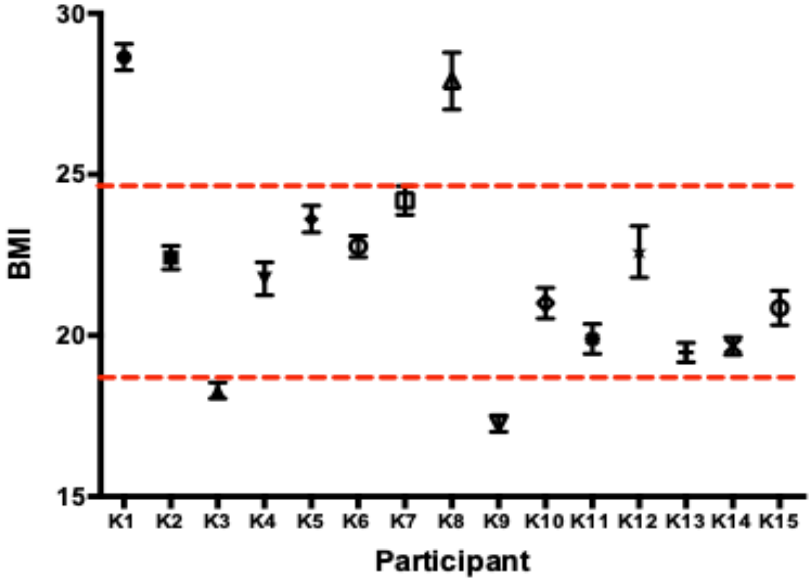


Figure 3.8 Average BMI for each participant over the study period. The data is presented as mean ± SD. The dashed red lines indicate the boundaries of the healthy weight range for BMI which falls between 18.5 and 24.9. Four participants had a BMI outside of the healthy range, with one participant classed as over-weight and two classed as underweight.

3.3.4.3 Heart rate and oxygen saturation

The mean heart rate and oxygen saturation for each participant over the course of the study is shown in Figure 3.9. Of note, participant K4's data varied widely for oxygen saturation, with several measurements falling outside of the normal physiological (e.g., SpO_2 >100%) or expected clinical range.

Furthermore, in comparison to the other participants, a notable lack in heart rate variability around the mean was also observed for the same participant. Taken together, these observations suggested a malfunctioning device as both heart rate and oxygen saturation are recorded on the same device. Therefore, heart rate (n = 174) and oxygen saturation (n = 174) measures for participant K4 were omitted from further analyses due to poor reliability.

3.3.4.4 Activity

The median number of steps recorded for each participant during the study is shown in Figure 3.10. Of note, no validation data for its use as a measure of step count in free-living conditions was available for the consumer-wearable activity tracker used in

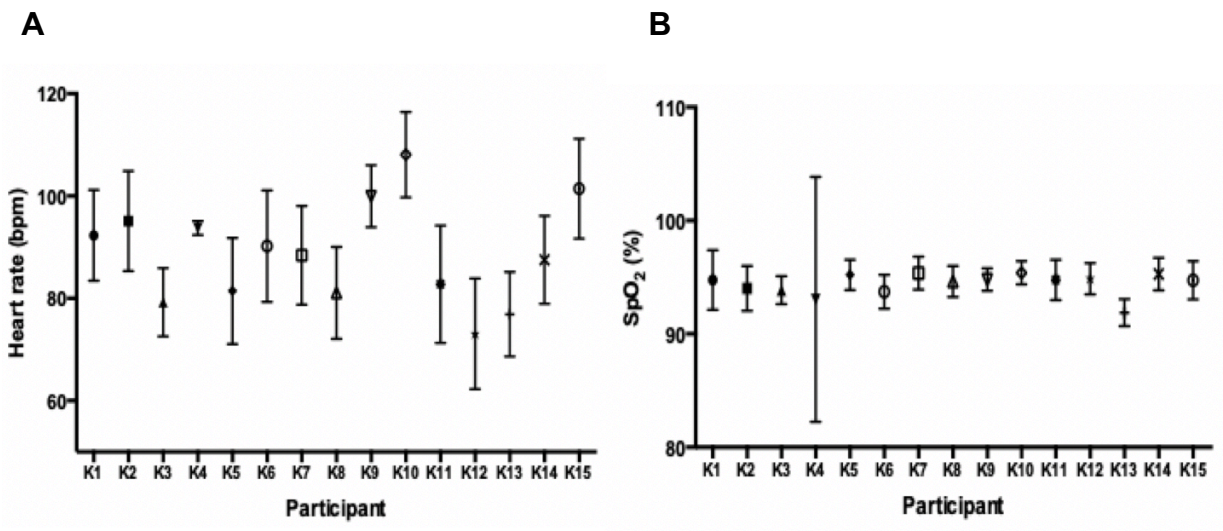


Figure 3.9. Average heart rate and oxygen saturation for each participant over the study period. (A) Heart rate (bpm). (B) Oxygen saturation (SpO_2, %). The data is presented as mean ± SD. The wide variability seen in the SpO_2 measures for participant K4 suggest a malfunctioning device with several measures falling outside acceptable clinical and physiological parameters. Given that heart rate and oxygen saturation were measured on the same device, these measures for participant 4 were excluded from further analyses due to a lack of reliability (n = 118 respectively).

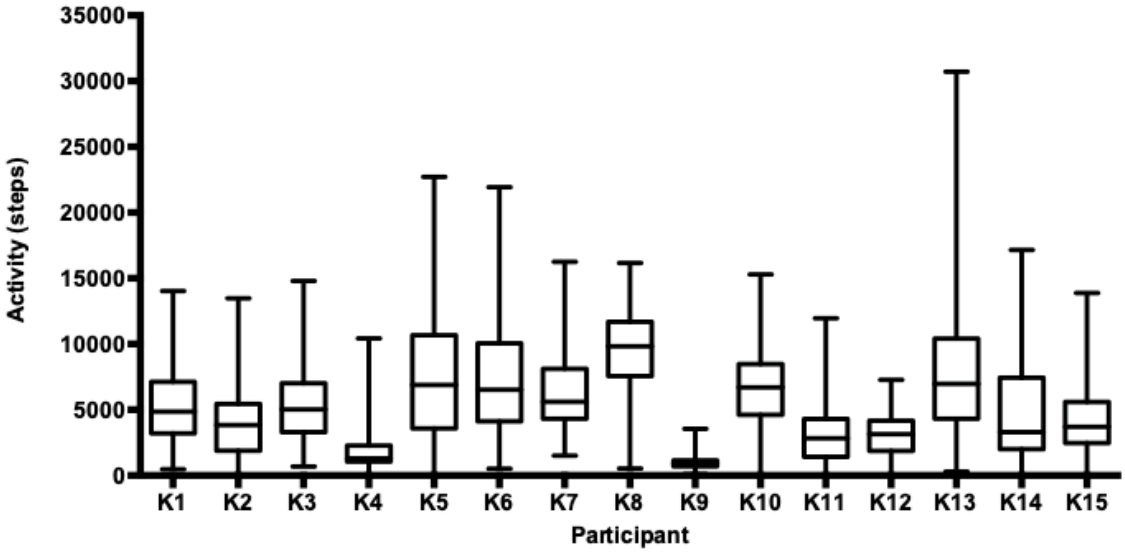


Figure 3.10. Activity levels (recorded in steps) for each participant through the study period. Each box encloses 50% of the data; median values are displayed as lines within the boxes. The tops and bottoms of the boxes mark the limits of ± 25% of the variation. The lines extending from the top and the bottom of each box mark the minimum and maximum values within the dataset. Activity levels varied widely between participants.

this study. The participant with the lowest baseline lung function in the cohort was also the least active (participant K9, 908 steps a day, range 107 to 3551) whilst the participant with the best lung function was the most active (participant K8, median 9830 steps a day, range 538 to 16161).

3.3.4.5 Symptom scores

Symptom scores for cough and wellness did not fluctuate greatly, with most participants' scores varying by either +1 or -1 around their median values (Figure 3.11).

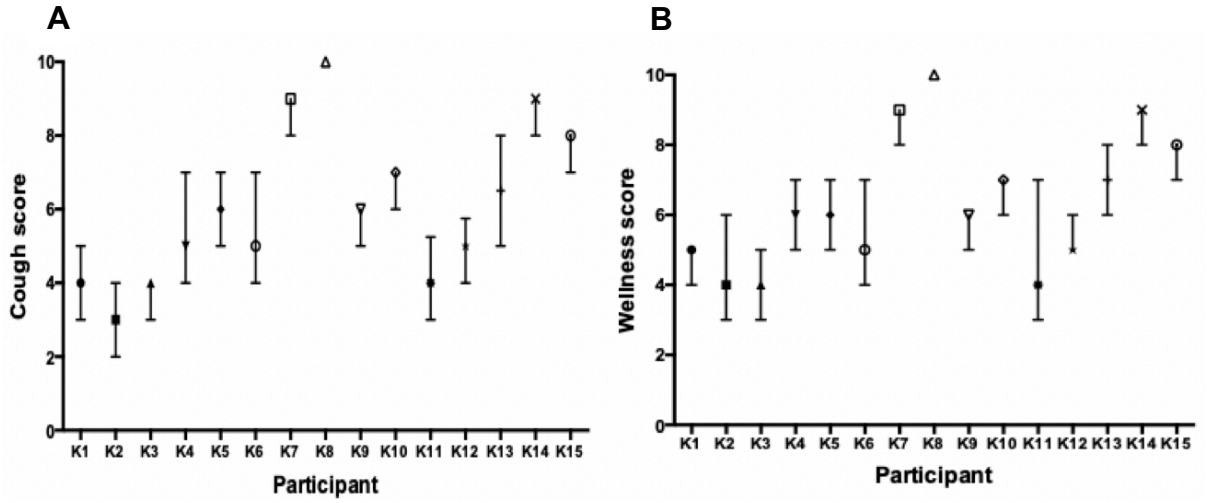


Figure 3.11. Variation in symptoms scores between participants. A) Cough score (B) Wellness score. Data is presented as median and interquartile range. Median values are displayed as symbols. The lines extending from the top and the bottom of each symbol mark the limits of ± 25% of the variation.

3.3.5 Correlations between Exotoxin A, lung function, frequency of exacerbations and markers of systemic inflammation

Exotoxin A was detected in all sputum samples collected. Significant variation in sputum Exotoxin A concentrations was observed between participants with median values ranging from 0.4 ng•mL^{-1} to 12.5 ng•mL^{-1}(Kruskal-Wallis test, $p < 0.0001$) (Figure 3.12).

To ascertain whether clinical markers known to be associated with CF disease severity

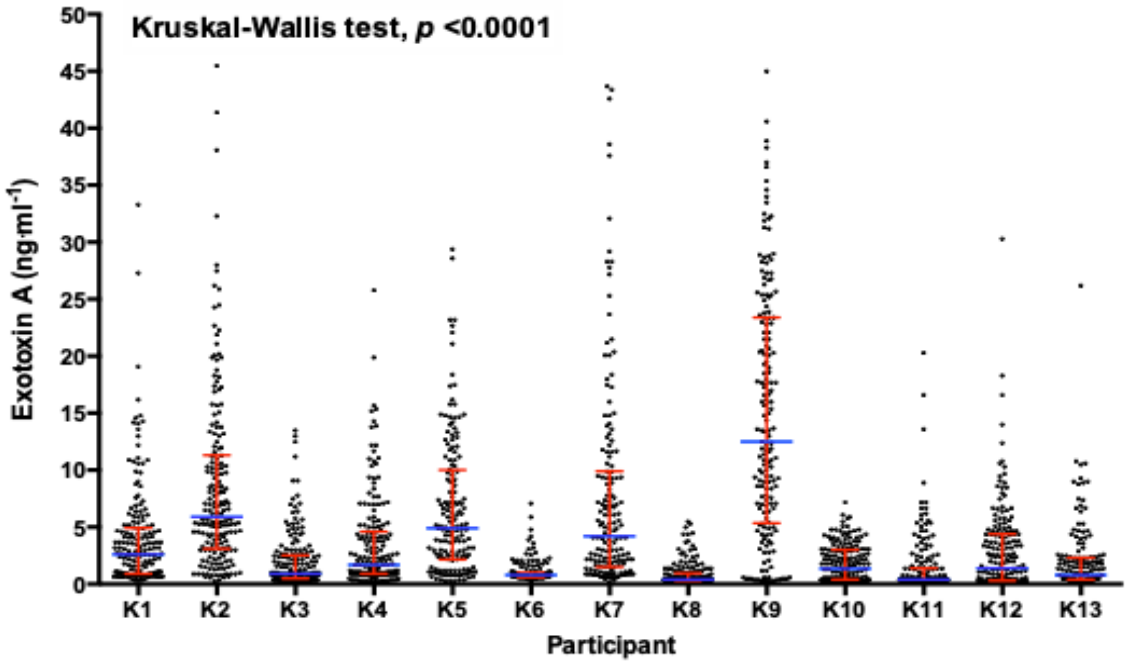


Figure 3.12. Exotoxin A concentration in sputum for each participant over the study period. Exotoxin A concentration was quantified in daily sputum samples provided by each participant. Median values are marked in blue. The top and bottom error bars in red mark the limits of ± 25% of the variation. Significant between-participant variability in median Exotoxin A concentrations was observed (Kruskal-Wallis test, $p < 0.0001$)

(e.g., baseline lung function, frequency of APEs) and degree of systemic inflammatory response at time of APE (e.g., CRP) were significantly associated with variation in Exotoxin A, I performed a correlation analysis. I did not identify significant relationships between either FEV_1 percent of predicted (Spearman r = -0.08, p = 0.4335) or CRP (Spearman r = -0.04, p = 0.6335), nor between frequency of exacerbations (Spearman r = 0.28, p = 0.3094) and Exotoxin A.

I next sub-divided the participants according to chronic infection with either an epidemic (MES or LES) or a non-epidemic strain type of *P. aeruginosa* to investigate for differences between the groups. I did not identify a difference in levels of Exotoxin A between the two groups (Mann-Whitney test, p = 0.0782) (Figure 3.13A). However, further comparison after sub-division of the epidemic cohort into MES- and LES-infected participants identified a statistically significant difference between groups, with Exotoxin A levels notably higher in participants chronically infected with the LES (Kruskal-Wallis test, p = 0.043) (Figure 3.13B). Although, the small number of

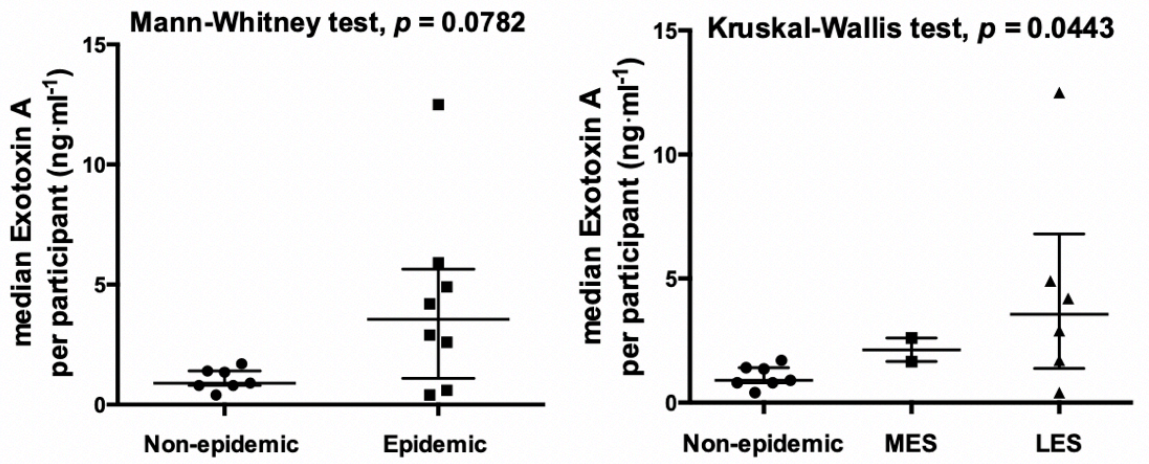


Figure 3.13. Comparison of median Exotoxin A concentration in sputum (A) between participants chronically infected with an epidemic strain of *P. aeruginosa* and those infected with a non-epidemic strain or (B) between participants chronically infected with a non-epidemic strain, the MES or the LES of *P. aeruginosa*. Data is presented as median and interquartile range. There was a statistically significant difference found between the three groups (Kruskal-Wallis test, $p = 0.0443$)

participants in this analysis constrains the drawing of broader inferences from these findings.

3.3.6 Correlations between Exotoxin A and home measures

To investigate for correlations between changes in the microbial biomarker Exotoxin A and home measures, I first generated line graphs for each participant's home monitoring daily data. This revealed a large and complex dataset, with substantial daily variation in physiological and symptom measures. In contrast to the discrete measures taken at clinic visits, daily collected home monitoring data provided a far more detailed, longitudinal overview of an individual's physiology and symptoms during periods of clinical stability and exacerbation. A representative example of a single participant's data capture through the 6-month study period is shown in Figure 3.14. Of note, when all APE episodes were assessed in aggregate, I observed deviations in several home measures around periods of antibiotic treatment for APE, suggesting possible correlations with APE onset.

Next, in order to identify any significant relationships between the seven home monitoring measures and Exotoxin A, I performed a correlation matrix (Figure 3.15). I

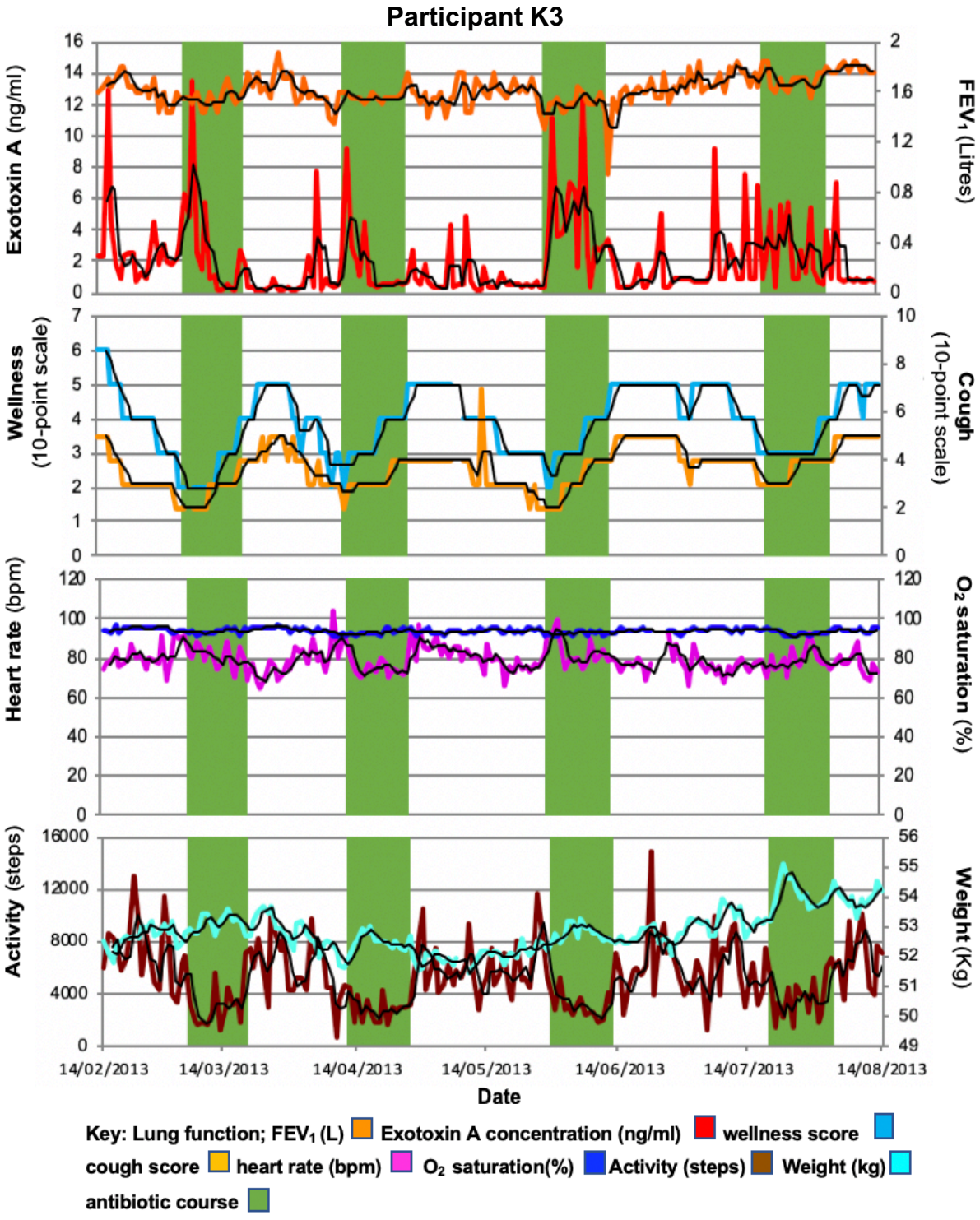


Figure 3.14. Representation of a single participant's daily data capture for each home monitoring parameter across the 6-month study. Green bars represent antibiotic treatment courses and correspond to each acute pulmonary exacerbation event. Deviations in several of the home measures were observed before and after each antibiotic course.

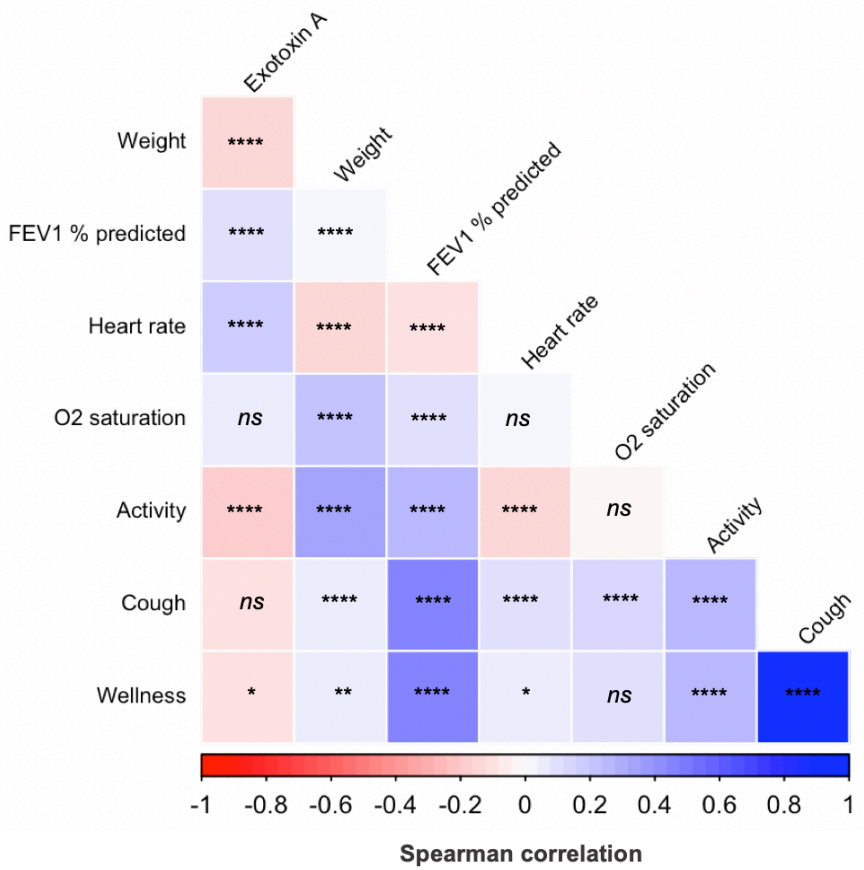


Figure 3.15. Correlation matrix of variable-variable associations as determined by Spearman correlation coefficient. Blue indicates a strong positive correlation between any given pair (Spearman coefficient closer to 1). Red indicates a strong negative correlation between any given pair (Spearman coefficient closer to -1). White indicates no correlation between pairs (0). Correlations were significant if $r > 0.35$ and $p \leq 0.05$ (*). *ns* = not significant, ** =$p < 0.001$, **** = $p < 0.00001$.

did not identify any relationships between Exotoxin A and any of the home monitoring parameters. However, FEV_1 (percent of predicted) was modestly positively correlated with symptom scores, with better lung function associated with fewer symptoms (cough: Spearman $r = 0.49$, wellness: Spearman $r = 0.5$, $p < 0.00001$).

Not surprisingly, I identified a strong positive correlation between cough and wellness symptom scores (Spearman $r = 0.91$, $p < 0.00001$). I also identified a positive, albeit weak, correlation between activity and each of the symptom scores (cough: Spearman $r = 0.27$, wellness: Spearman $r = 0.29$, $p < 0.00001$) and between activity and FEV_1 (percent of predicted) (Spearman $r = 0.27$, $p < 0.00001$).

3.3.7 Changes in Exotoxin A and home measures prior to the start of antibiotic treatment for an APE

I next investigated for evidence of a significant change in Exotoxin A levels prior to the initiation of antibiotic treatment for an APE. I did this by calculating, for the sample set as a whole, the median Exotoxin A levels for the 3-day, 7-day and 14-day period prior to the initiation of antibiotic therapy for an APE. I also calculated the median Exotoxin A levels for the start day (0-day) of antibiotic therapy. I included 20 out of a possible 39 pre-exacerbation periods in the 14-day analysis. However, I only included 19 pre-exacerbation periods in the 3-day and 7-day analyses, respectively, due to insufficient sputum samples available for exotoxin detection from participant K7 during these time periods. I excluded five pre-exacerbation periods (two from participant K14 and three from participant K15) as sputum samples were not provided for Exotoxin A detection by these participants during the TeleCF study. I also excluded a further eight pre-exacerbation periods (one each from participants K1, K2, K4, K5, K8, K9, K10 and K11 respectively) because these periods fell outside of Day 1 of the TeleCF study period. Finally, I excluded six pre-exacerbation periods (one each from participants K1, K2, K3, K5, K9 and K13) due to less than 28 days elapsing between antibiotic treatment courses for APE.

The median Exotoxin A concentrations calculated for the 14-day, 7-day, 3-day and 0-day intervals prior to initiation of antibiotic therapy for an APE were 2.2 (0.8-6.7) ng•mL^{-1}, 2.4 (0.8–8.4) ng•mL$^{-1,}$ 2.1 (0.7–7.0) ng•mL^{-1} and 2.2 (0.9-14.9) ng•mL^{-1} respectively. Accordingly, I did not find a statistically significant difference in median Exotoxin A levels between these intervals leading up to antibiotic treatment for an APE (Kruskal-Wallis test, p = 0.8338).

Similarly, I did not identify a statistically significant difference in the median or mean values for the seven home monitoring measures between any of the four intervals prior to starting antibiotic treatment for an APE (Table 3.4).

3.3.8 Changes in Exotoxin A and home measures between non-exacerbation periods and prior to initiation of antibiotic treatment for an APE

I next investigated for significant changes in either Exotoxin A or any of the home measures between periods of clinical stability (non-exacerbation) and 1) 14-days, 2)

Table 3.4. Comparison of home monitored measures prior to starting antibiotic treatment for an APE.

Home measure	Interval prior to antibiotic treatment for an APE (days)				*p* value
	14	7	3	0	
FEV_1 (% predicted) - mean	40 ± (12.2)	39 ± (12.8)	38 ± (13.8)	41 ± (12.2)	0.8554
Weight (kg) - mean	61 ± (9.7)	60 ± (9.8)	60 ± (9.8)	62 ± (9.8)	0.9105
Activity (steps) - median	4596 (2105 – 7386)	4498 (1622 – 7090)	4134 (1397 – 5942)	5035 (3597 – 6697)	0.3830
HR (bpm) - mean	89 ± (10.6)	89 ± (10.9)	90 ± (11.8)	86 ± (10.6)	0.7229
O_2 saturation (%) - mean	94 ± (1.4)	94± (1.6)	95 ± (1.6)	94 ± (1.6)	0.9010
Wellness score - median	4 (3 – 5)	4 (3 – 5)	3 (3 – 5)	4 (3 - 5)	0.2416
Cough score - median	4 (3 – 6)	4 (3 – 5)	3 (3 – 5)	4 (2 – 6)	0.4288

Differences between continuous variables were examined with one-way ANOVA for parametric data and Kruskal-Wallis test for non-parametric data. APE: acute pulmonary exacerbation, FEV_1: Forced expiratory volume in 1 second, HR: heart rate. Differences are significant if $p \leq 0.05$.

7-days and 3) 3-days prior to initiation of antibiotic therapy for an APE. The period of non-exacerbation that was used for comparison with each of the three pre-exacerbation periods prior to treatment initiation varied in duration and I defined this pragmatically as, the period starting from the end of a course of antibiotics received for an APE, to the start of either the 3-day (S1), 7-day (S2) or 14-day (S3) interval prior to antibiotic initiation for a subsequent APE. In three cases (APE1 for participants K6, K7 and K12), the previous antibiotic course fell outside of the first day of the TeleCF study for that participant. However, as more than 28 days had elapsed between the study start date to the first course of antibiotics for an APE, samples collected from the study start date up until the first antibiotic course for an APE were included in this analysis.

I did not find a statistically significant difference for the whole cohort in median Exotoxin A concentrations measured during periods of non-exacerbation and either the 3-day (Wilcoxon matched pairs test, p = 0.8203*)*, 7-day (Wilcoxon matched pairs test, p = 0.1055) or 14-day (Wilcoxon matched pairs test, p = 0.3008) period prior to starting antibiotics for an APE (Figure 3.16).

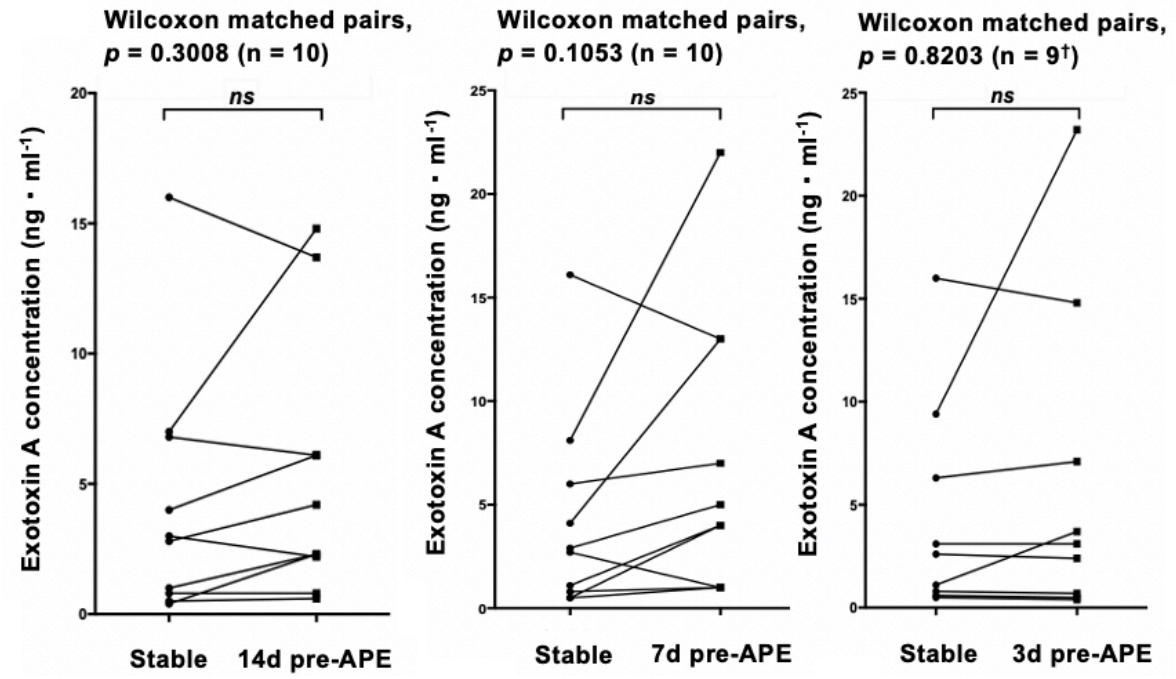


Figure 3.16. Comparison of Exotoxin A concentrations during clinical stability and prior to initiation of antibiotic treatment for an APE. Each pair of points represents a single subject's data and were handled as paired samples. Median Exotoxin A concentrations were compared between either the 14-day, 7-day and 3-day periods prior to initiation of antibiotic therapy for an APE and a period of clinical stability. This variable period of clinical stability was defined as the period starting from the end of a course of antibiotics received for an APE, to the start of either the 3-day, 7-day or 14-day interval prior to antibiotic initiation for a subsequent APE. A statistically significant difference was not found between Exotoxin A concentrations during periods of clinical stability compared with prior to initiation of antibiotics for an APE. Differences are significant if $p \leq 0.05$. (†) Insufficient samples for Exotoxin A detection were available from participant K7, for this analysis.

Next, I performed a similar analysis for each of the seven home monitored measures. Home measurement data was unavailable from participant K12 for several variables (heart rate, oxygen saturation, cough and wellness scores) for the 14-day period prior to an APE due to missed measures. Likewise, data was unavailable for weight in the

3-day and 14-day period prior to an APE for participant K11, and for lung function in the 3- , 7-, or 14-day period prior to exacerbation for participant K9. Heart rate and oxygen saturation data was excluded from participant K4's dataset, for reasons previously discussed in Heart rate and oxygen saturation, Section 3.3.4.3.

I identified a statistically significant difference between periods of non-exacerbation and prior to initiation of treatment for an APE for two of the home measures: lung function (FEV_1 percent of predicted) and cough score. For lung function, this difference was observed when comparing values during non-exacerbation with either the 7-day or 14-day period prior to treatment initiation for an APE (Wilcoxon matched pairs test, $p = 0.0357$ and $p = 0.0447$ respectively) (Figure 3.17).

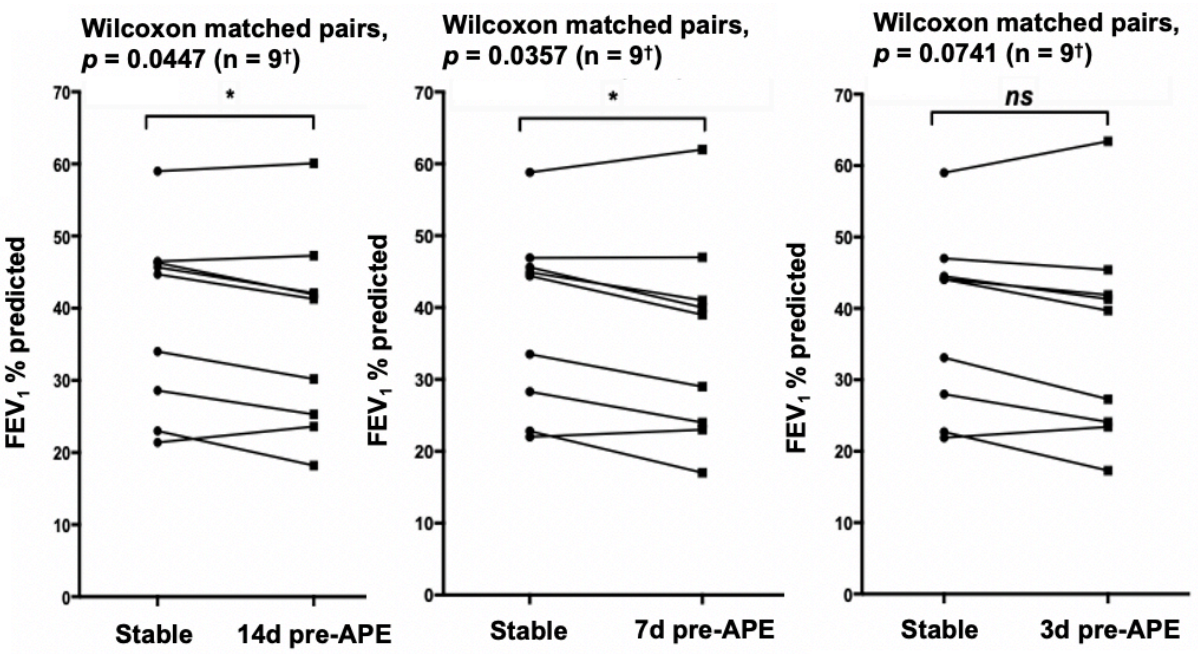


Figure 3.17. Comparison of lung function (FEV_1 % predicted) during clinical stability and prior to initiation of antibiotic treatment for an APE. Each pair of points represents a single subject's data and were handled as paired samples. Mean values for FEV_1 % predicted were compared between either the 14-day, 7-day and 3-day periods prior to initiation of antibiotic therapy for an APE and a period of clinical stability. This variable period of clinical stability was defined as the period starting from the end of a course of antibiotics received for an APE, to the start of either the 3-day, 7-day or 14-day interval prior to antibiotic initiation for a subsequent APE. A statistically significant difference was found between mean FEV_1 % predicted during periods of clinical stability compared with 7-days and 14-days prior to initiation of antibiotics for an APE. Differences are significant if $p \leq 0.05$. (†) No measures were available from participant K9 for this analysis.

For cough score this difference was noted when comparing values during non-exacerbation with either the 7-day or 3-day period prior to treatment initiation for an APE (Wilcoxon matched pairs test, p = 0.0313 and p = 0.046 respectively) (Figure 3.18).

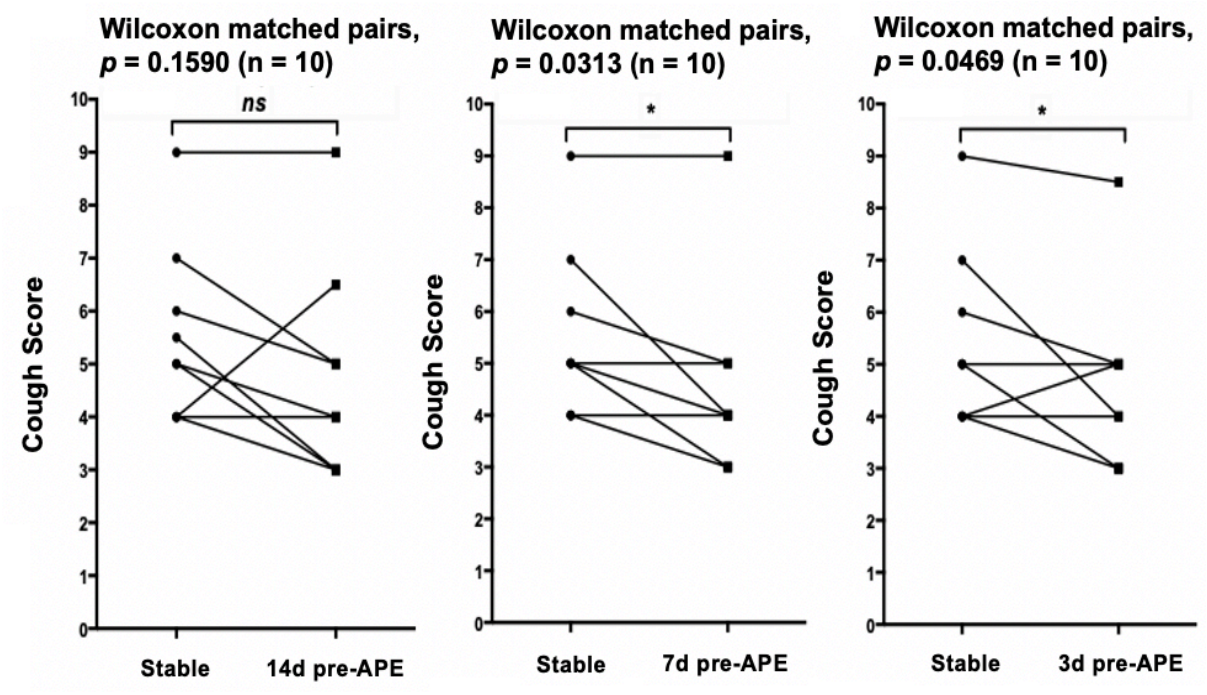


Figure 3.18. Comparison of cough score during clinical stability and prior to initiation of antibiotic treatment for an APE. Each pair of points represents a single subject's data and were handled as paired samples. Median values for cough score were compared between either the 14-day, 7-day and 3-day periods prior to initiation of antibiotic therapy for an APE and a period of clinical stability. This variable period of clinical stability was defined as the period starting from the end of a course of antibiotics received for an APE, to the start of either the 3-day, 7-day or 14-day interval prior to antibiotic initiation for a subsequent APE. A statistically significant difference was found between median cough score during periods of clinical stability compared with 3-days and 7-days prior to initiation of antibiotics for an APE. Differences are significant if $p \leq 0.05$.

3.3.9 Within-participant variation in Exotoxin A and home measures before treatment for an APE

I next sought to investigate whether Exotoxin A and/or home measures changed significantly, and consistently, between antibiotic treatment episodes for APE within the same participant.

To determine this, I calculated the median/mean values of the variables in the 3-day, 7-day and 14-day period prior to each APE and compared these to the median/mean values in the preceding period of non-exacerbation, as previously defined in Section 3.3.8.

For ease of reporting, I have assigned the following abbreviations: the period from the preceding course of antibiotics for an APE *up to* 3 days before antibiotics for the next APE is designated as S1, the period from the preceding course of antibiotics for an APE *up to* 7 days before antibiotics for the next APE is designated as S2 and the period from the preceding course of antibiotics for an APE *up to* 14 days before antibiotics for the next APE is designated as S3. I will also refer to the period including S1 *and* the 3 days prior to antibiotic initiation as interval-1, the period including S2 *and* the 7 days prior to antibiotic initiation as interval-2 and the period including S3 *and* the 14 days prior to antibiotic initiation as interval-3. For this analysis I have only included data from participants K1, K2, K3, K4 and K6, who each experienced at least three complete treatment-defined APEs within the study period (Figure 3.19). I took this decision in order to facilitate comparison of changes between sequential treatment-defined APEs. I excluded heart rate and oxygen saturation from this analysis, as they have previously been shown to have minimal correlation with any of the other variables in the correlation analysis.

For participant K1, comparison of the median/mean values of the variables for the three intervals before APE2 and APE3 only revealed statistically significant differences in mean FEV_1 percent of predicted in interval-1 ($p = 0.0167$) and interval-2 ($p < 0.0001$) prior to APE2, and in median wellness score in interval-2 ($p = 0.0079$) prior to APE2.

For participant K2, I found statistically significant differences in three variables prior to APE2: mean FEV_1 percent of predicted (interval-2 and interval-3, $p = 0.0180$ and 0.0385 respectively), median cough score (interval-1, interval-2 and interval-3, $p = 0.0304$, 0.0449 and 0.0034 respectively) and median wellness score (interval-3, $p = 0.0076$). Similarly, prior to APE3, I found statistically significant differences in two variables: median Exotoxin A (interval-2, $p = 0.0294$) and mean FEV_1 percent (interval-2 and interval-3, $p = 0.0011$ and 0.0309 respectively). For APE4, I found statistically significant differences in three variables: mean FEV_1 percent (interval-3, $p = 0.0061$),

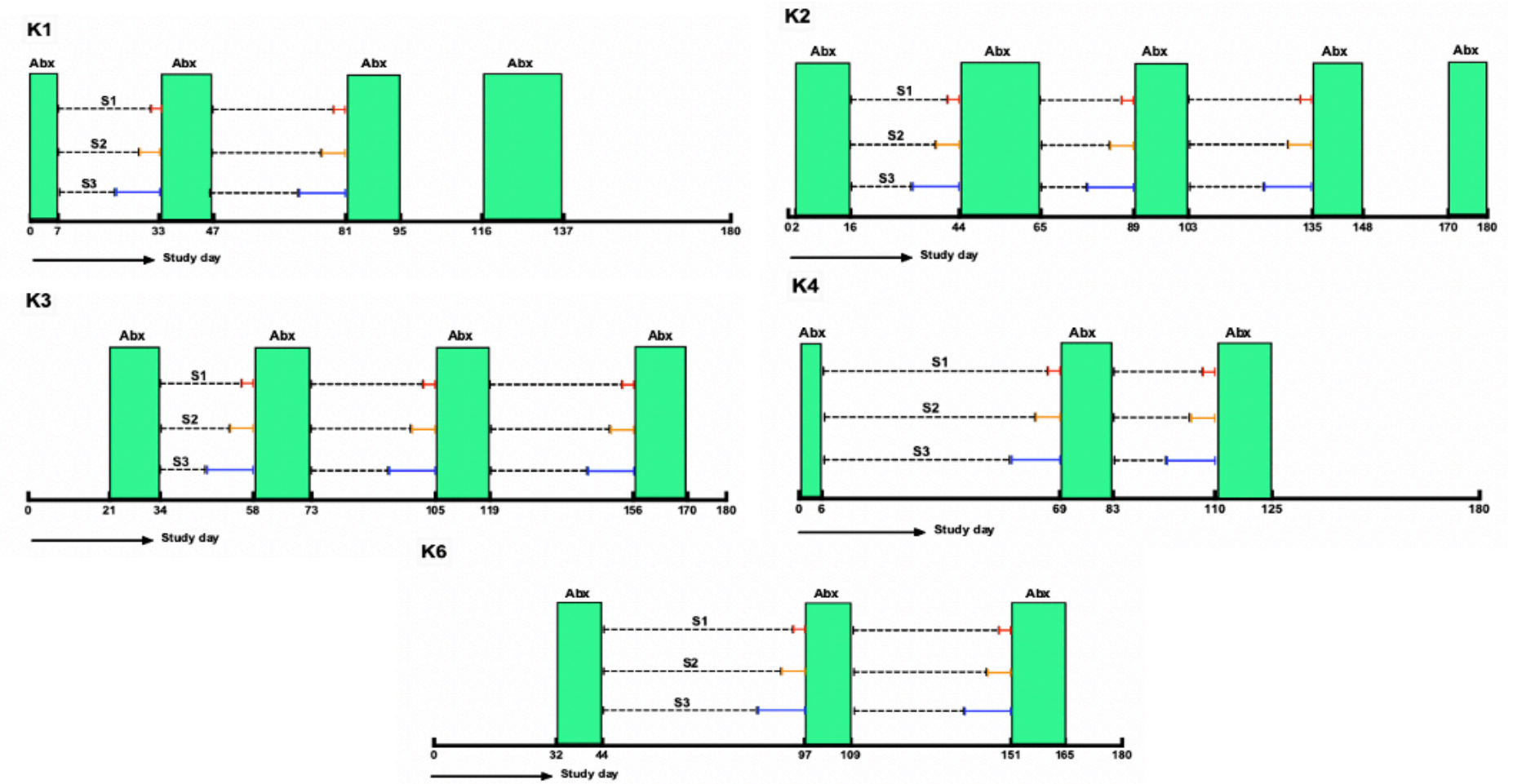


Figure 3.19. Sampling periods for the 5 participants included in the analysis of within-participant variation in Exotoxin A and home measures (FEV_1 % predicted, weight, cough score, wellness score and activity) prior to initiation of antibiotic treatment for an APE. Median/mean values for each of the variables were calculated for the 3-day (red line), 7-day (orange line) and 14-day (blue line) periods prior to antibiotic (Abx) treatment for an APE, and compared with median/mean values in the period extending from the preceding course of antibiotics to either 3-day (S1), 7-day (S2) or 14-day (S3) prior to antibiotics starting, respectively, for each inter-treatment period that fell within the study period. The green bars represent antibiotic treatment courses. Inter-treatment periods shorter than 21 days were excluded from this analysis.

cough score (interval-2, and interval-3, p = 0.0193 and <0.0001 respectively) and median wellness score (interval-3, p <0.0001).

For participant K3, I found a statistically significant difference in median cough scores in each of the three intervals, prior to all three of the APEs examined – APE2: interval-1, p = 0.0311; interval-2, p = 0.0003; interval-3, p = 0.0156; APE3: interval-1, p = 0.0099; interval-2, p = 0.0004; interval-3, p <0.0001, and APE4: interval-1, p = 0.0077; interval-2, p = 0.0042; interval-3, p <0.0001. In addition, I found statistically significant differences in two other variables in each of the three intervals prior to APE2: mean FEV_1 percent of predicted (interval-1, interval-2 and interval-3, p = 0.0059, 0.0032 and 0.0236 respectively) and median wellness score (interval-1, interval-2, and interval-3, p = 0.0056, 0.0008 and 0.0051 respectively). Furthermore, I found a statistically significant difference in median activity in interval-1 (p = 0.0305) and interval-3 (p = 0.0275) prior to APE2. For interval-2 and interval-3 prior to APE3, I found a statistically significant difference in the median wellness score (p = 0.0001 and <0.0001 respectively). In addition to the cough score, I also found statistically significant differences in three more variables prior to APE4: median Exotoxin A (interval-3, p = 0.0334), mean FEV_1 percent (interval-3, p = 0.0307), and median wellness score (interval-1, p = 0.0013; interval-2, p <0.0001; interval-3, p = <0.0059).

For participant K4, I found a statistically significant difference in median cough scores in all three intervals prior to APE2: interval-1, p = 0.0298; interval-2, p <0.0001; interval-3, p <0.0001. In addition, I found statistically significant differences in three other variables prior to APE2: mean FEV_1 percent of predicted (interval-2 and interval-3, p = 0.0329 and 0.0015 respectively), median wellness score (interval-2, and interval-3, p = 0.0168 and <0.0001 respectively) and median activity level (interval-2, and interval-3, p = 0.0124 and <0.0001 respectively). Finally, prior to APE4, I found a statistically significant difference in only one variable for interval-2: mean FEV_1 percent of predicted (p = 0.0059).

For participant K6, I found a statistically significant difference in mean FEV_1 percent of predicted (interval-3, p = 0.0003) and median wellness score (interval-2, p = 0.002) prior to APE2. In addition, I found a statistically significant difference in the mean/median values of four variables prior to APE4: median Exotoxin A (interval-2

and interval-3, p = 0089 and 0.0047 respectively), mean FEV_1 percent of predicted (interval-1 and interval-2, p = 0.0003 and 0.0020 respectively), median cough score (interval-1, p = 0.0077; interval-2, p = 0.0066; interval-3, p <0.0001) and median wellness score (interval-1, p = 0.0028; interval-2, p = 0.0024; interval-3, p <0.0001).

Taken together, these observations suggest that symptom scores, particularly cough score, change significantly in the period prior to treatment for an APE. Often, accompanying changes in FEV_1 percent of predicted are observed, although this change is less consistent between sequential APE events within the same participant. Furthermore, although significant differences in Exotoxin A and activity levels were observed these changes were very infrequent and not consistent between sequential APE events.

3.4 DISCUSSION

Unlike most Gram-negative bacteria, *P. aeruginosa* is able to secrete a variety of virulence factors into the extracellular milieu, among them Exotoxin A. Secretion of Exotoxin A enables tissue adherence and facilitates tissue damage for nutritional supply and dissemination (442). Previous studies have confirmed the presence of Exotoxin A in CF sputum (167). Furthermore, several authors have demonstrated that in CF patients chronically infected with *P. aeruginosa* and hospitalised for APE, exoprotein levels (Exotoxin A, elastase and alkaline protease) in sputum fall during and after antibiotic treatment (167,267). Therefore, it would seem plausible that Exotoxin A levels may likewise vary between periods of clinical stability and prior to antibiotic treatment initiation.

For the purposes of this study, the start of an APE was defined as a decision to initiate antibiotic therapy in response to a deterioration in respiratory symptoms. Given that changes in Exotoxin A levels likely precede patient-reported symptoms, a two-week period prior to initiation of antibiotic therapy was selected as this seemed reasonable to detect changes in Exotoxin A levels. Daily home monitoring of lung function, weight, heart rate, oxygen saturation, activity levels, cough and wellness scores were undertaken alongside sputum assessment of Exotoxin A levels.

This is the first study investigating changes in Exotoxin A levels prior to initiation of antibiotic treatment for an APE. Furthermore, this is the first study to combine home monitoring of physiological and symptom measures to enable objective establishment of periods of clinical stability and exacerbation, against which to analyse, the utility of a potential microbial biomarker to predict for the onset of APE.

The results from this single-centre study have enabled the following conclusions to be drawn. First, in contrast to previous work (443), poor correlations were found between Exotoxin A and markers of systemic inflammation (e.g., CRP) and CF disease severity, specifically severity of baseline lung function impairment and frequency of APEs. Second, significantly higher Exotoxin A levels were observed in patients chronically infected with the LES. This observation provides additional evidence in support of the hypervirulent phenotype associated with the LES and is in keeping with previous work describing overproduction of other secreted virulence factors (e.g., pyocyanin) by the LES (444). Third, median Exotoxin A levels do not change significantly two weeks, one week or three days prior to the initiation of antibiotics for an APE. Furthermore, Exotoxin A levels did not vary significantly between APE episodes within the same individual, suggesting that this single virulence factor is not likely to be responsible for triggering APEs and consequently has poor utility as a microbial biomarker to detect for APE onset.

In general, home monitoring using USB-enabled devices and a home personal computer for data transmission proved successful, with the majority of patients providing a high number of recordings across all the devices for the duration of the study. However, the study was incentivised which may have influenced retention rates. In addition, the number of participants in this study was small further limiting an assessment of acceptability and feasibility. Nonetheless, the large dataset of home-monitored measures collected in this study provided, for the first time, detailed resolution of the day-to-day variation in physiology and symptoms during periods of stability and decline for an individual with CF. However, despite observation of falling trends in several measures prior to treatment initiation for an APE no consistent change in any single home measure was identified prior to an APE. Still, in some individual participants, significant changes in some home measures, most consistently symptom scores and lung function, in the period prior to antibiotic treatment were

seen. These findings suggest that home monitoring of physiology and symptom scores may have potential to detect declining trends in lung health prior to presentation in the clinic. However, given the complexity of the dataset, with a combination of day-to-day variation in measures and occasional missing data points reducing the signal-to-noise ratio in the measurements, more advanced data analytics on a larger sample of participants will need to be undertaken to better understand the relationships between physiology, symptoms and APE.

This study, however, has limitations in its methodology that should be taken into account when interpreting these findings. It presents data from a single specialist centre with a small sample size. Furthermore, only individuals chronically infected with *P. aeruginosa* were included due to the nature of the biomarker under investigation, therefore limiting the generalisability of the home monitoring findings to the wider CF population. In addition, no assessment of user experience with regards to daily home monitoring was conducted, thereby limiting recommendations on feasibility and acceptability to the wider CF population. This study only analysed changes in the variables in the two-week window prior to antibiotic initiation for APE. Moreover, it was not possible to determine with certainty the start date for an APE, independent of the initiation of antibiotic treatment. Therefore, it remains possible that changes in both bacterial behaviour and host response precede this arbitrary cut-off, limiting the scope of the conclusions that can be drawn from the data. Finally, we only analysed a single bacterial marker. However, given the complex inter-relationships between virulence determinants associated with *P. aeruginosa*, investigation of a panel of biomarkers may yield more promising results. An appreciation of the limitations highlighted in this study has informed the rest of the work discussed in this dissertation.

4. Investigation of changes in the phenotypic composition of clonal populations of *P. aeruginosa* as a trigger for APE onset in adults with CF.

Summary:

P. aeruginosa isolates from chronic CF lung infections exhibit diverse phenotypic profiles. We hypothesised that changes in the phenotypic composition of *P. aeruginosa* clonal populations may be responsible for triggering APEs in CF.

Sequential sputum samples, provided over a 6-month period, were collected from 9 adults with CF before, during and after antibiotic treatment for an APE. Sets of 95 isolates per sputum sample were analysed for a series of phenotypic traits associated with *P. aeruginosa* virulence. We also investigated for differences in phenotypic composition between isolate populations of the *P. aeruginosa* LES, MES and local non-epidemic strains.

We characterised 6 traits (morphotype, auxotrophy, exoproduct secretion, motility, biofilm formation and QS AI production for a total of 4408 isolates. Most isolates lacked production of OdDHL, the cognate AI for LasR, the QS master regulator. Yet, typically LasR-regulated phenotypes were not predictably lost. Moreover, *rhl* signalling was preserved in two thirds of the isolates. LES isolates displayed significantly less phenotypic variation compared to MES and non-epidemic isolates. No significant change in the phenotype composition of *P. aeruginosa* isolate populations was observed in relation to exacerbation periods.

These findings provide further evidence for 're-wiring' of the traditional QS regulatory hierarchy in CF isolates, with the *rhl* subsystem playing a more central role in virulence expression in certain strain types. Importantly, APEs could not be linked to the emergence of a particular sub-population of morphotypic or phenotypic variants.

Statement of contribution:

I carried out all clinical data collection, experiments, analyses and interpretation in this chapter, barring multi-locus sequence typing which was carried out by Dr Sam Kidman (PhD, Parkhill Lab), who also provided invaluable bioinformatic training and support.

4.1 INTRODUCTION

The opportunistic, Gram-negative bacterium *P. aeruginosa* (reviewed in *P. aeruginosa*, Section 1.6) is considered the archetypal pathogen of the CF airway. It is often associated with intermittent airway infection during childhood. However, recalcitrant endobronchial infection becomes established (in most cases) by late adolescence, despite early aggressive antibiotic eradication strategies (445–448). The association between recurrent pulmonary exacerbation and prevalent *P. aeruginosa* culture positivity on respiratory sampling has consolidated the bacteria's position as a leading cause of increased morbidity and mortality in the CF population (124). Yet still, the mechanisms by which resident *P. aeruginosa* trigger dynamic worsening in respiratory status among individuals with CF remains unclear.

Genotypic evolution of *P. aeruginosa* during chronic infection is accompanied by the acquisition and expression of certain pathoadaptive phenotypic traits that distinguish CF-evolved isolates from the initial infecting environmental strain (reviewed in *P. aeruginosa* adaptive phenotypes and virulence determinants in chronic CF infection, Section 1.7). Early genetic studies, albeit based on small numbers of sequential isolates, suggested that this adaptation favoured selection for phenotypic traits consistent with a down-regulation or alteration in virulence expression (176,179,449). However, despite apparent pathoadaptive trait conservation in chronic infection, several studies have since reported broad phenotypic diversity in both clonal isolates from within the same patient and between clonal populations from different CF patients (177,271,325,326,444,450,451). Moreover, the phenotypic variation described by these authors has challenged the traditional concept of a reduction in virulence expression over time, suggesting that this model may be an oversimplification (174,334,452). Of interest, studies focused on changes in single phenotypic traits have proposed a link between bacterial behaviour and exacerbation states (167,267,444,453). This raises the possibility that the transition to APE (on a background of chronic airway infection) may arise as a consequence of the emergence of sub-populations of phenotypic variants expressing traits conducive to inducing acute exacerbations.

To date, few studies have endeavoured to characterise the nature of short-term variations in phenotypic expression of clonal populations of *P. aeruginosa* within the CF lung before, during and after transient worsening in clinical state needing intervention with antibiotic treatment. Prior studies have been limited by either low sampling depths (174–181,184) or small patient numbers, profiling of few timepoints (177,178,180–182) independent of changes in clinical state, assessment of limited phenotypes (121) or focus on single transmissible strains (121,271,325,326). This has hindered generalisability and lead to underestimation of population diversity and a failure to demonstrate correlations between phenotypic behaviour and APE.

In this work I propose to describe, in detail, the dynamic short-term changes in *in vitro* phenotypic diversity of both epidemic and non-epidemic clonal populations of *P. aeruginosa* through acute periods of clinical instability in nine adults with CF. I also aim to determine whether these changes in bacterial behaviour are reliable markers for the onset of APEs.

4.2 MATERIALS AND METHODS

4.2.1 Source of reagents

All reagents used in this study were of analytical grade and were sourced from Sigma-Aldrich, UK, unless otherwise stated.

4.2.2 Bacterial strains, preparation and maintenance

4.2.2.1 Clinical strains

I used clinical data on antibiotic treatment administration to identify periods of APE for nine patients who took part in the TeleCF study, previously described in Chapter 3. These nine patients were chronically infected with *P. aeruginosa* and had provided daily sputum samples over the six-month duration of the home monitoring study. These samples had been collected and stored as described in Sputum collection, processing and storage, Section 2.4.

I selected sputum samples, in relation to time from initiation of antibiotics for an APE (Figure 4.1). Where available, sputum samples were selected in sets of three, linked to one antibiotic treatment episode. A minimum of two samples were selected in relation to each antibiotic treatment episode. Sputum samples were not available to

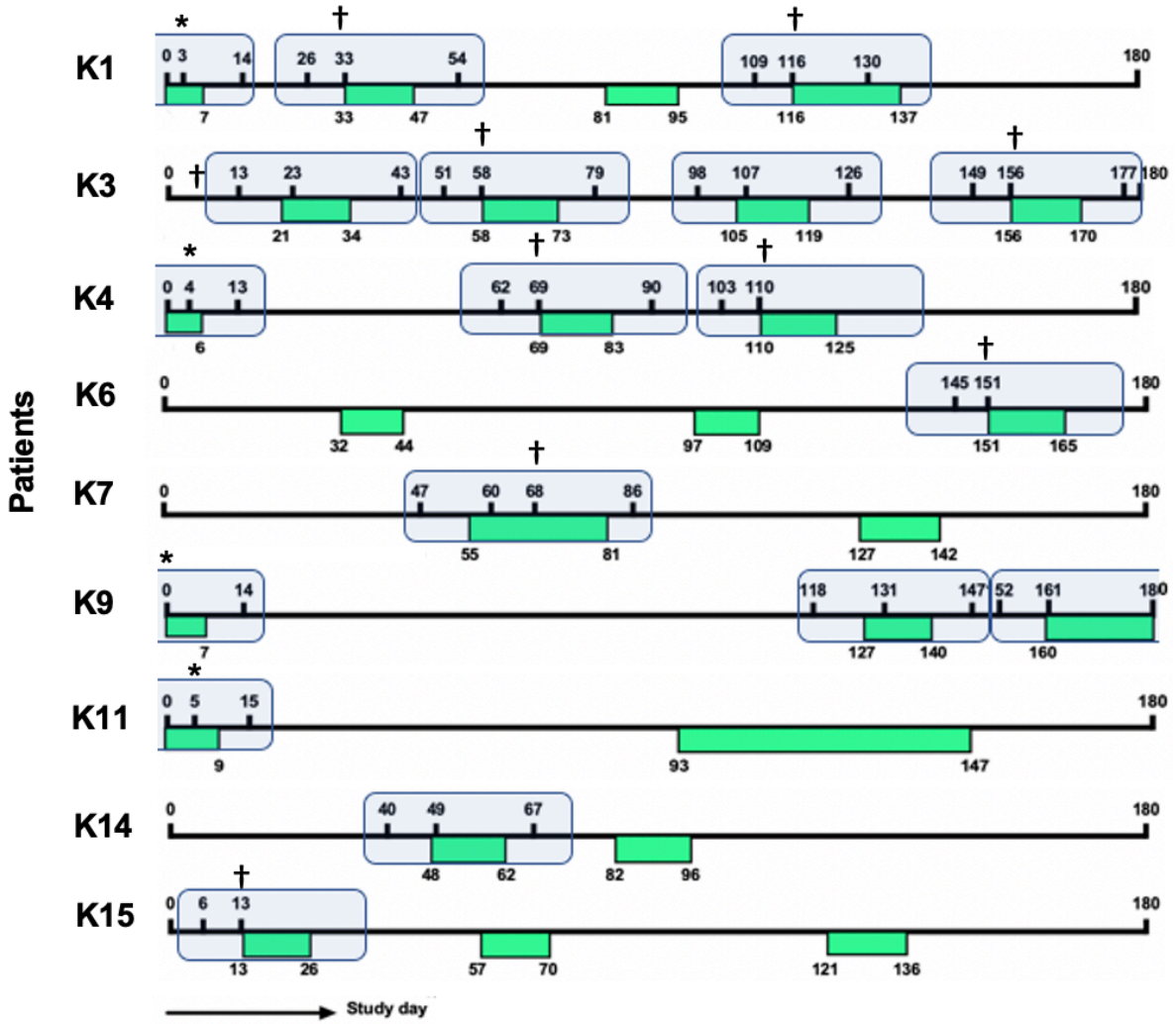


Figure 4.1. Timeline of sputa collection from 9 adult patients with CF. Samples were selected from the TeleCF sputum bank; a repository of frozen sputum samples collected daily over a 6-month period from adults with CF who took part in the TeleCF home monitoring study. Sputum samples were selected in sets of three (blue shaded boxes) in relation to one antibiotic treatment episode (in green) where possible: 1) a non-exacerbation sample (e.g., 7-14 days before initiation of antibiotic treatment for an APE), 2) an exacerbation sample (e.g., Day 0 of antibiotic initiation up to 5 days on treatment and 3) a recovery sample (e.g., 7-14 days following antibiotic therapy). Exceptions (*) were made to the criterion for the selection of exacerbation samples in four cases (see text). Sampling timepoints, in days from the start of the study, are noted on the upper axis whilst antibiotic stop and start timepoints are noted on the lower axis. Where possible, exacerbation samples were collected prior to initiation of antibiotic treatment (†), however, this was not the case for 9 out of 19 exacerbation samples.

complete a set of three for the following reasons: 1) a sample was not produced in the required time period, 2) samples had been analysed for the TeleCF study or 3) the sample yielded minimal colony growth after culture. The first sample of the set of three was taken to represent a period of non-exacerbation and was selected 7-14 days before initiation of antibiotic treatment for an APE. The second sample was selected, where possible, prior to starting antibiotic treatment (Day 0 up to 5 days on treatment) and was representative of a period of peak exacerbation (in terms of symptoms). I made exceptions to this criterion on four occasions: APE1 for patients K1, K4, K9 and K14, where, in each case, a course of antibiotics had commenced 9, 12, 7 and 10 days prior to sampling respectively. The third sample was selected 7-14 days after completion of antibiotic treatment and was taken to represent a period of recovery following antibiotic treatment. The sampling period for each set of samples related to one antibiotic treatment episode that was no longer than six weeks from first to last sample in the set.

I selected a total of 14 non-exacerbation, 19 exacerbation and 14 recovery samples from the TeleCF sputum bank. These samples were defrosted to room temperature, treated with equal volume 0.1% dithiothreitol, vortexed, incubated at room temperature for 15 mins then vortexed again to homogenise. Samples were handled under sterile conditions in a Class II Microbiological safety Cabinet.

I streaked processed sputum samples to purity and grew them on *Pseudomonas* selective agar base prepared plates with cetrimide and sodium nalidixate supplement (PCN agar; Oxoid). I incubated plates at 37°C for 48-72 h, to allow for the growth of slow-replicating and small colony variants.

Where possible, I harvested 95 single colonies from each sputum sample and selected colonies to proportionally represent all morphotypes present within each sputum sample. Two exacerbation samples yielded less than 95 colonies, one from patient K3 (n = 61) and one from patient K9 (n = 72). I arrayed the single colonies into a 96 deep well microplate containing 1 mL cetrimide broth and incubated the microplates statically at 37°C for a further 6 h to increase cell density. I recovered a total of 4408 pseudomonal strains from 47 sputum samples (Figure 4.2).

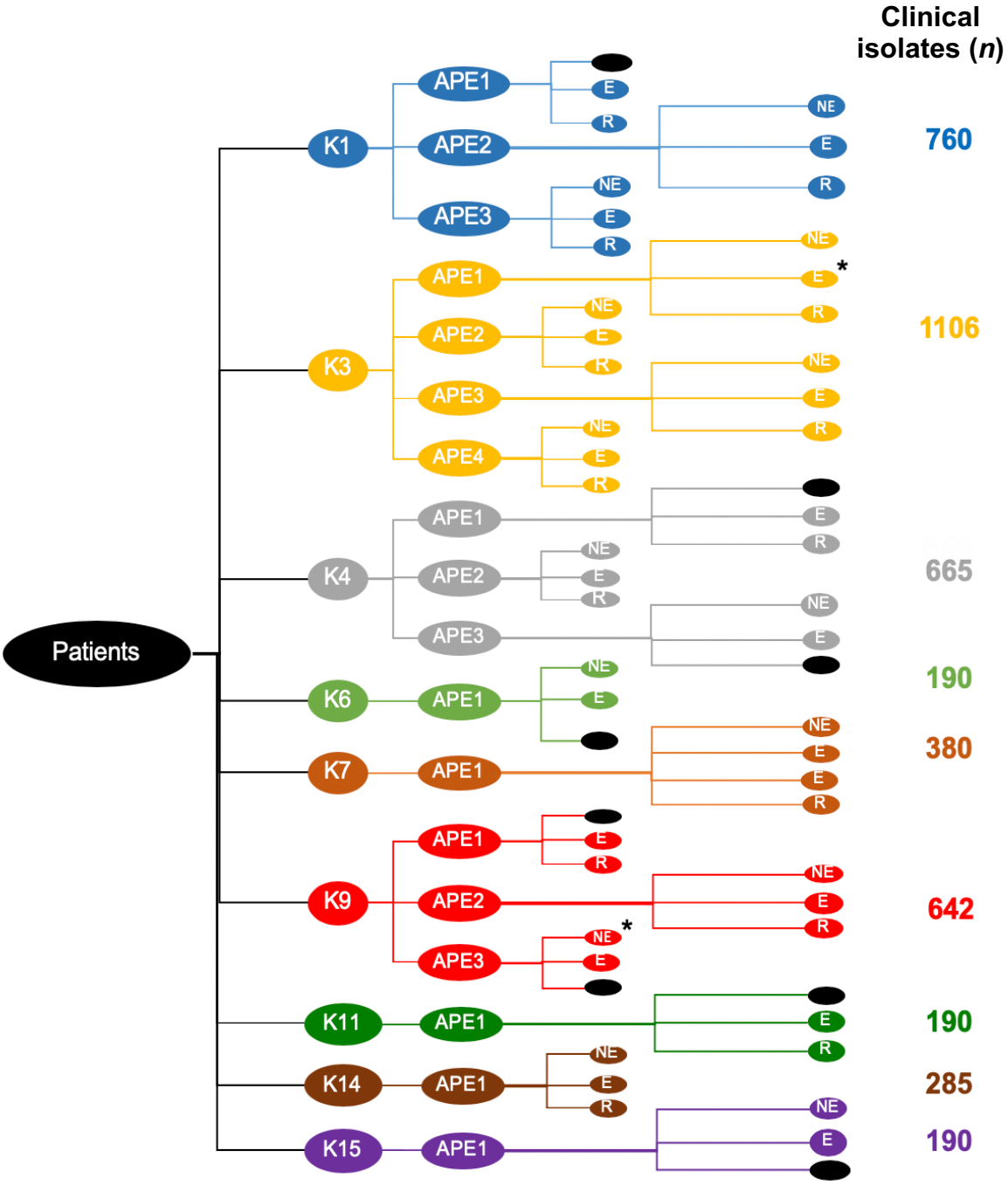


Figure 4.2. A breakdown of the number of APEs per patient, number of sputum samples (NE = non-exacerbation, E = exacerbation and R = recovery) per APE, and the number of clinical isolates per patient that were recovered for this study. Where possible, 95 isolates of *P. aeruginosa* were harvested from each sputum sample, with the exception of one exacerbation sample from patient K3 and from patient K9 (*), which yielded 61 and 72 isolates respectively. Missing samples, highlighted in black, were either due to inadequate sputum for processing or minimal colony growth. A total of 4408 pseudomonal clonal strains were collected from 47 sputum samples. Note, two exacerbation samples were taken from patient K7, but these samples were considered to be part of the same continued exacerbation as the first exacerbation sample was collected whilst the patient was taking oral antibiotics and the second was taken prior to transitioning to intravenous antibiotics.

To confirm that each isolate was *P. aeruginosa*, I re-streaked each of the 4408 harvested strains to purity and grew them again on PCN plates at 37°C for 48-72 h. I then picked single colonies and re-arrayed them into 96-deep-well microplates (one microplate per initial sputum sample) containing 1 mL cetrimide broth. Following static incubation at 37°C for 6 h, the strains were stored at -80°C in 25% (v/v) glycerol solution for long-term storage, to minimise potential for adaptation to the lab environment. Serial *in vitro* passage was limited to avoid inducing phenotype changes.

I grew overnight cultures statically at 37°C in 96-well microplates containing 100 μL/well of cetrimide broth, unless otherwise stated.

4.2.2.2 Laboratory strains and plasmids

Laboratory bacterial strains and plasmids used in this study are listed in Table 4.1.

Table 4.1. Laboratory bacterial strains and plasmids

Strain	Description	Reference
P. aeruginosa		
PAO1	Wild-type	Stover, *et al.,* 2000 (274)
$P_{pqsA::lux}$	Bioluminescent reporter for HHQ/PQS, *pqsA* promoter fused to *luxCDABE* and inserted into a neutral site in the chromosome of *pqsA* mutant	Fletcher, *et al.,* 2007 (454)
E. coli		
JM109 (pSB1142)	Bioluminescent reporter for OdDHL, contains *lasR* and promoter of *lasI::luxCDABE,* Tc^R	Wang, *et al.,* 2007 (506)
JM109 (pSB536)	Bioluminescent reporter for BHL, *ahyRI'::luxCDABE,* Cb^R	Swift, *et al.,* 1997 (455)

Unless otherwise stated, I grew *P. aeruginosa* and *E. coli* laboratory strains in Luria Broth (LB) at 37°C for 24 h.

I supplemented media with antibiotics when required: 10 μg/mL tetracycline for JM109 pSB1142 and 50 μg/mL carbenicillin for JM109 pSB536.

For short-term-storage (up to one week), I streaked cells from frozen glycerol stocks to form single colonies on 1.5% (w/v) agar, incubated overnight and kept at 4°C.

I grew overnight cultures in 10 mL screw-cap universal tubes or 50 mL falcon tubes inoculated from a single bacterial colony. I grew overnight cultures at 37°C for 12-16 h on a rotating drum or on a shaking platform at 200 rpm.

4.2.3 Media and solutions

Media and solutions used in this study are listed in Table 4.2. I carried out sterilisation of media, solutions and glassware by autoclaving at 121°C for 20 min prior to use, unless otherwise specified. I filter-sterilised the following growth factors using 0.22 μm syringe filters (Millipore): L-asparagine, L-cysteine, L-glutamic acid, L-tryptophan, L-tyrosine and diaminopimelic acid (DAPA). I prepared all media using deionised water, unless otherwise stated. Plates were poured and either used immediately or stored at 4°C for no more than a week prior to use, unless otherwise stated. Antibiotics were stored at -20°C. Growth factors were stored at 4°C.

4.2.4 Phenotypic screening assays

All phenotypic assays (described below) were performed by myself. I received short-term assistance from a laboratory assistant to facilitate mass production of certain stock solutions and media, and completion of the protease degradation, motility and biofilm formation assays. However, for each of the 4408 strains analysed, all phenotypes were assigned by myself. For each set of 95 isolates screened, the *P. aeruginosa* laboratory strain, PAO1, was used as a positive control for all assays, unless otherwise stated. I analysed each isolate for six phenotypic traits: colony morphology (8 features), metabolism (auxotrophy to 30 growth factors), exoproduct

Table 4.2. Composition of media and stock solutions

Media/Solution	**Components**	
Growth media		
Luria Broth (LB) (Formedium, Hunstanton, UK)	10 g/L Tryptone 5 g/L Yeast extract 5 g/L NaCl Dissolve 20 g in 1 L	
Cetrimide Broth	16 g/L Gelatin peptone 10 g/L Casein hydrolysate 10 g/L Potassium sulphate 1.4 g/L Magnesium chloride 0.2 g/L Cetrimide 10 mL/L Glycerol Dissolve 37.6 g of the dry components in 1 L	
Pseudomonas CN Selective Agar (Thermo Fisher Scientific Oxoid, ready-prepared plates, Basingstoke, UK)	16 g/L Gelatin peptone 10 g/L Casein hydrolysate 10 g/L Potassium sulphate 1.4 g/L Magnesium chloride 0.2 g/L Cetrimide 0.015 g/L Sodium nalidixate 11 g/L Agar 10 mL/L Glycerol	
Solid Agar	LB in 1.5% (w/v) Bacto agar (Becton Dickinson)	
Auxotroph pool assay		
Growth factor Stock Solutions	**10 mg/mL** L-Asparagine L-Cysteine (Melford) L-Glutamic Acid L-Tryptophan L-Tyrosine L-Histidine L-Glutamine	**5 mg/mL** Adenosine (0.1 M HCl) Guanosine (0.3 M HCl) Thymine Uracil

Table 4.2. (cont.) Composition of media and stock solutions

Auxotroph pool assay (cont.)		
Growth factor Stock Solutions (cont.)	**10 mg/mL** L-Leucine L-Serine L-Isoleucine L-Methionine (Duchefa) L-Lysine L-Threonine L-Aspartic Acid L-Valine L-Alanine L-Arginine L-Proline L-Phenylalanine (0.01 M HCl) Glycine	**300 μg/mL** DAPA **50 μg/mL** Pyridoxine Pantothenate Nicotinic Acid **1 μg/mL** Thiamine Biotin
5x M9 salts solution stock	56.4 g/L 5x M9 salts (Scientific Laboratory Supplies, Nottingham, UK)	
M9 Minimal Agar	200 mL/L 5x M9 Salts 20 mL/L 20% (w/v) glucose 2 ml/L 1 M $MgSO_4$ (Fisher Scientific, Loughborough, UK) 0.1 mL/L 1 M $CaCl_2$ 1.5% (w/v) Bacto agar	
Pool Plate Stock solutions (10 mL)	Mix together equal volumes of each growth factor stock solution:	
	Pool 1: Adenosine, Histidine, Phenylalanine, Glutamine, Thymine, Pyridoxine	
	Pool 2: Guanosine, Leucine, Tyrosine, Asparagine, Serine, Nicotinic Acid	
	Pool 3: Cysteine, Isoleucine, Tryptophan, Uracil, Glutamic Acid, Biotin	
	Pool 4: Methionine, Lysine, Threonine, Aspartic Acid, DAPA	

Table 4.2. (cont.) Composition of media and stock solutions

Pool Plate Stock solutions (10 m) (cont.)	Pool 4 (cont.): Pantothenate
	Pool 5: Thiamine, Valine, Proline, Arginine, Glycine, Alanine
	Pool 6: Adenosine, Guanosine, Cysteine, Methionine, Thiamine
	Pool 7: Histidine, Leucine, Isoleucine, Lysine, Valine
	Pool 8: Phenylalanine, Tyrosine, Tryptophan, Threonine, Proline
	Pool 9: Glutamine, Asparagine, Uracil, Aspartic Acid, Arginine
	Pool 10: Thymine, Serine, Glutamic Acid, DAPA, Glycine
	Pool 11: Pyridoxine, Nicotinic Acid, Biotin, Pantothenate, Alanine
Protease degradation assays	
Skim Milk Agar	50 g/L Tryptic Soy agar 2% (w/v) Skim Milk (Marvel)
Gelatin Agar	30 g/L gelatin 13 g/L nutrient broth (Melford) 1.6% (w/v) Bacto agar
Motility assays	
Swim Agar	20 g/L LB 0.3% (w/v) Bacto agar
Twitch Agar	20 g/L LB 1.5% (w/v) Bacto agar
Siderophore (Schwyn-Neilands) assay	
Chrome azurol S (CAS) stock solution	1.21 g/L Chrome azurol S (Fluka) in water
Fe stock solution	1 mM $FeCl_3$ 10 mM HCl
Hexadecyltrimethylammonium bromide (HDTMA) stock solution	1.825 g/L HDTMA

Table 4.2. (cont.) Composition of media and stock solutions

Siderophore (Schwyn-Neilands) assay (cont.)	
M9 salts stock solution (pH 7.0)	30 g/L KH_2PO_4, 50 g/L NaCl 100 g/L NH_4Cl
10% Casamino acids solution (CAS)(100 mL)	10 g casamino acids 3% (w/w) 8-hydroxyquinoline in equal volume chloroform solution
Piperazine-N, N'-bis (2-ethansulfonic acid) (PIPES) buffer (pH 6.8)	0.1 M PIPES (Melford)
CAS-HDTMA (Blue Dye) stock solution	500 mL/L CAS stock solution 100 mL/L Fe stock solution 400 mL/L HDTMA stock solution Stored in plastic container (foil wrapped)
CAS agar (1 L)	750 mL/L PIPES buffer 1.5% (w/v) Bacto agar 100 mL/L M9 salts 30 mL/L 10% casamino acids 10 mL/L 20% (w/v) glucose 1 mL/L 1M $MgCl_2$ 1 mL/L 100mM $CaCl_2$ 100 mL/L Blue Dye solution
Rhamnolipid (Siegmund-Wagner) assay	
Proteose-peptone-glucose-ammonium salts (PPGAS) medium (pH 7.2)	0.698 g/L NH_4Cl 1.491 g/L KCl 14.537 g/L Trizma Base 0.394 g/L $MgSO_4$ 1% (w/v) Proteose peptone (Difco) 0.5% (w/v) glucose 1.5% (w/v) Bacto agar (Formedium)
Cetyl trimethylammonium bromide (CTAB)	200 μg/mL CTAB 5 μg/mL Methylene Blue
Biofilm formation assay	
$MgSO_4$ stock solution (100ml)	1 M $MgSO_4 \bullet 7H_2O$
$FeSO_4$ stock solution (100ml)	2.5 g/L $FeSO_4$

Table 4.2. (cont.) Composition of media and stock solutions

Biofilm formation assay (cont.)	
CAS stock solution (100ml)	20% (w/v) Casamino acids
5x M63 medium	68 g/L KH_2PO_4 10 g/L $(NH_4)_2SO_4$ 2.5 mg/L $FeSO_4 \bullet 7H_2O$
M63 minimal medium	200 mL 5x M63 medium 10 mL 20% (w/v) glucose 5 mL 20% (w/v) Casamino acids 1 mL 1 M $MgSO_4 \bullet 7H_2O$

production (gelatinase, caseinase, siderophore and rhamnolipid), motility (swimming, twitching), biofilm formation and QS autoinducer (OdDHL, BHL and PQS) production. Due to the large number of isolates screened, phenotypic assays were chosen for reproducibility and ease of adaptation to a high through-put manner.

4.2.4.1 Morphotype screening

To determine colony morphotype cells I streaked cells from frozen glycerol stocks of the clinical strains to form single colonies on *Pseudomonas* selective agar base with cetrimide and sodium nalidixate supplement (PCN agar; Oxoid) and incubated at 37°C for 48-72 h. As previously described (with some modifications) (456), I assigned colony morphotype by visual inspection and description of eight identifiers: Pigmentation, mucoidy, colony lucency, surface texture, size, margin regularity, autolysis and surface sheen (Table 4.3). I defined each morphotype was as a unique combination of these identifiers with size being used to distinguish small colony variants.

4.2.4.2 Microplate QS bioassay

I examined production of the QS signal molecules OdDHL, BHL and PQS, as previously described, with some modifications (454,455). I quantified the AHLs in the cell supernatant using the two *lux* reporter strains; JM109 (pSB114) for OdDHL and JM109 (pSB536) for BHL. I quantified PQS using the *lux* reporter strain; $P_{pqsA::lux}$.

Table 4.3. Features used to classify colony morphology of the clinical isolates

Class	Sub-class
Pigmentation	White
	Green
	Yellow
	Orange
	Tan
Mucoidy	Mucoid
	Non-mucoid
Colony lucency	Opaque
	Translucent
Surface texture	Smooth
	Rough
Size*	Small
	Large
Margin regularity	Smooth
	Irregular
Autolysis	Halo
	No halo
Surface sheen	Iridescence
	No iridescence

*Colonies were considered small if colonial diameter was ≤ 3mm after growth on *Pseudomonas* selective agar base with cetrimide and sodium nalidixate supplement (PCN agar; Oxoid) and incubated at 37°C for 48-72 h.

In summary, I grew each clinical strain in 800 μL of buffered LB at 37°C for 24 h. I collected crude supernatants from planktonic stationary cultures by centrifugation at 4000 rpm for 15 min at room temperature. I co-incubated aliquots of 100 μL from the supernatants statically at 37°C for 3-5 h with 100 μL of overnight culture of JM109 pSB536, JM109 pSB1142 or $P_{pqsA::lux}$ reporter strains in Grenier Cellstar® black, 96 well, polystyrene, flat micro-clear bottom microplates. I measured the bioluminescence of individual wells as relative light units with the Fluostar Omega (BMG Labtech) microplate reader. I quantified AHL and AQ levels by comparison with dilutions of synthetic standards. The experiments for each QS signalling molecule were performed in duplicate.

4.2.4.3 Protease degradation assays

I detected caseinase activity (dependent mainly on LasB protease, alkaline protease and proteinase IV) was by spotting aliquots (5 μL) of overnight culture onto skim milk plates (457). I incubated plates at 37°C for 48 h before identifying protease activity by the formation of clearing zones around the colony growth due to casein hydrolysis. I scored protease activity qualitatively as either absent, low (halo diameter ≤ 1 cm) or high (halo diameter ≥ 1 cm).

I measured gelatinase activity (dependent mainly on LasA protease) in a similar manner, except that I spotted aliquots (3 μL) of overnight cultures on to gelatin agar plates and incubated the plates for 24 h (374). I identified protease activity by visualising proteolytic clearing zones, formed by gelatin hydrolysis, after flooding the plates with saturated ammonium sulphate solution. I scored protease activity qualitatively as either absent, low (halo diameter ≤ 1 cm) or high (halo diameter ≥ 1 cm).

4.2.4.4 Modified Schwyn-Neilands assay for siderophore activity

I achieved siderophore detection using chrome azurol S (CAS) indicator plates as previously described, but with a few modifications (458,459). Briefly, I spotted 5 μL aliquots of overnight strain culture onto CAS plates and incubated at 37°C for 48 h. I assessed siderophore production by visualisation of an orange/burgundy-coloured halo formation around the colony growth, due to the removal of Fe^{3+} from the chrome azurol S/iron(III)/hexadecyltrimethylammonium bromide complex by the chelating action of the siderophores secreted by the iron-starved bacterial colony. I classified production as absent, low (halo diameter ≤ 1 cm) or high (halo diameter ≥ 1 cm).

4.2.4.5 Modified Siegmund-Wagner assay for rhamnolipid activity

I detected extracellular rhamnolipid production using methylene blue agar plates containing the surfactant cetyltrimethyl ammonium bromide (CTAB), prepared by modifying a previously described protocol (460–462). I supplemented proteose peptone-glucose-ammonium salts (PPGAS) medium with 0.02% CTAB and 0.0005% methylene blue and solidified with 1.5% Bacto agar. In the presence of CTAB, the cationic dye methylene blue formed an insoluble complex with the anionic rhamnolipid

molecules causing the formation of a dark blue precipitate around the colony growth. I spotted aliquots of 5 μL from the overnight strain culture onto the rhamnolipid plates and incubated at 37°C for 48 h. Plates were then kept at room temperature for at least 24 h until visualisation of the blue halos, indicating biosurfactant production. I classified rhamnolipid production as absent, low (halo diameter ≤ 1 cm) or high (halo diameter ≥ 1 cm).

4.2.4.6 Motility assays

I assessed swimming (flagellar-based) and twitching (pili-based) motilities as described previously but with modifications (463). I poured, cooled and inoculated swim plates on the same day. I inoculated plates with 5 μL of overnight strain culture using a plastic disposable pipette tip that gently pierced the surface of the agar. I incubated plates for 8-12 h at 37°C. I assessed swim motility qualitatively by examining for the circular haze of growth/turbid zone formed by bacterial cell migration away from the point of inoculation. I scored turbid zones as absent, small (halo diameter ≤ 1 cm) or large (halo diameter ≥ 1 cm).

I poured twitch plates to a depth of 3-5 mm, cooled and inoculated them on the same day. I stab-inoculated plates with cells from overnight strain culture, using sterile 10 μL pipette tips, through the thin LB agar to the bottom of the assay plate. After incubation at 37°C for 48-72 h I visualised a hazy zone of growth at the interface between the agar and the polystyrene surface. I examined the ability of the bacteria to strongly adhere and form a biofilm on the polystyrene surface, consistent with interstitial colony expansion, by staining plates with 0.1% (w/v) crystal violet after removal of the agar. I scored twitch motility zones qualitatively as being either absent or present.

4.2.4.7 Microplate biofilm formation assay

I assayed biofilm formation using a method described previously, with some modifications (464). In summary, I diluted stationary-phase cultures 1:100 in fresh M63 minimal medium supplemented with 20% (w/v) glucose and 20% (w/v) casamino acids and seeded them into a 96 well polystyrene microplate, with each well containing a single isolate. I incubated microplates in static culture for 24 h at 37°C. Culture

supernatant and nonadherent cells were then removed and wells washed twice in sterile deionised water to remove residual planktonic bacteria. I stained the remaining adherent cells with 0.1% (w/v) crystal violet for 10 min at room temperature. I washed the wells a further two times and air-dried overnight. I then added one hundred and twenty-five microlitres of 30% (w/v) acetic acid to each well and incubated for 10–15 min to solubilise the biofilm. I quantified crystal violet-stained biomass by measuring the absorbance at 595 nm using an EZ Read 400 microplate reader. I performed all experiments at least four times on independent biological replicates.

4.2.4.8 Auxotroph pool assay

I achieved auxotroph detection using an auxotroph screening pool assay adapted from a method previously described (465). In summary, small volume inocula of overnight culture were replica transferred onto 11 pool plates using a 48-pin microplate replicator, with flame sterilisation between each transfer. The enriched minimal agar assay plates constituted of M9 minimal salts agar supplemented with 20% (w/v) glucose and a pooled combination of growth factors at physiologically relevant concentrations. I observed for growth after incubation at 37°C for 48 - 72h.

I identified auxotrophic variants by growth only on 2 of the 11 pool plates, with both plates sharing a common growth factor requirement (Table 4.4). In addition, I identified specific auxotrophs as follows: 1) purine mutants – growth on pools 1, 2 and 6 (requiring adenosine or guanosine) or growth only on pool 6 (requiring adenosine and thiamine), 2) pyrimidine mutants – growth only on pool 9, 3) aromatic amino acid early biosynthetic pathway mutants – growth only on pool 8, 4) isoleucine and valine mutants – growth only on pool 7 and 5) pleiotropic mutants – some mutations may cause pleiotropic effects resulting in failure to grow on any pool plate.

Due to the high volume of isolates screened I took a pragmatic approach to omit further testing of 1) isolates that grew on only one pool plate of plates 1-11 and 2) isolates that grew on more than one, but not all of the pool plates. These isolates were classed as 1) ‘single pool growth’ (*n* = 112, 3% of all isolates), and 2) ‘Multi-pool growth’ (*n* = 833, 19% of all isolates) respectively.

Table 4.4. Components of enriched minimal Pool Plates.

Pool Plate	1	2	3	4	5
6	Adenosine	Guanosine	Cysteine	Methionine	Thiamine
7	Histidine	Leucine	Isoleucine	Lysine	Valine
8	Phenylalanine	Tyrosine	Tryptophan	Threonine	Proline
9	Glutamine	Asparagine	Uracil	Aspartic Acid	Arginine
10	Thymine	Serine	Glutamic Acid	DAPA	Glycine
11	Pyridoxine	Nicotinic Acid	Biotin	Pantothenate	Alanine

The components of pools 1 to 5 are listed vertically; the components of pools 6 to 10 are listed horizontally. Interpretation of growth on a particular pool plate: If a strain grows on two pool plates, the auxotrophic requirement is for the component common to both pools. If a strain grows on one pool plate of plates 1 – 10, the strain must require more than one component from that pool. If a strain grows only on pool 11, each component of pool 11, must be tested individually for growth to identify the auxotrophy.

4.2.5 Computational quantitation of phenotypes

To enrich the scoring of semi-quantitative phenotypic assays further, I performed manual quantification was performed on captured plate images for caseinase, gelatinase, swim, twitch and siderophore activity using the software package ImageJ (LOCI, University of Wisconsin, USA). I captured all images under similar lighting conditions.

4.2.6 Multi-locus sequence typing of clinical isolates

Multi-locus sequence type (MLST) profiles for all the clinical isolates were inferred from whole genome sequence data assembled for each of the strains as part of a separate genomics study undertaken by Dr Sam Kidman (PhD, Parkhill Lab).

In brief, for each isolate, seven conserved housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA trpE*) were screened for an allele type and sequence type assignments completed. Each isolate has an allelic profile, determined by genetic polymorphisms in sequences at the loci of the seven genes. Isolates with the same allelic profile (7 of 7 alleles) comprise a specific sequence type (ST) (113).

4.2.7 Computational and statistical analysis

I performed statistical analyses using Microsoft Excel for Mac version 16.27 (Microsoft, Redmond, WA, USA) and Prism 6.0 for Mac OS X (Graphpad Software Inc, San Diego, CA, USA). I treated phenotypic traits as continuous variables for all traits except for auxotrophy, mucoidy, SCV morphotype and rhamnolipids.

Descriptive statistics of colonial morphotypes are expressed as mean (standard deviation [SD]) or median (interquartile range (IQR), minimum and maximum) for numerical variables and as number (percentage) for categorical variables. No attempt was made to substitute missing data.

I examined variations in the frequency of strains detected and phenotypic traits by Chi-squared (χ^2) tests or by Fisher's exact test where the predicted number of subjects in each group was less than five.

I determined correlation relationships between functional phenotypes by calculating Spearman's Rho for continuous variables and visualised using the tidyverse, Hmisc, corrplot, reshape2, ggplot2, survival, lattice and Formula packages in RStudio Version 1.1.456 (RStudio Inc, Boston, MA, USA).

I performed principal components analysis (PCA) on quantified phenotype traits using the FactoMineR package in Rstudio Version 1.1456 (RStudio Inc, Boston, MA, USA). A total of nine phenotypes were included: protease activity (caseinase and gelatinase), motility, (swimming and twitching), siderophore production, QS (OdDHL, BHL and PQS) and biofilm formation. Isolates with missing data (368/4408 isolates) were excluded from analysis.

All statistical tests were two-tailed and a p value of ≤ 0.05 was considered statistically significant.

4.3 RESULTS

To characterise the temporal variation in phenotypic composition of clonal isolate populations of *P. aeruginosa,* in relation to clinical state, I performed both qualitative and quantitative phenotypic screening studies on 4408 clinical isolates, recovered from 47 spontaneously expectorated sputum samples provided by 9 adults with CF. These individuals, who were chronically infected with *P. aeruginosa*, were participants in TeleCF, a 6-month home monitoring study previously described in Chapter 3. The demographic and clinical characteristics of these 9 patients (K1, K3, K4, K6, K7, K9, K11, K14 and K15) are previously detailed in Table 3.2.

In total, 1330 isolates were recovered from 14 non-exacerbation samples, 1748 isolates from 19 exacerbation samples and a further 1330 isolates from 14 recovery samples. At the time of sputum sample selection, results from the ML analysis (described in Chapter 5) were not available to enable objective determination of the onset of an APE (e.g., independent of a clinical decision to treat with antibiotics for a deterioration in respiratory status). Therefore, I pragmatically selected sputum samples (as described in Clinical strains, Section 4.2.2.1) in relation to antibiotic treatment courses to enable comparison of isolate phenotypes between periods of non-exacerbation, acute exacerbation and recovery following antibiotic treatment. Previous authors have adopted a similar methodology (183,271,325,326). Initially, the sampling timepoints chosen in this study appeared to be independently supported by daily home monitoring data. This data revealed, in general, a trend toward lower cough scores (reflective of increased cough) coincident with exacerbation samples and a comparative improvement in cough scores following antibiotic treatment (Figure 4.3). However, on further analysis, I did not identify a statistically significant difference in cough scores between the sampling timepoints (Kruskal-Wallis test, $p = 0.0743$).

MLST allelic profiles and ST assignments of the clinical isolates revealed that each patient was infected with a single *P. aeruginosa* strain type, although some patients shared the same strain type (Table 4.5).

Two epidemic strains were identified in this cohort. Patients K1 and K14 were both infected with ST217, otherwise known as the MES (108).

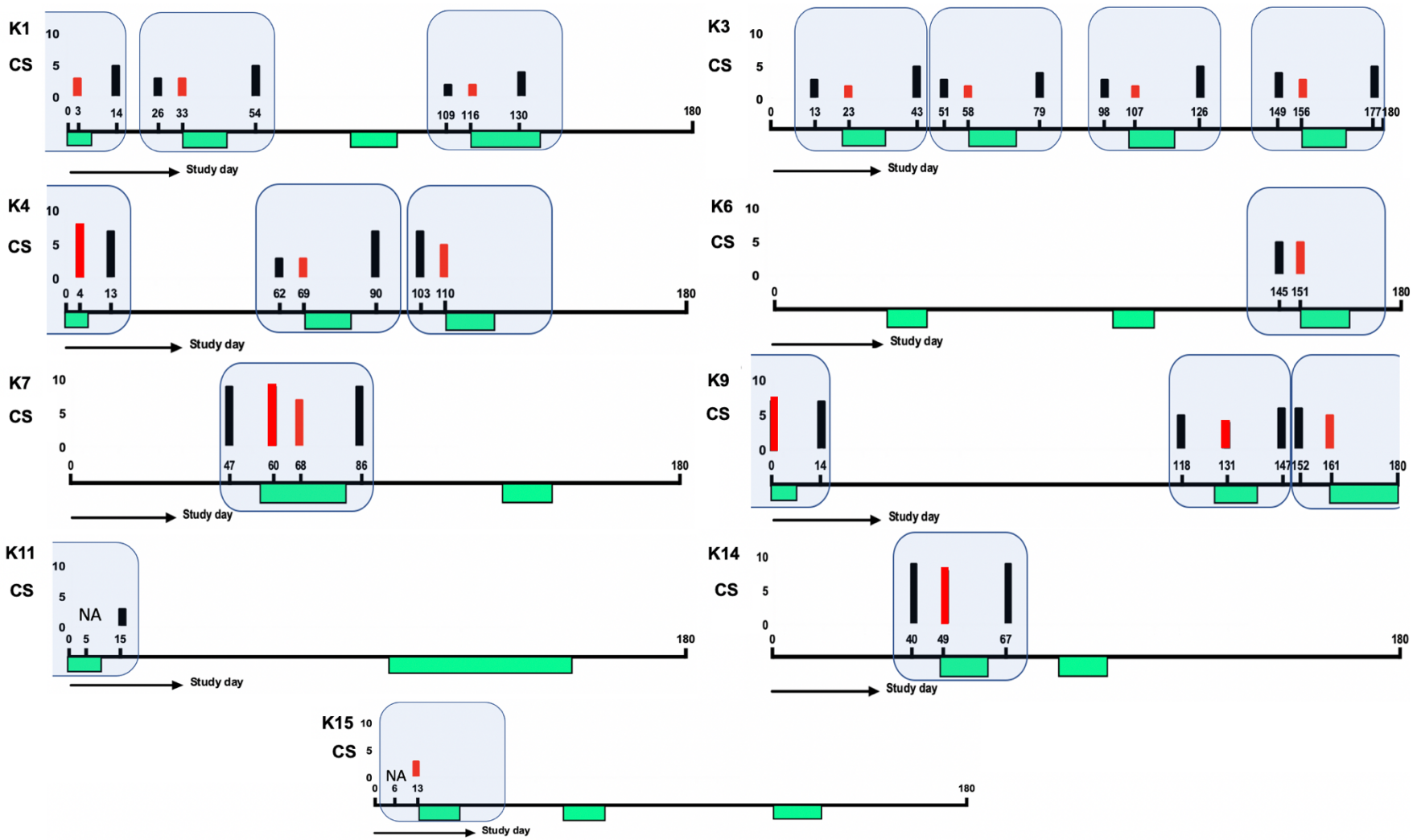


Figure 4.3. Sputum samples and corresponding cough scores (CS) on the day of sample selection. A 10-point scale was used to assess cough severity, rated from worst ever (1/10) to best ever (10/10). Sputum samples were selected in sets of three (blue shaded boxes) in relation to one antibiotic treatment episode (in green), where possible. Exacerbation samples are highlighted in red. Non-exacerbation (pre-treatment) and recovery (post-treatment) samples are represented by the black bars. Although in general, lower cough scores were associated with exacerbation samples, reflective of worse cough, the difference in cough score between the sampling timepoints was not statistically significant (Kruskal-Wallis test, $p = 0.0743$).

Table 4.5. MSLT analysis of *P. aeruginosa* clinical isolates

Patient	Allele Type							Sequence Type
	acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
K1, K14	28	5	11	18	4	13	3	ST217†
K3, K6	23	143	190	11	3	15	7	ST3307*
K4	36	5	12	3	4	4	52	ST3308*
K7, K9, K11, K15	6	5	11	3	4	23	1	ST146‡

(*) denotes a novel strain type identified in this study. (†) and (‡) denote MES and LES respectively. Data provided by Dr Sam Kidman (PhD, Parkhill Lab).

Patients K7, K9, K11 and K15 were all infected with ST146, more commonly known as the LES (307).

Patients K3 and K6 both share a genetically identical novel strain. However, this strain has not been demonstrated to be readily and frequently transmitted from one chronically infected individual to another nor reached a minimum threshold prevalence to be considered a potential epidemic strain.

MLST also confirmed contamination with the laboratory strain PAO1 of one exacerbation sample (linked to APE1) from patient K1. The results from these isolates (*n* = 55) were subsequently excluded from this study and phenotypic characterisation of the remaining 4353 clinical isolates will now be described.

4.3.1 Morphotypic diversity

I identified thirty-one unique colony morphotypes in this study following growth of isolates on *Pseudomonas* selective agar base with cetrimide and sodium nalidixate supplement (PCN agar; Oxoid) (Figure 4.4). I observed wide variation in morphotypic diversity between clonal *P. aeruginosa* populations from different patients (range 2 - 15 morphotypes per patient) (Figure 4.5). Similarly, variation was also noted in morphotypic diversity between coeval isolates, with a median number of colony

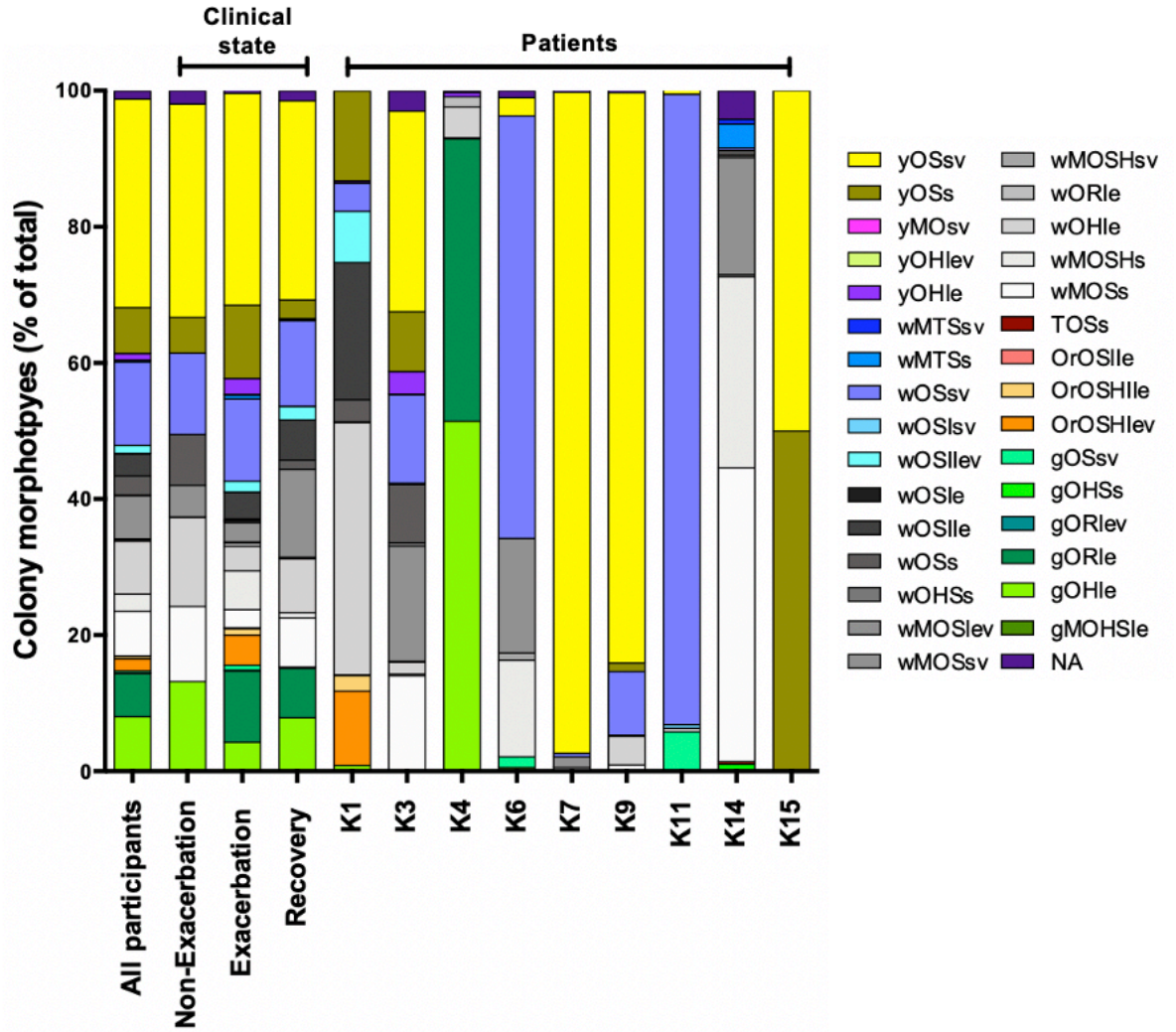
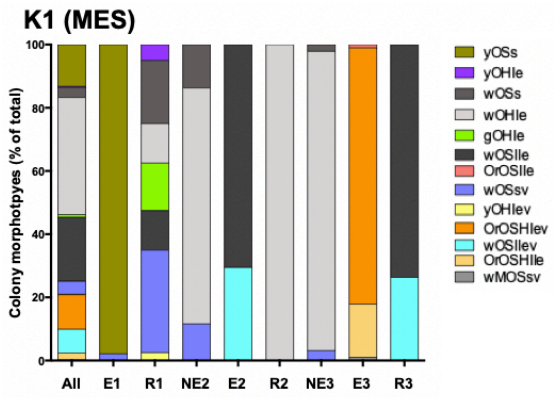
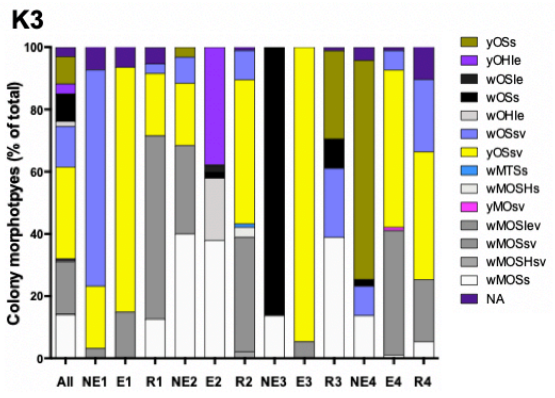


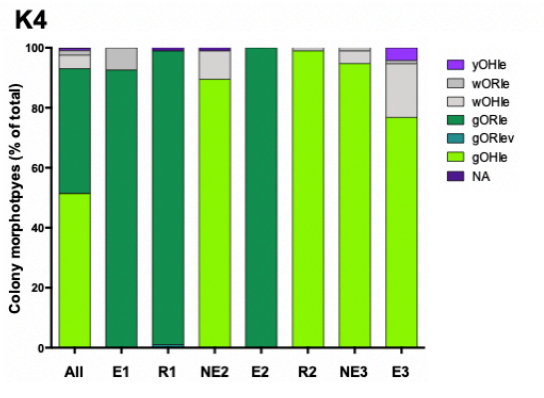
Figure 4.4. Relative abundance of colony morphotypes, and the variation observed between different clinical states (e.g., during periods of non-exacerbation, exacerbation and recovery following antibiotic treatment for an APE) and between different patients. Key: colour-coded unique colony morphotypes (extended definitions of morphotype assignments are found in Table 4.6).

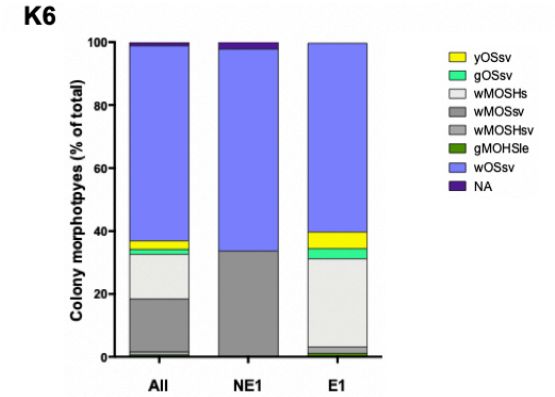
morphotypes identified within each sputum sample of three (range 1 – 9 morphotypes per sample).

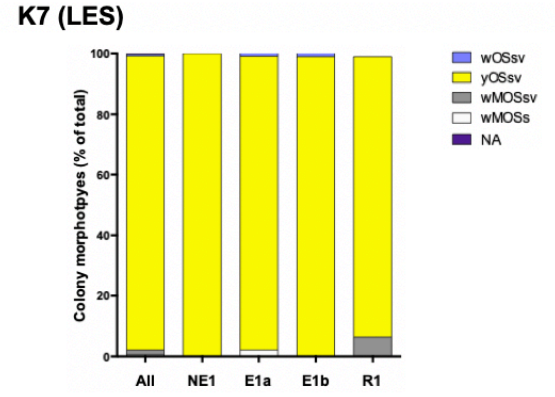
Next, to determine if morphotype abundance differed between patients harbouring unique strains and those infected with an epidemic clone I compared the median number of colony morphotypes between the two groups. I did not identify a significant difference between the two groups (Mann-Whitney test, p = 0.3095). However, a comparison of the median number of morphotypes per sputum sample between

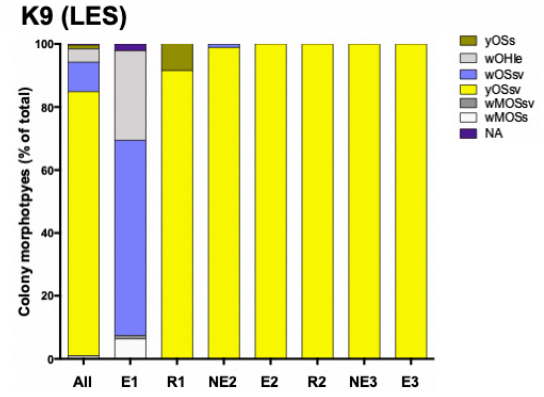

K1 (MES)
Colony morphotpyes (% of total)
All E1 R1 NE2 E2 R2 NE3 E3 R3
yOSs
yOHIe
wOSs
wOHIe
gOHIe
wOSIIe
OrOSIIe
wOSsv
yOHIev
OrOSHIev
wOSIIev
OrOSHIIe
wMOSsv

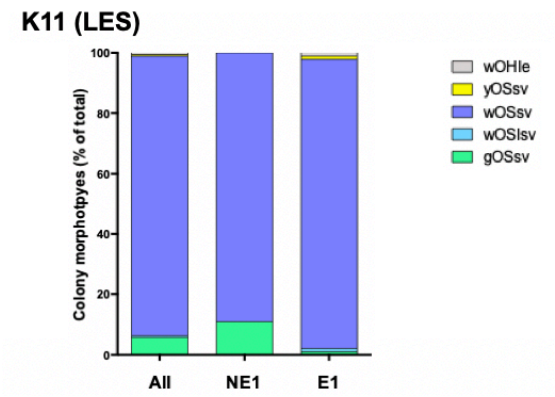

K3
Colony morphotpyes (% of total)
All NE1 E1 R1 NE2 E2 R2 NE3 E3 R3 NE4 E4 R4
yOSs
yOHIe
wOSIe
wOSs
wOHIe
wOSsv
yOSsv
wMTSs
wMOSHs
yMOsv
wMOSIev
wMOSsv
wMOSHsv
wMOSs
NA

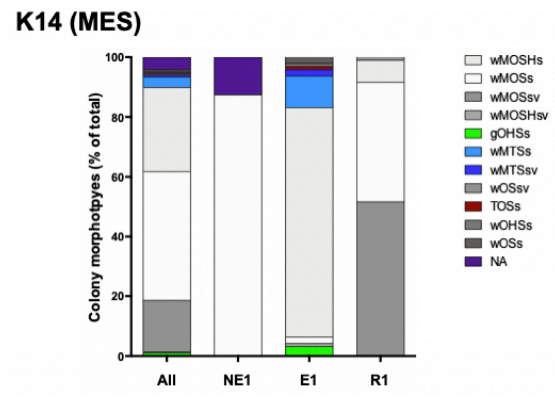

K4
Colony morphotpyes (% of total)
All E1 R1 NE2 E2 R2 NE3 E3
yOHIe
wORIe
wOHIe
gORIe
gORIev
gOHIe
NA


K6
Colony morphotpyes (% of total)
All NE1 E1
yOSsv
gOSsv
wMOSHs
wMOSsv
wMOSHsv
gMOHSIe
wOSsv
NA


K7 (LES)
Colony morphotpyes (% of total)
All NE1 E1a E1b R1
wOSsv
yOSsv
wMOSsv
wMOSs
NA


K9 (LES)
Colony morphotpyes (% of total)
All E1 R1 NE2 E2 R2 NE3 E3
yOSs
wOHIe
wOSsv
yOSsv
wMOSsv
wMOSs
NA


K11 (LES)
Colony morphotpyes (% of total)
All NE1 E1
wOHIe
yOSsv
wOSsv
wOSIsv
gOSsv


K14 (MES)
Colony morphotpyes (% of total)
All NE1 E1 R1
wMOSHs
wMOSs
wMOSsv
wMOSHsv
gOHSs
wMTSs
wMTSsv
wOSsv
TOSs
wOHSs
wOSs
NA

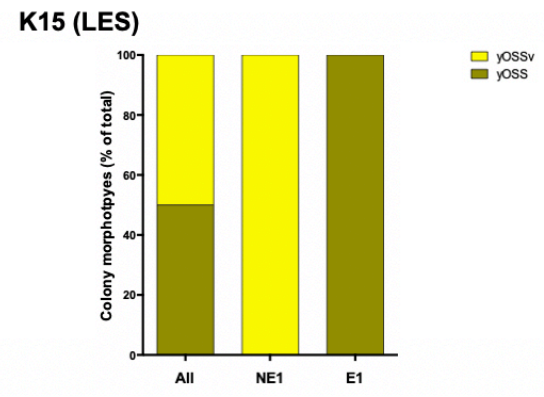

K15 (LES)
Colony morphotpyes (% of total)
All NE1 E1
yOSSv
yOSS

Figure 4.5. Within-patient and within-sample variation in morphotypic diversity of *P. aeruginosa* clonal strains collected from nine patients with CF. Sputum samples were collected at times of non-exacerbation (NE), exacerbation (E), and recovery (R) following antibiotic treatment for an APE. Numbers on the X-axis denote samples associated with the same antibiotic treatment event (e.g., NE1, E1 and R1 = the non-exacerbation, exacerbation and recovery samples associated with the first antibiotic treatment episode for an APE). LES: Liverpool epidemic strain, MES: Manchester epidemic strain. Key: colour-coded unique colony morphotypes (extended definitions of morphotype assignment are found in Table 4.6).

patients infected with a unique strain with those infected with the MES or the LES identified a significant difference in morphotype frequency per sample between the cohorts. There was a lower frequency of colony morphotypes per sample observed in patients harbouring the LES strain compared with those infected with a unique or the MES (Kruskal-Wallis test, $p = 0.0151$), as visualised in Figure 4.5.

In addition, I did not identify a significant difference in the morphotype abundance during periods of exacerbation compared to periods of non-exacerbation or recovery following antibiotic treatment for an APE (Kruskal-Wallis test, $p = 0.0607$). Importantly, no specific colony morphotype were found to be associated with periods of APE.

As was to be expected, I observed the manifestation of adaptive morphotypes typically associated with chronic pseudomonal CF airway infection (e.g., SCVs, mucoid phenotype), although the abundance varied widely between patients. 'Dwarf colony', or small colony variants, typically display colonial diameters ranging between 1-3 mm (after 48 h incubation on *Pseudomonas* selective agar) and have been described to confer a selective advantage *in vivo* (335). Their presence is associated with poorer clinical outcomes, particularly increased antimicrobial resistance (323,466). Just over one third (35%) of all isolates exhibited the SCV phenotype, however, the prevalence was significantly skewed towards patients infected with the LES (Figure 4.6) (χ^2 test, p <0.0001). I did not find a significant difference in the prevalence of SCV morphotype between non-exacerbation, exacerbation and recovery isolates (χ^2 test, p value 0.6794).

Table 4.6. Extended definitions of colony morphotype assignments for *P. aeruginosa* clinical isolates.

morphotype assignment	pigmentation	mucoidy	lucency	surface texture	size	margin regularity	autolysis	surface sheen
gMOHSIe	green	mucoid	opaque	smooth	non-SCV	irregular	halo	no sheen
gOHIe	green	non-mucoid	opaque	rough	non-SCV	irregular	halo	no sheen
gORIev	green	non-mucoid	opaque	rough	SCV	irregular	no halo	no sheen
gOSHs	green	non-mucoid	opaque	smooth	non-SCV	smooth	halo	no sheen
gOSsv	green	non-mucoid	opaque	smooth	SCV	smooth	no halo	no sheen
OrOSHIev	orange	non-mucoid	opaque	smooth	SCV	irregular	halo	no sheen
OrOSHIe	orange	non-mucoid	opaque	smooth	non-SCV	irregular	halo	no sheen
OrOSIIe	orange	non-mucoid	opaque	smooth	non-SCV	irregular	no halo	iridescent
TOSs	tan	non-mucoid	opaque	smooth	non-SCV	smooth	no halo	no sheen
wMOSs	white	mucoid	opaque	smooth	non-SCV	smooth	no halo	no sheen
wMOSHs	white	mucoid	opaque	smooth	non-SCV	smooth	halo	no sheen
wOHIe	white	non-mucoid	opaque	smooth	non-SCV	irregular	halo	no sheen
wORIe	white	non-mucoid	opaque	rough	non-SCV	irregular	no halo	no sheen
wMOSHsv	white	mucoid	opaque	smooth	SCV	smooth	halo	no sheen
wMOSsv	white	mucoid	opaque	smooth	SCV	smooth	no halo	no sheen
wMOSIev	white	mucoid	opaque	smooth	SCV	irregular	no halo	no sheen
wOHSs	white	non-mucoid	opaque	smooth	non-SCV	smooth	halo	no sheen
wOSs	white	non-mucoid	opaque	smooth	non-SCV	smooth	no halo	no sheen

Table 4.6 (cont). Extended definitions of colony morphotype assignments for *P. aeruginosa* clinical isolates.

morphotype assignment	pigmentation	mucoidy	lucency	surface texture	size	margin regularity	autolysis	surface sheen
wOSIIe	white	non-mucoid	opaque	smooth	non-SCV	irregular	no halo	iridescent
wOSIe	white	non-mucoid	opaque	smooth	non-SCV	irregular	no halo	no sheen
wOSIIev	white	NM	opaque	smooth	SCV	irregular	no halo	iridescent
wOSIsv	white	NM	opaque	smooth	SCV	smooth	no halo	iridescent
wOSvs	white	NM	opaque	smooth	SCV	smooth	no halo	no sheen
wMTSs	white	mucoid	translucent	smooth	non-SCV	smooth	no halo	no sheen
wMTSsv	white	mucoid	translucent	smooth	SCV	smooth	no halo	no sheen
yOHIe	yellow	NM	opaque	smooth	non-SCV	irregular	halo	no sheen
yOHIev	yellow	NM	opaque	smooth	SCV	irregular	halo	no sheen
yMOSv	yellow	mucoid	opaque	smooth	SCV	smooth	no halo	no sheen
yOSs	yellow	NM	opaque	smooth	non-SCV	smooth	no halo	no sheen
yOSsv	yellow	NM	opaque	smooth	SCV	smooth	no halo	no sheen
NA	Missing score							

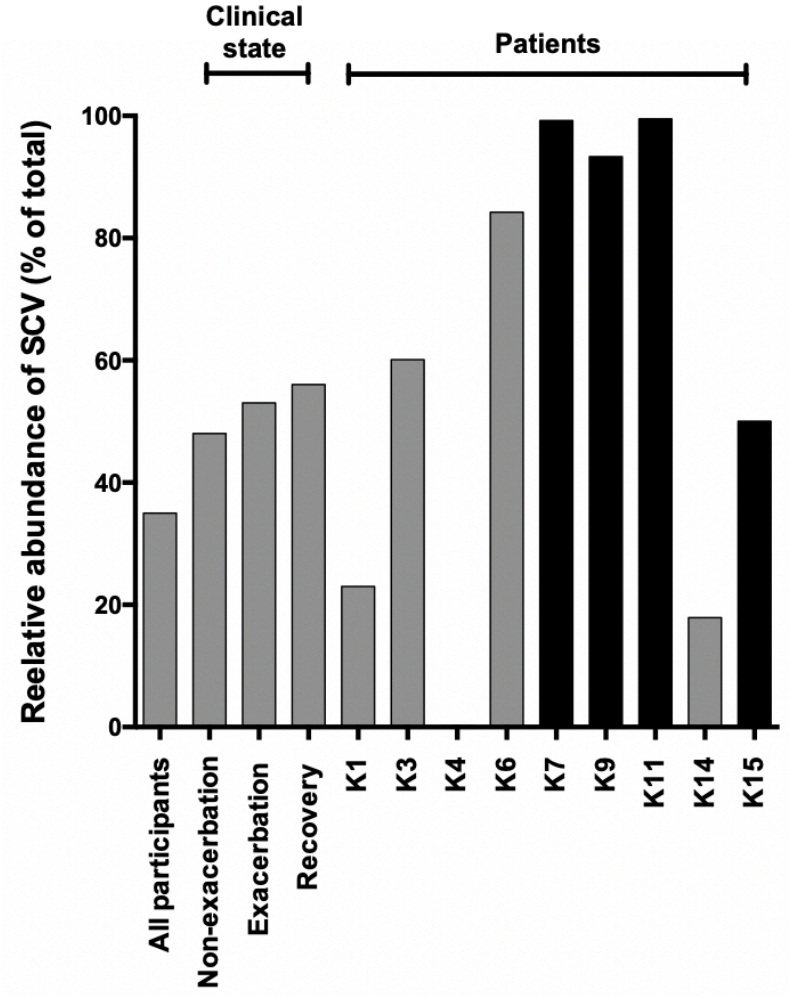


Figure 4.6. Relative abundance of SCV morphotype, and the variation observed between different clinical states (e.g., during periods of non-exacerbation, exacerbation and recovery following antibiotic treatment for an APE) and between different patients. No SCVs were identified in the strain collection from patient K4. Furthermore, the prevalence of SCV phenotype was found to be significantly higher among LES isolates (black bars) compared with their counterparts (χ^2 test, p <0.0001).

Alginate overproduction and consequent display of the mucoid morphotype is considered a classic hallmark of chronic *P. aeruginosa* CF infection and is associated with an increased resistance to various antimicrobial agents (reviewed in Morphology variants, Section 1.7.1). The presence or absence of mucoidy in the isolates was assessed at isolation on *Pseudomonas* selective agar base supplemented with cetrimide and sodium nalidixate. The prevalence of mucoid phenotype in chronic CF

isolates has been reported in some studies as high as 30% (181). However, in this study, mucoidy was evident in only 16% of all isolates (Figure 4.7). In general, LES isolates were non-mucoid, in line with previous reports of a higher prevalence of the non-mucoid phenotype in this strain type (121,271,325). No trend was observed among the MES isolates in this study. For example, all isolates from patient K1 were identified as non-mucoid, whereas, nearly all (93%) of the isolates from patient K14 exhibited the mucoid phenotype.

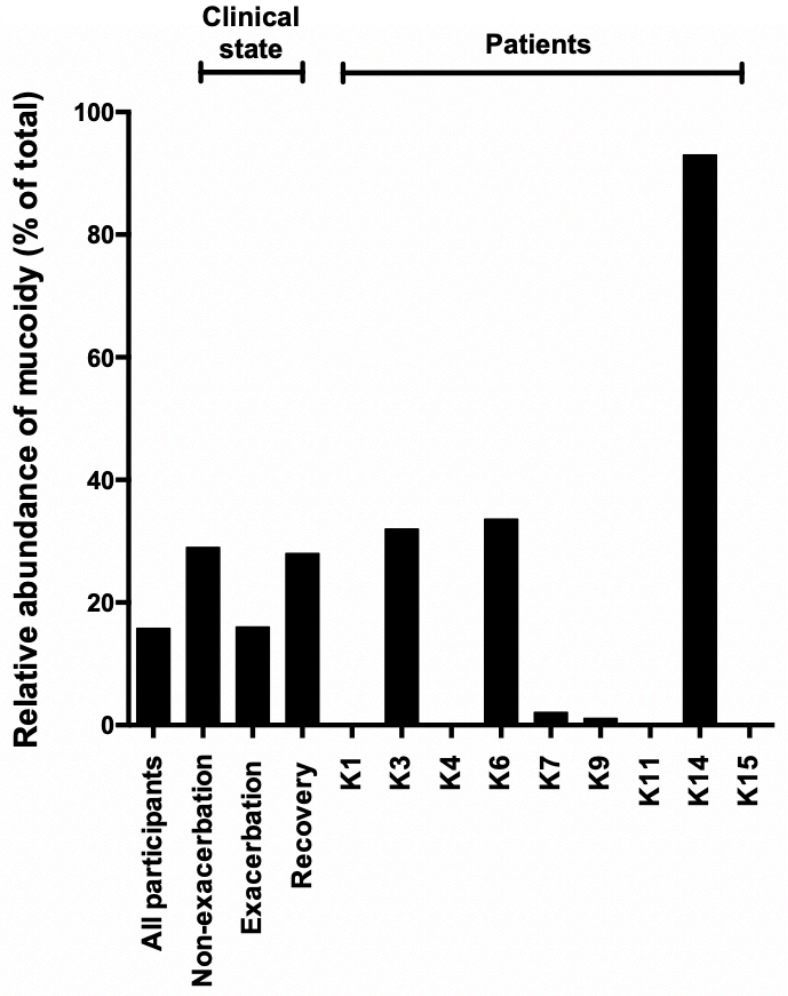


Figure 4.7. Relative abundance of mucoid phenotype, and the variation observed between different clinical states (e.g., during periods of non-exacerbation, exacerbation and recovery following antibiotic treatment for an APE) and between different patients. The highest proportion of mucoid isolates were recovered from patient K14 however overall, the prevalence of mucoidy expression among all isolates was low (16%), despite this being a hallmark of chronic adaptation of *P. aeruginosa* to the CF airway.

Notably, clonal isolates recovered from patient K4 were identified as morphotypically distinctive, with the majority (93%) expressing a green surface pigment, in contrast to their counterparts from the other patients. Furthermore, none of the K4 isolates were identified as SCVs or mucoid, features typically associated with chronic adaptation and persistence in the CF lung. This set of morphotypic features may be consistent with production of the exoproduct pyocyanin (usually green-hued on yellow-coloured plates) alongside colonial features more typically associated with acute (e.g., non-mucoid, large colonies) rather than chronic isolates, further highlighting the broad variation in colonial morphotypes present among these chronic isolates.

A comparison of the frequency of mucoid isolates recovered either during periods of non- exacerbation, exacerbation or following antibiotic treatment for an APE did not reveal a significant difference between the three groups (χ^2 test, p = 0.1161). This suggests that the mucoid phenotype is not prevalent during periods of acute exacerbation nor is it induced by the application of short-term antibiotic selection pressure during treatment episodes for these events.

4.3.2 Characterisation of QS signal production

Based on *in vitro* studies, virulence factor production in *P. aeruginosa* is widely understood to be under the control of a set of complex, interconnected QS systems: each activated by their own specific cognate autoinducer in response to changes in bacterial cell density. Three such systems, the LasIR, RhlIR and PQS systems, have been characterised for *P. aeruginosa* (reviewed in Quorum sensing, Section 1.7.2), two of which (LasIR and RhlIR) are activated by the AHLs; OdDHL and BHL, whereas the third system (PQS) is mediated by the AQ signal PQS. To investigate the presence or absence of QS in these CF isolates I assessed the production of each QS signal molecule (OdDHL, BHL and PQS).

In this study, the vast majority (81%) of clinical isolates screened were found to be OdDHL-deficient (Figure 4.8A). Based on laboratory studies in “domesticated” strains such as PAO1, the *las* signalling system directs transcriptional activation of the other two QS systems in a hierarchical manner (467). However, in the clinical isolates studied here, the absence of OdDHL production was not found to confer a loss of

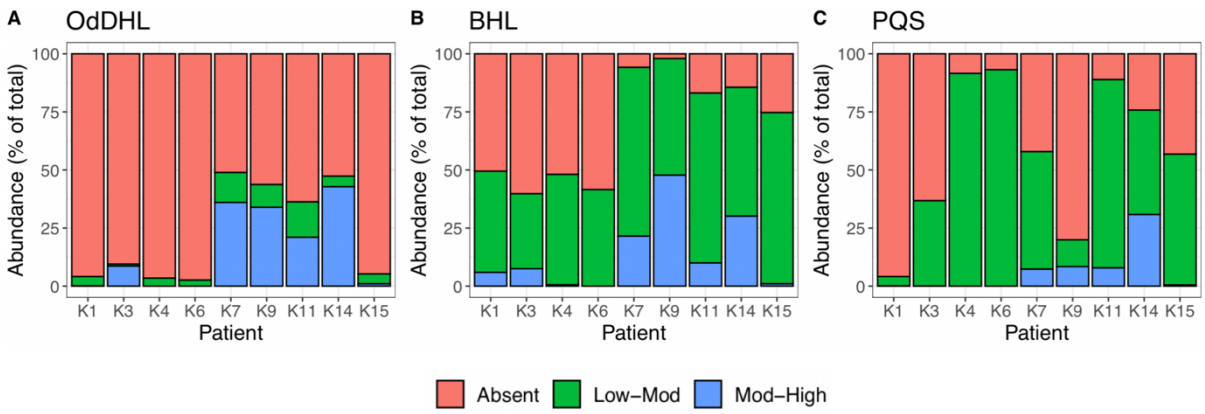


Figure 4.8. Prevalence of QS signal molecule production among *P. aeruginosa* isolates collected from nine patients with CF. (A) OdDHL, (B) BHL, and (C) PQS production. The majority (81%) of clinical isolates were deficient in OdDHL production.

activation of either the *rhl* or *pqs* systems. Indeed, a third (30%) of OdDHL-deficient isolates were still able to produce BHL alone (Figure 4.8B), whereas a lower proportion were able to produce both BHL and PQS together (25%), or PQS alone (17%) (Figure 4.8C). By comparison, just over half (53%) of all the isolates in the study were PQS-deficient. PQS-deficient isolates that were still able to produce OdDHL alone were extremely rare (*n* = 13 out of 2290), although a minority (11%) were still able to produce OdDHL and BHL together. Strikingly, a significant proportion of PQS-deficient isolates retained production of BHL alone (46%). A little over one third (38%) of all isolates were BHL-deficient. Very few (*n* = 13 out of 1634) were able to produce OdDHL in the absence of PQS, although the proportion of BHL-deficient isolates capable of producing OdDHL and PQS together was still very low (2%). However, a significant minority (37%) of BHL-deficient isolates were still able to produce PQS alone.

In this work, several clonal populations of *P. aeruginosa,* recovered from different patients, shared a very low prevalence of OdDHL-proficient isolates. Specifically, only 4%, 9%, 3%, 3% and 5% of isolates collected from patients K1, K3, K4, K6 and K15 respectively were identified as OdDHL-proficient. This may suggest possession of mutations in similar genes involved with OdDHL synthesis among isolates from these patients. Moreover, in contrast, greater variation was observed in the prevalence of either BHL and PQS production among isolates from these same patients. For example, preserved BHL was produced in nearly half (48%) of patient K4's isolates,

whereas PQS production was preserved in a much greater (93%) proportion. Similarly, the prevalence of BHL- and PQS-proficient isolates among the isolates from patient K6 was 42% and 93% respectively. However, in contrast, among isolates recovered from patient K3, the prevalence of BHL-proficient isolates was 40%, whilst the proportion of isolates identified as PQS-proficient was only 37%.

Of note, although patient K3 shared a similarly high proportion of QS-deficient isolates to patient K1 [47% and 48%, respectively, (Table S1, Appendix 2)], a substantially higher proportion (87% *versus* 23%) of the QS-deficient isolates recovered from patient K3 were capable of expressing at least one of the known QS-regulated phenotypes, suggesting that QS-regulation in this clonal population may not be necessary for the production of phenotypes traditionally considered to be under QS control.

In the sub-group of K3 isolates which were found to have preserved QS signal production in at least one of the three QS sub-systems investigated, the prevalence of BHL and PQS secretion was similarly high (75% and 70% of isolates respectively), whereas OdDHL secretion was low (18%), again suggesting the possibility of loss-of-function mutations affecting *lasI* in this cohort.

Interestingly, despite isolates recovered from patient K1 and K14 sharing similar genetic backgrounds (both are representative of the MES), the overall production of QS signal molecules was very different between these two clonal populations. In addition to a very low prevalence of OdDHL-proficient isolates, patient K1 also harboured the lowest prevalence of PQS-proficient isolates in the study (4%), Intriguingly, and despite the marked absence of OdDHL and PQS production, half (50%) of the K1 isolates were BHL-proficient, suggesting possible preservation of RhlI activity amongst a significant proportion of these isolates. Nonetheless, among this sub-group of BHL-producing isolates, QS-regulated phenotypes were maintained in only 50% of the cohort. By contrast, patient K14 had a much higher proportion of isolates which were OdDHL-, BHL- and PQS-proficient, with a prevalence of 47%, 86% and 76% respectively.

In comparison to the MES clonal populations, the LES clonal populations (from patients K7, K9, K11 and K15) shared similar prevalence rates for OdDHL- and BHL-producing isolates, with the exception of a comparatively lower frequency of OdDHL-proficient isolates from patient K15. However, greater variability was observed in the prevalence of PQS production (range 20% - 89%) in this cohort (Figure 4.8).

Given the broad variation in QS signal molecule production observed among isolates in this study I compared production levels between epidemic and non-epidemic clones to assess for significant differences between the two cohorts. Data for the LES and MES isolates were combined for these analyses. A higher proportion of epidemic clones were found to retain production of OdDHL and BHL compared with the non-epidemic isolates (χ^2 test, p <0.0001), whereas a higher proportion of non-epidemic isolates were found to be PQS-proficient (χ^2 test, p <0.0001) (Figure 4.9). Furthermore, the difference between the epidemic and non-epidemic strain cohorts was largely driven by the LES isolates, with a significant difference also found between LES and MES isolates in the prevalence of OdDHL-, BHL- and PQS-proficient isolates (χ^2 test, p <0.0001) (Table 4.7).

Taken together, these observations provided substantial evidence for *las*-independent *rhlI* and *pqsA-E* activation among the isolates analysed in this study. Moreover, given the signal specificity of OdDHL for its cognate receptor LasR and the low prevalence of OdDHL-producing isolates found in the isolate populations from 5 of the 8 patients,

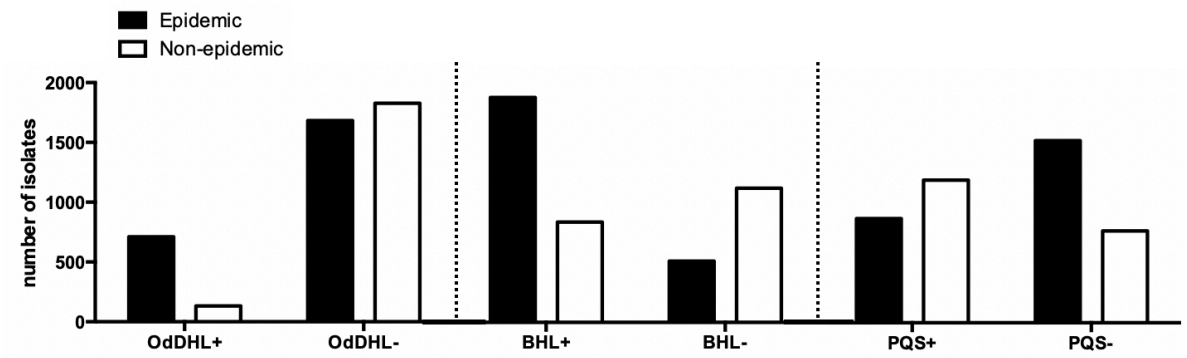


Figure 4.9. Differences in QS signal molecule production between epidemic and non-epidemic clonal isolates of *P. aeruginosa*. Significant differences were observed between the two cohorts with a higher prevalence of OdDHL- and BHL-proficient isolates found in the epidemic cohort (χ^2 test, p <0.0001). However, the proportion of PQS producing isolates was notably higher in the non-epidemic cohort (χ^2 test, p <0.0001). OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

Table 4.7. Comparison of the frequency of QS signal-producing isolates among LES and MES isolates of CF-evolved *P. aeruginosa*

Phenotype	LES (*n* = 1402)	MES (*n* = 990)	*p* value
OdDHL secretion	546 (40%)	164 (17%)	< 0.0001
BHL secretion	1287 (92%)	593 (60%)	< 0.0001
PQS secretion	546 (39%)	245 (25%)	< 0.0001

Differences between categorical data were examined with χ^2 tests. OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

I had expected to observe accompanying deficiencies in the production of typically LasR-regulated phenotypes (e.g., secreted proteases, PQS production and *rhlR*-associated phenotypes) across a significant proportion of this collection of isolates. However, this was not the case, as will be described in further detail below.

4.3.3 Frequency of virulence-associated phenotypes

Virulence-associated phenotypes were measured for all the isolates, including phenotypes whose presence or absence is understood to be specific to regulation by a particular QS sub-system (e.g the LasA protease (whose activity is reported through the gelatinase assay) is controlled by the *las* system, whereas rhamnolipid production is controlled by the *rhl* system) (374,468). Substantial phenotypic diversity has previously been reported in both epidemic and non-epidemic clonal isolates of *P. aeruginosa* from individuals with CF (271,325–327). In line with prior work, striking diversity was observed in the frequency of these virulence-associated phenotypes, both among clonal isolates collected from the same patient and between clonal populations collected from different patients. The prevalence of the various phenotypes is summarised in Figures 4.10 and 4.12. In general, greater within-patient phenotypic diversity was observed among clonal isolates collected from patients not infected with the LES (patients K1, K3, K4, K6 and K14). Furthermore, and in contrast to studies carried out with “domesticated” laboratory strains (342), QS-regulated phenotypes were not consistently abolished in isolates deficient in LasI-generated signal.

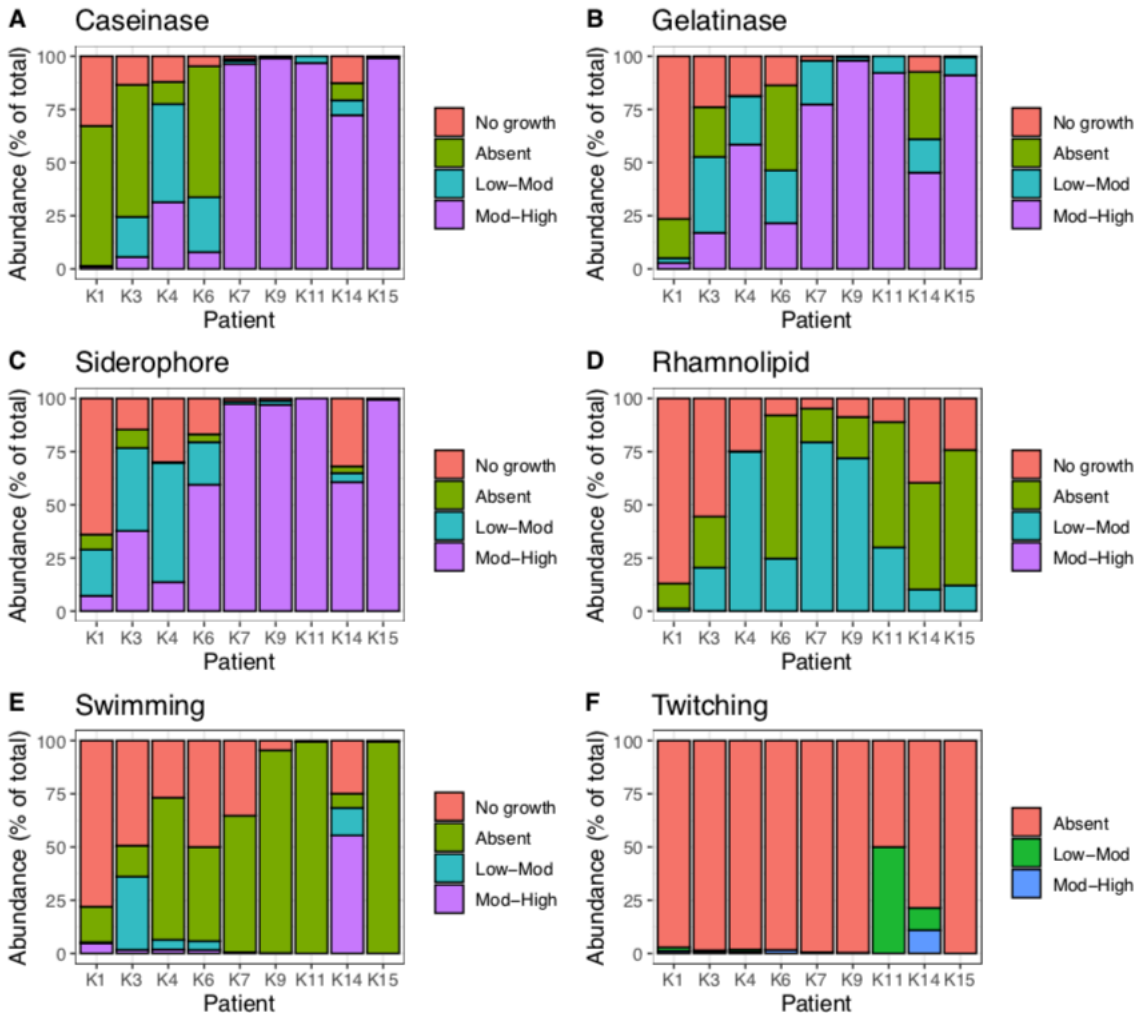


Figure 4.10. Variation in the prevalence of virulence-associated phenotypes among clonal isolates of *P. aeruginosa*, collected from nine patients with CF. For phenotypes A-E, the isolates are classified as either failing to grow (red), having a negative phenotype (green), displaying a low-moderate positive phenotype (blue) or a moderate-high expression of the phenotype. For phenotype F, isolates were classified as either having a negative phenotype (red), or displaying low-moderate (green) or moderate-high (blue) expression of the phenotype.

4.3.3.1 Protease secretion

Among MES isolates recovered from patient K1, a very low proportion of caseinase- (1%) and gelatinase-producing (5%) strains were identified, suggesting a substantial loss of secreted protease activity among this set of isolates. In contrast, 79% and 61% of the MES isolates from patient K14 were capable of caseinase and gelatinase secretion, respectively. The notable difference in prevalence of protease secretion observed between these two clonally-related MES populations is in keeping with their

QS signal production profiles (previously described in Characterisation of QS signal production, Section 4.3.2).

Among isolates recovered from patients infected with a non-epidemic strain of *P. aeruginosa* (patients K3, K4, K6) wide variations in secreted protease production was noted between patients' isolates. Fifty-two percent and 24% of all isolates recovered from patient K3 exhibited proteolytic activity on gelatin and on casein respectively. Interestingly, of this sub-group of protease-proficient isolates, 48% of those active on gelatin and 38% of those active on casein were also identified as completely lacking in QS signal production, providing potential evidence for QS-independent regulation of these phenotypes. For isolates recovered from patient K4, the overall prevalence of protease-proficient isolates was higher with 77% and 81% of isolates found to express caseinase and gelatinase respectively. By contrast, the frequency of protease-proficient isolates was comparatively lower among isolates recovered from patient K6, with only 34% and 46% of isolates capable of proteolytic activity on casein and gelatin respectively.

Little variation in protease-secreting potential was observed among the LES isolates with nearly all of the isolates recovered from patients K7, K9, K11 and K15 confirmed as proficient in both caseinase and gelatinase production (Figure 4.10A and 4.10B).

4.3.3.2 Siderophore secretion

The lowest (29%) frequency of siderophore-expressing isolates in this study was identified among the K1 isolates, and accompanied the low prevalence of protease- and QS-proficient isolates previously described for this cohort. Again, a comparatively higher (65%) proportion of siderophore-expressing isolates was identified among isolates recovered from patient K14 (also harbouring the MES) and the prevalence was similar to that observed among patients infected with a unique strain, with 77%, 70%, and 79% of isolates identified as siderophore producers from patients K3, K4 and K6 respectively.

In contrast to the non-LES isolates, siderophore production was maintained in nearly all of the LES isolates (range 99 – 100%), recovered from patients K7, K9, K11 and K15 (Figure 4.10C).

Curiously, a comparison of the halo colour on the siderophore screening plates for the K4 isolates with those generated by the other isolates revealed a distinctive halo phenotype (Figure 4.11A and 4.11B). Typically, halos generated by isolates in this study displayed an orange halo surrounding a central colony (Figure 4.11 C-M), consistent with the positive control, PAO1.

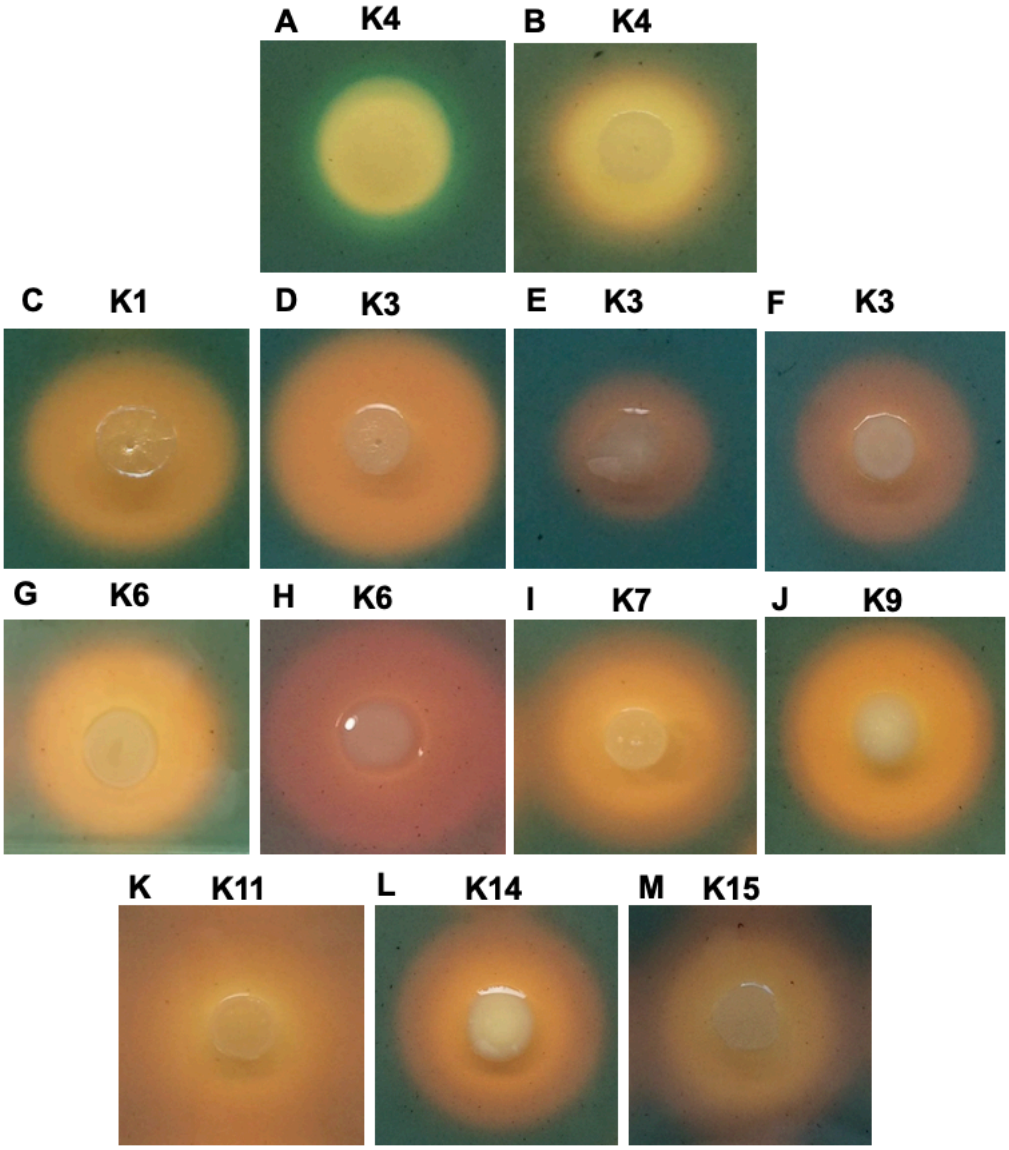


Figure 4.11. Screening for siderophore production of CF-evolved clonal isolates of *P. aeruginosa*. Production can be visualised by formation of an orange halo around a surrounding colony on CAS indicator plates. Isolates from one (patient K4) of the nine patients displayed a distinctive halo pattern (A, B) when compared with siderophore-producing isolates from the other patients (C-M). in most cases, siderophore-producing isolates from the same patient displayed the same halo phenotype, where this was not the case (e.g., patients K3, K4 and K6), representative examples of the prevalent halo phenotypes for that patient are included.

In contrast, K4 isolates produced yellow halos, either with a surrounding green ring or orange ring, with the majority of K4 isolates displaying halos represented in Figure 4.11A. This might suggest differential production in siderophore type between these isolates and their counterparts (from other patients) in this study.

4.3.3.3 Rhamnolipid secretion

Rhamnolipids are understood to play a significant role in the establishment and persistence of chronic *P. aeruginosa* infection in the CF airway, including the acute infiltration of airway epithelia and inhibition of the host's polymorphonuclear leukocytes (469). Yet little has been reported on the presence or absence of this phenotype among CF-evolved isolates of *P. aeruginosa,* including in relation to acute episodes of clinical deterioration.

In this study, the prevalence of rhamnolipid-producing isolates varied widely between patients (Figure 4.10D). However overall, the majority of patients harboured clonal populations that were lacking in rhamnolipid production. Interestingly, two thirds of all isolates which failed to produce rhamnolipids were proficient in production of the RhlIR signal molecule, BHL. This is somewhat surprising as rhamnolipid production is directly regulated by the *rhl* system (discussed in Rhamnolipids, Section 1.7.3.4). Among patients infected with the MES, only 1% of K1 isolates and 10% of K14 isolates were identified as rhamnolipid-producers.

Among the patients harbouring non-epidemic isolates, a relatively low prevalence of rhamnolipid-producing isolates was identified in two of the three patients, with only 21% of patient K3's and 25% of patient K6's isolates found to be rhamnolipid producers. By contrast, a greater proportion (75%) of isolates from patient K4 were identified as rhamnolipid-producing. Unexpectedly, half of these rhamnolipid-producing isolates were found to be lacking in BHL production.

Similarly, among the patients harbouring the LES, a high proportion of isolates from two of the four patients were rhamnolipid-producing (79% for patient K7 and 72% for patient K9), whereas a low prevalence was observed among isolates from patients K11 and K15 (30% and 12% respectively).

4.3.3.4 Swim and twitch motility

Isolates from chronic *P. aeruginosa* CF infection often display a non-flagellated, non-motile phenotype (324,470). Consistent with this, most patients in this study harboured isolates that lacked both swim (flagellar-based) and twitch (pilus-based) motility (Figure 4.10E and 4.10F), with a few exceptions.

Among the isolates recovered from patient K3, 36% exhibited swim motility. Unexpectedly, just over half (56%) of these isolates were also found to be lacking in production of all 3 QS signal molecules. A high proportion of motile isolates were also identified among the strains recovered from patient K14, with 68% and 21% of isolates displaying swim and twitch motility respectively. Nearly all (95%) of the swim-proficient isolates from patient K14 were observed to exhibit the mucoid phenotype. This was somewhat surprising as alginate overproduction is thought to be co-ordinately regulated with loss of flagellum expression (via the actions of AlgT) to yield mucoid non-motile variants. In addition, previous studies on LESB58 (a laboratory reference strain known to cause chronic infection and the earliest available LES isolate) as well as on clinical isolates of the LES (including those taken from the non-CF parent of a CF patient, who presented with pneumonia) have described this strain to be deficient in pilus-based motility (174,452,471). However, 50% of isolates recovered from patient K11 were found to exhibit twitch motility. Although swim and swarm motility have previously been reported for the LES (452), this the first report of preserved twitch motility in the LES.

4.3.3.5. Biofilm formation

Unexpectedly, given the chronic nature of *P. aeruginosa* infection in this study, the majority of isolates recovered from patients infected with either the MES or a non-epidemic strain of *P. aeruginosa* were found to lack surface attachment phenotype (on crystal violet staining), with the production of biofilm biomass detected in only 12%, 5%, 21% and 27% of isolates from patients K1, K3, K6 and K14 respectively. Moreover, none of the isolates from patient K4 were found to be biofilm-forming (Figure 4.12). In contrast, the prevalence of biofilm-forming isolates among the LES cohort was higher, with the exception of isolates from patient K11. Seventy-one

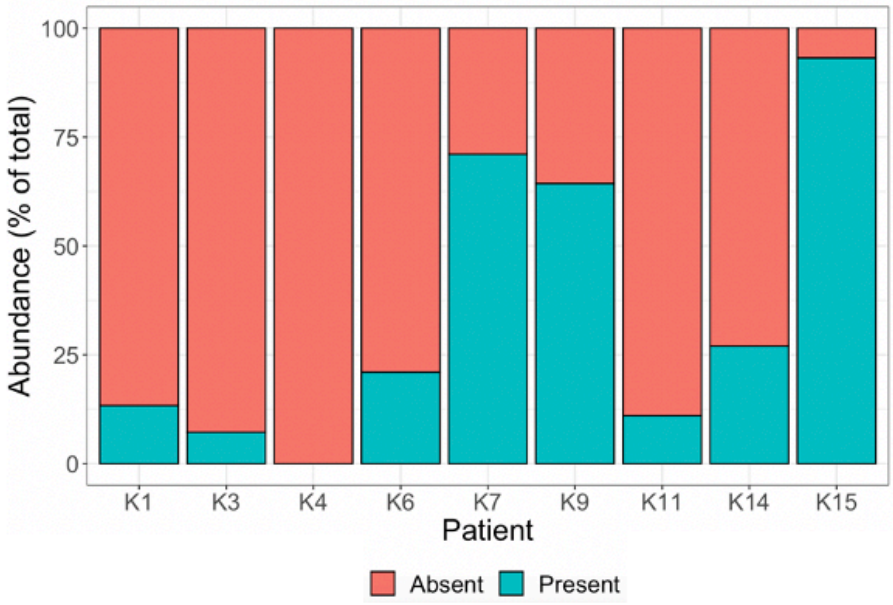


Figure 4.12. The prevalence of biofilm-forming isolates among clonal isolates of *P. aeruginosa* collected from nine patients with CF.

percent, 64% and 93% of isolates from patients K7, K9 and K15 respectively, were identified as biofilm-forming, whereas only 11% of patient K11's isolates were identified as biofilm producers. In some of these cases, the lack of biofilm formation may in part be due to reduced production of rhamnolipids, which have been reported to perform several roles in the establishment and maintenance of *P. aeruginosa* biofilms (reviewed in Rhamnolipids, Section 1.7.3.4).

4.3.4 Characterisation and frequency of auxotrophic variants

CF sputum is rich in amino acids and is capable of supporting the growth of auxotrophs (433,434). Prior studies have suggested an important role for arginine metabolism, whereas methionine auxotrophs have been associated with APEs, and *lasR* mutants with growth advantages on phenylalanine (184,453,472). In this study, prototrophs were identified among isolates recovered from only six of the nine patients with an overall prevalence of 23%. No patient was identified to be colonised exclusively with prototrophs. Furthermore, the majority (65%) of the prototrophs were identified among isolates recovered from patients not infected with the LES (patients K1, K3, K4, K6 and K14) (Figure 4.13).

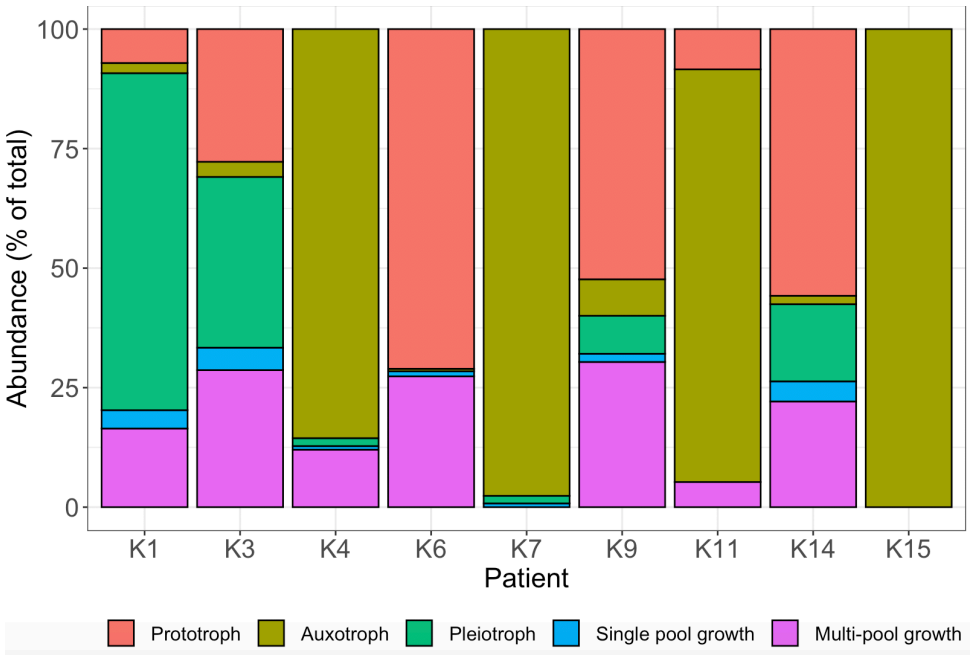


Figure 4.13. Prevalence of auxotrophic mutants among clonal isolates of *P. aeruginosa* collected from nine patients with CF.

Pleiotropic mutants, isolates which fail to grow on enriched minimal media, were also found to be more prevalent among patients not infected with the LES. Only 4% of isolates identified as pleiotropic mutants were found among the LES cohort. The majority (70%) were recovered from patient K1 whilst a lower proportion of isolates were identified as pleiotropic mutants from patient K3 and K14 (36% and 17% respectively).

A third (32%) of the isolates in this study were confirmed as auxotrophic variants, defective in the synthesis of a specific growth factor limiting growth on minimal medium. A further 19% of isolates were identified as unable to synthesise a combination of several factors, either amino acids or certain cofactors, as determined by growth on multiple instead of only two of eleven pools (e.g., multi-pool growth) of enriched minimal media. A small proportion (3%) of isolates grew on only one (e.g., single pool growth) of the eleven enriched minimal media pools, however, I was unable to identify which of the growth factors was needed for growth on enriched minimal media. Nonetheless, among the third of isolates confirmed as auxotrophs for a specific growth factor, the majority (94%) were identified as methionine auxotrophs. The next

most common single growth factor requirement identified was for aromatic amino acids (2%), followed by purine (1%) and pyrimidine (1%).

A significant difference was identified between epidemic and non-epidemic isolates in the prevalence of auxotrophic variants, with a higher prevalence of auxotrophs found among patients infected with an epidemic strain (χ^2 test, p <0.0001). This difference was largely due to a significantly higher prevalence of auxotrophs in the LES cohort (most of which were methionine auxotrophs) compared with the MES (χ^2 test, p <0.0001). Among patients not infected with the LES, patient K4 harboured the greatest proportion (86%) of auxotrophic variants, of which the majority (97%) were again identified as methionine auxotrophs.

4.3.5 A comparison of phenotype abundance between epidemic and non-epidemic clonal populations of *P. aeruginosa*

Given the diversity in composition of virulence-associated phenotypes observed in this study I wanted to determine if there were particular phenotypes that might differentiate epidemic strains from non-epidemic strains.

When I compared the epidemic strains against the non-epidemic strains, I found significant differences between the two groups in terms of the prevalence of phenotype-proficient isolates within each group (Table 4.8). A higher frequency of isolates proficient in protease production (χ^2 test, p = 0.0024), twitch motility (χ^2 test, p <0.0001) and biofilm formation (χ^2 test, p <0.0001), but deficient in flagellar-based motility (χ^2 test, p <0.0001) distinguished the cohort of epidemic isolates from the non-epidemic isolates. No difference was found between the two cohorts in the prevalence of isolates proficient in rhamnolipid or siderophore production (χ^2 test, p = 0.8945 and 0.0910 respectively).

Given the clinical significance of epidemic clones, which share notoriety for nosocomial transmission and high antibiotic resistance, I wanted to determine whether any phenotypes distinguished the LES from the MES isolates in this study. I found that the prevalence of several phenotypic traits was significantly different between the two cohorts, with the exception of twitch motility (χ^2 test, p = 0.9445) (Table 4.9). Overall,

Table 4.8. Comparison of the frequency of phenotype-proficient isolates among epidemic and non-epidemic isolates of CF-evolved *P. aeruginosa.*

Phenotype	Epidemic isolates (*n* = 2392)	Non-epidemic isolates (*n* = 1961)	*p* value
Protease secretion	1693 (71%)	1303 (66%)	0.0024
Siderophore production	1778(74%)	1462 (76%)	0.8945
Rhamnolipid production	882 (37%)	773 (39%)	0.0910
Twitch motility	180 (3%)	31(0.2%)	< 0.0001
Swim motility	237(10%)	447 (23%)	< 0.0001
Biofilm formation	1052 (45%)	120 (6%)	< 0.0001

Differences between categorical data were examined with χ^2 tests.

Table 4.9. Comparison of the frequency of phenotype-proficient isolates among LES and MES isolates of CF-evolved *P. aeruginosa.*

Phenotype	LES (*n* = 1402)	MES (*n* = 990)	*p* value
Protease secretion	1390 (99%)	298 (30%)	< 0.0001
Siderophore production	1389 (99%)	389 (39%)	< 0.0001
Rhamnolipid production	844 (60%)	38 (4%)	< 0.0001
Twitch motility	99 (7%)	81 (9%)	0.9445
Swim motility	5 (0.4%)	232 (23%)	< 0.0001
Biofilm formation	881 (63%)	171 (17%)	< 0.0001

Differences between categorical data were examined with χ^2 tests.

a higher prevalence of isolates proficient in protease secretion, rhamnolipid and siderophore production, swim motility and biofilm formation were found among the LES cohort (χ^2 test, p <0.0001) compared with the MES cohort.

I next examined whether the differences in phenotype prevalence observed between the LES and MES cohorts also distinguished the LES from the non-epidemic cohort of isolates. Again, the prevalence of isolates proficient in protease secretion, siderophore

production, rhamnolipid production, biofilm formation and swim and twitch motility was higher in the LES cohort compared with the non-epidemic cohort (χ^2 test, p <0.0001). A similar comparison between the MES and non-epidemic isolates, revealed a lower frequency of isolates proficient in protease secretion, siderophore and rhamnolipid production among the MES cohort (Table 4.10). However, the very low prevalence of all phenotypes among the K1 isolates likely accounts for the significant differences observed. Nonetheless, a higher frequency of isolates proficient in biofilm formation and swimming motility was observed among the MES isolates compared with the non-epidemic cohort.

Table 4.10. Comparison of the frequency of phenotype-proficient isolates among MES and non-epidemic isolates of CF-evolved *P. aeruginosa.*

Phenotype	MES (n = 990)	Non-epidemic isolates (n= 1961)	p value
Protease secretion	298 (30%)	1303 (66%)	< 0.0001
Siderophore production	389 (39%)	1462 (76%)	< 0.0001
Rhamnolipid production	38 (4%)	773 (39%)	< 0.0001
Twitch motility	81 (9%)	31 (0.2%)	< 0.0001
Swim motility	232 (23%)	447 (23%)	0.3436
Biofilm formation	171 (17%)	120 (6%)	< 0.0001

Differences between categorical data were examined with χ^2 tests.

4.3.6 Correlations between functional phenotypes

Despite the broad variation in functional phenotypes observed in this study, several relationships between phenotypes were identified (Figure 4.14). In line with previous studies, phenotypic traits that are often associated with virulence and early (rather than chronic) CF airway infection were correlated (327). These characteristics were also found to be inversely related to some, but not all, chronic adaptive phenotypes (e.g., biofilm formation, SCV phenotype, mucoidy). Furthermore, variation (e.g., stronger or weaker correlations) in the display of these phenotype-phenotype relationships was observed between populations of clonal isolates from different patients (Figure S1, Appendix 2).

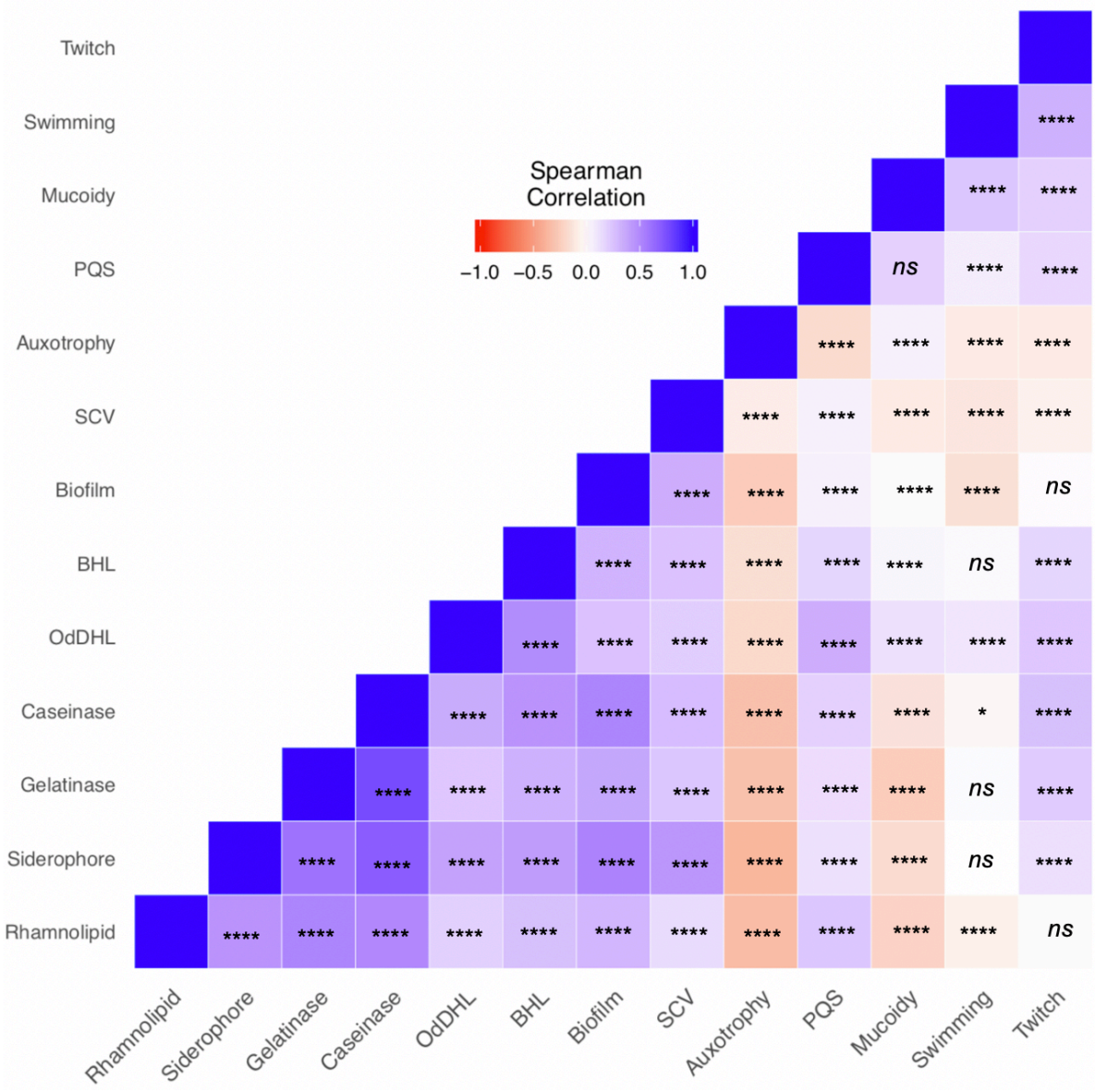


Figure 4.14. Correlation matrix of phenotype-phenotype associations for all isolates as determined by Spearman rank correlation coefficient. Blue indicates strong positive correlation between any given phenotype pair (Spearman rank coefficient closer to 1) while red indicates strong negative correlation between any given phenotype pair (Spearman rank coefficient closer to -1). White indicates no correlation between phenotype pairs (0). Correlations were significant if r >0.35 and p <0.05 (*). *ns* = not significant, **** = p <0.00001. SCV: small colony variant, OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

OdDHL production was modestly correlated with BHL production (Spearman $r = 0.49$, $p < 0.00001$), whereas PQS production was not associated with production of either of the two acyl-homoserine lactones (OdDHL: Spearman $r = 0.33$, $p < 0.00001$), BHL: Spearman $r = 0.17$, $p < 0.00001$). In general, PQS production correlated poorly with the presence/absence of the majority of functional phenotypic traits assessed ($p < 0.00001$).

Significant positive correlations were observed between the secreted virulence-associated phenotypes (e.g., rhamnolipid production, protease activity, siderophore production), with the strongest correlations noted between protease activity and siderophore production (e.g., of gelatinase: Spearman $r = 0.6$, $p < 0.00001$, of caseinase: Spearman $r = 0.69$, $p < 0.00001$). Siderophore production was also significantly, albeit modestly, correlated with the SCV morphotype (Spearman $r = 0.45$, $p < 0.00001$), biofilm formation (Spearman $r = 0.53$, $p < 0.00001$) and BHL production (Spearman $r = 0.41$, $p < 0.00001$). Taken together, these associations may suggest that isolates adapted to express the SCV morphotype are somewhat more likely to maintain iron-scavenging ability via siderophore secretion. Furthermore, these siderophore-producing isolates are more likely to maintain phenotypes typically associated with virulence and persistence in the CF airway.

Rhamnolipid production was moderately correlated with siderophore (Spearman $r = 0.45$, $p < 0.00001$), gelatinase (Spearman $r = 0.51$, $p < 0.00001$) and caseinase (Spearman $r = 0.5$, $p < 0.00001$) production. The overlap in QS regulatory pathways influencing both protease and rhamnolipid biosynthesis most likely accounts for this relationship. Interestingly, although rhamnolipids have been reported to be of importance in the maintenance of established biofilms, biofilm formation was not well correlated with rhamnolipid production in this study (Spearman $r = 0.31$, $p < 0.00001$). Similarly, no correlation was identified between mucoid phenotype and biofilm formation (Spearman $r = -0.01$, $p < 0.00001$). Moreover, mucoid phenotype, which is characteristic of chronic pseudomonal infection, was poorly associated with all of the secreted virulence factors.

Auxotrophic metabolism was weakly negatively correlated with some virulence-associated traits, including siderophore production (Spearman $r = -0.37$, $p < 0.00001$),

and rhamnolipid production (Spearman r = - 0.35, p <0.00001), and was not well correlated with protease secretion (gelatinase: Spearman r = - 0.31, p <0.00001, caseinase: Spearman r = - 0.32, p <0.00001). The association between biofilm formation (Spearman r = - 0.25, p <0.00001) and auxotrophy was negligible. However, of note, biofilm formation was positively correlated with several secreted exoproducts. For example, a weak association was observed with gelatinase (Spearman r = 0.37, p = 0.0134) and a moderate association with caseinase production (Spearman r = 0.52, p = 0.0025). Taken together, these relationships may suggest that acquiring defects in cell metabolism may play more of a role in virulence downregulation in *P. aeruginosa* chronic infection than the transition from a planktonic to a sessile lifestyle.

No relationship was observed in this study between swim motility and any of the phenotypes assessed. Neither were relationships identified between twitch motility and several phenotypes (e.g., biofilm formation, SCV morphotype and auxotrophic metabolism) commonly associated with chronicity. In a similar vein, biofilm formation also appeared to behave independently of the presence of the mucoid phenotype.

4.3.7 Influence of strain epidemicity on phenotype-phenotype associations

Given the similarities in phenotypic profiles observed between certain isolate cohorts, primarily among the LES isolates but to a lesser extent also among the non-epidemic isolates in this study, I examined whether phenotype-phenotype associations differed between the LES, MES and non-epidemic clonal populations.

In contrast to the associations identified in the global phenotype-phenotype analysis, phenotypes in the LES cohort were typically poorly correlated (Figure 4.15), suggesting that most phenotypes behaved independently of each other. Notably, siderophore production was no longer well correlated with both virulence-associated and adaptive phenotypes. Surprisingly, a moderate negative relationship was observed between biofilm production and the SCV phenotype (Spearman r = - 0.43, p <0.00001), despite the predominance of both SCV phenotype and biofilm forming isolates in the LES cohort. This observation runs contrary to the association of enhanced biofilm forming capacity associated with SCVs that has previously been reported in the literature (323,334).

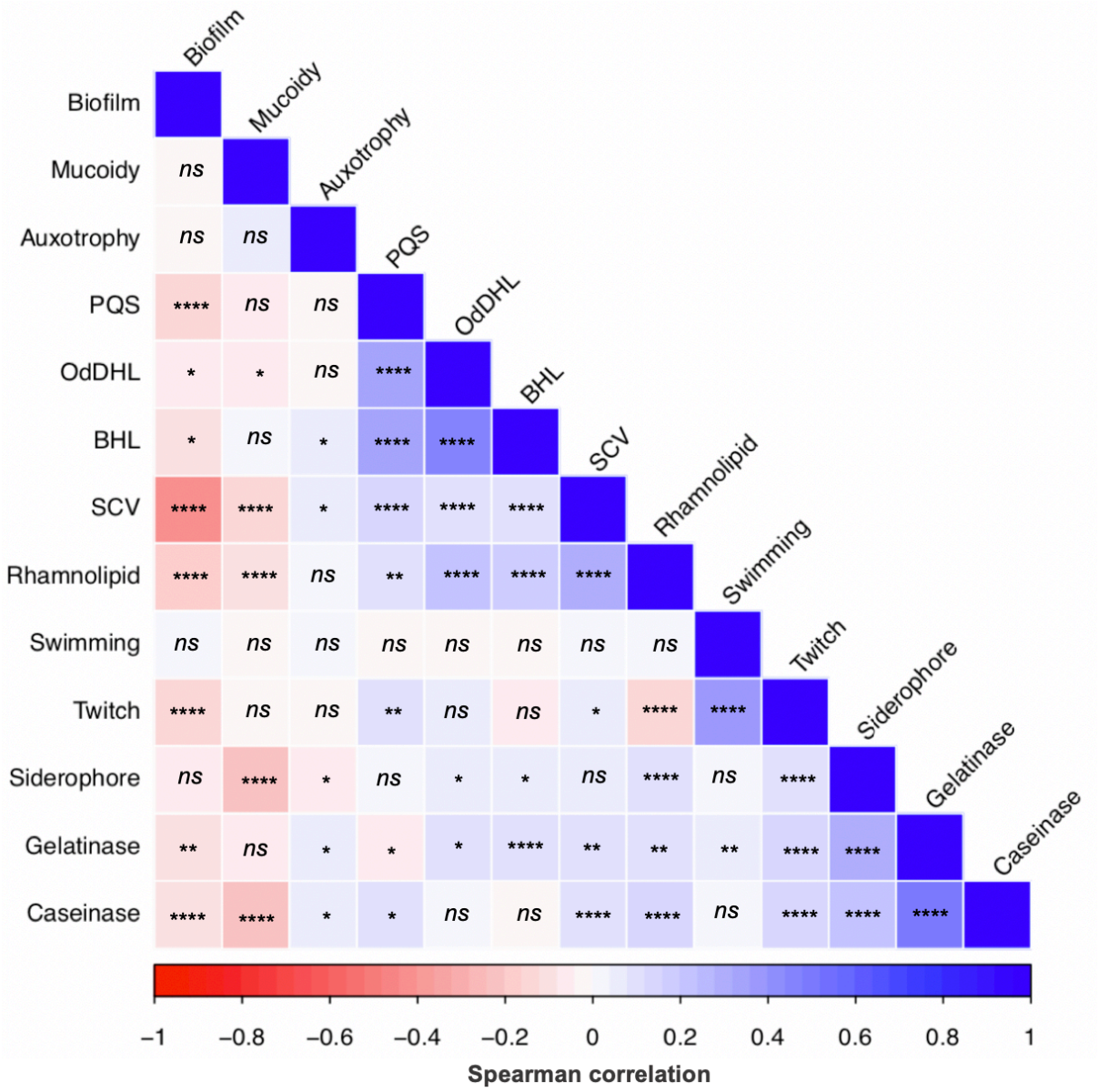


Figure 4.15. Correlation matrix of phenotype-phenotype associations for the LES isolates as determined by Spearman rank correlation coefficient. Blue indicates strong positive correlation between any given phenotype pair (Spearman rank coefficient closer to 1), red indicates strong negative correlation between any given phenotype pair (Spearman rank coefficient closer to -1) and white indicates no correlation between phenotype pairs (0). Correlations were significant if r >0.35 and p <0.05 (*). *ns* = not significant, ** = p <0.001. ***p <0.0001, **** = p <0.00001. SCV: small colony variant, OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

In addition, weak associations were found between some of the QS signal molecule pairs, with only a significant modest positive correlation observed between OdDHL and BHL production (Spearman r = 0.44, p <0.00001). However, no relationship was identified between OdDHL and PQS production (Spearman r = 0.33, p <0.00001). Of note, for the LES cohort, no relationship was identified between auxotrophic metabolism and any of the virulence-associated or persistence-related phenotypes, despite the predominance of methionine auxotrophs in this cohort.

In contrast to the LES cohort, a number of positive phenotype-phenotype relationships were identified among the MES isolates (Figure 4.16). Correlations identified between secreted exoproducts and QS signal molecule production were more consistent with traditional QS regulation of these factors. For example, positive associations were identified between OdDHL secretion and caseinase (Spearman r = 0.66, p <0.00001), or with rhamnolipid production (Spearman r = 0.55, p <0.00001) or with siderophore production (Spearman r = 0.71, p <0.00001); BHL secretion with caseinase (Spearman r = 0.48, p <0.00001), or with siderophore production (Spearman r = 0.51, p <0.00001); and PQS with caseinase (Spearman r = 0.54, p <0.00001) or with rhamnolipid production (Spearman r = 0.53, p <0.00001). Similarly, in keeping with their shared regulatory pathways, the production of several secreted exoproducts was correlated together. For example, of siderophore production with gelatinase (Spearman r = 0.42, p <0.00001), or with caseinase (Spearman r = 0.69, p <0.00001), or with rhamnolipid production (Spearman r = 0.65, p <0.00001). However, QS signal production was not well correlated with biofilm formation (e.g., with OdDHL, Spearman r = 0.33, p <0.00001; with BHL, Spearman r = 0.12, p <0.00001 or with PQS, Spearman r = 0.2, p <0.00001).

Positive correlations were also observed between swim motility and QS signal production (with OdDHL, Spearman r = 0.59, p <0.00001; with BHL, Spearman r = 0.53, p <0.00001, or with PQS, Spearman r = 0.53, p <0.00001).

Correlations between *las*, *rhl* and *pqs* signal molecules was also observed with positive associations found between BHL and PQS (Spearman r = 0.68, p <0.00001), OdDHL and BHL (Spearman r = 0.58, p <0.00001) and OdDHL with PQS production (Spearman r = 0.71, p <0.00001).

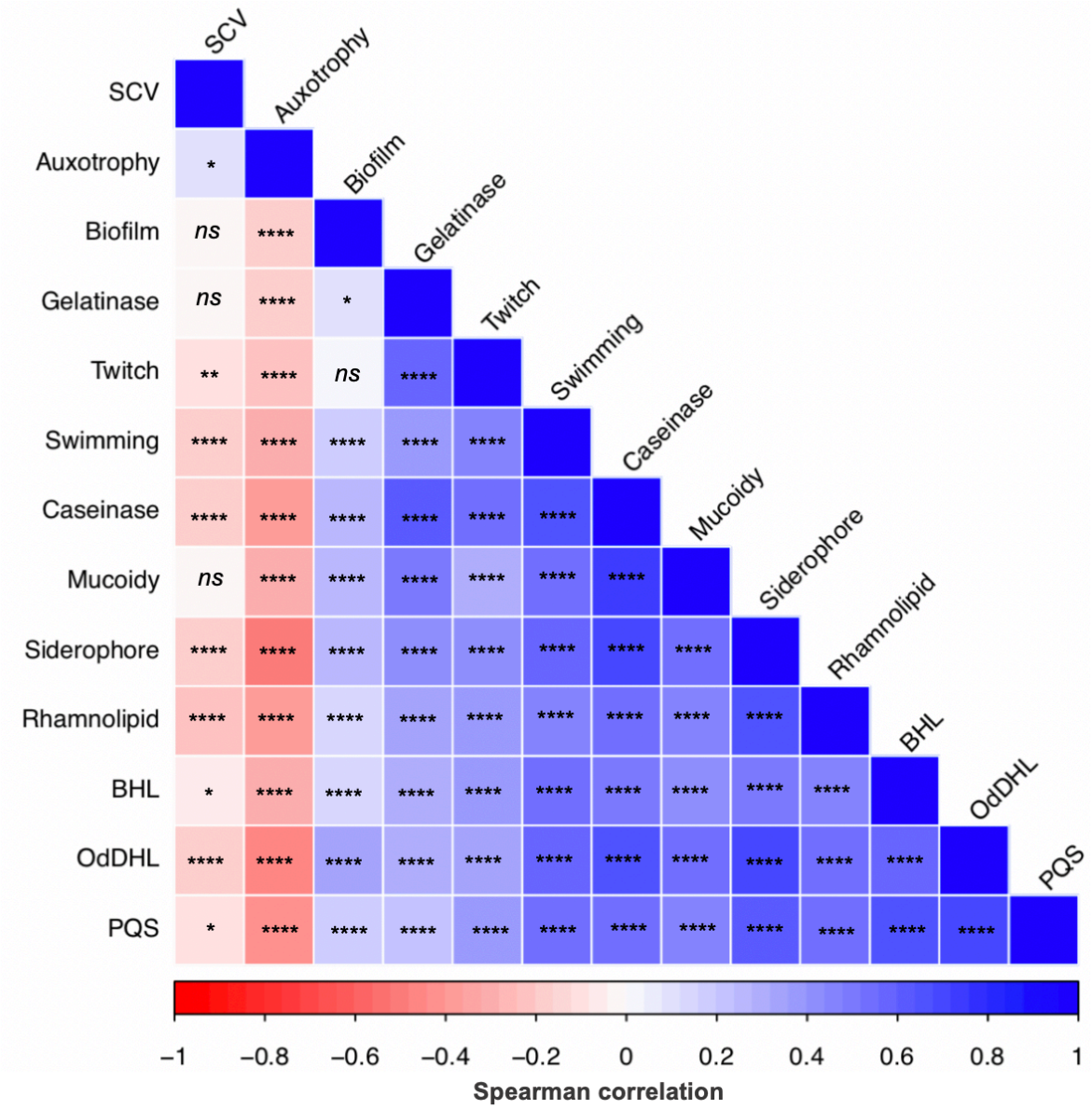


Figure 4.16. Correlation matrix of phenotype-phenotype associations for the MES isolates as determined by Spearman rank correlation coefficient. Blue indicates strong positive correlation between any given phenotype pair (Spearman rank coefficient closer to 1), red indicates strong negative correlation between any given phenotype pair (Spearman rank coefficient closer to -1) and white indicates no correlation between phenotype pairs (0). Correlations were significant if r >0.35 and p <0.05 (*). *ns* = not significant, ** = p <0.001. ***p <0.0001, **** = p <0.00001. SCV: small colony variant, OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

Interestingly, the production of certain virulence factors was found to be associated with the mucoid morphotype in this cohort, with positive correlations identified between mucoidy and caseinase (Spearman r = 0.73, p <0.00001), or with gelatinase (Spearman r = 0.5, p <0.00001), or with swim motility (Spearman r = 0.53, p <0.00001) or with siderophore production (Spearman r = 0.56, p <0.00001).

However, again unexpectedly, mucoid phenotype was poorly associated with biofilm formation (Spearman r = 0.26, p <0.0.0001). Although caution must be applied to interpretation of these results because mucoidy was assessed via visual inspection rather than by quantitation.

In contrast to findings with the LES cohort, auxotrophic metabolism was modestly negatively correlated with virulence-associated phenotypes among the MES cohort. These observations may suggest a trade-off between metabolic fitness and the maintenance of virulence-associated phenotypes among the MES isolates, as compared to their LES counterparts.

However, for the non-epidemic cohort of isolates, only five significant phenotype-phenotype associations were identified (Figure 4.17). Moderate positive correlations were observed between caseinase and gelatinase production (Spearman r = 0.67, p <0.00001) and between swim and twitch motility (Spearman r = 0.67, p <0.00001), whereas weaker positive correlations were identified between rhamnolipid and gelatinase production (Spearman r = 0.41, p <0.00001), and with caseinase production (Spearman r = 0.39, p <0.00001). In contrast to the MES cohort, gelatinase production was also found to be negatively associated with the mucoid morphotype (Spearman r = -0.46, p <0.00001).

These observations demonstrate that, in similarity with their LES counterparts, non-epidemic isolates displayed a considerable degree of phenotype independence. Furthermore, the lack of association between QS signal production and presence/absence of any of the phenotypes challenges the typical understanding of QS-regulated virulence production and further highlights the genetic complexity that underpins phenotypic expression in clonal isolates of *P. aeruginosa*.

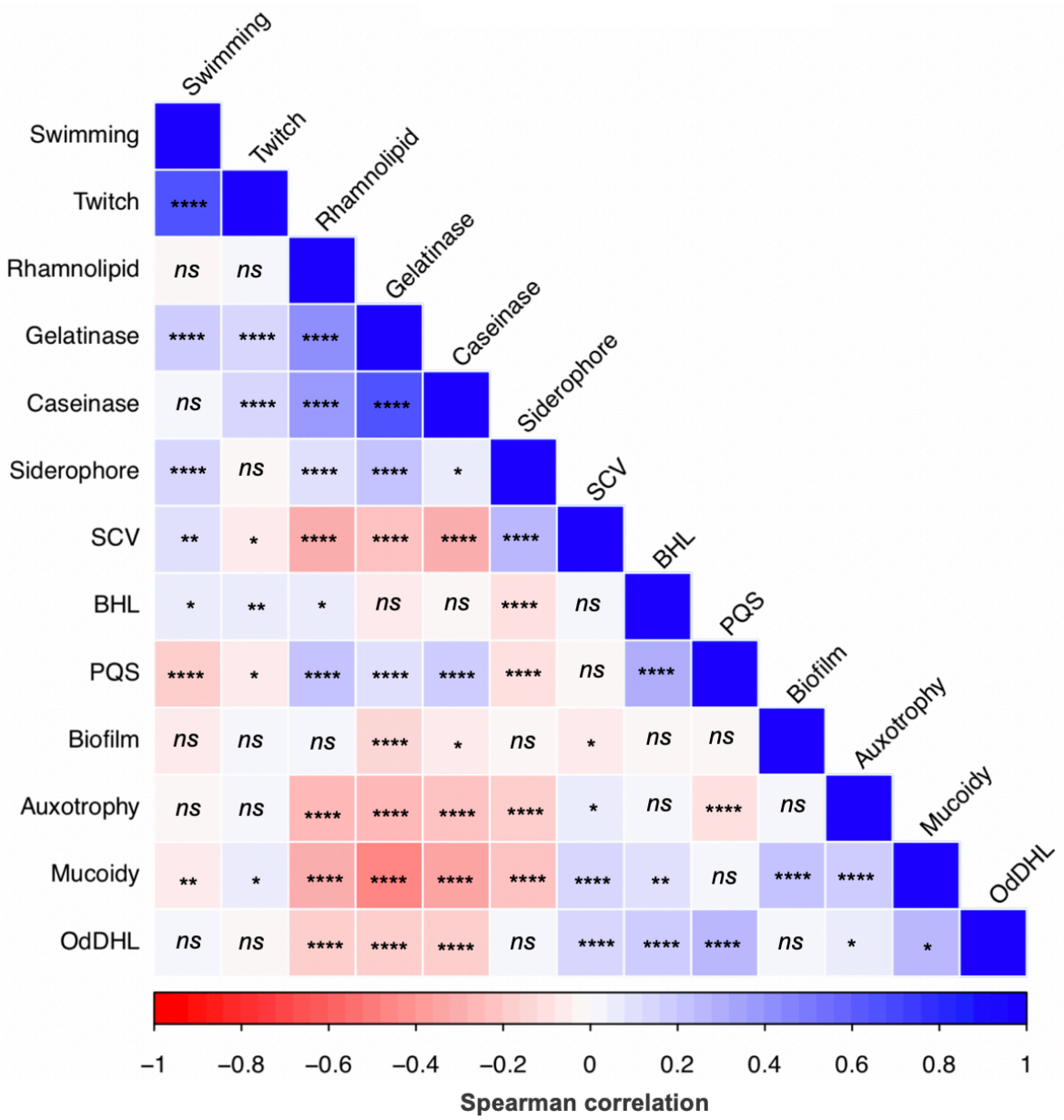


Figure 4.17. Correlation matrix of phenotype-phenotype associations for the non-epidemic isolates as determined by Spearman rank correlation coefficient. Blue indicates strong positive correlation between any given phenotype pair (Spearman rank coefficient closer to 1), red indicates strong negative correlation between any given phenotype pair (Spearman rank coefficient closer to -1) and white indicates no correlation between phenotype pairs (0). Correlations were significant if r >0.35 and p <0.05 (*). *ns* = not significant, ** = p <0.001. ***p <0.0001, **** = p <0.00001. SCV: small colony variant, OdDHL: *N*-3-oxo-dodecanoyl-homoserine lactone, BHL: *N*-butanoyl-homoserine lactone, PQS: *Pseudomonas* quinolone signal.

4.3.8 Covariation of functional phenotypes in CF-evolved clonal isolates of *P. aeruginosa*

I next wanted to investigate to what extent the multiple phenotypes in this large dataset covaried. To address this I conducted principal component analysis (PCA) on 9 of the 13 phenotypic traits. The traits selected accounted for QS signal production and several virulence-associated factors. Qualitative traits (e.g., SCV morphotype, mucoid morphotype, rhamnolipids and auxotrophy) were excluded from this analysis. The first two principal components, with eigenvalues greater than 1.3, explained approximately 54% of the overall variation in the selected phenotypes. PCA did not separate the clinical isolates into clusters independent of their patient of origin. However isolates from patients K1 and K3 did largely group separately from isolates from patients K7, K9, K11 and K15. In addition, tight clustering of several patients' isolates suggested low diversity in phenotype composition within that cluster. The PCA confirmed that isolates from patient K14 displayed the greatest phenotypic diversity, with phenotypic profiles much more divergent between the strains.

Although the total amount of variation explained by the global PCA was relatively low, several relationships could be inferred by the direction of the variable vectors on the PCA plot (Figure 4.18). In general, production of the secreted exoproducts (caseinase, siderophore and gelatinase) was strongly associated together, whereas QS signal production (OdDHL, BHL and PQS) was positively correlated with the motility phenotypes (swim and twitch). Furthermore, the PCA confirmed that higher exoproduct production was associated with a reduction in motility, and, exoproduct produciton was largely independent of whether isolates were PQS-secreting. Instead, exoproduct production was more closely associated with BHL production.

4.3.9 Differences in phenotype covariation in relation to strain epidemicity

I also performed separate PCAs on the 9 phenotypes after classifying the isolates according to the presence/absence of an epidemic strain type (LES, MES or non-epidemic), in order to determine whether the phenotypes covaried differently between epidemic and non-epidemic clonal populations. Interestingly, plotting of loading biplots for the first two principal components, with eigenvalues greater than 1.6, 1.2 and 1.5,

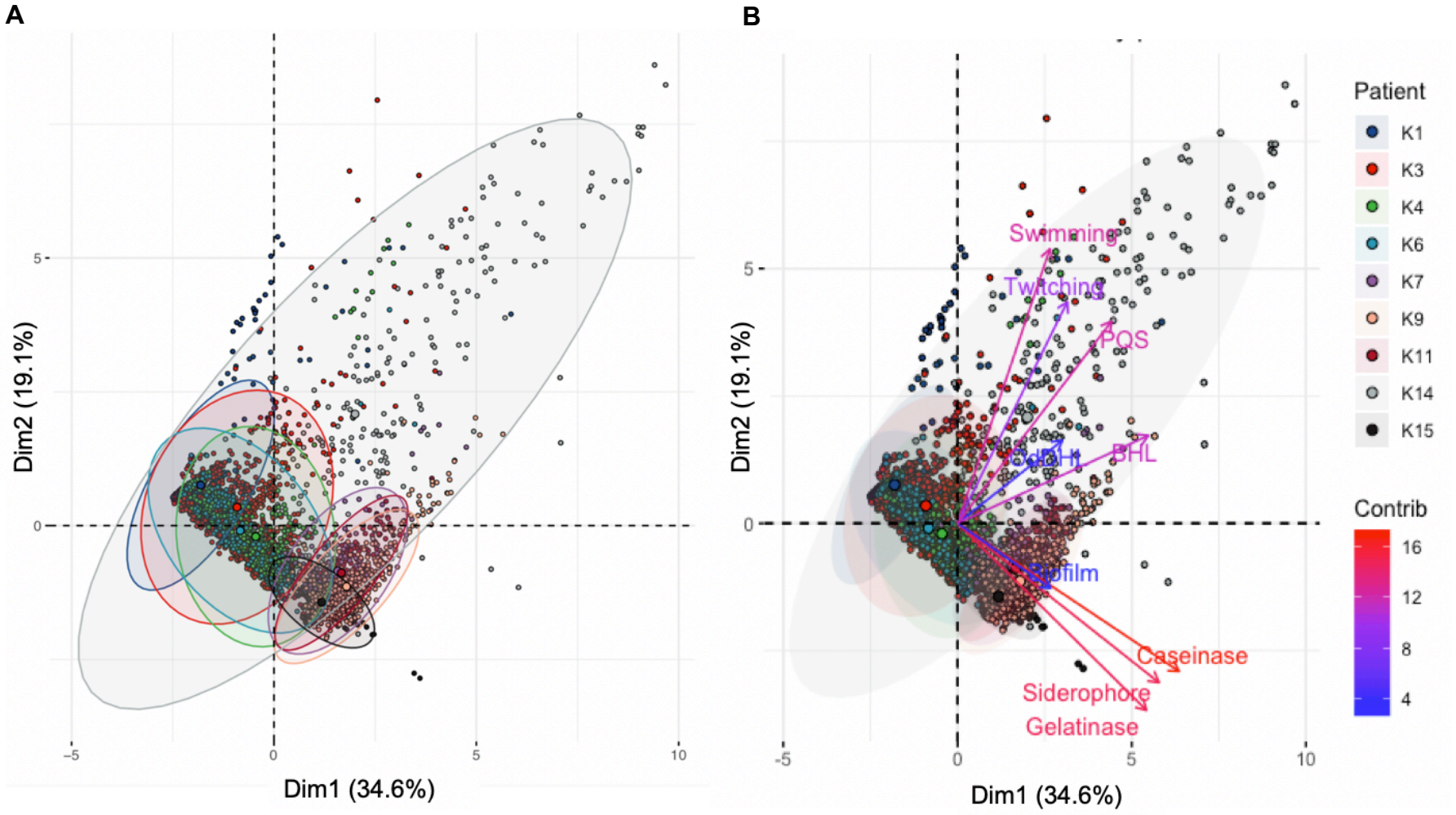


Figure 4.18. Principal component analysis of the nine measured quantitative phenotypic traits. (A) The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. (B) The first two components (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis.

for the LES, MES and non-epidemic cohorts respectively, revealed some differences in phenotype co-occurrence between the groups.

Based on PCA, clustering of phenotypic profiles among the LES isolates did not reveal unique groupings, with phenotypic patterns closely shared between isolates from different patients (Figure 4.19A). The amount of total variation in the LES phenotypes explained by the the first two principal components of the PCA was very low (40.3%) (Figure 4.19B). Nevertheless, the presence of motility and secreted virulence factors was tightly associated, which is reflected in the close association of the vectors of these variables on the PCA biplot. Interestingly, these traits were negatively correlated with biofilm formation, suggesting a tradeoff in production against each other in the LES cohort. Furthermore, and consistent with the results from the pairwise Spearman's rank correlation, QS signal production was poorly associated with biofilm formation, production of virulence exoproducts and motility in this cohort.

The PCA of the MES-associated phenotypic data, revealed that isolates from patient K14 displayed greater diversity in phenotypic profiles than isolates from patient K1. This is reflected in the looser clustering of individual isolates from patient K14 on the PCA plot (Figure 4.20A). The first two principal components in the PCA explained a reasonable amount (66%) of the total variation in the MES-associated dataset (Figure 4.20B). In contrast to the LES cohort, QS signal production was closely associated with swim motility, caseinase and siderophore secretion. Similarly, twitching and gelatinase secretion were closely associated although, unlike with the LES isolates, these phenotypes were less strongly correlated with siderophore and caseinase production. In addition, biofilm formation appeared to be expressed independently of protease production and twitching motility.

The PCA of the phenotypic data corresponding to the non-epidemic isolates (from patients K3, K4 and K6), revealed that the latent phenotypic expression patterns did not differentiate between clonal isolates from different patients (Figure 4.21A). The first two principal components explained only 39% of the total variation in the phenotypes (Figure 4.21B), which is very low. However, the PCA did show different

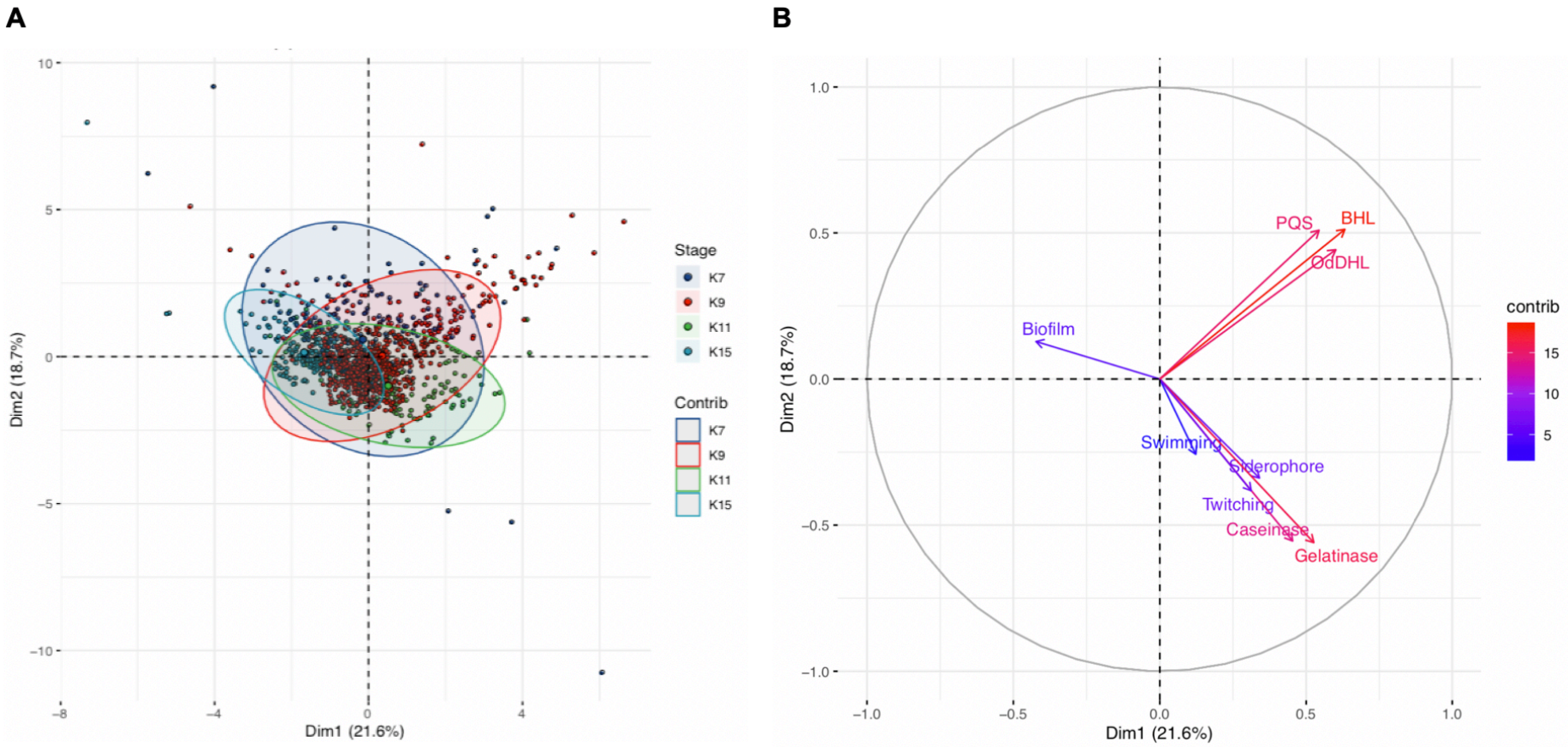


Figure 4.19. Principal component analysis of the expression profiles for the nine measured phenotypes in the LES isolates, collected from patients K7, K9, K11 and K15 (*n* = 1402). (A) The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. (B) The first two components (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis.

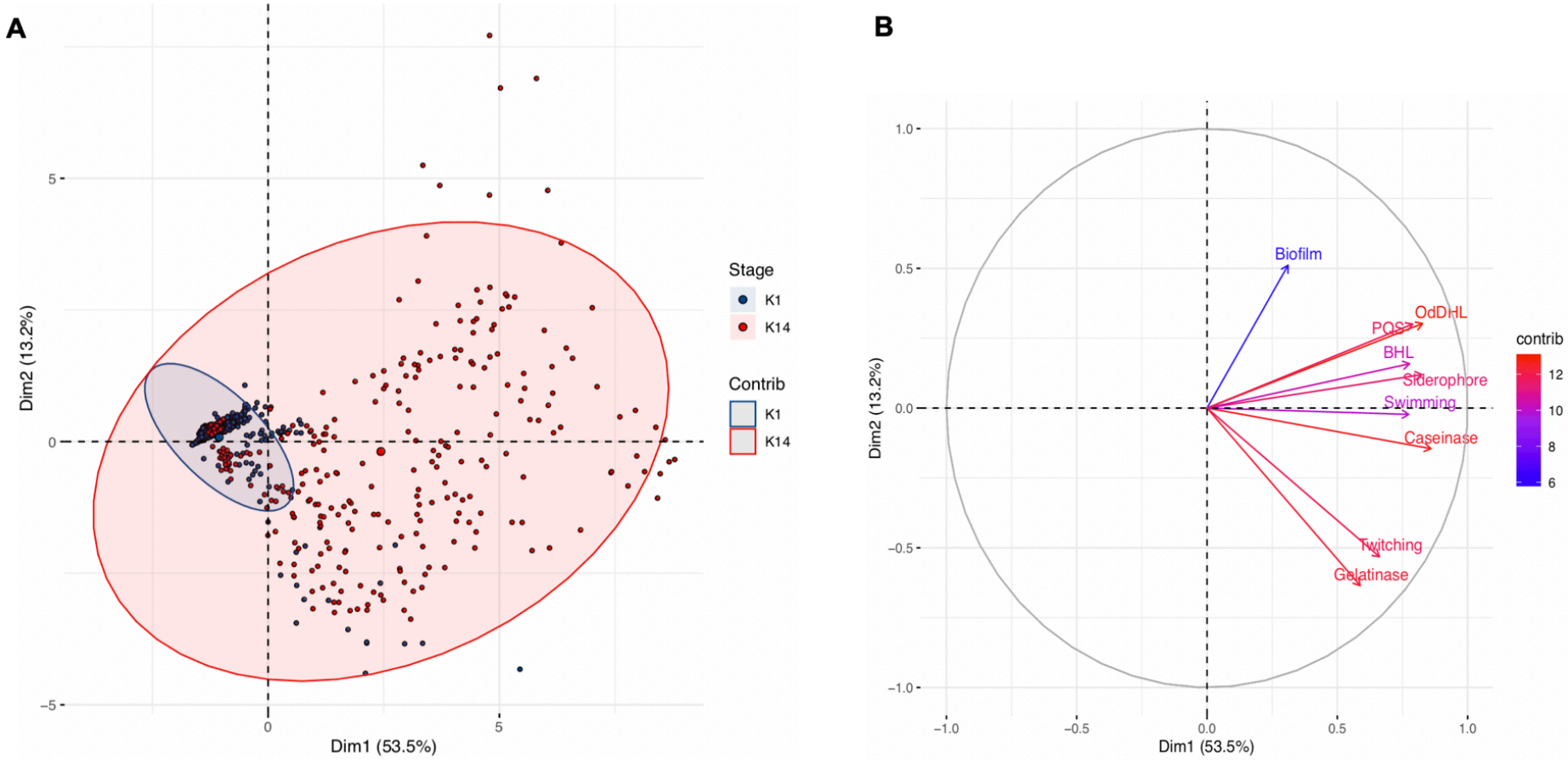


Figure 4.20. Principal component analysis of the expression profiles for the nine measured phenotypes in the MES isolates, collected from patients K1 and K14 (*n* = 990). (A) The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. (B) The first two components (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal

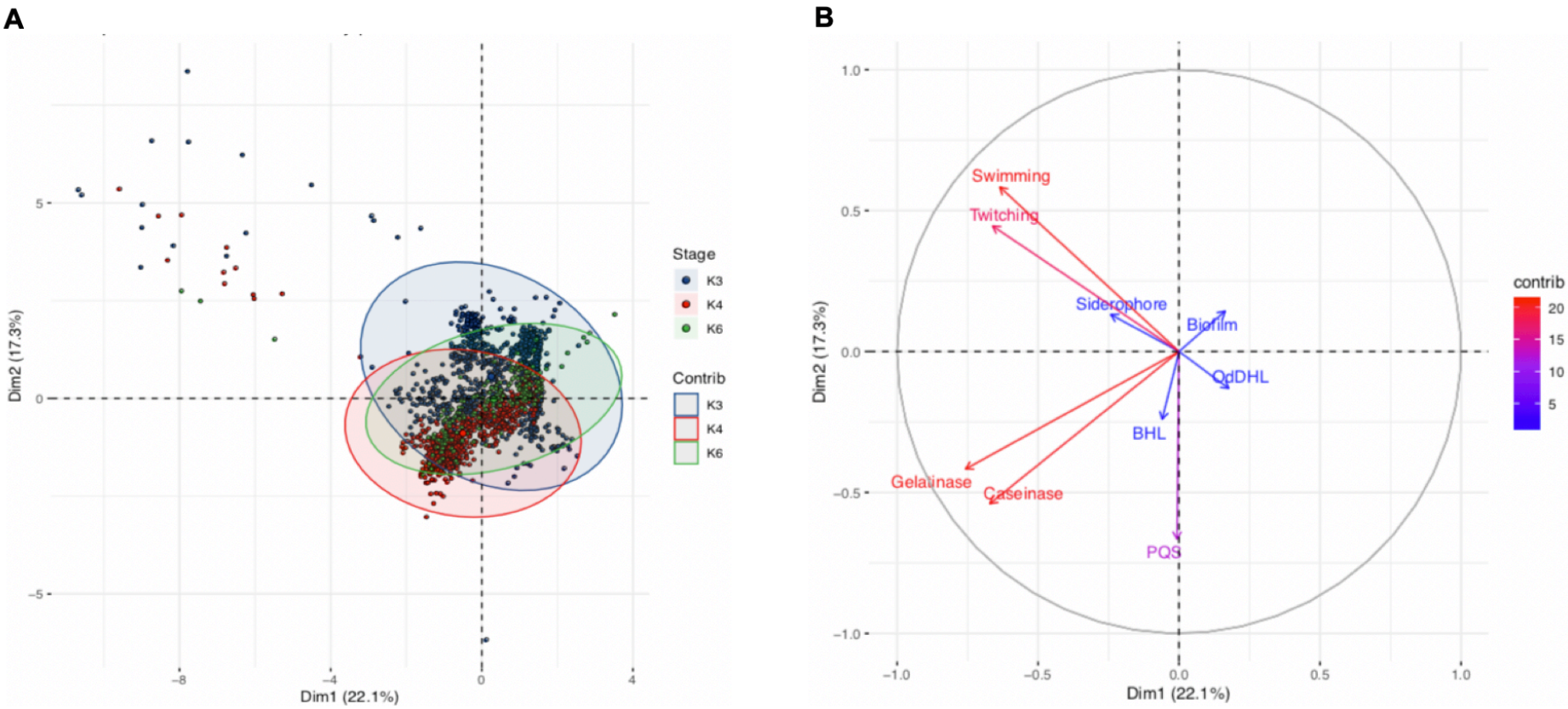


Figure 4.21. Principal component analysis of the expression profiles for the nine measured phenotypes in the non-epidemic isolates, collected from patients K3, K4 and K6 (*n* = 1961). (A) The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. (B) The first two components (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis.

patterns in co-varying phenotypes compared with the epidemic isolates. Overall, fewer of the phenotypes expressed by the non-epidemic isolates covaried together. In addition, although strong correlations were observed between commonly co-occuring phenotypes (eg. gelatinase with caseinase or swim with twitch motility), variable correlations were observed between these phenotypes and QS signal production. For example, BHL production was only weakly associated with protease production, and the motility phenotypes were negatively correlated with OdDHL production. Furthermore, in contrast to the MES isolates, but in accord with the LES isolates, protease production was also inversely correlated with biofilm formation.

4.3.10 Differences in phenotype covariation in relation to antibiotic treatment episodes for APE

I next wanted to determine whether co-variation of selected virulence-associated phenotypes differed at times of APE and if so, to what extent. Clonal sub-populations expressing, for example, differential QS signalling, invasive exoproducts and preserved motility may predominate during clinical decline compared with periods of clinical stability (non-exacerbation) or following recovery after administration of antibiotic therapy. To investigate whether this was the case I carried out PCA on all isolates, grouped in relation to APE (e.g., non-exacerbation isolates, exacerbation isolates and recovery isolates).

PCA confirmed that non-exacerbation isolates (Figure 4.22A) displayed greater dispersion in phenotypic profiles compared with exacerbation (Figure 4.22B) and recovery isolates (Figure 4.22C). This suggested reduced diversity in phenotypic composition among clonal isolates of *P. aeruginosa* during periods of exacerbation and following recovery. This fall in diversity during periods of exacerbation might reflect the emergence of dominant clones with distinct phenotypic profiles better capable of inducing acute deteriorations (in the setting of chronic infection) and withstanding antibiotic treatment.

However, at least half of the total variation in the phenotypic profiles, explained by the PCA for each cohort, was driven by covariation in similar phenotypes. In particular, swimming and twitching motilities were strongly correlated together in each of the cohorts. Similarly, protease and siderophore production were closely correlated

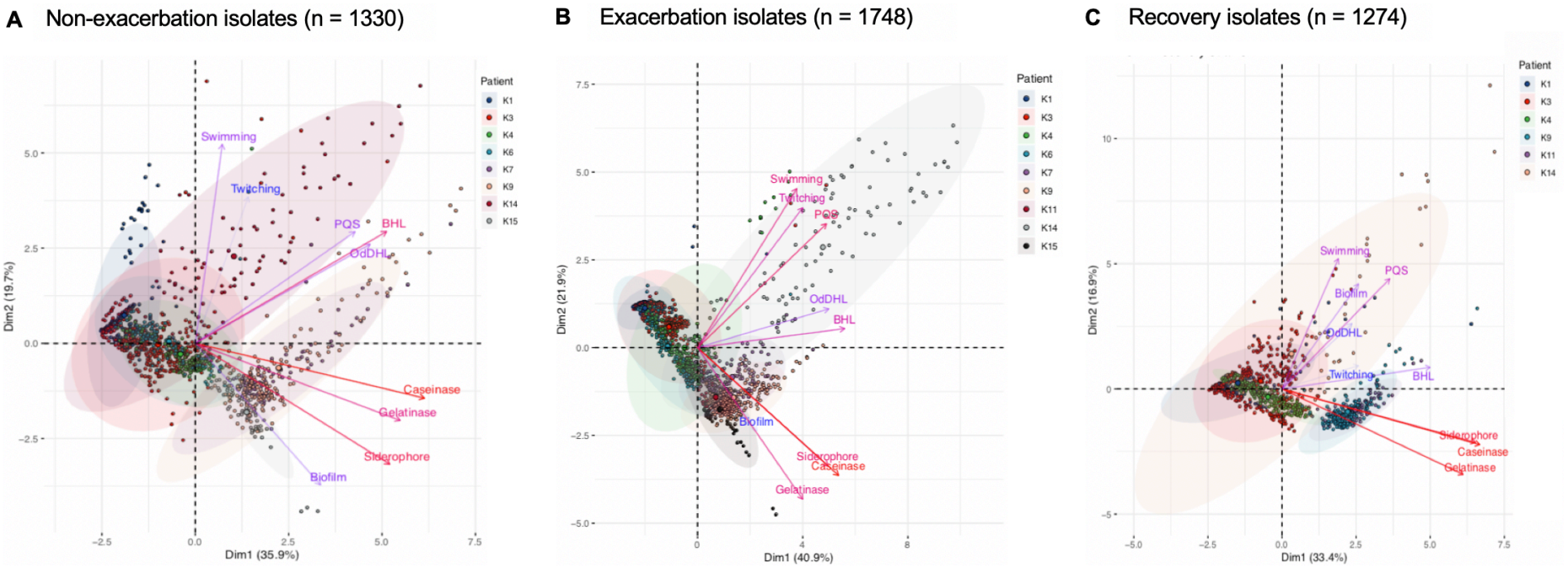


Figure 4.22. Principal component(s) analysis of the expression profiles for the nine measured phenotypes collected from nine CF patients during periods of (A) non-exacerbation, (B) acute pulmonary exacerbation and (C) recovery from acute pulmonary exacerbation following antibiotic treatment. The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. The first two components are shown (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis. Isolates were not recovered from every period for each participant. Patient K11 did not contribute any non-exacerbation isolates and patients K6, K7 and K15 did not contribute any recovery isolates.

together whilst neither protease nor siderophore production was well correlated with swimming motility in each of the groups. The PCA did not show a separation of isolates, based on the emergence of a dominant phenotype cluster, during an APE.

Nonetheless, PCA did highlight some differences in phenotype co-variation between non-exacerbation, exacerbation and recovery isolates. Biofilm production was associated with production of virulence exoproducts among the non-exacerbation isolates, less so among the exacerbation isolates, and not at all among the recovery isolates. Furthermore, the biplots of the non-exacerbation and exacerbation isolates showed that the phenotypic profiles of patient K1's isolates (lacking in production of siderophores, proteases, QS signal molecules, motility and biofilm) contrasted with those of isolates from patients K7, K9 and K15 (biofilm forming, protease- and siderophore-producing), whereas patient K3, K4 and K6's isolates shared phenotypic profiles which overlapped with isolates from all of the patients. Similarly, the biplot of the recovery isolates confirmed shared phenotypic profiles for isolates recovered from patients K1, K3, K4 and K14, which were distinct from the phenotypic profiles shared by the K9 and K11 isolates. These observations suggested that phenotypic co-variation might be influenced by strain type. To explore if this was the case I further grouped isolates according to the presence/absence of an epidemic strain type (e.g., LES, MES and non-epidemic strains). Exhibition of swimming and twitching motilities were excluded from the PCA of the LES exacerbation isolates whereas twitching motility was excluded from the PCA for the LES recovery isolates. Exclusion was on the basis of a lack of variation in these phenotypes with the majority of isolates deficient in the phenotype.

The top two principal components, with eigenvalues greater than 1.7, 1.6 and 1.8, explained, respectively, 47.3%, 50.8% and 53.8% of the total variation in phenotypes for, the non-exacerbation, exacerbation and recovery LES isolates (respectively). Although the PCAs explained a low amount of the total variation in each of the isolate groups several interesting correlations were noted.

Among the non-exacerbation LES isolates (Figure 4.23A), the PCA confirmed a tight correlation between production of all three QS signalling molecules, with isolates exhibiting high OdDHL, BHL and PQS production likely to be low producers of biofilm.

In this sub-group, gelatinase production, moreso than motility, was associated with QS signal production. In addition, biofilm formation was negatively, albeit weakly, correlated with gelatinase activity and high production of QS signal. Interestingly, non-exacerbation isolates from patient K15 were typically of a “high biofilm and low QS signal and exoproduct” phenotypic profile. This differed from the phenotypic profiles of the K7 and K11 non-exacerbation isolates, which tended towards lower biofilm but higher QS signal and exoproduct production. Principal component analysis of the phenotypes associated with the exacerbation LES isolates (Figure 4.23B) confirmed positive correlations between production of all three of the QS signalling molecules. However, QS signal production was not well correlated with either siderophore or protease secretion in this sub-group. In addition, biofilm formation was weakly negatively correlated with these virulence-associated phenotypes. Overlapping clusters on the PCA confirmed that phenotypic profiles were shared among the acute LES isolates, irrespective of the patient background.

For the recovery LES isolates, PCA revealed a separation of isolates from patient K9 and K11 (Figure 4.23C), suggesting that these isolates did not share similar phenotypic profiles. In general, K9 recovery isolates were biofilm forming, with preserved BHL and OdDHL production. In contrast, K11 isolates were comparatively deficient in biofilm formation but proficient in twitch motility, caseinase, siderophore and PQS production. Furthermore, exacerbation and recovery LES isolates showed substantially higher phenotypic diversity compared to non-exacerbation LES isolates, even though there were some areas of overlap among the exacerbation LES isolates.

Performance of separate PCAs on the MES-associated phenotypes after classification by clinical state, revealed that the eigenvalues for the first two principal components were greater than 1.6, 0.86 and 1.6 for the non-exacerbation, exacerbation and recovery MES isolates respectively. Furthermore, a reasonable amount of the total variation in the phenotypes at each clinical period was explained by the first two principal components: 60.5%, 79.9% and 61.9% for the non-exacerbation, exacerbation and recovery MES isolates respectively (Figure 4.24).

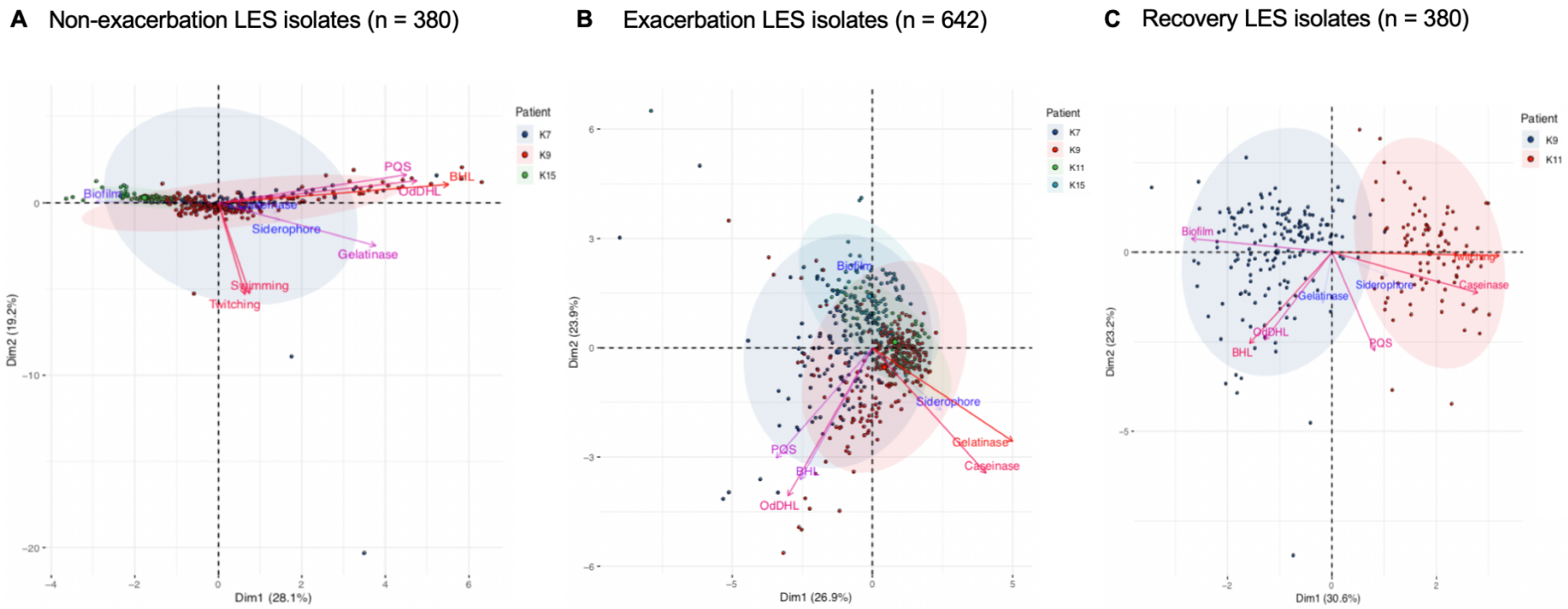


Figure 4.23. Principal component(s) analysis of the expression profiles of the LES isolates for the nine measured phenotypes collected from four CF patients during periods of (A) non-exacerbation, (B) acute pulmonary exacerbation and (C recovery from acute pulmonary exacerbation following antibiotic treatment. The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. The first two components are shown (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis. Patient K11 did not contribute any non-exacerbation isolates and patients K7 and K15 did not contribute any recovery isolates to this analysis.

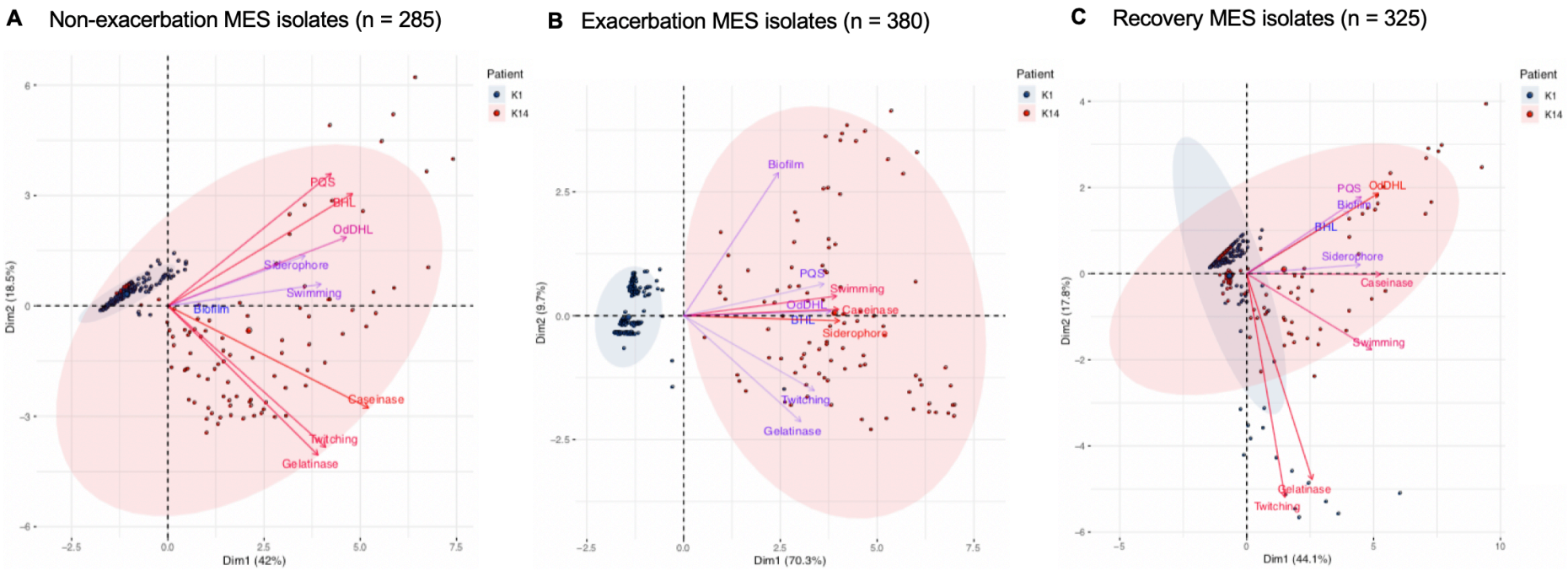


Figure 4.24. Principal component(s) analysis of the expression profiles of the MES isolates for the nine measured phenotypes collected from two CF patients during periods of (A) non-exacerbation, (B) acute pulmonary exacerbation and (C) recovery from acute pulmonary exacerbation following antibiotic treatment. The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. The first two components are shown (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis.

As noted previously, much greater diversity in the composition of phenotypic profiles was observed among isolates from patient K14 compared with isolates from patient K1 with K1 isolates largely deficient in the phenotypes assessed.

Quorum signal production, siderophore production and swim motility were correlated together among the non-exacerbation MES isolates (Figure 4.24A). Similarly, protease production was correlated with twitching motility but these phenotypes co-varied independently of QS signal production.

In contrast, among the exacerbation MES isolates (Figure 4.24B), although QS signal production, siderophore production and swim motility remained well correlated, these phenotypes were now positively associated with caseinase production, whereas exhibition of twitch motility and gelatinase production co-varied together and were less strongly associated with the other phenotypes.

For the recovery MES isolates (Figure 4.24C), PCA confirmed an association between biofilm formation and production of QS signal molecules, whilst no correlation was found between the presence of these phenotypes and the presence of motility phenotypes or gelatinase production. Siderophore and caseinase production remained well correlated, but less so with QS signal production, motility or gelatinase production.

Finally, I carried out PCA on the non-exacerbation, exacerbation and recovery isolates recovered from patients harbouring a non-epidemic strain of *P. aeruginosa*. The first two principal components of the PCA of the non-exacerbation isolates, with eigenvalues greater than 1.7, explained 43.5% of the total variation in the phenotypes (Figure 4.25A). Although this was low, PCA did confirm a positive correlation between the motility phenotypes, and between protease secretion and PQS production. A negative correlation between high protease secretion and high biofilm production could also be inferred. Furthermore, no relationship between protease production and either BHL production or motility could be inferred by the PCA.

Principal component analysis on the non-epidemic exacerbation isolates showed some differences in the pattern of co-varying phenotypes compared with the non-

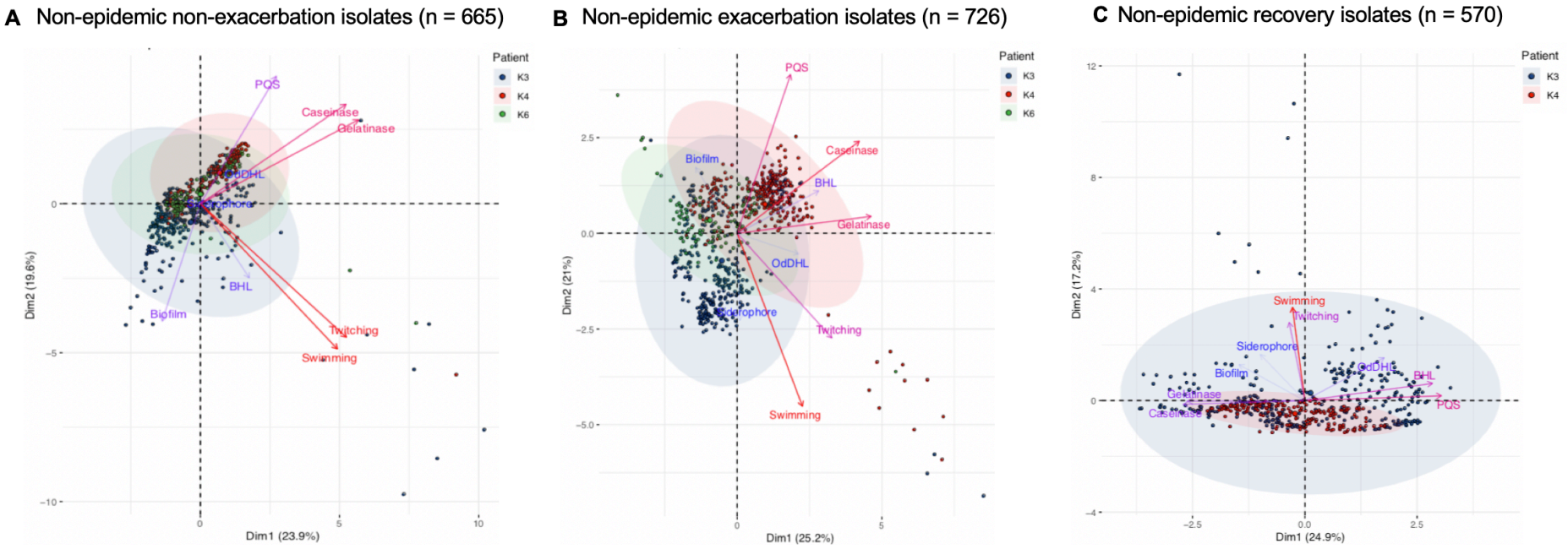


Figure 4.25. Principal component(s) analysis of the expression profiles of the non-epidemic isolates for the nine measured phenotypes collected from three CF patients during periods of (A) non-exacerbation, (B) acute pulmonary exacerbation and (C) recovery from acute pulmonary exacerbation following antibiotic treatment. The data points (individual isolates) are coloured according to the source patient. Ellipses are drawn to show clusters of patients' isolates. The shaded areas represent the 95% confidence ellipse for each cluster. The first two components are shown (Dim1 and Dim2) and the amount of variation (in percent) explained by each principal component is shown on the axis. Patient K6 did not contribute any recovery isolates to this analysis.

exacerbation isolates. The first two principal components, with eigenvalues greater than 1.8, explained 46.2% of the total variation in the phenotypes (Figure 4.25B). Again, the amount of variation in phenotypes explained by the PCA was low. However, it did confirm a weakly positive correlation between protease secretion and BHL production. Although a negative correlation between biofilm formation and the motility phenotypes, as well as between OdDHL, could be inferred, the association was relatively weak. PQS, caseinase and BHL secretion also appeared to be expressed independently of the motility phenoptypes.

Principal component analysis on the non-epidemic recovery isolates revealed further differences in co-varying phenoptypes in comparison to the other two cohorts (Figure 4.25C). The first two principal components, with eigenvalues greater than 1.5, explained 42.1% of the total variation in the phenotypes. Positive correlations were confirmed between protease and biofilm production, with isolates stongly expressing these phenotypes generally exhibiting weak QS signal production. Furthermore, no relationship could be inferred between QS signalling and motility, nor between motility and protease production in this subgroup.

4.4 DISCUSSION

Substantial phenotypic diversity has previously been described in CF-evolved clonal isolates of *P. aeruginosa* collected from patients chronically infected with this organism, however, relatively little is known about the short-term variation in the exhibition of phenotypic traits typically associated with *P. aeruginosa* virulence regulation and production, and how such variation may relate to acute periods of respiratory deterioration in individuals with CF. Existing studies that account for periods of acute exacerbation have focused solely on single strain clonal populations (hindering broader strain-strain comparisons) and have been limited either by low sampling depth and range of phenotypic traits examined (possibly under-estimating the degree of diversity present), or by the study of isolates taken only from a single patient (limiting wider generalisability) (121,325,326).

In this work, I have endeavoured to address these deficiencies by expanding on the depth of sampling, range of phenotypes characterised, study population size and distribution of strain types investigated, including accounting for the most prevalent strain types both within the UK (LES and MES) and worldwide (LES). This study, therefore, represents the largest such study of comparative phenotypic analyses in CF-evolved clonal isolates of *P. aeruginosa* to date, and offers an expansive picture of the inter-relationships between traditionally considered virulence-associated phenotypic expression, including QS signalling, and how this may relate to acute exacerbation events. My observations provide strong phenotypic evidence for the uncoupling of the traditional QS regulatory hierarchy in CF isolates of *P. aeruginosa* with the *rhl* subsystem playing a more central role in virulence expression in certain strain types. Importantly, APEs could not be linked to the emergence of a particular sub-population of morphotypic or phenotypic variants.

In line with previous work on the LES, Prairie epidemic strain (PES) and the unique strain ST-274, extensive morphotypic diversity was observed both between samples from the same patient, and between isolates from different patients (271,325–327). However, in this study, comparative analysis of morphotypic variation between epidemic (LES and the MES) and non-epidemic clones revealed significantly lower morphotype diversity among the LES, with a significant predominance of small colony variants in this population. The observation of a reduction in morphotype diversity, accompanied by dominance of the SCV colony morphotype, among the LES isolate population suggests a selection for and maintenance of this morphotype variant in this particular strain. Although attribution of causality is difficult, the association of the SCV morphotype with enhanced potential to form biofilm and increased antimicrobial resistance, particularly to aminoglycosides, is well recognised and may account for its increased prevalence among the LES, a strain notorious for its high antimicrobial resistance profile (333,473,474). Nonetheless, this is the first study to report a definitive association of this morphotype variant with the LES.

By contrast, despite its description as a pathognomonic trait of chronic *P. aeruginosa* infection in CF, the prevalence of mucoidy, a consequence of alginate overproduction, was unexpectedly low in this study. The most plausible explanation for this is the instability of the mucoid phenotype outside of the CF lung, particularly during growth

under anaerobic conditions, with *in vitro* reversion a commonly described phenomenon (475,476). Typically, such mucoid revertants maintain the original mutation in *mucA*, with reversion to the non-mucoid phenotype achieved via acquisition of second-site suppressor mutations in genes involved in the regulation of alginate biosynthesis, most commonly in *algT/U,* an alternative sigma factor AlgT/U essential for activation of alginate biosynthesis (476,477). In addition to alginate overproduction, establishment of chronic *P. aeruginosa* airway infection is phenotypically characterised by a loss of flagellar-based motility (324). Moreover, flagellum expression has been shown to be repressed by AlgT in mucoid variants, through inhibition of the flagellar regulator *fleQ* (179,388,393). However, in this study I found substantial evidence for the presence of mucoidy without loss of flagellar-based motility, predominantly among isolates recovered from patient K14. Either, alginate overproduction is insufficient alone to lead to loss of flagellar-based motility, or acquisition of secondary site mutations in *algT*, triggered by variations in environmental conditions during high-throughput screening, have led to re-activation of flagellar expression in these mucoid variants.

Quorum sensing plays a dominant role in the regulation of *P. aeruginosa* virulence factor production, as evidenced by attenuated virulence of constructed QS-deficient mutants in animal studies (166,478). *In vitro* studies in the laboratory strain PAO1 had originally shown the three-system QS regulatory network to operate in a hierarchical manner, with the *las* signalling system on top, controlling activation of the RhlR and PQS pathways. However, evidence for the uncoupling of this hierarchical model continues to emerge from studies on clinical and environmental isolates. For example, the identification of *lasR* mutants, with preserved expression of LasR-associated phenotypes, among *P. aeruginosa* isolates taken from chronically infected CF patients is not uncommon (177,184,363,364,479).

Several findings in this work provide additional evidence in support for a ‘re-wiring’ of the traditional model of QS regulation of *P. aeruginosa* phenotypes in CF-evolved isolates. I found a high prevalence of OdDHL-deficient isolates, in line with estimates from a previous, albeit much smaller, study (180). A logical assumption from loss of OdDHL production is the presence of loss-of-function mutations in *lasI.* However, in contrast to *lasR* mutants, *lasI* mutants are rarely reported in association with deficient

OdDHL production among CF isolates of *P. aeruginosa* (180,184). D'argenio *et al*. has shown that absent LasI-generated signal may be consistent with mutations in *lasR,* along with large colony morphotype and iridescent sheen, features that can be masked by more severe colony morphotypes such as SCV or mucoidy (184). On the other hand, OdDHL-deficient isolates, usually with accompanying loss in BHL production, have been described, and associated with mutations in *rhlI* (with or without mutations in *lasI*) (177). For example, a study by Bjarnsholt *et al.* examined the *lasI* and *rhlI* genes of 135 CF-evolved *P. aeruginosa* isolates deficient in OdDHL production and found conservation of the wild-type *lasI* sequences among all but one of the isolates, whilst the vast majority presented mutations in *rhlI* (363). Intriguingly, these mutations in *rhlI*, in contrast to the study by Wilder *et al.*, were not associated with a loss or reduction in BHL production (177). Nevertheless, if impairment of LasR function can be inferred from loss of OdDHL production, the majority of the isolates in this study did not incur predictable deficiencies in typically LasR-associated phenotypes, in agreement with prior reports (177,180,363,364). A likely explanation for the maintenance of QS-regulated phenotypes in the absence of functional LasR is compensatory activation of LasR-controlled functions by RhlR, given that expression of most QS-associated phenotypes is under the influence of both LasR and RhlR (467). The recovery of *rhlR*-mutant *P. aeruginosa* isolates from chronic infected CF lungs is rare and has been described specifically in hypermutable strains (363). In contrast, preserved *rhl* signalling and expression of QS phenotypes in CF isolates without functional LasR is frequently reported (177,178,180,364). Furthermore, functional studies on both non-CF and CF isolates have provided support for LasR-independent RhlR activity (356,364). For example, Dekimpe and Deziel showed that RhlR activation is only delayed (occurring during stationary phase) in a PA14 *lasR* mutant and that RhlR can initiate production of typically LasR-specific factors, including OdDHL and PQS (356). Later work by Feltner *et al.* also confirmed LasR-independent activation of RhlR in CF-evolved *P. aeruginosa* isolates, although the exact mechanism was not identified (364). Certainly, the retention of RhlI-generated signal by the majority of isolates in this study, likely in the setting of functional RhlR, suggests preferential conservation of this QS pathway thereby underscoring its importance in expression of QS-associated virulence determinants among CF-evolved *P. aeruginosa* isolates. Intriguingly, a substantial proportion of isolates were capable of producing PQS in the absence of either LasI- or RhlI-generated signal. This

observation suggests that in addition to LasR-independent transcriptional activation of *rhlIR*, PqsR-dependent activation of the *pqsABCDE* operon, required for the synthesis of the precursor of PQS, may not require input from the *lasI/lasR* signalling pathway.

Given the arguments made in favour of RhlI-RhlR in the maintenance of QS-regulated phenotypes when functional LasR is absent it must be highlighted that clonal isolates from patient K1 distinctly lacked expression of all phenotypes examined, despite a significant proportion retaining production of BHL. I considered isolate non-viability as an explanation, given that K1 isolates more often failed to grow on different assay media in comparison with their counterparts from other patients. However, growth failure on all assay media by the same isolate (a surrogate measure for isolate viability in the setting of high-throughput screening) was only observed in 13% of K1 isolates. Accounting for true loss of parental isolates, a plausible explanation for the marked loss in OdDHL and PQS secretion, protease activity, rhamnolipid production and biofilm formation observed might be mutations within a global regulator of QS such as VqsR (virulence and quorum-sensing regulator). Although constitutively expressed at low levels during growth in LB medium, inactivating mutations of *vqsR* have been reported to result in loss of production of acyl-homoserine lactones, impairment of proteolytic activity and reduction in rhamnolipid production (480). Furthermore, Juhas *et al.* reported on iron-independent VqsR regulation of siderophore gene expression, with a *vqsR* mutant also exhibiting down-regulation in both pyoverdine and pyochelin biosynthetic genes (480). However, preservation of BHL production in half of the isolates runs contrary to QS phenotypic variation associated with mutant VqsR. An alternate explanation may be the presence of combined mutations in *lasIR* and *rhlR* leading to the observed phenotypic characteristics. This may also better account for the preservation of BHL production seen in some of the K1 isolates. Work done by Dekimpe and Delziel on a PA14 *lasR rhlR* double mutant demonstrated complete loss of proteolytic activity, rhamnolipid and PQS secretion (356). Although siderophore secretion was not analysed in their study, it may be expected that loss of PQS in the double mutant might lead to secondary reduction in siderophore secretion given the role of PQS as a positive regulator of siderophore gene expression. It must also be considered that the extreme down-regulation in virulence-associated phenotypes observed in the K1 isolates may be a consequence of defects in major metabolic biosynthetic pathways, rather than in the QS regulatory system alone. Growth under

laboratory conditions may have exploited growth defects secondary to nutritional requirements better catered for *in vivo*. In all cases, confirmatory growth assays and accompanying gene sequencing and transcriptome analysis would be useful to determine the nature of the genetic determinants impacting on phenotypic expression.

The existence of QS-null variants, isolates deficient in QS signal production but with preserved expression of QS-controlled phenotypes, have been recognised for some time, yet remain a curious conundrum. Such variants have been previously reported among *P. aeruginosa* isolates recovered from mixed infections (urinary tract, wound and lower respiratory infection) or from patients with chronic CF infection suggesting these isolates are still capable of causing acute or chronic infection (177,468). One common theory proposed for the existence of these QS-null isolates is that such variants are "social cheats" and profit from the presence of "common" goods (e.g., extracellular autoinducers), produced by the QS-proficient members of the bacterial community (177,184). In this study, the overall prevalence of QS-null variants was relatively low, with the exception of isolates from patient K3. In this clonal population of isolates almost half were identified as QS-null variants. Surprisingly most retained siderophore production, and close to half retained swim motility and protease production. Curiously, and in contradiction to observations in previous work (177), isolates in this study were not recovered from sputum samples containing mixed populations of QS-null and QS-proficient isolates, but rather all isolates within the same sputum sample were found to be QS-null variants. Whether this reflects sampling of specific sub-populations resident in the CF lung at different timepoints from within the same patient is not clear. Initially, I considered failure of the reporter bioassay as a plausible explanation for my results. However, signal molecule production was maintained by the positive control (PAO1) excluding this possibility. It remains intriguing what QS-independent functions could be involved in setting up chronic infection in the QS-null isolates seen in this current study and further, what genetic mutation(s) may account for the loss of QS signal production observed.

The substantial within-host diversity of virulence-associated phenotypes observed across all isolates in this study is in broad agreement with published work (177,271,326,327). However, amidst the variation observed, certain phenotypes were noted to be globally expressed by all clonal isolates unique to only one patient. This

prompted me to speculate as to the potential genetic drivers leading to uniform presence/absence of a particular phenotype within a clonal isolate population. For example, isolates from patient K4 were distinctive for production of a green colony pigment (when grown on PCN agar), yellow medium discoloration resulting from siderophore secretion on the CAS assay, a predominance of methionine auxotrophs, and absent manifestation of biofilm. It is likely that the green colony pigment expressed is due to pyocyanin production, as this phenazine is known to contribute to the green colour of *P. aeruginosa* cultures (481). The production of pyocyanin in turn implies the presence of functional RhlR (a positive regulator for both phenazine operons) and PqsE (necessary for the induction of each operon), both required for pyocyanin production. The lack of biofilm formation may be linked to the high prevalence of methionine auxotrophs. This association seems plausible based on work done by Joachim *et al* (482). In this study the authors examined biofilm formation by wild-type PA14 and isogenic methionine auxotrophic mutants grown on M9 agar plates containing 30 to 100 μM methionine and Congo red. Congo red staining, indicative of biofilm matrix presence, was observed to be dependent on methionine concentration in the auxotrophic mutant colonies. Furthermore, biofilm formed by the mutant strains in defined medium supplemented with methionine had significantly lower biomass compared with the WT and these biofilms easily dispersed when transferred to methionine-deficient conditions. However, the exact mechanisms underpinning biofilm dependence on methionine availability remain unclear. An alternate explanation for the absent biofilm phenotype observed for the K4 isolates may include loss of pyoverdine production. I inferred pyoverdine loss from the yellow medium coloration produced by siderophore secretion on the CAS agar assay, which contrasted with the orange medium coloration produced by the other isolates (including the WT PAO1) in this study. The colour change associated with the CAS assay can be used to differentiate which siderophore type is secreted by the organism. *P. aeruginosa* secretes two main iron-scavenging siderophores, pyoverdine and pyochelin, under iron-limited conditions. Pyoverdine, as well as activation of its cognate receptor (FpvA), has been shown to be necessary for the development of biofilms *in vitro*, whilst pyochelin is not (408). Therefore, it seems plausible that the presence of pyoverdine-negative mutants among the K4 isolates may also account for the lack of biofilm production among this clonal population.

This study is the largest comparative phenotypic analysis to date between epidemic and non-epidemic isolates local to the UK. Moreover, it is the first study to include for comparison, detailed phenotypic characterisation of clonal isolates of the Manchester epidemic strain. Epidemic clones, comprised of the LES and the MES, were more likely to exhibit protease activity, produce LasI- and RhlI-generated signal, be motile and form biofilm compared with non-epidemic clonal isolates. Moreover, LES isolates were distinguished from both MES and non-epidemic isolates by a higher frequency of isolates with preserved QS signal production, exoproduct secretion (proteases, rhamnolipids and siderophores), biofilm formation and loss of flagellar-based motility. Several longitudinal studies, have demonstrated accumulation of mutations in genes responsible for these virulence-associated phenotypes, suggesting expression is down-regulated in chronic *P. aeruginosa* infection (174,179,181,184,324,483). However, my observations suggest that QS (particularly via RhlI signalling) and QS-associated phenotypes are largely maintained by the LES during chronic CF airway infection. Furthermore, results presented here support observations in prior studies which have described an 'overproduction' phenotype for pyocyanin and LasA protease among some LES isolates (174,444,452). However, as yet, no specific mutation has been identified to explain this overproduction phenotype. Nonetheless, taken together, my findings imply that the maintenance of QS and the expression of phenotypes under QS control in chronic infections of the LES in CF likely contribute towards its enhanced potential for transmissibility and persistence within the CF lung. By comparison, MES isolates were less distinguishable phenotypically from non-epidemic isolates and differed solely on the higher prevalence of isolates with preserved biofilm phenotype and swimming motility. This suggests that the initial differences identified between the epidemic and non-epidemic isolates were driven predominantly by phenotypic features (e.g., genetic configuration) unique to the LES. One important observation highlighted by this analysis is that epidemic strains cannot be assumed to be phenotypically similar, despite their comparable impact on clinical outcomes. Grouping such strains together can falsely ascribe characteristics unique to one strain type to the whole cohort and confound efforts to find a useful phenotypic biomarker.

I observed few pairwise correlations between phenotypes across the cohort of LES and non-epidemic isolates. Previous studies of clinical LES isolates have demonstrated similar observations, and likewise, so has a study of isolates of the PES

where phenotypes were shown to be expressed largely independently of each other (271,326,452). By contrast, across the set of MES isolates multiple phenotype pairs were observed to occur more frequently together in the same isolate than would be expected by chance alone. Positive associations were observed between traits typically considered to be expressed together due to common regulatory pathways (e.g., OdDHL with BHL, rhamnolipids with proteases, OdDHL with proteases, BHL with rhamnolipids). These observations are more in line with a study by Clark *et al.*, which described co-occurrence of phenotypes typically considered associated with earlier rather than chronic *P. aeruginosa* infection (e.g., secreted exoproducts, motility phenotypes) (327). However, Clark's study (which focused on non-epidemic isolates) also described negative associations between phenotypes considered reflective of persistent CF *P. aeruginosa* infection (e.g., biofilm, mucoidy) and those associated with earlier/invasive infection, which was not observed in this work.

PCA on this large dataset confirmed several relationships highlighted in the Spearman rank analyses but also revealed additional associations between phenotypes which differed between the LES, MES and non-epidemic isolates. This work is the first to report specifically on correlations between QS signal molecules and a broad range of QS-associated phenotypes for the LES and the MES. Notably, for the LES isolates, production of all three QS signal molecules was well correlated, but intriguingly, the expression of phenotypes normally considered linked via QS (e.g., exoproteases, siderophores, biofilm formation, swim and twitch motility) was often independent of QS signal release (362,484). I found strong positive correlations between each QS-linked phenotype, with the exception of biofilm formation, which was negatively correlated with these phenotypes. The trade-offs with biofilm formation were not surprising as evidence exists to support a reduction in expression of several of these QS-associated phenotypes during chronic *P. aeruginosa* infection, whereas biofilm formation is preserved (177,184,324,483,485). However, it warrants further investigation whether acquisition of shared mutational events is responsible for the QS-independent expression of phenotypes otherwise understood to be under QS regulation. Certainly, my observations highlight that previously described phenotypic correlations based on studies of laboratory strains of *P. aeruginosa* may not accurately reflect the biology of CF-evolved isolates. Furthermore, from a clinical perspective, a focus on QS targets to manage chronic CF airway infection may prove futile if expression of virulence

determinants is uncoupled from QS, particularly in a strain known to be highly transmissible.

In contrast, for the MES isolates, phenotypic expression was largely linked with QS signal molecule release, with particularly strong associations found between OdDHL, BHL, PQS, caseinase and siderophore production and swim motility. It may be that phenotypic expression among these MES isolates reflects a much shorter duration of clinically defined chronic infection, and that decoupling of QS signal expression from target gene expression has not yet occurred. Alternatively, MES isolates may evolve towards variants with preserved expression of phenotypes under QS regulation during chronic *P. aeruginosa* infection. If this were the case, such selection may contribute to the MES's ability to spread between patients and persist in the CF airway.

Trade-offs were also found between phenotypes expressed by the non-epidemic isolates. These trade-offs are in line with existing evidence in support of both a down-regulation in expression of several of the phenotypes tested and a transition to BHL-associated expression of typically LasIR-regulated phenotypes among clinical isolates during *P. aeruginosa* chronic infection (180). For example, motile and protease-secreting isolates were associated with low production of biofilm whereas protease secretion was associated with BHL but negatively correlated with OdDHL. Overall, key implications from this analysis are that for the LES, QS activities appear to influence the expression of traditionally QS-controlled phenotypes in a non-traditional manner to our current understanding of *P. aeruginosa* biology. However, for the MES neither virulence nor QS signal expression appear attenuated during chronic infection. This is not the case for non-epidemic isolates, where trade-offs exist between traits typically considered persistence-related and virulence-associated.

In this work, I was interested in identifying a dominant phenotypic variant associated with periods of acute pulmonary exacerbation. However, as has been demonstrated by others, despite the broad range of virulence-associated phenotypes assessed no single morphotypic or phenotypic variant distinguished between periods of acute pulmonary exacerbation, relative clinical stability or recovery following treatment with intravenous antibiotics (325–327). Moreover, even among *P. aeruginosa* sub-types recognised for their shared genetic background and enhanced capacity for

transmission, a dominant phenotypic variant was not identified. What is clear from my findings is that the emergence of sub-populations of *P. aeruginosa* phenotypic variants which are more active in the expression of QS-regulated virulence determinants is an unlikely explanation for the transition from relative clinical stability toward APE. Rather, it continues to be apparent that the factors responsible for initiation of an APE are likely to be more complex than a change in single-pathogen phenotypic behaviour. In line with this thinking, recent studies have highlighted the importance of the non-classical pathogens comprising the CF microbiome in driving diversification of *P. aeruginosa* populations (486). Moreover, there is growing evidence to support characterisation of microbial interactions (the interactome) within the CF lung as more effective in identifying key taxa or community functional states which have a stronger influence on fluctuations in clinical status, than examination of simple correlations of taxa abundance of one or several traditional CF pathogens (507, 508). However, whilst inferring complex pathogen-pathogen interaction networks is a crucial step towards determining if they drive APEs, the factors influencing these ecological interactions must also be taken into account, such as the role of nutrient availability, host-microbiome dynamics and environmental factors (e.g., antibiotic exposure). How these factors interplay and impact on the CF microbiome is not well understood but is critical to understanding the degree to which microbiome dynamics are context-dependent (509), which in turn may facilitate the development of patient-specific biomarker signatures and microbial targets for earlier detection and treatment of APEs.

I acknowledge several limitations when interpreting the conclusions made in this study. As others have highlighted (326,327,487), the use of spontaneously expectorated sputum samples may under-estimate the true phenotypic diversity within the lung, due to uncertainty regarding the spatial distribution of sampled populations within the lung. Nevertheless, more invasive sampling (e.g., bronchoalveolar lavage) is impractical for a study of this kind. Moreover, culture-based *in vitro* phenotyping in the manner used in this study cannot adequately replicate the environmental conditions present *in vivo* which therefore may not be a true characterisation of *P. aeruginosa* phenotypic expression. Similarly, the potential for *de novo* acquisition of mutations or selection for particular variants during repeated sub-culturing cannot be excluded. Furthermore, it must also be acknowledged that reliance on a treatment-

defined definition for APE onset may limit detection of significant changes in microbial community behaviour prior to the 'true' onset of these events. Finally, due to the method of high-volume screening adopted, observations have been drawn largely on prevalence of expression and not on quantitative output, which may also lead to an under-estimation of the degree of phenotypic diversity present within.

Nonetheless this study provides a detailed comparative analysis of the extensive phenotypic diversity present in CF-evolved clonal populations of both epidemic and non-epidemic *P. aeruginosa* isolates and how these phenotypes may vary in relation to periods of relative clinical stability, acute pulmonary exacerbation and recovery following IV antibiotics.

5. SMARTCARE: A feasibility analysis of home-monitored physiology and symptoms using smart devices in adults with CF to better understand and predict for changes preceding acute pulmonary exacerbations

Summary:

Sudden deteriorations in lung health, termed acute pulmonary exacerbations, are a major driver of mortality and morbidity in CF. Advanced identification of impending APEs would permit pre-emptive interventions and allow home monitoring to safely replace hospital-based physician consultations.

We enrolled 147 adults with CF into a 6-month study of home monitoring based at seven UK specialist CF Centres. Subjects were asked to undertake daily measurements of lung function, oximetry, pulse rate, weight, and activity (using sensors Bluetooth-linked to mobile phones), and provide daily self-reported symptom scores of cough frequency and general wellness. Linked-anonymised data were then analysed using ML methods to define the profile of APEs and predict their onset.

End of study questionnaires revealed that 92% of participants found home monitoring easy to use and 77% found it helpful or very helpful in tracking their health over time. Unsupervised ML analysis uncovered the typical signal profile of an APE and revealed three distinct classes of APE. We developed an ML predictive classifier that can detect an impending APE on average 11 days earlier than current clinical practice.

High frequency home monitoring in CF is feasible, reveals distinct types of APEs, and permits accurate prediction of future APEs.

Statement of contribution:

I was responsible for the study design, study management and data collection. Data verification and quality control was completed by myself with help from Damian Sutcliffe (PhD student, Floto Lab). I carried out all data analyses and interpretation. Machine learning analysis (including figure generation) and model development was completed by Damian Sutcliffe.

5.1 INTRODUCTION

Increasingly, telehealth solutions have been shown to be an effective means of monitoring and managing chronic disease at a distance (488). Advances in remote sensor technologies, data transmission devices and internet-based monitoring systems are re-designing the delivery of home-based care. Systematic reviewers have judged it as promising for improving the clinical effectiveness of patient care in diabetes and respiratory and cardiac disease (209–211). In the CF population however, studies to date have been few and of variable quality (198,217–219).

In order to stay well individuals with CF patients must engage in an onerous burden of lifelong treatment, punctuated by frequent visits to specialist centres (even when well) for reassessment and if needed, intensification of care (441). Clinic visits increase in frequency and duration during APEs and may lead to in-patient treatment. The disruption to everyday life that ensues for individuals with CF and their carers can lead to delays in reporting of declining health, to the detriment of long-term fitness and wellbeing (124).

Despite the wide availability of wearable and blue-tooth-enabled sensor devices for health tracking, no study has examined their use in CF care as a means to augment self-management, detect early deteriorations in clinical state and reduce unnecessary contact with the healthcare environment. The potential to mitigate cross-infection risk by reducing unnecessary time in hospital is particularly attractive. Data is also lacking which describes the daily variation in physiological state for an individual with CF during clinical stability, and through the course of an APE. Home-based health monitoring can facilitate longitudinal capture of significant information and trends about an individual's health that may be informative for both individuals with CF and their care teams alike.

I hypothesised that daily collection of symptom scores and physiologic measures, using commercially available sensor devices, would be feasible and enable rapid collection of data for analysis using ML techniques. This would enable examination of the relationships between symptoms and physiological variables, better characterisation of APE patterns and identification of changes in these home-based

measures that may be indicative of an impending APE. In addition, this study will facilitate the development of a monitoring platform for future work investigating whether intervention based on home monitoring data will enable prevention of clinical deterioration and thereby preserve health and well-being for individuals with CF.

5.2 METHODS

5.2.1 Study design and participants

The Standardised Multi-Centre Analysis of Remote Monitoring in CF Adult Patients to Reduce Pulmonary Exacerbations (SMARTCARE) was a prospective cohort, non-interventional, feasibility study (ClinicalTrials.gov number NCT02416375). We conducted the study in accordance with the protocol which received National Ethical approval from the NRES Committee East of England-Norfolk (14/EE/1244) and site-specific approval and monitoring from each participating Hospital R&D Department.

The study design was peer reviewed and regularly monitored by a trial steering committee, which was chaired by myself and was comprised of the principal investigator (PI), study investigators from each participating site and the Research Director of the Cystic Fibrosis Trust UK. I maintained frequent communication and conducted regular meetings to update the sites and receive feedback on progress and any site-specific issues,

In addition to inclusion and exclusion criteria previously detailed in General Methods, Section 2.0, prospective participants met eligibility criteria if they had a history of at least one APE (defined in Definition of an acute pulmonary exacerbation, Section 2.5) in the 12 months prior to screening. I excluded participants if they experienced less than one acute pulmonary in the 12 months prior to screening or were in receipt of a lung transplant.

We received funding for this study from the Cystic Fibrosis Trust UK. A software developer (Fat Fractal), was contracted to develop software for the home monitoring platform used in this study and had no role in the study design, data collection or analysis.

5.2.2 SMARTCARE network

We recruited study participants from seven specialist adult CF centres across England (based at Bristol, Royal Brompton, Frimley Park, King's College, Leeds, Royal Papworth and Southampton University Hospitals). Multiple sites were selected on the basis of their organisational capability and enthusiasm for participating in this study, as well as to enhance generalizability of the study's results, with particular attention to geographic and socio-economic diversity in participants.

The participating sites were responsible for recruiting participants, collection of clinical data (detailed in Data collection schedule, Section 5.2.9) and management of all clinical queries generated during the course of the study.

A research nurse from each study site was designated "site coordinator" and retained primary responsibility for overseeing execution of the study protocol at that site.

Royal Papworth Hospital (Cambridge, UK) was the designated coordinating centre for the study and conducted set-up visits, distribution of study equipment and enrolment of study participants on to the telemonitoring system. The Royal Papworth study team was responsible for reviewing and managing information from the home monitoring system.

5.2.3 Recruitment, screening and enrolment

Prospective participants were identified by individual site coordinators following review of clinical databases and physician notes. Patient information letters detailing the study objectives and protocol were sent to eligible participants. Positive responses were consented and enrolled into the study between the 3rd of August 2015 and the 6th of May 2017.

Participants were withdrawn from the study if they withdrew consent. I considered participants lost-to-follow-up (LTF) if they failed to complete the study-specific questionnaire, despite several attempts by the site coordinator to re-engage the participant in the study. If a participant withdrew consent, was lost-to-follow-up or passed away during the study period their demographic information was kept for

comparison with those for participants who remained in the study and formed part of the feasibility analysis.

We excluded participants' data from the ML analysis if they provided insufficient telemonitoring data. Insufficient data was defined as either 1) less than forty days duration between the first and last measurement, 2) less than thirty-five days with more than one measurement or 3) more than half of the study days with 0 or 1 measure. Data exclusion criteria, as described, were defined post hoc following development and optimisation of the ML model. Telemonitoring data with few data points (as defined above) contributed little information to the development of the ML model.

5.2.4 Home monitoring equipment

Site coordinators provided participants with all study equipment at study inclusion. Participants received the Motorola™ Moto G android smartphone, Vitalograph® blue-tooth spirometer (lung monitor BT), Nonin 3230 *Bluetooth*® Smart Fingertip Oximeter, Pally™ Smart Wireless scale and the Misfit Flash activity tracker. The specifications of each device are listed in Table 5.1. Participants also received a rucksack for transportation of their devices, a cooler bag for transportation of sputum samples, clinell™ anti-microbial wipes for equipment cleaning, nose clips and, if required, a mini-freezer for temporary storage of sputum samples.

Participants received instruction on use of the study devices and were provided with a step-by-step user manual and contact details for technical support. A SMARTCARE device tutorial was also recorded on YouTube (https://www.youtube.com/watch?v=YU21aB6Z-Bk&feature=youtu.be) to provide participants with access to device demonstration and instructions at any time through the study.

5.2.5 SMARTCARE home monitoring system: structure and functionality

The telemonitoring system consisted of an android smartphone application, HTML5/Java Script web application and secure central server. This bespoke system (described in detail below) was built by the software developer Fat Fractal, as no existing commercially available system met the desired requirements for this study.

Table 5.1. Features of the home monitoring devices used in the SMARTCARE study

Device	Manufacturer features
Motorola™ Moto G 	• Model X1032 • Android 4.3 • Bluetooth 4.0 LE • 129.9 x 65.9 x 11.6mm • 143g
Vitalograph® lung monitor BT 	• Model 4000 • Bluetooth 4.0 LE • Display parameters: FEV_1, FEV_6, FEF_{25-75} and FEV_1/FEV_6 • Sensor: Stator rotor • Manufacture-stated accuracy: better than ± 3% • Performance standards: ISO 26782:2009, ISO 23747:2007 and ATS/ERS 2005 • Design and manufacture standards: ISO 13485:2003 and FDA 21CFR820 • Medical safety standard: Medical Device Directive 93/42/EEC
Nonin 3230 *Bluetooth*® Smart Fingertip Oximeter 	• Bluetooth 4.0 LE • Long battery life – up to 2200 spot checks on 2 AAA batteries • Manufacturer-stated accuracy: ± 2 digits in the range 70-100% for oxygen saturation and ± 3 digits in the range 25-250 BPM for heart rate. • Design and manufacture standard ISO (Biological evaluation of medical devices) 10993-1
Pally™ Smart Wireless scale 	• Bluetooth 4.0 LE • 3000 measurement storage • Battery life – more than 5 years on 4 x AA batteries
Misfit Flash activity tracker 	• Bluetooth 4.0 LE • Sensors: 3-axis accelerometer • Battery life ~ 6 months • Measures: steps, calories, distance, sleep activity

5.2.5.1 Smartphone application

The android application enabled the participant to rapidly collect sensor data daily via wireless technology from Bluetooth-enabled monitoring devices. The graphical user interface facilitated easy navigation between several views within the application including 1) User Login, 2) Home Screen with widgets for navigation to other screens, 3) Biometrics recording sequence 4) Biometrics history, 5) “Other events” screen and 6) Help drop-down menu. A series of screen grabs of the user views is illustrated in Figure 5.1. The application was also designed with the ability to work “offline”, enabling participants to collect data collection from the monitoring devices independent of data transmission to the central server.

The user interface of the Biometrics recording sequence screen featured a “traffic light” system alongside the widgets for the various measures that needed to be collected each day. “Green” indicated successful collection of data and transmission to the server, “amber” indicated successful collection of data from the device but pending transmission to the server and “red” indicated data was yet to be collected. The order of the biometrics list corresponded to the order in which participants had been requested to collect their daily data, acting as an aide memoir. A screen grab of the android app’s Biometrics recording screen is shown in Figure 5.2 and illustrates that some measures have been collected and transmitted whilst others are still awaiting collection.

Manual data entry was only required for three measures: collection of sputum, cough and wellness scores (Figure 5.3), significantly simplifying data collection for the study participants.

Automatic pairing of the sensor device with the smartphone occurred once the participant clicked on the relevant biometric widget on the Biometric recording screen. On the subsequent screen the participant would have access to a set of reminder instructions on data collection from that sensor. The list of instructions would cycle from black to green font once each instruction was completed, reassuring the participant that data collection for that biometric measure was proceeding correctly (Figure 5.4).

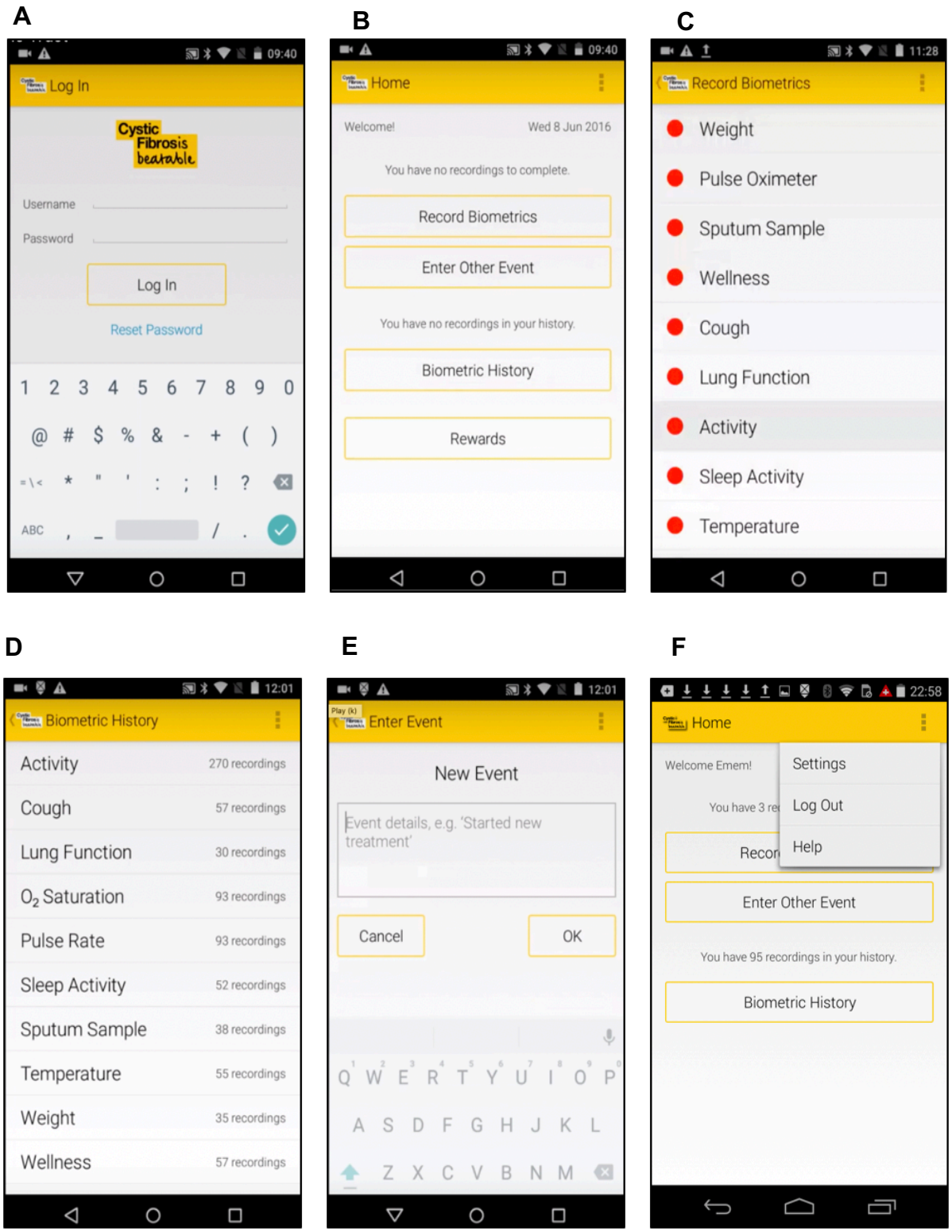


Figure 5.1. Screen views of the graphical user interface for the SMARTCARE android application. A) Log In screen, B) Home screen, C) Biometrics recording sequence, D) Biometrics History, E) "Other event" recording screen and F) drop-down menu including Help menu.

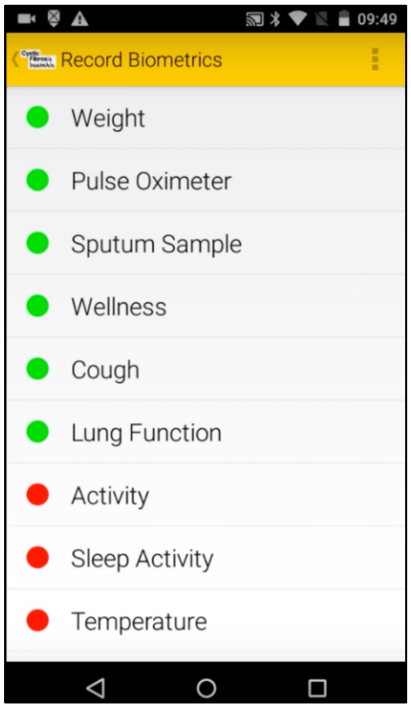


Figure 5.2. Screen grab of the Biometrics recording sequence screen for the SMARTCARE application. Alongside each biometric widget was a "traffic light" indicator to alert the participant to the status of data collection for the day. "Green" indicates successful collection and transmission of data from the sensor to the server, "amber" (not shown) indicates successful data collection from the sensor but pending transmission to the server and "red" indicates data collection is yet to be completed from the sensor.

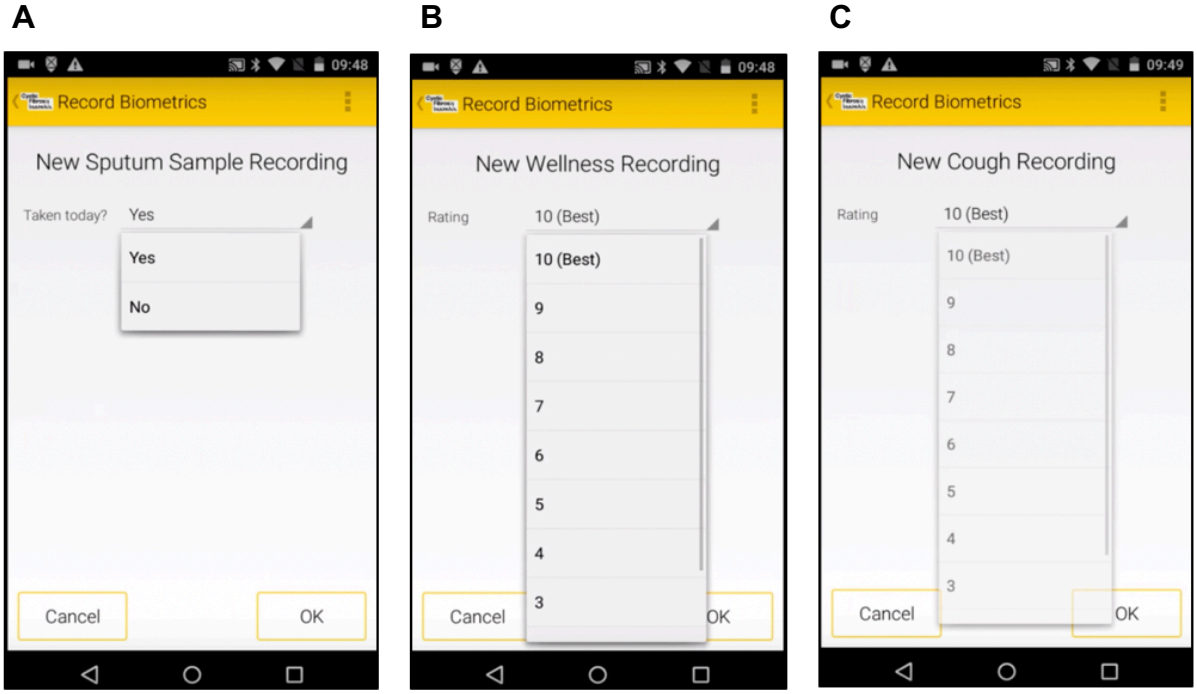


Figure 5.3. Screen grabs of the recording screens for the biometrics that required manual entry within the SMARTCARE application. A) Sputum collection record B) Wellness symptom score C) Cough severity symptom score.

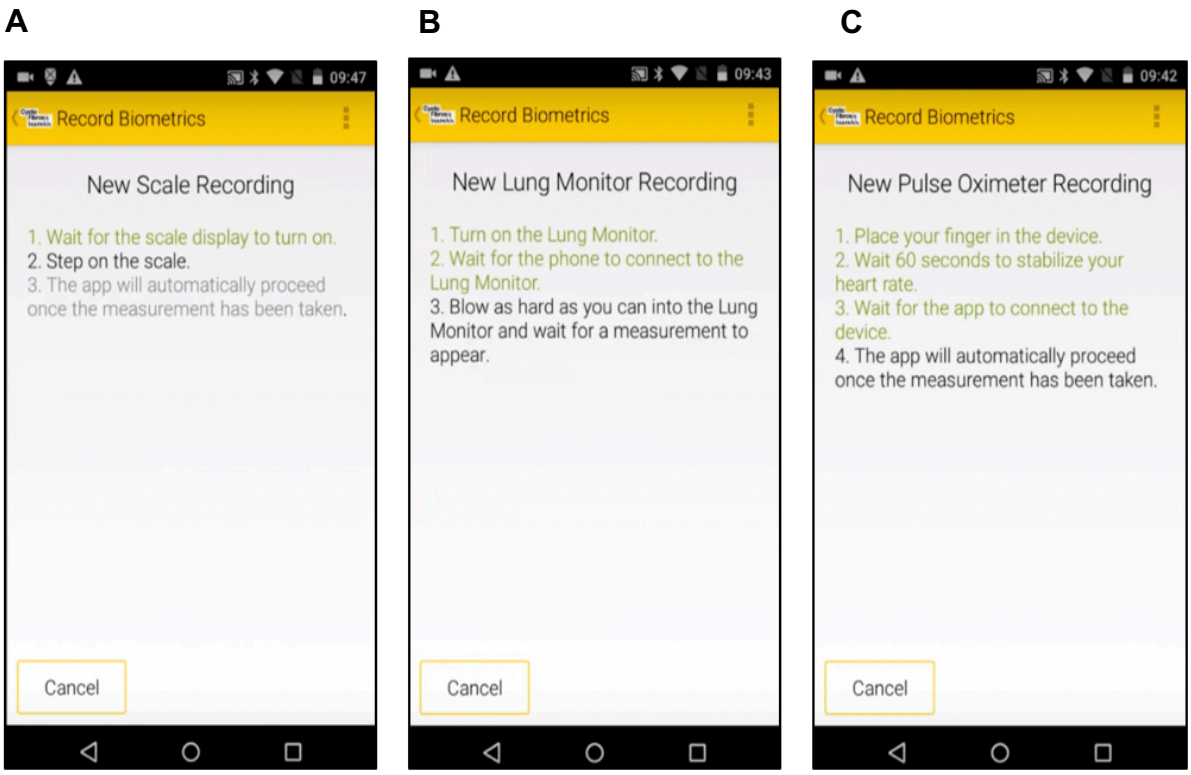


Figure 5.4. Screen grabs of the recording screens within the SMARTCARE application for data collection from three of the Bluetooth-enabled devices. (A) Weight (B) Lung function (C) Heart rate and oxygen saturation. The instruction list cycles from black to green font as each step is completed, reassuring the participant that data collection was proceeding correctly.

Participants were able to view their longitudinal data within the app as time-series charts, with view options of either a week, month or total time in the study. An example of the time-series data capture for one participant is shown in Figure 5.5. Once data collection was completed from the sensor, the captured value would be displayed for the participant to view and accept. Data stored in the smartphone application was automatically transferred to the secure central server whenever the participant was connected to a WiFi network. Participants were also able to manually record events that may have impacted on their measurements (e.g., missed airway clearance sessions) in the “Other Events” screen.

5.2.5.2 Web application

The HTML5/JS web application was designed to provide the Royal Papworth study team and site coordinators with the ability to view (Figure 5.6), analyse and export

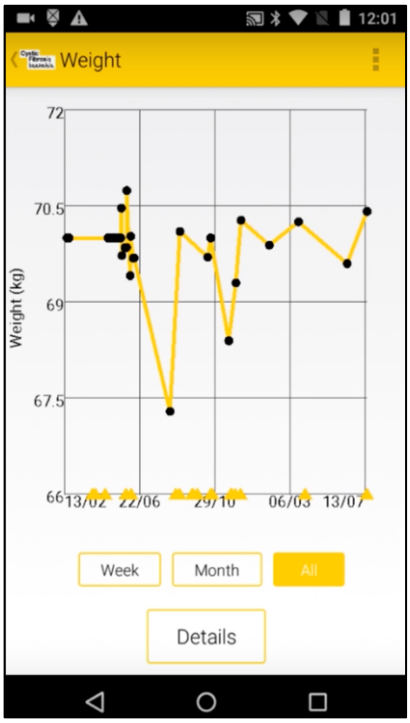


Figure 5.5. Illustration of the time-series graphical representation of one participant's weight measures within the SMARTCARE application. The user interface has the option to view cumulative results over one week, one month or the duration of the study to help participants identify trends in their personal data.

participant data. Registration of study participants onto the web application and access to data from all study participants was limited to the Royal Papworth study team. Password-permissions enabled site coordinators to only review data from their own participants.

Data transfer between the smartphone application, secure backend server and the web application was encrypted and link-anonymised in both transmission and rest.

Participant data was able to be exported in .csv format when required for data analysis.

The web-based application was accessible from the registered website name www.cftrust.fatfractal.com.

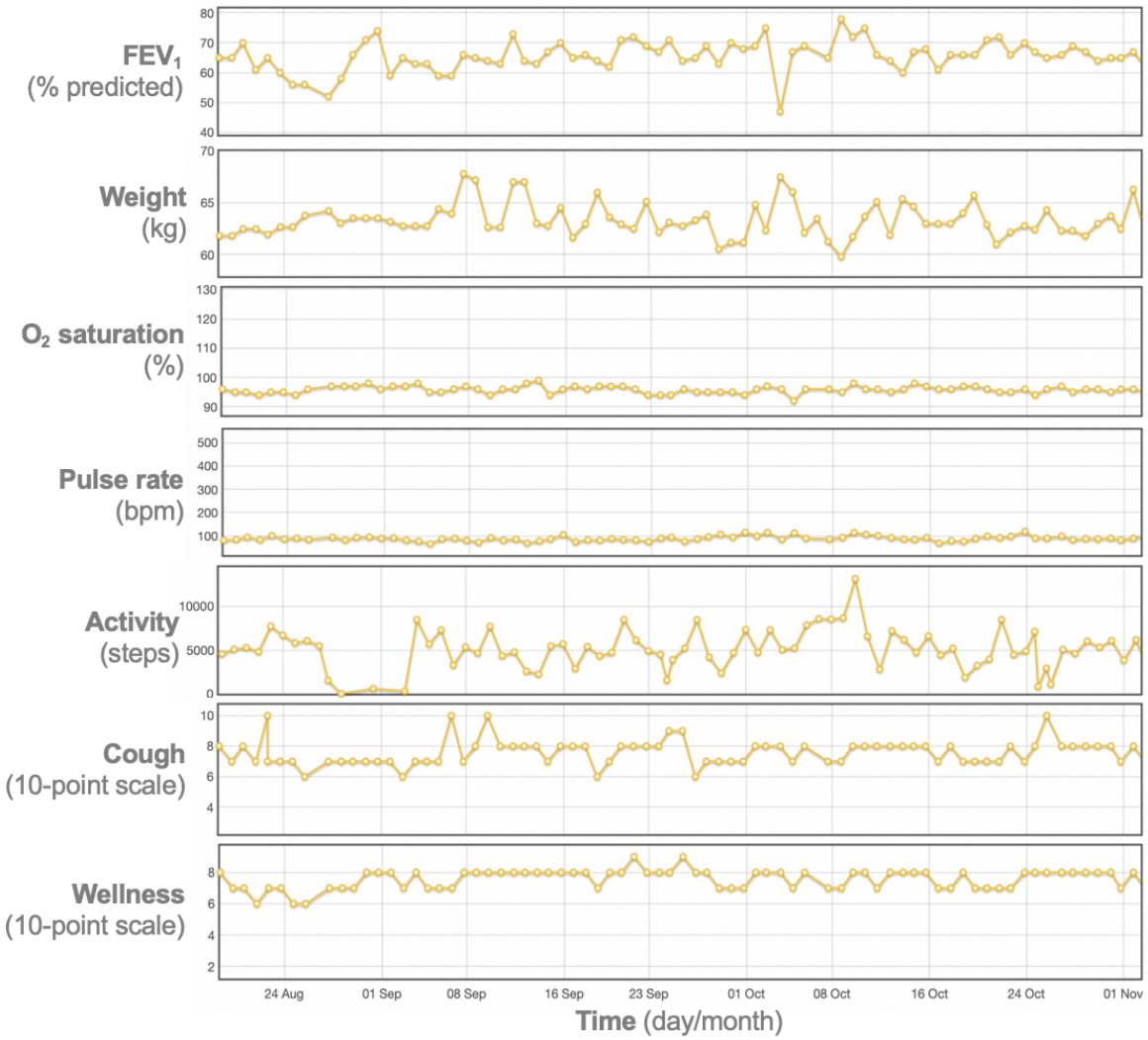


Figure 5.6. A screen grab of the time-series graphical representation of measures for one participant captured by the SMARTCARE web application. Toggle buttons on the user interface enabled the user to increase or decrease the view time interval and review trends over time. A 10-point scale was used to assess wellness and cough severity, rated from worst ever (1/10) to best ever (10/10).

5.2.5.3 Secure central server

The secure central server enabled participant data from their biometric measurements to be stored securely, in line with NHS N3 compliance standards (489), for collation and processing. The central server also enforced role-based data access controls in line with user identity and role.

5.2.6 Home monitoring protocol

I requested that participants perform home monitoring and sputum collection as detailed in Trial Protocols, Section 2.3. They were instructed to collect and record their data in the following order: 1) weight (kg), 2) Pulse rate (beats/minute) and oxygen saturation (%), 3) 10-point scale of wellness [reported as “worst ever” (1/10) to “best ever” (10/10)], 4) 10-point scale of cough quality [reported as “worst ever” (1/10) to “best ever” (10/10)], 5) lung function (FEV_1 in litres) and 6) physical activity (step count),

Site coordinators provided first line technical support for issues with the telemonitoring system. Unresolved issues were escalated and managed by myself and the Royal Papworth site coordinator. Additional technical support was provided by the software developer, Fat Fractal. Site coordinators were encouraged to review their own site’s telemonitoring data regularly and monitor for adherence. If patients did not enter data for more than two weeks, we instructed site coordinators to make contact to encourage participation and promote adherence with home monitoring.

The primary adverse event I anticipated with telemonitoring was an increase in patient anxiety due to accessibility and intensity of clinical monitoring. I have addressed risk mitigation under Usual care guidance, Section 5.2.7.

5.2.7 Usual care guidance

SMARTCARE was a non-interventional study therefore study site clinicians were blinded to the telemonitoring datasets. The anticipation was that participant-driven interactions with their clinicians might increase, prompted by access to their monitoring data. In order to minimise the risk of undue anxiety generated by access to their monitoring data, we instructed participants to address clinical concerns to their CF Centre and to respond to changes in their health as they would normally in the absence of home monitoring.

Treatment of APEs during the study occurred in accordance with local CF Centre guidelines.

5.2.8 Study questionnaires

5.2.8.1 SMARTCARE survey

I created a non-validated study-specific questionnaire to assess participant experience, satisfaction, preferences and attitudes of daily home monitoring using Bluetooth-enabled devices and smartphone technology. The seventeen survey questions were also designed to gauge user feedback on the technical quality of the telemonitoring platform and to assess the impact of home monitoring on their QOL and self-management. Items were scored either as a frequency response on a 5-point scale, a usability rating on a 5-point scale, an impact rating on a 10-point scale, a choice rating on a 5-point scale, a helpfulness rating on a 5-point scale or an acceptability rating on an 8-point scale. A free-text section provided opportunity for feedback on an individual participant's experience with home monitoring. A copy of the SMARTCARE survey is found in Appendix 3.

5.2.8.2 Cystic Fibrosis Questionnaire-Revised (CFQ-R)

I used a validated disease-specific questionnaire to assess the longitudinally impact of home monitoring on health-related quality of life (HRQoL) for the study cohort.

The CFQ-R consists of 35-50 items divided into 7-9 domains (depending on age group) which encompass general domains of HRQoL: physical functioning, vitality (energy and well-being), emotional functioning, social functioning, role functioning and health perceptions (490). The questionnaire also includes three symptom domains specific to CF: body image, eating disturbances, treatment burden and respiratory and digestive symptoms.

Items are scored on five distinct 4-point Likert scales. The scores for each HRQoL domain range from 0 to 100 with higher scores corresponding to better health. A minimal clinically important difference, the smallest clinically relevant change a patient can detect, of 4.0 has only been determined in stable patients for the respiratory symptom domain (491). A copy of the CFQ-R is found in Appendix 3.

5.2.9 Data collection schedule

I requested study participants complete the CFQ-R (Version 2.0) questionnaire twice during the study, at inclusion and on exit from the study. Additionally, at study exit, I asked participants to complete the SMARTCARE survey.

I requested site coordinators to prospectively record participant-specific-information on: 1) demographics and clinical characteristics (e.g., co-morbidities, medications, corticosteroid use), 2) dates and details of antibiotic requirement during the study period - the initiation of either oral or intravenous antibiotics to treat a deterioration in respiratory status was used to record the occurrence of a clinician-defined APE, 3) hospital-based measurement of C-reactive protein during the study period, 4) dates and details of hospital admission for APEs, 5) dates and details of out-patient clinic visits, 6) positive sputum microbiology during the study period, 7) hospital-based measurement of Forced expiratory volume in one second (FEV_1 in litres and percent of predicted) 8) hospital-based measurement of weight and 9) non-clinic encounters and reason(s) for initiation of contact with the medical team.

Data collection was completed via a bespoke web application and stored on an NHS N3 network-compliant SQL database. The website was hosted on a server at Royal Papworth Hospital. Password-permission access was provided to site coordinators and the Royal Papworth study team. User accounts for the site coordinators were linked only to their study site. Access to data from all the study sites was only permitted to the Royal Papworth study team.

The database was exported in excel format at the end of the study for analysis.

5.2.10 Outcome measures

I assessed feasibility by a review of enrolment and retention characteristics (e.g., number of participants referred, number consented, drop-out rate), acceptability and compliance (e.g., survey responses, adherence with monitoring), and analysis of the functionality of the telemonitoring system (e.g., sensor functionality, success of data upload, frequency of requests for technical support) and data characteristics (e.g., duplicate data, missing data, variance in measures).

A secondary outcome of this work was to be able to better characterise the relationship between symptom scores and physiological measures leading up to and during the course of an APE by applying ML techniques to gain new insights from the high frequency data generated in the SMARTCARE study.

5.2.11 Quality assurance

Prior to screening the Royal Papworth site coordinator and myself completed a visit of each study site to provide set-up support and ensure that the study protocol was able to be completed at the study site. During this visit, we demonstrated operation of the study equipment, reviewed the study and provided a site file and electronic copy of all study documents.

I completed rigorous data checks for consistency and completeness on the data entered into the electronic clinical database and the telemonitoring web application at the end of the study, with additional data management support from Damian Sutcliffe. I referred inconsistencies in the clinical data collection back to the study site and updated amendments when received. A detailed description of the management of data applied to the home monitoring data is found in Technical quality of the home monitoring system, Section 5.3.2.2.

5.2.12 Compensation

Participants were given £50 as a one-off inconvenience payment for their time and effort with the study and had the option of keeping all study equipment at the end of the study. Compensation was not contingent on time spent in the study or amount of data provided.

5.2.13 Sample size

Due to a lack of existing data on the application of ML analysis to telemonitoring data in CF it was not possible to perform a power calculation to determine sample size for this study. We therefore based the sample size on the feasibility of recruitment from the pool of potential participants as well as on sample sizes in similar ML-based tasks in other conditions (492,493). The sample size target was 200 participants.

5.2.14 Statistical analysis

I performed statistical analyses using Microsoft Excel for Mac version 16.27 (Microsoft, Redmond, WA, USA), Prism 6.0 for Mac OS X (Graphpad Software Inc, San Diego, CA, USA) and the tidyverse, Hmisc, corrplot, reshape2, ggplot2, survival, lattice and Formula packages in RStudio version 1.1.456 (RStudio Inc, Boston, MA, USA).

Descriptive statistics of the baseline characteristics of participants are expressed as mean (standard deviation [SD]) or median (interquartile range (IQR), minimum and maximum) for numerical variables and as number (percentage) for categorical variables. I did not attempt to substitute missing data.

I examined between-study site and between-cohort (grouping based on days of recordings completed or impact of home monitoring on anxiety levels) differences for continuous variables by one-way analysis of variance (ANOVA) for parametric data, Mann-Whitney U-test and Kruskal-Wallis tests for non-parametric data and Chi-squared (χ^2) tests for categorical data.

I analysed correlation relationships between continuous and discrete variables by Spearman's Rho for non-parametric data and Pearson's correlation coefficient for parametric data.

I assessed for comparability of in-clinic and home-based spirometric data using a Bland-Altman analysis of same-day in-home and in-clinic FEV_1 (% predicted) to establish the level of agreement between these two measurements (440).

In addition to describing user experiences, satisfaction and use of the home monitoring system qualitatively, I compared user experience of home monitoring between survey respondents and non-respondents using unpaired two-tailed t-test for parametric data and unpaired Mann-Whitney signed rank test for non-parametric data. Categorical variables were compared using the Fisher's exact test.

All statistical tests were two-tailed and a p value of < 0.05 was considered statistically significant.

5.2.15 Machine learning analysis

ML analysis was completed by Damian Sutcliffe (PhD student, Floto Lab) using telemonitoring data collected from a subset of the study participants (*n* = 104). Data from the remaining study participants (*n* = 43) was excluded due to either insufficient data (defined in Recruitment, screening and enrolment, Section 5.2.3) for the ML tasks or because participants withdrew study consent. A total of 147 antibiotic treatment episodes were captured for ML analysis of which 97 were used. Fifty records were excluded due to the following criteria: 1) less than 15 days with measures in the 40 days prior to treatment, 2) an average of less than 2 measurements per day or 3) less than 35% total data completeness in the data record.

In brief, the onset of each APE was considered a hidden (or *latent*) variable. An unsupervised ML approach (a probabilistic generative model) was used 1) to learn the latent variable for each measurement across all the APE periods, 2) to align the records of each measurement type (e.g., lung function, weight, heart rate, O_2 saturations, activity, wellness and symptom scores), based on the learnt latent variable, for all the APE periods (*n* = 97) in the dataset, and 3) to learn the average profile for each measurement type during an APE (which was also assigned a latent profile) (Figure 5.7). Probability distributions of the latent quantities were inferred iteratively using an Expectation Maximisation algorithm. In order to generate the typical ('average') profile of an APE, the model was constrained to allow only one set of latent measurement curves to be learnt from the dataset. Subsequently, the model was extended to learn multiple sets of latent measurement curves, each representative of a different class of APE. For the predictive classifier, a supervised ML approach was used, specifically a probabilistic regression classifier. The dataset was subdivided and four-fold cross validation was used to train and test the classifier model. Twenty percent of the participants' records were randomly selected at the outset and kept back as a final test set for once the model had been fully optimised.

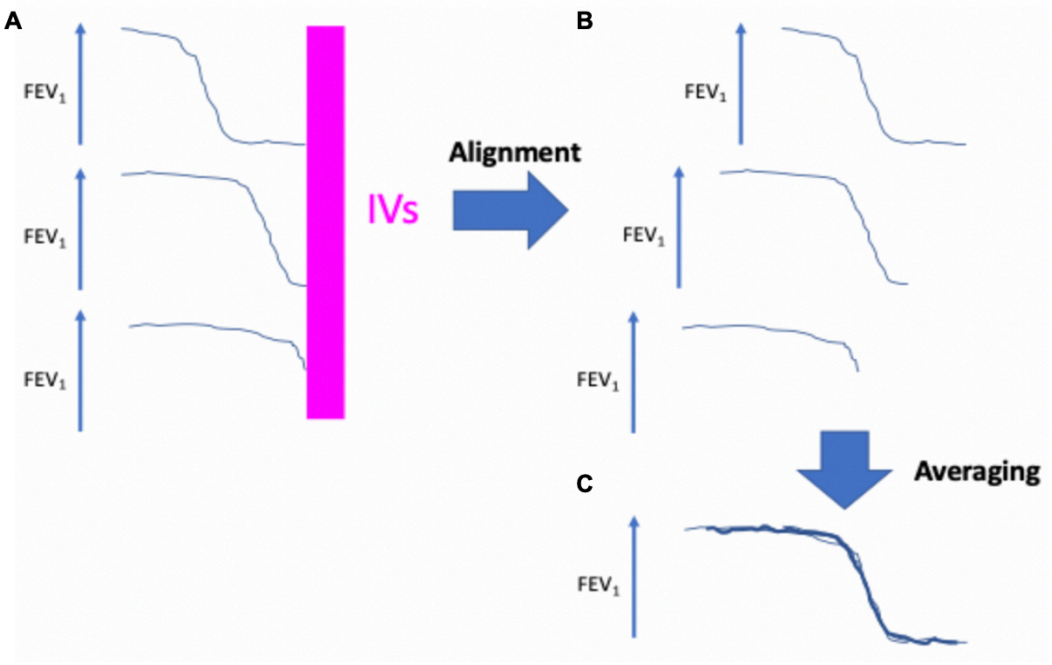


Figure 5.7. An illustration of the desired signal alignment behaviour of the ML alignment model using FEV_1 data records from 3 participants as an example. We observed differing time delays between the start of decline in a home measure (in this example, FEV_1) to the start of treatment (IVs) between different APEs (A). In order to produce an average APE profile, we first need to shift the data for each APE, so that the start of an APE is aligned across all FEV_1 examples. This is done using an unsupervised ML approach, where the model learns the unobserved (e.g., hidden or 'latent') variable for the relative start time of an APE. (B). The model then averages them (in this illustration for FEV_1) to generate the average latent profile of an APE despite the variable times to treatment (C). Once the shape of the latent change curves for each signal type is learnt, the start of an APE can be marked as the consensus inflexion point across all signal types (Figure courtesy of RA Floto).

5.3 RESULTS

5.3.1 Participants

We screened 183 potential participants for inclusion in SMARTCARE. Of these, nineteen were ineligible and ten declined to participate. One hundred and forty-seven participants were enrolled in the study. Three deaths occurred during the course of the study. I included data from the remaining 144 participants in the feasibility analysis of this study (Figure 5.8). We excluded data from forty-three participants from the ML tasks for these reasons: five (3%) participants withdrew their consent, thirty-eight

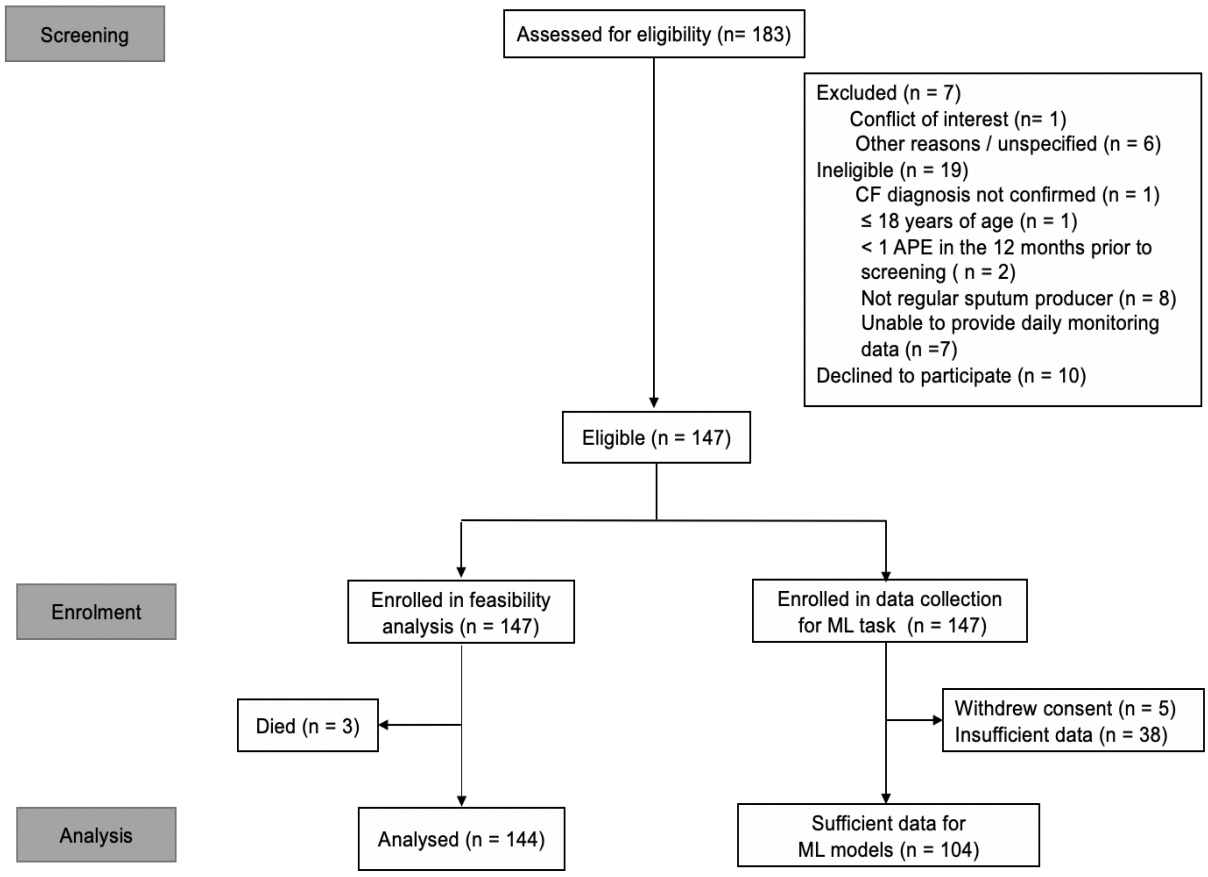


Figure 5.8. Screening, enrolment and follow-up of the SMARTCARE study participants. ML: Machine learning.

(26%) participants recorded insufficient data (defined in Recruitment, screening and enrolment 4.2.3), one of whom passed away during the study period.

Seventy-three (50%) participants completed more than five months of home monitoring, of which two thirds (47 out of 73) provided daily data for the full 6 months of the study period. Eight (5%) participants completed 4 to 5 months of home monitoring, nine (6%) completed 3 to 4 months and thirteen (10%) completed 2 to 3 months of monitoring. Forty-four (30%) participants completed less than two months of data. The average number of measures recorded per day was 6.3 (range 0 – 7). One hundred and four (71%) participants provided sufficient data for the ML tasks, including two participants who passed away during the course of the study.

Twenty-three of the 43 participants who discontinued home monitoring in the first month of the study did not complete the study-specific survey, one of whom had

passed away during the course of the study. Of the 57 participants who discontinued home monitoring between months 1 and 6, 39 completed the study-specific survey, providing feedback on their experience. Of the 47 participants who provided all 6 months of home monitoring data, only 4 participants did not complete the study-specific survey. In total, I received survey feedback from 102 (69%) participants and this data formed part of the feasibility analysis. 82 (56%) participants provided both sufficient data for the machine learning models as well as survey feedback on the home monitoring experience.

Demographic and clinical characteristics for the SMARTCARE cohort are summarised in Table 5.2. Participants had clinical characteristics broadly representative of the UK CF population. The mean age of the cohort was 31.6 years. Marginally more women (56%) took part in the study. Nearly two thirds (62%) of the participants were homozygous for the CFTR F508del mutation. Almost half (48%) had moderately impaired lung function ($FEV_1 \geq 40$ to < 70 % of predicted), whilst a quarter (25%) of the cohort had severely impaired lung function (FEV_1 < 40% of predicted). Just over half (52%) of the participants had chronic airway infection with *P. aeruginosa*. The majority of participants (86%) were prescribed some form of nebulised mucolytic (e.g., dornase alpha, mannitol or hypertonic saline) for airway clearance, with just over half (56%) of the cohort also taking an inhaled antibiotic as part of their daily treatments. Close to two thirds (59%) of participants were in either part-time or full-time employment.

Seven adult CF centres took part in this study with the number of participants recruited from each site ranging from eleven to thirty-five. The clinical characteristics and treatment burden at each site are summarised in Table 5.3. A total of 229 antibiotic treatment events were captured across all participants during the course of the study. Half (48%) of the participants experienced one to two treatment-defined APEs, whilst a quarter (24%) experienced between three to six APEs during the study period (Figure 5.9).

Of note, only a small minority of participants (7%) were on a CFTR modulator during the study period: 3% on monotherapy and 4% on first generation dual therapy.

Table 5.2. Baseline characteristics of the SMARTCARE study population

Characteristic	**SMARTCARE (*n* = 147)**
Age (yr) at screening - mean	31.6 ± 9.3
Female sex - no. (%)	83 (56)
Genotype – no. (%)	
F508del homozygous	91 (62)
F508del heterozygous	48 (33)
Other	8 (5)
Percentage of predicted FEV_1	
Mean	56.1 ± 21.9
Subgroup – no. (%)	
< 40%	37 (25)
≥ 40 to < 70%	70 (48)
≥ 70 to < 90%	27 (18)
≥ 90%	13 (9)
Body Mass Index - mean	23± 3.4
Chronic infection with *P. aeruginosa* – no. (%)	77 (52)
CF-related diabetes – no. (%)	61 (41)
Prescribed medications – no. (%)	
Inhaled antibiotic	93 (63)
Inhaled bronchodilator	103 (70)
Inhaled mucolytic	128 (87)
Azithromycin	72 (49)
Employment status – no. (%)	
Fulltime/Part-time work	87 (59)
CFQ-R treatment domain score at screening - median	44 (33 – 67)

FEV_1: Forced expiratory volume in 1 second.

Table 5.3 Comparison of clinical characteristics and treatment burden across each study centre

	Whole Cohort (*n* = 147)	Royal Papworth (*n* = 35)	Leeds (*n* = 30)	Bristol (*n* = 23)	Southampton (*n* = 16)	Kings College (*n* = 18)	Royal Brompton (*n* = 14)	Frimley (*n* = 11)	*p* value
Age (yr) - mean	32 ± 9.3	32 ± 10	35 ± 9	28 ± 7	29 ± 9	34 ± 9	35 ± 9	27 ± 5	**0.0413**
Female sex – no. (%)	83 (56.5)	19 (54.2)	14 (46.7)	14 (60.9)	11 (68.8)	12 (66.7)	6 (42.9)	7 (63.3)	0.6470
FEV_1 (% predicted) - mean	56.1 ± 21.9	65 ± 21	45 ± 18	62 ± 24	48 ± 22	53 ± 21	57± 16	61 ± 22	**0.0060**
Subgroup – no. (%)									
< 40	37 (25.2)	5 (14.3)	13 (43.3)	6 (26.1)	4 (25.0)	4 (22.2)	2 (14.3)	3 (27.3)	0.1321
≥ 40 to < 70	70 (47.6)	17 (48.6)	14 (46.7)	6 (26.1)	9 (56.3)	11 (61.1)	9 (64.3)	4 (36.4)	0.2233
≥ 70 to < 90	27 (18.4)	6 (17.1)	2 (6.7)	7 (30.4)	3 (18.8)	2 (11.1)	3 (21.4)	4 (36.4)	0.2305
≥ 90	13 (8.8)	7 (20.0)	1 (3.3)	4 (17.4)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	**0.0073**
BMI – mean	23± 3.4	23 ± 3.6	24 ± 4.0	23 ± 2.8	22 ± 3.6	22 ± 3.0	23 ± 2.7	22 ± 2.1	0.6983
PI – no. (%)	126 (85.7)	32 (91.4)	29 (96.7)	14 (60.9)	11 (68.8)	18 (100.0)	13 (92.9)	9 (81.8)	**0.0100**
CFRD – no. (%)	61 (41)	15 (42.9)	13 (43.3)	10 (43.5)	5 (31.2)	5 (27.8)	4 (28.6)	7 (63.6)	0.5035
Antibiotic treatment events – median	1 (0 - 2)	1 (0 – 1.5)	3 (1.3 – 3)	2 (1 – 2)	2 (1 – 3)	0 (0 – 1)	0.5 (0 – 1.8)	1 (0.5 – 1.5)	**< 0.0001**
Clinic visits - median	5 (3 – 6.5)	6 (4 – 7)	6 (4.2 – 9)	3 (2 – 4.5)	3 (2 – 4.3)	3 (2 – 4)	6 (4.3 – 6.8)	5 (4.5 – 5.5)	**< 0.0001**
Admissions - median	1 (0 – 2)	0 (0 – 1.5)	2 (1 – 3)	0 (0 – 1)	1.5 (1 – 2)	0 (0 – 1)	0 (0 – 1)	0 (0 – 0.5)	**0.0004**
Admission days - median	5 (0 – 21)	0 (0 - 19)	18 (7.8 – 31)	0 (0 – 17)	11.5 (8.8 - 20.3)	0 (0 – 8)	0 (0 – 12.8)	0 (0 – 4.5)	**0.0027**
Intravenous antibiotic days - median	17 (0 – 29)	14 (0 – 24)	32 (21 – 46.3)	27 (4 – 28)	26 (14 – 40)	0 (0 – 14)	0 (0 – 18)	14 (6.5 – 19.5)	**< 0.0001**
Inhaled bronchodilator - no. (%)	103 (70.0)	30 (85.7)	17 (56.7)	18 (78.3)	11 (68.8)	10 (55.6)	10 (71.4)	9 (81.8)	0.1209
Inhaled antibiotic - no. (%)	93 (63.3)	33 (94.3)	16 (53.3)	16 (69.6)	7 (43.8)	9 (50.0)	11 (78.6)	5 (45.5)	**0.0005**
Dornase alpha - no. (%)	109 (74.1)	20 (57.1)	29 (96.7)	14 (60.9)	13 (81.3)	15 (83.3)	14 (100)	9 (81.8)	**0.0008**
HTS – no. (%)	44 (29.9)	19 (54.3)	3 (10.0)	6 (26.1)	1 (6.3)	10 (55.6)	5 (35.7)	0 (0.0)	**< 0.0001**
Azithromycin – no. (%)	72 (49.0)	16 (45.7)	8 (26.7)	18 (78.3)	7 (43.8)	8 (44.4)	4 (28.6)	10 (90.9)	**0.0004**

Differences between continuous variables were examined with one-way ANOVA for parametric data and Kruskall-Wallis test for non-parametric data. Differences between categorical data were examined with χ^2 tests. FEV_1: forced expiratory volume in 1 second, BMI: body mass index, PI: pancreatic insufficiency, CFRD: CF-related diabetes, HTS: hypertonic saline. Where relevant, some percentages might not add up to 100% due to rounding.

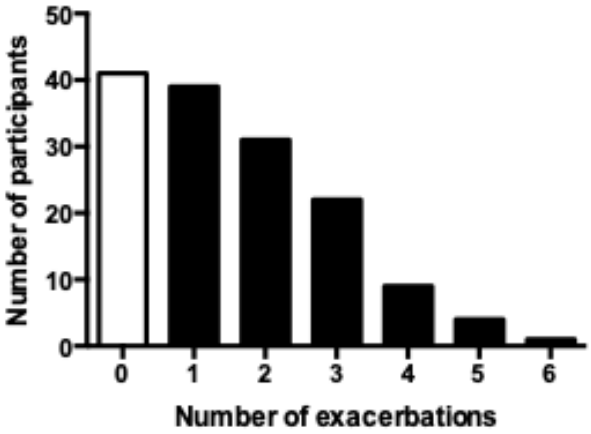


Figure 5.9. Distribution of treatment-defined pulmonary exacerbations across the study period. The total number of APE during the study period was determined for each participant (*n* = 147). An APE was defined based on the initiation of antibiotic therapy (intravenous or oral) by the treating clinician for a deterioration in clinical state from baseline.

Clinical characteristics between the sites were fairly similar, with a few exceptions. There were significant differences in the average lung function (FEV_1 % predicted) values for participants at each of the study sites (ANOVA, $p = 0.0060$) (Figure 5.10).

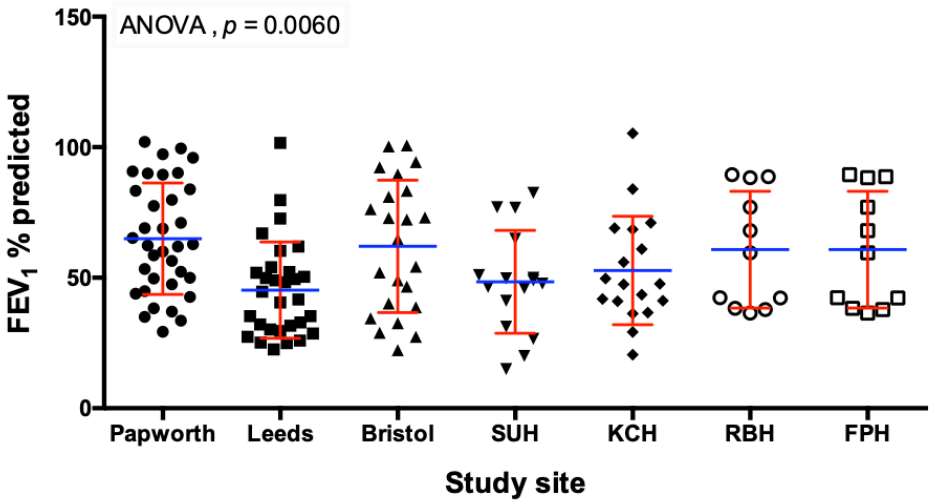


Figure 5.10. Comparison of lung function results between study sites. The data is presented as mean (in blue) ± SD (in red). There was a significant difference in mean lung function between the study cohorts, with participants from Leeds and Southampton University Hospitals, on average, having lower lung function than participants from the other study sites (ANOVA, $p = 0.0060$). SUH: Southampton University Hospital, KCH: King College Hospital, RBH: Royal Brompton Hospital, and FPH: Frimley Park Hospital.

The mean age (ANOVA, $p = 0.0413$) and pancreatic insufficiency status (ANOVA, $p = 0.0100$) of participants also varied significantly between the centres. I also found significant differences in treatment burden between the centres, both in acute and maintenance treatments, with the Leeds cohort, in general, requiring more admissions and intravenous antibiotics than their study counterparts (Table 5.3).

5.3.2. Correlations between home-monitored measures

I next performed a correlation analysis on the home-measured dataset to identify any significant relationships between the physiological variables and symptom scores (Figure 5.11). I did not observe a significant association between lung function and activity levels (Spearman $r = 0.25$, $p < 0.00001$), nor between activity levels and either cough or wellness scores (Spearman $r = 0.25$ and 0.27 respectively, $p < 0.00001$).

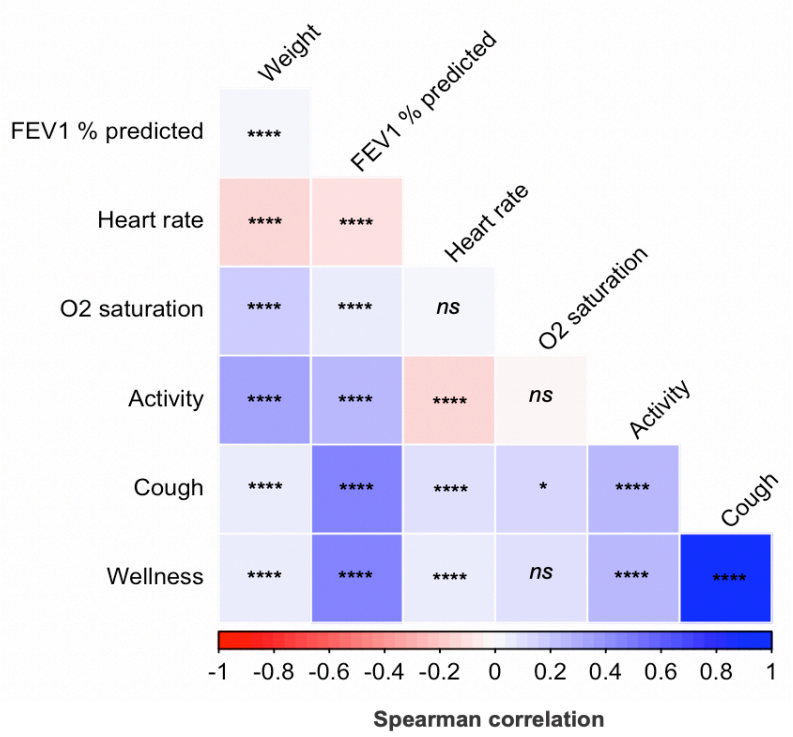


Figure 5.11. Correlation matrix of variable-variable associations as determined by Spearman correlation coefficient. Blue indicates a strong positive correlation between any given pair (Spearman coefficient closer to 1). Red indicates a strong negative correlation between any given pair (Spearman coefficient closer to -1). White indicates no correlation between pairs (0). Correlations were significant if $r > 0.35$ and $p < 0.05$ (*). *ns* = not significant, **** = $p < 0.00001$.

Similarly, I did not identify a strong correlation between weight and activity (Spearman $r = 0.33$, $p < 0.00001$) measures. Lung function was only modestly correlated with both cough and wellness scores (Spearman $r = 0.44$ and 0.46 respectively, $p < 0.0001$). Lung function testing is highly technique- and effort-dependent and measures performed in the home setting (without the support of a specialist respiratory physiologist) may be more susceptible to these factors influencing correlations with an individual's symptom perception (254). Unsurprisingly, as these two measures are likely to be internally consistent, I observed the strongest correlation between the two patient-reported measures of cough and wellness (Spearman $r = 0.91$, $p < 0.00001$).

5.3.3 Home monitoring feasibility analysis

5.3.3.1 Enrolment rate, retention and participant characteristics

The median recruitment time across the sites was 7 months (range 6-18 months). The recruitment rate at each site was, on average, 3 participants a month. As we expected, larger centres were able to recruit more participants into the study.

To assess for explanatory factors that may have influenced study retention and compliance with daily home monitoring, I divided participants into four groups based on the number of days of recordings that they provided during the study period: 1) insufficient (either less than forty days duration between the first and last measurement or less than thirty-five days with more than one measurement or more than half of the study days with 0 or 1 measure), 2) between 41 and 90 days 3) between 91 and 150 days and 4) more than 150 days of home measures.

The participant, treatment burden and home monitoring characteristics of these four groups are summarised in Table 5.4. Comparisons between the groups showed similarity for most of the features. However, participants' age (ANOVA, $p < 0.0001$) and BMI (ANOVA, $p < 0.0001$) may have had an influence on adherence with monitoring. Notably, my findings suggest that older participants are more likely to be adherent with home monitoring. I did not identify any other associations between home monitoring compliance and cohort characteristics, particularly with regards to degree of lung function impairment or treatment burden.

Table 5.4. Comparison of participant characteristics, treatment burden and home monitoring activity between cohorts based on number of days of recordings provided during the 6-month study period.

	Insufficient days (*n* = 43)	41 – 90 days (*n* = 15)	91 – 150 days (*n* = 17)	>150 days (*n* = 72)	*p* value
Age (yr) - mean	27.3 ± 7.5	27.0 ± 5.3	31.5 ± 5.8	35.4 ± 10.1	**< 0.0001**
Female sex – no. (%)	17 (39.5)	11 (73.3)	11 (64.7)	43 (59.7)	0.0581
BMI - mean	22.3 ± 2.9	21.6 ± 4.5	23.5 ± 5.1	22.9 ± 2.9	**< 0.0001**
FEV_1 (% predicted) - mean	54.4 ± 22.1	62.6 ± 23.5	54.3 ± 24.0	56.1 ± 21.0	0.6413
Subgroup – no. (%)					
<40	11 (25.6)	2 (13.3)	4 (23.5)	20 (27.8)	0.7046
≥ 40 to < 70	20 (46.5)	6 (40)	9 (52.9)	35 (48.6)	0.8983
≥ 70 to < 90	11 (25.6)	5 (33.3)	1 (5.9)	10 (13.9)	0.0911
≥ 90	1 (2.3)	2 (13.3)	3 (17.6)	7 (9.7)	0.2266
CFRD – no. (%)	14 (33)	6 (40)	10 (59)	30 (42)	0.3181
Part-time or full-time employment – no. (%)	24 (56)	10 (66)	7 (41)	45 (63)	0.3741
Intravenous antibiotic days - median	21 (13.5 - 39.5)	14 (0 – 31)	24 (0 – 43.5)	14 (0 – 28)	0.0896
Inhaled antibiotics and/or mucolytics – no. (%)	37 (86)	15 (100)	17 (100)	66 (92)	0.1894
Measures per day - mean	NA	6.3 ± 1.1	5.9 ± 1.1	6.4 ± 1.1	0.2010

Differences between continuous variables were examined with one-way ANOVA for parametric data and Kruskal-Wallis test for non-parametric data. Differences between categorical data were examined with χ^2 tests. FEV_1: forced expiratory volume in 1 second, BMI: body mass index, CFRD:CF-related diabetes. Where relevant, some percentages may not add up to 100% due to rounding.

5.3.3.2 Technical quality of the home monitoring system

115813 recordings were collected across the study period (Figure 5.12). 13608 of these recordings were not physiological or symptom measures but rather a record of the sputum samples collected during the study.

Empty measurements, which we defined as time-stamped entries with no measurement logged, were captured for activity, cough and wellness scores. In total, we identified 1308 (1%) empty measurements and these were removed from the dataset. A further 122 recordings were excluded due to resting values that were outside of the expected physiological range (e.g., FEV_1 % predicted < 10% or > 130%, O_2 saturation < 80% or > 100%, Heart rate < 50 bpm or > 150 bpm or weight < 35kg or > 125kg).

We identified repeated measures using the following definitions: Class 1) exact date, time and value, Class 2) identical/near identical measures within a short time frame (e.g., <12 mins for activity recordings, < 30 mins for non-activity recordings) and Class 3) multiple measures on the same day.

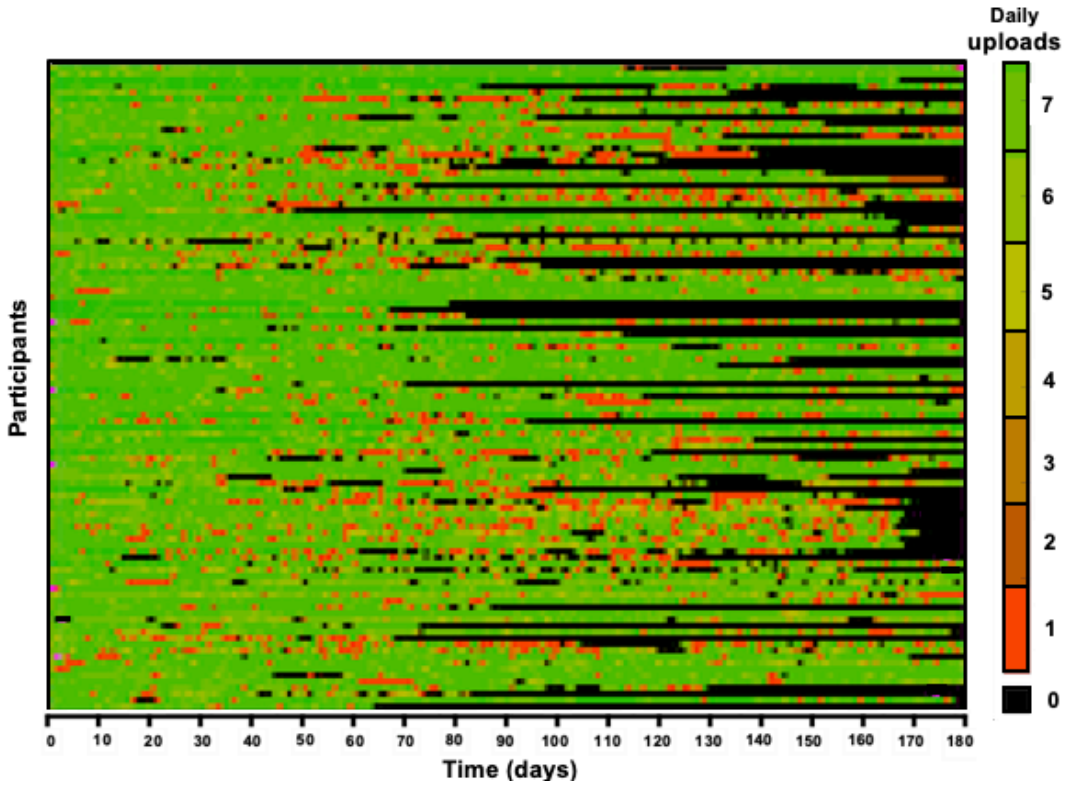


Figure 5.12. Data capture for each participant (*n* = 147) across the study period. Each row represents a single participant. The colour bar codes for the number of measures recorded on a given day. A maximum of 7 recordings could be made per day: lung function, weight, activity (steps), cough score, wellness score, heart rate, O_2 saturation. (Figure courtesy of Damian Sutcliffe).

In total, we identified 4161 (4%) repeated measures. Measurements of Class 1 were collapsed to a single measure and duplicates removed. For measurements of Class 2, we accepted the last chronological measure with the exception of FEV_1, where the best measure was selected, in line with clinical practice. We handled Class 3 measurements by averaging the values to produce a composite single measure for that day.

In addition, we excluded recordings that fell outside of the study range. These were defined as follows: 1) outlier first measures: recordings taken at the set-up visit followed by a gap (up to 3 weeks) before active measuring was commenced by the participant and 2) outlier last measures: recordings that continued after a participant had exited the study but still persisted to use some of their monitoring devices on an *adhoc* basis. In total, 275 outlier measures for 45 participants and 1802 last measures for 56 participants were identified and excluded from the dataset.

Although repeated values occurred at relatively low frequency for the automated sensor devices, the activity monitor was comparatively the worst performing of the group. Interrogation of the server backend log of the raw activity recordings revealed a higher proportion of multi-replicate values (e.g., same time stamp and value) than any other sensor. In every case, the server accepted the value with a success status code. It is likely that the re-sending of the same value from the device was secondary to a fault in the activity monitor or an issue with network connectivity leading to storage and re-transmission of data.

Currently, FEV_1 remains the gold standard physiological biomarker for use in exacerbation diagnosis in clinical practice. It features in several exacerbation definitions in the clinical trial setting (254). Therefore, I evaluated the agreement between home-measured FEV_1 and in-clinic FEV_1 (performed using different equipment) by carrying out a Bland-Altman analysis. The comparison of home and clinic values recorded on the same day in 99 participants on 327 occasions is shown in Figure 5.13. On average, clinic values for FEV_1 tended to be slightly higher than the home values with a mean difference of 4% of predicted. The wide range for the limits of agreement

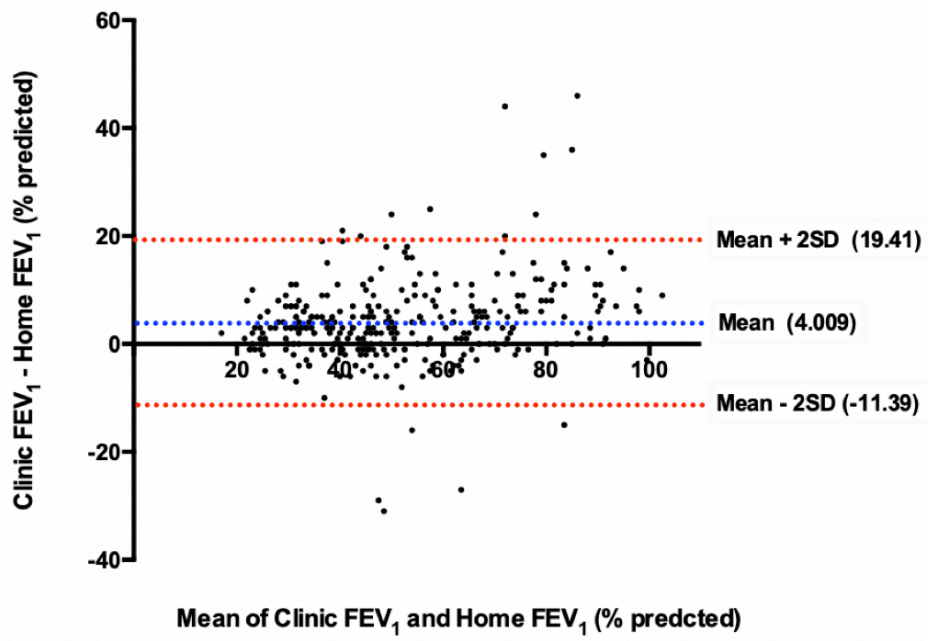


Figure 5.13. Comparison of clinic and home measurements for FEV_1 obtained on the same day on 327 occasions for 99 participants. The difference between clinic and home measurements is on the *y* axis and the average of clinic and home measurements is on the *x* axis. The blue dashed line indicates the mean difference for all data points and the red dashed lines indicate the limits of agreement (Upper line: mean + 2SD), lower line: mean -2SD).

(-11.39, 19.41) suggest that the home measurements may either underestimate FEV_1 by up to approximately 11% of predicted or overestimate FEV_1 by up to 19% of predicted. Home measurements also tended to under-estimate lung function at higher lung function values.

5.3.3.3 User experience of daily home monitoring

More than two thirds (102, 69%) of participants completed the SMARTCARE survey permitting first ever quantitative and qualitative assessment of user experience with daily home monitoring in adults with CF.

The clinical characteristics, treatment burden and home monitoring activity between survey respondents and non-respondents are compared in Table 5.5. The non-respondent cohort tended to be younger (unpaired t test, p = 0.0012) and less compliant with home monitoring (Fisher's test, p = 0.0002) and completion of the second study questionnaire; the CFQ-R (Fisher's test, p <0.0001). Gender, a pre-

Table 5.5. Comparison of participant characteristics, treatment burden and home monitoring activity between SMARTCARE survey respondents and non-respondents.

	Respondents (*n* = 102)	Non-respondents (*n* = 45)	*p* value
Age (yr) - mean	33.4 ± 9.7	28.0 ± 7.4	**0.0012**
Female sex – no. (%)	54 (52.9)	28 (62.2)	0.3682
BMI - mean	22.9 ± 3.2	22.2 ± 3.9	0.2783
FEV_1 (% predicted) - mean	56.6 ± 22.0	54.9 ± 21.5	0.6530
CFRD - no. (%)	46 (45.1)	15 (33.3)	0.2068
Pre-existing anxiety/depression - no. (%)	6 (5.9)	4 (8.9)	0.4954
Part-time or full-time employment, no. (%)	63 (61.8)	24 (53.3)	0.3664
CFQ-R treatment domain score available - no. (%)	81 (79.4)	7 (15.6)	**<0.0001**
Pre-study treatment domain score – mean	48.0 ± 21.7	49.3 ± 25.0	0.8855
Post-study treatment domain score – mean	45.6 ± 23.9	55.7 ± 14.6	0.2750
Inhaled antibiotics and/or mucolytics - no. (%)	97	40	0.1755
Intravenous antibiotic days - median	16 (0 - 29)	20 (6 – 38)	0.2440
Insufficient recording days – no. (%)	20 (19.6)	23 (51.1)	**0.0002**

Differences between continuous variables were examined with unpaired t test for parametric data and unpaired Mann Whitney signed rank test for non-parametric data. Differences between categorical data were examined with Fisher's tests. FEV_1: forced expiratory volume in 1 second, BMI: body mass index, CFRD:CF-related diabetes, CFQ-R: Cystic Fibrosis Questionnaire – Revised.

existing history of depression/anxiety or treatment burden did not differ significantly between the two groups.

Of the subset of respondents who were poorly compliant with home monitoring (20 out of 102), various reasons were provided for discontinuing monitoring but primary reasons cited were: increased time commitment/treatment burden which was harder to maintain when unwell and the daily frequency of measurements provoking increased anxiety about their health.

5.3.3.4 User interaction with the home monitoring technology

I assessed the usability of the home monitoring system by analysis of survey responses to the following questions: how easy is the technology to use (Q1), how frequently did you contact technical support for help (Q2), how often did you have problems measuring data (Q3) and how often did you have problems uploading data (Q4).

Survey responses to Q1 and Q2 are summarised in Figure 5.14. Most respondents (> 90%) found the components of the home monitoring system easy, or very easy, to use and did not need to contact technical support for assistance in data collection or transmission more frequently than monthly. More than half did not need technical support at all with either data collection (53%) or data transmission (69%).

Survey responses to Q3 and Q4 are summarised in Figure 5.15. Comparatively, a higher proportion of survey respondents experienced issues with data collection (85%) from sensor devices onto the SMARTCARE app than with data transmission (66%) from the app to the backend server. Of these sub-groups, respondents most commonly experienced data collection problems on a monthly basis (38% of respondents) whilst data transmission issues were more frequent, occurring weekly in 27% of respondents.

5.3.3.5 User perception of impact and ideal frequency of home monitoring

I assessed the acceptability of daily home monitoring by analysis of survey responses to the following questions: to what extent did daily home-monitoring interfere with your

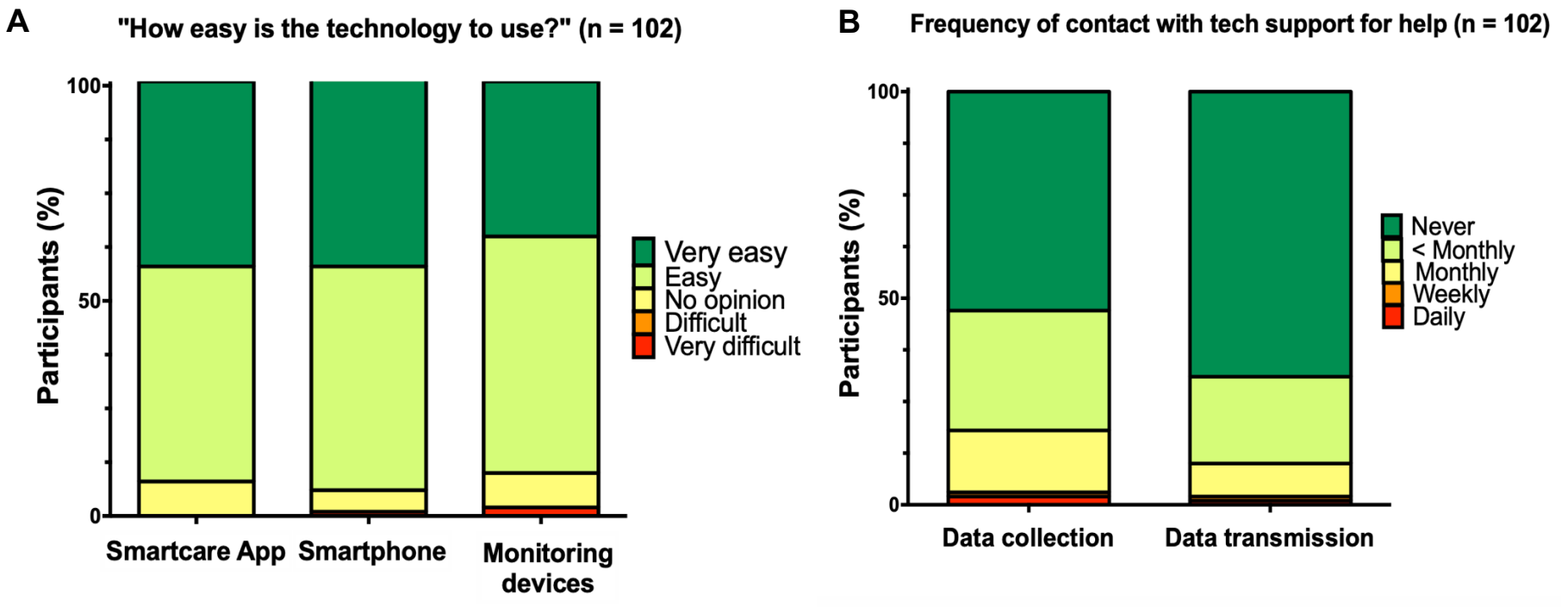


Figure 5.14. Survey responses from participants (*n* = 102) assessing (A) how easy the telemonitoring system was to use and (B) how often they needed technical assistance with collecting or transmitting their home measures. The majority of respondents found the technology easy to use and did not require frequent technical assistance to resolve issues with data collection or transmission.

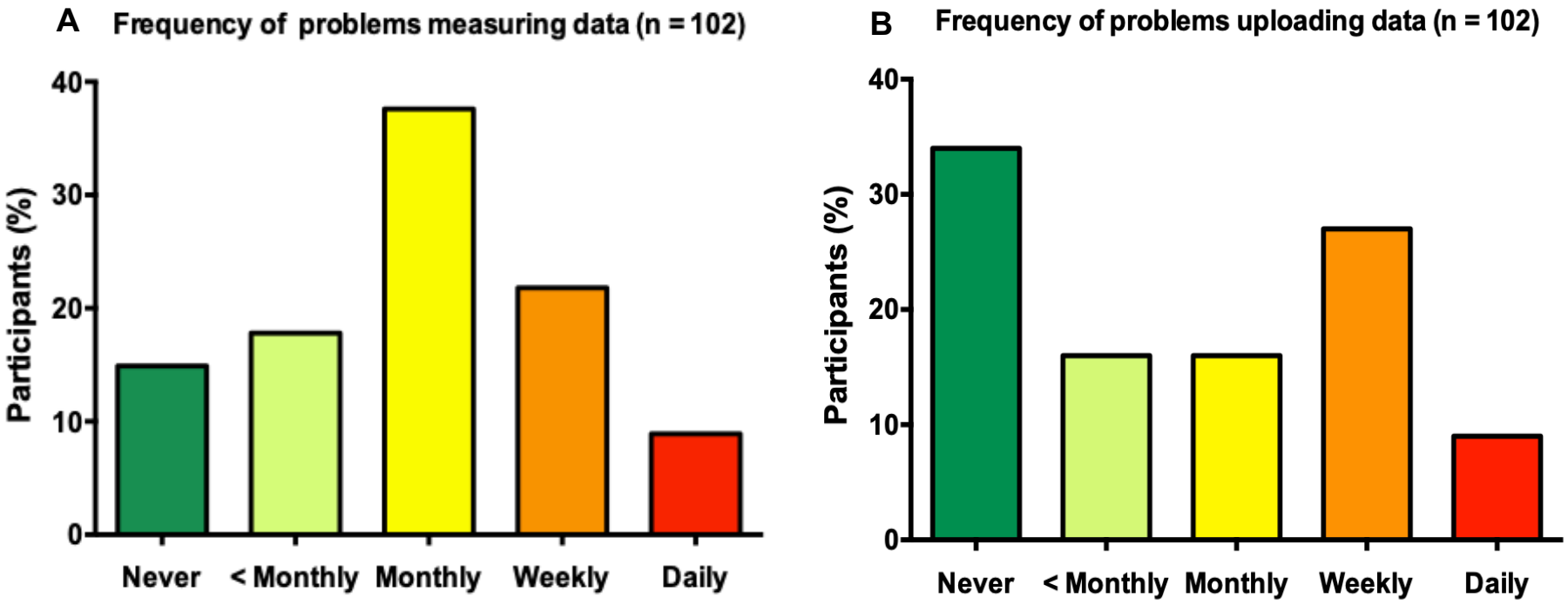


Figure 5.15. Survey responses from participants (*n* = 102) assessing (A) how often they encountered problems with data collection from their sensor devices and (B) how often they encountered problems with data transmission to the web server. A higher proportion of respondents experienced problems with data collection (85%) than with data transmission (66%), however overall, data collection problems occurred less frequently with the majority experiencing problems on a monthly basis compared with on a weekly basis for data transmission.

usual activities (Q1), how frequently would you like to complete home monitoring? (Q2), how helpful did you find it to be able to monitor your breathing, activity and weight at home (Q3) and what impact did home monitoring have on your ability to manage your health, anxiety levels and quality of life? (Q4).

Half of the respondents (50%) declared that daily home monitoring interfered only slightly with their usual activities whilst a further 15% felt that daily home monitoring was not an added daily imposition (Figure 5.16A). Of note, spirometry and sputum collection were considered most burdensome. Interestingly despite this, the majority of respondents (46%) said they would prefer to monitor their health only on a weekly basis rather than on a daily basis (Figure 5.16B). A considerable proportion of respondents (15%) offered alternate options for frequency of monitoring with the most common suggestion being less frequent (e.g., weekly) monitoring when well and increased frequency when unwell. However, some participants suggested only performing home monitoring when unwell, or no more frequently than thrice weekly, whilst others suggested daily monitoring for some measurements (e.g., lung function) and less frequent monitoring for others (e.g., weight).

Despite previous studies suggesting poor uptake of home monitoring in CF (reviewed in Detecting APEs using home monitoring, Section 1.2.3), the vast majority (77%) of survey respondents found home monitoring a helpful activity to do (Figure 5.17A). Interestingly, of the subset of respondents (20 out of 102) who provided insufficient days of recordings, the majority (75%) still declared that they found it helpful to be able to monitor their breathing, activity levels and weight at home. The main reasons provided for not continuing with daily monitoring included difficulties balancing daily life with remembering to complete regular monitoring and loss or breakage of equipment.

Respondents' perceptions of the impact of home monitoring on their ability to manage their own health were very encouraging with more than half (58%) declaring that their self-management was made better by being able to monitor their health at home (Figure 5.17B). More importantly, only a small minority (5%) felt that their self-management was made worse. Furthermore, over a third (37%) of respondents felt that their general quality of life was improved by home monitoring.

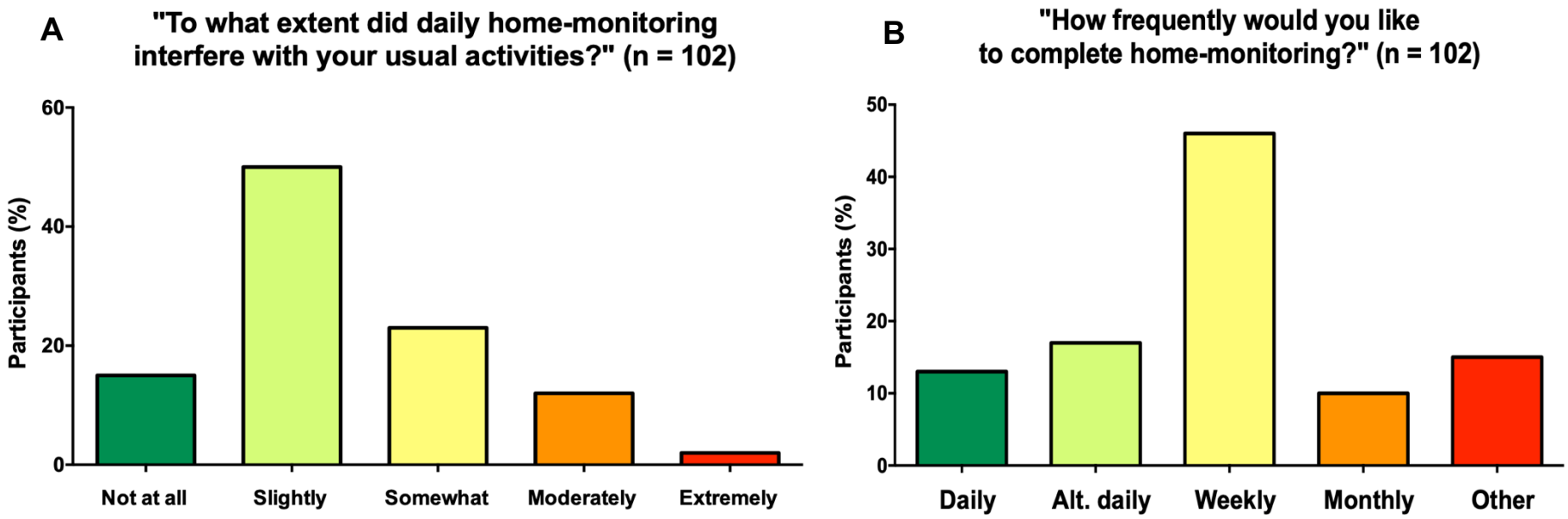


Figure 5.16. Survey responses from participants (*n* = 102) assessing (A) the impact of daily home monitoring on their usual activities and (B) their preferences on the ideal frequency of home monitoring. Most respondents (65%) declared that daily home monitoring interfered only slightly, if at all, with their usual activities. However, most (46%) preferred to complete home monitoring only on a weekly basis. Alt. daily: Alternate daily

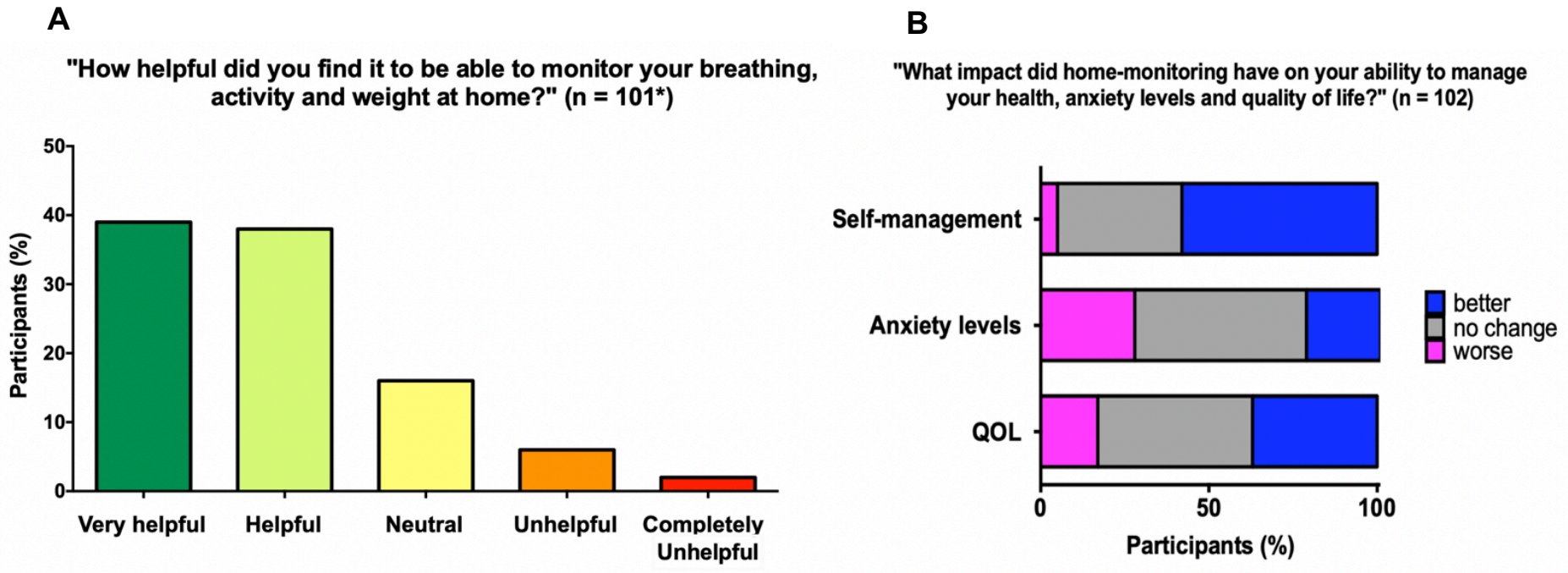


Figure 5.17. Survey responses from participants (*n* = 102) assessing (A) the helpfulness of being able to monitor their health at home and (B) the impact of home-monitoring on their self-management, anxiety levels and quality of life (QOL). The vast majority (75%) found it helpful to be able to monitor their breathing, activity and weight at home and most (58%) found that home monitoring improved their self-management. Although the majority (73%) of respondents felt that home monitoring either improved or had no impact on their anxiety levels, a significant proportion (28%) found that home monitoring made their anxiety levels worse. Over a third (37%) of respondents felt that home monitoring contributed to an improvement in their QOL. (*):one respondent missed answering this question

Although the majority of respondents (46%) did not feel that home monitoring had any impact on their overall QOL a small proportion (15 out of 102, 17%) declared that home monitoring negatively impacted on their QOL (Figure 5.17B). The main reasons provided for this were: 1) technical difficulties (e.g., faulty sensors (with inconsistent/unreliable recordings or missed recordings) causing frustration (e.g., with having to redo measurements) or in some cases leading to increased anxiety due to misleading results and 2) daily frequency of monitoring became tedious over time, especially on top of their usual treatment burden.

Increased levels of user anxiety (due to focus on the disease condition and regular observation of negative health trends) are anecdotally often cited as a key reason to reconsider frequent home monitoring in CF with the possibility that intensive health tracking may lead to negative feedback and behaviour change. This study is the first to qualify user feedback on this particular issue. Almost a quarter (22%) of respondents felt that their anxiety levels were improved by home monitoring whilst half (51%) declared that home monitoring had no impact on their anxiety levels. Although these results are reassuring, a number of respondents (28%) felt that home monitoring made their anxiety levels worse. Discordance between in-home and in-clinic lung function results were cited most commonly as a cause for increased anxiety and dissatisfaction with the home monitoring process. Comparison of clinical and home monitoring characteristics between the three cohorts (e.g., anxiety better, anxiety unchanged, anxiety worse) revealed some significant differences between the groups with regards to age, the presence of pre-existing anxiety/depression and the proportion of respondents who were non-compliant with home monitoring, however of note, these differences were not skewed toward the cohort who reported increased anxiety from home monitoring (Table 5.6).

5.3.3.6 Change in disease-specific health-related QOL scores with home monitoring

The response rates for the CFQ-R were considerably lower than for the study-specific survey, with only eighty-eight (59%) participants completing both pre-and post-questionnaires to allow for comparison. Of note, two participants from this cohort completed the survey despite withdrawing from the study. The mean CFQ-R domain scores at study initiation (Pre-CFQ-R) and study end (Post-CFQ-R) and the change in

Table 5.6. Comparison of demographic, clinical and home monitoring characteristics between groups of SMARTCARE survey respondents based on the impact of home monitoring on their anxiety levels.

	Anxiety better (*n* = 22)	Anxiety unchanged (*n* = 98)	Anxiety worse (*n* = 28)	*p* value
Age (yr) - mean	37.7 ± 8.2	29.8 ± 8.7	34.0 ± 10.0	**0.0004**
Female sex – no. (%)	9 (41)	53 (54)	20 (71)	0.0885
BMI - mean	23.5 ± 2.1	22.5 ± 3.7	22.7 ± 2.9	0.5128
FEV_1 (% predicted) - mean	46 ± 12.1	57.8 ± 22.9	57.9 ± 22.1	0.0638
CFRD - no. (%)	12 (54)	35 (73)	14 (50)	0.1549
Pre-existing anxiety/depression - no. (%)	0 (0)	11 (11)	0 (0)	**0.0482**
Part-time or Full-time employment - no. (%)	12 (54)	59 (60)	17 (61)	0.8776
CFQ-R treatment domain scores available - no. (%)	21 (95)	45 (46)	22 (79)	**<0.0001**
Pre - mean	45.4 ± 20.7	51.4 ± 19.2	44.5 ± 26.8	0.3682
Post - mean	35.9 ± 25.2	52.9 ± 20.1	44.1 ± 24.5	**0.0169**
Inhaled antibiotics and/or mucolytics - no. (%)	22 (100)	91 (93)	27 (96)	0.3642
Intravenous antibiotic days - median	14 (0 – 27.8)	15 (0 – 29)	19 (0 - 38.3)	0.87029
Insufficient recording days – no. (%)	1 (5)	30 (31)	3 (11)	**0.0073**

Differences between continuous variables were examined with one-way ANOVA for parametric data and Kruskal-Wallis test for non-parametric data. Differences between categorical data were examined with χ^2 tests. FEV_1: forced expiratory volume in 1 second, BMI: body mass index, CFRD:CF-related diabetes.

scores from baseline are summarised in Table 5.7. Somewhat unexpectedly, given the responses obtained from the SMARTCARE survey questions assessing the impact of health monitoring on general QOL, the majority of the CFQ-R domain scores did not show a statistically significant improvement following home monitoring. Furthermore, I identified a significant deterioration in emotional and social functioning domain scores. Of the 83 respondents who completed both the SMARTCARE survey and CFQ-R, only a minority (14%, 12 out of 83) had declared on the SMARTCARE survey that their QOL was made worse by home monitoring. The discordance between survey results may suggest that assessment of impact of home monitoring on treatment burden was inadequately addressed by the non-validated tool. In any case, this warrants further investigation to clarify if home monitoring is an explanatory factor for the deterioration in health-related QOL scores obtained on the CFQ-R.

5.3.3.7 User preferences on home monitoring to replace clinic attendance

In line with best practice guidelines, individuals with CF must attend clinic on a regular basis (at least three- to four-monthly), even when stable (79,80,441). This is often not without significant disruption to their everyday lives. Participants were therefore assessed on whether they would prefer to use home monitoring rather than attend routine clinic visits. Although the combined majority of participants (40%) were probably, if not definitely, in favour of replacing clinic attendance with home monitoring, the largest single proportion (28%) of respondents were not in favour of doing so (Figure 5.18). A key reason given against replacing clinic visits was inaccurate recordings or faulty equipment.

5.3.4 Using an ML approach to define the onset of an APE

In order to better understand the relationships between changes in home monitored measures and exacerbation events we applied ML approaches to learn new insights from the complex dataset. First, in order to generate a typical ('average') profile of the rise/fall in home measures in the period preceding treatment for an APE, we needed to define the onset of an APE, which we considered a hidden/*latent* variable. In the absence of a consensus definition for the onset of an APE (reviewed in Defining a CF exacerbation, Section 1.2.2) we defined the start of an APE as the point at which a change in the home-monitored measures is sustained, eventually culminating in an antibiotic treatment course.

Table 5.7. Domain scores at study initiation and study end for the CFQ-R respondents (*n* = 88).

CFQ-R domain	Pre-study (mean, SD)	Post-study (mean, SD)	Δ CFQ-R (mean, SD)	*p* value
Physical functioning	48 ± 22	47 ± 23	-2 ± 19	0.4371
Role perception	62 ± 23	62 ± 25	0 ± 18	0.8759
Vitality	42 ± 19	41 ± 21	-2 ± 20	0.4677
Emotional functioning	72 ± 18	67 ± 21	-4 ± 17	**0.0188**
Social functioning	61 ± 19	56 ± 20	-5 ± 17	**0.0056**
Body image	69 ± 25	68 ± 25	-1 ± 23	0.6635
Eating disturbance	83 ± 21	85 ± 20	2 ± 20	0.4184
Treatment burden	48 ± 22	46 ± 23	-2 ± 19	0.3904
Health perception	48 ± 22	45 ± 26	-3 ± 28	0.4184
Weight	73 ± 32	72 ± 34	-1 ± 36	0.6864
Respiratory symptoms	51 ± 20	51 ± 20	0 ± 18	0.7505
Digestive symptoms	78 ± 21	78 ± 19	0 ± 21	0.8181

Differences between pre-study and post study CFQ-R domain scores were examined with paired t-tests. CFQ-R: Cystic Fibrosis Questionnaire – Revised. Δ CFQ-R: change in CFQ-R domain score over time.

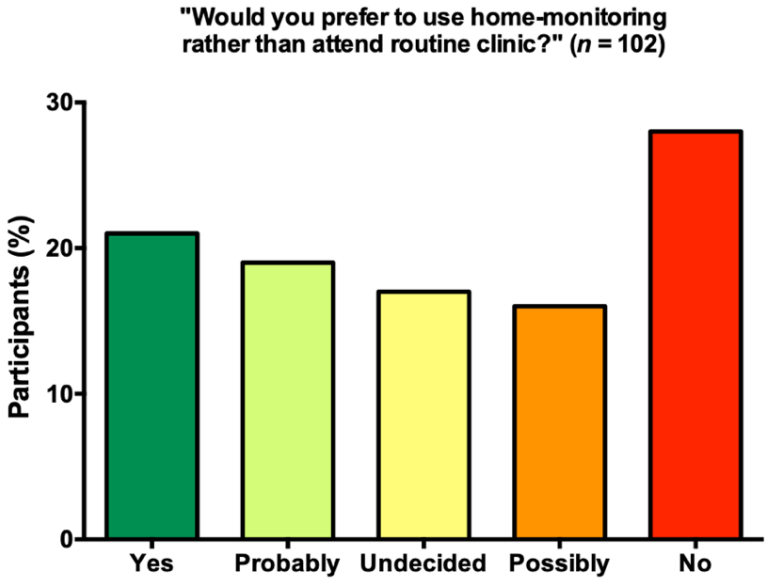


Figure 5.18. Survey responses from participants (*n* = 102) assessing whether they would prefer to use home monitoring instead of attending a routine clinic appointment for clinical review. More than a quarter (28%) of participants were not in favour of replacing routine clinic with home monitoring, however, the majority (40%) were probably, if not definitely, in favour of doing so.

However, between antibiotic treatment episodes, we observed considerable variation in the time from the point of a change in the home-monitored measures to antibiotic treatment initiation. An example of this variation is shown in Figure 5.19.

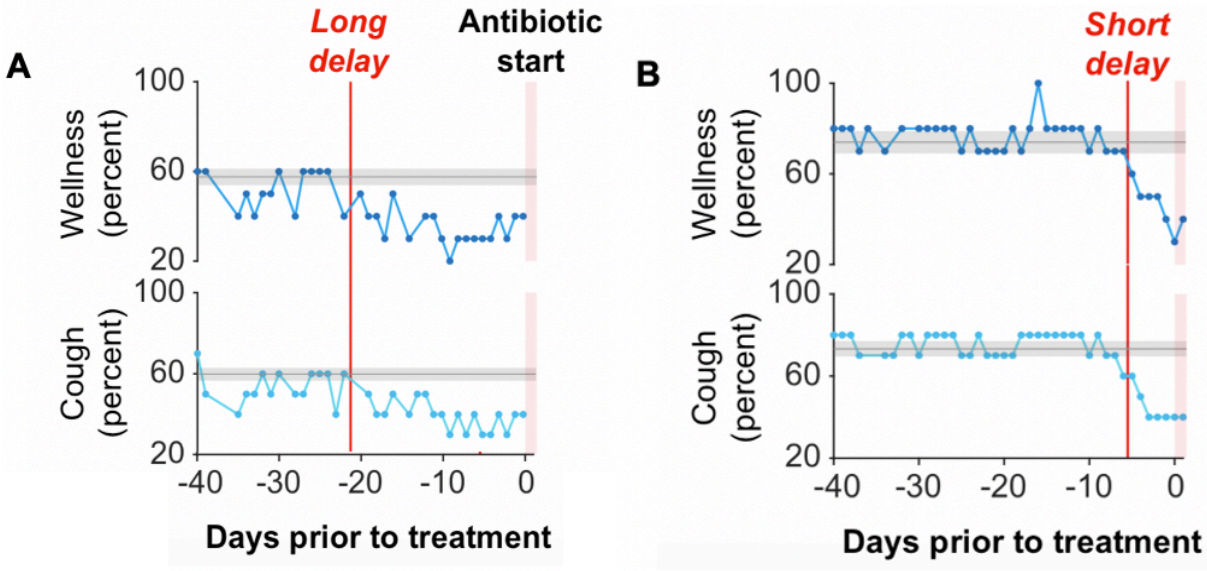


Figure 5.19. Variation in the time to treatment from the onset of a sustained change in home measures (illustrated for cough and wellness scores). In a 40-day window prior to starting antibiotics, the time from clinical deterioration (e.g., fall in cough and wellness symptom scores) to starting antibiotic treatment was longer for one APE episode (A) than for the other (B). Cough and wellness scores expressed as percentages with zero = worst ever and 100 = best ever) (Figure courtesy of Damian Sutcliffe).

We applied an unsupervised ML approach, using a probabilistic generative model, to learn the latent variable (onset of the APE) for each measurement record across all the APE episodes in the dataset. The average value of each measurement type during an APE was also assigned a latent profile in the model. Probability distributions for these latent quantities were inferred using an iterative algorithm [Expectation Maximisation (494)]. From the output of this process, the model was able to align the individual records of each measurement type, (accounting for the variations in time to treatment initiation from the onset of clinical decline) and generate a typical, temporally aligned profile for each measurement type over the period of an average APE (Figure 5.20). From these inferred APE profiles for each measurement type, we were able to determine the start of an APE using the consensus inflexion point across all the measurement profiles. This consensus point revealed that all measurement types

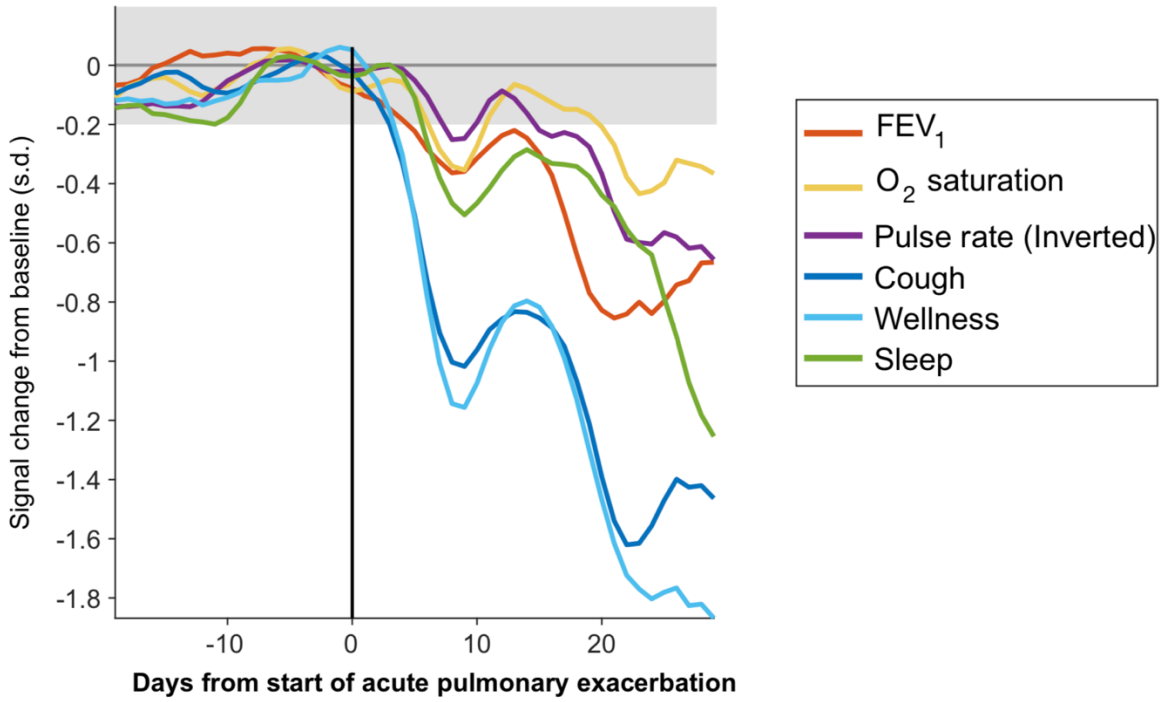


Figure 5.20. Inferred average profiles of the change in home monitored measures over the course of an average APE using an unsupervised ML model. The likely onset of the APE (black line) is taken as the consensus inflexion point at which sustained changes (as determined by the ML model) in all the measurements begins. The measurements are relatively stable, with some mild daily variation, prior to the onset of the APE. From the start of the APE, the start of decline occurs with relatively consistent timings across all measurements (e.g., within a 3-4 day window) with an earlier fall observed in FEV_1 and O_2 saturations than for the other measures. Larger deviations from baseline were observed for the self-reported measures (cough and wellness) than for the device-recorded measures. A non-sustained partial recovery in physiology and symptoms was also observed 10 days after the APE onset. (Figure courtesy of Damian Sutcliffe).

began to fall together within a 3-4 day window. Notably, we also observed a transient partial recovery around 10 days from the start of an APE (possibly reflective of patient-driven self-management in order to prevent further decline). This transient partial recovery suggests that interventions other than antibiotic treatment may have the potential to avert an APE. However, this partial recovery was then followed by a further prolonged decline, with recovery eventually coinciding with starting antibiotic treatment. In addition, the start point of the APE (that we determined from the learnt latent average change profiles generated by the model) could be mapped back to the

original measurement records to provide a start date label for each APE. The labelled dataset could then be used, with a supervised ML approach, to generate a predictive model for earlier detection of an APE (discussed in section 5.3.6).

5.3.5 Characterising different types of APEs

Our initial alignment model inferred a consensus picture of an average APE. We wondered, however, whether different types of APEs might exist. To explore this possibility, we next extended our model to variously allow for 2, 3, or 4 distinct sets of profiles, each representing a different class of APE. We found that we were able to classify APEs into three different classes, each exhibiting a unique average profile (Figure 5.21): Class 1 (51% of all APEs) was characterised by a synchronous fall in all signals interrupted by a transient partial recovery period (similar to the consensus profile; Figure 5.20); Class 2 (25% of all APEs) exhibited an early decline in cough and wellness alone, followed by a partial interim recovery, and then a fall in all signals; while Class 3 (24% of APEs) showed recovery in signals prior to the onset of the APE followed by a steep decline in all signals without an interim recovery period.

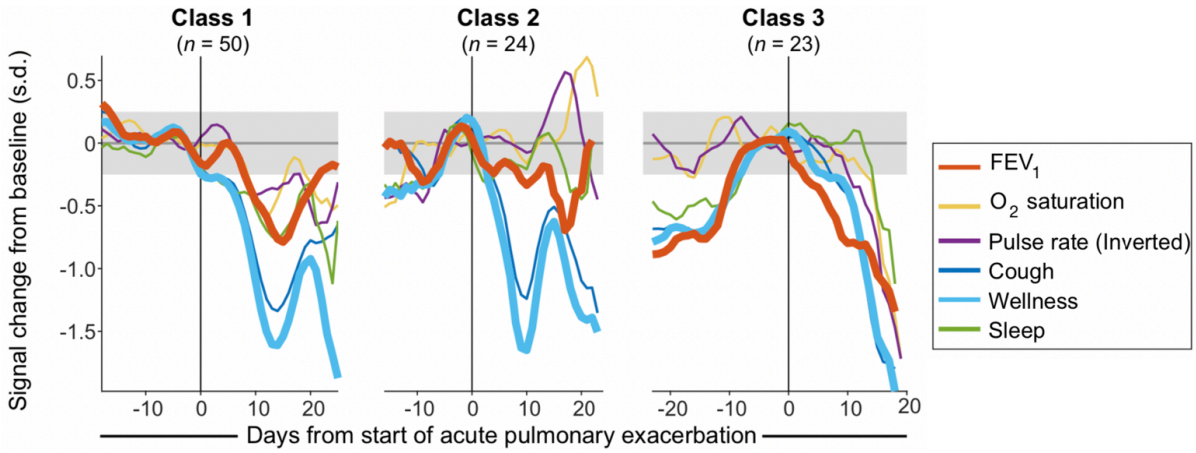


Figure 5.21. Categorisation of acute pulmonary exacerbations into different classes. (Figure courtesy of Damian Sutcliffe).

We also investigated for associations between class membership and other clinical variables of interest and found that: Class 1 APEs were more commonly associated with individuals who were chronically infected with *P. aeruginosa* (χ^2 test, p = 0.03) (Figure 5.22A); Class 2 APEs were associated with lower C-reactive protein (CRP) at the start of intravenous antibiotics (Mann-Whitney U-test, p = 0.03) (Table 5.8) and

with individuals who had fewer treatment courses during the study period (χ^2 test, p = 0.006) (Figure 5.22B, Table 5.8); and Class 1 and Class 3 APEs were associated with individuals who had a second APE within four weeks of the previous APE (χ^2 test, p = 0.04 and p = 0.008 respectively) (Table 5.8). It must, however, be acknowledged that it is difficult to be certain if this was a new APE or a continuation of a previous episode.

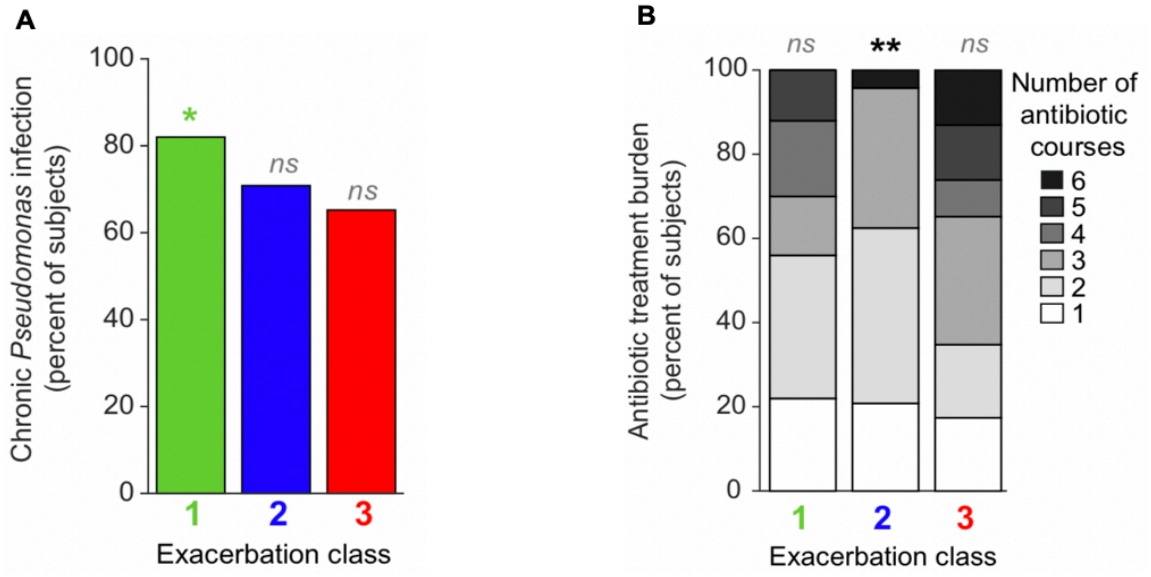


Figure 5.22. Associations between exacerbation class and clinical variables of interest. (A) Class 1 exacerbations were more commonly found in individuals chronically infected with *P. aeruginosa* (χ^2 test, p = 0.03). (B) Class 2 exacerbations were more commonly associated with individuals who experienced fewer treatment courses over the study period. (χ^2 test, p = 0.006). (Figure courtesy of Damian Sutcliffe).

Of note, exacerbation class membership was not associated with stable FEV_1, body mass index (BMI) or age. Furthermore, we found that some individuals tended to experience predominantly only one class of APE, whilst others went through multiple types of APE (Figure 5.23).

5.3.6 Using ML to predict for an APE

Next, we sought to develop a predictive algorithm for the onset of an APE; a function that might augment clinical decision support for individuals with CF and their care teams. We used the output from our single class alignment model (described in Using an ML approach to define the onset of an APE, Section 5.3.4) to label each day in a participant's data record as either occurring during an APE or not. Days occurring

Table 5.8. Comparison of clinical and demographic characteristics between exacerbation classes.

	Exacerbation Class		
	Class 1	**Class 2**	**Class 3**
Stable FEV1 (median ± IQR)	51 ± 33	53 ± 22	51 ± 24
p value	0.96	0.21	0.23
BMI (median ± IQR)	22 ± 5	22 ± 5	22 ± 4
p value	0.67	0.67	0.94
Age (median ± IQR)	31 ± 9	33 ± 12	33 ± 20
p value	0.35	0.81	0.40
CRP on admission (median ± IQR)	14 ± 27	6 ± 22	24 ± 39
p value	0.22	0.03	0.48
CRP Stable (median ± IQR)	1 ± 8	0 ± 6	0 ± 10
p value	0.46	0.70	0.63
Gender (%)	64	79	65
p value	0.20	0.14	0.50
Chronic *P. aeruginosa* infection (%)	82	71	65
p value	0.03	0.76	0.21
Chronic *S. aureus* infection (%)	14	25	13
p value	0.37	0.10	0.43
Number of antibiotic courses (mean)	2.6	2.3	3.2
p value	0.06	0.006	0.25
Repeat exacerbation < 4wk (%)	35	38	64
p value	0.04	0.34	0.008

Differences between continuous variables were examined with Mann-Whitney U-test for non-parametric data. Differences between categorical data were examined with χ^2 tests. (Table courtesy of Damian Sutcliffe).

during antibiotic treatment courses (or between two closely associated sequential treatment courses) were excluded. Using a supervised ML approach, we employed a probabilistic regression classifier and used subsets of the data to iteratively train and then test the algorithm (using four-fold cross-validation).

The classifier used features derived from a trailing window of measurements on a subset of the data and aimed to predict the exacerbation label for the day following the measurement window. We ensured the model had no visibility of future data (to simulate the requirement for use in the clinical setting). We defined the true positive rate of the classifier as the proportion of APE periods where we correctly identified an APE, and the false positive rate was defined as the proportion of the stable periods where we incorrectly identified an APE. For each APE episode, we defined early warning as the number of days between the first day the prediction exceeded the

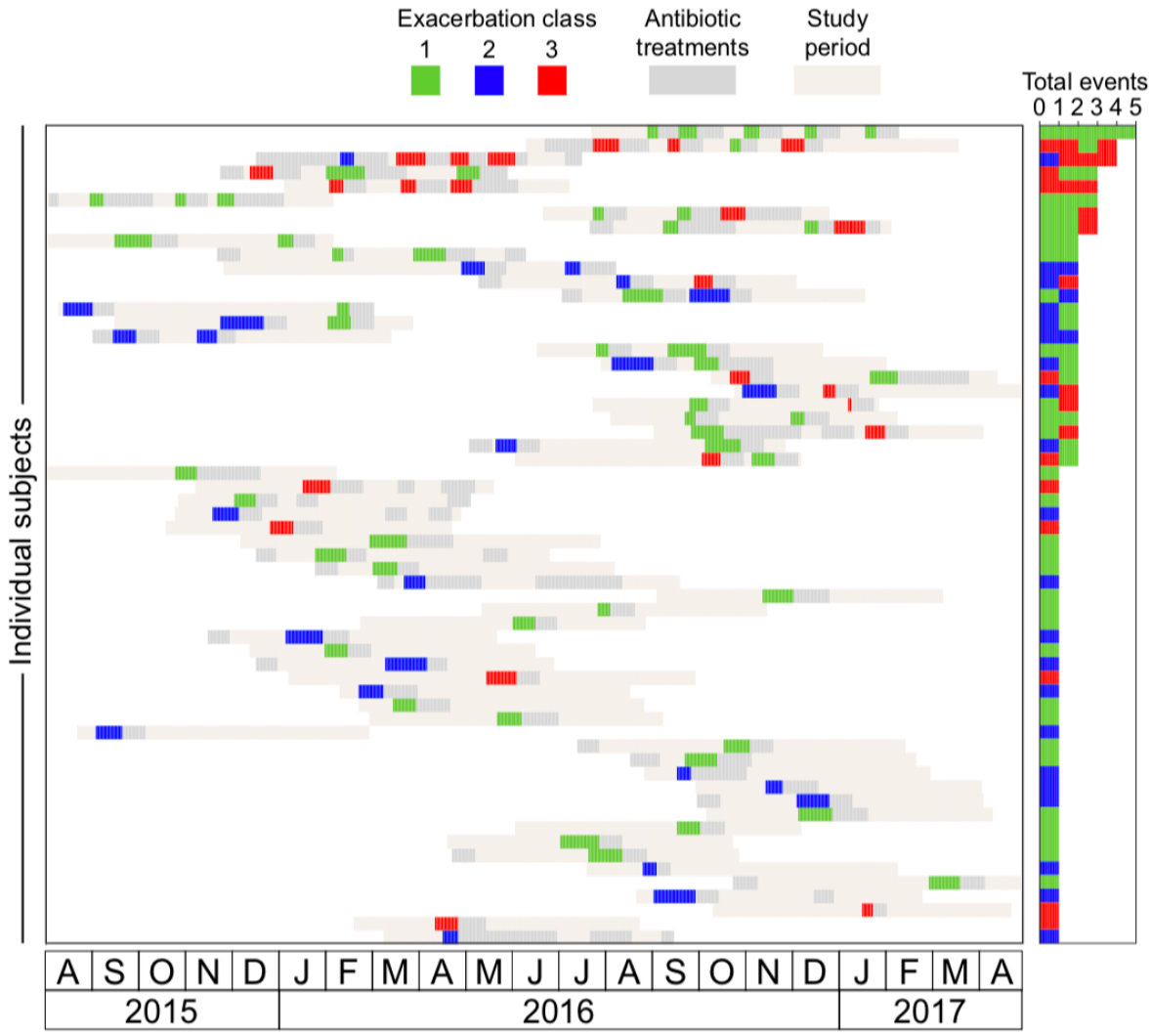


Figure 5.23. Summary of acute pulmonary exacerbation events experienced by participants over the course of the study period, colour coded according to exacerbation class (Class 1 in green, Class 2 in blue and Class 3 in red). The grey bars denote antibiotic treatment immediately following an exacerbation (note, some treatment episodes were not associated with exacerbation events due to insufficient data). A summary of the exacerbation types experienced by each participant is illustrated in the colour bar on the right-hand side of the plot. (Figure courtesy of Damian Sutcliffe).

trigger threshold and the day treatment was administered. We then calculated the average over all APE episodes. The classifier performed well, with an area under ROC curve of 88.9% (Figure 5.24A), and was able to provide 11 days advance warning of an impending APE at a clinically acceptable false positive rate of 22% (Figure 5.24B). The false positive rate was deemed clinically acceptable as the consequence would be a conversation with the clinical team, at which point an appropriate management plan would be initiated (e.g., no further action required). We also examined the relative

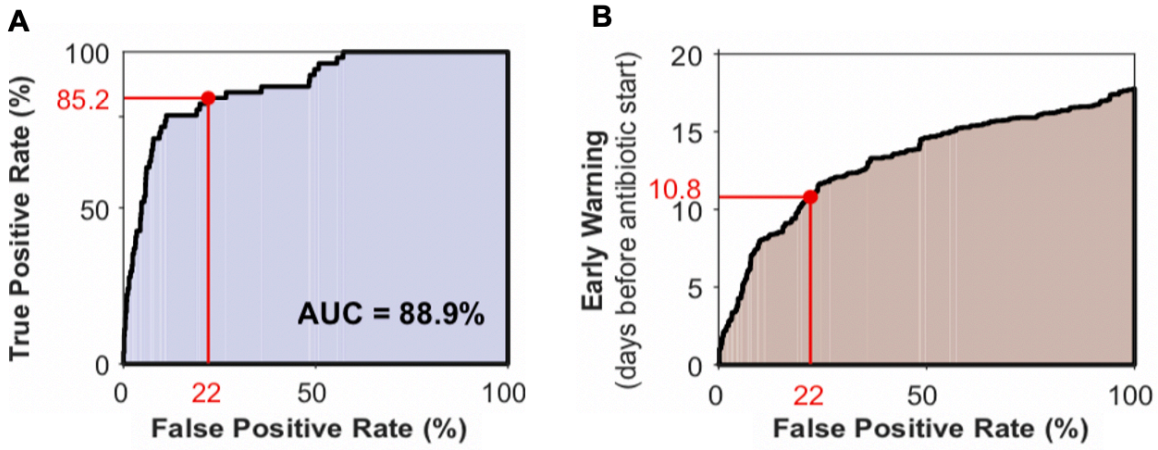


Figure 5.24. Evaluation of the predictive classifier. (A) ROC plot for the ability of the predictive algorithm to accurately detect an APE. (B) Early warning plot of the average time from detection of an APE to initiation of antibiotic treatment. (Figure courtesy of Damian Sutcliffe).

contributions of individual features to the performance of the predictive classifier (Figure 5.25) and found that symptom features (predominantly 'wellness' rather than 'cough') provided considerable episodic prediction value over the physiological measurements (FEV_1, O_2 saturations, and pulse rate). The classifier performed best when both symptom features and physiological features were used together.

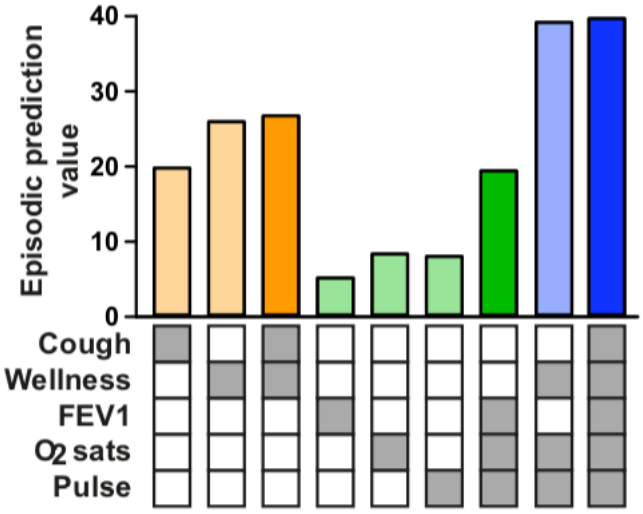


Figure 5.25. Relative contributions of individual features (wellness, cough, FEV_1, O_2 saturations, and pulse rate to the performance of the predictive classifier. (Figure courtesy of Damian Sutcliffe). The Episodic Prediction Value is a quality measure that rewards high predictions for episodes during an exacerbation (e.g., with true labels), and low predictions for episodes during stable periods (e.g., with false labels). First, the Average True Prediction and Average False Prediction are calculated as the average episodic predictions during true and false episodes respectively. The Episodic Prediction Value is the difference between them. In grey are the features contributing to each Episodic Prediction Value. (Figure courtesy of Damian Sutcliffe).

Of note, the predictive performance of the model was nearly as good when using just wellness, O_2 saturations and pulse rate, which suggests that home monitoring may not have to incorporate regular spirometry, which is time consuming, effort dependent and can cause discomfort when performed.

5.4. DISCUSSION

Cystic fibrosis care has seen remarkable advances over the last five decades that have led to major improvements in life expectancy (73). However, for individuals with CF this has been accompanied by a high daily treatment burden and regular visits to the clinic, even when stable. Periods of ill health, most often triggered by APEs, require intensification of treatment and clinical monitoring, which adds to the disruption of everyday life and negatively impacts wellbeing (129). Moreover, delays to recognition and treatment of APEs accelerate decline in lung health and adversely affects survival outcomes (127).

The rapid advances in digital technology and widespread adoption of connected wearable sensor devices and health apps to track and change health behaviours by the general population has led the healthcare sector to consider leveraging their use for chronic disease management, particularly as a means to enable timely personalised care, empower self-management, improve patient outcomes and reduce unnecessary healthcare utilisation. In CF, unique scope exists for the application of home-based, self-monitoring strategies, including technologically-supported self-management, earlier detection and treatment of APEs and nosocomial infection risk reduction by minimising routine hospital visits when well.

This multi-centre, non-interventional study is the first of its kind to investigate the feasibility and acceptability of using digital technology, via commercially available Bluetooth-enabled sensor monitoring devices and a smartphone application, to complete daily home monitoring in adults with CF. Furthermore, this is also the first study to apply ML approaches to high frequency physiological and symptom-based data, collected with home monitoring, in order to better understand and identify APEs in adults with CF.

A key priority of this study was to streamline health monitoring and minimise any impact on the existing daily treatment burden for an individual with CF. This was achieved with the development of a smartphone- and web-based home monitoring system. The choice of home monitoring parameters was guided by the findings of an earlier pilot study (TeleCF) (discussed in Chapter 3) and in keeping with currently used measures for the diagnosis of APE in clinical practice. Data collection was simplified through use of a unified interface on a smartphone, providing participants with easy access to their health data. Data capture was excellent, with a very low percentage of missed or repeated measurements logged by the monitoring devices.

Previous criticisms of home monitoring in CF have included concerns regarding the reliability and accuracy of electronically monitored home-based lung function in the absence of supervision by a dedicated respiratory physiologist (495). Notably, in this study, the agreement between in-home and in-clinic spirometry measurements was very good, with only a 4% of predicted difference in FEV_1 found between the two devices. However, there was a tendency for the in-home device to underestimate lung function at lower values, with the converse true at higher values. This may be related to procedural issues (e.g., poor effort or user technique) in unsupervised recordings or to differences between the two instruments. However, by selecting the highest lung function measurement for each day, according to BTS criteria, we reduced the impact of suboptimal efforts on our analysis. Nonetheless, future use of this system for diagnostic and monitoring purposes will need to take this into account, particularly as data unreliability was cited as a source for discontinuation and increased anxiety by a minority of participants.

This study has demonstrated that daily home monitoring is technically feasible and helpful for adults with CF. Users found the smartphone application and monitoring devices easy to use and, for the majority, technical issues with the monitoring system were infrequent. Adherence to electronic monitoring in this study was significantly better than adherence rates reported in previous CF monitoring studies (203,207), with the majority of participants (48%) completing daily measures for at least five months of the 6-month study period. Furthermore, older participants were more likely to adhere to the monitoring schedule than their younger counterparts. We also did not observe any differences in clinical characteristics, including severity of lung function

impairment and treatment burden, between participants who adhered to monitoring and those that did not. It is likely that the factors influencing adherence to home monitoring are not adequately captured by assessment of cohort demographics and clinical characteristics, and warrants a more detailed exploration.

Studies evaluating the impact on self-management of health-monitoring using digital technologies (such as smartphone applications, health trackers) in CF are lacking. However, recent work in asthma and diabetes has demonstrated improvement in health outcomes and self-management as a consequence of their application (496,497). This is the first study to report user feedback on the impact of home monitoring using newer technologies in adults with CF. The majority of participants reported minimal imposition on their day-to-day activities from taking part in daily home monitoring with this technology. Furthermore, most considered it helpful to be able to track their symptoms and physiology at home as this assisted in their self-management. Nonetheless, it should be noted that most preferred to reduce the frequency of health monitoring to at least weekly, particularly when well. The reasons for this were not immediately clear. However, it is likely that it reflects a normal desire to minimise the dominance of the disease in an individual's life when stable.

A common criticism raised against home monitoring by CF clinicians and caregivers is that frequent tracking of health measures, in addition to the existing care activities that individuals with CF must undertake, may reinforce the presence of CF in their lives and may exacerbate or precipitate anxiety or unhelpful health behaviours (495,498). These concerns were not borne out in this study. Most participants experienced no change in their baseline anxiety levels with some reporting improved anxiety levels as a result of home monitoring. Participants who reported such improvements tended to be older than those who reported no change. This may suggest that for older individuals with CF, anxiety may be more closely related to health status whilst for younger individuals the drivers are multifactorial.

Unexpectedly, findings from this study did suggest that daily home monitoring had a negative impact on perceptions of social and emotional functioning domains as captured by the disease-specific assessment tool; CFQ-R. It is not entirely clear if this can be linked to home monitoring and it may be that alternate assessment tools are

required to explore these domains further. However, perceptions of treatment burden did not change following introduction of home monitoring. Our results contrast with that of a similar analysis recently reported by Lechtzin *et al* (215). In their large-scale, randomised, controlled trial, which evaluated the benefits of electronic home monitoring of FEV_1 and respiratory symptoms in early detection of pulmonary exacerbations among adolescents and adults with CF, participants randomised to the early intervention arm (twice weekly spirometry and respiratory symptom scores via an electronic monitoring system over a 12-month period) received twice as many protocol-defined review visits and more treatments than the usual care group, despite no association with a slower rate of decline in FEV_1. Furthermore, the intervention arm encountered several technical issues during this study. Therefore, not unexpectedly, an increased treatment burden was associated with the home monitoring intervention. Although our study, as a non-interventional trial, will have a lesser impact on treatment burden comparatively, it is noteworthy that technical issues were a significant deterrent to adherence in the study by Lechtzin *et al.*, underscoring the importance of streamlining technology to simplify care and maintain engagement.

Given the impact of APEs on the survival and wellbeing of individuals with CF, there is an urgent need to better characterise the changes that precede APEs in order to be able to identify their onset promptly and enable timely intervention. Using unsupervised ML methods, this study is the first to report on a characteristic profile of the temporal changes in physiology and symptoms associated with an average APE in adults with CF. This derived profile enabled us to define an accurate start date for an average APE, which provides a label to train ML-based predictive algorithms that can be integrated into clinical decision support systems. The average APE profile also revealed a characteristic partial recovery after about 10 days from the start of the exacerbation. This transient improvement in health may be due to the institution of self-management strategies by the individual, such as increased/improved airway clearance or adherence with routine medications, to avoid the need for antibiotic treatment. This suggests that home monitoring, supported by ML-based algorithms, has the potential to enable behaviour change and provide opportunities for non-antibiotic-based interventions. Extension of our initial alignment model permitted the identification of three different classes of APE: Class 1 exacerbations were similar to the consensus average profile and associated with chronic *P. aeruginosa* infection;

Class 2 exacerbations showed symptomatic decline prior to falls in lung function (possibly as a consequence of an initial upper respiratory tract infection, were associated with lower systemic inflammation (as measured by CRP) and more prevalent in individuals with low exacerbation frequencies; and Class 3 exacerbations were associated with a recent previous APE and a steep clinical deterioration, with no interval improvement, possibly due to overwhelming infection. Further work is needed to understand whether these exacerbation classes reflect distinct pathological states, different intrinsic host responses to lung infection and inflammation, or different initiating triggers of exacerbation. Using a supervised ML approach and the output from our alignment model, this is the first study to report on a predictive algorithm for earlier detection of APEs in adults with CF. Using our predictive model, we identified APEs in advance, by around 11 days. If incorporated into a home monitoring platform this function offers the potential for early intervention, thereby possibly reducing cumulative inflammatory lung damage.

This study has several limitations that may influence the interpretation of our findings. First, qualitative feedback on feasibility and acceptability of home monitoring was collated only from survey respondents, which may confound conclusions made due to selection bias. Survey respondents may be more receptive of the home monitoring process overall. Indeed, contrary to expectations that younger participants may be more technologically-literate and therefore engage better with the monitoring system, non-respondents tended to be younger and less compliant with monitoring. Barriers to adherence with treatments are well documented among younger individuals with CF therefore further consideration of user priorities in this group would be beneficial to the longer-term application of this system. Second, this was a non-interventional study therefore no comment can be made on the clinical impact of home monitoring on usual clinical outcomes, specifically in terms of its utility in predicting for and minimising APEs. Third, no assessment or recommendation can be made on the practical impact home monitoring may have on clinical care provision, how it may be received by clinicians and the wider CF multi-disciplinary team and how it may be integrated into the current models of CF care. Fourth, our smartphone application was developed only for an android operating system. Although an android smartphone phone was provided to participants during this study, this feature limits its accessibility and

potential for integration with other sensor devices that may enhance home monitoring capabilities.

Future work in this area will need to focus on determining what the optimum frequency of home monitoring is across the parameters assessed in order to minimise treatment burden and maintain clinical utility; developing a smartphone application that is device agnostic improving accessibility, ensuring that technical issues are overcome, refining and testing the predictive algorithm on larger cohorts and assessing its utility against current clinical practice; and exploring the cost benefit of integrating home monitoring into the clinic setting, and how this may affect clinical interactions (both from the perspective of the patient and the clinician) and delivery of best patient care. Currently, a large-scale, multi-centre trial (Project Breathe) is underway to address many of these questions.

6. CONCLUSIONS

6.1 A restatement of the research aims

Acute pulmonary exacerbations are the primary driver of morbidity and mortality in the CF population. This makes understanding what triggers them and developing strategies to optimise earlier detection a key priority. Despite their clinical significance little is known about what factors precipitate these acute deteriorations in lung health from a stable baseline.

The aim of this dissertation was to examine whether fluctuations in the expression of certain phenotypic traits by clonal populations of *P. aeruginosa*, the dominant CF airway pathogen, were responsible for triggering exacerbation events. Moreover, before this work, very little was known about the changes in physiology and symptoms that precede the onset of an APE. Previous studies have tried to address this question but were limited by use of analytical techniques that dealt poorly with multi-dimensional datasets. This work aimed to apply ML techniques to daily home monitored data of patient physiology and symptoms to better understand the changes which precede exacerbation onset, and from these new insights, assess for predictive signals that may facilitate earlier detection of exacerbation onset.

6.2 Key findings

6.2.1 TeleCF: Investigating the use of home monitoring and single biomarker profiling in CF sputum to predict for acute pulmonary exacerbations

This study was the first to examine CF sputum samples for changes in the levels of, Exotoxin A, a secreted *P. aeruginosa* virulence factor, before, during and after antibiotic treatment for an APE. The results confirmed that Exotoxin A alone is an unreliable biomarker of exacerbation onset, and, in contrast to earlier studies, does not correlate well with CF disease severity or markers of systemic inflammation.

In addition, this prospective, pilot study of 15 participants, was the first to document the extent of day-to-day variation in symptoms (cough and wellness) and several physiological markers (lung function, oxygen saturation, heart rate, weight, activity levels) during illness and health in adults with CF. Whilst analysis suggested there

might be signal changes, that would be predictive of exacerbation onset, using a combination of home-measured parameters, establishing a robust prediction algorithm was not possible using conventional statistical methods due to the complex nature of the dataset.

The findings of this study, in the form of an abstract and poster presentation (awarded Best Poster presentation), were presented at the 37th European Cystic Fibrosis Conference (Gothenburg, Sweden) (499).

6.2.2 Investigation of changes in the phenotypic expression of clonal populations of *P. aeruginosa* as a trigger for exacerbation onset in adults with CF.

This work reported on the phenotypic analysis of the largest collection (*n* = 4353) of longitudinally collected co-eval, *P. aeruginosa* isolates from chronically infected adults (*n* = 9) with CF. We found no evidence for the emergence of a particular sub-population of phenotypic variants associated with periods of acute pulmonary exacerbation. Specifically, we have shown categorically that the production of QS-associated regulatory and virulence factors is not significantly associated with these events, irrespective of the genetic background (e.g., epidemic versus non-epidemic) of the *P. aeruginosa* clonal population.

This work, in addition, was the first to report on a comparative analysis of differences in functional phenotypes between non-epidemic CF isolates of *P. aeruginosa* and the two most prevalent, epidemic CF strains in the UK: the LES and the MES. We reported on the first ever characterisation of LES isolates as predominantly, non-motile, small colony variants with a greater tendency to retain production of biofilm and QS-associated virulence factors, in comparison to the MES or non-epidemic isolates.

Quorum sensing is currently understood to play an essential role in the regulation of *P. aeruginosa* virulence factor production, important for the initiation and maintenance of infections. Yet, a puzzling observation is that *P. aeruginosa* isolates collected from chronic CF infections appear to frequently lose expression of *lasR*, commonly described as the ‘master regulator’ of the hierarchical QS network. Increasingly,

evidence for a 're-wiring' of QS regulatory circuitry in CF-evolved *P. aeruginosa* isolates suggests that the *rhlIR* system plays an indispensable role (which may compensate for loss of LasR activity) in controlling QS activity. In support of these observations, this study reported on a very high prevalence (81%) of OdDHL-deficient isolates (suggestive of mutations in *lasIR*) without accompanying deficiencies in traditionally LasR-controlled phenotypes (including preserved RhlI-generated signal). We also found that although QS-signalling (in particular RhlI-generated signal) and QS-associated phenotypes were more likely to be maintained by LES isolates, QS signal production was typically independent of the production of QS-associated virulence factors. Whether this suggests a decoupling of QS signalling from gene transcription in the LES remains to be determined.

6.2.3 SMARTCARE: A feasibility analysis of home-monitored physiology and symptoms using smart devices in adults with CF to better understand and predict for changes preceding acute pulmonary exacerbations

The availability and use of new digital technologies (connected wearable monitoring devices, web-based and mobile applications) to manage chronic disease at a distance is increasing and has been accelerated in the wake of the Covid-19 pandemic. Until now, robust, large scale, feasibility and acceptability studies of digital technology-enabled home monitoring in CF has been lacking. This multi-centre study of 147 participants was the first to examine the use of blue-tooth-enabled monitoring devices and smartphone technology to track daily symptoms and physiology in an adult CF population. Moreover, it was the first study to report on user feedback on the home monitoring experience, much of which was positive.

The current model of CF care is largely hospital-based with patients having to travel to their CF centre for review at least every 2-3months. However, the increasing recognition of cross-infection as a risk, further heightened by the SARS-CoV-2 pandemic, has necessitated the consideration of new ways to limit unnecessary hospital contact. Home monitoring, with its potential to be incorporated into video clinics, provides a novel means of triaging which patients really need to attend clinic for an in-person review. This not only addresses cross-infection risks but improves convenience for individuals with CF, enabling a reduction in travel burden and time off work and education.

These new digital technologies also hold benefits for the CF clinician. Information gathering and analysis through home monitoring systems provides an opportunity to augment clinical decision-making through enabling more timely identification of intervention points (possibly minimising the need for more aggressive therapies), and offering real-time feedback on the efficacy of starting new interventions and therapies. This can also provide an opportunity to stop ineffective therapies and reduce the treatment burden for individuals with CF.

Moreover, through the application of ML approaches to high frequency home monitored data, it was possible to characterise, for the first time, a typical profile of the changes in physiology and symptoms taking place during an APE. Extending on this work, we identified three distinct exacerbation profiles: Class 1 associated with chronic *P. aeruginosa* infection, Class 2 associated with lower systemic inflammation and low exacerbation frequency, and Class 3 associated with a recent previous exacerbation. It is not yet known whether these distinct classes represent different pathophysiological processes, initiating factors or host responses to infection and inflammation. The generation of a typical profile of an average APE also allowed us to develop an ML-based predictive model that was able to provide advance warning of an APE by around 11 days. Incorporation of this ML-based predictive algorithm for APEs into a home monitoring platform could not only support more patient-centred, clinical decision-making but also encourage, support and empower responsive self-management, for the benefit of improved well-being and long-term lung health.

Part of the findings of this work have been submitted for publication [Sutcliffe D, Ukor E *et al.* 2020 Machine learning predicts acute pulmonary exacerbations in cystic fibrosis (under review)].

6.3 Future directions

Future work on home monitoring technologies in CF should build upon the work presented here and explore the feasibility of incorporating these systems into routine clinical care. Cost benefit analyses and longer-term assessments of the impact on health outcomes, health care usage, satisfaction with care and patient-reported quality of life will need to be undertaken. Future studies would also benefit from a mixed-methods approach in which feedback is sought from both patients and clinicians. This

can provide insights into aligning care goals between individuals with CF and their care team.

Further work on this large collection of epidemic and non-epidemic CF-evolved *P. aeruginosa* isolates should initially focus on characterising the genetic basis for the preservation of traditionally LasR-regulated phenotypes in isolates deficient in *las* signalling. Particular focus should be paid to isolates in whom preferential conservation of the *rhl* signalling system was observed. Combining proteomic and metabolomic studies with genotype analysis of this sub-group of isolates may help with elucidating whether *in vivo* QS is RhlR-dependent in chronic CF infections.

If time had permitted, I would have liked to undertake growth rate analysis on this large isolate cohort. This would have enriched this phenotypic screen further by providing insights on *P. aeruginosa* fitness. Of interest would have been to further characterise the auxotrophic isolates observed. Moreover, comparative evaluation of this subgroup of isolates following growth in synthetic CF sputum medium (which more closely reflects the *in vivo* nutritional environment of the CF lung) may have provided helpful insights into the nutritional cues that influence cell-cell signalling and *P. aeruginosa* pathogenesis.

Furthermore, if time had permitted, I would have liked to apply the treatment-independent definition of APE onset learned from the ML analysis to more accurately select sputum samples for phenotypic screening. This may have led to the detection of significant changes in microbial behaviour prior to the onset of an APE that was otherwise missed.

Finally, given the availability of the large sputum biobank generated by the SMARTCARE study, of interest may be completion of detailed phenotypic characterisation of populations of *P. aeruginosa* clonal isolates collected from daily sputum samples from a single individual with CF over time. Such an analysis, combined with genotypic, metagenomic, proteomic or metabolomic analyses may provide unique insights into the day-to-day variation in clonal population behaviour

and structure of *P. aeruginosa* in relation to periods of clinical stability and exacerbation in the CF lung.

6.4 Closing comments

For the modern CF population with increasing access to highly effective modulator therapies, a majority will experience a dramatic reduction in their respiratory symptom burden with consequent stability in baseline health and well-being. This poses unexpected challenges as traditional biomarkers of disease stability become less sensitivity (e.g., lung function, frequency of APEs) and non-invasive airway microbial surveillance is reduced as patients become unable to expectorate sputum. Such challenges, not encountered specifically in this work given the very low numbers of participants on CFTR modulator therapy, may be addressed in future studies building on the progress made in this work.

This work has provided evidence for the feasibility, acceptability and utility of home monitoring using digital technologies in adults with CF. It has also applied ML approaches to high frequency home monitored data to provide novel insights into the changes in symptoms and physiology occurring during the course of an acute pulmonary exacerbation. Furthermore, it has shown how ML-based approaches can be used to identify and consequently predict for the onset of acute pulmonary exacerbations, providing a means to augment clinical-decision making and support self-management in CF. With scope to be integrated with validated patient-reported outcome measures, opportunity exists to improve on diagnostic paradigms for APEs in the face of a potentially changing symptom landscape. Finally, this work has revealed strong phenotypic evidence for the uncoupling of the traditional QS regulatory circuitry in CF isolates of *P. aeruginosa* and, given the scale of phenotypes and isolates analysed, shown that the emergence of a particular phenotypic variant is not a trigger for acute pulmonary exacerbations in adults with CF.

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APPENDIX 1

NHS
Health Research Authority
NRES Committee East of England - Hertfordshire
Victoria House
Capital Park
Fulbourn
Cambridge
CB21 5XB

Telephone: 01223 597733
Facsimile: 01223 597645

05 December 2012

Dr Andres Floto
Welcome Trust Senior Clinical Fellow and Honorary Consultant
Papworth Hospital NHS Foundation Trust
Papworth Hospital
Papworth Everard
Cambridge
CB23 3RE

Dear Dr Floto

Study title:	**A home-based, rapid and quantitative test for bacterial respiratory infections in patients with Cystic Fibrosis, to reduce admissions and length of hospital stay and to improve healthcare outcomes.**
REC reference:	**12/EE/0462**
IRAS project ID:	**110454**

Thank you for your letter of 21 October 2012, which we received on 22 November 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Miss Anna Bradnam, nrescommittee.eastofengland-norfolk@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter	From Jane Elliott CF R&D Manager	18 September 2012
Investigator CV	Rodrigo Andres Floto	28 June 2012

Letter of invitation to participant	2	21 November 2012
Participant Consent Form	2.0	21 November 2012
Participant Information Sheet	20	21 November 2012
Protocol	Version 1.0	09 August 2012
REC application	Submission Code: 110454/364761/1/516	18 September 2012
Response to Request for Further Information from Dr Andres Floto		21 October 2012
Summary/Synopsis	Version 1.0	30 July 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EE/0462 Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

Dr Steve Eckersall
Chair

Email:nrescommittee.eastofengland-norfolk@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Email to: *Dr Andres Floto* andres.floto@papworth.nhs.uk

Copy to: *Dr Ian Smith* ian.smith@papworth.nhs.uk

Mrs Victoria Stoneman, Papworth Hospital NHS Foundation Trust
victoria.stoneman@papworth.nhs.uk

PATIENT CONSENT FORM (Home wellness study)

TITLE OF STUDY: A home-based, rapid and quantitative test for bacterial respiratory infections in patients with cystic fibrosis, to reduce admissions and hospital stay length and to improve healthcare outcomes

Name of researcher: Dr Andres Floto

Please initial each box & sign at bottom

1.	I confirm that I have read and understand the 'Patient Information Sheet' version 20 dated 21 November 2012 for the above study and I have had an opportunity to ask questions.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
3.	If I should withdraw my consent I am willing for my study data to be retained by the researchers.	
4.	I understand relevant sections of my medical notes and data collected during the study may be looked at by authorized individuals from the regulatory aurthorites and Papworth Hospital NHS Foundation Trust where it is relevant to my taking part in this research. I give permission to these individuals to have access to my records.	
5.	I understand that if I withdraw from the study at anytime I must return the equipment and computer technology to a member of the research team as soon as possible.	
6.	I accept that I will be paid £1 per day for each daily set of data/samples that I provide in compensation for participating in the study.	
7.	I agree that I will only use any computer and internet access services provided, for my own benefit and so as to take part in this study and not for business purposes. I also agree that the computer and internet access will not be used in breach of UK law. I also agree to using the internet service provided in a fair and reasonable way (ie no file sharing or downloading of movies etc) to within a monthly allowance of 1 Gb/month.	
8.	I consent to the storage of my study-related data for three years after the study has finished in accordance with the Data Protection Act 1998.	
9.	I consent to my sputum samples being stored in the Papworth Hospital Tissue bank at the end of the study for use in future research studies which have been authorized by a research ethics committee.	
10	I agree to take part in the study.	

……	……	……
Name of patient *(please print)*	Date	Signature
……	……	……
Researcher *(please print)*	Date	Signature

Patient Information Sheet

Study Title: A home-based, rapid and quantitative test for bacterial respiratory infections in patients with cystic fibrosis, to reduce admissions and hospital stay length and to improve healthcare outcomes

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.
Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study.
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

1. What is the purpose of the study?

The purpose of this study is to find out if we can predict the onset of chest infections in cystic fibrosis before they become symptomatic e.g. before you have an increase in sputum, increased cough, decrease in lung function or feel unwell.

We wish to measure the levels of substances known as biomarkers which occur in sputum to find out how the levels change before, during and after chest infections. We have some evidence which shows that levels of biomarkers increase before it is obvious that a chest infection is happening. We now wish to gather more evidence to prove that measuring biomarkers is an accurate method of predicting the onset of chest infections in cystic fibrosis and whether we can combine this test with other home-based assessments of well being.

If the results of this study prove that biomarkers of infection are an accurate way of predicting chest infections we are planning to develop a test for patients to use at home as an early warning system of a chest infection before the patient starts to feel unwell. The ability to diagnose chest infections early could mean that patents are able to start antibiotics much earlier than they currently do and thereby reduce the amount of damage to the lungs.

2. Why have I been invited?

You have been chosen because you have cystic fibrosis and are at least 18 years of age and grow the bacterium Pseudomonas aeroginosa in your sputum.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw or a decision not to take part will not affect your medical care or legal rights. If you choose to withdraw we would like, with your consent, to keep your study data.

4. What do I have to do?

If you agree to take part you will be asked to complete and sign a consent form. The duration of your participation in the study will be 6 months; further details are given in section 5 below.

Expenses and payments for any study visits which are additional to your routine clinic or ward visits.

We are able to offer an inconvenience payment of £1 for each study day which you complete i.e. all data collection and sputum sample.

5. What will happen to me if I take part?

Whilst you are in the study we will ask you to do the following every day for 6 months:

- Daily Sputum Sample: you will collect a sample of sputum every day to store at home in either your own freezer or a mini freezer which we can provide for you. We will ask you to bring the sputum samples to your routine clinic appointments to give to a member of the research team. We will provide you with a cool box to carry the samples to clinic.

- Peak Flow Measurements: we will provide a hand held device called a spirometer for you to measure your peak flow measurements. You will breathe in and then blow out as hard and as fast you can into the hand held device. After you have measured your peak flow you will attach the spirometer to a lap top computer, which we will provide, to upload the peak flow measurement to the research study's website.

- Pulse Rate and Oxygen Saturation Levels: We will provide you with a small machine, about 4cm x 2cm, which will have a clip that will fit onto one of your fingers to measure your pulse rate and the levels of oxygen in your blood. When the measurement is complete you will attach the machine to the lap top computer to upload the measurements to the research study's website

- Activity: We will ask you to wear a step counter, a small device which can be clipped to a belt, waistband or carried in a pocket, during the day time. Once a day you will attach it to the laptop computer to upload the data to the research study's website

- Weight: we will ask you to measure your weight every day using the scales which we will provide. Once a day you will attach the scales to the lap top computer to upload the measurement to the research study's website

We will provide training, support and all the equipment including a 3G enabled PC lap top for the duration of the trial so the data can be transmitted to the study's website. If you complete the study we will let you keep to the laptop. If you choose to keep it you will be responsible for its running costs and maintenance. If you decide to withdraw from the study we will ask you to return all the equipment including the computer and modem to the research team as soon as possible.

If you are admitted to hospital at any time whilst you are participating in this study please inform a member of the research team so we can make appropriate arrangements to collect the above measurements and your sputum samples.

Further details about confidentiality are given in part 2 of this information sheet.

6. What are the possible disadvantages and risks of taking part?

Apart from your time commitment, space for a mini freezer for the duration of this study there are no other disadvantages or risks.

7. What are the possible benefits of taking part?

If the results of this study prove that early measurement of biomarkers in sputum and other home-based test are a useful way of predicting chest infections this could lead to the development of a hand held device which future patients could use at home to diagnose the early onset of chest infections.

8. What if there is a problem?

Whilst you are in the study, a member of the research team will be available should you have any concerns. You may ask for your involvement in the study to stop at any time.

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

9. Will my taking part in the study be kept confidential?

All the information about your participation in this study will be kept confidential. The details are included in Part 2.

10. Contact Details:

Please contact either Dr Floto 01480 830541 or Judy Ryan on 01480 364116 or judy.ryan@papworth.nhs.uk. You will be given 24 hour contact numbers for the research team at the start of the study.

If you have any concerns during the study and wish to speak to an independent person, please contact: The Research and Development Department Tel: 01480 364997.

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

11. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Floto tel: 01480 830541). If you wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the Patient Advice and Liaison Service (PALS), Tel: 01480 364896 or 08003899092.

Papworth Hospital NHS Foundation Trust holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove Papworth Hospital NHS Foundation Trust is at fault. This does not affect your legal right to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator Dr Floto 01480 830541. The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Papworth Hospital NHS Foundation Trust Research Governance Office 01480 36997.

12. Will my taking part in this study be kept confidential?

All information about you will be kept strictly confidential and will only be accessed by the research team. Each participant will be allocated a unique study number in order to ensure records and stored sputum samples are anonymised. Access to your computer and the study's website will be password protected. All the data which you upload will be stored on the study's website and not on the hard drive of the computer. Only members of the research team will be able to see the data which have been saved onto the website. Your study number, records and study data will be kept securely and will be stored for 15 years. The custodian for study-related paper records and electronic data will be Dr Andres Floto. The procedures for handling, processing, storage and destruction of study data are compliant with the Data Protection Act 1998. Responsible individuals from Papworth Hospital NHS Foundation Trust or staff from regulatory bodies may need to access your medical records. This is to check the research is being performed in accordance with national

guidelines. Individuals who monitor for this purpose will be suitable qualified and authorised and will not disclose any personal information about you.

13. Will my general practitioner be informed of my participation?

We will not inform your general practitioner that you are taking part in this research study.

14. What if relevant new information becomes available?

If any new information on the study medication becomes available which may influence your decision to continue in the study you will be told.

15. What happens when the study stops?

We will ask you to return all the study equipment but might be able to allow you to keep the lap top.

16. What will happen to the results of the research study?

Dr Andres Floto in conjunction with the research team will analyse the results of the study. The research team plans to publish the results in scientific journals and to present the results at scientific meetings. You will not be identified in any report or publication. The data we obtain will be stored for three years after we have completed the study. With your permission, your sputum samples will be retained in the Papworth Hospital Tissue Bank for use in future research projects which have been approved by a research ethics committee.

17. Who is organising and funding the research?

Papworth Hospital NHS Foundation Trust has taken on the role of ensuring the study is conducted in accordance with the relevant legislation.

Health Enterprise East has provided the funding to conduct this study.

16. Who has reviewed the study?

This study has research ethics approval from the Hertfordshire Research Ethics Committee.

If you decide to participate in this study you will be given a copy of the information sheet and a copy of the consent form.

Thank you for taking time to read this sheet and for considering taking part in this study.

Dr Andres Floto
Consultant Physician

[Date]

[Recipient's name and address]

Dear [Name]

Re: A Research Study to find out if a home-based, rapid and quantitative test for bacterial respiratory infections in patients with cystic fibrosis can reduce admissions and hospital stay length and to improve healthcare outcomes

I'm writing to see if you might be interested in participating in a new research study we're doing over the next few months.

The Papworth Cystic Fibrosis unit is leading a new research study to find out if we are able to predict when chest infections are likely to occur before patients become symptomatic.

We are currently recruiting volunteers to participate in this research study and have included the patient information sheet (which gives the details of the study) with this letter. If you decide not to take part or withdraw from the study this will not affect your legal rights or medical care in any way.

We are able to offer an inconvenience payment to you for the additional time required to conduct some of the tests.

If you're interested in taking part or just want to find out more about this or other CF research projects taking place at Papworth, contact Dr. Andres Floto (01480 364763) or Judy Ryan our Research Nurse (01480 364116 / judy.ryan@papworth.nhs.uk).

Many thanks and best wishes

Yours sincerely

Dr Andres Floto
Consultant in Respiratory Medicine

Health Research Authority
NRES Committee East of England - Norfolk
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS
Telephone: 01158839390

10 December 2014

Professor RA Floto
Professor of Respiratory Biology, Research Director and Honorary Consultant Physician
Papworth Hospital NHS Foundation Trust
Cambridge Centre for Lung Infection
Papworth Hospital
Papworth, Everard, Cambridge
CB23 3RE

Dear Professor Floto

Study title:	**Smart Care - A Standardized Multi-centre Analysis of Remote Monitoring in CF Adult patients to Reduce pumlmonary Exacerbations**
REC reference:	**14/EE/1244**
IRAS project ID:	**163484**

Thank you for your letter of 24th November 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Assistant, Tad Jones, NRESCommittee.EastofEngland-Norfolk@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [REC letter of response]	1.0	30 December 2014
GP/consultant information sheets or letters [Smartcare GP Letter]	Version 1.0	26 October 2014
IRAS Checklist XML [Checklist_31102014]		31 October 2014
IRAS Checklist XML [Checklist_01122014]		01 December 2014
Letters of invitation to participant [Smartcare Participant Invitation Letter]	Version 2.0	26 December 2014
Non-validated questionnaire [Smartcare Patient Survey]	Version 1.0	26 October 2014
Other [Summary CV (Research Nurse)]	Version 1.0	31 October 2014
Participant consent form [Smartcare Participant Consent Form]	Version 2.0	26 December 2014
Participant information sheet (PIS) [Smartcare Participant Information Sheet]	Version 2.0	26 December 2014
REC Application Form [REC_Form_01122014]		01 December 2014
Research protocol or project proposal [Smartcare Study Protocol]	Version 2.0	26 December 2014
Summary CV for Chief Investigator (CI) [Summary CV (CI)]	Version 1.0	31 October 2014
Summary CV for student [Summary CV (EU)]	Version 1.0	29 October 2014
Validated questionnaire [B-IPQ Survey (Smartcare)]	Version 1.0	26 October 2014
Validated questionnaire [CFQ-R]	Version 2.0	26 December 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/EE/1244	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Dr Robert Stone
Vice Chair

Email:NRESCommittee.EastofEngland-Norfolk@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Dr Victoria Stoneman, Papworth Hospital NHS Foundation Trust*

Re-issued 11.08.15 due to document list error.

NRES Committee East of England - Cambridgeshire and Hertfordshire
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839435
Fax: 0115 8839294

25 June 2015

Dr Emem-Fong Ukor
Clinical Research Fellow
Cambridge Centre for Lung Infection
Papworth Hospital NHS Foundation Trust
Papworth Everard
Cambridge
CB23 3RE

Dear Dr Ukor

Study title:	**Smart Care - A Standardized Multi-centre Analysis of Remote Monitoring in CF Adult patients to Reduce pumlmonary Exacerbations**
REC reference:	**14/EE/1244**
Amendment number:	
Amendment date:	**25 June 2015**
IRAS project ID:	**163484**

Thank you for your letter of 25 June 2015, notifying the Committee of the above amendment.

The Committee does not consider this to be a “substantial amendment“ as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters	3.0	14 June 2015
Letters of invitation to participant	4.0	14 June 2015
Notice of Minor Amendment		25 June 2015
Other [Patient Instruction Manual]	1.0	17 June 2015
Participant consent form	5.0	14 June 2015
Participant information sheet (PIS)	5.0	14 June 2015
Research protocol or project proposal	4.0	14 June 2015

Statement of compliance

Re-issued 11.08.15 due to document list error.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

14/EE/1244:	**Please quote this number on all correspondence**

Yours sincerely

Miss Georgia Copeland
REC Manager

Email: nrescommittee.eastofengland-cambsandherts@nhs.net

Copy to: *Dr Victoria Stoneman, Papworth Hospital NHS Foundation Trust*

Professor RA Floto
Professor of Respiratory Biology, Research Director and Honorary Consultant Physician
Papworth Hospital NHS Foundation Trust
Cambridge Centre for Lung Infection
Papworth Hospital
Papworth, Everard, Cambridge
CB23 3RE

PATIENT CONSENT FORM (Home-monitoring study)

TITLE OF STUDY: **A multi-centre feasibility study of remote monitoring in adult CF patients**

Name of researcher: **Professor Andres Floto**

Please initial each box & sign at bottom

1.	I confirm that I have read and understand the 'Patient Information Sheet' version **5.0** dated **14 June** 2015 for the above study and I have had an opportunity to ask questions.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
3.	If I should withdraw my consent I am willing for my study data to be retained by the researchers.	
4.	I understand that authorized individuals from the regulatory authorities and Papworth Hospital NHS Foundation Trust may look at relevant sections of my medical notes and data collected during the study where it is relevant to my taking part in this research. I give permission to these individuals to have access to my records.	
5.	I agree that I will only use the smartphone and data allowance provided, for my own benefit and so as to take part in this study and not for business purposes. I also agree that the smartphone and data allowance will not be used in breach of UK law. I also agree to using the Internet service provided in a fair and reasonable way (e.g. no file sharing or downloading of movies) to within a pre-defined monthly allowance.	
6.	I consent to my sputum samples being appropriately stored at the end of the study for future analysis.	
7.	I consent to the storage of my study-related data, which is in accordance with the Data Protection Act 1998.	
8.	I agree to collect my data and use the study devices as per instructed.	
9.	I agree to my GP being informed of my participation in the study.	
10.	I agree to take part in the study.	

…….	……	……
Name of patient *(please print)*	Date	Signature
…….	……	……
Researcher *(please print)*	Date	Signature

Patient Information Sheet

Study Title: A multi-centre feasibility study of remote monitoring in adult CF patients

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

1. What is the purpose of the study?

The purpose of this study is to find out if we can use remote monitoring of breathing, weight and activity to keep individuals with CF at home as much as possible.

The first step is to check that we can record lung function, oxygen saturations, activity, sleep, temperature, weight and symptoms using home-monitoring equipment with a smartphone to transmit the information back to the CF Centre. The information obtained during this study will not change how you are looked after, but will allow us to develop a software program that will work out what the signals are that predict when a chest infection is going to happen, that is, before you have an increase in sputum, increased cough, drop in lung function or feel unwell.

This should allow us, in the future, to let patients who are stable stay at home and not come to clinic for review if they don't need to. At the same time, we hope to predict when patients are about to become ill and start treatment earlier to hopefully keep lungs healthier for longer.

We also wish to measure the levels of substances known as biomarkers, which occur in sputum, to find out how the levels change before, during and after chest infections. We have some evidence that shows that levels of biomarkers increase before it is obvious that a chest infection is happening. We now wish to gather more evidence to determine which biomarkers can provide an accurate method of predicting the onset of chest infections in cystic fibrosis and investigate whether we can combine this test in the future with other home-based assessments of well being.

2. Why have I been invited?

You have been invited to participate because you have cystic fibrosis, are at least 18 years of age and have experienced at least one acute pulmonary exacerbation (chest infection) in the last 12 months.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw or a decision not to take part will not affect your

medical care or legal rights. If you choose to withdraw we would like, with your consent, to keep your study data.

4. What do I have to do if I agree to take part?

If you agree to take part you will be asked to complete and sign a consent form. The duration of your participation in the study will be 6 months.

Whilst you are in the study we will ask you to do the following **every day** for 6 months:

- Weight: we will ask you to measure your weight using a set of scales that we will provide. Once a day you will transmit the data via Bluetooth to the smartphone, to upload the data to the research study's website

- Pulse Rate and Oxygen Saturation levels: we will provide you a small machine, about 4cm x 2 cm, which will have a clip that will fit on one of your fingers to measure your pulse rate and the levels of oxygen in your blood. When the measurement is complete you will transmit the data via Bluetooth to the smartphone to upload the data to the research study's website.

- Daily Sputum Sample: you will collect a sample of sputum to store at home in either your own freezer or a mini freezer, which we can provide for you. We will ask you to bring the sputum samples to your routine clinic appointments to give to a member of the research team. We will provide you with a cool box to carry the samples to clinic.

- Wellness and Cough scores: we will ask you to score (1) how well you are feeling and (2) the quality of your cough on a scale from 1 (worst ever) to 10 (best ever). You will be able to record your scores on the study-specific App that you can access on the smartphone. The data will be transmitted to the research study's website.

- Lung function test: we will provide a hand-held device called a spirometer for you to measure your lung function. You will breathe in and then blow out as hard and as fast you can into the hand-held device until your lungs are empty. After you have measured your lung function you will transmit the data via Bluetooth to a smartphone, which we will provide, to upload the measurements to the research study's website.

- Activity: we will ask you to wear a slim-line wristband during the daytime. Once a day you will transmit the data via Bluetooth to the smartphone, to upload the data to the research study's website.

We will provide training, support and all the equipment above, including a 4G-enabled smartphone for the duration of the trial so the data can be transmitted to the study's website. You will be allowed to keep the SIM free smartphone and the home-monitoring devices when you complete the study. If you choose to keep the devices you will be responsible for their running costs and maintenance. If you are admitted to hospital at any time whilst you are participating in this study please inform a member of the research team so we can make appropriate arrangements to collect the above measurements and your sputum samples.

We will also ask you to complete two questionnaires (one at the beginning and the end of the study and the other only at the end) to help us collect feedback from you on the impact of taking part in this study.

Further details about confidentiality are given in part 2 of this information sheet.

5. What are the possible disadvantages and risks of taking part?

Apart from your time commitment and space for a mini freezer for the duration of this study there are no other disadvantages or risks.

6. What are the possible benefits of taking part?

If the results of this study prove that home-based monitoring of physical signs and symptoms of health is possible and acceptable to patients with CF, we may be able to reduce the time you spend attending hospital when you are stable.

If we can also discover which biomarkers in sputum, when measured early in combination with home-based monitoring, are a useful way of predicting chest infections this could lead to the development of a hand-held device and software program which future patients could use at home to diagnose early the onset of chest infections. The results of this analysis will not have any impact on your routine clinical care during this study.

7. What if there is a problem?

Whilst you are in the study, a member of the research team will be available should you have any concerns. Technical support will also be available to you both during working and after hours. You may ask for your involvement in the study to stop at any time.

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

8. Will my taking part in the study be kept confidential?

All the information about your participation in this study will be kept confidential. The details are included in Part 2.

9. Contact Details:

Please contact ***[insert site-specific details]***. You will be given 24-hour contact numbers for the research team at the start of the study.

If you have any concerns during the study and wish to speak to an independent person, please contact: The Patient Advice Liaison Services Tel: ***[xxx insert site-specific details]***.

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

10. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions ***[insert site-specific Lead PI]***. If you wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the Patient Advice and Liaison Service (PALS), Tel: ***[xxx insert site-specific details]***.

Every reasonable effort will be made to prevent any injury that could result from this research. If you believe you have an injury that is directly related to your participation in the study, you should inform the study doctor.

If you are harmed due to someone's negligence, then you may have grounds for legal action for compensation against ***[insert site-specific details],*** but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the ***[insert site-specific Lead PI]***.

11. Will my taking part in this study be kept confidential?

All information about you will be kept strictly confidential and will only be accessed by the research team. If you join the study you will be allocated a unique study number in order to ensure records and stored sputum samples are linked-anonymised. Access to your smartphone and the study's website will be password-protected. All the data which you upload will be stored in encrypted form on the study's website and saved onto a secure NHS-approved web-based site. Only members of the research team will be able to access this data, Authorised personnel from Papworth Hospital NHS Foundation Trust or staff from regulatory bodies may need to access your medical records and the study data for quality control and audit purposes.

Your study number, records and study data will be kept securely for 15 years. The procedures for handling, processing, storage and destruction of study data are compliant with the Data Protection Act 1998.

12. Will my general practitioner be informed of my participation?

With your consent, your General Practitioner (GP) will be informed about your participation in the study. We would do this to ensure that we and your GP can best manage your overall healthcare and this can be done by providing written information about the study to your GP.

13. What if relevant new information becomes available?

If any new information on the study devices, or related monitoring technology becomes available which may influence your decision to continue in the study you will be informed.

14. What happens when the study stops?

On completion of the study you will be able to keep the SIM free smartphone and the home-monitoring devices.. If you chose to keep the devices for personal use you will be responsible for their running costs and maintenance.

15. Expenses and payments

On completion of the training period for the study we are able to offer you a one -off inconvenience payment of £50.00 for your time and effort with this study.

16. What will happen to the results of the research study?

The Chief Investigator in conjunction with the research team will analyse the results of the study. The research team plans to publish the numerical results in scientific journals and to present the results at scientific meetings. You will not be identified in any report or publication. The data we obtain will be stored for a maximum of 15 years after we have completed the study. With your

permission, your sputum samples will be prepared and stored in a freezer with a unique study number to protect your identity. Your name will not be attached to the samples.

17. Who is organising and funding the research?

Papworth Hospital NHS Foundation Trust has taken on the role of ensuring the study is conducted in accordance with the relevant legislation.

The Cystic Fibrosis Trust UK has provided the funding to conduct this study.

16. Who has reviewed the study?

This study has research ethics approval from the ***NRES Committee East of England-Norfolk***.

If you decide to participate in this study you will be given a copy of the information sheet and a copy of the consent form.

Thank you for taking time to read this sheet and for considering taking part in this study.

Professor Andres Floto (Chief Investigator)
Consultant Physician

<<Date>>

<<Name of participant>>
<<Address>>
<<City, Postcode>>

RE: **A multi-centre feasibility study of remote monitoring in adult CF patients**

Dear <<Name>>,

We are delighted to inform you of a new research study being undertaken in patients with cystic fibrosis.

We are writing to see if you would consider participating in a new innovative research study using blue-tooth technology home-based monitoring devices to detect the early onset of chest infections in order to guide antibiotic treatment, with the aim of reducing time spent in hospital for patients with cystic fibrosis.

This is a multi-centre UK study lead by Professor Andres Floto, with Papworth Hospital Adult Cystic Fibrosis Centre as the lead site. There are another **six** leading Adult Cystic Fibrosis Centres in the UK participating in this study and your centre ***(insert centre name)*** is one of them. The Cystic Fibrosis Trust is funding this study.

We are aiming to recruit a total of 200 male and female Cystic Fibrosis patients that are 18 years of age or older from the seven study centres.

Your participation in the study will be approximately six months, during which time you will be expected to collect daily, via Bluetooth-enabled devices the following:

- Pulse rate and oxygen saturation
- Spirometry (breathing) measurements
- Weight
- Physical Activity
- Wellness and cough scores

You will also be expected to provide daily sputum samples.

This linked-anonymised data will then be transmitted from the Bluetooth-enabled devices, via a Smartphone, to a secure NHS approved web-based site, to be analyzed.

If you feel you may be interested in participating in this study and would like to know more about this project, please telephone / email: ***(insert site specific details).***

By asking for more information this does not in any way commit you to participating in this study and if you decide not to take part or withdraw from the study this will not affect your legal rights or medical care in any way.

If you are interested in participating we are happy to provide further written information to you in a detailed Patient Information Sheet about the study for you to consider.

We would like to thank you for considering this study and we look forward to hearing from you.

Yours sincerely,

(Site-specific Lead PI)

Consultant Physician

Papworth Hospital NHS Foundation Trust

MATERIAL TRANSFER AGREEMENT

THIS AGREEMENT is made on **BETWEEN:**

(1) FRIMLEY HEALTH NHS FOUNDATION TRUST ("the Trust")

(2) PAPWORTH HOSPITAL NHS FOUNDATION TRUST of Papworth Everard, Cambridge CB23 3RE ("the Recipient")

WHEREAS the Trust agrees to supply to the Recipient certain tissues (or tissue derivatives) and in consideration of such supply the Recipient has agreed to comply with the terms of this Agreement.

(1) Supply of Tissues

1.1 The Trust agrees to supply the following tissues (or tissue derivatives) **("sputum")** to the Recipient, for a period of two (2) years (the "Term") from the date of execution:

List of Tissues
Sputum

1.2 Risk in the Tissues shall pass to the Recipient who should have either:

1.2.1 project specific ethical approval for the use of such tissue according to a REC approved research protocol *OR*

1.2.2 generic ethical approval for the use of such tissue according to a Trust approved research protocol and REC approved conditions *OR*

1.2.3 an appropriate HTA licence for the storage of such tissues.

1.3 The Trust represents that all Tissues supplied under the terms of this agreement have been obtained in compliance with all relevant UK laws and guidelines.

1.4 The Tissues shall be used by the Recipient for the following project **"SmartCare":**

Project Title:	***SmartCare: A Standardized Multi-centre Analysis of Remote Monitoring in CF Adult Patients to Reduce Pulmonary Exacerbations***
Project Description	A UK based prospective cohort study investigating the feasibility and acceptability of remote monitoring in an adult CF population, and its potential to detect the onset of pulmonary exacerbation before patient-reported symptoms
Names of Investigators:	Prof A. Floto
Name of any Project Collaborators outside the Recipient:	1. Cystic Fibrosis Trust UK 2. Microsoft Research 3. Papworth Hospital NHS Foundation Trust 4. King's College Hospital NHS Foundation Trust 5. Leeds Teaching Hospitals NHS Trust 6. Royal Brompton & Harefields NHS Foundation Trust 7. Southampton University Hospital NHS Trust 8. University Hospitals Bristol NHS Foundation Trust

1.5 Recipient will own all rights; title and interest in Project results, and the Trust will retain the right to use Project results for research purposes only.

1.6 If applicable, the recipient shall supply to the Trust a copy of the REC application for project specific approval and a copy of the letter of approval.

2 Obligations of the Recipient

2.1 The Recipient shall not transfer or sell all or part of the Tissues to any third party. It shall be the responsibility of the Recipient to ensure that the investigators and collaborators referred to above, comply with the terms of this Agreement in all respects as though they were parties to it.

2.2 The Recipient shall ensure that the Tissues are used only for the purposes of the Project and not otherwise. The Recipient shall seek authorisation from the Trust in writing, before using the Tissues or any part of the Tissues for a purpose other than the specified Project and shall not use such Tissues for such additional purposes without the prior written consent of the R&D Unit at and appropriate regulatory approvals.

2.3 The Recipient represents that its use of the Tissues will be in compliance with all applicable UK laws and regulations.

2.4 The Recipient shall comply with all reasonable instructions of the Trust concerning the treatment, storage, transport and care of the Tissues. The Recipient shall be responsible for organising and arranging the collection of the Tissues from the Trust, provide appropriately labelled containers, packaging and storage medium and arrange their safe transport to the Recipient's premises or other location for the purposes of the Project.

2.5 The Recipient shall at all times keep the Tissue safe and secure and shall use reasonable endeavours to prevent the theft of or unauthorised use or interference with the Tissues.

2.6 (a) If the Recipient commits any material breach of the terms of this Agreement or becomes insolvent or has a receiver, administrator or administrative receiver appointed of its undertaking, then:

(b) If the Trust for any reason believes that the Tissues are in jeopardy or otherwise in its absolute discretion thinks fit, subject to prior notification of Recipient and reasonable discussion between the parties of steps to address any such concerns within 60 days of notification thereof, then:

the Trust shall be entitled to terminate this Agreement by not less than thirty (30) days written notice to the Recipient, whereupon the Recipient's entitlement to possession of the Tissues shall terminate.

2.7 The Recipient undertakes to keep confidential all information about the Trust and its operations which it learns by reason of this agreement save for information which is in the public domain otherwise than by reason of a breach of this clause by the Recipient.

2.8 The Recipient shall reimburse to the Trust all reasonable costs and expenses incurred by the Trust for this transfer as set out in Annex A.

2.9 That NHS Foundation Trust shall be acknowledged as the source of the Tissues in any publication or presentations resulting from work on the samples provided by the Trust.

3. Disclaimer

3.1 The Trust gives no warranty or assurance of any kind to the Recipient or any third party that the Tissues are free from infection (including, without limitation, HIV, hepatitis B or tuberculosis). No warranty (statutory or otherwise) or representation is given by the Trust that the Tissues are of any particular quality or fit for any particular purpose. It shall be the sole responsibility of the

Recipient to ensure that the Tissues are of satisfactory quality, free from infection and fit for the purpose of carrying out the Projects (or any other purpose).

3.2 In no event shall the Trust be liable for any use by the Recipient or Recipient Investigators or Project Collaborators of the Tissues transferred under this Agreement. The Recipient shall ensure that all appropriate precautions are taken by its employees and any other persons coming into contact with the Tissue. No liability is accepted by the Trust to the Recipient or any third party for any loss, claim, damage or liability, of whatsoever kind or nature, due to or arising from the use, handling, storage or disposal of the Tissues by the Recipient, except when caused by the gross negligence or wilful misconduct of the Trust.

4. General

4.1 If any provision of this agreement is found by any court, tribunal or administrative body of competent jurisdiction to be wholly or partly illegal, invalid, void, voidable, unenforceable or unreasonable, it shall to the extent of such illegality, invalidity, voidness, voidability, unenforceability or unreasonableness to be deemed severable. The remaining provisions of this agreement and the remainder of such provision shall continue in full force and effect.

4.2 Failure by the Trust in enforcing or partially enforcing any provision of this agreement will not be construed as a waiver of any of its rights under this agreement. Any waiver by the Trust of any breach of, or any default under, any provision of this agreement will not be deemed a waiver of any subsequent breach or default and will in no way affect the other terms of this agreement.

4.3 The parties to this agreement do not intend that any term of this agreement will be enforceable by virtue of the Contracts (Right of Third Parties) Act 1999 by any person that is not a party to it.

4.4 This agreement sets out the entire understanding between the parties in relation to its subject matter and supersedes any prior agreements (written or oral) between the parties.

4.5 English law shall govern the formation, existence, construction, performance, validity and all aspects of this agreement. The parties shall submit to the exclusive jurisdiction of the English courts.

SIGNATURES ON NEXT PAGE

Signed for any on behalf of Papworth Hospital NHS Foundation Trust

Signed: **Signed:**

Name: **Name:**

Date: **Date:**

Signed for any on behalf of add recipient details

Signed:

Name:

Date:

List of Documents Reviewed by Papworth Hospital NHS Foundation Trust:

Document	*Version*	*Date*
Study Protocol	2.0	26/11/14
Patient invitation letter	2.0	26/11/14
Patient information sheet	2.0	26/11/14
Patient Consent Form	2.0	26/11/14
GP information letter	1.0	26/10/14
SmartCare Patient Survey	2.0	26/11/14
Validated Questionnaire (CFQ-R)	2.0	
REC Approval letter		10/12/14

ANNEX A

Papworth Hospital NHS Foundation Trust

Support Agreement.

WHEREAS the Trust has agreed to supply to the Recipient certain tissues (or tissue derivatives) as outlined in the agreement and in consideration of such supply the Recipient agrees to compensate the Trust in respect of this costs incurred through the collection, storage and issues of said tissues.

The Trust undertakes to provide the Tissues, with relevant anonymous clinical/pathological details, for approved research projects.

The Trust warrants that all Tissues supplied under the terms of this agreement have been obtained in compliance with all relevant UK laws and guidelines.

The handling charges in relation to the supply of tissues will be covered as follows:

The cost of courier fees for specimen transport to the lead site (Papworth Hospital) will be covered by the lead site's funding for the study.

The lead site study team will arrange the schedule for specimen transfer.

APPENDIX 2

Table S1. Proportion of QS-null clonal *P. aeruginosa* isolates per patient.

Patient	K1	K3	K4	K6	K7	K9	K11	K14	K15
QS-null isolates (%)	48	47	5	3	5	2	7	6	11

The proportion of isolates within each patient's collection of clonal strains that failed to secrete any of the three QS molecules assayed (OdDHL, BHL or PQS) but were still able to express traditionally QS-associated phenotypes.

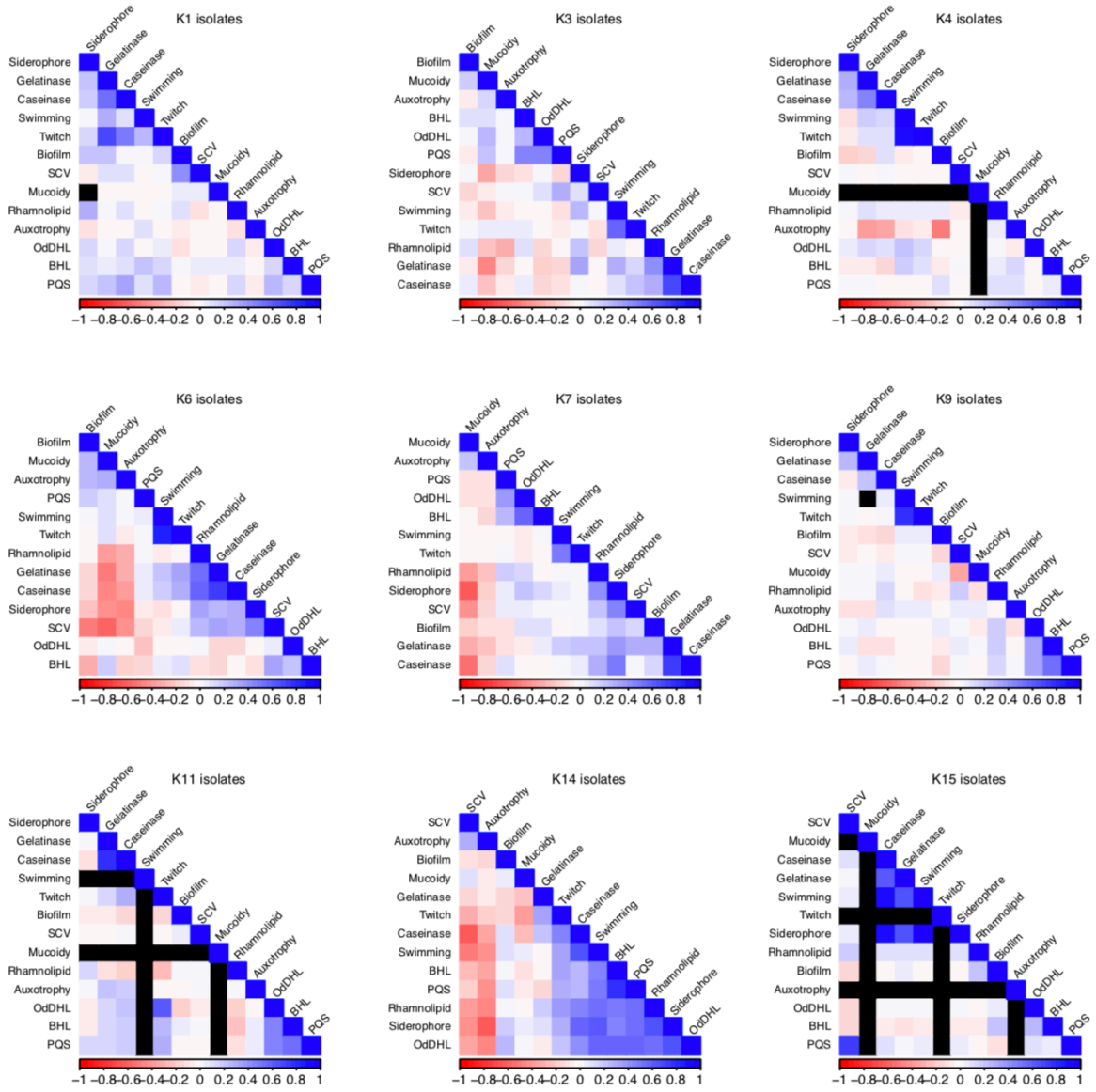

Figure S1. Correlation matrix of phenotype-phenotype associations for each patient's set of isolates, as determined by Spearman rank correlation coefficient. Blue indicates strong positive correlation between any given phenotype pair (Spearman rank coefficient closer to 1), red indicates strong negative correlation between any given phenotype pair (Spearman rank coefficient closer to -1) and white indicates no correlation between phenotype pairs (0).

APPENDIX 3

SMARTCARE PATIENT SURVEY

1. How did you find using the monitoring devices?

Very easy	Easy	Neutral	Difficult	Very Difficult
☐	☐	☐	☐	☐

2. How did you find using the **smartphone**?

Very easy	Easy	Neutral	Difficult	Very Difficult
☐	☐	☐	☐	☐

3. How did you find using the **Smartcare app**?

Very easy	Easy	Neutral	Difficult	Very Difficult
☐	☐	☐	☐	☐

4. How often did you have problems with measuring results?

Never	Daily	Weekly	Monthly	Less than monthly
☐	☐	☐	☐	☐

5. How often did you have problems with uploading results?

Never	Daily	Weekly	Monthly	Less than monthly
☐	☐	☐	☐	☐

6. How often did you have to contact someone for help with measuring results?

Never	Daily	Weekly	Monthly	Less than monthly
☐	☐	☐	☐	☐

7. How often did you have to contact someone for help with uploading results?

Never	Daily	Weekly	Monthly	Less than monthly
☐	☐	☐	☐	☐

8. If you had to contact someone for help measuring or uploading results, how did you find this process?

Very easy	Easy	Neutral	Difficult	Very difficult
☐	☐	☐	☐	☐

9. To what extent did **daily** home-monitoring interfere with your usual activities?

Not at all	Slightly	Somewhat	Moderately	Extremely
☐	☐	☐	☐	☐

10. Please score the tests on a scale of 1 to 8 (1 = most acceptable, 8 = unacceptable) in terms of acceptability to perform (Tests may have the same score)?

☐ Weight

☐ Sputum collection

☐ Oximetry (heart rate & oxygen saturation)

☐ Wellness Score

☐ Cough score

☐ Spirometry (lung function)

☐ Activity/Sleep and Temperature Monitor

11. What impact has home-monitoring had on your quality of life?

0 1 2 3 4 5 6 7 8 9 10

Worse No impact Better

12. Ideally, how frequently would you like to do home-monitoring?

Daily	Every other day	Weekly	Monthly	Other
☐	☐	☐	☐	☐

If other, please specify

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13. How helpful did you find being able to monitor your breathing, activity and weight at home?

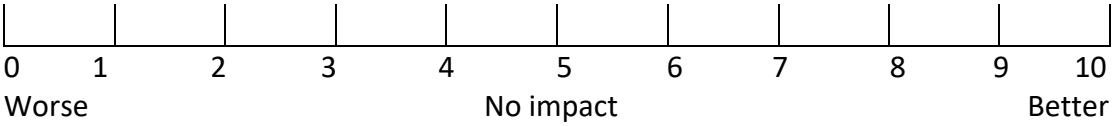
Very helpful	Helpful	Neutral	Unhelpful	Completely unhelpful
☐	☐	☐	☐	☐

14. What impact has home-monitoring had on your anxiety levels?

0 1 2 3 4 5 6 7 8 9 10

More Anxiety No impact Less Anxiety

15. What impact has home-monitoring had on your ability to cope with managing your health?



16. Given the choice, would you prefer to use home-monitoring rather than attend routine clinic?

Definitely	Probably	Undecided	Possibly	Definitely not
☐	☐	☐	☐	☐

17. Please let us know if you had any specific comments about your experience of doing home-monitoring.

Thank you for your answers!