Evaluation of host-pathogen interactions for the design of novel therapies against *Pseudomonas aeruginosa*

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Statement of original authorship

I hereby certify that the submitted work is my own work, was completed while I was registered as a candidate for the degree stated on the front page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Candidate signature: Date: 08 May, 2024

Research Outputs

Peer-reviewed manuscripts

1. Jurado-Martín, I.; **Sainz-Mejías, M**.; McClean, S. *Pseudomonas aeruginosa*: An Audacious Pathogen with an Adaptable Arsenal of Virulence Factors. Int. J. Mol. Sci. 2021, 22, 3128. https://doi.org/10.3390/ijms22063128. Citations: 262 (MDPI metrics, Certificate of Publication: Highly cited Papers IJMS 2021).
2. **Sainz-Mejías, M.;** Jurado-Martín, I.; McClean, S. Understanding *Pseudomonas aeruginosa*–Host Interactions: The Ongoing Quest for an Efficacious Vaccine. Cells 2020, 9, 2617.https://doi.org/10.3390/cells9122617. Citations: 45. (MDPI metrics).

Oral Talks

1. **Annual Conference, 2024. Microbiology Society**, Edinburgh, Scotland. Identification of host-pathogen interactions: The role of PDIA1 and PDIA3 as novel receptors for *P. aeruginosa.*
2. **BactiVax Summer School**. **Budapest, 2023**. Understanding *Pseudomonas aeruginosa*–Host Interactions for the design of novel therapies.
3. **Research Day Competition, 2023.** University College Dublin. Dublin, Ireland. Understanding host-pathogen interactions for the design of novel therapies against *Pseudomonas aeruginosa* respiratory infections.
4. **BactiVax Summer School in Ischia, 2022.** Vaccinology course. Institute of Biostructures and Bioimaging of the National Research Council, Naples, Italy. Understanding *Pseudomonas aeruginosa*–Host Interactions for the design of novel therapies.
5. **BactiVax Meeting in Bilbao, 2021.** Methodologies in Vaccine Development. CIC bioGUNE, Bilbao, Basque Country, Spain. Optimisation of novel adjuvants for therapeutic vaccinations against *Pseudomonas aeruginosa.*
6. **BactiVax ESR Kick-Off Meeting, 2020.** Vaccines & Vaccinology Course. Online, Ludwig Maximilian University of Munich, Germany. *Pseudomonas aeruginosa.* Optimisation of novel adjuvants for therapeutic vaccinations against *Pseudomonas aeruginosa.*

Poster Presentations

1. Annual Conference, 2024. Microbiology Society, Edinburgh, Scotland. **Sainz-Mejías, M.;** Berisio, Rita.; Anathy, Vikas; McClean, Siobhán. Identification of host-pathogen interactions: The role of PDIA1 and PDIA3 as novel receptors for *P. aeruginosa.*
2. Conway Festival 2023. University College Dublin. Dublin, Ireland. **Maite Sainz-Mejías**, Alessio Romero, Chaoying Ma, Julen Tomás-Cortázar, Irene Jurado-Martín, Ana Rita Franco, Francesco Peri, Siobhán McClean. Evaluation of Synthetic Glycolipids as molecular adjuvants in anti- Pseudomonas aeruginosa vaccine formulations.

3. British Society for Immunology Congress, 2023. **Maite Sainz-Mejías**, Alessio Romero, Chaoying Ma, Julen Tomás-Cortázar, Irene Jurado-Martín , Ana Rita Franco, Francesco Peri, Siobhán McClean. Evaluation of Synthetic Glycolipids as molecular adjuvants in anti-*Pseudomonas aeruginosa* vaccine formulations.
4. Conway Festival 2022. University College Dublin. Dublin, Ireland. **Maite Sainz-Mejías,** Siobhán McClean. Understanding *Pseudomonas aeruginosa*–Host Interactions for the design of novel therapies.
5. *Pseudomonas* 2022 Biannual Conference. Atlanta, Georgia, USA. **Maite Sainz-Mejías,** Siobhán McClean. Understanding *Pseudomonas aeruginosa*–Host Interactions for the design of novel therapies.
6. Conference 2022 Microbiology Society, Belfast, Northern Ireland. **Maite Sainz-Mejías,** Siobhán McClean. Identification of host-pathogen interactions for the design of novel therapies.
7. Conway Festival 2021. University College Dublin. Dublin, Ireland. **Maite Sainz-Mejías**, Siobhán McClean**.** Identification of host-pathogen interactions for the design of novel therapies.
8. Annual Conference 2021. Microbiology Society, Belfast, Northern Ireland. **Maite Sainz-Mejías**, Siobhán McClean. Identification of host-pathogen interactions for the design of novel therapies.

Manuscripts in progress:

1. **Sainz-Mejías, M.;** Berisio, Rita.; Anathy, Vikas; McClean, Siobhán. Identification of host-pathogen interactions: The role of PDIA1 and PDIA3 as novel receptors for *P. aeruginosa.*
2. **Sainz-Mejías, M.;** Romerio, Alessio.; Tomás Cortázar, Julen.; Ma, Chaoying.; Franco, Ana Rita.; Peri, Francesco.; McClean, Siobhán. Evaluation of TLR4 ligands as adjuvants for novel vaccines against *P. aeruginosa.*

Abstract

Pseudomonas aeruginosa is a highly antimicrobial-resistant pathogen that causes difficult-to-treat acute and chronic infections. Hence, new antibiotic therapies against *P. aeruginosa* are urgently needed. Understanding host-bacterial interactions is critical for the design of innovative treatments against infections. This project evaluated the interplay between *P. aeruginosa* and the human host from different perspectives: (a) the effect of the versatility and adaptability of *P. aeruginosa* within the host by the *in vitro* characterisation of host immune responses stimulated by *P. aeruginosa* sequential isolates from two CF patients (b) the elucidation of host receptors for *P. aeruginosa*; and (c) the evaluation of TLR4 ligands as adjuvants for vaccines against *P. aeruginosa.* The immune responses against CF sequential isolates were independent on their status as "early" or "late" isolates, confirming a divergent evolution of the isolates within the host. One of the main achievements was the identification and validation of the role of the human protein disulfide isomerases (PDI) A1 and PDIA3 in *P. aeruginosa* attachment to epithelial cells using a novel unbiased 2D proteomic approach. The pre-treatment of human bronchial epithelial cells (16HBE14o^{-}) with the PDI inhibitor, LOC14, decreased *P. aeruginosa* attachment to these cell lines by 2.5 fold (p= 0.0188), *P. aeruginosa* attachment to HEK293T cells overexpressing PDIA1 and PDIA3 was 6.01 and 6.52 fold higher than the control (empty plasmid), respectively (p=0.0344), and bacterial attachment to CRISPR cell lines A549 *pdia3*$^{-/-}$ was 1.71 fold lower than to wild-type A549 cells (p=0.0344). This discovery might lead to the design of novel antimicrobial therapies or the use of currently available drugs that target human PDIs. Finally, rOprF (recombinant outer membrane protein F) in combination with FP18 adjuvant was validated as a suitable adjuvant-antigen system for vaccines against *P. aeruginosa*. FP18 reduced bacterial counts in the lungs and dissemination of bacteria to spleens, it enhanced the production of antibodies and IFNγ stimulation, relative to immunisation with antigen alone in a mice model of acute *P. aeruginosa* pneumonia. All the results together might lead to novel host-directed therapies against the challenging pathogen, *P. aeruginosa.*

Abbreviations

3GC: Third-generation cephalosporins.

4GC: Fourth-generation cephalosporins.

A549: Adenocarcinoma human alveolar basal epithelial cells.

Adj: adjuvant.

Ag: antigen.

AHL: Acyl homoserine lactose.

Al $(OH)_3$: Aluminium hydroxide.

$AlPO_4$: Aluminium phosphate.

ANOVA: Analysis of variance.

AnvM: Anaerobic and virulence modulator.

APCs: Antigen-presenting cells.

AS: Adjuvant system.

ASC: Antibody-secreting plasma cells.

ASL: Airway surface liquid.

BL: β-lactamase.

BLI: β-lactamase inhibitors.

BSA: Bovine serum albumin.

BSIs: Bloodstream infections.

CCC: Chemokine

CF: cystic fibrosis.

CFBE: Cystic fibrosis bronchial epithelial cells.

CFTR: CF transmembrane regulator.

CFU: Colony Forming Units.

CHINET: China Antimicrobial Surveillance Network .

Cif: CFTR inhibitory factor.

CifR: CFTR inhibitory factor repressor.

CLEC5A: Myeloid C-type lectin domain family 5 member A.

DAMPs: Danger-associated molecular patterns.

DCs: Dendritic cells.

DMEM: Dulbecco's Modified Eagle's Medium.

dmLT: Detoxified mutants LT.

DS: Delivery systems.

Dsb: Disulfide bond forming enzymes.

DTR: Difficult-to-treat resistance.

E. coli: *Escherichia coli.*

ENaC: Epithelial sodium channel.

ETA: Exotoxin A.

FBS: Fetal bovine serum.

FDA: Food and Drug Administration.

GLP: Glycolipoprotein.

HAP: Hospital-acquired pneumonia.

HBE: Human bronchial epithelial cells.

HCM: human cytomegalovirus.

HDT: Host-directed therapies.

HEK: Human embryonic kidney cells.

HRP: Horseradish peroxidase.

IAV: Influenza

IC: Immune complex.

ICU: Intensive care unit.

i.d.: Intradermal.

Ig: Immunoglobulin.

IL: Interleukin.

i.m: Intramuscular.

IMS: Immunostimulators.

i.n.: Intranasal.

i.p.: Intraperitoneal.

IPTG: Isopropyl β-d-1 thiogalactopyranoside.

i.t.: Intratracheal.

LB: Luria-Bertani.

LDH: Lactose dehydrogenase.

LPS: Lipopolysaccharide.

MDR: Multi-drug resistance.

MEM: Modified Eagle's Medium.

MEP: mucoid exopolysaccharide.

MHC: major histocompatibility complex.

MIC: Minimum Inhibitory concentration.

MOI: multiplicity of infection.

MPLA: Monophosphoryl lipid A.

MW: Molecular weight.

NA: Neuraminidase.

NF-κB: Nuclear factor binding near the κ light- chain.

NHE-RF1: Na(+)/H (+) exchange regulatory cofactor.

NLR: NOD like receptors.

ODN: Oligodeoxynucleotide

OM: Outer membrane.

OMPs: Outer membrane proteins.

OMVs: Outer membrane vesicles.

OprF: Outer membrane protein F.

OPS: O- polysaccharide.

PAMPs: Pathogen associated molecular patterns.

PBS: Phosphate Buffered Saline.

PCN: Pyocyanin.

PDI: Protein Disulfide Isomerase.

PGLYRPs: Peptidoglycan-Recognition-Proteins.

pI: Isoelectric point.

PilA: pilin A.

PMN: Polymorphonuclear leukocytes.
NS: Not specified.

PRRS: Pattern recognition receptors.

QS: Quorum sensing.

R5: Receptor 5.

rOprF : Recombinant OprF.

RT: Room Temperature.

s.c.: Subcutaneous.

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SEC: Size Exclusion Chromatography.

SNV: single nucleotide variant

SS: Secretory Systems.

T4P: Type 4 pili.

TCA: Tricarboxylic acid cycle

TCR: T-cell receptor.

Th: T-cell helper.

TLR: Toll-like receptor.

TNF: Tumor Necrosis Factor.

Trx: Thioredoxin.

UTI: Urinary tract infection.

VAP: Ventilator-associated pneumonia.

wgMLST: whole genome multilocus sequence typing.

WHO: World Health Organisation

Measurement Units

°C	Degree Celsius
%	Percentage
× g	g-force
bp	Base pair
cm	Centimetre
G	Gauge
g	Gram
h	Hours
Hz	Hertz
kDa	KiloDalton
L	Litre
M	Molar
mA	Milliamperes
mg	Milligram
min	Minutes
mL	Millilitre
mM	Millimolar
ng	Nanograms
pg	picograms
rpm	Revolutions per minute
s	Seconds
V	Volt
µg	Microgram
µm	Micrometre
µL	Microlitre
µM	Micromolar

Chapter 1

General Introduction

1. Pseudomonas aeruginosa: genus and characteristics

The genus *Pseudomonas,* described by Migula in 1894, is one of the most complex and is currently the genus of Gram-negative bacteria with the highest number of species (1). *P. aeruginosa* is a motile bacillus (0.5-3.0 µm in size), and a non-fermentative bacterium that, in aerobic situations, uses the glycolytic pathway for glucose degradation, with oxygen as the final electron acceptor. However, it can use nitrogen as an electron acceptor under anaerobic conditions. It is catalase and oxidase positive (2). *P. aeruginosa* survives in a wide range of environmental conditions as it can exploit a variety of carbon, nitrogen and energy sources, and a range of growth temperatures between 4º and 42ºC (3).

2. *Pseudomonas* aeruginosa infections: epidemiology, acute and chronic infections.

The great diversity and adaptability of *P. aeruginosa* enables it to ubiquitously inhabit non-clinical and clinical environments (1, 4). Reservoirs in urban communities include hot tubs and swimming pools. Hence, *P. aeruginosa* can cause community-acquired infections like folliculitis, and puncture wounds leading to osteomyelitis, pneumonia or otitis externa. *P. aeruginosa* is also an important opportunistic pathogen causing nosocomial infections. Reservoirs in the hospital setting include potable water, taps, sinks, and disinfecting solutions, among others (5). *P. aeruginosa* can cause acute infections mainly in immunocompromised people, but it can also adapt to the host causing chronic infections (Table 1):

2.1 *P. aeruginosa* acute infections:

P. aeruginosa causes severe acute infections such as respiratory tract infections (RTI), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), keratitis and corneal ulcers in contact lens-wearing individuals, urinary tract infection (UTI), bloodstream infections (BSIs), osteomyelitis, and endocarditis (6-10) (Figure 1 and 2). *P. aeruginosa* has a prevalence of 7.1%–7.3% among all healthcare-associated infections (11, 12). Pneumonia is the most common presentation of *P. aeruginosa* infection, accounting for up to 18% of nosocomial pneumonia cases (11), making it one of the most frequently isolated pathogens in intensive care unit (ICU)

patients. A large international observational point-prevalence study of infections in ICU patients found that *P. aeruginosa* was the cause of 23% of all ICU-acquired infections, with a respiratory source being the most common site of infection (13). A meta-analysis of 11 studies of VAP cases after post-cardiac surgery showed that *P. aeruginosa* was the causative agent in 23.2% of cases (14).

In UTIs, particularly in people with catheters, *P. aeruginosa* infections can lead to life-threatening pyelonephritis (15). Regarding BSIs, results from a 13-year (2002–2015) prospective cohort study at Duke University Medical Centre indicated a significantly increased mortality rate associated with BSI caused by *P. aeruginosa* as compared to other bacterial pathogens, including *Staphylococcus aureus* (16). Importantly, the long-term sequelae of BSIs include very serious and life-threatening complications, such as elevated risks for venous thromboembolism, myocardial infarction and stroke (17). The SENTRY Antimicrobial Surveillance Program released a 20-year investigative report on the microbiology of BSIs (18). *P. aeruginosa* was found to be the fourth leading cause of BSIs behind *S. aureus*, *E. coli*, and *K. pneumoniae*, accounting for 5.3% of all infections (18).

Finally, data from the China Antimicrobial Surveillance Network (CHINET) (http://www.chinets.com/) identified clinically isolated pathogenic strains and found that *P. aeruginosa* was the fourth cause of nosocomial infections, accounting for 7.96% (19, 20). Globally, in 2019, *P. aeruginosa* was associated with more than 500,000 of deaths (Figure 1) (21). Overall, the data strongly indicates that *P. aeruginosa* is a global major threat to human health, being especially important in respiratory tract infections.

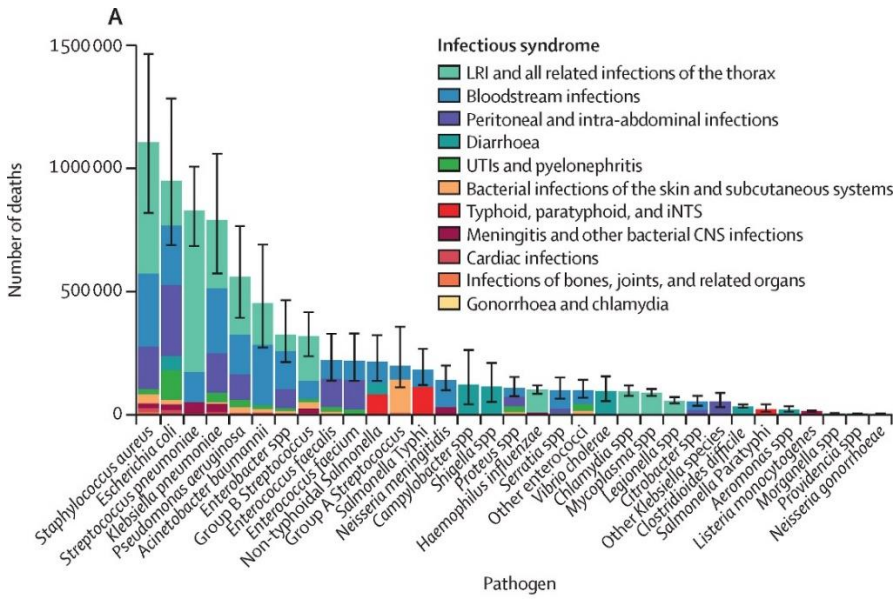

Figure 1. Global number of deaths by pathogen and infectious syndrome (2019). Columns show total number of deaths for each pathogen, with error bars showing 95% uncertainty intervals, with the bars split into infectious syndromes. Lower respiratory infection. iNTS: invasive non-typhoidal *Salmonella*. *Salmonella* Typhi: Salmonella enterica serotype Typhi. Salmonella Paratyphi = Salmonella enterica serotype Paratyphi. UTI=urinary tract infection. Taken from: Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. (2022) (22).

2.2 Chronic Infections:

2.2.1. P. aeruginosa and Bronchiectasis:

Bronchiectasis is a chronic endobronchial suppurative disease, characterised by irreversibly dilated bronchi damage due to repeated polymicrobial infection and neutrophilic airway inflammation (23). *P. aeruginosa* remains one of the predominant bacterial species present in the lower respiratory tract of patients with bronchiectasis, accounting for 21.4% of cases with bacterial colonisation, according to a meta-analysis with data derived from 21 observational cohort studies comparing *P. aeruginosa* colonised bronchiectasis patients with those without *P. aeruginosa* colonisation (24). *P. aeruginosa* colonisation in patients with bronchiectasis is associated with accelerated lung function decline, poorer quality of life, greater risk of hospitalisation and exacerbations, and increased mortality (24). The British Thoracic Society guidelines highlighted the importance of prompt evaluation of *P. aeruginosa* infection for implementing an optimal management of bronchiectasis (25).

2.2.2. P. aeruginosa burn-wound infections:

Wound infection is one of the main clinical complications associated with wound care, with 75% of all deaths from burns resulting from infection (26). *P. aeruginosa* is recognised as a critical cause of mortality and morbidity among burn patients being responsible for as high as 77% of burn wound mortalities, due mainly to its capacity to form biofilms in the burn site (27, 28). *P. aeruginosa* can cause cross-transmission and outbreaks within hospitals and localised outbreaks in burn treatment centres (29). *P. aeruginosa* was responsible for burn infections 64% of the time from 1999–2009 in paediatric burn ICUs (30).

2.2.3.P. aeruginosa and Cystic Fibrosis (CF):

CF is the most common autosomal recessive genetic disorder among Caucasians. It is caused by mutations in the CF transmembrane conductance regulator (CFTR), responsible for chloride ion transport across apical membranes of epithelial tissues (31, 32). Hence, CFTR deficiency leads to diminished chloride transport and increased sodium transport through the epithelial sodium channel (ENaC), resulting in a dehydrated airway surface liquid (ASL) and the production of mucopurulent secretions that are difficult to clear (31). *P. aeruginosa* chronic infection is the leading cause of lung function decline in people with CF (33).

The percentage of CF individuals with a positive culture for *P. aeruginosa* has continued to decline over time (Figure 2). According to the 2022 report from the USA Cystic Fibrosis Foundation, the largest decrease was observed among individuals younger than 18 years (43.8% had a positive culture in 2002 compared with 13.5% in 2022). Among the individuals with CF and a culture that grew *P. aeruginosa* in 2022, 12.7% were reported to have multi-drug resistance (MDR)-*P. aeruginosa* (resistance to all antibiotics tested in two or more antibiotic classes in a single culture) (Figure 2) (34).

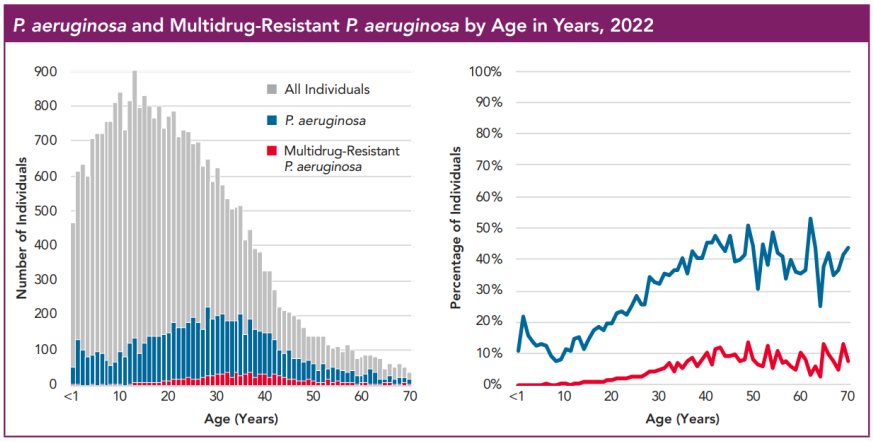

Figure 2. Prevalence of *P. aeruginosa* in individuals with CF in the United States in 2022. Source of data: Cystic fibrosis patients under care at CF Foundation-accredited care centers in the United States, who consented to have their data entered. USA Cystic Fibrosis Foundation. Permission is granted for the use of the image (34).

The CFTR defect and the dehydration of ASL in CF leads to many changes that facilitate *P. aeruginosa* colonisation of the CF lung, including effectively poor mucociliary clearance, low pH, and impaired antimicrobial and immune response mechanisms (35). The acidic environment of the CF airways results in the improper folding of the carbohydrate side chains of mucins, hampering their ability to bind foreign particles and making them more likely to bind to the cell-tethered mucins MUC1 and MUC4, glueing the mucous layer to the epithelium and preventing mucociliary clearance (36). The low pH is also associated with an altered O-glycosylation and sulfation of the airway mucins, mainly due to the alkalization of the cell compartments in CF. This unique phenotype of sputum O-glycosylation increases the ability of *P. aeruginosa* to adhere to and colonise the host's respiratory tract (37).

The recruitment of neutrophils in the CF environment can lead to a reduction of O_2 in the airway mucus due to the intensive consumption of O_2 by polymorphonuclear leukocytes (PMN) for superoxide and nitric oxide production. PMNs exert a bacteriostatic effect on aggregate bacteria since the growth rate of *P. aeruginosa* in CF mucus is inversely correlated with the amount of PMNs. Given that the most effective production of adenosine triphosphate (ATP) by *P. aeruginosa* occurs by aerobic respiration, the lack of O_2 may contribute to the inactive and therefore tolerant state of this pathogen in mucus (38).

The frequent use of antibiotics in CF can represent one of the biggest challenges that *P. aeruginosa* faces and the primary driver of decreasing bacterial diversity in the respiratory tract of CF patients. Quin et al. observed that CF metabolism existed in two states: one in severely diseased patients who had higher diversity of metabolites and more *P. aeruginosa* and another in patients with better lung function, lower metabolite diversity, and fewer pathogenic bacteria. They concluded that in cases of severe CF, there is an amino acid-rich environment due to proteolysis by host enzymes which become dominated by *P. aeruginosa* as amino acid richness provides the pathogen its preferred carbon source (39).

The study of the respiratory tract environment in CF is very complex. In addition to the impact of CFTR, "modifier genes" affect the CF phenotype and generate variability in pulmonary severity among patients (40). Some of these genes also impact *P. aeruginosa* infections in CF patients. Among them is *SLC6A14*, which is expressed in respiratory epithelial cells and transports L-arginine out of ASL. Di Paola et al. suggested that *SLC6A14* plays a role in modifying the early stages of *P. aeruginosa* infection in the airways by altering the level of L-arginine in ASL, which in turn affects *P. aeruginosa* adhesion (41). The added complexity in CF airways is the CF gender gap (42-44). Women with CF are at increased risk for the mucoid conversion of *P. aeruginosa*, which contributes to a sexual dichotomy in disease severity. Chotirmall et al. concluded that estradiol and estriol induced alginate production in PAO1 and clinical isolates obtained from patients with and without CF (45). Interestingly, a review of the CF Registry of Ireland suggested that the use of oral contraceptives was associated with a decreased need for antibiotics (45, 46).

Table 1. Summary of the prevalence of acute and chronic *P. aeruginosa* infections

	***P. aeruginosa* infection**	**Prevalence**	**References**
Acute Infections	Health care- associated	7.1-7.3%	(11, 12)
	General pneumonia	18%	(11)
	ICU-acquired infections	23%	(13)
	Ventilator associated pneumonia	23%	(14)
	BSIs	5%	(18)
Chronic Infections	Bronchiectasis	21%	(24)
	Burn wound	64%	(27, 28)
	Cystic Fibrosis	10-55%	(34)

In summary, *P. aeruginosa* can cause infections in almost any part of the human body (Figure 3). It is an important pathogen in both acute and chronic infections, suggesting high adaptability to the host. The prevalence of *P. aeruginosa* and the high mortality rates of its infections justifies and motivates the search for therapies against this pathogen.

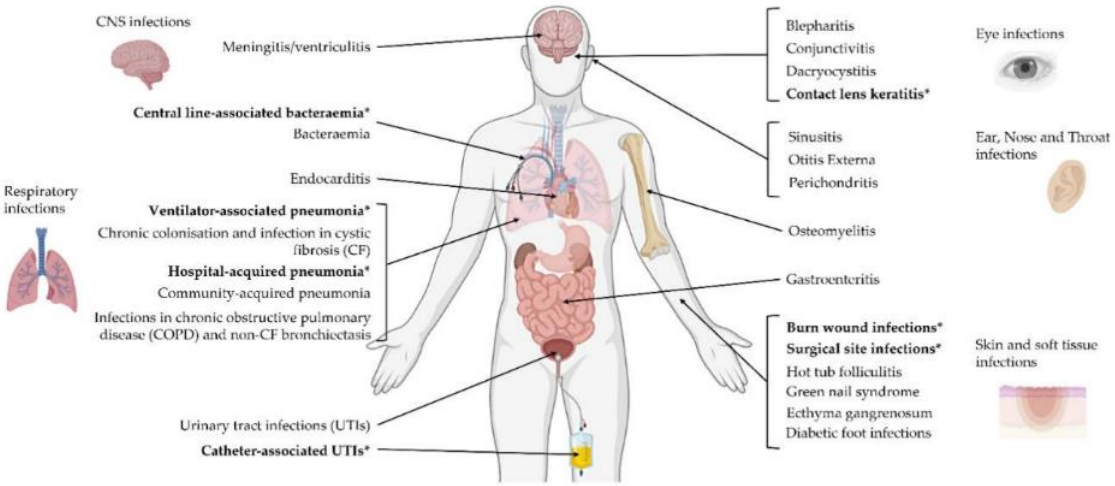

Figure 3. The many sites of *P. aeruginosa* infection throughout the body. Labels in bold and starred (*) are those infections associated with healthcare or healthcare devices (47) .

3. *P. aeruginosa* antimicrobial resistance: genome and virulence factors

In 2017 *P. aeruginosa* was classified as one of the MDR ESKAPE pathogens, comprising ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K***lebsiella *pneumoniae,* ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* (48, 49). In that year, MDR *P. aeruginosa* caused 32,600 infections among hospitalised patients and 2,700 estimated deaths in the United States (50-53). The World Health Organisation (WHO) has included carbapenem-resistant *P. aeruginosa* in the group of "critical" priority pathogens, for which new antibiotics are urgently needed (54). Importantly, hospital-acquired infections continue to produce resistance to conventionally effective antibiotics (20, 55). In 2019, six pathogens were each responsible for >250 000 AMR-associated deaths: *E coli, Staphylococcus aureus, K pneumoniae, S pneumoniae, Acinetobacter baumannii and P. aeruginosa*, in order of number of deaths (Figure 4 and 5) (22).

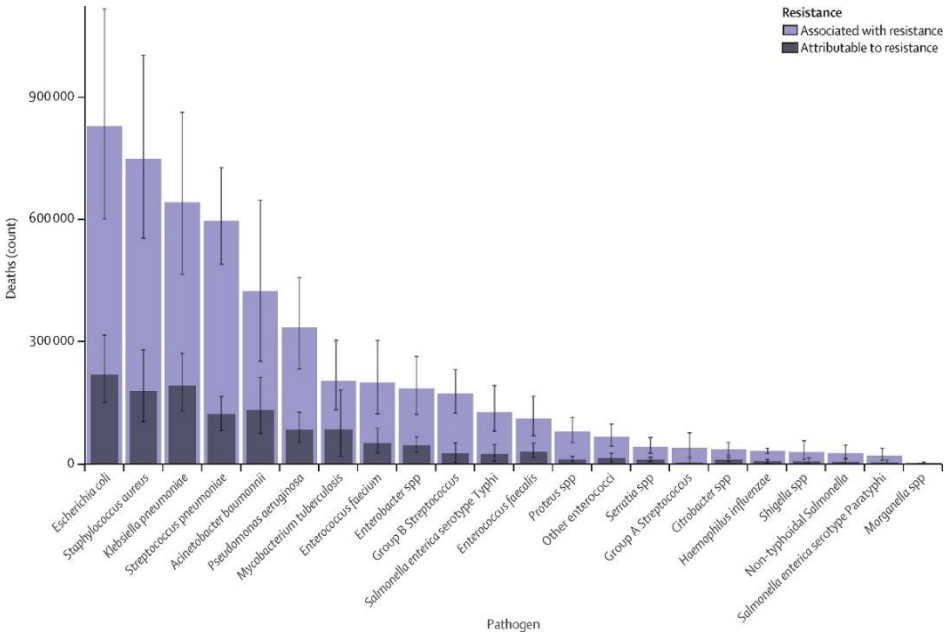

Figure 4. Global deaths (counts) attributable to and associated with bacterial antimicrobial resistance by pathogen (2019). Estimates were aggregated across drugs, accounting for the co-occurrence of resistance to multiple drugs. Error bars show 95% uncertainty intervals ¡. Taken from: Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. (2022) (22).

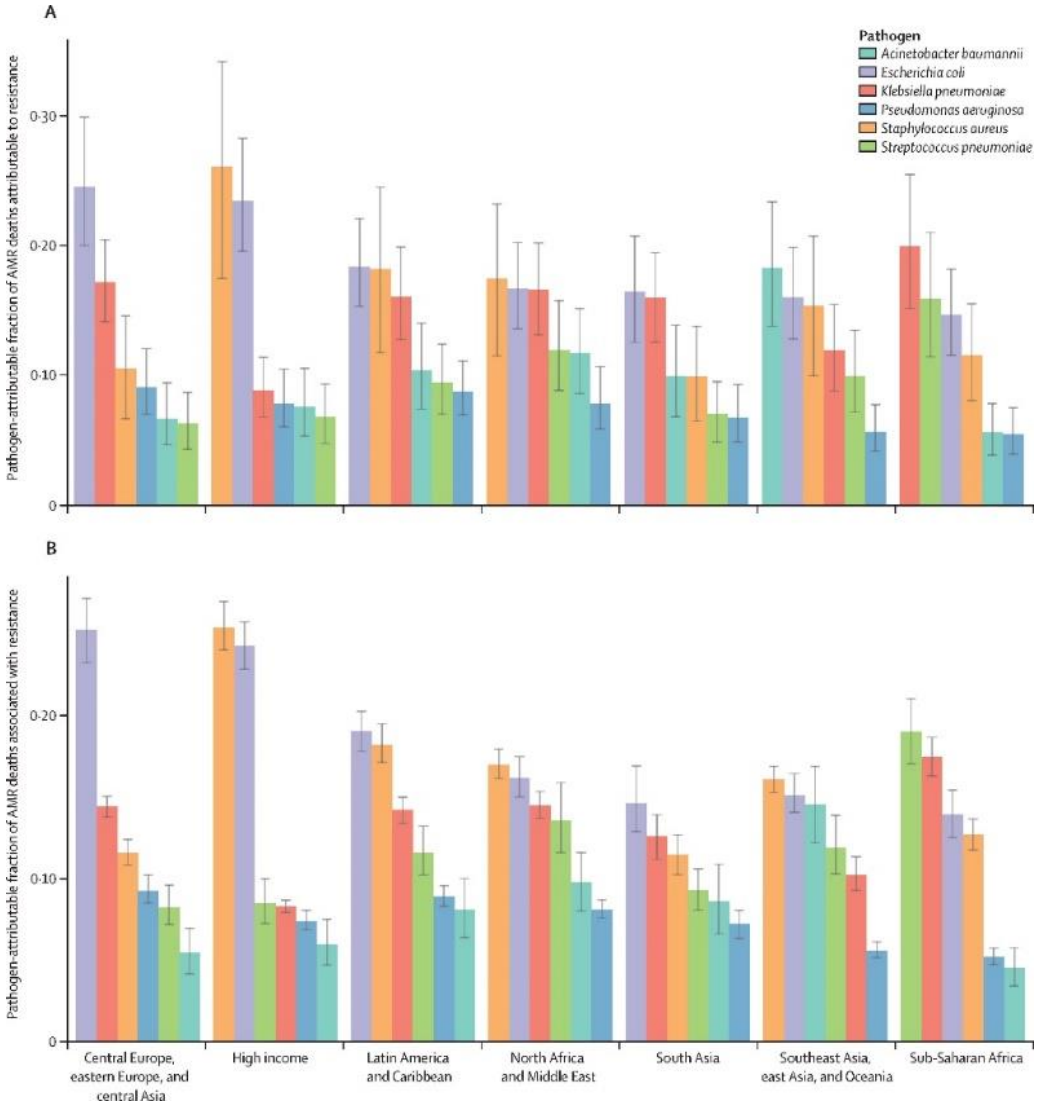

Figure 5. Pathogen-attributable fraction of deaths attributable to (A) and associated with (B) bacterial AMR for the six leading pathogens by GBD super-region (2019). Error bars show 95% uncertainty intervals. AMR=antimicrobial resistance. GBD=Global Burden of Diseases, Injuries, and Risk Factors Study Taken from: Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. (2022) (22).

The pathogenic profile of *P. aeruginosa* stems from the large and variable arsenal of virulence factors and antibiotic resistance determinants harboured in its genome (56-58). The complete genome sequence of the *P. aeruginosa* PAO1 strain was published by Stover et al. in 2000 and is used as a reference for comparison with the genomes of other strains (59). *P. aeruginosa* PAO1 has a very large and complex genome of about 6.3 Mbp (G+C content of 66.6%), encoding 5,700 genes, including 5,584 predicted open reading frames (ORFs). Moreover, an estimated 150 of the genes identified in *P.*

aeruginosa PAO1 encode outer membrane proteins related to adhesion, movement, antibiotic and virulence factor production, representing a much larger number compared to other bacterial genomes (4).

P. aeruginosa has a mosaic genome, composed of many core genes interspersed by strain-specific gene blocks. The *P. aeruginosa* pan-genome contains approximately 54,272 genes, 665 of which are core, 26,420 are accessory genes, and 27,187 are unique genes (present in one strain only) (4). The accessory genome tends to cluster at certain loci, rather than being randomly distributed throughout the core genome. These loci are often referred to as genomic islands (>10 kb) which are very relevant for their clinical implications. For example, the genomic island of *P. aeruginosa* PAPI-2 contains the gene encoding ExoU, a type-III-secreted effector protein linked to increased virulence in animal models and human patients. The intrinsic determinants of antibiotic resistance in *P. aeruginosa*, such as efflux pumps and β-lactamases, are in the core genome. However, genes of acquired antibiotic resistance are present in the accessory genome (4). Some of the main virulence factors encoded by the *P. aeruginosa* genome are:

3.1 Lipopolysaccharide (LPS):

The cell envelope of Gram-negative bacteria is composed of two membranes that are separated by the periplasm, an aqueous compartment that includes a peptidoglycan cell wall (60-63). The inner membrane (IM) is a symmetric phospholipid bilayer, and the outer membrane (OM), is asymmetric; and mainly composed of LPS molecules (60-64). The structure of LPS comprises three covalently attached domains: the lipophilic lipid A, the hydrophilic core oligosaccharide, and the hydrophilic O-antigen (65, 66). The lipid A moiety is the most conserved and toxic portion of LPS (63, 67, 68), while the O-antigen (O-Ag) consists of oligosaccharide repeating units (up to 40) each having 3–8 sugar residues, it is the most variable constituent of LPS and determines the serological specificity (61, 69). Lipid A is embedded in the outer leaflet of the bacterial outer membrane (OM) while the core oligosaccharide and the O-antigen are extended outward (65, 70) (Figure 6)

The O-antigen is synthesised separately from the lipid core A and subsequently bound to it, hence not all lipid core A molecules bind to the O-antigen before export,

creating a heterogeneous outer membrane surface, with LPS with and without O-antigen (71). Intraspecies variation of the specific O-antigen presented on the cell surface has allowed serotyping of *P. aeruginosa* (72, 73). *P. aeruginosa* can simultaneously produce two O-antigens: the common polysaccharide antigen (CPA) and the O-specific antigen (OSA, formerly termed B band) (72, 74). CPA has a common and conserved structure while the structure of OSA is variable and is therefore the determinant used in serotyping to segregate *P. aeruginosa* species into different groups (75, 76). The International Antigenic Typing Scheme (IATS) is the standardised serotype classification of *P. aeruginosa,* which includes 20 different serotypes. This classification system has generally been effective in classifying *P. aeruginosa* strains that are wild-type organisms producing smooth LPS with all three domains (lipid A, core oligosaccharide and O antigens). This is usually not the case in clinical settings, as many of these isolates partially or completely lack O-Ag. Serotyping of chronic bacterial isolates from CF patients for epidemiological studies was especially problematic because a very high proportion of these bacteria proved to be polymorphable by more than one serotyping antiserum or no typeable (72). As a virulence factor, LPS is essential as a surface structural component to protect the bacteria's external leaflet and poison host cells. The endotoxicity of lipid A enable tissue damage, attachment, and recognition by host receptors (77).

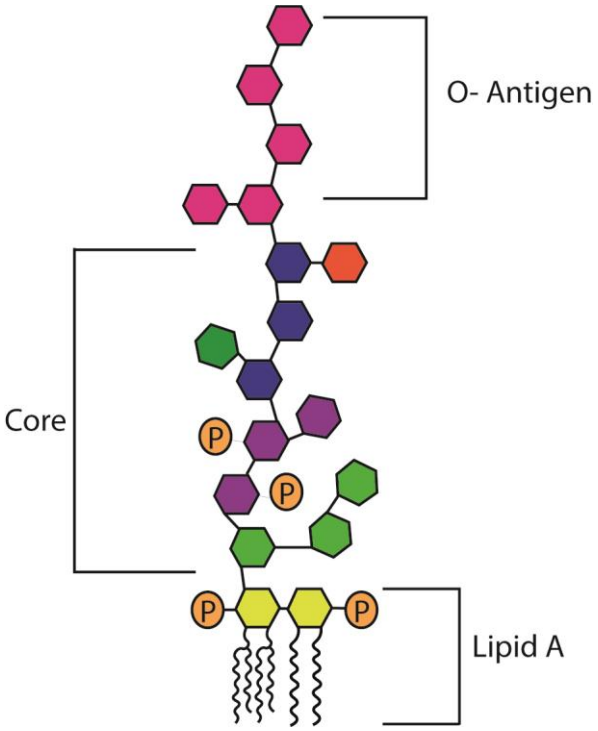

Figure 6. Structure of the lipopolysaccharide of Gram-negative bacteria. Image adapted from: Maeshima et al. (78).

3.2 Outer membrane proteins (OMPs):

The OMPs are essential for nutrient exchange, antibiotic resistance or adhesion of *P. aeruginosa* and all Gram-negatives (79). The OM of *P. aeruginosa* is characterised by very low permeability, which contributes to the high intrinsic and induced resistance to antibiotics. There are 26 identified porins in *P. aeruginosa*, including outer membrane protein F (OprF), which is the major non-lipoprotein OMP, and the homolog of OmpA of *E. coli* (79).

OprF porin allows nonspecific diffusion of ionic species and small polar nutrients (80). It has high importance for *P. aeruginosa* virulence being involved in quorum sensing, toxin secretion and cell adhesion (81). It is partially exposed to the external surface of the bacteria. Interestingly, the interaction of OprF with IFNγ was shown to stimulate the production of two bacterial virulence factors: the lectin PA-1L and the phenazine pyocyanin (82, 83), suggesting that OprF could be a sensor of the host immune system (84).

3.3 Flagellum and type IV pili:

Flagella enable bacteria to swim in liquid media and swarm on semisolid surfaces. In Gram-negative bacteria, this apparatus encompasses the inner and outer membranes. Flagella allow bacteria to move toward more favourable conditions or escape a harmful environment (85). A polar flagellum enables *P. aeruginosa* to swim and swarm and is essential for host colonisation. Like LPS, it is involved in the adhesion of *P. aeruginosa* to host surfaces (86). FleQ is the flagellar master regulator in *Pseudomonas* species (85). Another component that enables *P. aeruginosa* motility is the type IV pili (T4P), located in retractile appendages. They are crucial for host cell adherence as an initial step in colonisation, they polymerise and depolymerise, leading to rapid cycles of extension and retraction that generate considerable mechanical force (87). At the onset of infection, flagella and T4P control motility and adhesion to *P. aeruginosa* host cells (86). That adhesion led to changes in host signal transduction pathways, as flagellin binds to TLR-5 (88), which is discussed later in the section "7.3 Recognition of *P. aeruginosa* by human cell receptors that trigger immune responses".

3.4 Secretory systems:

P. aeruginosa has six secretory systems (SS) (89, 90). These include flagella (associated with T6SS), pili (T4SS), and multi-toxin component secretion system type 3 (T3SS), which are reported to be essential for host colonisation, adhesion, swimming, and swarming in response to chemotactic signalling. Based on the secretion pathways of the transporter proteins, the secretion systems are divided into two main classes, the one-step secretion system (T1SS, T3SS, T4SS and T6SS) and the two-step secretion system (T2SS and T5SS). The one-step secretion system directly secretes proteins from the bacterial cytosol to the surface, whereas the two-step secretion system requires a brief periplasmic sojourn of the secreted proteins in the export pathway and then releases the proteins (91).

The *P. aeruginosa* T3SS, which plays a key role in virulence such as quorum sensing, is one of the most studied secreted toxins (92, 93). The T3SS is similar to a molecular syringe, containing: the needle complex, the translocation apparatus, the regulatory proteins, the effector proteins and the chaperones. T3SS secrete virulence

effectors (ExoS, ExoT, ExoY and ExoU) into eukaryotic host cells to disrupt intracellular signalling and ultimately cause cell death (91).

3.5 Secretory molecules:

Exotoxin A (ETA) is the most toxic product released by *P. aeruginosa*, as it inhibits host protein synthesis due to its ADP-ribosylating activity, ultimately leading to irreversible cell death (94). Moreover, LasA and LasB elastases, alkaline protease (AprA), LipC lipases, phospholipase C and esterase A enzymes comprise a large group of lytic enzymes that modulate other virulence factors (95). Also, antioxidant enzymes, such as catalases (KatA, KatB and KatE), alkyl hydroperoxide reductases and superoxide dismutases, neutralise the activity of reactive oxygen species (ROS) in phagocytic environments to prevent bacterial clearance, as observed by Dar et al. using co-cultures of epithelial cells and macrophages (91, 96-99).

3.6 Quorum sensing (QS):

The regulation of all *P. aeruginosa* virulence factors is cell density-dependent through the release of autoinducers from four QS systems (Las, Rhl, Pqs and Iqs). They are hierarchically interconnected, creating a highly adaptive network that responds to external stressors and provides *P. aeruginosa* with extraordinary plasticity that facilitates the successful colonisation of a wide range of niches (100). Acyl homoserine lactose (AHL) molecules are the main QS molecules. The concentration of AHL molecules increases as the bacterial population grows (101, 102). When the population density reaches the "quorum", these AHL molecules exceed the threshold concentration and are recognized by LasR receptors on *P. aeruginosa*. R proteins bind to AHLs and directly regulate the transcription of target genes (101, 102).

4. Mechanisms of antimicrobial resistance in *P. aeruginosa*:

The mechanisms that *P. aeruginosa* use to resist antibiotics can be divided into intrinsic, acquired and adaptive antimicrobial resistance. Intrinsic antimicrobial resistance is related to the use of virulence factors: i) limiting antibiotic penetration by manipulating OMPs (103); ii) the use of efflux pumps, such as MexAB-OprM and MexXY-OprM, crucial for developing carbapenem-resistant *P. aeruginosa* strains (104) or; *iii)* antibiotic-inactivating enzymes (hydrolase) to degrade or alter antibiotics. In contrast,

acquired resistance is related to mutations or horizontal gene transfer, for example, mutations of DNA gyrase (GyrA) cause resistance to quinolone antibiotics (105). And finally, adaptive resistance is caused by environmental stimuli, thus a transient change in gene and/or protein expression (91). In *P. aeruginosa*, the formation of biofilms is the most frequent strategy to acquire adaptive antibiotic resistance. Biofilms are complex communities of microbes that are embedded in a self-produced extracellular macromolecular matrix produced by the residents (106, 107).

5. Host immune response against *P. aeruginosa*: innate and adaptive immune responses and pathogen recognition:

5.1. Innate Immune Response:

The recognition of *P. aeruginosa* pathogen-associated molecular patterns (PAMPs) elicits a potent inflammatory response, which is critical for the recruitment of neutrophils and macrophages, facilitating bacterial clearance. An optimal host response is essential; a weak response with inefficient infiltration of phagocytic cells leads to unsuccessful bacterial killing and clearance, while an excessive response causes host tissue damage (108-114).

Massive recruitment of neutrophils into the infected respiratory tract is a hallmark of *P. aeruginosa* infection. In neutropenic mice, the lethal dose of *P. aeruginosa* was 100,000 times lower than that required by mice with normal neutrophil levels. These observations were replicated in both rabbits and humans (114-117). The factors that regulate the recruitment of neutrophils to *P. aeruginosa* infected sites have not been well characterised, but the recruitment is mediated, at least in part, by the production of chemokines, which are critical for neutrophil chemotaxis and activation (111), such as IL-8 (115, 118). The neutrophils act to kill *P. aeruginosa* but can also contribute to host lung damage due to the production of ROS and the release of bactericidal proteins from their acidophilic granules. Hence, the appropriate level of neutrophil recruitment is essential to achieve bacterial clearance without causing excessive tissue damage (119).

Macrophages are also essential for the clearance of *P. aeruginosa* infection as they are the first immune cells to encounter *P. aeruginosa* in the lung. They act as effector cells and regulators of neutrophil recruitment (120). Alveolar macrophages are not only

responsible for the internalisation and killing of *P. aeruginosa* but also for the phagocytosis of dying neutrophils, thus limiting neutrophil-induced tissue damage (121). Intracellular bacteria may eventually induce macrophage lysis, in an ExoS-dependent manner. Therefore, T3SS and ExoS, whose expression is modulated by MgtC and OprF, are key actors in the intramacrophage lifestyle of *P. aeruginosa* (122). The complement system is also necessary for the survival of mice after pulmonary infection with *P. aeruginosa* (113), as OprF porin in the OM of *P. aeruginosa* acts as a binding acceptor molecule for C3b to initiate the formation of the membrane attack complex (MAC).

In summary, the innate immune response against *P. aeruginosa* is driven mainly by neutrophils, macrophages and the complement system. It is essential for the control of *P. aeruginosa* infections; however, the relative importance of these pathways and how they are integrated *in vivo* remain unclear.

5.2. Adaptive immune response :

5.2.1. Cellular responses:

The sensor pattern recognition receptors (PRRs) from antigen-presenting cells (APCs), such as dendritic cells (DCs), sense *P. aeruginosa* antigens and trigger intracellular signalling events that promote phagocytosis, maturation, and secretion of cytokines (123, 124). Once the *P. aeruginosa* antigens are internalised, they are digested into peptide fragments, and displayed on a set of cell surface receptors: major histocompatibility complex (MHC) (124). The APCs and T-cells interact through MHC/TCR (T-cell receptor) binding, leading to the differentiation and proliferation of naïve T-cells into effector cells (125). Activated $CD4^+$ T-cells proliferate and differentiate into different immune cell subsets: T-helper (Th)1, Th2, Th17, T follicular helper (Tfh), and regulatory T-cells (Treg) (126) (Figure 7).

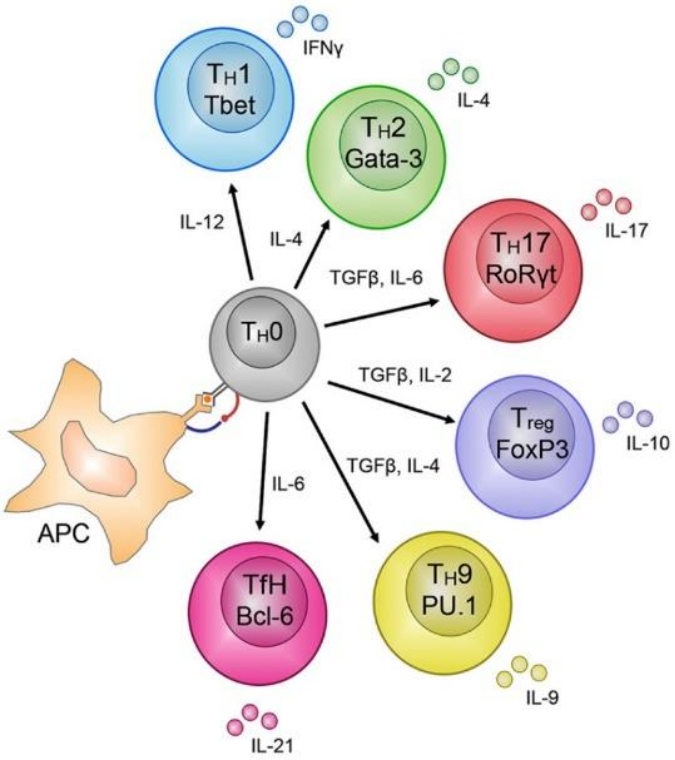

Figure 7. CD4⁺ Th cell subset differentiation. CD4⁺ T cells differentiate into different subsets based on the soluble molecules secreted by antigen-presenting cells (APcs). Interleukin (IL)-12 or Interferon- gamma (IFN-γ) induces Th1 cell differentiation that leads to the production of IFN-γ, which stimulates macrophages and cytotoxic T lymphocytes (CTLs) (127). IL-4 induces Th2 cell differentiation, the cells then produce IL-4, IL-5, and IL-13, mobilising eosinophils, basophils, and mast cells to combat helminthic parasites (128). Th17 cell differentiation is induced by TGF-β plus IL-6 (or IL-21). Th17 cells produce IL-17A, IL-17F, and IL-22, stimulating GM-CSF production in epithelial cells, fibroblasts, and stromal cells, then recruiting neutrophils and inducing inflammation that is related to extracellular bacteria and fungi clearance (129). And, finally, Treg cells can be differentiated by TGF-β, which activates (130-134). Image taken from: Russ et al. (126).

The resolution of the acute inflammatory response by *P. aeruginosa* requires attenuation of pro-inflammatory pathways. Regulatory T-cells (Tregs) inhibit the secretion of pro-inflammatory cytokines and secrete anti-inflammatory cytokines, while DCs initiate adaptive responses, characterised by a Th2-skewed response with the upregulation of IL-5 and IL-13; higher B-cell sensitivity to IL-4; low levels of IFN-γ and elevated levels of IL-10, which further downregulate IFN-γ. This response hinders antigen presentation and the successful immune response of the host against *P. aeruginosa* infections (135). Importantly, the release of IFN-γ can improve lung function due to a Th1-like response (136, 137). CF patients chronically colonised with *P. aeruginosa* have greater levels of IL-3, IL-4, and secreted IgA, and lower IFN-γ secretion, compared with intermittently colonised patients or those without *P. aeruginosa* (138). However, the appropriate balance of the Th1/Th2/Th17 responses has not yet been elucidated.

In recent years, studies have focused on the Th17 response and its role in the mucosal immune response to respiratory pathogens (139). Several studies in murine models of acute pneumonia have demonstrated the protective effects of the Th17 response against *P. aeruginosa*, reflected in the reduction in bacterial counts within the lungs of *P. aeruginosa*-infected mice relative to the control groups (140, 141). These data are important in the context of vaccines against *P. aeruginosa*, but it is also worth highlighting that Th17 immune responses may only be slightly effective in the absence of the Th1 pathway (142). Also, despite promising results on the protective effects of Th17 responses against *P. aeruginosa* in murine models of acute infection, studies have failed to clarify its role in chronic *P. aeruginosa* infections. Bayes et al. highlighted the key role of IL-17 in mouse survival and prevention of *P. aeruginosa* chronic infection (143). However, the Th2–Th17 axis in CF may predispose to the development of *P. aeruginosa* lung infection. Hence, it is possible that vaccine-induced Th17 responses are ineffective in the CF lung and may even exacerbate the neutrophilic airway inflammation of CF (144).

In summary, the role of the Th17 immune response in *Pseudomonas aeruginosa* infections is not fully understood and requires further evaluation. While it shows protective effects in acute pneumonia by reducing bacterial counts, its effectiveness may be limited without the Th1 pathway. In chronic infections, the role of Th17 remains unclear. Experiments with B cell-deficient mice demonstrated that B cell production of IL-17 or natural antibodies did not protect against chronic P. aeruginosa infection (143). This highlights IL-17, rather than antibodies, as crucial for host defense in chronic pulmonary infections (143). However, in cystic fibrosis (CF), the Th2-Th17 axis may worsen lung inflammation, potentially making Th17-based vaccines less effective or harmful in CF patients.

5.2.2. Humoral responses:

Adaptive humoral immune responses require the activation of resting B cells following antigen recognition, which leads to the generation of high-affinity memory B cells and antibody-secreting plasma cells (ASC). High antibody production followed by immune complex (IC) formation is a hallmark in *P. aeruginosa* infections (135). Production of IgG antibodies during chronic *Pseudomonas* infection, especially in CF

patients, has been associated with the high expression of the nuclear factor binding near the κ light- chain (NF-κB); however, the response against specific antigens appears to depend on the infection stage, with some antigens provoking a more intense response in the acute phase, while others are more targeted during the chronic stage. For example, the level of specific antibodies increases in the presence of the *P. aeruginosa* mucoid phenotype, and it is associated with poor prognosis (135). Also, despite the presence of the potent B-cell activator (BAFF), chronic colonisation is common, suggesting that this response is ineffective (145). The reasons for the inefficient antibody response against *P. aeruginosa* infections remain unclear, and better knowledge of the underlying mechanisms, such as maturation of avidity/affinity, class change, memory formation, and cytokine synthesis, is needed to understand this phenomenon (135).

Secretory immunoglobulin A (sIgA) is also of great importance in the humoral response against *P. aeruginosa* respiratory infections, as it is the predominant antibody isotype in the mucosal immune system, lining the respiratory tract (146). The concentration of sIgA against *P. aeruginosa* in nasal secretions and saliva correlated with the infection status of CF patients (i.e., not colonised, intermittently colonised or chronically infected with *P. aeruginosa*) (147, 148). The role of B cells in the defence against *P. aeruginosa* is still far from being understood. However, high antibody production is a characteristic of *P. aeruginosa* infections, which has not only proven to be ineffective in the clearance of the pathogen but also detrimental when the infection becomes chronic (135). A better understanding of the humoral response, such as IgG avidity for antigens or the role of IgA in mucosal immunity, is needed to find better methods of diagnosis and treatment of acute and chronic infections generated by *P. aeruginosa*, thus reducing the morbidity and mortality of susceptible individuals such as CF patients (149).

5.3 Recognition of *P. aeruginosa* by human cell receptors that trigger immune responses:

PRRs recognise and bind their respective ligands, recruiting adaptor molecules through their effector domains, and initiating downstream signalling pathways to exert effects. The study of these interactions between PRRs and their ligands has suggested new ways to treat infections(150). The main PRRs involved in *P. aeruginosa* infection are (Figure 8):

5.3.1 Toll-like receptors (TLRs):

P. aeruginosa expresses powerful agonists of Toll-like receptors (TLR), TLR2, TLR4, TLR5 and TLR9, which recognise lipopeptides, LPS, flagellin, and non-methylated bacterial CpG DNA, respectively (113, 151, 152).

5.3.1.1 TLR-2:

The major TLR-2 ligands known from *P. aeruginosa* are lipoprotein, components of the extracellular capsule and the secreted toxin ExoS. Lipopeptide I (OprI) of *P. aeruginosa* binds to TLR2 and TLR4 in DCs. Mucoid *P. aeruginosa* expresses high levels of lipoproteins, which induce TLR2-mediated cytokine responses in airway epithelial cells (152). *P. aeruginosa* protein, AnvM (anaerobic and virulence modulator, PA3880 protein), binds directly to TLR2 and TLR5. Deletion of AnvM resulted in increased host survival, decreased bacterial burdens, reduced inflammatory responses, and fewer lung injuries in challenged mice hosts (153, 154).

5.3.1.2 TLR- 4:

TLR4 binds to the lipid A component of LPS, a glycolipid component of the cell wall of Gram-negative bacteria. This event induces a potent immune response, potentially underlying severe inflammation and sepsis in the host (155). TLR4 also appears to share several *P. aeruginosa* ligands with TLR2, including possibly LPS, certain OMPs, ExoS, alginate capsule and, to a lesser extent, slime- glycolipoprotein (GLP) (155).

The binding of *P. aeruginosa* LPS to TLR4 depends on the structure of lipid A, which is strain specific. (152, 156). Many laboratory and environmental strains express a pentacylated lipid A, which does not activate human TLR4 (157). However, strains adapted to chronic infection of CF patients often produce hexacylated lipid A, which is a more potent TLR4 agonist. However, in murine macrophages, TLR4 appears to react to pentacylated and hexacylated forms equally, whereas human TLR4 signalling complexes react strongly to the hexacylated form. This difference in specificity is the result of sequence variations in a hypervariable region of the TLR4 binding site (152).

5.3.1.3 TLR- 5:

TLR-5 specifically binds to flagellin, the major component of bacterial flagella (158). The TLR5-binding site of *P. aeruginosa* flagellin is located at amino acid residues

88-97, and mutations in this region can drastically reduce the interaction of the molecule with TLR5 without affecting bacterial motility (159). A loss of glycosylation of *P. aeruginosa* flagellin is also sufficient to decrease TLR5-mediated responses, although glycosylation does not appear to affect TLR5 recognition of flagellin in other species. Loss of motility, even without a complete loss of the flagellum, may be a mechanism of immune evasion for *P. aeruginosa*, given that many clinical isolates are not motile (152, 160).

TLR5 expression in non-hematopoietic cells is an important component of innate immunity against *P. aeruginosa* (161). TLR4, together with TLR5, is critical for resistance to *P. aeruginosa* lung infection in healthy mice. Lack of TLR4/LPS or TLR5/flagellin interactions results in impaired neutrophil chemokine, tumour necrosis factor (TNF) and IL-6 responses of murine airway epithelial cells and alveolar macrophages. It is important to consider this redundancy between TLR4 and TLR5 in the experimental design of *P. aeruginosa* - host interaction studies (152, 162).

5.3.1.4. TLR-9

In contrast to the three previously discussed TLRs, TLR9 functions intracellularly, where it detects abundant unmethylated CpG motifs in bacterial DNA (163, 164). The role of this TLR in the response to *P. aeruginosa* has not been extensively characterised and is thought to be variable depending on cell type, for example, neutrophils responded to *P. aeruginosa* DNA in a TLR9-independent manner (165).

5.3.1.5 MyD88

The role of MyD88, an adaptor molecule for almost all TLRs, is especially important since several studies have shown that it is necessary for the rapid recruitment of neutrophils to the site of infection (166). Blockage of multiple TLR pathways in mice (e.g., TLR2/TLR4/TLR5) did not compromise their response to *P. aeruginosa* as mice lacking MyD88 (167). Hussain et al. demonstrated that TLR5 is physically associated with TLR4, diverting TLR4 signalling to the MyD88 pathway. After exposure of primary murine macrophages to ultra-pure LPS, TLR5 was co-immunoprecipitated with MyD88, TLR4 and LPS, suggesting an updated paradigm for TLR4/TLR5 signalling (168).

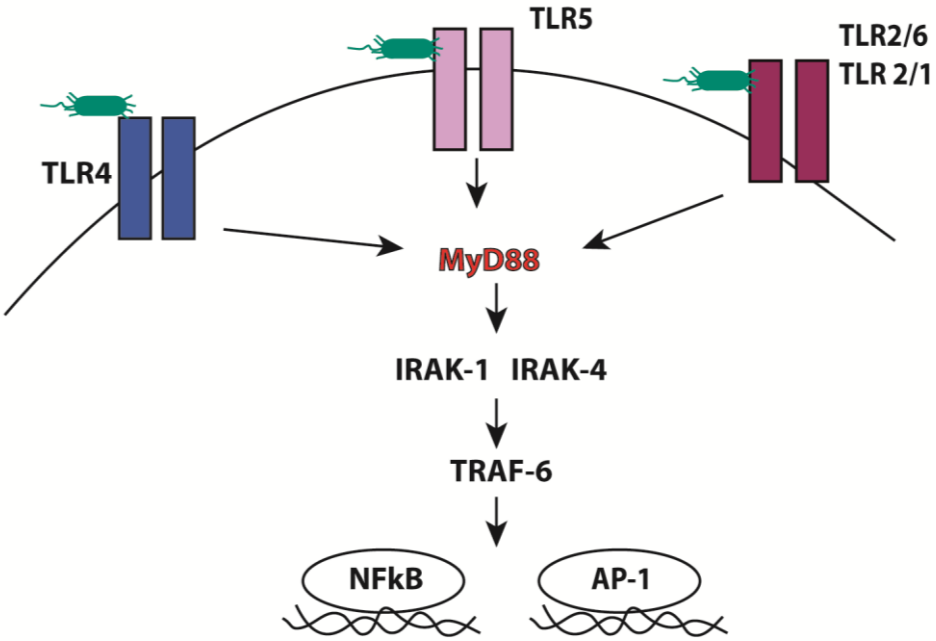

Figure 8. Main TLRs involved in *P. aeruginosa* recognition. Adapted from McIsaac et al. (152).

5.3.2 Nod-like Receptors (NLRs):

The contribution of NLRs to the bacterial immune response is less well-defined compared to TLRs. However, it has been recognized that NLR activation is an essential part of innate immunity and consequently mediates host cellular responses leading to inflammation (169). Currently, the main NLRs involved in the immune response against *P. aeruginosa* are:

5.3.2.1. NOD-1 AND NOD-2:

NOD-1 and NOD-2 are cytosolic receptors for muropeptides of bacterial peptidoglycan. They associate with NF-κβ activation upon stimulation with peptidoglycan (170-173). Some studies suggest that *P. aeruginosa* is internalised in human epithelial cells (174) and NOD-1 is activated by *P. aeruginosa* peptidoglycan, which activates NF-κβ and helps bacterial killing (175). Another study identified a novel mechanism of *P. aeruginosa* to transport peptidoglycan to NOD-1 in host cells via outer membrane vesicles (OMVs) (175, 176).

5.3.2.2. NLRC4:

NLRC4 activates caspase-1 in *P. aeruginosa*-infected macrophages (177, 178). Such caspase-1 activation requires a functional bacterial secretion system, like T3SS and T4SS. Flagellin is also important for NLRC4 inflammasome activation. Since delivery of purified flagellin to the macrophage cytosol triggers caspase-1 activation through NLRC4 (179, 180), it had been thought that NLRC4 is activated in macrophages through leakage of small amounts of flagellin through a T3SS during *P. aeruginosa* infection (181). Flagellin-independent activation has also been proposed because while NLRC4 inflammasome activation is dependent on flagellin and T3SS, flagellin-deficient *P. aeruginosa* strains are still able to induce inflammasome activation (182).

5.3.2.3 NLRP3:

NLR3 is one of the most important types of NLRs as it is activated by numerous pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (183) and does not interact directly with their activators, but its activation is triggered through an intermediate cellular signal such as NF-κB, TNF, IL-1β and SYK (a non-receptor tyrosine kinase). Pre-treatment of human macrophages with the SYK inhibitor R406 has been suggested to result in significant inhibition of SYK phosphorylation and down-regulation of IL-1β production in *P. aeruginosa-infected* cells (184). Deng et al. (2016), demonstrated NLRP3 activation in human macrophages with *P. aeruginosa* and explored a novel mechanism of this bacterial pathogen to escape intracellular death of macrophages through NLRP3 activation (185).

5.3.2.4 C-Type Lectin receptors:

Myeloid C-type lectin domain family 5 member A (CLEC5A) interacts with *P. aeruginosa* LPS and is responsible for *P. aeruginosa-induced* NET (neutrophil extracellular traps) formation and lung inflammation. Blockade of CLEC5A attenuated *P. aeruginosa*-induced NETosis and lung injury. Simultaneous administration of anti-CLEC5A mAb with ciprofloxacin increased the survival rate and decreased collagen deposition in the lungs of mice challenged with a lethal dose of *P. aeruginosa* (186). Singh et al. suggested that carbohydrates present in *P. aeruginosa* biofilms may contribute to immune recognition of *P. aeruginosa* biofilms by binding C-type lectins. The authors demonstrated the binding of dendritic cell-specific intercellular adhesion molecule-3-

non-integrin (DC-SIGN, CD209), mannose receptor (MR, CD206) and Dectin-2 to *P. aeruginosa*, identifying the presence of ligands for three important C-type lectins on *P. aeruginosa* biofilm structures and purified biofilm carbohydrates, and highlighting the potential of these receptors to influence immunity to *P. aeruginosa* infection (187).

5.3.2.5 AIM2- like receptors

The AIM2 inflammasome recognises foreign cytosolic double-stranded DNA. The role of these receptors in *P. aeruginosa* infection has not been extensively studied, however, Pang et al. reported that *P. aeruginosa* infection induced AIM2 protein degradation in macrophages, and a similar level of IL-1β, IL-6 and TNF production in wild-type and AIM2-deficient mice. Similarly, no significant differences in bacterial clearance, neutrophil infiltration and NF-κβ activation were observed between wild-type and AIM2-deficient mice after *P. aeruginosa* lung infection, suggesting that the AIM2 inflammasome is dispensable for host defence against *P. aeruginosa* infection (188).

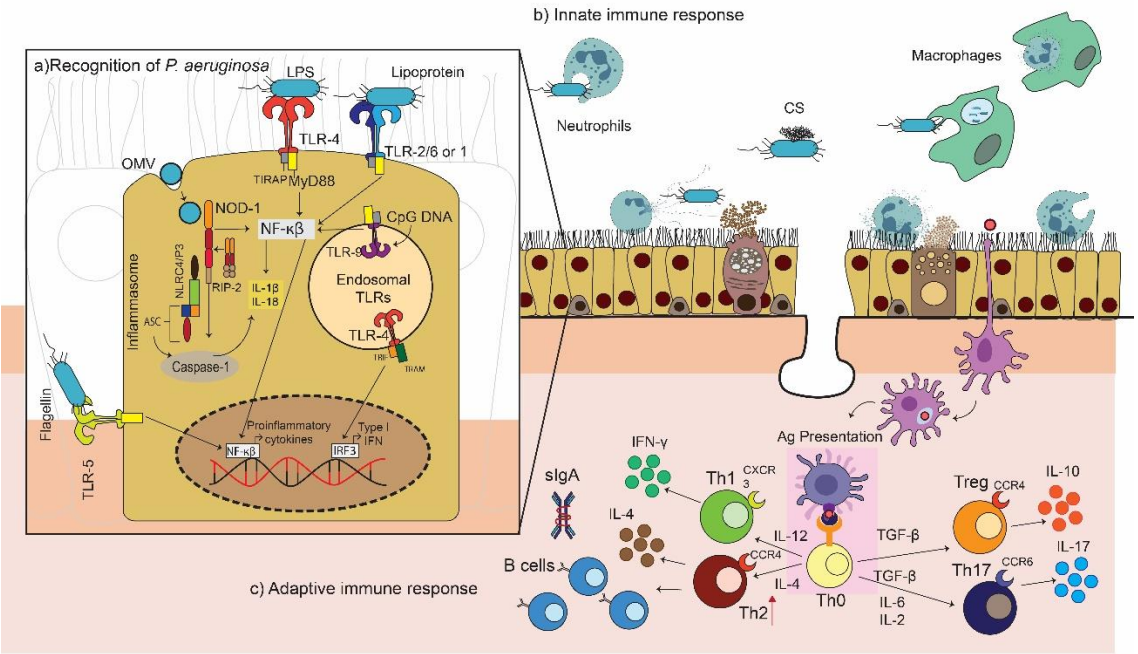

Figure 9. Host immune response against *P. aeruginosa* infections in the pseudostratified respiratory epithelium. (**a**) Recognition of *P. aeruginosa*. Pathogen recognition receptors (PRRs) located on the immune cells (TLR2, TLR4, TLR5, TLR9) recognise the PAMPs of *P. aeruginosa* (Lipoprotein, LPS, flagellin, and CpG-DNA, respectively) and trigger the production of pro-inflammatory cytokines and chemokines. OMV endocytosis activates the NF-κB pathway. (**b**) Innate immune response. (i) Neutrophils, recruited in response to *P. aeruginosa* (ii) Macrophages, which phagocytise bacteria or dying neutrophils (iii) complement system (CS). (**c**) Adaptive immune response. A skewed Th2 response occurs during *P. aeruginosa* infection with high but inefficient antibody production. In addition, the production of IgA seems to be relevant, as its levels may correlate with the status of *Pseudomonas* infection.

6. Cystic Fibrosis as a model for the study of *P. aeruginosa* adaptation within the host:

6.1 *P. aeruginosa* adaptation to the CF environment:

The prevalence of *P. aeruginosa* in CF patients allows the study of long-term adapted strains. Hence, many genomic and phenotypic studies have been done using *P. aeruginosa* isolates from CF patients to explore host-pathogen interactions within the host and how *P. aeruginosa* can overcome hostile conditions. Following initial infection with environmental isolates, specific bacterial phenotypes are selected within the CF airway environment (189). *P. aeruginosa* adaptation traits are manifested in both children and adults and include loss of flagellar-dependent motility (190), loss of O-antigen (191), auxotrophy (192), less pyocyanin production (193), antibiotic resistance (194), biofilm formation (195), among others (Figure 10).

The loss of flagellar expression within the CF airway seems to make *P. aeruginosa* less phagocytosed by alveolar macrophages and polymorphonuclear leukocytes (190, 196), allowing *P. aeruginosa* to evade detection and clearance by host defence mechanisms and causing chronic lung infection (197). The loss of O-antigen and other changes in LPS, common in *P. aeruginosa* CF-adapted strains, seems to also protect bacteria from phagocytosis (191, 198). Some first studies with CF-adapted strains suggested that serum from some patients with chronic bronchiectasis inhibited the clearance of *P. aeruginosa* due to increased IgG2 anti-O antigen antibody titres. The inhibitory effect correlated with decreased lung function, finding an association between elevated IgG2 anti-O antigen with poor prognosis in CF patients (199, 200). More recently, Torrens et al. confirmed that CF strains were more serum-susceptible than those from bacteraemia, although there was no trend when comparing the early/late isolates from the same patient. However, they observed that late CF strains had increased susceptibility against lysozyme and Peptidoglycan-Recognition-Proteins (PGLYRPs) (immune human proteins) when combined with colistin treatment. So, they proposed attacking some *P. aeruginosa* cell-wall biology-related elements to increase the activity of the innate weapons (201, 202).

The increased auxotrophy is also characteristic of CF *P. aeruginosa* isolates. Auxotrophy is the inability to synthesise one or more specific metabolites required for

growth, which frequently occurs due to the high availability of those metabolites in the extracellular environment. As the biosynthesis of amino acids represents an energetic cost for bacteria, losing the ability to synthesize them when they are available in the environment, creates a positive selective advantage of auxotrophic over prototrophic strains (192). The amino acids are essential carbon sources for *P. aeruginosa* and the CF mucus is rich in these metabolites. Hence, methionine, leucine, and arginine auxotrophies are frequently observed among clinical CF isolates of *P. aeruginosa.* Analyses of more than 200 sputum samples from 60 CF patients with reduced lung function showed that in more than 60% of the patients, the increased amino acid concentration promoted the development of auxotrophy from pre-existing prototrophic bacteria (203, 204).

During CF lung infection *P. aeruginosa* secretes high amounts of pyocyanin (PCN), a redox-active phenazine. As pyocyanin is proinflammatory, it plays an important role in mediating pneumonia development (205). Pyocyanin is produced by 95% of tested *P. aeruginosa* isolates (206), and it allows *P. aeruginosa* to manipulate the redox micro-environment during infection. Pyocyanin induces ROS production by host cells and blocks the host expression of catalase, an enzyme that neutralizes ROS through NADPH oxidation (205). Sustained exposure to ROS results in host cell damage and a decrease in host defences, which allows bacteria to establish a chronic infection (36). PCN-deficient mutants were found to be attenuated in their ability to infect mice lungs in an acute pneumonia model of infection when compared with isogenic wild-type bacteria (205). Thus, the production of PCN appears to confer a growth and/or survival advantage in mixed culture settings (193).

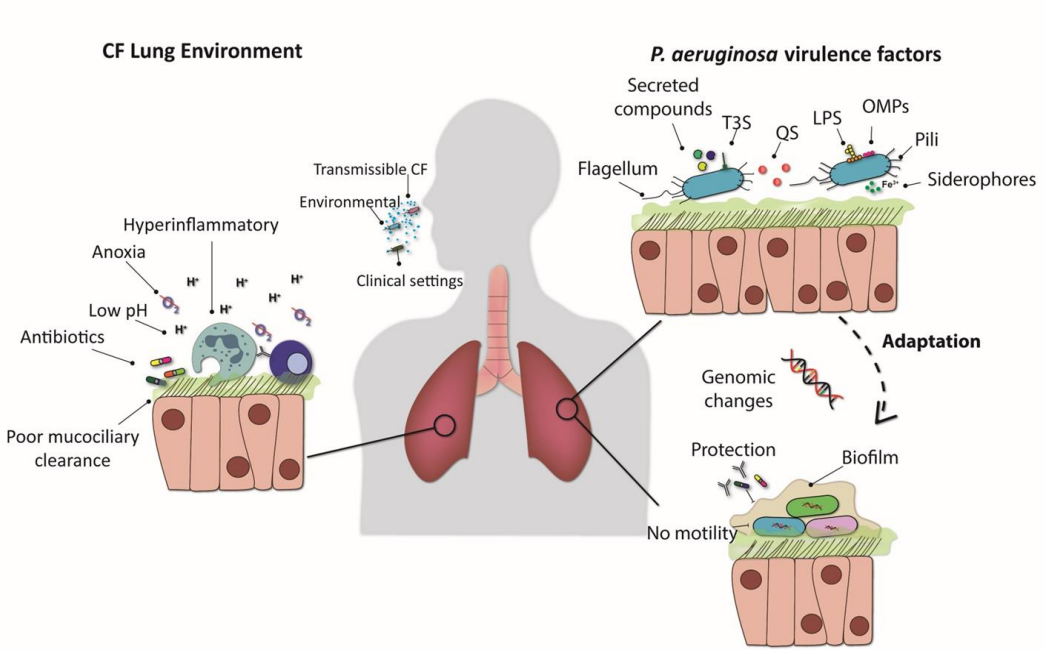

Figure 10. Representation of *P. aeruginosa* adaptation to the cystic fibrosis (CF) lung throughout infection. In the early stages of infection, *P. aeruginosa* isolates are fully equipped with virulence factors (flagellum, secreted proteins, quorum sensing, LPS, pili, among others) that allow respiratory tract colonisation and lung injury. The stressful environment of the CF airway drives *P. aeruginosa* pathoadaptative changes that enable long-term colonisation and establishment of recalcitrant infections. That adaptation is reflected in the genomes of P. aeruginosa CF-adapted strains (6).

Antibiotic resistance (194) and the formation of biofilms (195), among other adaptations, are also associated with CF *P. aeruginosa* strains. Many of these adaptations are observed in *P. aeruginosa* isolates from the airways of older patients with CF. Bacterial biofilms require a substrate to bind to, like that of a wound, or the surface of a foreign medical device, like a urinary catheter. However, in the lungs of individuals with CF, bacteria often self-aggregate, forming clusters of communities suspended within the airway mucus (117). *P. aeruginosa* can express a mucoid phenotype through copious alginate production, which encases bacterial communities, providing a barrier to antimicrobials and host defences (207, 208). Alginate is one of the exopolysaccharides comprising the extracellular matrix of the biofilm and is overexpressed in many, but not all, chronic isolates of *P. aeruginosa* from the airways of patients with CF. Such a phenotypic change is an example of a survival mechanism that can be driven by factors inherent in the CF lung, but also by external factors, including antibiotic administration (209, 210).

6.2 Understanding *P. aeruginosa* adaptation with genome analysis:

The sequencing of sequential and longitudinal isolates from CF patients has provided valuable information on how *P. aeruginosa* manages to evolve and persist in the host by favouring some virulence factors over others. Among the most mutable genes identified in longitudinal isolates from CF patients are those linked to a biofilm-associated lifestyle (*mucA, algU,* and *morA*), decreases in antibiotic susceptibility (*mexZ, nfxB, mexR, gyrA, gyrB,* and *mpl*), reduced virulence factor production (*ykoM* and *mpl*), and different regulatory systems (*rpoN, nfxB, mexR, gacA,* and *gacS*), in different patient lineages despite different clonal backgrounds (32, 211, 212) (Table 2).

The sequencing of bacterial genomes has been a key to demonstrating the evolution of bacterial clones through mutational changes in pre-existing genes, a mechanism also known as pathoadaptive mutation (213, 214) (Table 2). This is especially evident with the sequencing of *P. aeruginosa* genomes from CF patients (215). For example, the sequencing of 474 longitudinal clinical isolates of *P. aeruginosa* from 34 children and young adults with CF, identified 36 *P. aeruginosa* lineages and convergent molecular evolution in 52 genes. The main mutated genes were *asR*, *mexA*, *mexS*, *nex, yecS, algU, gyrA, gyrB, mexB, oprD, pela,* and *rbdA*, which suggested an adaptation towards the acquisition of antibiotic resistance and loss of extracellular virulence factors. But importantly, the study highlighted the importance of clinical collections from chronically infected patients in understanding the convergence and evolutionary contingency of pathogens *in vivo* for the design of future therapeutic strategies (213).

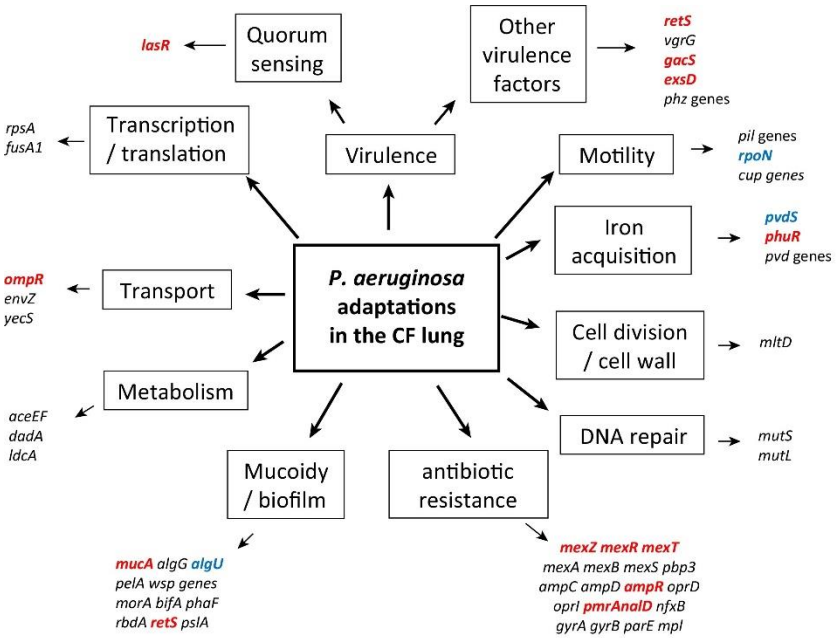

Figure 11. Pathoadaptive Mutations in *Pseudomonas aeruginosa*. Genes encoding regulatory proteins are highlighted in red. Genes encoding sigma factors are highlighted in blue. Image taken from: Winstanley C, O'Brien S, Brockhurst MA. *Pseudomonas aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. Trends Microbiol. 2016 May;24(5):327-337. doi: 10.1016/j.tim.2016.01.008. Epub 2016 Mar 3. PMID: 26946977; PMCID: PMC4854172.

Genomic studies have provided insights into mechanisms of antibiotic resistance. Greipel et al. examined 17 antimicrobial susceptibility and resistance loci in an international strain collection of 361 *P. aeruginosa* isolates from 258 CF patients, identifying 1,112 sequence variants that were not present in the genomes of strains representative of the 20 most common clones in the global *P. aeruginosa* population. A high frequency of variants was observed in *spuE, mexA, gyrA, rpoB, fusA1, mexZ, mexY, oprD, ampD, parR, parS,* and *envZ* (*amgS*), which appear to be involved in the response of *P. aeruginosa* populations to antimicrobial load in CF (Table 2) (216). Another study showed the mutational profile of the resistome of a hypermutator lineage of *P. aeruginosa* by performing longitudinal and cross-sectional analyses of isolates collected from a CF patient over 20 years of chronic infection, demonstrating that mutations in antibiotic resistance genes were positively selected, driven by antibiotic treatment. The infection progressed towards the establishment of a population consisting of genotypically diversified co-existing sub-lineages, all converging towards multi-drug resistance (217) (Table 2). Ahmed et al. combined genomics with *in vitro* studies and demonstrated that the pathways of developing Ciprofloxacin (CIP) resistance are growth

mode-dependent, and they suggested evolved phenotypic and genotypic changes that paralleled the evolution of CIP resistance. Cross-resistance to β-lactam antibiotics was associated with mutations in genes involved in cell wall recycling (*ftsZ, murG*) and could also be explained by mutations in TCA cycle genes (*sdhA*) and genes involved in arginine catabolism (218). Other features such as the expression of the T3SS and QS, were identified by genomics as highly discriminatory in the context of *P. aeruginosa* virulence by the analysis of genome-wide extended multi-locus sequence typing (wgMLST) of four *P. aeruginosa* strains of environmental and clinical origin, compared to the wgMLST of PAO1 and PA14 type strains (219).

The studies mentioned above demonstrated how genomics is essential for the identification of genes that can explain the establishment of MDR *P. aeruginosa* strains; a knowledge that is essential for diagnostic and therapeutic purposes. But importantly, genomic differences observed in different clones within the same CF patient are not always reflected in phenotypic studies. La Rosa et al. analysed 26 clinical isolates of *P. aeruginosa* belonging to three different clone types, exhibiting naïve, intermediate, and adapted phenotypes, sampled from a single CF patient over 8 years of infection. Evolution within the patient involved convergent metabolic specialisation characterised by loss of non-essential metabolic functions, independent of clone type, genomic composition, or mutation pattern (220). Bartell et al. also highlighted the value of classical phenotype-based investigations to complement genomic approaches. Using statistical modelling, they examined eight infection-relevant phenotypes of 443 longitudinal *P. aeruginosa* isolates from 39 young CF patients over 10 years. They identified emergent patterns of bacterial phenotypic changes across the patient cohort that deviate from expected evolutionary trajectories, estimating a period of initial rapid adaptation during which bacteria move from a “naïve” to an “evolved” phenotypic state. They proposed new associations between observed phenotypic phenomena and genetic adaptation. Multi-trait modelling can map complex, patient-specific evolutionary trajectories that will allow an understanding of pathogen persistence and how to prevent it (221)

Overall, genomics has been key for the study of *P. aeruginosa* evolution within the CF environment and the transmissibility of strains between patients. It gives insights into

how *P. aeruginosa* genes regulating virulence factors and AMR are conserved or acquired. But, as demonstrated by La Rosa et al. and Bartell et al. it is essential to combine genomics studies with phenotypic analyses to truly understand functional changes during *P. aeruginosa* adaptation, which is crucial for the development of novel diagnostic and therapeutic strategies against *P. aeruginosa* infections.

Table 2. Examples of *P. aeruginosa* genomic evolution and adaptation studies.

Type of Study	Source of Isolates	Results	Mutated Genes	Function	Ref.
In vivo evolution study using whole genome sequencing	474 longitudinal CF clinical isolates from 34 children and young individuals.	36 lineages with convergent evolution in 52 genes	*asR*, *mexA*, *mexS*, *nex, yecS, algU, gyrA, gyrB, mexB, oprD, pela,* and *rbdA*	Host adaptation, AMR, and loss of extracellular virulence factors	(213, 222)
In vivo evolution study of 17 AMR loci	361, independent CF isolates collected from 30 CF centres.	1112 sequence variants not present in the 20 most common PA clones	*spuE, mexA, gyrA, rpoB, fusA1, mexZ, mexY, oprD, ampD, parR, parS, and envZ (amgS),* and *pagL*	Unrelated. Translation, transport, LPS modification, and AMR	(216)
In vivo longitudinal and evolution analysis	14 isolates from the same clonal lineage of a CF patient (20 years of the infection).	Different evolutionary pathways affecting genes of the same functional categories	*ampC, ftsI*	Codification of β-lactamase and penicillin-binding protein 3 (AMR)	(217)
In vitro biofilm and stationary-phase planktonic culture evolution study	57 CIP-evolved populations and 35 control.	CIP-resistance development depends on the bacterial lifestyle	*ftsZ*, *murG, sdhA*	Cell-wall recycling, TCA cycle, and arginine catabolism	(218)
Real-time *in vivo* evolution, metabolic and genomic study.	26 from a single CF patient (8 years of infection).	Convergence at the phenotypic level but different mutational patterns	Not specified (functional grouping)	Amino acid transport and metabolism, defence, signal transduction and translation	(220)
In vivo genome analysis (wgMLST)	2 environmental, 1 veterinary and a CF clinical isolates with a defective Las QS system	Identification of ten highly discriminatory *loci* between the studied strains and the PAO1 and PA14 strains	*exsA, rsmN,* and *hopJ*	T3SS and QS-regulated virulence traits.	(219)
Screening of 8 infection-relevant phenotypes (*In vivo* evolution)	443 longitudinal isolates from 39 young cystic fibrosis patients over 10 years	Identification of phenotypic changes that deviate from expected evolutionary trajectories	*mexZ, nfxB, nalDmucA, algU, retS/gacAS/rsmA gyrA and gyrB47*	Drug efflux pumps, mucoid regulators, ciprofloxacin resistance	(221)

Abbreviations: AMR, antimicrobial resistance; CF, cystic fibrosis; CIP, ciprofloxacin; QS, quorum sensing; LPS, lipopolysaccharide; PA, *P. aeruginosa*; TCA, tricarboxylic acid cycle; T3SS, Type 3 secretion system; wgMLST, whole genome multilocus sequence typing.

7. Therapies against *P. aeruginosa* infections: antibiotics, phage therapy, small molecules, vaccines.

7.1 Antibiotics

The treatment of *P. aeruginosa* infections is challenging, and adequate initial therapy is crucial to improve survival. Conventional β-lactams with antipseudomonal activity and fluoroquinolones are normally used, but newer antibiotics, such as ceftolozane/tazobactam, ceftazidime-avibactam, imipenem/ relebactam, cefiderocol, finafloxacin and delafloxacin appear to also be effective in the treatment of *P. aeruginosa* (223). MDR-*P. aeruginosa* is defined as the non-susceptibility of *P. aeruginosa* to at least one antibiotic from at least three classes of antibiotics for which *P. aeruginosa* susceptibility is generally expected: penicillin, cephalosporins, fluoroquinolones, aminoglycosides and carbapenems. Also, “difficult-to-treat" resistance (DTR) is defined as *P. aeruginosa* exhibiting-susceptibility to all: piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem-cilastatin, ciprofloxacin and levofloxacin (224).

Ceftolozane-tazobactam and ceftazidime-avibactam, in general, are safe, efficient, and carbapenem options against DTR strains (224). However, resistance against both compounds is emerging, suggesting that the drugs will lose their efficacy soon. Cefiderocol might be an option when more complex mechanisms of resistance interact together, and they could also be stable against most carbapenemases (225). Imipenem-cilastatin-relebactam could be considered a reasonable treatment option against emerging ceftolozane-tazobactam- non-susceptible isolates, but more clinical data is necessary. Combination therapy with old drugs remains an option in case of deep-seated infections when MICs are high (225). In summary, the conventional antipseudomonal antibiotics have been used successfully for a long time, but the emergent *P. aeruginosa* antimicrobial resistance forces the development of newer antimicrobial agents as AMR is causing deaths around the world, especially carbapenem-resistant *P. aeruginosa* strains, which caused 38,100 deaths in 2019 (22). (Figure 12).

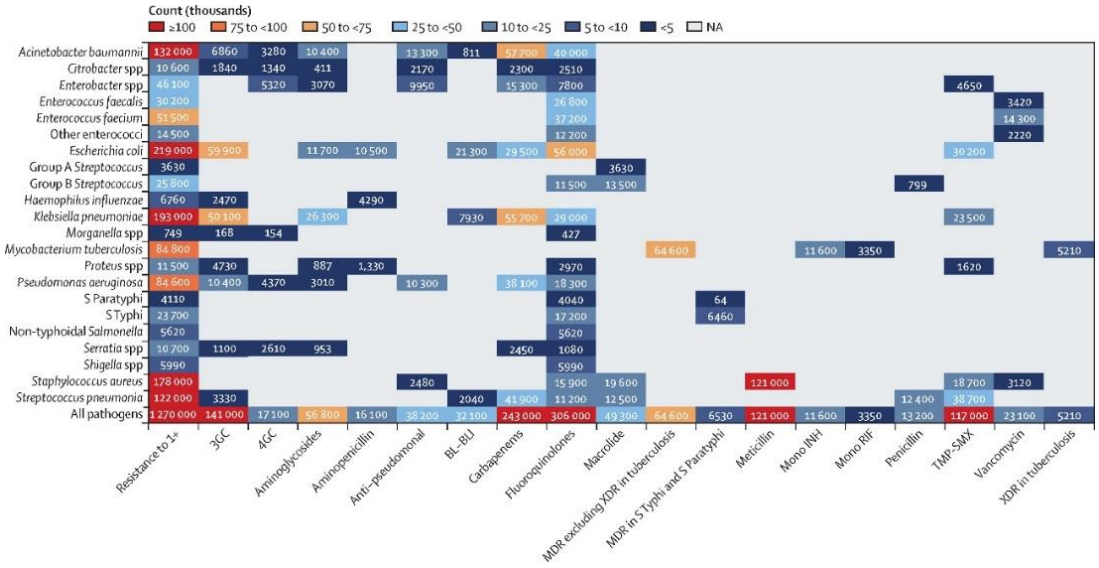

Figure 12. Global deaths (counts) attributable to bacterial antimicrobial resistance by pathogen–drug combination, 2019. 3GC=third generation cephalosporins. 4GC=fourth generation cephalosporins. Anti-pseudomonal=anti-pseudomonal penicillin or beta-lactamase inhibitors. BL-BLI=β-lactam or β-lactamase inhibitors. MDR=multidrug resistance. Mono INH=isoniazid mono-resistance. Mono RIF=rifampicin mono-resistance. NA=not applicable. Resistance to 1+=resistance to one or more drug. S Paratyphi=Salmonella enterica serotype Paratyphi. S Typhi=S enterica serotype Typhi. TMP-SMX=trimethoprim-sulfamethoxazole. XDR=extensive drug resistance Taken from: Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. (2022) (22).

7.2 Phage Therapy

For patients who are not responding to conventional antibiotics, phage therapy is becoming a promising treatment. Bacteriophages target specific bacteria by migrating towards the site of infection and adhering to the cell surface of the targeted bacteria (226). There have been over 700 phages, identified and sequenced, infecting *P. aeruginosa* (227). Waters et al. demonstrated that the phage PELP20 can penetrate and kill bacteria within a biofilm-associated CF lung-like environment. In their novel mice model of *P.* a*eruginosa* LESB65 chronic lung infection, phage therapy with PELP20 was highly effective against an established 6-day lung infection, the treatment completely cleared bacteria from the lungs of 70% of mice, and significantly reduced CFU counts in the other 30%, relative to the controls (228).

Recently, it was demonstrated that the combination of genetically diverse *Pseudomonas* phages, such as SPA01 and SPA05 with jumbo phages like PhiKZ, improved the efficacy of the phage cocktail against *P. aeruginosa* since bacterial isolates resistant

to SPA01 and SPA05 are susceptible to PhiKZ. The number of viable *P. aeruginosa* PA01 when treated with just SPA01-SPA05 was around 4 to 6 -log higher than those treated with the phage cocktail SPA01-SPA05-PhiKZ. However, phage-resistant isolates emerged in both treatments, highlighting a possible limitation of this type of therapy (229). Interestingly, in a murine model, Ashworth et al. observed that 48 h post-infection, *P. aeruginosa* isolates collected from the organs of non-phage-treated mice exhibited greater phage resistance than the input strains. Experimental evolution studies to understand this phenomenon suggested that bacterial adaptation to oxygen availability and the presence of mucin and polyamines, common stressors in the lung environment, contributed to the development of phage resistance. Importantly, the authors used a pan-resistant *P. aeruginosa* infection model and demonstrated that phage steering therapy was an effective strategy against systemic *P. aeruginosa* infection. Treatment with a phage cocktail (PELP20, PNM, PT6, and 14/1) completely cleared bacteria from mice blood, kidneys, and spleen. Furthermore, the remaining bacteria in the lungs and liver were re-sensitized to a wide range of antibiotics, demonstrating that sequential administration of phage therapy and antibiotics could help combat pan-resistant *P. aeruginosa* infections (230). Another study isolated a *P. aeruginosa* phage named Paride, which replicates on deep stationary-phase cultures of laboratory and clinical strains of *P. aeruginosa*. Paride could sterilize deep-stationary phase cultures of *P. aeruginosa* if combined with the β-lactam meropenem via a phage-antibiotic synergy. Paride specifically exploits weak spots in the resilient physiology of dormant bacteria that could be targeted as Achilles' heels by new treatment options (231).

In the last years, one clinical trial (NCT04636554) on personalised phage therapy in patients with COVID-19 and bacterial co-infection (including *P. aeruginosa* bacteraemia/sepsis) is ongoing (reports still not available). There are several limitations to the approval of phage therapies and for now, it is still experimental, as each case needs approval from the US Food and Drug Administration (FDA) in a single-use Investigational New Drug application. Also, it has only been allowed in life-threatening and caused by MDR infections. It is important to consider that phages can multiply, so treatment dosage needs to be carefully considered for any clinical trial, also phage resistance can occur (232)

7.3 Anti- virulence strategies:

The T3SS is crucial for *P. aeruginosa* virulence so it has been proposed as a target for novel drugs. The current drug candidates targeting the T3SS are primarily modified antibodies and screened/designed small molecules: MEDI3902 (BiS4αPa), a bivalent human immunoglobulin IgG1κ monoclonal antibody. MEDI3902 reduced *P. aeruginosa* infection in animal models (233), and phase I clinical trial in healthy subjects confirmed the safety and efficacy of MEDI3902 (NCT02255760) (234), but in phase II studies the results were unsatisfactory (NCT02696902). KB001-A, a recombinant PEGylated Fab also targets T3SS, but it showed ineffectiveness in phase I and II clinical studies despite its safety record (235). Other bacterial surface structures targeted are flagella, pili and LPS. KBPA-101, a human monoclonal antibody against the LPS O-polysaccharide moiety of *P. aeruginosa* serotype IATS O11, reported full protection of mice infected by *P. aeruginosa* at low doses (< 5 μg/mouse), and it was safe in a human phase I trial, but the results from the phase II study are not yet published (236-239). A possible limitation of targeting single pathogen mechanisms is that resistance might quickly appear.

7.4 Vaccines against *P. aeruginosa*

Despite 50 years of research, there is still no vaccine in the market against *P. aeruginosa*. Multiple antigens have been already evaluated with mixed results (Figure 13):

7.4.1 LPS:

Lipopolysaccharide is possibly the most widely characterised vaccine candidate and although highly immunogenic, the first trials also suggested unacceptable levels of cytotoxicity and no eradication of *P. aeruginosa* infection (240-245). Alternatives, such as introducing the complete-core LPS in liposomes, helped to induce strong antibody responses while being non-toxic and non-pyrogenic (246, 247). However, several heterogeneous O- polysaccharide (OPS)-based *P. aeruginosa* serotypes are responsible for life-threatening infections, so monovalent vaccines did not seem to be the best option. A heptavalent version with different OPS serotypes was evaluated but it elicited limited levels of opsonic anti-LPS antibodies (248). Then combinations of LPS with other *P.* aeruginosa components were evaluated to enhance the protective effect of LPS. Aerugen® (Crucell, Leiden, the Netherlands), an octavalent OPS-ETA conjugate vaccine,

underwent phase II evaluation with 59 healthy volunteers. However, the magnitude of the immune response among the various serotypes was quite variable (249). When it was evaluated in 30 young patients with CF, a single dose was sufficient to elicit antibodies to all serotypes, but a drop-off in antibodies was detected. In the first clinical trial for Aerugen®, IgG antibody concentrations increased significantly following vaccination and remained elevated for 12 months. A booster dose administered at 12 months triggered an anamnestic response. However, a significant drop in antibody levels was observed between the second and third years of follow-up, which correlated with an increased infection rate in immunized patients. A retrospective study after 10 years of the first immunisation was used to assessed serological responses, following initial vaccination, annual boosters were given except at year 2. The study suggested that protection could extend beyond 10 years, especially with annual booster doses. The initial immunizations at months 0 and 2 led to a rapid rise in specific serum IgG levels, which declined quickly but were sustained at moderate levels over the 10 year period in immunized, noninfected patients through yearly boosters. (250, 251). It appeared partially protective as after 10 years, was concluded that a reduction in the frequency of *P. aeruginosa* chronic infection (35% vs. 72% in immunised vs. non-immunised patients, respectively) was associated with better preservation of lung function (252-254); however, it failed to show sufficient efficacy in a phase III trial involving 476 CF patients, as no difference between the vaccinated and control groups was achieved (255).

7.4.2: Alginate:

The mucoid exopolysaccharide (MEP) or alginate, has been also widely studied as a vaccine candidate. In trials with healthy volunteers, high molecular polymers of MEP raised IgG titres for up to two years, but the immunogenicity of MEP was moderate, with only 35% of the volunteers producing measurable titres (256). Conjugates of MEP with other antigen candidates have been also studied, and a polymannuronic acid-type-a flagellin conjugate vaccine protected mice against mucoid and non-mucoid strains (257). Similar results were obtained coupling forms of MEP with diphtheria toxoid (258), OMVs of *Neisseria meningitidis* serogroup B (259) or PLGA (polymer poly-lactic-co-glycolic acid) nanoparticles (260). No clinical trials are currently being carried out with alginate as a vaccine antigen for *P. aeruginosa* vaccines, however, a study in 2021 demonstrated that

solid lipid nanoparticles (SLN) containing alginate might be suitable as a nano vaccine against *P. aeruginosa*, as mice immunised intraperitoneally with alginate-SLN had lower CFU counts in the spleens (0.8 log less) than mice immunised with just alginate (261).

7.4.3. Flagellum and pili

The use of flagellum as an antigen showed high protection in pre-clinical studies with a mouse burn model (262, 263). Monovalent flagellar preparations induced long-lasting antibody titres on all 220 healthy volunteers, in a phase I clinical trial (264). Similar results were observed with a phase II study of a monovalent type-b flagellin vaccine (265). Finally, a randomised, double-blind, placebo-controlled, multicentre phase III clinical trial (17585011) with 483 European CF patients on a bivalent vaccine containing three type-a subtypes and type-b serotype failed to achieve the primary outcome of 66% protection, reporting 34% of protection against acute infection and 51% in chronic infection (266). Major pilin (PilA)-based approaches have also been assessed. Mice intratracheally immunised with pilin protein showed a significant improvement in survival (80–100%) (267). Importantly, survival rates were lower (23.5–47%) when mice were challenged with clinical strains, compared to the laboratory strain (PAO1). A bivalent vaccine composed of type-b FliC and PilA also proved to be protective (87% survival) in a murine model (268). Later a trivalent vaccine, type-b FliC, PilA and type-a flagellin showed higher survival rates (92–100%) (269). No other clinical trials are currently being carried out with flagellin as a vaccine antigen for *P. aeruginosa*.

7.4.4. Outer membrane proteins

Regarding outer membrane proteins, OprF and lipoprotein I (OprI) are the most studied as vaccine antigens. OprF-OprI hybrid antigen was initially developed by Intercell AG (Vienna, Austria) and named IC43 (recently renamed as VLA43) and successfully evaluated in a phase I, randomised, placebo-controlled, blinded trial with healthy adult volunteers; and in a phase II study in mechanically ventilated ICU patients (270) (NCT00778388, NCT01563263,). However, although the differences were not statistically significant, a higher infection rate was observed in the treatment group (11.2–14%) than in the placebo group (6.1%), suggesting that IC43 vaccine may affect *P. aeruginosa* virulence rather than clearance (270). A confirmatory, randomised, multicentre, placebo-controlled, double-blind phase II-III with 799 ICU patients, showed

that IC43 had no clinical benefit over the placebo group in terms of mortality. This was mainly due to the intrinsic heterogeneity of ICU patients, the introduction of patients from different European countries, and *P. aeruginosa* infection before the development of effective IgG immune response (270). The mucosal formulation of the OprF-OprI hybrid protein vaccine was examined when loaded into mannose-modified chitosan microspheres, it showed promise as a delivery system, since nasal immunisation of mice demonstrated 75% protective efficacy and a strong specific humoral response. Influenza virus (271), cowpea mosaic virus (272, 273), tobacco mosaic virus (274) and adenovirus (275-278) have all been used as vectors.

Another OMP evaluated was OprL which was identified as an early immunogenic protein in CF patients (279) and elicited strong IL-17 secretion in a murine pneumonia model(144). Vaccination with 50 µg of OprL plus curdland conferred Th17-dependent and serotype-independent protection in mice (survival rate of the rOprL plus curdlan group was 60% at the end of the observation period, significantly higher than PBS ($p= 0.0014$) and Curdlan ($p=0.0004$) groups (280, 281). Finally, OprH was also assessed. A screen of two LPS-heterologous *P. aeruginosa* strains in a murine acute pneumonia model showed that OprH was the most highly expressed OMP, but immunisation of mice with OprH using curdlan as adjuvant rendered no significant protective efficacy, nor did co-administration of iron acquisition proteins or a prepared trivalent mixture (282). In contrast, vaccination with OprH refolded in micelles elicited specific opsonic antibodies and conferred protection against the two strains used (282), highlighting the importance of the final vaccine formulation in eliciting a protective response. Mice immunised intranasally with 70 µg of OprH plus curdlan, resulted in in 40% survival after lung challenge with PA14 (serogroup O19), compared to 10% in the control group. Recently, Bianconi et al. identified various outer membrane and periplasmic proteins, with both known and unknown functions, that significantly increase the survival rate among challenged mice when given in combination. Three of those OMPs, MotY, PA5340 and PA3526, gave the maximum protection (50% survival) (283).

7.4.5. T3SS:

PcrV was the first protein of the T3SS translocation apparatus used as a vaccine antigen (284, 285). Immunisation with Mab166 epitope (PcrV144-257) provided

comparable survival rates with immunisation with whole PcrV (65%), with a significant increase in IgG1 and IgG2 titres (286). PopB, also from the translocation apparatus of the T3SS, elicited Th17 responses and mice protection with recombinant PopB and its chaperone, PcrH, encapsulated in PLGA nanoparticles (141, 144).

7.4.6. Iron uptake systems and QS:

Iron uptake system proteins, including FpvA, FoxA, and HasR were highly expressed during infection in a murine acute pneumonia model (282). However, they did not show protective efficacy when mice were immunised intranasally either separately or in combination (282). In addition, vaccines involving components of the QS system seem to have limited potential as a vaccine candidate (287).

7.4.7. DNA vaccines:

DNA vaccines have also been assessed. Gong et al. compared four DNA vaccines with *oprL* and *oprF* genes as monovalent vaccines, a fusion OprL-OprF vaccine or a divalent combination (OprL and OprF). They all induced antibodies, IL-2 and IFN-γ, and protected chickens against *P. aeruginosa* challenge (80% survival)(288). Also, a plasmid encoding PilA incorporated in a multivalent DNA vaccine that also contained other plasmids encoding a fusion OprF-OprI fusion and PcrV protein showed 100% survival in mice (288).

7.4.8 mRNA vaccines:

Recently mRNA vaccines have also been tested against *P. aeruginosa*. mRNA-PcrV induced significantly stronger antigen-specific humoral and cellular immune responses and a higher survival rate compared with the OprF-I after the challenge. The survival rate of PcrV-immunized groups (5 μg and 25 μg) was as high as 100%, compared to 75% survival rate by immunisation with 25 μg of OprF-I and 5% with 5 μg of OprF-I in a burned infection model. All mice in the control group died. The results suggested that mRNA-PcrV as well as the mixture of mRNA-PcrV and mRNA-OprF-I are promising vaccine candidates for the prevention of *P. aeruginosa* infection (289)

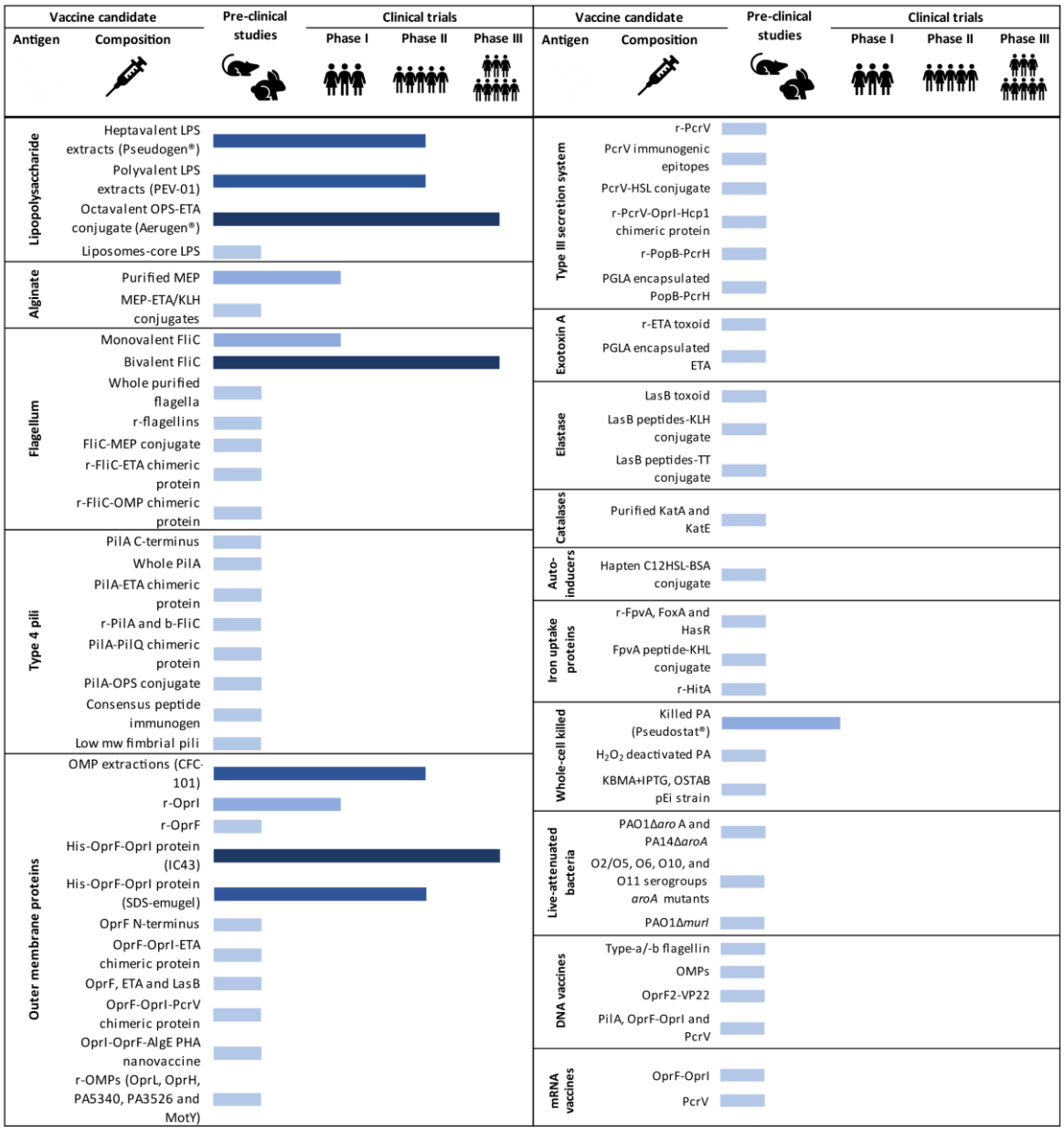

Figure 13. Progress in the development of a vaccine against *P. aeruginosa* infections. The darker the colour of the bar, the further the vaccine candidate proposed in preclinical and clinical studies. Adapted from Sainz-Mejías et al. (149).

In summary, the high adaptability of *P. aeruginosa* has hampered the development of antimicrobial therapies, thus *P. aeruginosa* infections remain a major health threat. Due to its antimicrobial resistance, vaccination is one of the most

promising strategies to tackle *P. aeruginosa*. Prophylactically, vaccines can decrease the number of infectious disease cases, thus reducing antibiotic use and the spread of AMR. In contrast, antibiotics and other therapies explained above, which are commonly administered therapeutically, act on established infections, increasing the probability that resistant clones emerge. However, despite multiple efforts in the search for a vaccine against *P. aeruginosa*, no vaccine has been licensed, which is clear evidence of the great challenge involved and the need for further progress in understanding the complex *P. aeruginosa*-host relationship. The emergence of multi-resistant strains of antibiotics and the susceptibility of vulnerable groups to *P. aeruginosa* infections motivate the search for effective systems for the generation of a vaccine against this challenging pathogen.

8. Project rationale, aim, and objectives.

As mentioned above, *P. aeruginosa* is a critical pathogen due to its great capacity to adapt to the host. Worldwide, *P. aeruginosa* strains resistant to multiple antibiotics are emerging, with resistance to carbapenems being of particular concern and the cause of a high number of deaths globally. Therefore, the development of novel therapies against *P. aeruginosa* is urgent. Normally, the study of new antimicrobial therapies focuses mainly on the study of the pathogen in searching for new therapeutic targets. This traditional approach has not been successful against *P. aeruginosa*, as with such a challenging pathogen the search for host-relevant therapeutic options must be approached from different perspectives.

The understanding of host-pathogen interactions is indispensable to have a global view of *P. aeruginosa* infection, and how both pathogen and host suffer changes during infection that complicate the treatment of its infections. The understanding of host-pathogen interactions opens the possibility of multiple avenues towards new, unconventional treatments against *P. aeruginosa* through a rational design of therapies that will consider not just the pathogen, but also the host and the interplay of host-pathogen. This project therefore aims to explore *P.* aeruginosa-human cell interaction through different methods and apply that knowledge in the development of novel approaches for the treatment of *P. aeruginosa* infections.

Objectives:

1. Evaluation of host immune responses against *P. aeruginosa* CF sequential isolates *in vitro*, for a better understanding of *P. aeruginosa* adaptation and versatility within the host.

2. Identification of the host receptors with which *P. aeruginosa* interacts during infections for a better understanding of *P. aeruginosa's* interaction with the human cell.

3. Evaluation of novel TLR4 ligands as adjuvants for vaccines against *P. aeruginosa* in an acute *P. aeruginosa* pneumonia mouse model.

Chapter 2

P. aeruginosa versatility and adaptability into the human host

Introduction

1. CF sequential isolates for the study of *P. aeruginosa* within-host adaptation

Long-term chronic lung infections in CF patients are characterised by adaptation and phenotypic diversification (212). As *P. aeruginosa* is the leading cause of chronic infection in CF, the sequencing of sequential and longitudinal isolates from CF patients has provided valuable information on how *P. aeruginosa* manages to evolve and persist in the host. As described in the section "8. Cystic Fibrosis as a model for the study of *P. aeruginosa* adaptation within the host", genomics facilitated the elucidation of the adaptive mechanisms of *P. aeruginosa*, but its integration with phenotyping studies is needed to support a better interpretation of the evolutionary dynamics of the pathogen within the host (290). Understanding this within-host pathogen evolution and diversification during *P. aeruginosa* chronic infections is crucial for the design of novel therapeutic intervention strategies.

A hallmark in *P. aeruginosa* research is the use of common laboratory strains which leads to conclusions that are not relevant to the clinical setting, due to the high diversity and adaptability of *P. aeruginosa* (291). Hence, a well-established international panel of *P. aeruginosa* strains was developed by members of our research group and others to avoid the use of isolates with limited availability and to potentially prevent unnecessary repetition across laboratories. The availability of a standardised reference panel can improve efficiency and coordinate the collective efforts of the *P. aeruginosa* research community, facilitating the search for improved therapeutic approaches (292). The *P. aeruginosa* panel was genomically (293) and phenotypically (291) characterised. The panel included three series of sequential *P. aeruginosa* isolates from CF patients, "early" and "late" in an infection. In this project, two of those sets of sequential isolates from Europe and North America (Figure 14, Appendix A) were selected. Those isolates represent approximately 7 years of colonisation between the first bacterial isolate (early) and the two subsequent late isolates per patient: **Patient 1:** AA2 (early, 0.6-year-old), AA43 (late mucoid, 7.5 years old), AA44 (late non-mucoid, 7.5 years old); **Patient 2:** AMT0060-3 (early isolate, 7 years old), AMT0060-2 (late isolate, 14.5 years old) and AMT0060-1(late isolate 14.5 years old) (Figure 14) (292, 294-296).

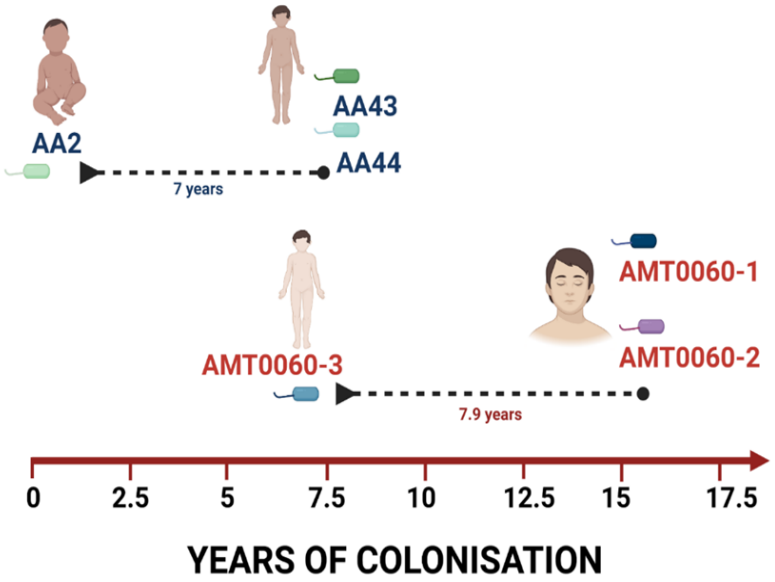

Figure 14. *P. aeruginosa* CF sequential isolates from an International Reference Panel. Two panels of *P. aeruginosa* sequential isolates from a strain collection (292) were characterised for genotypic and phenotypic traits (291, 293). The years of lung colonisation at the moment of their isolation from the patient are shown by the red arrow.

2. Phenotypic traits of CF sequential isolates from the International *P. aeruginosa* Reference Panel:

The CF sequential isolates were characterised by virulence in *Galleria mellonella*, motility, production of alginate, pyocyanin, LPS, biofilm formation, quorum sensing (AHL production), antibiotic resistance and phage susceptibility (291) (Table 3). Only reduced virulence over the time of colonisation was related to late isolates. The result was consistent with previous studies about *P. aeruginosa's* adaptation to the CF lung environment (296, 297). Regarding motility, the early isolate AMT0060-3 showed more swarming, swimming and twitching motilities relative to the late isolate, AMT0060-2. However, the other series of isolates showed a similar motility between early and late isolates. AHL levels did not correlate with time of colonisation in sequential CF strains, for example, early strain AA2 and the late strain AA43 both expressed low levels of AHL, while the signal from another late isolate (AA44) was much higher. AA44 expressing higher levels of AHL also produced strong biofilms. AMT0060-1, AMT0060-2 and AA44 produced stronger biofilms relative to their respective early isolate, while AA43, also a late isolate, showed less biofilm formation than AA2. Pyocyanin production also differed between the two sets of isolates. While AA2 produced more pyocyanin than A443 and

AA44, AMT0060-1 produced similar amounts of pyocyanin than the early isolate AMT0060-3. The LPS of the sequential strains AA2 (early isolate), AA43 and AA44 (late isolates) were complete and showed an identical O-antigen repeating unit in all three strains. Importantly, it was possible to distinguish the presence of a B-band in AA2 and AMT0060-3 isolates but not in the late isolates, except AA43. The early CF strain, AA2, showed lower susceptibility to phage infection compared to late strains (AA43, AA44). In contrast, AMT0060-3 showed higher susceptibility than the late isolate. Finally, the mucoid strains AMT0060-2 showed increased alginate production, relative to the other isolates from that series.

Table 3. Comparison of phenotypic traits between early and late isolates of *P. aeruginosa* CF sequential isolates from an International Reference Panel (291).

	Patient 1		**Patient 2**	
Early Isolate	**AA2**		**AMT0060-3**	
Late CF Isolates	**AA43**	**AA44**	**AMT0060-2**	**AMT0060-1**
Virulence in *Galleria mellonella*	↓	↓	↓	↓
Motility	=	=	↓	=
AHL levels	=	↑	↓	↓
Biofilms	↓	↑	↑	↑
Pyocyanin levels	↓	↓	↓	=
LPS	**B band**	**No B band**	**No B band**	**No B band**
Susceptibility to phage infection	↑	↑	↓	↓

3. Genomic analysis of CF sequential isolates from the International *P. aeruginosa* Reference Panel:

Previous core genome single nucleotide variant (SNV) phylogeny analysis of the isolates AMT0060-1,2,3 confirmed they are sequential isolates (293) (Figure 15). However, the genomic data of strains AA43 and AA44 were not sequenced at the moment of the study and were not collected as requested by the original suppliers of these isolates. The core genome single nucleotide variant (SNV) phylogeny analysis of the panel strains alongside genome sequence data from strains publicly available on NCBI, distributed the strains from the panel into two main groups (group 1 and group 2). All the selected isolates are distributed in group 1, the AMT0060 series is closer to the

reference strain *P. aeruginosa* LESB58 while AA2 is closer to the reference strain *P. aeruginosa* PAK.

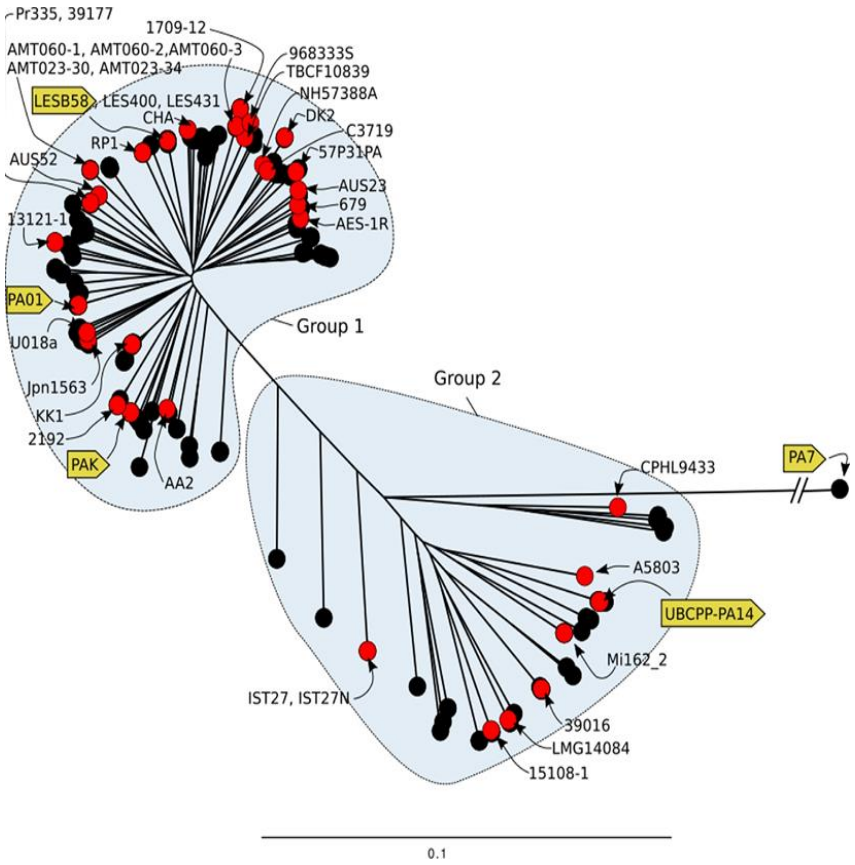

Figure 15. Core genome phylogeny showing AMT0060-1,2,3 as sequential CF isolates. Red dots identify panel strains, while black dots identify strains from NCBI. Commonly studied reference strains are identified by yellow boxes. The two main groups that define the population structure of *P. aeruginosa* are highlighted in light blue. Taken from: Luca Freschi et al. (291-293).

The genomes of the sequential CF isolates AA2, AA43 and AA44 all contain the same frameshift mutation in *mexA*. Another commonly reported CF adaptation is the occurrence of mucoid colonies, usually due to *mucA* mutations leading to overproduction of alginate. AMT0060-2 and AMT0060-1 have a frameshift in *mucA* (293). The GacA/GacS two-component regulatory system has been implicated in the switch between acute and chronic infection lifestyles and plays a key role in virulence. AMT0060-2 has a frameshift in *gacA* (293) (Table 4).

Table 4. Genomic data available for the *P. aeruginosa* isolates included in the study.

Strain	Genome size (bp)	Mutations
AA2	6258	*mexA* mutant (1 bp frameshift)
AMT0060-3	7036	Not reported
AMT0060-2	7037	several mutants (1 bp frameshift) and 2 bp frameshift (*gacA*)
AMT0060-1	7033	*mucA* mutant (1 bp frameshift)

Abbreviations, bp: base pairs.

4. Epithelial cell lines for the study of *P. aeruginosa* host-pathogen interactions:

The human cystic fibrosis bronchial epithelial cell line (CFBE41o$^-$) has been widely used in CF studies. It is an immortalised cell line created from the bronchial epithelium of a CF patient homozygous for $F508_{del}$ (298). CFBE41o$^-$ cells have been used to study CFTR function and the response to small molecules due to their clinical relevance to CF and their ability to polarise and form tight junctions (299, 300). In contrast to CFBE41o$^-$ cells, the 16HBE14o- human cells express high levels of CFTR, mRNA and protein (301). 16HBE14o$^-$ is a human bronchial epithelial cell line isolated from a 1-year-old male heart-lung patient and immortalised with the origin-of-replication defective SV40 plasmid (pSVori). The cell line conserves the traits of normal differentiated bronchial epithelial cells including a cobblestone morphology, cytokeratin expression, the ability to form tight junctions, and directional ion transport (301).

Aims and Objectives:

CF sequential isolates from a *P. aeruginosa* International Reference Panel have been phenotypically and genomically characterised. Overall, there is no specific trend in the traits of "early" and "late" isolates, which suggests a divergent evolution of the isolates within the host. Hence, we wondered whether these phenotypic differences between the sequential *P. aeruginosa* isolates could also be observed in the host immune responses against those isolates, *in vitro,* or if the immune responses are dependent on the "early" or "late" status of the isolates. It is still unclear how CF-adapted *P. aeruginosa* strains shape the host response to favour their persistence and this is difficult to address in humans or animal models due to confounding factors (microbiota, genetic variability, among others). Hence, this chapter aimed to understand the *in vitro* immune responses against *P. aeruginosa* CF sequential isolates to determine how natural selection of some *P. aeruginosa* strains and host immunity interact to move toward a state of immune evasion or an inflammatory state; this knowledge will be essential to lead the development of drugs against chronic *P. aeruginosa* infections, especially treatments for CF disease.

Objectives:

1. To evaluate the attachment to 16HBE14o$^-$ and CFBE41o$^-$ human epithelial cells of two sets of CF sequential isolates from a *P. aeruginosa* international panel reference.

2. To evaluate the production of proinflammatory cytokines, IL-6 and IL-8, from human epithelial cells when stimulated with the different CF sequential *P. aeruginosa* strains from an international panel reference.

Methods

Cell culture and media: 16HBE14o$^-$ and CFBE41o$^-$ lung epithelial cells were generous gifts from Dieter Gruenert (UCSF). The human bronchial epithelial cell line 16HBE14o$^-$ was routinely grown in Minimal Essential Medium (MEM, M2279-500) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% l-glutamine, and 1% sodium pyruvate and incubated in a 5% CO_2 environment at 37°C.

Bacterial strains and culture: *P. aeruginosa* strains were purchased according to their LMG code (Appendix A) from the Belgian Co-ordinated Collection of Micro-organisms (BCCM)/LMG Bacteria Collection, Ghent University, Gent, Belgium (http://bccm.belspo.be/about-us/bccm-lmg). Strains were routinely grown on Luria–Bertani agar (LB; Sigma-Aldrich® L3147) overnight (16–18 h) at 37 °C. Overnight broth cultures were prepared by inoculating 5 mL LB broth (LB; Sigma-Aldrich® L3022) with fresh growth from a pure streak plate. Cultures were grown for 16–18 h at 37 °C, shaking at 200 rpm.

Growth curves: Bacterial growth curves were performed in LB media, 37 °C and 200 rpm. Overnight cultures of *P. aeruginosa* strains (5 mL) were diluted in LB to an OD_{600}= 0.1. For the evaluation of OD_{600} Vs time, 5 µl of the diluted culture was used to inoculate 96 microtiter wells containing 195 µl of LB broth and the automatic measures were taken in a microplate reader (Thermo Fisher Scientific, Hemel Hempstead, UK). Duplicates were inoculated for each strain tested. For CFU Vs. OD_{600} the same procedure was performed but the cultures were diluted in 100 mL of LB and measures of 100 µL were taken manually each 30 min to perform serial dilutions in phosphate-buffered saline (PBS). The dilutions were plated in LB agar plates, the plates were incubated overnight at 37ºC and CFU counting was performed the next day to determine the CFU/mL (Appendix A). This was calculated from the mean CFU duplicate plates, using the following formula: (Average CFU * dilution factor)/ mL plated. The standard growth curves were obtained by plotting the absorbance values (OD_{600}) versus the CFU/mL, and the equations and R^2 values were obtained. This information was used in subsequent experiments to calculate bacterial inoculum.

Attachment of *P. aeruginosa* strains to human epithelial cells: Host cell attachment was determined according to previously published methods (302). Human 16HBE14o$^-$ or CFBE41o$^-$ epithelial cells were seeded at a concentration of 4×10^5 cells/well overnight in Cellstar® 24-well plates (Greiner Bio-one, Austria) in fresh media. Bacteria were grown to the mid-logarithmic phase and diluted according to the equation of the growth curve (Appendix A) to get a multiplicity of infection (MOI): 5. The cells were washed with 1 ml of warm media before the addition of bacteria in a final volume of 500 µl in duplicate. The 24-well plates were centrifuged at 700× *g* for 5 min to promote adhesion and incubated for 30 min at 37 °C. The wells were washed three times with warm sterile PBS before the incubation with 500 µL lysis buffer (125µL Triton X-100 in 50 mL PBS) for 20 min at room temperature. The cells were removed by scraping with a pipette tip, serially diluted, plated onto LB agar and incubated overnight at 37 °C, after which colonies were counted.

The percentage of bacterial attachment was determined by calculating the average colony-forming units (CFU) per milliliter (CFU/mL) in each well after incubation. Briefly, the CFU/mL in each well was calculated using the formula: CFU/mL = (Average CFU × Dilution Factor) / Volume (mL). The average CFU/mL from the experimental wells was then compared to the initial CFU/mL of the inoculated bacterial suspension (control). The percentage of bacterial attachment was calculated using the following formula: % of Control (Bacterial Attachment) = (Experimental CFU/mL / Initial CFU/mL) × 100.

Stimulation of epithelial cells with sequential *P. aeruginosa* CF isolates: Human 16HBE14o$^-$ or CFBE41o$^-$ epithelial cells were seeded at a concentration of 4×10^5 cells/well overnight in 24-well plates, media was changed 24 h later for a second 24 h incubation of the cells in FBS-antibiotic- free media. Then the cells were stimulated with the six strains ($OD_{600} = 0.6$) at a MOI:5 for 2 h (non-cytotoxic conditions, Appendix A). After 2 h incubation, the media was changed to fresh media containing 200 µl/mL of polymyxin B and incubated for 24h to allow cytokines production. Growth media were collected at the end of the incubation, centrifuged and stored at -80°C for analysis of IL-6 and IL-8, by ELISA.

ELISA for IL-6 and IL-8 analysis of the collected supernatants: Human BioLegend's ELISA Max™ kit was used for the quantification of IL-8 and IL-6 in the collected supernatants of previously stimulated human cells.

Statistical Analysis: Statistical analysis was performed by One-Way Analysis of Variance (ANOVA) using Prism software with the Kruskal-Wallis test when the data did not pass the Shapiro-Wilk normality test. The graphs and heat maps were done using GraphPad Prism 8.0.2.

Results

1.Evaluation of growth kinetics of the CF *P. aeruginosa* sequential isolates

To ensure consistent results, the growth dynamics of each isolate were evaluated (Appendix A, Figure 1A). As changes in growth dynamics could be an adaptation of *P. aeruginosa* to the CF environment, the growth of both early isolates (AA2 and AMT0060-3) against their respective late isolates were compared (Figure 16). Interestingly, differences between isolates from patient 1 and patient 2 were observed. While the growth curves for late isolates AA43 and AA44 were comparable to their respective early isolate (AA2), a slower growth of both late isolates from patient 2 (AMT0060-1 and AMT0060-2), relative to the early isolate (AMT0060-3) was observed. AMT0060-1 and AMT0060-2 isolates may be more adapted to the CF environment than AA43 or AA44 isolates, which showed a growth similar to the early isolate AA2.

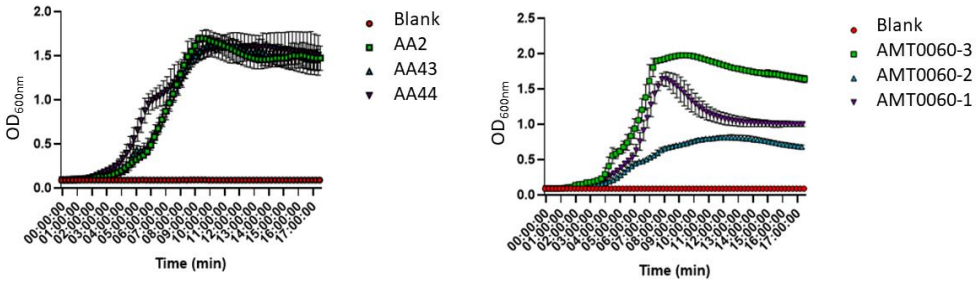

Figure 16. Growth kinetics of *P. aeruginosa* sequential isolates from two different CF patients. The growth of each set of isolates is presented separately (**patient 1**, AA2, AA43 and AA44; **patient 2**, AMT0060-1, AMT0060-2 and AMT0060-3). Bacterial growth curves were performed in LB media at 37 °C and 200 rpm. The "y" axis shows the OD_{600} each 30 min ("X" axis), starting with an OD_{600}: 0.1, approximately. Duplicates were inoculated for each strain tested. "Blank" corresponds to wells containing LB media used to dilute the isolates to guarantee sterile growth was achieved. Early isolates are represented with green squares and late isolates with either blue or purple triangles.

Antibiotic resistance is a characteristic of CF-adapted strains and a consequence of slow growth. Hence, the growth of the isolates in 200 µg/mL of amikacin, gentamicin or polymyxin B was assessed. All the isolates were resistant to amikacin and sensitive to polymyxin B at the concentration tested. But, importantly, AMT0060-2 slowed growth in LB without any antibiotic (Figure 16) and was also the only isolate resistant to gentamicin (Figure 17) suggesting that antimicrobial resistance might be due to the observed slower growth.

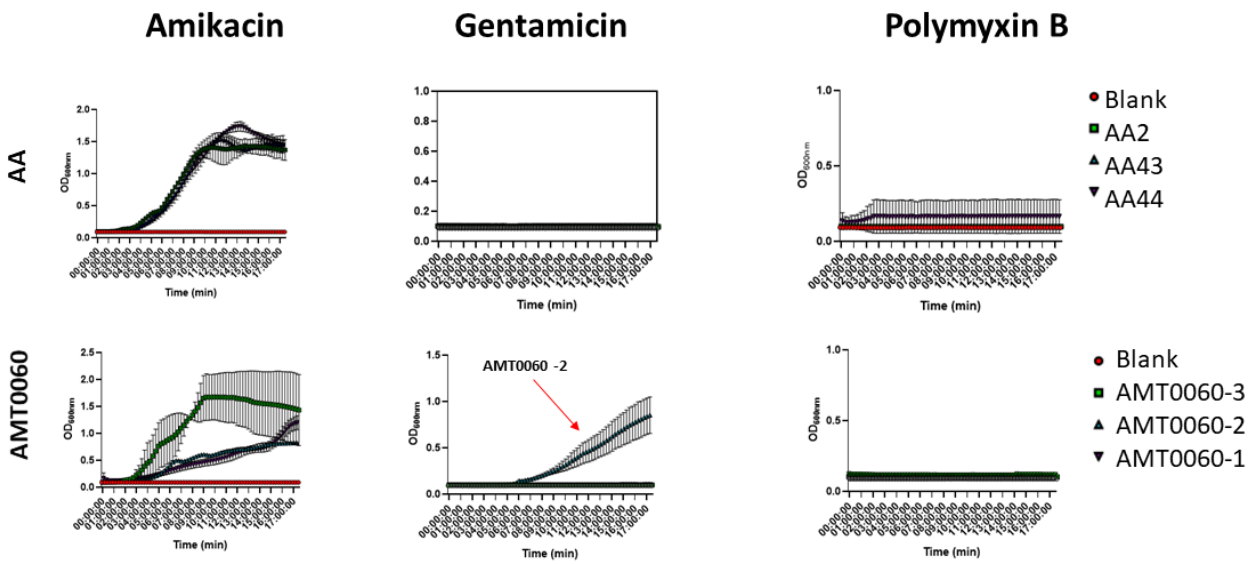

Figure 17. Growth kinetics of *P. aeruginosa* sequential isolates from two different CF patients. The growth of each set of isolates with three different antibiotics was determined by repeating the same procedure as in media without antibiotics (Figure 16). The growth was tested in 200µg/mL of amikacin, 200µg/mL of gentamicin or 200µg/mL of polymyxin B. All strains were resistant to amikacin and sensitive to polymyxin B. The only strain resistant against gentamicin was AMT0060-2 (highlighted with a red arrow).

2. Attachment of *P. aeruginosa* CF isolates to human epithelial cells

Host-cell attachment is the first stage of host-pathogen interaction during infection and an essential step for bacteria to achieve host colonisation. Hence, the ability of *P. aeruginosa* CF sequential isolates to attach to 16HBE14o$^-$ and CFBE41o$^-$ cells was assessed to examine differences between early and late isolates, using an MOI: 5, and determining the number of bacteria by CFU counting (Figure 18). An increase in the attachment to CFBE41o$^-$ ($p= 0.0482$) and to 16HBE14o$^-$ ($p= 0.0005$) was observed only between the late isolate AMT0060-1 and the other two isolates of the same set,

AMT0060-3 (early) and AMT0060-2 (late). But not in the other series (Figure 18). This could suggest an adaptation of AMT0060-1 to the host. Therefore, it was concluded that the attachment of *P. aeruginosa* to 16HBE14o$^-$ and CFBE41o$^-$ cells appears to be isolate-dependent and independent of the infection stage (early or late). It is noteworthy, that the attachment of all the isolates to CFBE41o$^-$ cells was higher than to 16HBE14o$^-$ cells (Figure 18).

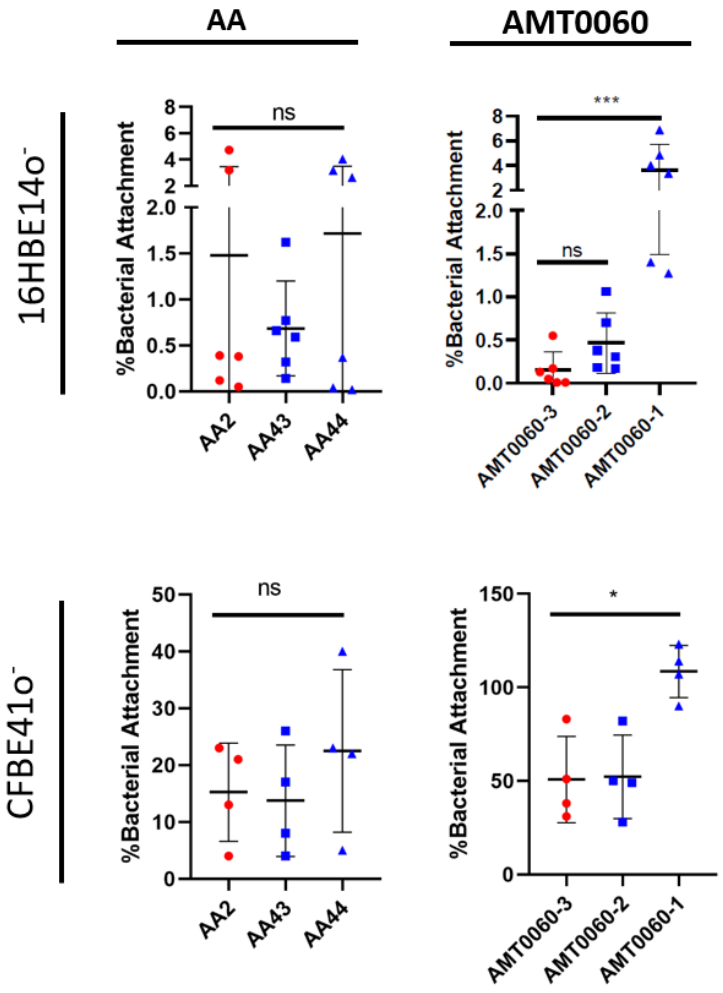

Figure 18. Attachment of *P. aeruginosa* CF sequential isolates to human bronchial epithelial cells (16HBE14o-) and CF bronchial epithelial (CFBE41o-) cells. The graph shows at least two biological replicates for each isolate. All the early isolates are represented with red dots and late isolates with blue squares or triangles. AMT0060-1 isolate was the only one with a significantly higher attachment relative to its respective early isolate (Kruskal-Wallis test p= 0.0482 (CFBE41o-) and p= 0.0005 (16HBE14o-)). All isolates had a higher attachment to CFBE41o- than to 16HBE14o-. The percentage of bacterial attachment was calculated by determining the average colony-forming units (CFU) per milliliter (CFU/mL) in each well: (Average CFU × Dilution Factor) / Volume (mL). This was compared to the initial CFU/mL of the inoculum (control), and the percentage of bacterial attachment was calculated as: (Experimental CFU/mL / Initial CFU/mL) × 100.

3. Stimulation of pro-inflammatory cytokines by *P. aeruginosa* CF isolates.

To evaluate if the immune responses of epithelial cells to *P. aeruginosa* were different between early and late isolates, proinflammatory cytokines IL-6 and IL-8 were measured in response to *P. aeruginosa* exposure (Figure 19). The late isolates from patient 1 (AA2, AA43 and AA44) stimulated lower levels of IL-6 ($p=0.0022$(AA44)) and IL-8 ($p= 0.0270$ (AA43); $p<0.0001$ (AA44)) in 16HBE14o$^-$ cells relative to the early isolate AA2, except AA43, which showed no significant difference in the stimulation of IL-6 relative to the early isolate.

Interestingly, in CFBE41o$^-$ cells the AA43 isolate also showed a lower stimulation of IL-6 ($p=0.027$) and IL-8 ($p=0.0235$) relative to the early isolate. However, AA44 showed higher stimulation of the pro-inflammatory cytokine IL-8 ($p=0.0034$) by CFBE41o$^-$ cells and no significant difference in the stimulation of IL-6, relative to the early isolate AA2. The second set of CF sequential isolates (patient 2, AAMT0060-1,2,3) did not show differences in the stimulation of IL-6 or IL-8 by 16HBE14o$^-$ cells but interestingly in CFBE41o$^-$ cells they showed a similar trend than isolates from patient 1; AMT0060-2 isolate showed a similar stimulation of IL-6 relative to the early isolate AMT0060-3 and highest stimulation of IL-8 ($p<0.0001$); while AMT0060-1 showed lowest stimulation of IL-6 ($p<0.0001$)) and the same low stimulation of IL-8, relative to the early isolate AMT0060-1 (Figures 19 and 20).

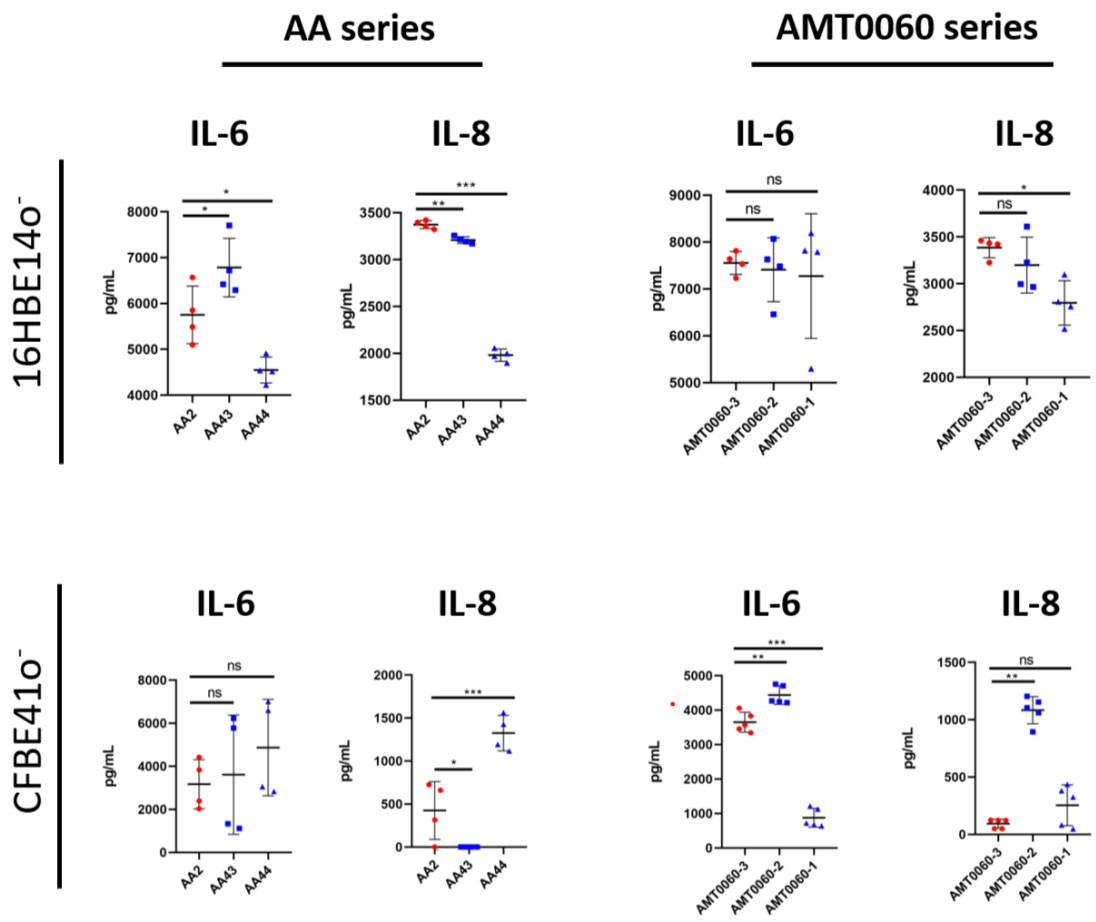

Figure 19. IL-6 and IL-8 production by human epithelial cells after stimulation with *P. aeruginosa* CF sequential isolates. 16HBE14o$^-$ cells and CFBE41o$^-$ were stimulated with *P. aeruginosa* isolates for 2 hours at a MOI: 5 (non-cytotoxic conditions) and IL-6 and IL-8 were measured by ELISA. The graphs show at least two biological replicates for each isolate. All the early isolates are represented with red dots and late isolates with blue squares or triangles.

To compare the levels of IL-6 and IL-8 in response to *P. aeruginosa* exposure, a heatmap summarising the data was created. The intensity of the red indicates stronger levels of the respective cytokine stimulation. Overall, similar to what was observed during the attachment assays, the stimulation of proinflammatory responses in 16HBE14o$^-$ cells and CFBE41o$^-$ was isolate-dependent and independent of the infection stage (early or late) (Figure 20).

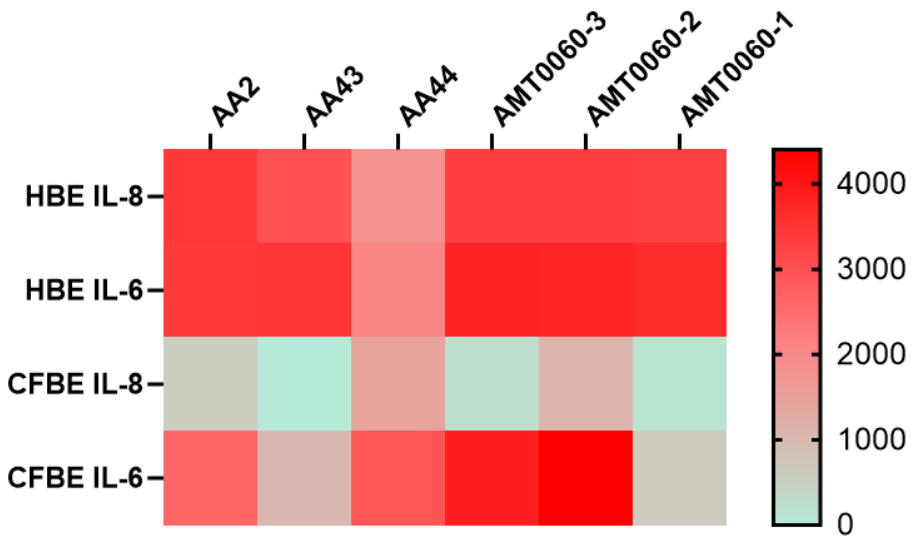

Figure 20. Heatmap of IL-8 and IL-6 release after stimulation of human epithelial cells with *P. aeruginosa* CF isolates. Summary of IL-6 and IL-8 stimulation in the different cell lines (16HBE14o$^-$ and CFBE41o$^-$ cell lines). The intensity of the red indicates the level of cytokine detected by ELISA (pg/mL). The more intense the red, the more concentration of the cytokine was detected, while grey means low or no detection of the Interleukin.

Discussion

Several studies have analysed sequential isolates from CF patients to understand the mechanisms of the adaptation that *P. aeruginosa* populations undergo during chronic lung infections (297, 303-305). Two series of well-characterised "early" and "late" isolates from European and North American CF patients were selected to evaluate if the ability to attach to human epithelial cells and to stimulate pro-inflammatory cytokines (IL6 and IL8), differed between early and late isolates, suggesting adaptations that affect host interactions during the course of infection (Table 5).

The long-term *P. aeruginosa* infection in the CF airways was reported to cause a reduction in bacterial growth rate (220, 306). In this study, the growth of all the strains was similar except for the late isolate AMT0060-2, which was also the only one resistant to gentamicin. These phenotypes might indicate an adaptation that helped AMT0060-2 to establish chronic infections in the antibiotic-treated airways of CF patients, as it has been demonstrated that reverting the slow growth phenotype of *P. aeruginosa* clinical strains to a high growth rate can increase antibiotic susceptibility (220, 306). The slow growth of AMT0060-2 might also be due to its high production of alginate, which represents a fitness cost to *P. aeruginosa* (220). Importantly, AMT0060-2 has a 2bp frameshift in the gene *gacA* (Table 2), involved in antibiotic resistance by increasing biofilm formation, matching with the previously reported phenotypes (307). Noteworthy, reduced growth rate might be a phenotypic marker of evolution progression from naïve/environmental strain to adapted/clinical strain, which could help to determine the stage of the infection during the diagnosis of the patient (306).

The attachment of all the isolates to 16HBE14o$^-$ cells was lower relative to the attachment to CFBE41o$^-$ cells. The attachment to 16HBE14o$^-$ was consistent with those reported for A549 cells by the PA01 isolate (308), showing the consistency of these results with the available literature. The relatively higher attachment to CFBE41o$^-$ observed in all the isolates relative to the attachment to 16HBE14o$^-$ was also observed by DiPaola et al. (309). The higher attachment of AMT0060-1 to epithelial cells relative to the early isolate AMT0060-3 may indicate adaptations of that late isolate. Overall, the

results suggest that the ability to attach to epithelial cells is isolate-dependent instead of dependent on the infection stage, as no other significant differences were observed either between AA2 and AA43 or AA44; or between AMT0060-3 and AMT0060-2.

Table 5. Comparison between *P. aeruginosa* late and early isolates from two different CF patients.

	Patient 1		**Patient 2**	
Early Isolate	**AA2**		**AMT0060-3**	
Late CF Isolates	**AA43**	**AA44**	**AMT0060-2**	**AMT0060-1**
Growth	=	=	↓	↓
Attachment HBE	=	=	=	↑
Attachment CFBE	=	=	=	↑
IL-8 16HBE14o$^-$	↓	↓	=	=
IL-6 16HBE14o$^-$	=	↓	=	=
IL-8 CFBE41o$^-$	↓	↑	↑	=
IL-6 CFBE41o$^-$	↓	=	=	↓

Abbreviations: IL, Interleukin; HBE, human bronchial epithelial; CFBE. Cystic fibrosis bronchial epithelial.

The stimulation of cytokines in epithelial cells by early and late isolates did not show a clear trend (Table 4). It has been suggested that *P. aeruginosa* shape the immune response by lowering the pro-inflammatory response induced by *P. aeruginosa*-adapted variants (310). Although a decrease in IL-8 with AA43, AA44 by 16HBE14o$^-$, an AMT0060-1 by CFBE41o$^-$ was observed; AMT0060-2 and AA44 induced higher concentrations of IL-8 in CFBE41o$^-$ cells, contradicting that hypothesis. The results may be explained by the fact that *P. aeruginosa* populations in CF lungs exhibit high levels of phenotypic diversity in motility, virulence factors production, siderophore production, antibiotic resistance, auxotrophy, and hypermutability. Hence, although some members of the population have acquired mutations affecting some phenotypes, the mutants can coexist in patients alongside other genotypes that have not (212). By sequencing *P. aeruginosa* populations from a group of patients infected with the *P. aeruginosa* LES

strain, the coexistence of divergent sublineages within individual patients was shown, strongly suggesting the likelihood of ongoing transmission between patients (212). Data from this study also demonstrated that, although mutations in some genes are common, they are not always present in all patients, nor carried by all members of the *P. aeruginosa* population within individual patients (212). This matches the variable results between isolates from the same patient.

Previous assessment of motility (swimming, swarming and twitching) showed similar phenotypes between isolates from patient 1 (AA2, AA43 and AA44 isolates) and between AMT0060-3 and AMT0060-3, but AMT0060-2 has lost the ability to swarm and twitch (291). It stimulated IL-6 and IL-8 in both cell lines tested, suggesting that stimulation of proinflammatory cytokines is not dependent on bacterial motility and other virulence factors still stimulate inflammatory responses even when the strains have lost motility. For example, AMT0060-2 produces high amounts of alginate, which has immunostimulatory properties. This is characteristic of CF-adapted strains, as the overproduction of alginate enables mucoid *P. aeruginosa* strains to form persistent infections in the lungs of CF patients. Finally, it is important to highlight that for both patients at least one of the late isolates stimulated one or both of the studied pro-inflammatory cytokines suggesting that stimulation of inflammatory responses is always present in chronic *P. aeruginosa* infection. This is of great relevance for the development of new drugs against *P. aeruginosa* and for the treatment of individuals with CF. For example, there is currently great interest in anti-inflammatory therapies to treat CF, however, it is still necessary to understand whether *P. aeruginosa* infection would affect these therapies, as well as whether these treatments may be detrimental in *P. aeruginosa*-infected patients since the natural host response against some late isolates is inflammation (311, 312).

Phenotypic diversity across the *P. aeruginosa* sequential isolates was apparent by their previous phenotypes examined (291), agreeing with the variability in the stimulation of proinflammatory cytokines. Here, we demonstrated that *P. aeruginosa* CF-adapted variants attached and stimulated proinflammatory cytokines in epithelial cells, independently of their status as early or late CF isolates**.** The ability to switch between

different lifestyles allows bacterial pathogens to thrive in diverse ecological niches. However, a molecular understanding of their lifestyle changes within the human host is lacking. Combining `-omics studies with host-pathogen interactions analysis will help to better understand the adaptation of *P. aeruginosa* in the host and might accelerate the discovery of more effective ways to tackle infections caused by the pathogen. For example, proteomic studies of the six strains evaluated here were performed by our laboratory (Drabrinska et al., manuscript in preparation). This opens the possibility of finding proteins that might be involved in the evaluated host immune responses against *P. aeruginosa.*

The most practical clinically relevant consequence of *P. aeruginosa* phenotypic diversity is the diagnosis of infection and the choice of the proper treatment strategy, which is typically carried out on either single isolate (313). An understanding of the evolution dynamics of *P. aeruginosa* pathogens within infected hosts is critical, as it has important consequences for how bacterial infections are understood and interfered with. For example, contrary to the results from this study, Cigana et al. observed that *P.* aeruginosa CF-adaptive variants shaped the innate immune response towards a lower stimulation of proinflammatory cytokines (310). This may be due to the wide variability of *P. aeruginosa* between patients, as observed in this study. Hence further studies with more sets of CF sequential isolates, and more "early" isolates per patient, will be needed to clarify if there is a pattern in the host immune responses against *P. aeruginosa* CF-adapted isolates or, as demonstrated here it is isolate-dependent. This is essential for the design of new therapeutic strategies, not only against *P. aeruginosa* infection, but also for the treatment of CF patients or others chronically affected by *P. aeruginosa*, as drugs could shift the host immune response towards a response that is efficient for the disease but inefficient to combat *P. aeruginosa.*

Chapter 3

Elucidation of novel human host receptors for *P. aeruginosa*

Introduction

The epithelium of the respiratory tract is the first line of defence against pathogens. Since the first interaction of *P. aeruginosa* with the epithelium, the cells of the respiratory tract recognise the components and virulence factors of *P. aeruginosa* and activate an immune response to prevent or resolve the infection. In the early stages of infection, *P. aeruginosa* attaches to host cell surfaces to sense and signalling a host. Normally, planktonic bacteria use their flagella to swim close to a surface and their pili to attach to that surface. Although, the dynamics of this process are poorly understood (314, 315). Among the main virulence factors of *P. aeruginosa* that are recognized by the epithelium are the appendages of motility and adhesion, pili and flagella, that are localised on the bacterial surface, and mainly responsible for movement and adhesion to host cells (88). Other important virulence factors that work as adhesins to human cells are LPS, which enables tissue damage, attachment, and recognition by host receptors (77); retractile type 4 pili, powering adhesion to biotic surfaces; type 3 secretion system (T3SS) (113) and OprF (316).

Bacteria may carry more than one adhesin for more than one target on the cell surface. Also, multiple adhesins can act in a concerted way and are expressed at different stages during the infection (317). Once adhesion is established, exopolymeric substances are secreted by bacterial cells generating a microenvironment to help the dividing bacterial cells initiate the formation of bacterial microcolonies or biofilms. Finally, bacteria secrete enzymes to digest the exopolymeric substances in the centre of those microcolonies, and the newly flagellated bacterial cells are released, initiating adhesion to newly exposed surfaces, and leading to host colonisation (318). Thus, understanding attachment is critical to research. Bacteria detect the host surface to initiate contact and correctly adhere to the human cells, while molecules on the host, present in the epithelial cells and the components of the human extracellular matrix, sense *P. aeruginosa* adhesins or virulence factors and initiate the innate immune response. Proteins reported to be important, specifically, in the interaction of *P. aeruginosa* with human epithelial cells are the cytosolic annexin A2 (AnxA2) protein, autophagy-related protein 7 (ATG7), NLRC4, and non-coding RNAs (lncRNA and microRNA) (319-321). Access of *P. aeruginosa* to the basolateral epithelium is crucial for initiating infection and

is the common link across the various infections caused by this pathogen (91). However direct interactions between *P. aeruginosa* and specific receptors in human epithelial cells are still not well understood.

Alternative therapies against *P. aeruginosa* are urgently needed. Blocking bacterial adhesion, quorum sensing, biofilm formation and virulence might be some of them, as they inhibit pathogenicity without killing the bacteria, hindering the development of further resistance in a bacterial population by removing the selective pressure for such mutations, which provides key advantages over classical drugs (317). In addition, microbial adhesins that consistently pair with host receptors, are normally exposed to other cells and available to be presented to the immune system, representing important potential vaccine candidates. Moreover, the identification and characterisation of receptor/ligand pairs will also expand the understanding of how the attachment of *P. aeruginosa* to human cells modulates mechanisms associated with its pathogenesis (317, 322). The identification of receptor/ligand associations is a difficult task. Generally, they are identified by screening for gene deletions in either the host or pathogen, looking for defects in the adhesion/colonization phenotype, e.g. with CRISPR libraries; or by pull-down affinity approaches (323). However, with these targeted approaches, the identity of one of the two molecules involved in the interaction must usually be known. Also, they may fail to consider the complex multifactorial host-pathogen interactions. The development of less biased techniques is essential to accelerate the discovery of novel molecules involved in host-pathogen interactions (323).

Due to the importance of *P. aeruginosa* attachment for host colonisation and the lack of techniques to study this critical step of the infection, we developed a novel proteomic approach for the recognition of human receptors for *P. aeruginosa*. The technique helped us to identify the human disulfide isomerases, PDIA1 and PDIA3, as receptors involved in the adhesion of *P. aeruginosa* to the human epithelium. Our findings may lead to the development of new drugs that prevent this essential step of infection, the adhesion of *P. aeruginosa* to the host, as well as understanding in a more specific way this key process.

Aims and Objectives:

The attachment of *P. aeruginosa* to the host is the crucial step for host colonisation and there is a lack of unbiased techniques for studying this step of the infection. Hence, this chapter aimed to develop a novel unbiased approach for the identification of human receptors for *P. aeruginosa.*

Objectives:

1. To design and optimise a proteomic 2D blot approach for the identification of human receptors for *P. aeruginosa.*

2. To validate the receptor candidates *in vitro* to confirm their role in *P. aeruginosa* attachment to the human cells.

Methods

Isolation of the membrane proteins: 16HBE14o$^-$ cells were grown to confluency (80%), the medium was removed, and the attached cells were washed once with 5 mL of PBS/flask. The cells were then collected from the flask by scraping using Nunc^{TM} Cell Scrapers (Thermo Fisher, 179963) and washed again with 5 mL of PBS. The cells were collected from the flask and centrifuged at 1200 rpm for 5 min. The pellets were collected by removing the supernatant. For protein extraction, the Mem-PER^{TM} Plus Membrane Protein Extraction Kit (Thermo Fisher, 89842) was used following the manufacturer's instructions. The membrane fraction was washed with a 2D Clean-Up Kit (Cytiva, 80648451) following the manufacturer's instructions before the membrane fraction was separated by 2D electrophoresis. Importantly, cOmplete Mini EDTA-free protease inhibitors (Merck, ref 11836170001) were added to all the buffers following the manufacturer's instructions.

Electrophoresis 2D: Membrane proteins were solubilised for isoelectric focusing (IEF) in a rehydration buffer containing 8 M Urea, 2 M Thiourea, 4 %CHAPS, 1 % Triton X, 10 mM Tris Base, 65 mM DTT, 0.8 % immobilised pH gradient (IPG) buffer (pH 3-11NL) and a trace of bromophenol blue. IPG dry strips (Cytiva ImmobilineTM DryStrip, REF 17600373), 7 cm long, (pH 3-11NL) (Cytiva) were rehydrated overnight at RT with 120 µl of the rehydration buffer (previously used for protein solubilisation) containing 150-180 µg of protein, covered with IPG cover fluid. The IEF step was carried out for five hours with 300V for 30mins, 1,000V for 30mins and 5,000V (gradient) for four hours on an Ettan IPGphor IEF system. Following IEF, IPG strips were equilibrated at RT in a reducing buffer containing 30 % glycerol, 2 % SDS, 6 M Urea, 50 mM Tris and 2 % DTT for 15 minutes. IPG strips were then alkylated for 15 minutes in Equilibration buffer, (30 % glycerol, 2 % SDS, 6 M Urea, 50 mM Tris, 2.5 % iodoacetamide and with a trace of bromophenol blue). IPG strips were placed on 10% or 12 % SDS-PAGE gels, sealed using 1% agarose in 1x running buffer with a trace of bromophenol blue. Four microlitres of protein marker Precision Plus Protein^{TM} Standards (250-10 kDa) were added next to each strip. The separation was performed at 110V, 100 mA until the bromophenol blue reached the bottom of the gel. Proteins were visualised using Page Blue protein staining.

Probing with bacterial cells: The proteins were transferred from the gel to PVDF membranes (pre-equilibrated in 30 s in methanol and 5 s in dH_2O) using a wet transfer method at 30mA overnight (18h). After protein transfer, the membranes were blocked overnight at 4°C with 5% BSA and 3% Marvell milk in PBS with gentle rotation. Five mL of LB broth were inoculated with a single colony of *P. aeruginosa* AA43 strain and cultured for 16 h. Subsequently, 50 ml of LB was inoculated with the bacteria overnight culture (OD_{600nm}: 0.1) until it reached an OD_{600nm}: 0.6. The membranes were incubated in 2 mL of the bacteria culture mixed with 18 mL of MEM and one tablet of cOmplete Mini EDTA-free protease inhibitors (Merck), 4°C, 24h on a rocker (Stuart gyro-rocker SSL3, 40 rpm). The membranes were rinsed five times with 20 ml of 0.05% (v/v) PBS-T, and any attached bacteria were fixed with 4% paraformaldehyde dissolved in PBS for 8 min, RT. After that, the membranes were incubated with an anti-*Pseudomonas* horseradish peroxidase (HRP) rabbit polyclonal antibody (Invitrogen PA1-73118, 1:1000 in 5% (w/v) BSA in 0.04% (v/v) PBS-T) overnight at 4°C with gentle rotation on a rocker. The membranes were then washed 5 times with 0.05% (v/v) PBS-T before chemiluminescence detection was carried out by Pierce™ ECL Western Blotting substrate (Thermo Fisher, 32109) followed by using the VILBER imager.

Matching spots: The developed 2D blots images were matched with the parallel gels and negative controls using Adobe *Illustrator* ® which allows the use of rulers. The spots were selected based on their shape, the distance between spots, molecular weight and isoelectric point. Once the spots in the membrane were matched against the respective parallel gel, they were excised from the gel to be analysed by LC/MS mass spectrometry (Appendix B).

In-gel trypsin digestion and sample purification: The selected spots were individually excised from the corresponding 2D gels with a clean scalpel and in-gel digested following the protocol by Shevchenko et al. (324). Briefly, the spots were destained with 100 mM ammonium bicarbonate (AB)/acetonitrile (ACN) (1:1) by 30 min-incubation and occasional vortexing. The destaining solution was removed and gel pieces were incubated with neat ACN until shrank. Gel pieces were saturated with trypsin buffer (13 ng/µL trypsin in 10 mM AB containing 10% ACN) for 2 h at 4ºC, after which 100 mM AB were added to follow an overnight incubation at 37ºC. The following day, the digested

peptides were extracted from the gel by incubating the pieces in 1:2 (vol/vol) 5% formic acid/acetonitrile at 37ºC for 15 min. The supernatants containing the peptides were transferred to LoBind microcentrifuge tubes (Eppendorf, 13-698-794) and dried in a vacuum. To redissolve tryptic peptides, 0.1% trifluoroacetic acid (TFA) was added and the tubes were sonicated for 5 min, then centrifuged for 10 min at 10,000 rpm, and finally dried by vacuum. The peptide samples were individually purified by zip-tipping using ZipTip®Pipette tips (Merck Millipore) following the manufacturer´s instructions.

Protein identification by LC-MS and shortlisting process: After trypsin digestion and subsequent clean-up, peptide samples were resuspended in 0.1% FA and analysed in the Mass Spectrometry Core service at the Conway Institute in University College Dublin (UCD). The protein spectra were obtained by LC-MS using Dionex UltiMate® 3000 HPLC System and the Thermo LTQ-Orbitrap. The protein identification was performed using MASCOT with Uniprot/SwissProt release 7.6 as the search database, using a human reference proteome. The proteins identified in at least two gels were filtered, corresponding to 56 proteins, out of the 377 proteins identified from all spots. Proteins with more than 10% of sequence coverage were short-listed.

Bioinformatic Analysis: *Uniprot* and *The Human Protein Atlas* tools were used for the identification of the molecular size and isoelectric point of PDIA1 and PDIA3 and the STRING tool was used for the evaluation of the interactomes of all the short-listed proteins and the PDIA1 and PDIA3 proteins.

LOC14 inhibition: 16HBE14o- cells were seeded in 24-well plates (4 × 105 cells/well) and incubated at 37°C, 5% CO_2 overnight. The cells were pre-treated two hours before the experiment with increasing doses of the PDIA3 inhibitor LOC 14 (Merck, 538765): 3 µM, 6 µM and 20 µM. LOC14 was dissolved in DMSO and the concentration of DMSO (Merck, D1435) contained in the highest dose (20 µM of LOC14) was used as a control (~0.08%). Subsequently, cells were incubated with *P. aeruginosa* AA43 cultures in antibiotic and FBS free-MEM for 30 min at a MOI 5. Cells were gently washed three times with phosphate-buffered saline (PBS) for 5 min each and incubated for 10 min in lysis buffer (PBS- 5% Triton 100). Cells were scrapped and serial dilutions in PBS were performed and plated in LB agar plates. The plates were incubated for 24 h and the bacteria CFU were counted. Before the experiment, bacterial growth curves with higher doses of the

inhibitor LOC14 in the LB media were performed to ensure the drug did not affect bacterial growth. The *P. aeruginosa* AA43 strain was cultured in LB broth at 37 °C and diluted in LB to an OD_{600} = 0.1, and 5 µl of the diluted culture was used to inoculate 96 microtiter wells (Thermo Fisher Scientific, Hemel Hempstead, UK) containing 195 µl of LB broth with a range of LOC14 concentrations. Duplicates were inoculated for each strain tested. The cell viability was also tested using an LDH assay CyQUANT™ LDH Cytotoxicity Assay (Invitrogen™, C20300) following the manufacturer´s instructions.

Transfection of HEK293T cells: HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen ™, 11668019) following the manufacturer´s instructions. Briefly, one day before transfection, $2.25x10^5$ cells were plated in 12 well plates (in triplicate) in 1mL of growth medium (DMEM) so that cells would be 70-90% confluent at the time of transfection. For each transfection sample, the complexes were prepared as follows: 2µg of DNA in 100 µl of Opti-MEM Reduced Serum Medium, and 4µL Lipofectamine 2000 were gently mixed before use, and diluted in 100 µl of Opti-MEM I Medium. The complexes were incubated for 5 min at RT. After the 5 min incubation, the diluted DNA was combined with diluted Lipofectamine 2000 (total volume = 200 µl), mixed gently and incubated for 20 min at room temperature. After the incubation, 200 µl of complexes were added to each well, containing cells and medium. The cells were incubated for 24 h at 37°C in a CO_2 incubator before testing for transgene expression and performing attachment assays. The host-cell attachment was examined as described previously using MOI 5 and 30 min of incubation with *P. aeruginosa* AA43. The plasmids used were purchased from Addgene: PDIA3-bio-His (Plasmid #52070), P4HB-bio-His (Plasmid #52069) and as a control the plasmid expressing just the tags was acquired, CD4d3+4-bio (Plasmid #32402). Protein expression was ascertained by Western blot using the anti-6x histidine tag monoclonal antibody (Invitrogen, MA1-21315).

PDIA3-deficient cells: A549 and A549 pdia3/- cells were generous gifts from Vikas Anathy (Department of Pathology and Laboratory Medicine, Larner College of Medicine, The University of Vermont, USA). The host-cell attachment by *P. aeruginosa* AA43 to A549, A549 *pdia3*$^{/-}$, A549 *pdia3*$^{-/-}$ complemented with a plasmid containing *pdia3* was examined as described previously. The results represent triplicates of at least two independent experiments. The data were analysed by the Kruskall-Wallis test. PDIA3

expression was determined by Western blot using the antibody ERp57 polyclonal antibody (1:300, PA3-009, Thermo Fisher) as primary antibody and a mouse anti-rabbit IgG-HRP as secondary antibody (1:2000, 31464, Thermo Fisher).

Confocal Microscopy: 16HBE14o- cells were seeded in 24-well imaging plates (Miltenyi Biotec) for 24 h. The medium was removed, and the wells were either incubated overnight in an antibiotic-free medium. The cells were then washed and incubated with bacteria at an MOI 5:1 or media alone (no bacteria control) the plates were centrifuged at 700 g for 5 min and incubated for 30 min at 37°C, 5% CO_2. Wells were then washed with PBS and adherent bacterial cells were fixed with 4% of paraformaldehyde and blocked with 5% skimmed milk. Cells were then incubated with a rabbit anti- *P. aeruginosa* FITC antibody (Invitrogen, PA1-73117) in 1% BSA PBS (1:1000 dilution) overnight at 4°C, followed by PBS washing and incubation with anti-ERp57 (PA3-009) or anti- PDIA1 (PA3-009) in 1% BSA PBS (1:300 dilution) or 1% BSA PBS or just 1% BSA PBS overnight at 4°C. An Alexa fluor® 647-conjugated secondary goat anti-rabbit antibody (Invitrogen) in 1% BSA PBS (1:1000 dilution) for 1 h was added to all the wells, in the dark at RT. Epithelial cell plasma membranes and nuclei were counterstained with 0.5× Cell Mask™ Orange Tracking Stain (Invitrogen) for 10 min, at 37°C in the dark, followed by a 15 min incubation with 0.25 µg/ml DAPI (Merck) at RT in the dark. Wells were finally covered with 1 ml PBS, and images were taken using an automated Opera Phenix™ High Content Screening (HCS) confocal microscope.

Statistical Analysis: Statistical analysis of host cell attachment was performed by One-Way Analysis of Variance (ANOVA) using Kruskal-Wallis tests and GraphPad Prism 8.0.2

Results

1. Development of an unbiased proteomic approach for the identification of novel human receptors for *P. aeruginosa.*

To identify the host receptors for *P. aeruginosa* an unbiased 2D proteomic approach was developed. The major novelty of the approach is its unbiased nature, meaning there is no need to previously know either the adhesin from the bacteria or the receptor in the human cells. For this, the cell-blot technique (302) previously used for the successful identification of novel bacterial adhesins was "flipped over" (Figures 21 and 22).

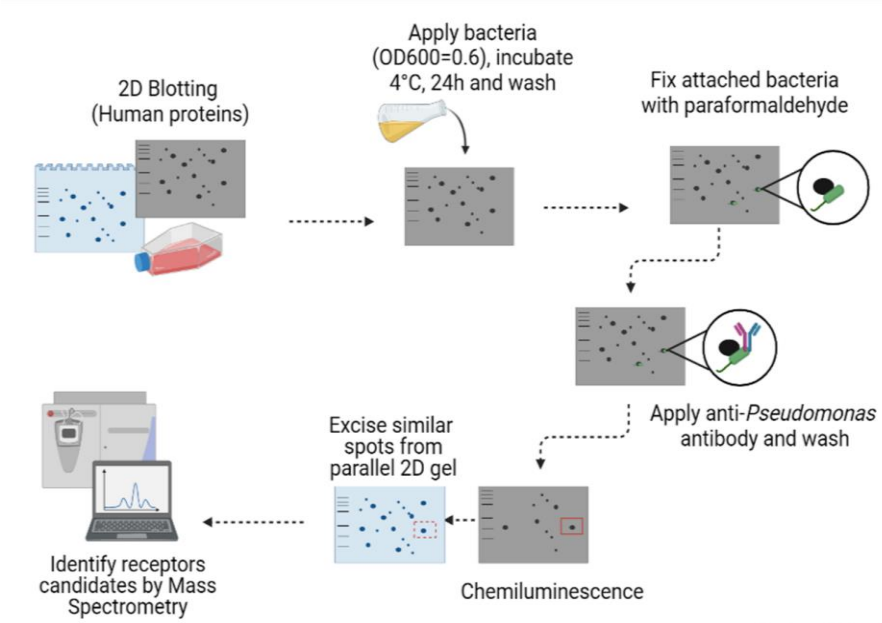

Figure 21. 2D-Blot technique for the identification of human host cell receptors with which *P. aeruginosa* interacts during infection. 16HBE14o$^-$ membrane proteins were separated by 2D electrophoresis on pairs of gels run in parallel. One of each pair was transferred to PVDF membranes and probed with *P. aeruginosa* AA43 (2D Blot) or bacteria-free media (negative control). After fixing, the attached bacteria were detected with an anti-Pseudomonas-HRP antibody followed by chemiluminescence detection. Similar spots between blots and parallel gels were identified by Mass Spectroscopy (LC/MS).

Several spots were identified on the cell blots that could be matched with the corresponding gels. To match the spots on the membrane (in black) with those on the gel (in blue) only spots (i) at the same position (same molecular weight and pH), (ii) similar distances between close spots, as well as (iii) similar shapes, were considered (Figure 22 and appendix B). After discarding the spots closer to those observed in the

negative control (membrane incubated without bacteria), ten spots were selected and are shown with red arrows (Figure 22). Mass Spectrometry (MS) of the excised spots (from 2 different gels, Appendix B) enabled the identification of 377 proteins in total. Of those 377 proteins, just 56 were identified in two of the best 2 blots achieved (Appendix B). The 56 short-listed proteins were filtered by sequence coverage and the proteins achieving more than 10% in two gels were selected, comprising 15 proteins (Table 6; Figure 23). Gene Ontology analysis was used to identify the proteins predicted to be localised on the cell surface (Figure 23), and consequently are most likely to be human receptors for *P. aeruginosa,* getting to a list of seven proteins. In summary, they achieved the highest intensity and 10% of sequence coverage, were reproducibly identified in two 2D blots, and are present on the cell surface of the human cell, meaning they are available for interaction with *P. aeruginosa.* The fifteen shortlisted proteins are listed in Table 6.

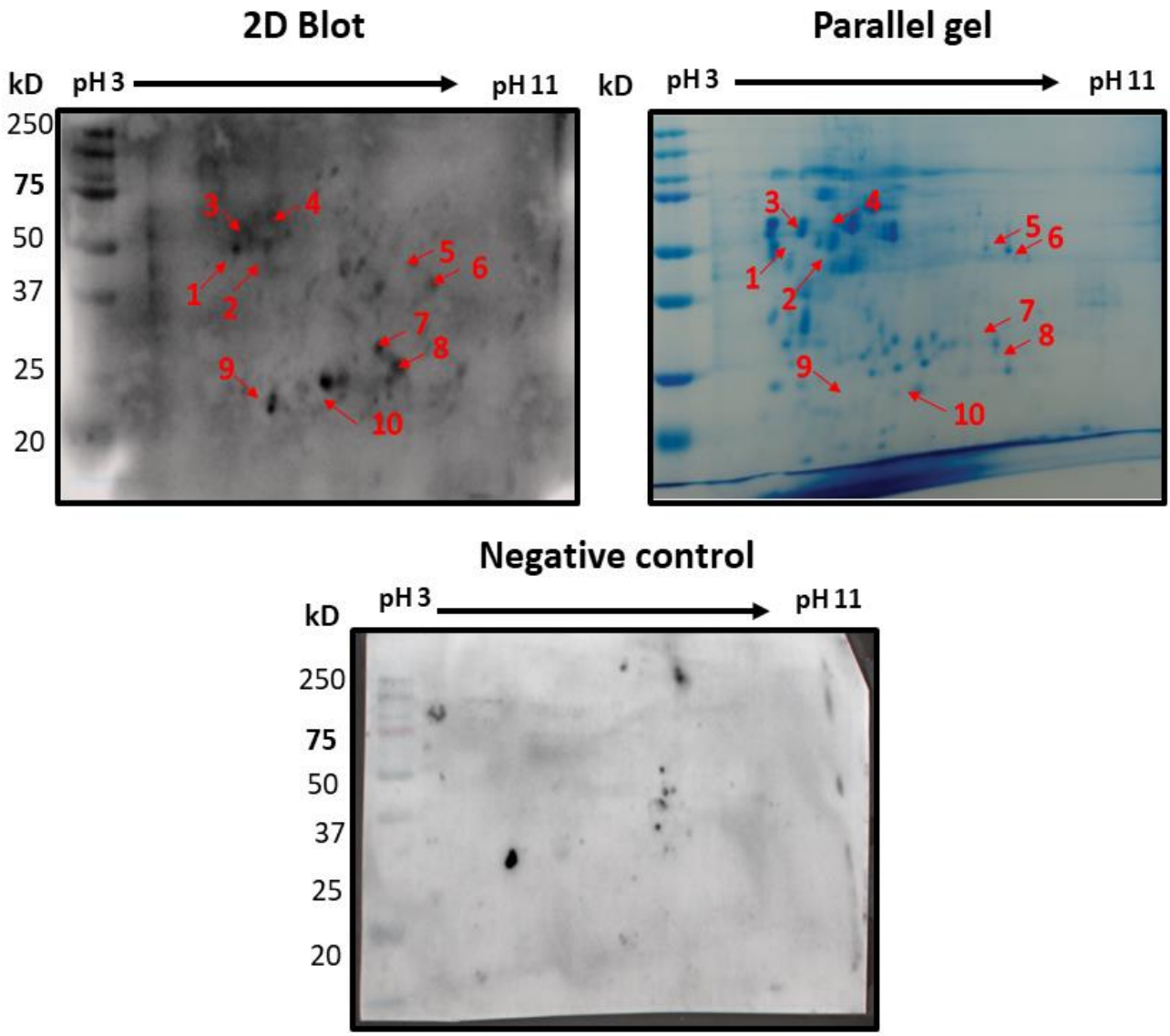

Figure 22. 2D- Blots for the identification of novel human proteins that interact with *P. aeruginosa*. Two-dimensional (2D) separation of membrane human proteins from 16HBE14o$^-$ cells ("Parallel gel") by 2D-SDS-PAGE, and electro transferred onto membrane blots ("2D Blot") that were subsequently incubated with *P. aeruginosa* live cells (OD_{600nm}: 0.6), or media alone ("Negative control") during 24 h at 4°C. *P. aeruginosa* -binding protein spots are highlighted with the red arrows, 10 spots. Denoted numbers to the right of the protein ladder in each picture indicate size in kilodaltons. The "pI" and numbers denoted at the top of each picture indicate the isoelectric point. All images are of full-length gels or 2D blots.

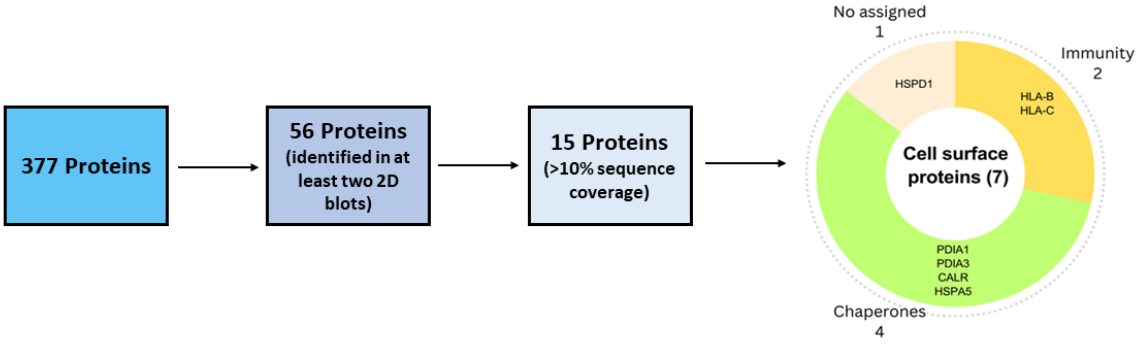

Figure 23. Short-listing method for the identified proteins by LC-MS of the excised spots. The excised spots were in-gel digested and sent for LC-MS analysis. MS identified 377 proteins, and the ones identified in at least 2 gels were selected (Appendix B); obtaining 56 proteins that were filtered by more than 10% sequence coverage in 2 gels, reducing the proteins to 15 (Table 6). The gene ontology analysis using GO Ontology identified seven proteins localised on the cell surface. The pie chart shows the molecular function of the seven proteins.

Table 6. Short-listed human proteins identified by the 2D approach.

Protein Name	Gene	Intensity 1*	Intensity 2**	Seq. Cov 1*	Seq. Cov 2*	MW (kDa)
60 kDa heat shock protein	HSPD1	34,69	35,54	53,00	49,00	61.10
Protein disulfide-isomerase	P4HB	32,75	33,98	29,00	38,00	61.00
75 kDa glucose-regulated protein	HSPA9	33,72	32,10	48,00	41,00	66.60
Protein disulfide-isomerase A3	PDIA3	22,89	28,70	29,00	38,00	56.80
Protein disulfide-isomerase A6	PDIA6	26,12	33,21	11,00	29,00	48.10
Calreticulin	CALR	29,01	31,18	13,00	18,00	48.10
Endoplasmic reticulum chaperone BiP	HSPA5	25,78	29,42	11,00	26,00	72.30
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1	24,56	28,81	14,00	32,00	38.90
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	26,67	28,45	19,00	24,00	51.00
Succinate Co-A ligase	SUCLG2	24,96	28,73	11,00	26,00	46.50
HLA class I histocompatibility antigen	HLA-C	27,01	27,62	16,00	16,00	44.40
Peroxisomal membrane protein PEX14	PEX 14	26,48	27,18	10,00	16,00	41.20
Nuclear migration protein nudc	NUDC	27,90	26,38	27,00	15,00	38.20
Cystatin A	CSTA	23,84	23,48	11,00	11,00	7.10
HLA class I histocompatibility antigen	HLAB	22,10	21,58	12,00	12,00	40.30

Abbreviations: Seq. Cov, sequence coverage, *Gel 1, ** Gel 2.

The interactions between the short-listed proteins were analysed by the STRING tool (325). Out of the 15 proteins, 11 were predicted to interact with each other, suggesting that instead of identifying single interactions with *P. aeruginosa*, interactions between *P. aeruginosa* and a complex of human proteins (bacteria-multiple proteins: HLA-B, HLA-C, HSPA5, HSPD1, PDIA1, PDIA3, PDIA6, HSPA9, HNRNPK and NUDC) were

identified, while interactions with SLC93R1, PEX14 and SUCLG2 might be individual interactions (bacteria-human protein) (Figure 24).

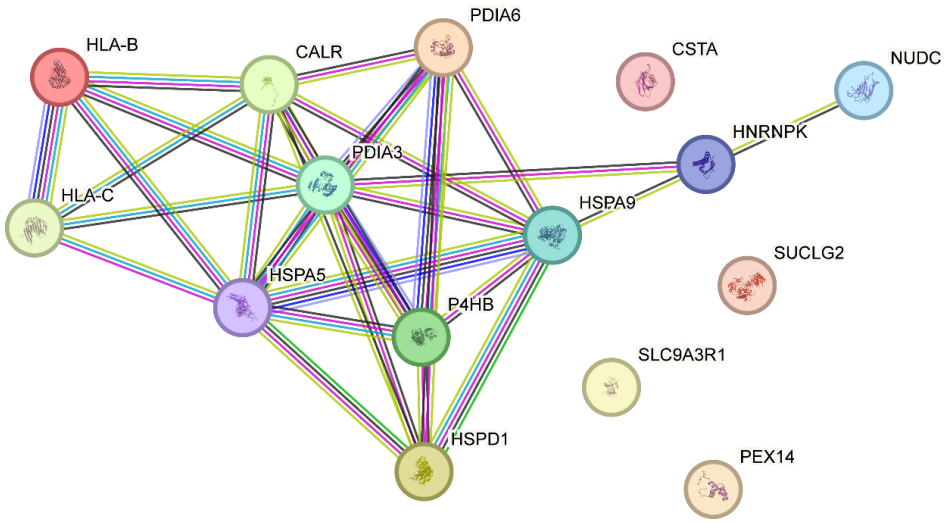

Figure 24. Predicted interactions between the short-listed proteins. The STRING tool was used to predict possible interactions between 15 proteins identified by the 2D approach. No interactions were predicted for CSTA, SUCLG2, SLC9A3R1 and PEX14. Light blue lines indicate known interactions from curated databases; pink lines, known interactions experimentally determined; green lines, predicted interactions; and dark blue lines, predicted to be co-expressed (325).

The protein HSPD1 was at the top of our list of potential receptors for *P. aeruginosa.* HSPD1 is an important mitochondrial chaperone. Jeong et al. (2017) identified that overexpression of HSPD1 increased p38 MAP kinase activity in human cells. Cytosol-localised HSPD1 was shown to be physically bound to and stabilise SEK-1/MAP kinase 3, which in turn up-regulated p38 MAP kinase and enhanced immunity against *P. aeruginosa* PA14 (30). The authors suggested that mitochondrial chaperones protect eukaryotic hosts from pathogenic bacteria by up-regulating cytosolic p38 MAPK signalling. Furthermore, the Na (+)/H (+) exchange regulatory cofactor (NHE-RF1), eighth in our short-listed proteins, has already been suggested to interact with *P. aeruginosa* during *in vitro* studies with the 16HBE14o$^-$ cell line (32).

The reproducible matches between the stained gels and the 2D blots, as well as the identification of relevant human proteins involved in *P. aeruginosa i*nfections such as HSPD1 and NHE-RF1 by the novel 2D blot technique, validate this approach to study human protein-whole bacteria cell interactions *in vitro*, which has always been a major

challenge for proteomics and molecular biology. The protein disulfide isomerase (PDI) A1 and PDIA3 were selected for further evaluation as novel human receptors for *P. aeruginosa*, as the gene ontology analysis suggested they are present on the cell surface of the human cell and their role in *P. aeruginosa* infections hadn't been established to date.

2. Bioinformatic analysis of PDIA1 and PDIA3

The predicted molecular size and isoelectric point of the two main candidates, PDIA1 and PDIA3 were analysed (Figure 25). PDIA1 and PDIA3 are 57 kDa proteins with an isoelectric point of 4.76, matching the spots observed on the PVDF membranes and parallel gels (Figure 22, arrows 1, 3 and 4). Furthermore, to verify that PDIA1 and PDIA3 are expressed in human lung cell lines, the expression of the proteins according to the Human Protein Atlas was verified (326, 327). Both PDIA1 and PDIA3 are highly expressed in the human lung, thus matching with the cell line used for their identification (Figure 25). The structure of the proteins was also analysed, showing that both PDIs have similar structures, but they differ in their signal sequences for their retention in the endoplasmic reticulum (KDEL Vs. QEDL).

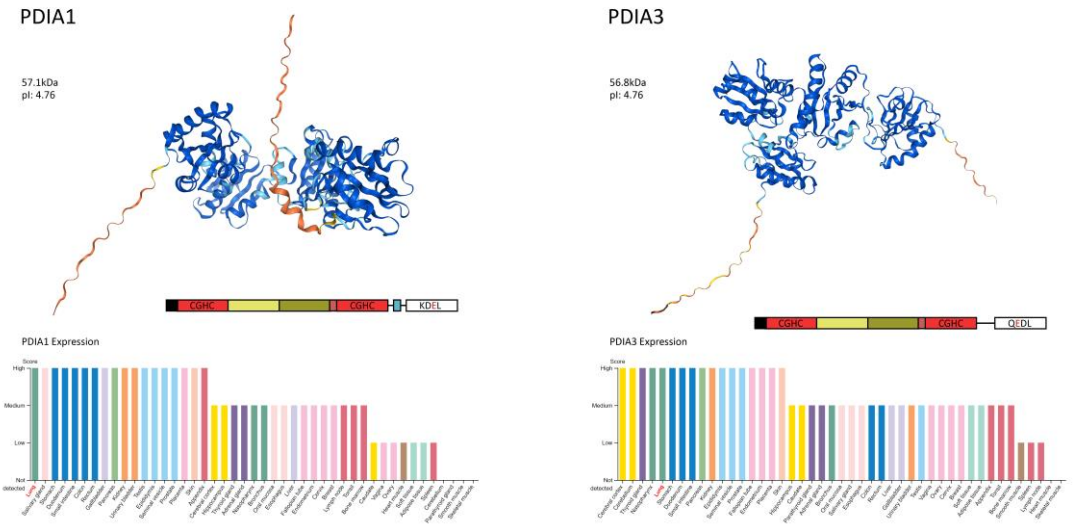

Figure 25. Bioinformatic analysis of PDIA1 and PDIA3. Both PDIA1 and PDIA3 have a molecular weight of ~57 kDa and an Isoelectric point of 4.76. They are both highly expressed in the human lungs (Human Protein Atlas) and have similar protein structures apart from their signal sequence.

The interactomes of PDIA1 and PDIA3 were also analysed (Figure 26). The proteins calreticulin (CALR), HSPA5, and HSP90B1 (Table 6) are components of either PDIA1 or PDIA3 interactomes and were also identified in the MS data. Overall, the bioinformatics analysis confirmed PDIA1 and PDIA3 as good candidates for *P. aeruginosa* receptors. Hence, further validation of the roles of PDIA1 and PDIA3 as ligands involved in *P. aeruginosa* attachment to lung cells was performed.

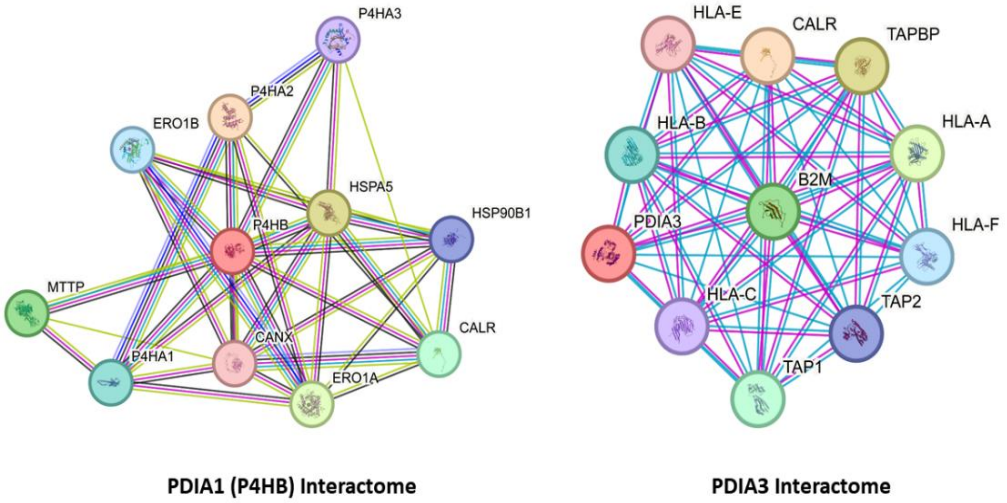

Figure 26. Interactomes of PDIA1 and PDIA3. Calreticulin (CALR), HSPA5, HSP90B1, and Calnexin (CANX), also identified in our mass spectrometry are part of PDIA1 (P4HB) and PDIA3 interactomes. Red balls indicate the studied proteins (PDIA3 and P4HB).

3. Inhibition of PDIs in 16HBE14o$^-$ cells with LOC14.

To confirm the role of PDIs in the attachment of *P. aeruginosa* to epithelial cells a pharmaceutical approach was first used (Figure 27). 16HBE14o$^-$ cells were pre-treated with increasing concentrations (3µM, 6µM and 20µM) of the potent PDI inhibitor, LOC14 (328), and the attachment of *P. aeruginosa* AA43 strain to the human cells was measured. Pre-treatment of 16HBE14o$^-$ cells with LOC14 showed a concentration-dependent decrease in *P. aeruginosa* attachment to 16HBE14o$^-$ cells**.** In particular, epithelial cells with 20µM of LOC14 significantly decreased the attachment of *P. aeruginosa* to 16HBE14o$^-$ by 2.5-fold, relative to the treatment with the DMSO control ($p=0.0188$). These results strengthen the likelihood that human disulfide isomerases are involved in the attachment of *P. aeruginosa* to human epithelial cells. Importantly, to

avoid seeing any artefact due to the killing of bacteria or human cells, the lack of effect from LOC14 on either the growth of the bacteria or the viability of human cells was confirmed (Figure 27, B and C).

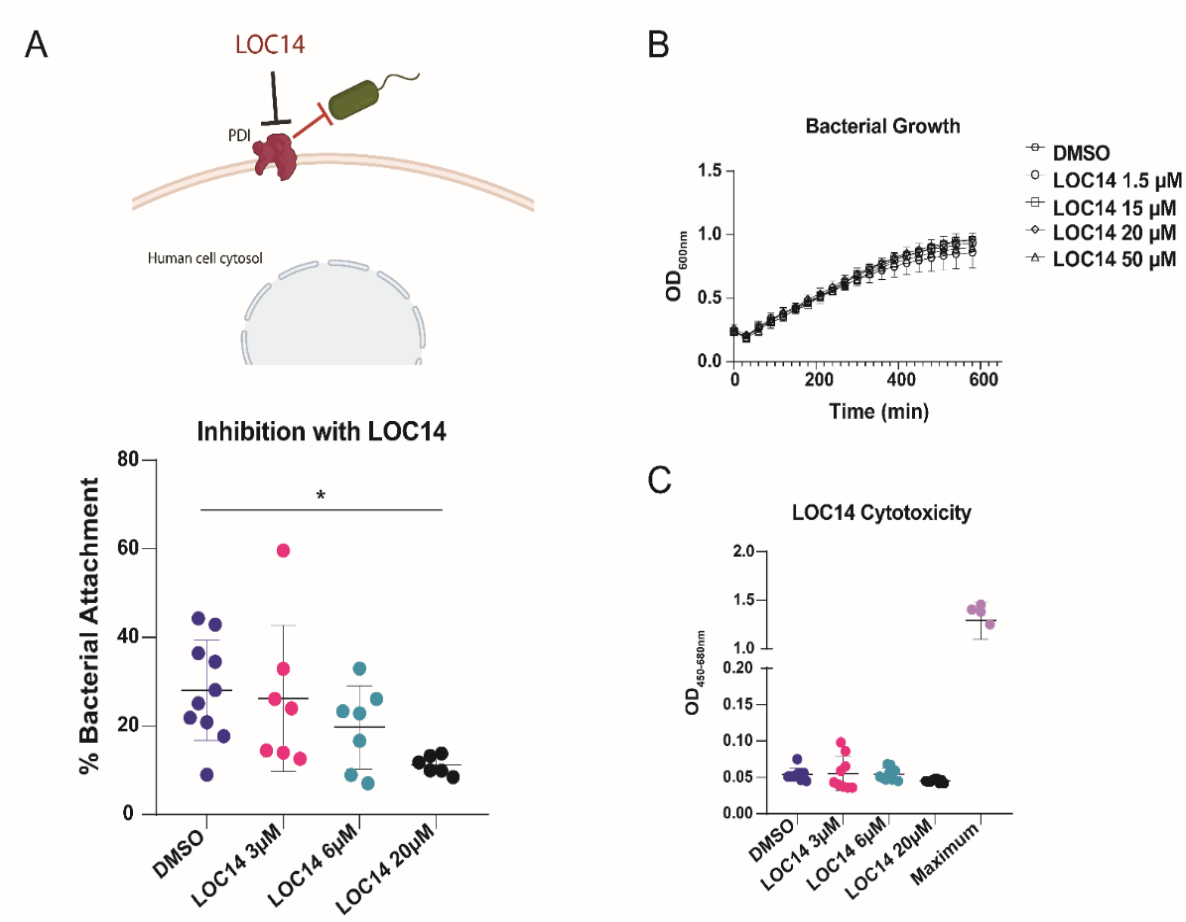

Figure 27. PDI inhibitor LOC14 decreases *P. aeruginosa* attachment to 16HBE14o⁻. A. Schematic representation of the hypothesis. If *P. aeruginosa* binds to PDI, inhibitors of PDI such as LOC14 will decrease *P. aeruginosa's* attachment to epithelial cells, relative to the control (DMSO, vehicle). Treatment of human epithelial cells with the PDI inhibitor LOC14 decreased *P. aeruginosa* attachment to the cells in a dose-dependent way (Kruskall–Wallis test, p=0.0188). The graph represents data from at least three independent experiments with biological duplicates. **B.** LOC14 does not affect *P. aeruginosa* growth relative to the control (DMSO). **C.** The pre-treatment of 16HBE14o- cells with LOC14 does not affect the viability of the cells relative to the control (DMSO). Cell viability was measured with CyQUANT™ LDH Cytotoxicity Assay, with "Maximum" representing the 100% of Lactose Dehydrogenase (LDH) release (100% of cell death).

4. Overexpression of PDIA1 and PDIA3 in HEK293T cells increased *P. aeruginosa* attachment to epithelial cells.

As human cells express 21 different disulfide isomerases (329), the inhibition with LOC14 may not inhibit PDIA1 and PDIA3 specifically. To confirm the specific role of PDIA1

(*p4hb*) and PDIA3 (*pdia3*), they were recombinantly expressed in HEK293T cells and the expression was confirmed by Western Blot. *P. aeruginosa* AA43 showed 6.01-fold and 6.52-fold higher attachment to both cells overexpressing PDIA3 and PDIA1, respectively, relative to the control (empty plasmid) (Figure 28). The results further strengthen the specific role of PDIA1 and PDIA3 in the attachment of *P. aeruginosa* to human lung cells.

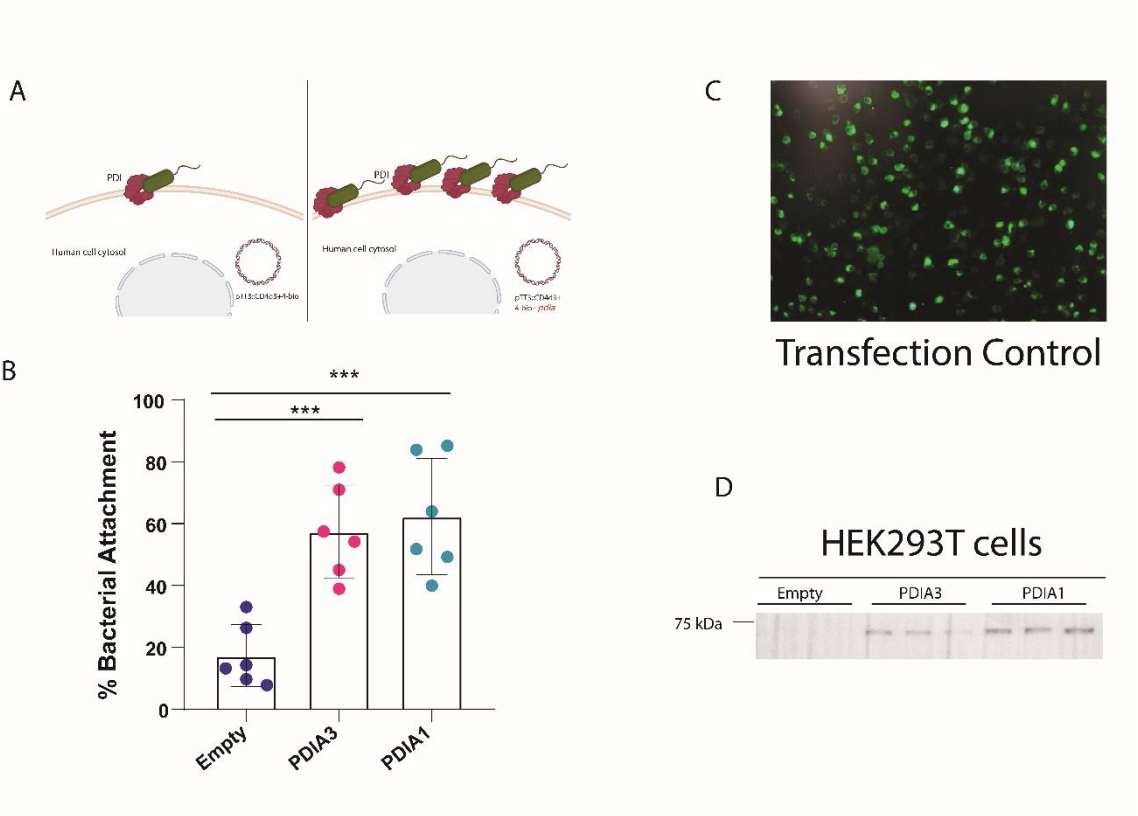

Figure 28. PDIA3 and PDIA1 are involved in *P. aeruginosa* attachment to human epithelial cells. A. Graphic summary of the experiment. **B.** *P. aeruginosa* attachment to HEK293T cells containing the plasmids pTT3::CD4d3+4-bio+*pdia3* (PDIA3) and pTT3::CD4d3+4-bio+*p4hb* (PDIA1) is higher (6.01 and 6.52 fold, p= 0.0360, 0.0132, respectively. Kruskall – Wallis test) relative to cells containing pTT3::CD4d3+4-bio (empty, plasmid control). The graph represents data from at least two independent experiments with biological triplicates. **C.** A plasmid containing a GFP was used to verify the transfection was always effective (transfection control). **D.** The western blot using an anti-6x histidine tag monoclonal antibody confirmed the lack of expression in the control cells and the expression of the PDIs (PDIA3 and PDIA1) in transfected cells.

5. Decreased *P. aeruginosa* attachment to PDIA3-deficient A549 cells

To overcome any possible non-specific effects from LOC14 drug treatment or transfection of the cells, the attachment of *P. aeruginosa* AA43 to PDIA3-deficient cell was evaluated. *P. aeruginosa* attachment to A549 *pdia3*$^{-/-}$ cells was 1.71-fold lower than the attachment of *P. aeruginosa* to A549 wild type (WT) cell lines, and 0.98-fold less than

the complemented control (PDIA3-deficient A549 cells transfected with pTT3::CD4d3+4-bio+*pdia3*), further confirming the involvement of PDIA3 in *P. aeruginosa* attachment (Figure 29). The Western Blot analysis confirmed the lack of PDIA3 expression in A549 *pdia3*$^{-/-}$ (Figure 29, C)

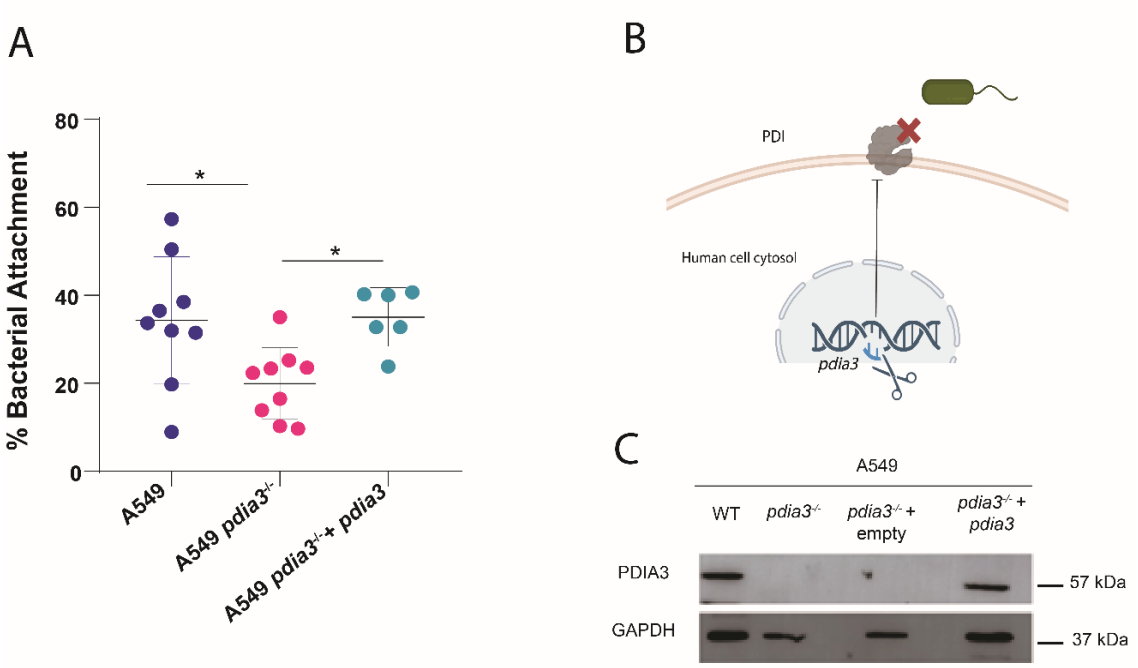

Figure 29. Attachment of *P. aeruginosa* AA43 to PDIA3-deficient A549 cells. A. PDIA3-deficient A549 cells were infected with *P. aeruginosa* AA43. The PDIA3-deficient cells showed 1.71 fold decrease in the attachment to epithelial cells relative to the A549 WT cells (p= 0.0344) or the PDIA3 deficient cells+ pTT3:: CD4d3+4-bio+*pdia3.* **B.** Schematic of the workflow. By using CRISPR cell lines (A549 *pdia3*$^{-/-}$) PDIA3 is not available for *P. aeruginosa*, so bacterial attachment to A549 *pdia3*$^{-/-}$should be less than to A549 WT and the complemented control. **C.** Western Blot analysis confirmed the lack of PDIA3 expression in the PDIA3-deficient cells.

6. Confocal microscopy suggests the co-localisation of PDIA1 and PDIA3 with *P. aeruginosa* AA43.

Once the role of PDIA1 and PDIA3 in *P. aeruginosa* attachment to epithelial cells was confirmed, confocal microscopy was performed to examine if the interaction between the proteins and the pathogen was direct or indirect, for example, due to the proteins being involved in pathways that might affect bacterial attachment. *P. aeruginosa* AA43 cells were incubated with the 16HBE14o$^-$ cells and examined by confocal microscopy to confirm the co-localisation of bacteria and PDIA1 and PDIA3. Cells were incubated with just media (no bacteria) and without the anti-PDI antibody, to consider possible non-specific interactions from the antibodies. Confocal microscopy confirmed a co-localisation of *P. aeruginosa* with PDIA1 and PDIA3 which might indicate a direct interaction between the pathogen and these human disulfide isomerases on the cell surface of the cell (Figure 30).

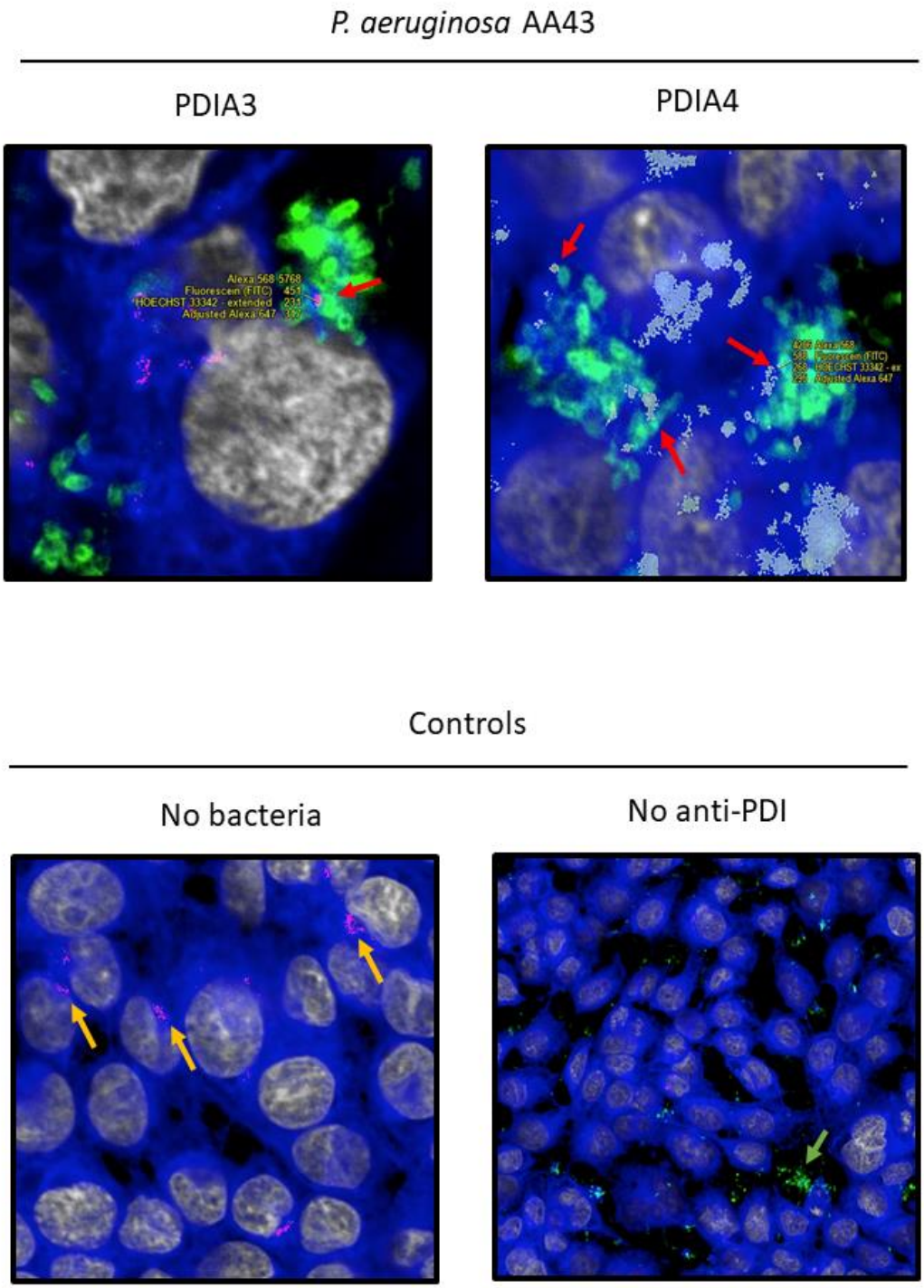

Figure 30. Co-localisation of PDIA1 and PDIA3 with *P. aeruginosa*. Co-localisation of *P. aeruginosa* AA43 with the 16HBE14o$^-$ cells. The red arrows highlight the zones where *P. aeruginosa* was co-localised with PDIA3 and PDIA1. As negative control cells were incubated without bacteria ("No bacteria", yellow arrows show PDIA3 in pink but no bacteria), or anti-PDI antibody (Ab) ("No anti-PDI", green arrow shows *P. aeruginosa* cells in green but no PDI). Representative images of epithelial cells stained with CellMask™ Orange (blue) and counterstained with DAPI (grey,4',6-diamidino-2-phenylindole) with bacterial strains stained with *anti-P. aeruginosa* antibody FITC polyclonal antibody (green) and PDIs with an anti-ERp57 (PDIA3) polyclonal antibody and anti-PDIA1 polyclonal antibody, that were observed for the binding of the secondary antibody Alexa 647 (represented in pink for PDIA3 image and light blue for PDIA1) using the automated Opera Phenix™ microscope.

***7. P. aeruginosa* might hijack the host cells.**

Having confirmed the role of human PDIs in *P. aeruginosa* attachment to epithelial cells, possible mechanisms for the interactions were investigated. The sequences and structures of PDIA1 and PDIA3 were analysed and compared with their homologs in *P. aeruginosa*, the disulfide bond-forming enzymes (Dsb) A, B, C, D, E and G (Figure 31). This work was performed by Dr Rita Berisio at the Consiglio Nazionale delle Ricerche, Istituto di Biostructure e Bioimmagini (Table 7, figures 31, 32, 33). In Gram-negative bacteria, the formation of disulfide bonds in extracytoplasmic proteins is catalysed by the Dsb protein system composed of numerous proteins located in the periplasm and inner membrane (330, 331).

Table 7. Homology between *P. aeruginosa* (PA) disulfide bonds forming enzymes and human PDIA1 and PDIA3*

PA	PDIA1 (residues)	PDIA3 (residues)
DsbA	31.4% identity (62.7% similar) in 51 aa overlap (64-111:135-185) CxxC4	36.4% identity (57.6% similar) in 33 aa overlap (49-80:6-38) CxxC1
DsbB	Poor	Poor (<20)
DsbC	25.6% identity (53.8% similar) in 39 aa overlap (40-78:120-151) CxxC1	23.2% identity (54.4% similar) in 125 aa overlap (304-422:29-146) CxxC4
DsbD	32.1% identity (56.8% similar) in 81 aa overlap (32-108:483-562) CxxC1	25.3% identity (53.5% similar) in 99 aa overlap (394-485:492-589) CxxC4
DsbE	23.5% identity (58.8% similar) in 51 aa overlap (386-436:62-107) CxxC4	23.1% identity (58.5% similar) in 65 aa overlap (364-422:25-89) CxxC4

*Performed by our collaborator Dr. Rita Berisio.

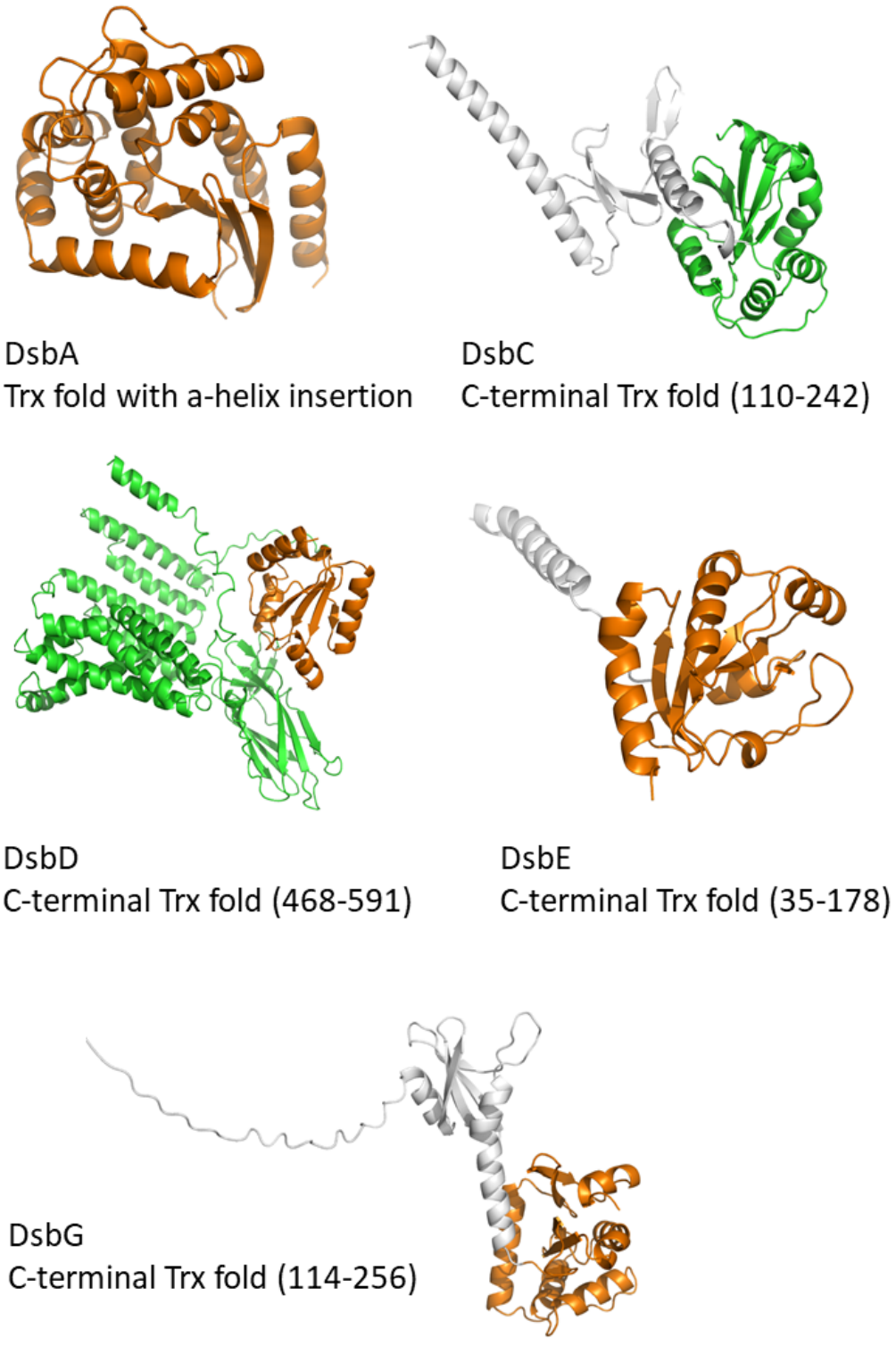

Figure 31. Structure of *P. aeruginosa* disulfide bond forming enzymes (Dsb). Dsb proteins are the homologs of human PDIs in *P. aeruginosa.* All *P. aeruginosa* Dsbs have conserved thioredoxin (Trx) domains. Structural modelling was performed by our collaborator Dr Rita Berisio.

Dsb domains share the highest sequence identity with either domain 1 or domain 4 of PDIA1/PDIA3 (containing CxxC motifs) (Table 7). *P. aeruginosa* DsbA and DsbD showed the highest identity and similarity with PDIA1 and PDIA3. The structure of *P. aeruginosa* DsbD is the most similar to human PDIA1 among all the Dsb proteins. The superposition of PDIA1 and DsbD showed similar Thioredoxin (Trx) folds, although the orientation of the helix embedding CxxC differs (black arrows, Figure 32). Hence, a BLASTP search to look for other homologous proteins was performed against all *P. aeruginosa* sequences. Thioredoxin (Trx) proteins (TrxC and TrcGI) showed better matches with human PDIs (39% of identity) and the superposition of PDIA1 with TrxC and TrcGI models (from *P. aeruginosa*) suggests that Trx folds and the conformation of the CGxC catalytic site are almost identical. The latter suggests a possible hijacking by *P. aeruginosa* to the human cells due to the outstanding similarities of PDIA1 and PDIA3 to TrxC and TrxGI proteins from *P. aeruginosa* (Figure 33).

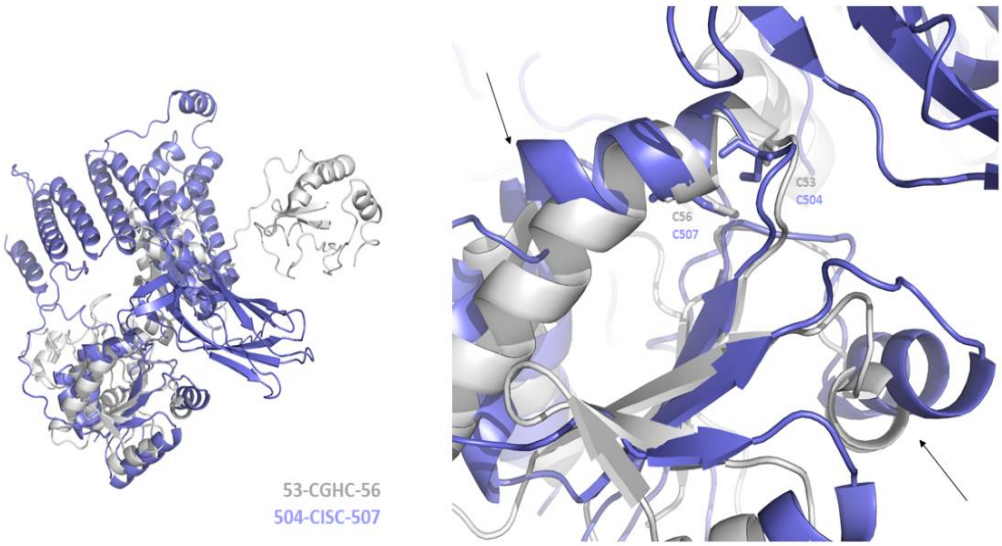

Figure 32. Superposition of human PDIA1 and *P. aeruginosa* DsbD. The human protein PDIA1 is showed in grey, and *P. aeruginosa* DsbD in purple. The black arrows highlight the lack of superposition in the helix embedding the CxxC catalytic site.

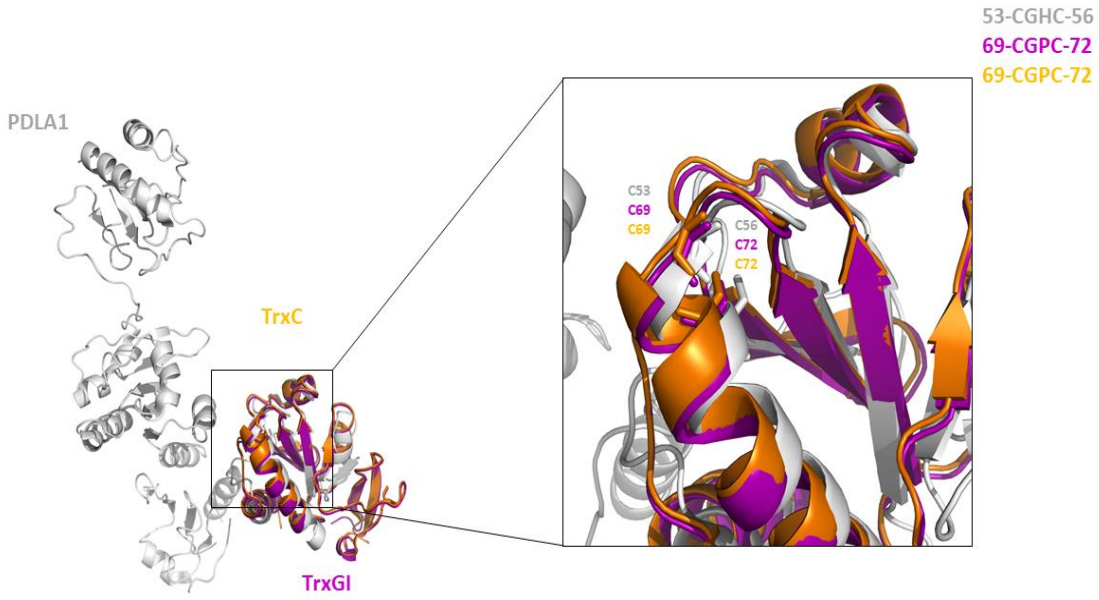

Figure 33. Superposition of PDIA1 and *P. aeruginosa* TrxC and TrxGI models. The human protein PDIA1 is showed in grey, *P. aeruginosa* TrxGI in purple and, *P. aeruginosa* TrxC in orange. The square highlights the perfect superposition in the helix embedding the CxxC catalytic site between PDIA1 and the bacterial proteins.

Discussion

Pathogen attachment to human cells is a crucial step for infection. Bacteria must quickly and effectively attach to host cells to avoid being removed from the organism and to exert effects on host cells (332). In this study, a novel 2D gel approach was successfully developed, leading to the discovery of PDIA1 and PDIA3 as novel human proteins involved in *P. aeruginosa* adhesion to epithelial cells. This novel technique will be useful for the elucidation of other human receptors for other pathogens. Also, the future validation of the other candidates identified in this study might help to discover more receptors for *P. aeruginosa.* For example, Succinyl Co-A (SUCLG2), which were also identified by the technique, have been less studied in *P. aeruginosa* and seems to be a promising receptor. SUCLG2, is a key enzyme in the tricarboxylic acid (TCA) cycle, converting succinyl-coenzyme A (CoA) to succinate. Succinate is a preferred carbon source for environmental *P. aeruginosa* (333). Thus, SUCLG2 might also be involved in interactions with *P. aeruginosa*. This needs to be further validated.

The technique enables the identification of host-pathogen interactions between molecules on a PVDF membrane and whole-cell bacteria. The main advantage is not needing to previously know either the host receptor or the bacterial ligand. The results are a proof of concept that mimicking the cell surface *in vitro* and getting *P. aeruginosa* bound to the proteins in the membrane is a real possibility. However, it could be adjusted to increase the capacity to detect more receptors. For example, one of the biggest challenges of the applied technique is the separation of human plasma membrane proteins and their enrichment in the sample, thus we used the Mem-PER™ Plus Membrane Protein Extraction Kit to facilitate this process. However, the enrichment of plasma membrane proteins was not high (just seven of the final short-listed proteins were predicted to be on the cell surface), this might be overcome by longer cell fractionation. Also, 2D electrophoresis by isoelectrofocusing (IEF) and SDS-PAGE could affect the human proteins on PVDF membranes, further affecting the analysis of interactions. This limitation might be easily overcome by adapting the 2D electrophoresis step. For example, blue native polyacrylamide gel electrophoresis (BN-PAGE) is a separation technique performed under non-denaturing conditions and allows the characterisation of intact protein complexes (334-336). Overall, current approaches for

the identification of host receptors require previous knowledge of either human receptors or bacterial ligands and, the most recent whole-cell techniques limit the study to a protein-protein interaction using biotinylated approaches of the whole surface. Here, the technique could be adapted for the study of any molecule, for example, carbohydrates on the PVDF membrane, making it not just an unbiased, but a flexible technique.

The 2D Blot technique led to the study of the PDIA1 and PDIA3 role in *P. aeruginosa* attachment. PDIs are a family of enzymes that catalyse the formation, reduction or isomerisation of disulfide bonds of newly synthesised proteins in the endoplasmic reticulum (ER). They act as chaperones, forming part of a quality control system for the correct folding of proteins in the ER as part of the MHC I complex, which is essential for a proper immune response as it binds and presents short antigenic peptides from endogenously or exogenously sources (337, 338).

PDIA3 and PDIA1, together with calreticulin and tapasin, recruit and stabilise peptide-responsive MHC I clients, helping to ensure that only MHC I molecules loaded with optimal peptide epitopes are released from the PLC (339, 340). The literature highlights the critical role of PDIA1 and PDIA3 in the antigen presentation process and their potential to be a target for pathogens as immune evasion strategies. For example, the human cytomegalovirus (HCMV) US3 protein can prevent the transit of MHC-I molecules to the cell surface, by enhancing protein disulfide isomerase (PDI) degradation indefinitely (338).

PDIs have been implicated in the entry of human immunodeficiency virus (HIV) (341, 342), Sindbis virus (343), mouse polyomavirus (344), Newcastle disease virus (345) and Influenza virus (346) to the human cells. PDIA3 is necessary for effective influenza pathogenesis *in vivo*, and pharmacological inhibition of PDIs might be a novel anti-influenza therapeutic strategy (347, 348). PDIs are implicated in the attachment to human cells of some bacteria from different species of *Chlamydia* (349-351). Also, PDIA3 was reported as a major PDI involved in the interaction between *Burkholderia cenocepacia* and human epithelial cells (352). It is noteworthy, that PDIA1 and PDIA3 are highly expressed in the apical side of CF epithelial cells where *P. aeruginosa* is one of the most prevalent pathogens. Airway epithelial inflammation in CF increases the expression

levels of ER chaperone proteins, e.g., calreticulin, GRP78/BIP, and PDI (353). Studies suggest that chronic airway infection/inflammation-dependent increases in protein synthesis induce an adaptive ER stress response associated with an increased ER protein folding capacity in CF epithelia, increasing the ER chaperones BIP and PDI in CF bronchial epithelia (354).

Regarding their structure, the common feature among these proteins is the presence of at least one TRX-like domain, which can be catalytically active (a or a') or inactive (b or b'). Although the enzymatic function of these domains is not conserved. PDIA1 has four domains (a, b, b' and a') like thioredoxin (Trx), which form a compact U (54). The a and a' domains contain a Cys-X-X-Cys motif as a redox-active site essential for thiol-disulfide exchange reactions with substrate proteins. For recruiting clients, the b' domain provides the major substrate binding sites based mainly on a hydrophobic pocket including Phe240, Phe249 and Phe304. It is involved in the binding/release of various substrates such as cholera toxin, antigenic peptides and the α-subunit of prolyl-4-hydroxylase (P4-H) (355-358). PDIA3 contains two **a-**type domains (Cys-Gly-His-Cys and Cys-Gly-His-Cys active sites), one b and one b’ type domain and an ER retention sequence, Gln-Glu-Asp-Leu (329, 359).

PDIA1 and PDIA3 (ERp57) have considerable overlap in their protein structure, but importantly, they have different roles in cellular homeostasis. PDIA1 catalyses the oxidative folding of non-glycoproteins while PDIA3 promotes the oxidative folding of glycoproteins selectively in concert with the lectin chaperones calnexin (CNX) and calreticulin (CRT) (358, 360, 361), as unlike PDIA1, PDIA3 cannot control redox-dependent conformations and client recruitment as it lacks the Arg300 of human PDIA1, which is essential for redox-driven conformational change (358). Along with PDIA1 and PDIA3, calreticulin was identified, as essential for PDIA3 binding to its targets. Also, both PDIA1 (361) and PDIA3 (362) can be found in the cell membrane, meaning they are available for *P. aeruginosa* to bind on the surface of the human cell (363).

Advances in PDI research allowed the identification of novel small-molecule inhibitors such as LOC14. The inhibitor reversibly binds to a region adjacent to the active site of PDI, induces the protein to adopt an oxidised conformation, and inhibits its reductase activity (328). LOC14 inhibited recombinant rPDIA3 at an IC50 of

approximately 5 µM in epithelial cells (347, 348). Here, a concentration-dependent decrease in the attachment of *P. aeruginosa* to human bronchial epithelial cells by the pre-treatment of the cells with LOC14 was observed (Figure 27), which was further confirmed by molecular approaches (Figure 28, 29). Hence, drugs targeting PDIs may reduce *P. aeruginosa* colonisation *in vivo.*

Many bacteria and viruses mediate their pathogenesis by molecular mimicry of human proteins, for example, they may be homologous to receptors on human cells, thus the most homologous system to human disulfide isomerases was evaluated *in silico* to determine whether *P. aeruginosa* might share homology with human PDIs. In Gram-negative bacteria, the formation of disulfide bonds in extracytoplasmic proteins is catalysed by the Dsb protein system composed of numerous proteins located in the periplasm and inner membrane (330, 331). They transport bacterial virulence factors from the periplasm or cytoplasm to the outer membrane, medium or eukaryotic cell. Hence, the inactivation of Dsb genes reduces the pathogenicity of *P. aeruginosa* (330, 364). The most similar homologous protein from *P. aeruginosa* to PDIA1 and PDIA3 is DsbD. However, the orientation of DsbD helix embedding CxxC differs from PDIA1. Exploring other proteins from *P. aeruginosa* with even higher homology to PDIA1, *P. aeruginosa* TrxC and TrcGI were found. They have Trx folds and a catalytic sites almost identical to PDIA1, suggesting a possible hijacking by *P. aeruginosa* to human cells.

Overall, our findings indicate that human PDIA1 and PDIA3 are involved in *P. aeruginosa* attachment to human epithelial cells. As previously said, attachment to human cells is critical for *P. aeruginosa* pathogenesis and a common step between the different types of *P. aeruginosa* infections, thus our results might lead to the design of novel antimicrobial therapies in several ways: (a) drug inhibitors or antibodies targeting these proteins, as already suggested with LOC14 for the treatment of Influenza, (b) by discovering novel adhesins by future pull-down assays, meaning novel targets for the design of antibiotics, (c) exploring if this interaction might be a mechanism of immune evasion, as observed with some virus, will be a big step in understanding *P. aeruginosa´s* immune evasion strategies; and essential for the design of novel antimicrobials, including vaccinations that will help to tackle *P. aeruginosa* infections. The results demonstrated that is possible to elucidate host receptors without any previous

knowledge about the host-pathogen interaction. And that the elucidation of the host receptors can lead to novel treatments to prevent colonisation.

Chapter 4

Evaluation of TLR-4 ligands as adjuvants for vaccines against *P. aeruginosa*

Introduction

1. Adjuvants as vaccine components: definition, benefits and types

Adjuvants enhance and tailor the immunogenicity of vaccines when administered in conjunction with vaccine antigens (365)**.** They can range from synthetic small molecule compounds to complex natural extracts and particulate materials. Contrary to live-attenuated or inactivated vaccines, recombinant subunit vaccines do not contain live components of the pathogen, but only its antigenic parts. Currently, subunit protein vaccines are attractive due to their high safety profile; however, they show a reduced immunogenicity which necessitates the inclusion of adjuvants (366-368). Adjuvants have facilitated the development of vaccines against pathogens for which live-attenuated or inactivated vaccines are ineffective or unsafe. The correct identification and selection of an adjuvant, appropriate for the infection and the protective immune response, is crucial for novel effective and safe vaccines (368).

Adjuvants can be classified as delivery systems (DS) and immunostimulators (IMS), or a combination of both. DS work as carriers with which antigens are associated, creating local pro-inflammatory responses that recruit innate immune cells to the injection site, such as liposomes or virosomes (369). IMS, on the other hand, includes innate immune receptor ligands such as TLRs, NLRs or C-type lectins. Among the more advanced compounds are the TLR4 Monophosphoryl lipid A (3-*O*-desacyl-4'-monophosphoryl lipid A, MPLA) ligand (AS04), a detoxified form of bacterial lipopolysaccharide, which is part of the adjuvant system of the Cervarix HPV vaccine (from GSK), and the CpG oligodeoxynucleotide (ODN) of the TLR9 ligand (367, 369) (Appendix C).

Although many molecules have adjuvant effects, only a few have been licensed for use in humans (370). Aluminium salts (alum) were the only approved adjuvant for more than a century, and they continue to be the most widely used adjuvants. Antigens are adsorbed on alum, improving antigen uptake and presentation by antigen-presenting cells (371). Recently, other adjuvants have been approved for use in human vaccines such as MPLA, oil-in-water emulsions (MF-59), combinations of AS (adjuvant systems), e.g., AS03 is used in pandemic influenza vaccines (Pandemrix, Arepanrix, Adjupanrix,

GSK). Finally, virosomes, spherical lipid layers assembled *in vitro* with viral proteins, are currently used in influenza and hepatitis A human vaccines (370, 372). The approval of so few adjuvants for use in human vaccines has been attributed to the lack of knowledge of their action mechanism. Thus, understanding the mode of action of adjuvants is fundamental for assessing their safety at the development and regulatory stages (368).

2. Adjuvants in vaccine formulations against *P. aeruginosa*

A considerable limitation of early *P. aeruginosa* vaccine development is that many studies either did not consider the impact of the adjuvant in the *P. aeruginosa* vaccine formulation or did not specify the contribution of these molecules to the protective effect of the immunisation. Hence, knowledge about the role of adjuvants in *P. aeruginosa* vaccines is limited (149). However, studies have suggested that a Th1/Th17 balance might help to achieve protection against *P. aeruginosa* in murine models (137, 140, 141, 373) (Table 8).

Aluminium hydroxide ($Al(OH)_3$) and aluminium phosphate ($AlPO_4$), commonly referred to as “alum”, are the most widely used adjuvants in preclinical and clinical trials of *P. aeruginosa* vaccines (269, 270, 374). Alum generally stimulates a strong Th2-mediated immune response and is a poor inducer of cellular immune responses (373). Studies comparing alum with other adjuvants in murine models, such as naloxone (NLX, an opioid receptor antagonist) or deoxygenated lipooligosaccharide (dLOS), a TLR4 agonist derived from an *E. coli* LPS, have been evaluated (269, 375). NLX with alum administered with recombinant *P. aeruginosa* PilA (r-PilA), generated more robust Th1 and Th2 type responses (IgG1 and IgG2a), compared to the use of rPilA with alum. The addition of NLX did not alter IL-4 production, but it significantly increased INF-γ and IL-17, suggesting NLX shifts the alum Th2 response towards a more balanced Th2/Th1, which was reflected in better protection against *P. aeruginosa* (75% of mice survived after 7 days of infection) compared to immunisation with r-PilA with alum only (approximately 40% survival) (269). Mice immunised with OMP with dLos showed 60 to 90% survival after 8 days of infection with *P. aeruginosa*, while mice given OMP with alum showed 20% to 50% survival. The improved protective effect of the dLos adjuvant was proposed to be due to elevated Th1 and Th17 cell responses (375). Importantly, Al

$(OH)_3$ was the adjuvant used in the phase II study of the *Pseudomonas* IC43 (OprF/I) vaccine, and it did not improve its immunogenicity (270).

The licensed adjuvant, CpG ODN, a short, single-stranded synthetic DNA fragment containing an unmethylated CpG sequence has also been evaluated in *P. aeruginosa* vaccines (376). This adjuvant mimics the immunostimulatory effects of bacterial DNA via TLR9, stimulation in DC and plasmacytoid B cells produces inflammatory cytokines and activates (natural killer) NK cells, monocytes, and neutrophils (377). Intranasal vaccination with PcrV-CpG showed efficacy against *P. aeruginosa* pneumonia, with 73% of immunised mice surviving, compared with 30% of the mice immunised with PcrV-alum or CpG alone. Anti-PcrV IgG titres (IgG1, IgG2a, and IgG2b) and anti-PcrV IgA titres were significantly higher in mice immunised with PcrV-CpG than in the other groups, suggesting a Th1/Th2 response (373). Other FDA-approved adjuvants for use in human vaccines that have been used in pre-clinical studies of *P. aeruginosa* infections include AS04 (MPLA with alum) and MF59® (378, 379). However, in a study on a trivalent vaccine, PcrV28-294-OprI25-83-Hcp11-162 (POH), no improvement was observed with either relative to the use of alum in murine pneumonia models (similar survival rates and antibody titres) (380), consequently they do not stand out as promising options for effective *P. aeruginosa* vaccines.

Wu et al. evaluated whether the exopolysaccharide curdlan enhanced the protective effect of the Th17 response-stimulating antigen PopB, against *P. aeruginosa*. Curdlan is a component of the inner fungal cell wall and a mast cell chemoattractant (MC) (381), mediated by Dectin-1, which activates DC cells directing $CD4^+$ IL-17 differentiation, producing Th17 effector cells (382). Immunisation using PopB/PcrH with curdlan-induced a systemic and mucosal Th17 responses, but it did not increase the protective effect of immunisation relative to immunisation with antigen alone (comparable survival rate, 62.5%). This protective efficacy was antibody-independent but IL-17-dependent (144, 383). Schaefers et al. found that PopB-encapsulated PLGA nanoparticles also elicited Th17 responses. Immunisation with PLGA-entrapped PopB/PcrH or curdlan-PopB/PcrH resulted in Th17 responses that were three to four times higher than in mice immunised with PLGA or PopB/PcrH alone. This was reflected in the protective effect of immunisation after 6 days of infection (~75% survival PLGA-entrapped PopB/PcrH, ~50%

curdlan-PopB/PcrH, 0% PopB/PcrH) (in contrast to Wu et al.). Schaefers et al. also reported a lack of antibodies with opsonophagocytic killing activity against *P. aeruginosa* suggesting Th17-mediated protection (141, 144). The mucosal adjuvant LT (R192G/L211A) or detoxified mutants (dm)LT, an 84 kDa polymer protein derived from the thermolabile enterotoxin (LT), induces strong IL-17 secretion and antigen-specific Th17 responses (140, 384). Baker et al. (2019) showed that mice immunised with OMPs and dmLT produced significantly more antigen-specific IgG and Th1 and Th17 $CD4^+$ memory T cells in the lung compared with the control groups (adjuvant alone or sham). Immunisation with OMPs with dmLT protected the mice against lethal *P. aeruginosa* lung infection and was associated with the early production of IFN-γ and IL-17 (Th1/Th17 responses). Mice immunised with OMPs+dmLT showed 53% survival in 10 days, while all other groups completely succumbed to infection within 1–3 days.

3. Adenoviral vectors in *P. aeruginosa* vaccines:

The contribution of adjuvants to the protection against chronic *P. aeruginosa* infection has been examined with the use of adenoviral vectors. Krause et al. evaluated the systemic and mucosal immunogenic properties of a non-human primate-based adenovirus vector, AdC7, expressing *P. aeruginosa* OprF (AdC7OprF) and compared it with a human serotype (Ad5OprF), using an agar-beads chronic infection model in mice (277). Intramuscular immunisation of mice with AdC7OprF induced similar levels of anti-OprF IgG in serum and mucosa but superior levels of anti-OprF IgA, compared to Ad5OprF vector. AdC7OprF-induced anti-OprF IgG antibodies that were predominantly IgG1 and IgG2b isotypes, followed by IgG2a and IgG3, with lower titres of IgG2a, IgG2b and IgG3 compared to Ad5-immunised animals (277). Immunisation with either Ad5OprF or AdC7OprF resulted in 100% of mice being protected against *P. aeruginosa*, and 0% survival in the controls after 6 days of infection.

The intratracheal administration of AdC7OprF vaccine induced: (i) protective immunity against *P. aeruginosa* despite the presence of lower total systemic anti-OprF IgG titres, (ii) an OprF-specific IFN-γ response in lung $CD3^+$ T-cells, (iii) OprF-specific IgG and IgA, and (iv) lung T-cell OprF-specific INF-γ compared to immunisation with Ad5OprF. In general, the favourable mucosal immune responses in mice following immunisation

with the AdC7 vaccine vector favour the further development of adenoviral vectors based on non-human primates as vaccines to induce protective lung mucosal immunity against *P. aeruginosa* (277).

4. Live-attenuated pathogens in *P. aeruginosa* vaccines:

The use of live attenuated pathogens such as *Salmonella* or *Francisella tularensis* as antigen delivery systems stands out among the attempts to improve the mucosal vaccine responses against *P. aeruginosa*. Preclinical studies (385, 386) and a phase I-II clinical trial (19887136)(387) involving *Salmonella* species for the nasal or oral delivery of *Pseudomonas* OprF or OprI, induced IgG and IgA antibodies. In murine models, subcutaneous vaccination with an S. Typhimurium LH430 expressing OprF-OprI achieved the highest levels of protection and specific immunoglobulin titres than oral immunisation (s.c. vaccination achieved 77.78% protection, in contrast to 41.18% via oral administration) (388). *F. tularensis* has also been used for the delivery of *P. aeruginosa* PilA, OprF, and FliC. Mice immunised with a live vaccine strain (LVS) expressing FliC, produced high levels of antibodies specific for *P. aeruginosa*, demonstrating the potential of this bacterial strain for the delivery of *P. aeruginosa* antigens (389).

Taken together, Th2 cell responses are not optimal for achieving protection against *P. aeruginosa* and a shift to Th1 or Th17 responses may improve the efficacy of *P. aeruginosa* vaccines. Previous studies have evaluated several antigens that have been protective in murine models, but we still lack more information on the mechanisms through which adjuvants might enhance the effect of the already identified *P. aeruginosa* antigens, to improve the rational design of vaccines against *P. aeruginosa.* Importantly, only three vaccine candidates have reached phase III trials: His-tagged outer membrane protein hybrid OprF-OprI protein (IC43) (270), a bivalent flagellin preparation (266) and an octavalent *O*-polysaccharide-exotoxin A conjugate (Aerugen®) (256). No vaccine achieved licensure. Hence, there is a need for the study of novel effective antigen-adjuvant systems for vaccines against *P. aeruginosa.*

Table 8. Examples of *P. aeruginosa* adjuvanted vaccine evaluated in pre-clinical studies.

Ag/Adj	Route	*P. aeruginosa* strains	Immunisation (N° x dose, Ag, Adj)	TR	Ref
PopB+PcrH/ Curdlan	IN	ExoU$^+$ PAO1 ($7x10^5$ CFU/mouse)	3x35 µg/dose, 10mg/mL	Th17	(144)
PopB+PcrH/Curdlan	IN	ExoU$^+$ PAO1 and live-attenuated PAO1Δ*aroA* (C^+) ($7x10^5$ CFU/mouse)	3x35 µg/dose, 10mg/mL	Th17	(141)
OMPs / dmLT	ID	PA01 ($1.4x10^7$ CFU/mouse)	3x1 µg/mouse, 1 µg	Th1/17	(140)
OMP/CIA06	IM	GN-H3 and PA103 (10 LD_{50})	2x5 µg/mouse, 0.5 µg dLOS + 25 µg of alum in 100µL of PBS	Th2/1/17	(390)
r-PilA/ alum+NLX	SC	PA01 and a clinical isolate ($3-5x10^6$ CFU)	3x5 µg/mouse, 6 mg/g body weight (NLX) and 200 mg of alum	Th2/Th1/ Th17	(269)
PcrV/ FA, alum, or CpG ODN	IP	PA103 *exoS$^-$, exoT$^+$, exoU$^+$ and exoY$^+$* ($1.5x10^6$ CFU)	3x10 µg/dose, 100µL/dose FCA/FIA, 100µl/dose alum or 10µg/dose of CpG ODN	FA and CpgODN: Th1/Th2. Alum: Th2	(373)
PcrV/CpG ODN	IN	PA103 strain (ATCC 29260, $1.5x10^6$ CFU/ mouse)	3x5 µg/mouse, 6 mg/g body weight and 200 mg of alum	Th2/Th1	(377)
OprF/AdC7 (AdC7OprF)	IM, IT	PA encapsulated in agar beads ($5x10^6$ CFU/mouse).	$1x10^9$ - 10^{11} pu/animal	Th2/Th1	(391)
SseJ-PcrV and SseJ-OprF-I/ Attenauated S.Typhimurium SV9699	IP	PAO1 ($9x10^6$ CFU/mouse)	$1x2×10^5$ CFU/ animal	NS	(392)
PilA$_{Pa}$, OprF$_{Pa}$, FliC$_{Pa}$/*Attenuated F. tularensis* LVS	IN	PA1244 (PilA$_{Pa}$) and PA14 (OprF$_{Pa}$, and FliC$_{Pa}$)	1x~100 CFU / animal	NS	(389)

Abbreviations; Adj: adjuvant, Ag: antigen, ID: intradermal, IM: intramuscular IN: intranasal, IP: intraperitoneal, IT: intratracheal, NS: Not specified, SC: subcutaneous, TR: Type of response.

5.Novel TLR-4 ligands as adjuvants:

Adjuvants that bind to TLR4 are immunostimulants that initiate signalling via the MyD88 pathway to activate NF-κB, which induces pro-inflammatory cytokine secretion and drives naive T-cells towards Th1-type cell polarisation. They can also signal through the TIR domain-containing adaptor inducing interferon-β (TRIF), leading to the activation of interferon regulatory factor 3 (IRF3), producing limited amounts of type I interferons (140, 393). Lipid A binds to TLR-4 inducing a massive immune response that can cause a septic shock, so even with high adjuvant activity, it is unsuitable for safe use in humans (394, 395). MPLA was developed to solve this issue, being a potent but less toxic lipid A-based immunomodulator. It was approved as part of the alum-containing AS04 Adjuvant System in vaccines against HPV (396) and hepatitis B (397, 398); and combined with

other adjuvants such as QS-21 (i.e., AS15, AS02 and, especially, the clinically approved AS01) (399, 400). Access to these AS04 adjuvants is restricted by GSK.

The total synthesis of MPLA is complex (around 24 chemical steps), expensive, and not chemically homogeneous, making it difficult to assess its quality. To address this limitation, our collaborators Prof. Francesco Peri and team developed a series of TLR 4 ligands, which are referred to here as FP compounds. They are composed of a glucosamine core functionalised with a phosphate and three fatty acid (FA) chains of variable length (10 to 14 carbons). They are selective TLR-4 agonists with proinflammatory properties. Their synthesis in six steps is significantly shorter than that of MPLA, resulting in a reduced cost, thus supporting their production on a global scale (394, 401, 402). The absence of hydroxylated fatty acid chains greatly simplifies FP chemical synthesis, compared to MPLA or other monosaccharide lipid A mimetics, such as SDZ MRL 953, making the production of these compounds scalable at an industrial level (394, 401, 402).

The FP18 compound behaves as a selective TLR4 agonist and is not active on TLR2 (403). FP18 bound TLR4 at sub-micromolar affinities and stimulated the MyD88-dependent pathway in human THP-1 cells, ultimately stimulating the release of TNF, IL-1β, and IL-6 cytokines. FP18 showed a higher potency than MPLA in inducing IL-1β release due to a greater ability to activate the NLRP3 inflammasome. Also, FP18 activates a TRIF-dependent pathway leading to a type I IFN response (403). Finally, FP18 lacks obvious *in vivo* and *in vitro* toxicity, and the straightforward synthesis procedure compared to MPLA supports the preclinical and clinical development of FP molecules as novel lead compounds to produce effective vaccine adjuvants.

A second generation of FP compounds was also produced to investigate whether mimicking the oligosaccharide core by addition of a carbohydrate moiety to FP compounds could increase the affinity for the receptor and its biological activity (Figure 34) (402). The new series of FP20 compounds showed a dramatic increase in the activity of glycosylated FP20, compared to the parent molecule and suggested a specific role of the added glycosyl moiety in the interaction with the receptor. However, the pharmacodynamic effect, probably based on sugar interaction with the receptor, has not been established yet. Overall, the synthesis of this panel of compounds is much shorter

than MPLA, which is a huge advantage in terms of industrial scalability, waste production, and final cost (394, 401, 402).

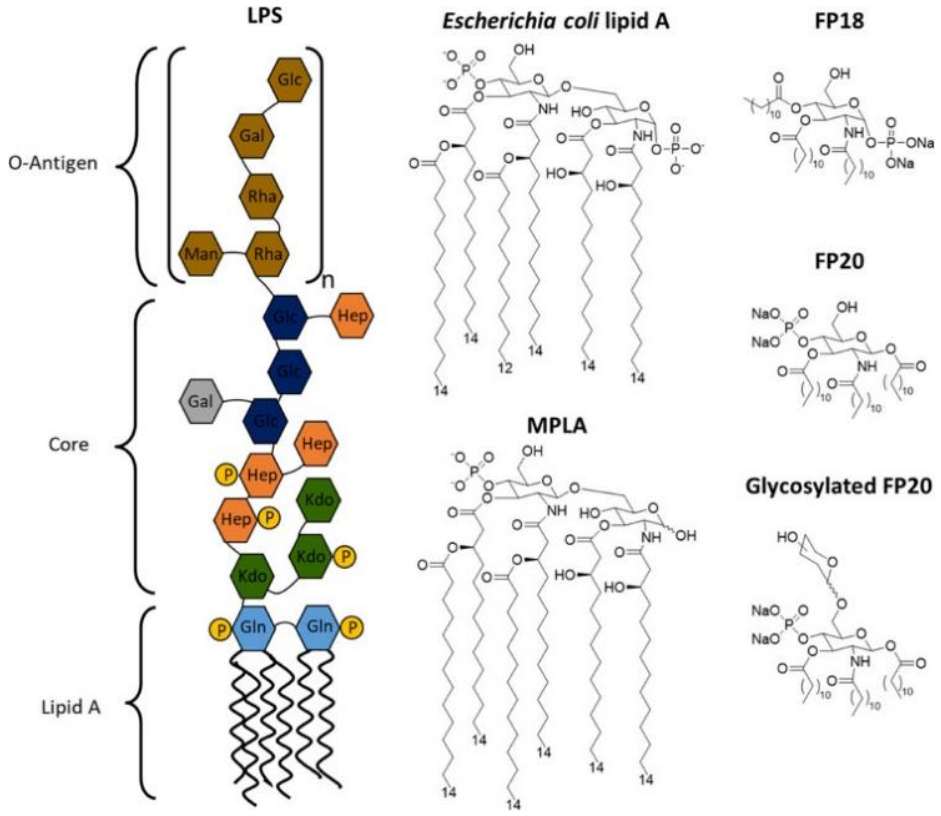

Figure 34. The structures of the novel TLR-4 agonists FP18, FP20Rha and the glycosylation analogues compared with LPS and MPLA. (402).

6.OprF is one of the most studied and evaluated antigens for *P. aeruginosa* vaccines.

The use of OprF, alone or fused to OprI, as a vaccine candidate has been evaluated in several formats such as recombinant subunit vaccines (404, 405), DNA vaccines (406), viral and bacterial vector systems (277, 388) and a dendritic cell vaccine (407). In addition, Weimer and colleagues used the C-terminal OprF epitope 8 (OprF311-341) as a fusion to OprI and flagellins to protect against non-mucoid *P. aeruginosa* which usually initiates colonisation of CF patients (323, 408). Recently, an mRNA-OprF-I vaccine showed promising results for the prevention of *P. aeruginosa* infection, eliciting Th1/Th2 mixed or slighted Th1-biased immune response and reducing bacterial burden and inflammation in murine models of burn and systemic *P. aeruginosa* infection. Overall,

OprF is an excellent candidate to be used in several vaccine platforms against *P. aeruginosa* (289) (Figure 35).

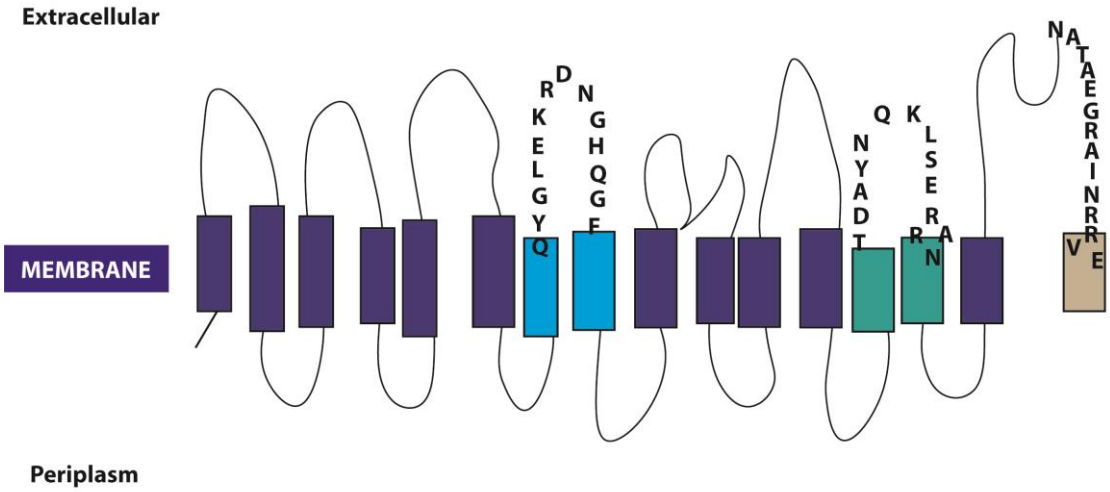

Figure 35. Structure of *P. aeruginosa* OprF in the bacterial membrane. The highlighted epitopes have shown immunogenicity (276).

Aim and Objectives:

Due to *P. aeruginosa's* high antimicrobial resistance, vaccines represent one of the best alternatives to combat infections, but despite several efforts, no vaccine against *P. aeruginosa* has been approved. Adjuvants can improve the immunogenicity and efficacy of subunit vaccines, however, $Al(OH)_3$ failed to improve the immunogenicity of a leading *P. aeruginosa* vaccine candidate, IC43 (OprF/I). Thus, there is still a need for effective antigen-adjuvant systems against *P. aeruginosa.* MPLA is a clinically approved adjuvant that stimulates TLR4. However, it lacks availability, and its synthesis poses manufacturing challenges. Hence, the evaluation of novel synthetic simplifications of MPLA based on monosaccharides (FP compounds) to improve the efficacy of *Pseudomonas* OprF antigen, which has already demonstrated its safety in human trials, might speed up the development of novel effective vaccine candidates against *P. aeruginosa* infections.

Objectives:

1. To evaluate the protective effect of FP18 and FP20Rha compounds as adjuvants, in combination with rOprF, in a murine model of *P. aeruginosa* acute pneumonia.

2. To assess the humoral responses stimulated by the subcutaneous immunisation of mice with FP18 or FP20Rha adjuvanted rOprF.

3. To examine the T-cell responses stimulated by the subcutaneous immunisation of mice with FP18 or FP20Rha adjuvanted rOprF.

Methods

Ethical statement:

All the work involving the animals was approved by the UCD Animal Research Ethics Committee (AREC-21-19), and mice were maintained according to the regulations of the

Health Products Regulatory Authority (Directive 2010/63/EU and Irish Statutory Instrument 543 of 2012), under authorisation number AE18982/P209.

Recombinant OprF_his expression:

Genomic DNA was extracted from *P. aeruginosa* PAO1 strain and the *oprF* sequence was amplified by PCR with specific primers: 5'-CGCGGATCCAAACTGAAGAACACCTTAGGCGTTGTC-3' (Fw) and 5'-CCCAAGCTTTTACTTGGCTTCGGCTTCTACTTCGGC-3' (Rev). The *oprf* gene fragment was cloned (BamHI and Hind III) into the pET28a expression vector (Novagen) with the gene for kanamycin resistance. It has a Histidine (His6) affinity tag at the 5' end of the polylinker that functions as a high-affinity nickel-binding domain in the translated protein. The plasmid was then sequenced, and the sequence was compared with the sequence reported in GenBank using SnapGene software. The gene fragment is flanked by BamHI and HindIII restriction enzyme sites and is under the strong T7 promoter. The 6xHis tag is located in the N terminal domain (Figure 36).

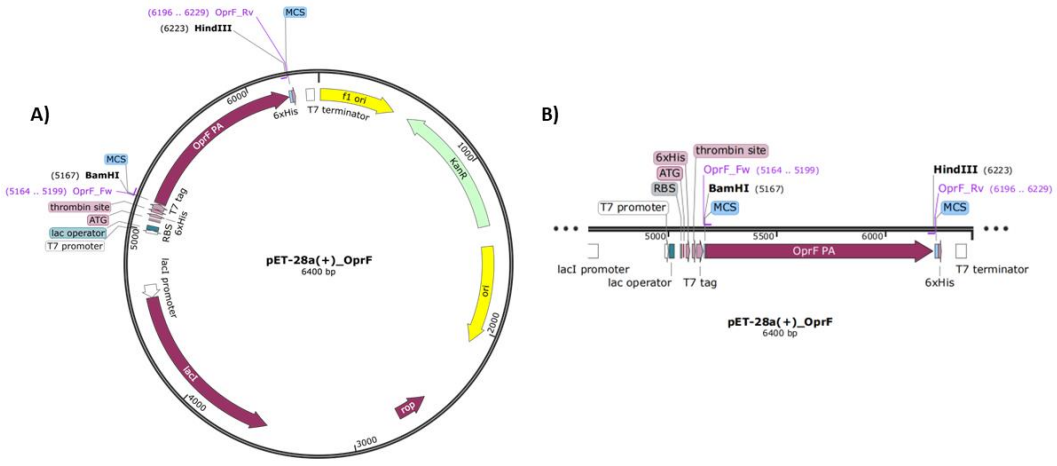

Figure 36. Expression of rOprF in a pET-28 a(+) vector. A) Map of the plasmid used for rOprF expression. B) Linear representation of the plasmid region where *oprf* was inserted (1050 bp).

The Qiagen expression host cells, *E. coli* BL21 (DE3) (Quiagen), were made chemically competent and transformed with the resulting plasmid pET28a-oprFhis following manufacturer´s instructions by the heat-shock method. Before protein purification, overnight cultures (100 mL) containing *E. coli* BL21 (DE3) cells, transformed with pET28a-oprFhis vectors containing *oprf*, were re-inoculated into 2 litres of LB broth

supplemented with 1% of glucose and 50 µg/mL kanamycin and cultured to the mid-log phase at 37 °C with shaking at 200 rpm. To determine the optimal conditions for OprF expression, overnight cultures (5mL) containing *E. coli* BL21 (DE3) cells transformed with pET28a-*oprf*_his vectors were re-inoculated into 50 mL of LB broth supplemented with 1% of glucose and 50 µg/mL kanamycin and cultured to the mid-log phase at 37 °C with shaking at 200 rpm. Cultures were induced with 1mM IPTG for 3h, 5h or overnight (ON) at either 37ºC or 20ºC. A sample before induction with IPTG was taken (pre-induction). Samples taken at each time point (1mL) were pelleted at 2500× *g* for 10 min at 4 °C, and the cell pellets were resuspended in 100 µL lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 8) supplemented with 1 mg/mL lysozyme and 1X complete mini EDTA-free protease inhibitor cocktail (Roche, Switzerland). The bacterial pellets were incubated for 30 min at 37ºC with gentle shaking, and then centrifuged for 30 min at 16,000 × g and 4ºC. The supernatants (S, Soluble fraction) were separated from the pellets (I, insoluble fraction). Western Blot analysis with an anti-6x histidine tag monoclonal antibody (Invitrogen, MA1-21315) confirmed the expression of rOprF_his.

Recombinant OprF_his purification:

The optimised conditions of rOprF_his expression were used for the purification of the antigen. Overnight cultures (5mL) containing *E. coli* BL21 (DE3) cells transformed with pET28a-*oprf*_his vectors were re-inoculated into 1L (x6) of LB broth supplemented with 1% of glucose and 50 µg/mL kanamycin and cultured to the mid-log phase at 37 °C with shaking at 200 rpm. The cultures were then induced with 1 mM IPTG for 20 h at 20°C at 200 rpm (optimised conditions, Figure 38). Bacterial cells were pelleted at 2500× *g* for 10 min at 4 °C, and the cell pellets were resuspended in 100 mL Ni-NTA lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 8; 50 mL/1 L culture) supplemented with 1 mg/mL lysozyme and 1X complete mini EDTA-free protease inhibitor cocktail (Roche, Switzerland). The bacterial pellets were incubated for 30 min at 37ºC with gentle shaking, and then sonicated on ice 10 times for 30 s at 20% amplitude, with 30 s rest intervals. The crude lysates were then centrifuged for 40 min at 16,000 × g and 4ºC, and the supernatants (soluble fraction) were separated from the pellets (insoluble fraction). The pellets were washed with 2% of Triton X- 100 in PBS and then washed with PBS. Recombinant OprF_his was extracted from the membrane, by

resuspending the pellets in 20 mL of denaturing buffer (basic buffer with 8 M urea), and then incubated, sonicated, and centrifuged as before. Purification was performed using HisPur™ Ni-NTA (nickel-nitrilotriacetic acid) Superflow Agarose (Thermo Fisher) resin in a gravity flow column. The resin (2 mL/ 1L culture) was equilibrated with the denaturing buffer (10 mM imidazole) and washed twice with 20 mM imidazole (1 column volume). Finally, rOprF_his was eluted with 350 mM of imidazole. Aliquots of each fraction were collected for analysis by SDS-PAGE. Urea and imidazole were removed by extensive dialysis using a 10K MWCO (molecular weight cut-off) SnakeSkin™ Dialysis Tubing (Thermo Fisher), firstly incubating 4h into a buffer containing 2M urea, 50 mM NaH_2PO_4 and 300 mM NaCl, and then performing overnight dialysis to a buffer containing 50 mM NaH_2PO_4 and 300 mM NaCl, supplemented with 2% glycine to avoid protein precipitation in the absence of urea. The antigen was further purified by Size Exclusion Chromatography (SEC) on a pre-equilibrated Hi load 16/60 Superdex 75 SEC column on an AKTA Vivo instrument. Fractions were pooled and concentrated by centrifugation (4,000 × g, 4ºC, 1 h) in an Amicon® Ultra 15 mL centrifugal device with Ultracel® 10 MWCO filter unit (Sigma-Aldrich). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit, and protein identity was confirmed by LC-MS as described before.

Quantification of endotoxin levels (LPS):

The endotoxin levels of the purified protein batches were quantified using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher), following the manufacturer's instructions. Sterile 96-well, flat-bottomed plates were pre-warmed at 37ºC for 10 min, and all the steps were performed at this temperature. Endotoxin standard dilutions, blanks, and samples were added to each well in duplicates (50 µL/well). Then, the Amebocyte Lysate Reagents were reconstituted immediately before their use by gently swirling the lyophilised powder in 1.7 mL endotoxin-free water; then, 50 µL/well was added, the plate was gently tapped ten times on the side to mix the reagents, and incubated for six min. The Chromogenic Substrates were reconstituted with 3.4 mL endotoxin-free water, mixed gently by tilting and swirling the vials and pre-warmed at 37ºC for five min before use. After the incubation time, the substrate was added (100 µL/well), and the chromogenic reactions were stopped after six min by

adding the stop solution (25% acetic acid, 50 µL/well). Finally, regression lines were produced from the standard absorbances and used to determine the LPS concentrations of the samples. A cut-off of <2 EU/mouse is recommended for mice immunisation (409).

Immunisation of mice with rOprF_his and FP compounds:

Female C57BL/6J mice (6-8 weeks old; n=7) were purchased from Charles Rivers (UK), randomly grouped and housed into individually ventilated cages upon arrival to the Biomedical Facility in UCD, and acclimatised for one week to their new environment. Food and water were available *ad libitum*. Sigma Adjuvant System® (SAS) vials were resuspended as per the manufacturer's instructions in protein buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) and mixed 1:1 with either the saline buffer alone or 50 µg/mouse of rOprF_his. SAS is a commercial adjuvant and each dose contains 12.5 µg/mouse of MPLA from *Salmonella minnesota* and 12.5 µg/mouse of synthetic Trehalose Dicorynomycolate in 2% oil (squalene)-Tween® 80-water. FP compounds were prepared in the same buffer as SAS and rOprF_his, adding 25 µg/mouse from a stock previously solubilized in dimethyl sulfoxide (DMSO, concentrations of DMSO were normalized in all the groups, assuring 11.8% of DMSO/ dose). Mice were immunised subcutaneously three times, two weeks apart. Mice were separated with 4 or 3 mice per cage (n=7 per group), and then immunised subcutaneously with 100 µl containing 50 µg rOprF_his alone, 50 µg rOprF alone + 25 µg FP18, 50 µg rOprF alone + 25 µg FP20Rha or 50 µg rOprF + SAS (~25 µg of active component). Mice were manually restrained by the scruff of their back and carefully immunised subcutaneously in their right flank.

Serum IgG Antibodies Determination

Blood was collected from each mouse via cheek bleeds one week after the third booster for serological analysis. The blood was allowed to stand at 4 °C overnight before being centrifuged for 30 min to isolate serum, which was transferred to freshly labelled tubes and stored at −80 ºC until required. Microtiter plates were coated overnight with 100 µL of purified rOprF_his antigen (0.5 µg/mL) in 0.2 M sodium carbonate pH 9.6 at 4 °C. Plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS containing 1% FBS for 1 h at room temperature. Five-fold dilutions of sera were added to wells in triplicate and incubated for two hours at room temperature. The wells were washed 3 times in PBS-T and incubated in 100 µL of HRP-conjugated anti-

mouse antibodies specific for total IgG, IgG1 or IgG2c (diluted 1:5000 in PBS containing 1% FBS) at room temperature for 60 min. Plates were washed again as before, followed by two additional washes containing 400 µL/well of 1 X PBS. TMB substrate (100 µL) was added to each well, and the plates were incubated at room temperature until a colour change was observed, at which time reactions ceased by using 100 µL/well of 2M sulfuric acid. Plates were read at an absorbance of 450 nm within 30 min. Sera from previous immunisations with adjuvants only (data not shown) were used as the control for each group immunised with adjuvants. The area under the curve was calculated for each triplicate and used to compare the antibodies between groups, using GraphPad Prism 8.0.2.

Bacterial challenge and determination of organ colonization:

Two weeks after the last immunisation, mice were challenged by oropharyngeal aspiration with $5.9x10^6$ CFU/mouse of *P. aeruginosa* CF KK1 isolate (297) and culled after 24 h. Lungs, spleens and stomachs were aseptically harvested in PBS, weighed, and homogenised in the TissueLyser II (Qiagen) for 15 min at the maximum frequency (30 Hz/s) using 3.2-mm stainless-steel beads. Organ homogenates were then serially diluted in PBS, plated onto LB agar in duplicate, and incubated at 37ºC. After 24 h, CFU were counted. Statistically significant differences between control and immunised mice were analysed using the unpaired non-parametric Kruskal-Wallis test (p-value<0.05) when the data did not pass the Shapiro-Wilk normality test.

Immunophenotyping by splenocyte *ex vivo* restimulation assays and flow cytometry

Female C57BL/6J mice (6-8 weeks old; n=7) were immunised subcutaneously once. After 14 days, mice were humanely sacrificed, and the individual spleens were aseptically harvested in PBS, mechanically disrupted, and filtered with a 70-µm cell strainer. Erythrocytes were depleted by hypotonic lysis with an Ammonium-Chloride Potassium (ACK) buffer. The splenocytes were then counted using the automated counter Countess™ 3 FL (Invitrogen) and seeded in triplicate into round bottom, 96-1pen/strep. Cells were stimulated with 10 µg of rOprF_his for 60 h at 37ºC and 5% CO_2. Afterwards, the cells were incubated for 5 h (37ºC, five per cent CO_2) with 5 µg/mL brefeldin A, then harvested by centrifugation, washed with PBS containing 1% FBS, and incubated with rat anti-mouse CD16/CD32 (1:100; BD Biosciences, USA) for 5 min on ice. The cells were

then centrifuged and washed, and extracellular markers were labelled for 30 min on ice with a master mix of fluorophore-conjugated antibodies (50 µL/well, table 3) in PBS containing 1% FBS mixed 1:1 with Brilliant Stain Buffer (BD Biosciences) and the ViaKrome 808 Fixable Viability Dye (1:100, Beckman). The wells were washed as before and then the cell membranes were permeabilised with BD Cytofix/CytopermTM (BD Biosciences) for 20 min on ice. Then, wells were washed with 1X BD Perm/Wash™ Buffer (BD Biosciences) and then cells were incubated for 30 min on ice with a master mix of intracellular fluorophore-conjugated antibodies (table 3) in 1X Perm/Wash™ Buffer with Brilliant Stain Buffer (1:1) for immunophenotyping. Finally, the cells were washed with 1X BD Perm/Wash™ Buffer and stored overnight at 4ºC. The following day, the cells were washed again and analysed using a Beckman Coulter CytoFLEX LX (NUV full configuration) cytometer. Quality control (QC) of the instrument was performed using Beckman Coulter Daily QC beads and IR Daily QC as per the manufacturers' specifications. Data analysis was performed with Beckman Coulter CytExpert v.2.4 software. Fluorescence minus one (FMO) control was prepared using splenocytes from one mouse in the control group and used for every single marker (<0.5%) to generate the gating strategies. Data were extracted as the percentage of parental cells. The $CD3^+$ marker was used to identify T-cells, and then $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells percentages were quantified. Shapiro-Wilks normality test was used to evaluate data distribution. Statistically significant differences (p-value<0.05) were evaluated using paired One-way ANOVA for data with normal distribution, or Wilcoxon matched-pairs signed rank test for data that did not follow normal distribution.

Table 4. Composition of the Ab master mix (BD Biosciences) used to label both intracellular and extracellular receptors and cytokines for their analysis by flow cytometry.

Target location	Ab target	Fluorophore	Reading channel	Dilution
Extracellular	CD3	BV786	V763	1:100
	CD4	BUV395	V405	1:200
	CD8	APC-H7	R763	1:100
	CD25	BV421	V450	1:100
	CD44	PE-Cy7	Y763	1:200
	CD49b	FITC	B525	1:100
	CD62L	BV480	V252	1:200
	γδ	R718	R712	1:100
Intracellular	IFN-γ	BV650	V660	1:100
	IL-4	BV605	V610	1:100
	IL-17A	PE-CF594	B610	1:100
	IL-22	PE	Y585	1:100
	TNF	BB700	B690	1:100
	FoxP3	AlexaFluor 647	R660	1:100

Results

1. Purification of the recombinant OprF-His Protein:

To evaluate the effect of the adjuvants in combination with the rOprF_his antigen, the *oprf* gene was cloned into a pet28a(+) vector and its correct insertion was verified by colony PCR. A band of 1050 bp confirmed the successful cloning and transformation of *E. coli* BL21 (DE3) containing pET-28 a(+)::*oprf.* The best conditions for rOprF_his expression were evaluated at three different times (3h, 5h and overnight) and two different temperatures (37ºC and 20ºC). Optimal rOprF_his expression was observed 20 hours after induction with 1 mM IPTG at 20ºC, as indicated by a more intense band in the insoluble fraction (Figure 38). However, expression at 20ºC after 5h was comparable with the chosen conditions.

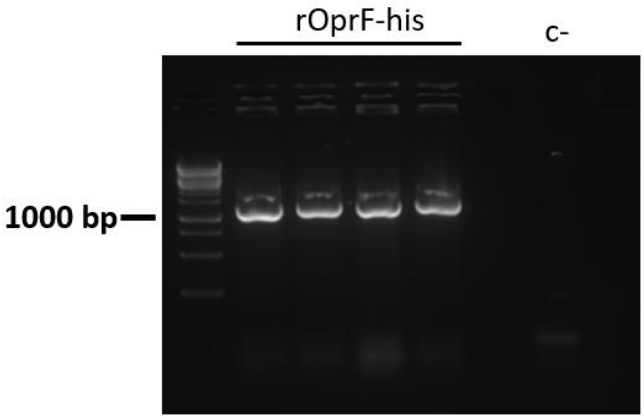

Figure 37. Colony-PCR of the *E. coli* BL21 (DE3) containing pET-28 a(+):: *oprf* indicating the successful insertion of the gene into the expression vector.

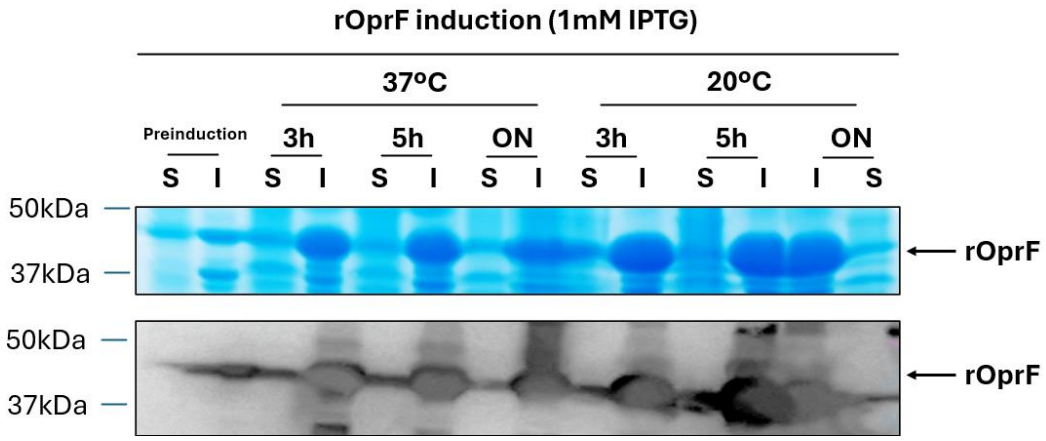

Figure 38. Optimisation of the rOprF expression in *E.coli* BL21 (DE3). Cultures of *E.coli* BL21 (DE3) pet28::*oprf* were induced with 1mM IPTG for 3h, 5h or overnight (ON) at either 37ºC or 20ºC. A sample before induction with IPTG was taken (pre-induction). The supernatants (S, Soluble fraction) were separated from the pellets (I, insoluble fraction). Western Blot analysis with an anti-6x histidine tag monoclonal antibody (Invitrogen, MA1-21315) confirmed the expression of rOprF_his.

Once the optimal conditions for rOprF_his expression were determined, rOprF_his was purified in two steps by purification on a Ni-NTA purification system followed by SEC, as indicated by a single large peak on chromatograms between fractions 25 and 30 mL representing the rOprF protein (Figure 39A), and its purity was determined by SDS-PAGE (Figure 39B). A pure fraction was achieved, which was apparent by observing the lack of other bands in the SDS PAGE gel (Figure 39C) or peaks during SEC. The rOprF eluted at a fraction volume of 40mL, equivalent to a molecular weight of 80kDa, indicating the dimerisation of the proteins, a smaller peak also eluted at a fraction volume of 60mL equivalent to a molecular weight of 40kDa, thus the monomer of rOprF_his. The identification of the purified antigen was further confirmed by MS analysis (35 peptides matched; sequence coverage 78%) and the endotoxin levels were below the cut-off suggested per mice (~0.20 EU/ dose) taking >2 EU/ dose as the limit for immunisation (409) (Appendix C).

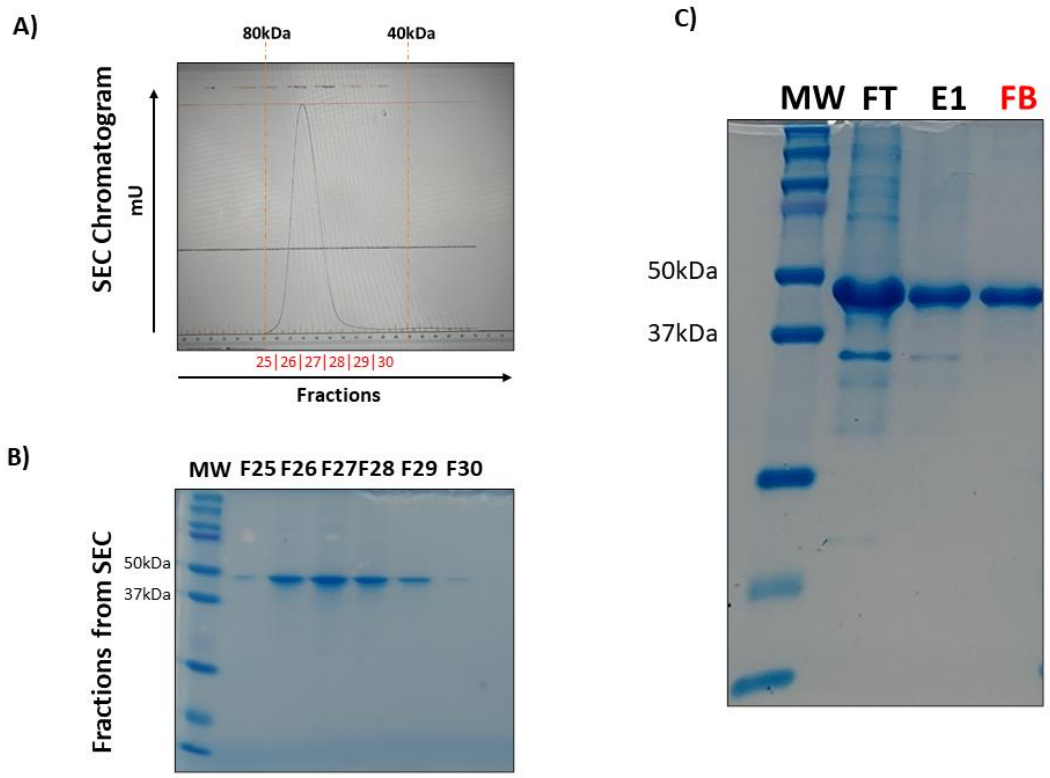

Figure 39. Purification of rOprF-his (41kDa) by nickel affinity column continued by size exclusion (SEC) chromatography. A) The purification of expressed and affinity-purified rOprF_his using a HiLoad® 16/600 Superdex® 75 pg column 16/60**.** Fractions collected representative of the large peak at a run volume of 40-52 mL (fractions 25-30). **B)** Purity of fractions 25-30 was visualised on 12% SDS gel. **C)** The purity and identity of the pooled SEC fractions were confirmed by SDS-PAGE. FT, Flow-through of the first purification by nickel affinity chromatography; E1, elution after nickel affinity chromatography; FB, final batch, after purification by SEC (pooled fractions 25-30).

2. Bacterial clearance in lung and dissemination to spleen after immunisation with rOprF_his combined with FP adjuvants

To examine whether FP18 and FP20Rha adjuvants enhanced the protective efficacy of rOprF against *P. aeruginosa*, they were assessed in a *P. aeruginosa* KK1 acute pneumonia model in C57BL/6J mice (n=7). Either FP18 or FP20Rha were combined with rOprF_his (OprF+Fp18, OprF+FP20Rha). The Sigma Adjuvant System (SAS) in combination with the antigen rOprF_his (OprF+SAS) was used as a positive control and the three groups were compared to immunisation with rOprF_his in the absence of any adjuvant (Figure 40). Neither FP18 nor FP20Rha showed any obvious detrimental effect on the mice, as determined by lack of weight loss. Generally, immunisation with OprF+SAS caused higher weight loss than immunisation with rOprF alone or combined with FP18 and FP20Rha adjuvants (Figure 41). All the mice recovered and gained weight

after completion of all immunisations. There were no significant differences in weight loss after the bacterial challenge between the groups (Figure 41).

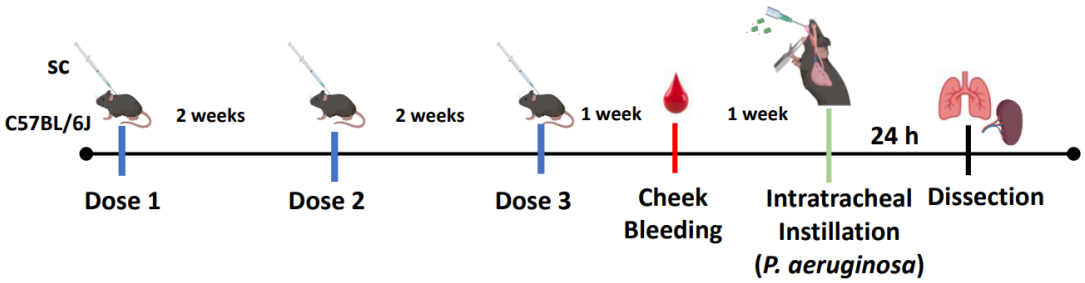

Figure 40. Timeline of mice immunisation, blood collection and *P. aeruginosa* challenging.

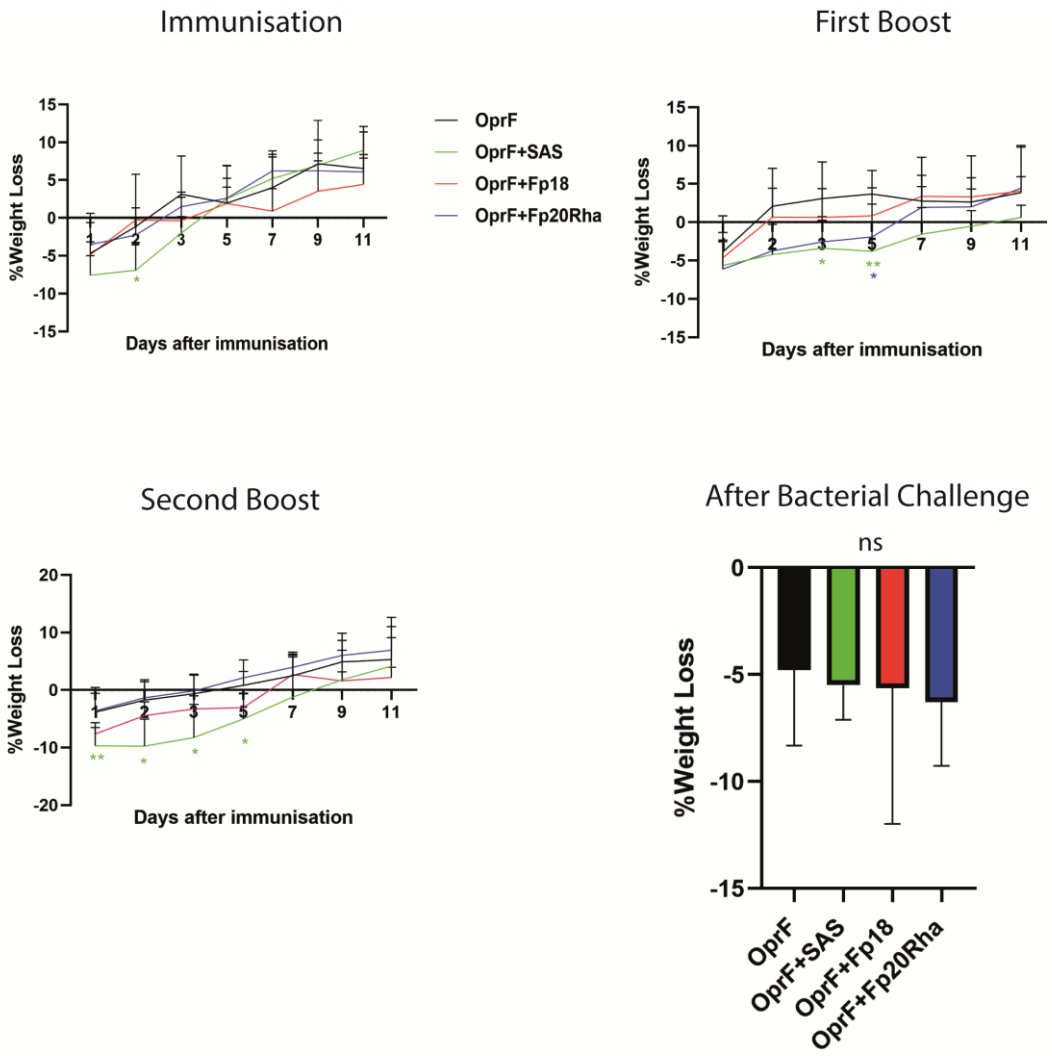

Figure 41. Weight loss during the mice immunisation study. The mean ± SD weight change in mice at each dose post-immunisation is shown (Immunisation, First Boost and Second Boost). **C)** Weight loss after bacterial challenge.

Bacterial lung colonisation was significantly decreased by 1.5 log (p=0.003) in the group immunised with OprF+ SAS and 1.15 log (p=0.026) in the group immunised with OprF+ FP18, relative to immunisation with OprF alone; indicating that FP18 was almost as protective as SAS when used as an adjuvant (Figure 42). The immunisation with OprF+ FP20Rha showed only a 0.91 reduction in lung colonisation, which was not significantly different relative to immunisation with antigen alone (p=0.417). Surprisingly, immunisation with OprF+FP18 significantly reduced bacterial dissemination to spleens by 1.09 log (p=0.003). In contrast, immunisation in combination with either SAS or FP20Rha did not show a significant change in spleen dissemination relative to immunisation with OprF alone (0.43 and 0.32, respectively, Figure 42).

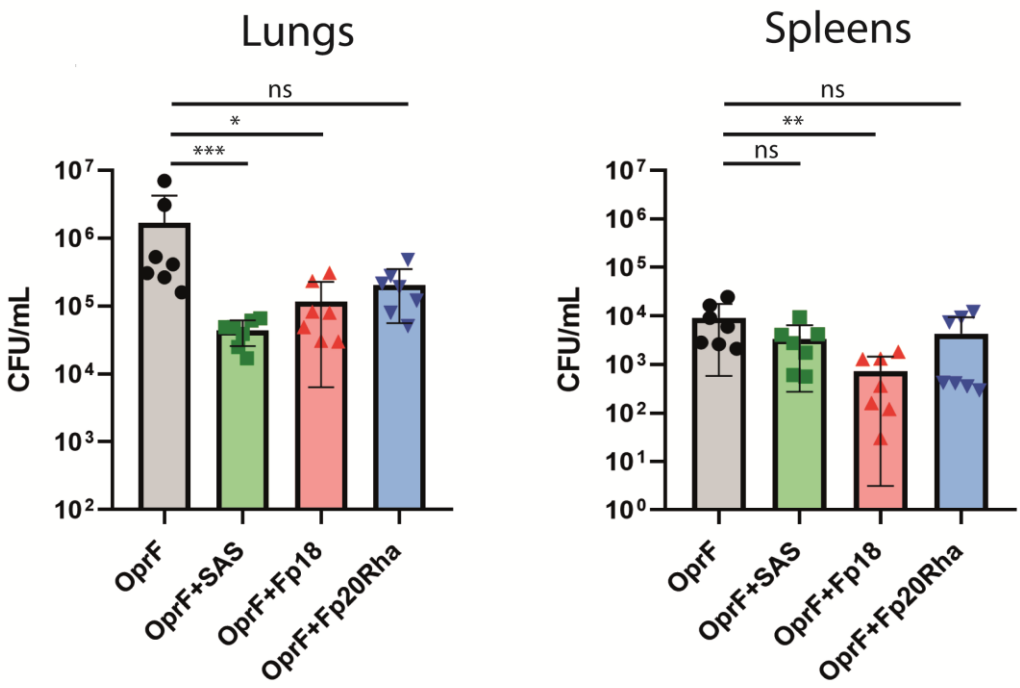

Figure 42. Bacterial clearance after immunisation with rOprF_his in combination with FP18 and FP20Rha or SAS adjuvant. Immunisation of OprF in combination with SAS and FP18 significantly decreased *P. aeruginosa* colonisation in the lungs by 1.5 and 1.15 log, respectively (Kruskal Wallis test, ***p=0.003 and *p=0.026). rOprF +FP18 significantly decreased bacteria dissemination to the spleens relative to the immunisation with antigen alone (Kruskal Wallis test, **p=0.003). Each point represents a single mouse (n=7) and the mean ± SD.

3. Evaluation of humoral responses after immunisation with rOprF and FP compounds

To determine whether the adjuvants enhanced specific humoral responses against rOprF_his, we collected serum one week after the second boost, and total IgGs

and specific IgG1 and IgG2c anti-rOprF were quantified. Groups immunised with rOprF+SAS and rOprF+FP18 ($p<0,0001$, One-way ANOVA), expressed high levels of antigen-specific IgG, but not in the group immunised with rOprF+FP20Rha, relative to immunisation with antigen alone (Figure 43). IgG1 levels were higher in all the groups immunised with rOprF_his adjuvanted relative to the group immunised with rOprF_his alone. The groups immunised with the antigen combined with SAS or FP18 showed the highest levels of IgG1 and were also the only adjuvants showing significantly more antigen-specific IgG2c levels than OprF alone (Figure 44).

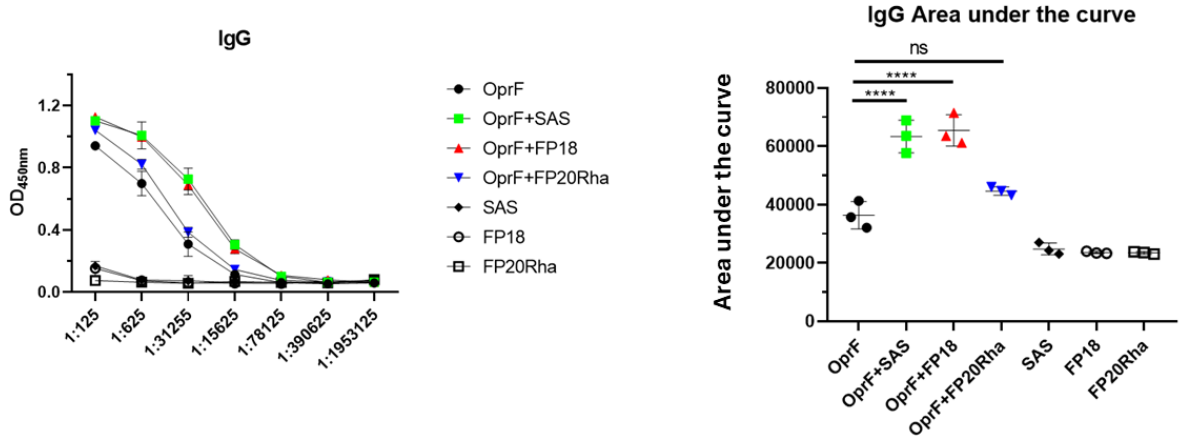

Figure 43. IgG antibodies against rOprF at 35 days post-immunisation. Values represent the mean ± SD. Kruskal Wallis tests (with an alpha of 0.05) were utilised to compare the areas under each curve. *$p < 0.05$; ****$p < 0.0001$. Sera from the seven mice was pooled together and technical triplicates were performed.

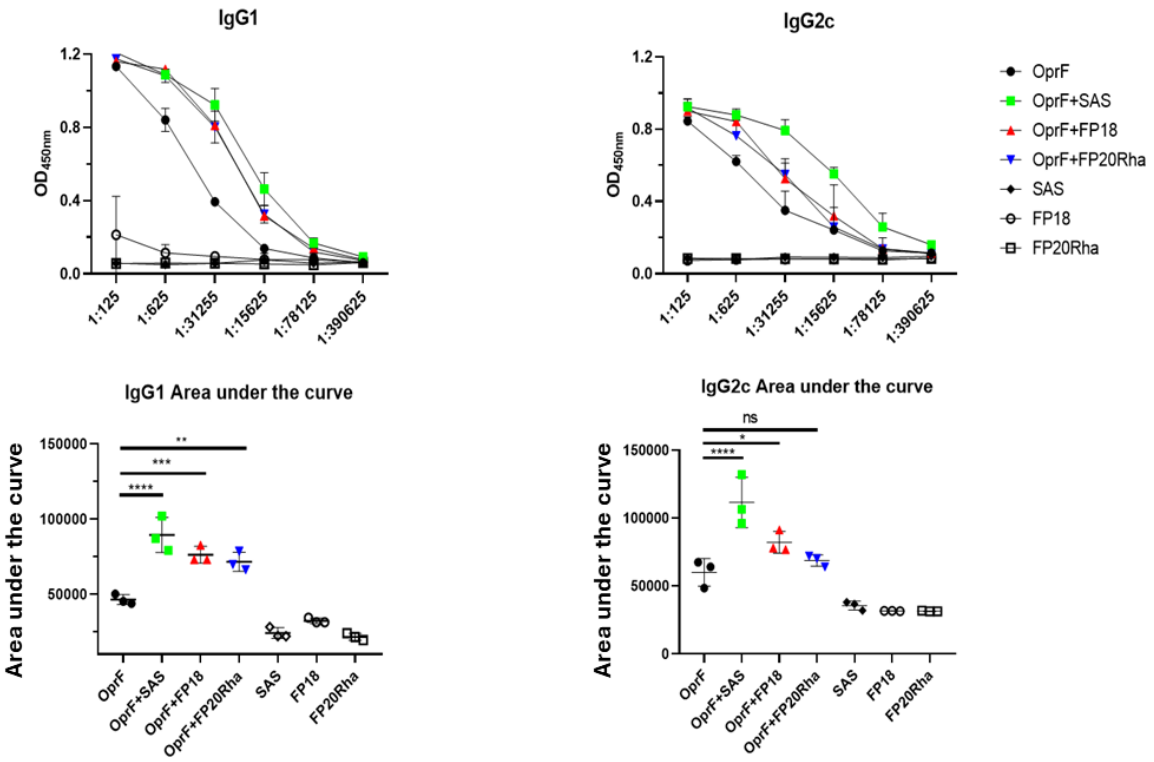

Figure 44. IgG1 and IgG2c antibodies against rOprF 35 days post-immunization. Values represent mean ± SD. Kruskal Wallis tests (with an alpha of 0.05) were utilised to compare the areas under each curve. *p < 0.05; ***p < 0.001; ****p < 0.0001. Sera from the seven mice was pooled together and technical triplicates were performed.

4. Evaluation of cellular responses after immunisation with FP compounds

The recall responses from splenocytes collected 14 days after one subcutaneous immunisation of C57BL/6J mice (same groups as in the previous mice trial) were assessed by flow cytometry (Figure 43).

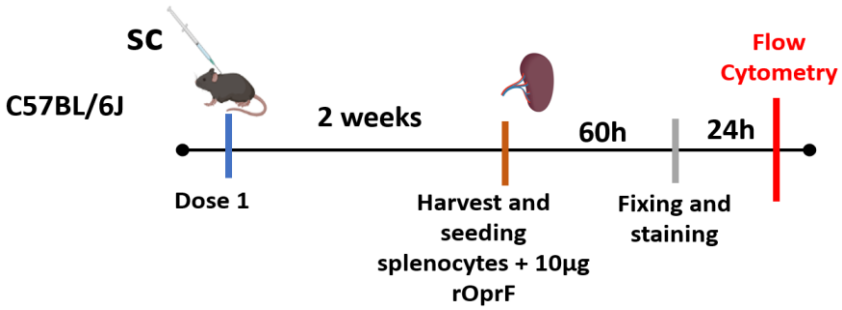

Figure 45. Timeline for the evaluation of recall responses from splenocytes in C57BL/6J mice.

Mice immunised with any of the three adjuvants showed significantly more $CD4^+$ T-cells, relative to immunisation with antigen alone. Lower levels of $CD8^+$ T-cells were observed in mice immunised with OprF+SAS and OprF+FP18, relative to immunisation with rOprF_his alone (Figure 44), while immunisation with rOprF+FP20Rha showed similar levels of $CD8^+$ T-cells relative to immunisation with antigen alone.

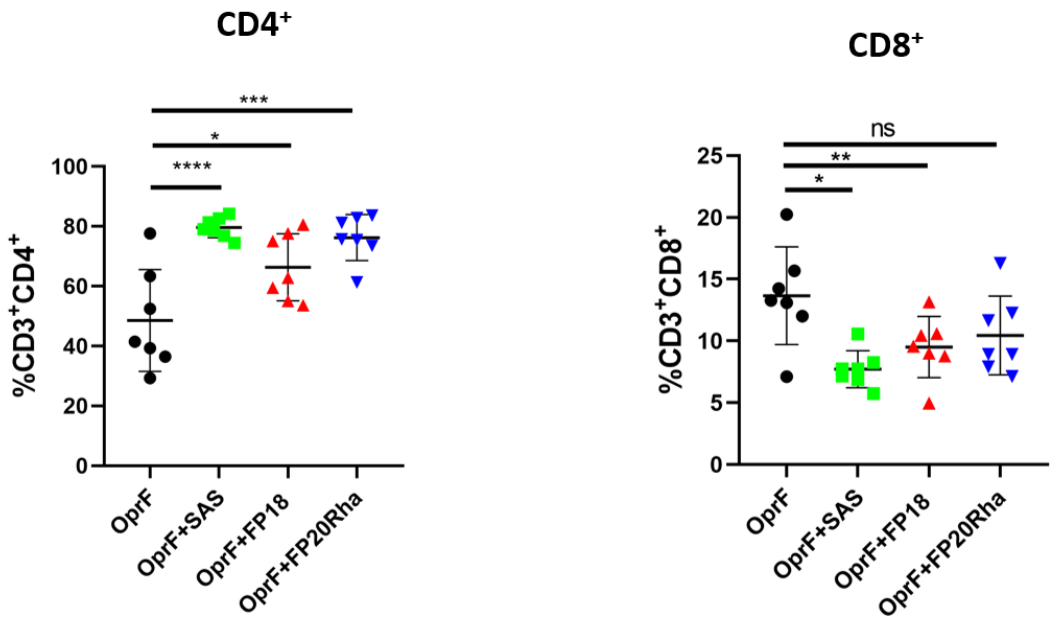

Figure 46. Cellular immune responses in C57BL/6J mice immunized with rOprF in combination with FP compounds. $CD3^+$ marker was used to identify T-cells. They were classified into $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells. Each dot in the graph indicates one mouse (n=7). *p < 0.05; **p < 0.01; ***p < 0.001.

The subpopulations of $CD4^+$ T-cells were then classified into naïve or effector T-cells according to the expression of CD62L and CD44 markers ($CD62L^+CD44^-$, naïve; and $CD62L^-CD44^+$, effector T-cells). Immunisation with OprF+SAS and OprF+FP20Rha showed higher levels of naïve T-cells relative to immunisation with antigen alone, while OprF+FP18 showed similar levels to rOprF_his. Mice immunised with FP18 and FP20Rha showed higher levels of total $CD44^+$ T-cells relative to immunisation with antigen alone or OprF+SAS (*p < 0.05; **p < 0.01, respectively). However, all the groups showed lower levels of effector T-cells relative to immunisation with rOprF_his alone (rOprF+SAS, p <

0.001; rOprF+FP18, *p < 0.05; and FP20Rha **p < 0.01). Finally, all the mice immunised with rOprF and any of the adjuvants showed higher levels of central memory T-cells (Figure 47).

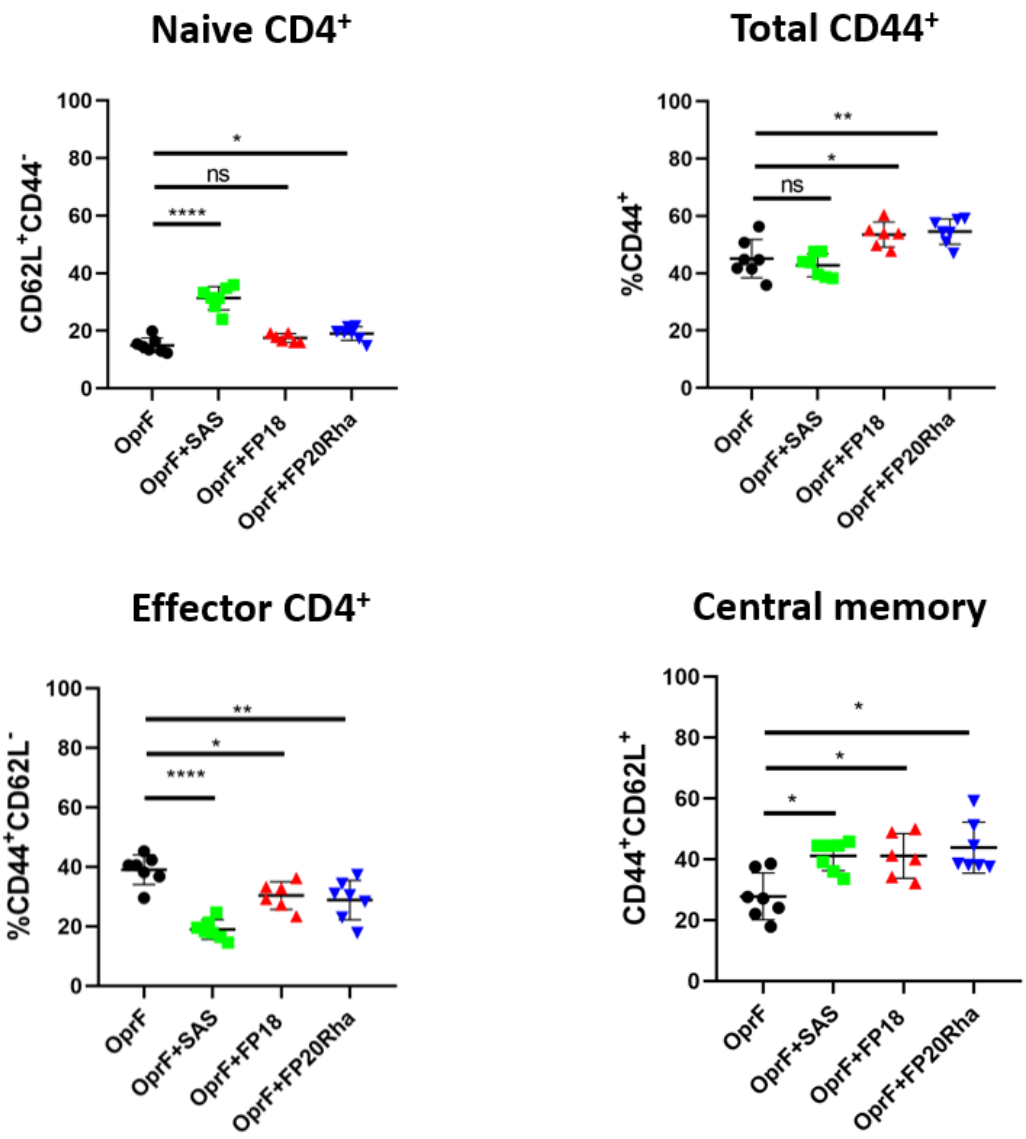

Figure 47. Subpopulations of T-cell CD4⁺ in C57BL/6J mice immunized with rOprF in combination with FP compounds. Subpopulations of CD4⁺ T-cells were classified into naïve or effectors T-cells according to the expression of CD62L and CD44 markers (CD62L⁺CD44⁻, naïve; and CD62L⁻CD44⁺, effector T-cells). Central memory T-cells were classified as CD62L⁻CD44⁺. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.001.

Finally, IFN-γ and IL-4 from CD4⁺ T-cells were evaluated to determine the type of Th response (Figure 46). Immunisation with OprF+FP18 (*p< 0.05) and OprF+FP20Rha (**p<0.01) showed significantly higher levels of IFN-γ production, relative to immunisation with rOprF_his alone, although the response was highly variable between

mice. No group showed production of IL-4, characteristic of a Th2 response, suggesting a Th1-biased response was elicited by FP compounds in combination with rOprF_his (Figure 48).

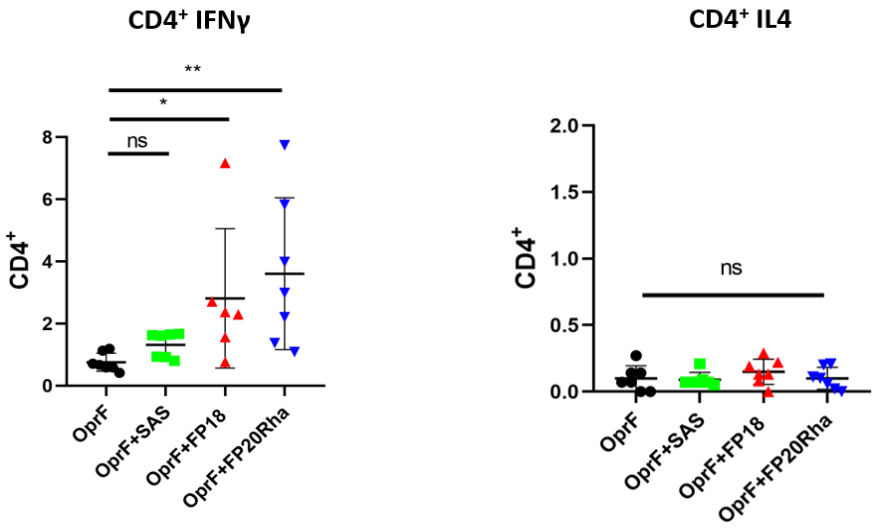

Figure 48. IFNγ and IL-4 elicited by CD4⁺ T-cell responses in C57BL/6J mice immunized with rOprF in combination with FP compounds. *p < 0.05; **p < 0.01; ***p < 0.001. FP compound production of IFNγ suggests a skewed Th1 response.

Discussion

P. aeruginosa is among the top priority pathogens against which new therapies are urgently needed. Its virulence and antibiotic resistance enable challenging *P. aeruginosa* to cause infections in immunocompromised patients. For example, Lansbury et al. (410) identified *P. aeruginosa* as the second most common bacterial co-infection isolated from patients with COVID-19 (47). Antibiotics have been key in the treatment of these infections but currently, one of the major threats to global health is the emergence of antimicrobial resistance (411).

Vaccines have a major role in fighting antimicrobial resistance. Prophylactically, vaccines can decrease the number of infectious disease cases, thus reducing antibiotic use and the spread of AMR. In contrast, antibiotics act on established infections increasing the probability that resistant clones will emerge. Also, antibiotics are used to target single bacterial mechanisms, while vaccines can target multiple antigens (polyclonal antibodies), and thus the emergence of vaccine escape variants would require several mutations impacting different epitopes (412).

FP18 and FP20Rha, two chemically simplified TLR-4 agonists derived from MPLA (401, 402), were evaluated in combination with one of the most successful *P. aeruginosa* vaccine antigen candidates, OprF. The reduction of bacterial colonisation achieved with OprF in combination with FP18 was comparable to the one observed by immunisation with rOprF+SAS, a more complex adjuvant. Importantly rOprF+FP18 immunised mice were the only group showing a reduction in bacterial dissemination to the spleen. The three adjuvants enhanced the production of IgG1 but only immunisation with rOprF+SAS or OprF+FP18 enhanced the production of IgG2c. This might explain the lack of protective effect within the rOprF+ FP20Rha group, as IgG2c is related to a Th1 response, which has been demonstrated to be protective in other evaluated vaccine candidates such as PopB encapsulated in PLGA (141, 144), among others (141, 144, 269, 375, 390). IgG1 can efficiently trigger the complement system (413), which has been demonstrated to be essential for *P. aeruginosa* clearance. So, the enhancement of specific anti-OprF IgG1 may explain the decrease in CFU observed in all the groups immunised with the adjuvants relative to immunisation with antigen alone (although no significant for FP20Rha). For example, mice deficient in the complement system (C5-deficient mice),

showed defective lung clearance of *P. aeruginosa* due to a reduction in the number of phagocytes recruited to the lung (414, 415). Mishra et al., demonstrated that an OprF-deficient *P. aeruginosa* mutant had lower C3b deposition than the parental strain. By binding less C3b, the OprF-deficient mutant was phagocytosed by neutrophils less efficiently than the parental strain. Moreover, the heterologous expression of OprF significantly enhanced C3b binding and increased serum-mediated bactericidal effects in complement-susceptible Escherichia coli (416). IgG1 is generally associated with Th2 responses while IgG2 suggests Th1 skewed responses (417). Th1 responses elicited by previous vaccine formulation candidates against P. aeruginosa were also shown to be beneficial such as PopB encapsulated in PLGA (141, 144) or PcrV/CpG ODN (377). The IgG profile observed in OprF-immunised mice matches the previous analysis of the FP compounds during OVA immunisation (401). Recently, OprF was used in an mRNA vaccination candidate against *P. aeruginosa* and elicited IgG1 and IgG2a isotypes suggesting a mixed Th1/Th2 response (slightly skewed Th1) that was also protective against *P. aeruginosa* infection (75% survival rate by immunisation with 25 µg of OprF-I) (289). Overall, the FP18 compound elicited Th1-skewed humoral responses beneficial for *P. aeruginosa* clearance, while FP20Rha did not stimulate IgG2c antibodies, which might be reflected in less bacterial clearance.

Cellular responses were also evaluated as they are required for an effective vaccine against *P. aeruginosa*, (149). Interestingly, mice immunised with FP20Rha showed higher levels of IFNγ, relative to immunisation with OprF alone, although OprF+FP18 also showed an enhancement of this cytokine. It is possible that a balance between humoral responses and cellular responses must be achieved to observe protection against *P. aeruginosa,* and cellular responses alone are not effective as observed with FP20Rha. Recent studies indicate that *P. aeruginosa* binds to human IFNγ through OprF, enhancing its virulence (418). Sera from OprF/I-immunised mice inhibited *P. aeruginosa* binding to IFN-γ, suggesting an alternative mechanism by which the OprF/I vaccine confers protection against *P. aeruginosa* infection (82, 83). IFNγ stimulates macrophages and cytotoxic T lymphocytes (CTLs), essential to clear *P. aeruginosa* infection (418).

Therefore, immunisation with FP18 as an adjuvant showed, (i) bacterial clearance in the lung, (ii) lower bacterial dissemination to the spleen, (iii) higher IgG1 and IgG2c anti-rOprF antibodies and (iv) higher production of IFNγ, relative to immunisation with antigen alone; making it a highly promising adjuvant to be included in vaccine formulations against *P. aeruginosa.* Most *P. aeruginosa* vaccine candidates that have shown protective effects against *P. aeruginosa* were those with Th1 responses suggested by the production of IFNγ and the generation of both IgGs isotypes (IgG1 and IgG2) (141, 144, 269, 375, 390). Overall, the FP18 adjuvant showed similar effects to the currently widely used in research adjuvant SAS, and consequently is a promising adjuvant candidate for further development in human vaccinations but also for use in research.

The development of novel, improved adjuvants is challenging, but here we showed that the rational development of adjuvants combined with already identified antigens can lead to effective vaccine formulations. The previous lack of knowledge into the mechanisms of developed adjuvants that were hampering the design of novel adjuvants is now being filled by studies like this, which might lead to novel vaccine formulations as well as clarifying the mechanisms of protection that could lead to effective vaccinations against *P. aeruginosa* and/or other pathogens.

Overall, this is the first study showing the effectiveness of the novel adjuvant FP18 in an *in vivo* infection model. Although this study is focused on *P. aeruginosa,* it may also be suitable for other vaccine formulations against other ESKAPE pathogens. The incorporation of easily scalable molecules into vaccine formulations will be crucial to enable non-marketable vaccine candidates to reach their licensing. The COVID-19 pandemic highlighted the need to have effective vaccine platforms to have a quick response against future pandemics, adjuvants will play a major role in achieving that goal and thus, fighting against antimicrobial resistance.

Final Discussion and Future Perspectives

The discovery of antibiotics was the greatest medical breakthrough of the 20th century. The production and characterisation of penicillin, in 1928, resulted in the discovery of several different classes of antibiotics that completely changed modern medicine. The pre-antibiotic era was characterised by high morbidity and mortality due to minor infections. Life expectancy has risen from around 47 years old before the introduction of antibiotics to ~78 years, today in the United States (419-421). However, almost 100 years after the discovery of antibiotics, antimicrobial resistance has become one of the biggest threats to human health (22). The World Health Organization (WHO) predicted a surge of 1.2 trillion USD in annual health expenditure by 2050, reducing the global domestic product (GDP) by 1.1–3.8%, due to antimicrobial-resistant pathogens (422). In 2023, a United Nations report estimated that drug-resistant microbes could lead to ten million deaths per year by 2050 (423). Hence, alternative strategies have become crucial to combat bacterial infections.

The study of host-directed therapies (HDT) to combat antibiotic resistance has emerged in recent years. The development of HDT coupled with new antibiotic agents is expected to create a more robust treatment strategy than current therapies (424, 425). HDT can enhance host cellular responses to pathogens, activate innate and adaptive protective immune responses, or modulate excessive inflammation, reducing morbidity, mortality, and organ damage (424, 425). The main advantages of HDT over conventional therapies are their effectiveness against both, antibiotic-resistant and antibiotic-susceptible bacteria; their reduced ability to produce bacterial resistance to drugs, and their ability to synergise with or shorten antibiotic treatment by targeting different pathways (426).

This project explored *P. aeruginosa* interactions with the host for a better understanding of *P. aeruginosa* pathogenesis, and the development of novel therapies in three different ways: i) the characterisation of attachment and inflammatory responses of human epithelial cells after exposure to CF *P. aeruginosa* “early” and “late” isolates, to determine if there is a common pattern in the host immune responses during early and late stages of *P. aeruginosa* infections that could lead to novel treatment strategies; ii) the elucidation of novel host receptors, and their validation by a

pharmaceutical approach; and iii) the use of TLR4 ligands to enhance the stimulation of immune responses against *P. aeruginosa* through vaccination.

The development of therapies against *P. aeruginosa* requires an understanding of the versatility and plasticity within the host. This has been widely studied in CF patients, where *P. aeruginosa* is a dominant pathogen. In this project, it was demonstrated that "early" and "late" isolates attached and stimulated proinflammatory cytokines in epithelial cells in an isolate-dependent way and independent of their time of colonisation. The study confirmed that the divergent evolution, previously observed in the virulence factors of the selected sequential CF isolates, was also reflected in the host responses. The attachment varied across isolates, and it was not necessarily dependent on the infection stage (early vs. late) or the phenotypes previously observed. A notable observation is that the late isolate AMT0060-2 displayed slow growth and was resistant to gentamicin, with a two-base-pair frameshift in the gene *gacA*, which likely contributes to its ability to establish chronic infections in the antibiotic-treated CF airways. The study also showed that some late isolates could still stimulate pro-inflammatory responses, countering the hypothesis that *P. aeruginosa* CF-adapted variants generally lower inflammation. The attachment patterns of isolates to different epithelial cells (16HBE14o- and CFBE41o-) and the production of alginate in the AMT0060-2 isolate are discussed as contributors to phenotypic variation, which impacts infection progression and treatment response. The reduced motility in some strains did not correlate with a diminished inflammatory response, suggesting that virulence factors other than motility could still drive immune stimulation. This is consistent with the diversity of the within-patient *P. aeruginosa* population in the CF environment, as suggested by several studies (212, 427-430). For example, one hypothesis is that bacterial isolation drives divergent evolution of *P. aeruginosa* in CF infections, as clonally related *P. aeruginosa* from different lung regions differed phenotypically. Phylogenetic analyses showed that regional bacteria evolve in isolation, and mixing is limited. This might be due to physical separation by factors such as thick mucus, airway obstruction, or the large distances between regions (427).

The identification of the molecular requirements for acute versus chronic infection would be important to tailor therapeutic strategies against *P. aeruginosa*, for example, recently a small RNA, *sicX*, was identified as a biomarker for the transition from chronic-to-acute *P. aeruginosa* infections (431). The small RNA *sicX* might be used to direct the treatment strategies in patients, predicting when the infection will become acute (431). Another example of how biomarkers of acute versus chronic *P. aeruginosa* infections have been used to direct therapies is the identification of the *P. aeruginosa* protein, TesG, by Zhao et al. as a significantly upregulated gene under chronic infection conditions in mice models (432). A series of elegant experiments suggested that TesG inhibits cellular functions designed to protect the host, including inflammation and phagocytosis, and is involved in the establishment of chronic *P. aeruginosa* infection (432). Hence, TesG protein levels could be used as a predictive biomarker of chronic infection. Most importantly, TesG studies might suggest that inflammatory responses are essential to avoid the establishment of chronic infection, so the current efforts to develop anti-inflammatory treatments for people with CF might affect the treatment of *P. aeruginosa* infection (433). This project also suggests that even when there is no specific trend between *P. aeruginosa* CF early and late isolates, in all the cases at least one of the late isolates elicited inflammatory responses, meaning *P. aeruginosa* might evolved divergently keeping the possibility to either evade the immune responses or to elicit those pro-inflammatory responses. Understanding what type of response against *P. aeruginosa* prevails in the lung of the CF individuals, will be essential in the development of treatments to control either *P. aeruginosa* infections or CF disease.

Experiments that lead to deciphering how the evolution of *P. aeruginosa* modulates host responses are essential for the design of therapeutic strategies, as explained above. Mouse models, although useful, introduce variables that are difficult to study. Hence, -`omics and phenotypic assays, *in vitro*, allow the association of specific genes with certain phenotypes more clearly. In this study, the characterisation of the immune responses against sequential CF isolates combined with the previous genomics analysis (293), and the virulence factors phenotyping (291), did not associate a specific gene or bacterial phenotype with a specific host response. For example, although AMT0060-2 showed lower motility, relative to the early isolate AMT0060-3, differences

in the stimulation of IL-6 and IL-8 were not observed. Flagellin, associated with motility (434), stimulates proinflammatory responses but, alginate, associated with lack of motility (435), seem also to stimulate proinflammatory responses; suggesting that pro-inflammatory cytokines might be stimulated during all the stages of *P. aeruginosa* infection, which, as stated above, might complicate the development of anti-inflammatory treatments in individuals with CF or other inflammatory disorders. Currently, anti-inflammatory treatments are in the Cystic Fibrosis Foundation (CFF) drug development pipeline (436). Hence, knowing which *P. aeruginosa* virulence factors modulate the immune response toward an inflammatory state is crucial.

Recently, our laboratory performed the proteomics analysis of the CF sequential isolates evaluated in this study (manuscript in preparation), identifying common proteins expressed by all the isolates, such as CifR, an epoxide-responsive repressor of the CFTR inhibitory factor (Cif). Future experiments directed towards the assessment of *P. aeruginosa* mutants in those common proteins for all the isolates or common for either early or late isolates, and the study of host immune responses with those mutants, *in vitro*, will provide a better understanding of how *P. aeruginosa* modulates host immune responses. That knowledge could be then applied to tailor the therapeutic strategies to treat *P. aeruginosa* infections.

Overall, the findings emphasise the need for further research on CF-adapted *P. aeruginosa* isolates, including more extensive studies on the host immune responses to better inform drug development and treatment approaches for CF patients dealing with chronic *P. aeruginosa* infections. In the search for new therapies against *Pseudomonas aeruginosa*, the complexity of its adaptation in cystic fibrosis (CF) patients highlights key approaches for exploration. The phenotypic diversity observed in sequential isolates suggests that treatments must account for the bacteria's various adaptations. Immunomodulatory strategies should aim to reduce inflammation without compromising infection control. Furthermore, combining antimicrobial treatments with therapies that specifically target these adaptive mechanisms may offer a more effective approach. Finally, genomic and proteomic studies will be crucial in identifying new therapeutic targets to combat chronic *P. aeruginosa* infections in CF patients.

In the early stages of infection, *P. aeruginosa* attaches to host cell surfaces. The adherent state is advantageous for bacterial survival (317). Hence, *P. aeruginosa* attachment through host receptors is a crucial step for the colonisation of the host. Consequently, a better understanding of this step might lead to novel therapeutic strategies. Thus, finding novel host receptors is crucial for both, the understanding of immune responses against the pathogen and the development of novel therapies against *P. aeruginosa.*

The importance of elucidating host receptors is exemplified by the radical change in immunology after the discovery of TLRs, recognised with the awarding of the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffmann and Bruce Beutler. The discovery of Toll-like receptors changed how innate immunity was seen and led to several treatments such as interfering with TLR responses to treat pathogen infections and the development of TLR ligands as vaccine adjuvants (393, 437). There are not a lot of specific human receptors for *P. aeruginosa* currently identified, although proteins such as annexin A2 (AnxA2) protein, autophagy-related protein 7 (ATG7), NLRC4, and non-coding RNAs (lncRNA and microRNA) (319-321) have been already suggested as important in the interaction of *P. aeruginosa* with human epithelial cells. Hence, due to the importance of the elucidation of host receptors and the lack of methods for their study, we developed a proteomic approach for the identification of human receptors for *P. aeruginosa* (438).

The 2D blot approach developed in this study demonstrated the possibility of discovering novel host receptors without previous knowledge about the identity of any of the two molecules (bacteria or human proteins) involved in the interaction. Consequently, it could be used for the study of any host-pathogen interaction. Furthermore, as it is based on the fact that proteins can be transferred to a PVDF membrane, it is not limited to protein interactions, but it has the potential to be adapted for carbohydrates or any other molecule that could be transferred to a PVDF membrane. Thus, the method will be useful to speed up the discovery of novel host receptors for more pathogens and human cell lines. However, further optimisation of the method should be directed to the use of native gels for the separation of the host proteins, as

having the native structure of receptors might enhance the opportunity to discover biologically relevant proteins. Also, the use of different *P. aeruginosa* strains and human cell lines might help to select receptors used by different bacterial strains and in different sites of infections in an early stage of the research. Nevertheless, the 2D approach allowed the identification of human PDIA1 and PDIA3 proteins, and several other proteins, as receptors involved in *P. aeruginosa* attachment, and their interaction with *P. aeruginosa* was validated by three main approaches: (a) the use of inhibitors (b) overexpression of human PDIs, and (c) CRISPR deletion of PDIA3.

The importance of the discovery of PDIA1 and PDIA3 as receptors for *P. aeruginosa* has two potential impacts: (i) As PDIA3 is part of the MHC-II complex, *P. aeruginosa* targeting proteins in the MHC-II might indicate a mechanism of immune evasion that would be worth it to explore in the future; and (ii) as there are inhibitors of human PDIs already available for the treatment of other diseases, HDT therapies with PDIs inhibitors might be relatively easy to develop.

The recent advance in PDI research is the identification of novel small-molecule inhibitors. PDI inhibitors have been identified as neuroprotective and anti-cancer agents, such as SK053, PACMA 31, and CCF642 for treating acute myeloid leukaemia, ovarian cancer, and multiple myeloma, respectively (439-441). Other studies also identified novel PDI inhibitors targeting mainly PDIA1, PDIA3 and PDIA4 with different specificity (442) (juniferdin(443) origamicin (444), 16F16 (445), securinine (446)) to treat HIV-1 infection, neurodegenerative diseases, or glioblastoma. The flavonoid antioxidant quercetin-3-rutinoside, ML359 and bepristats are also potent PDI inhibitors (447, 448). The specificity, cell-permeability, and *in vivo* efficacy of these drugs should be further investigated, but it would be of interest to test whether they can reduce *P. aeruginosa* colonisation in murine models.

Influenza (IAV) neuraminidase (NA) is a glycoprotein required for the viral exit from the cell. Chamberlain et al. demonstrated that PDIA3 is required for the proper activity of IAV-NA. The inhibition of PDIA3 in murine models using the drug LOC14 attenuated the influenza-induced inflammatory response in mice including the overall viral burden (347). The results with LOC14 led the authors to several patents, including

treatments with LOC14 and other PDIA3 inhibitors for Influenza, SARS-CoV-2 virus, and cardiovascular diseases, among others (449, 450). We are currently collaborating with Anathy´s research group to test whether the pre-treatment of mice with the drug LOC14 can decrease *P. aeruginosa* lung colonisation *in vivo*, opening the possibility of novel treatments for *P. aeruginosa* infections.

The milestone of this project was to develop a new method for elucidating host receptors and to validate it *in vitro*, reaching also the *in vivo* testing of potential drugs targeting the identified proteins. Hence, demonstrating that our methodology has the potential to not just elucidate novel host receptors but to help in the design of novel treatments against different infections. To further increase and explore the potential of PDIs as receptors involved in *P. aeruginosa* attachment, pull-down assays were performed and are being analysed by MS/MS to find the possible bacterial adhesins involved in the interaction between *P. aeruginosa* and human PDIs. For example, the validation of those proteins in two-hybrid assays in yeast might lead to the discovery of a full new mechanism of *P. aeruginosa* interaction with the human cells, and novel bacterial proteins as targets for drugs against *P. aeruginosa*.

Targeting bacterial adhesion will undoubtedly reduce the selective pressure on antimicrobial resistance (451). PDI inhibitors will not represent such a strong selective force as antibiotics. However, this strategy can lead to the selection of isolates capable of bypassing the adhesion requirement and persisting. The anti-adhesion therapies may become less effective, and their limitations must be considered when developing therapeutic strategies (452). Overall, attractive approaches that interfere with the ability of bacteria to adhere to host tissues are considered good therapeutic options. Bacteria resistant to anti-adhesion agents are expected to emerge, but since the agents do not act by killing or stopping the growth of the pathogen, it is reasonable to assume that the spread of the resistance will occur significantly less frequently than that of antibiotic-resistant bacteria (322). For example, targeting virulence instead of direct toxicity to the pathogen has led to promising anti-virulence strategies, as an adjunctive therapy to traditional antimicrobials (453).

The study highlights the importance of discovering novel host receptors, such as PDIA1 and PDIA3, for *P. aeruginosa*, which could lead to new therapeutic strategies targeting bacterial adhesion. The identification of PDIs as receptors opens the potential for Host-Directed Therapies (HDTs) using existing PDI inhibitors, which have already shown promise in treating various diseases. The development of therapies, like creams, containing inhibitors of those human PDIA1 and PDIA3 could be explored for burn wound infections caused by *P. aeruginosa*, to prevent bacterial colonisation and reduce the need for antibiotics. Testing these treatments in mouse models and human clinical trials could offer effective alternatives for managing complex infections and mitigating antimicrobial resistance.

The therapies targeting pathogen adhesion appear to be promising, however, infection is sometimes unavoidable. Therefore, other therapies such as vaccination, might be the only way to a radical stop of antimicrobial resistance, as observed with the eradication of smallpox, the almost complete elimination of poliomyelitis, and the decrease of more than 95% in the incidence of diseases such as diphtheria, tetanus, pertussis, measles, mumps and rubella (412). Despite substantial efforts, over 50 years, no licensed vaccines against *P. aeruginosa* are available. Even the most promising OprF–OprI fusion protein showed disappointing clinical efficacy results (270). Some target populations may not mount a strong immune response as some individuals are likely to be immunocompromised. Thus, vaccine research should assess adjuvants that can improve specific immune responses (412). Adjuvants contribute in different ways to enhance the effect of the vaccine: (a) it may increase the number of human receptors stimulated by the antigen, increasing the immune response to the given vaccine by enhancing the antibody responses; (b) to increase seroconversion rates in individuals with diminished responsiveness due to age, disease or therapeutic interventions, the MF59 adjuvant in the influenza vaccine increased response in elderly individuals (23, 24); and (c) to reduce the antigen dose and number of boosters of vaccine antigens required (25-27), since by better targeting the antigen, lower doses of vaccine are needed to obtain the desired effect. Due to the importance of adjuvants, and the promising results of the IC43 vaccine against *P. aeruginosa*, novel TLR4 ligands were evaluated to see if they could enhance the effect of the well-studied *P. aeruginosa* antigen, OprF.

The compound FP18 showed promising results as an adjuvant, showing reduced bacterial lung colonisation and bacterial dissemination to the spleen, enhanced IgG1 and IgG2 antibody titres and increased production of IFNγ, all relative to immunisation with antigen alone. Thus, FP18 is a novel good candidate as an adjuvant for vaccine candidates against *P. aeruginosa.* This project represents the first preclinical study of FP18 in an infection scenario. It was previously characterised using OVA antigen, but this is the first report of its ability to enhance antigen protectiveness and reduce bacterial burden in lungs and spleens after a bacterial challenge. The results of this study are consistent with what was previously reported for FP18: no toxic effects were observed and FP18 enhanced OVA-specific IgG titres (401).

The ideal vaccine adjuvant should induce the desired adaptive immune response while avoiding excessive innate immune response; it should be minimally toxic; low-cost and stable for long-term storage. The compound FP18 was optimised for a simpler chemical synthesis than the FDA-approved adjuvant MPLA, so taken together, FP18 achieved all the characteristics of an ideal adjuvant. This represents an advancement in vaccinology, not just for vaccines against *P. aeruginosa* but for any other vaccine (454)

FP18 and FP20 compounds have been recently licensed to CRODA Pharma, and they will be soon available in their catalogue of adjuvants. This will facilitate the use of FP18 by other researchers to demonstrate its non-toxic effects in animal models, helping the compound to reach the human trials stage. The promising results observed with FP18 in combination with OprF might encourage other researchers to also use FP18 in mice trials, thus the importance of the study is not just the demonstration of a novel vaccine candidate against *P. aeruginosa* (OprF+FP18) but highlights the importance of adjuvant research as a way to make available several antigens that are promising but might need the incorporation of other molecules in the vaccine formulation to reach human trials (454).

The further development of prophylactic vaccines against *P. aeruginosa* and multivalent vaccinations should be considered. The use of a single antigen in this study was mainly to facilitate the analysis of the adjuvant mechanisms without adding several

variables. But, as previously discussed and considering the lessons from Chapter 2, the multiple virulence mechanisms expected by *P. aeruginosa* will require the development of multivalent vaccines. The combination of OprF+FP18 with other good antigen candidates, in the future, might lead to an even better vaccine candidate against *P. aeruginosa* infections. Finally, although this study evaluated the adjuvants in a prophylactic model of acute *P. aeruginosa* pneumonia, a pilot study of a chronic infection model in mice was also attempted to allow investigation of antigen-adjuvant systems as therapeutic vaccines for chronic *P. aeruginosa* infection (Appendix C). As *P. aeruginosa* is an important pathogen in chronic infections, vaccine development should also be directed towards designing therapeutic vaccinations and/or testing in other mice models such as those that mimic CF. Adjuvants such as the FP18 compound will be highly important in this context, as a modulation of the immune system's machinery towards a response that successfully clears *P. aeruginosa* infection will be required.

Overall, the thesis explored several aspects of the interactions between *P. aeruginosa* and the human host. The divergent evolution of *P. aeruginosa* strains, reflected in the stimulation of the human cell responses, was confirmed. The project led to three major achievements: a) A novel unbiased method for the elucidation of host receptors; b) the discovery of the role of human PDIA1 and PDIA3 in *P. aeruginosa* attachment; and c) the demonstration of OprF+FP18 as a promising antigen-adjuvant system for *P. aeruginosa* vaccinations. The further development of this project may lead to:

a) A deeper insight into the drivers of the inflammatory response during *P. aeruginosa* infection

b) Novel drugs targeting PDIA1 and PDIA3 that might decrease *P. aeruginosa* colonisation. For example, Maraviroc (Selzentry/Celsentri; Pfizer), which inhibits the human C-C chemokine receptor type 5 (CCR5) and is essential for the entry of HIV-1 virus into the human cells was approved as a first-in-class CCR5 inhibitor in 2007 (455-457), demonstrating the efficacy of host-directed therapies.

c) Pull-down assays to determine which adhesins are involved in the interaction of *P. aeruginosa* with PDIA1 and PDIA3 may lead to further therapeutic targets and a better understanding of the identified interaction.

(d) the evaluation of more antigens alongside FP18 that demonstrate its effectiveness and non-toxicity in mice trials may quickly prompt FP18 to reach human trials.

The emerging therapies from this project could focus on host-directed therapies that enhance immune responses against *Pseudomonas aeruginosa* infections, potentially in combination with antibiotics. Anti-adhesion strategies, such as targeting PDIA1 and PDIA3 receptors with specific inhibitors, could block bacterial attachment, preventing the bacterium from establishing infections; or therapies that disrupt the bacterial adhesins and prevent biofilm formation. Additionally, therapies aimed at Toll-like receptor (TLR) ligands could serve as adjuvants to improve vaccine efficacy, while targeting inflammatory pathways could help control excessive inflammation, particularly in conditions like cystic fibrosis. These combined approaches, offer a promising future for treating *P. aeruginosa* infections, especially those caused by antibiotic-resistant strains.

In summary, while developing canonical antimicrobials targeting *P. aeruginosa* remains imperative, pursuing additional approaches is critical (424, 425). Overall, this project highlighted the importance of understanding infection as a complex interplay between host and pathogen that evolves differently in each patient. To combat formidable pathogens effectively, there is an imminent call for a more rational drug design, one that comprehensively addresses the inherent complexity of *P. aeruginosa* infections.

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Appendix A– Chapter 2

Table 1A. Characteristics of two sets of *P. aeruginosa* isolates from an International Reference Panel.

Strain	Description	LMG No	Characteristics	Reference
AA2	Early CF, Germany	27630	LPS and PGN studied; in vivo virulence	(292-295).
AA43	Late CF, Germany	27631	LPS and PGN studied; in vivo virulence	(292,295).
AA44	Late CF, Germany	27632	LPS and PGN studied; in vivo virulence	(292,295).
AMT0060-3	Early CF, Seattle, WA	27637	Early isolate from033 the same patient as AMT 0060-1 and -2 were isolated	(294)
AMT0060-2	Late CF, Seattle, WA	27635	Late isolate identifying hip mutants; late isolates showed high-level persister cells; mutation in mexZ repressor affecting MIC to Ofloxacin, Carbenicillin and Tobramycin	(294)
AMT0060-1	Late CF, Seattle, WA	27636	Late isolate identifying hip mutants; late isolates showed high-level persister cells; mutation in mexZ repressor affecting MIC to Ofloxacin, Carbenicillin and Tobramycin	(294)

Abbreviations: CF, cystic fibrosis; Washington; LPS, lipopolysaccharide; PNG, poly-N- acetyl glucosamine.

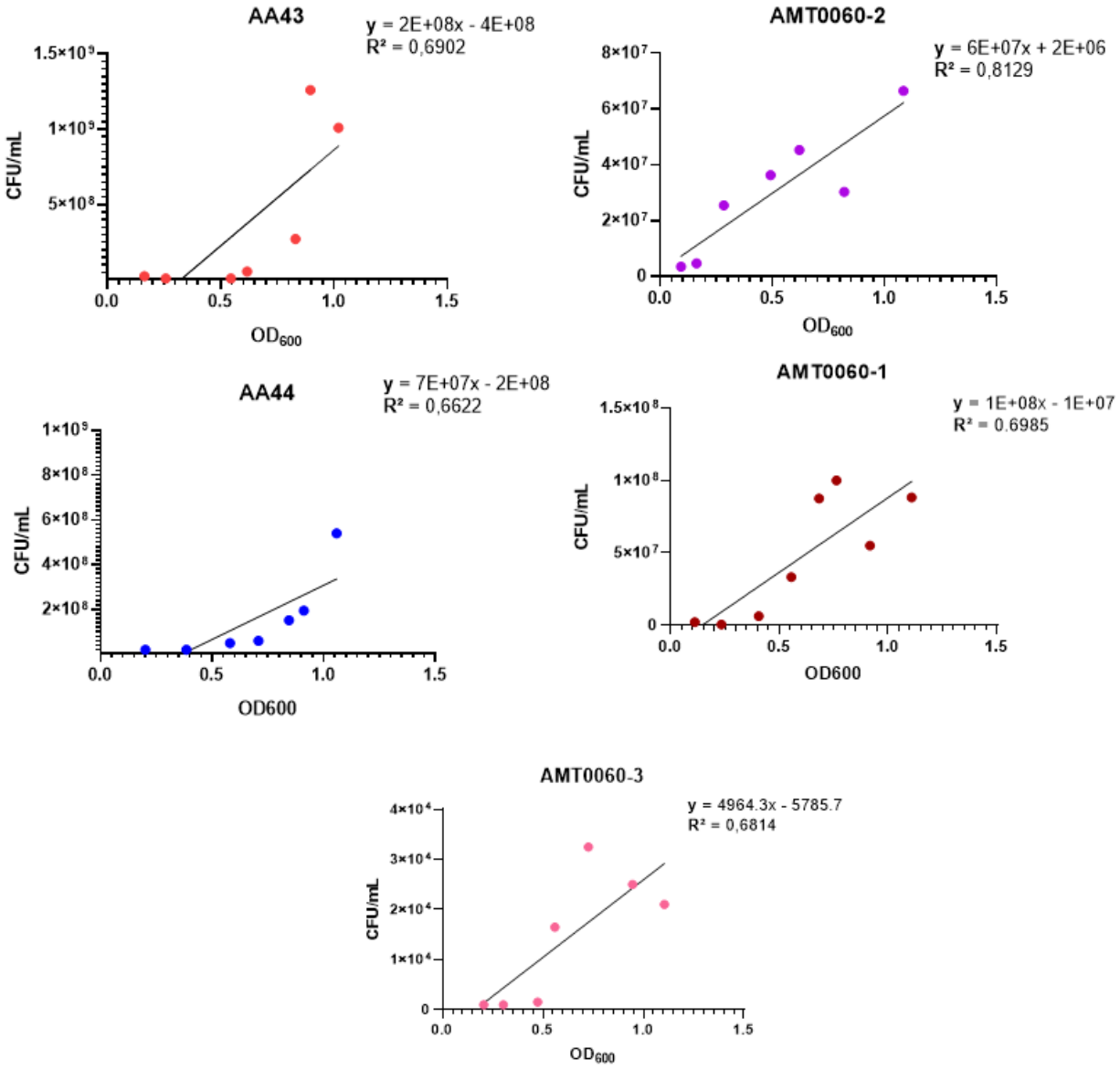

Figure 1A. Growth kinetics (CFU Vs. OD_{600}) of *P. aeruginosa* sequential isolates from two different CF patients. The growth of each set of isolates is presented separately, except for AA2 which was previously characterised for growth. The "y" axis shows the CFU/mL at each OD_{600} ("X" axis), starting with an OD_{600}: 0.1, approximately. The equation of the curve is shown for each graph. For AA2 the equation of the curve was already calculated (y=8E+08x+4E+07) in previous experiments, so it is not presented in this figure.

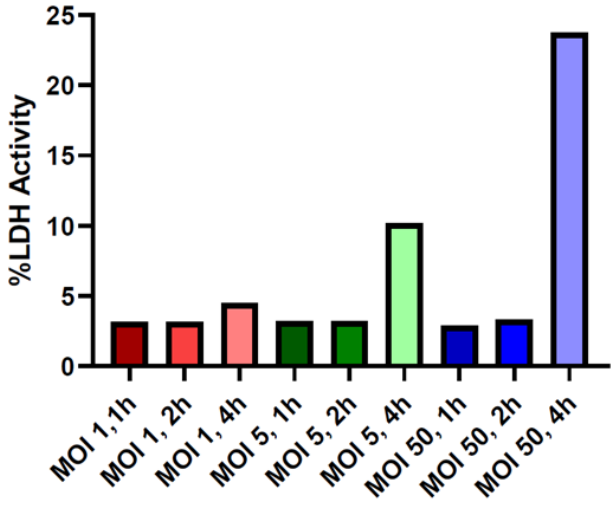

Figure 2A. Cytotoxicity of *P. aeruginosa* AA43 to 16HBE14o$^-$ cells. The cytotoxicity of human cells was determined by the percentage of Lactose Dehydrogenase ("y" axis, LDH) of 16HBE14o$^-$ cells under exposure to different MOI (1,5 and 50) and times (1, 2, 4h) of *P. aeruginosa* cultures. The highest the LDH%, the highest was the cytotoxicity.

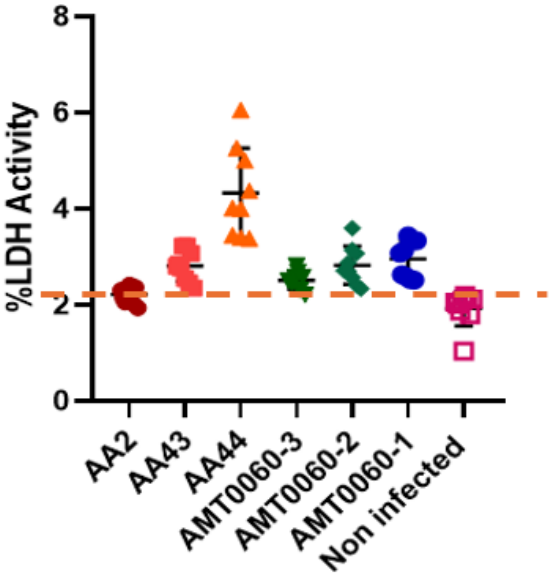

Figure 3A. Determination of non-cytotoxic conditions for six CF *P. aeruginosa* isolates. The cytotoxicity of human cells was determined by the percentage of Lactose Dehydrogenase ("y" axis, LDH) of 16HBE14o- cells under exposure to the different. *P. aeruginosa* isolates at MOI 5 and after 2h of stimulation. The highest the LDH%, the highest the cytotoxicity. The orange line highlights the difference between cells stimulated with bacteria relative to the control (non-infected).

Appendix B- Chapter 3

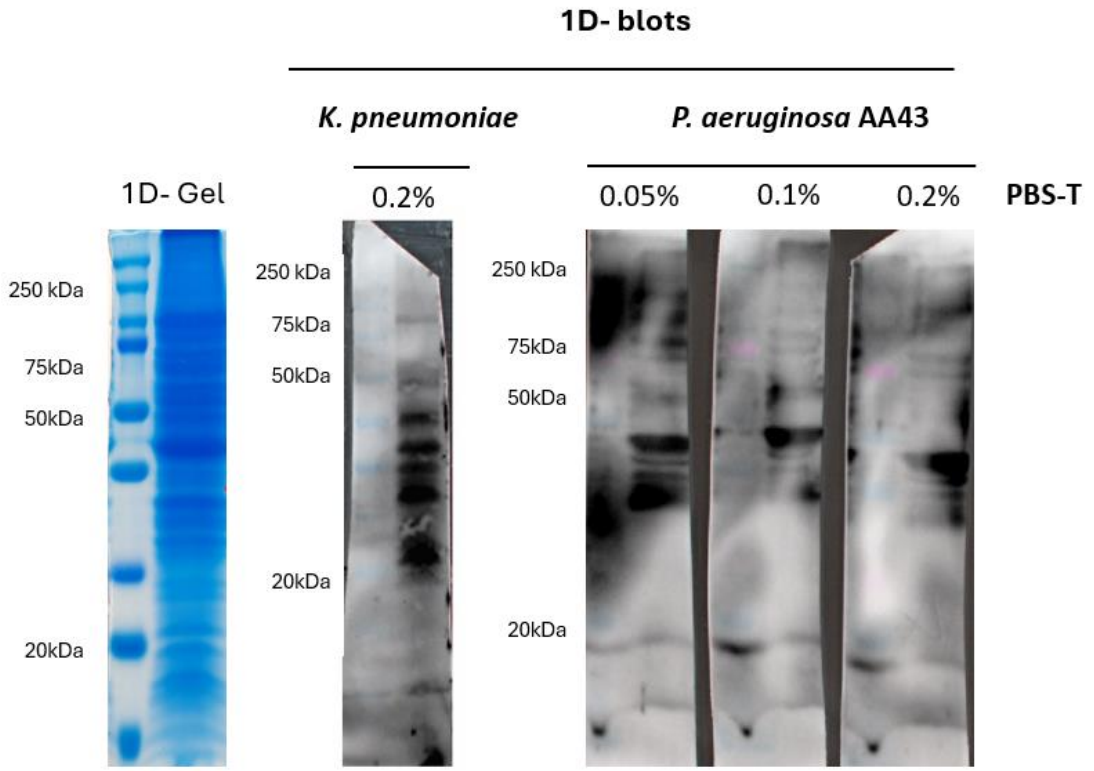

Figure 1B. Optimisation of the identification of novel human proteins that interact with P. aeruginosa by 1D blots. One-dimensional (1D) separation of membrane human proteins from 16HBE14o– cells ("1D-gel") by 2D-SDS-PAGE, and electrotransferred onto membrane blots ("1D Blot") that were subsequently incubated with *P. aeruginosa* live cells (OD600: 0.6), or *K.pneumoniae* during 24h at 4°C on a rocker. The membranes were rinsed five times with 20 ml of 0.05%, 0.1 or 0.2% (v/v) PBS-T, as indicated above each blot. Any attached bacteria were fixed with 4% paraformaldehyde, and subsequently incubated with an anti-*Pseudomonas* HRP Rabbit polyclonal antibody (1:1000) overnight at 4°C with gentle rotation on a rocker. The membrane was then washed, and chemiluminescence detection was carried out followed by using the VILBER imager. Denoted numbers to the right of the protein ladder in each picture indicate size in kilodaltons (kDa).

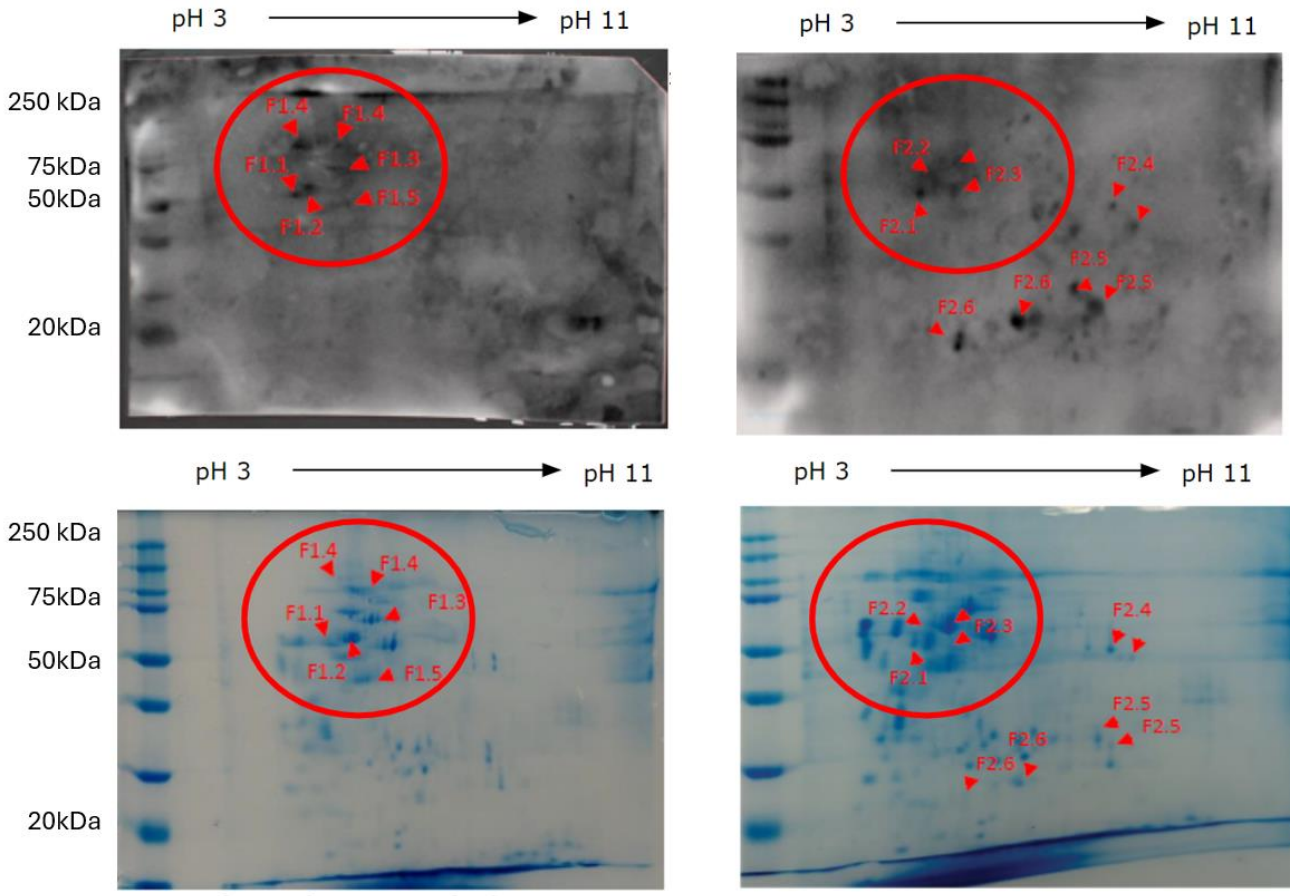

Figure 2B. Comparison of two different 2D blots for the identification of novel human proteins that interact with *P. aeruginosa*. Red circles indicate the most similar spots between gels, and red arrows the matching spots between gels and blots. Denoted numbers to the right of the protein ladder in each picture indicate size in kilodaltons (kDa). Numbers denoted at the top of each picture indicate the isoelectric point. All images are of full-length 12% SDS-PAGE gels or 2D blots.

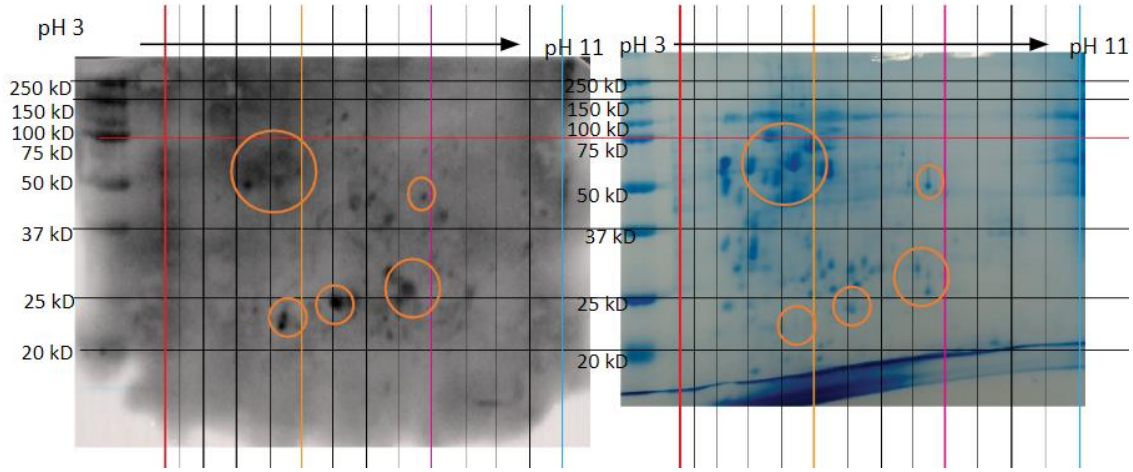

Figure3B. Mapping of spots matching between the PVDF membrane and the gel. The lines indicates the zones with similar molecular weight and pH. The red lines highlights were the pH 3 zone starts and the light blue pH 11. Orange circles highlight the most similar spots between the 2D blot and its parallel Coomassie blue-stained gel.

Table 1B. Proteins with the highest intensity by LC-MS analysis of spots in the 2D blot approach.

Uniprot	T: Protein Names	Gene	Intensity blot 1	Intensity blot 2
P10809	60 kDa heat shock protein, mitochondrial	HSPD1	34.6924	35.5442
A0A7P0TA35	Protein disulfide-isomerase	P4HB	32.7519	33.9849
A0A7I2V2G2	75 kDa glucose-regulated protein	HSPA9	33.7191	32.1009
P30101	Protein disulfide-isomerase A3	PDIA3	27.4955	31.058
Q15084	Protein disulfide-isomerase A6	PDIA6	26.1171	33.2061
P27797	Calreticulin	CALR	29.0051	31.1782
P06576	ATP synthase subunit beta, mitochondrial	ATP5F1B	23.9809	32.8071
P11021	Endoplasmic reticulum chaperone BiP	HSPA5	25.7779	29.4243
Q02818	Nucleobindin-1	NUCB1	24.7738	27.1288
P11142	Heat shock cognate 71 kDa protein	HSPA8	29.6756	24.8603
Q2L696	Nucb2 splice variant	NUCB2	23.6021	30.1873
Q8NBS9	Thioredoxin domain-containing protein 5	TXNDC5	23.764	30.7653
P17066	Heat shock 70 kDa protein 6	HSPA6	27.1102	25.8275
P14625	Endoplasmin	HSP90B1	23.5229	27.5619
H7BZJ3	Protein disulfide-isomerase A3 (Fragment)	PDIA3	22.8856	28.7023
P0DMV9	Heat shock 70 kDa protein 1B	HSPA1B	28.9088	24.6336
P50502	Hsc70-interacting protein	ST13	23.0296	29.9127
Q9BS26	Endoplasmic reticulum resident protein 44	ERP44	29.49	27.8464
O14745	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1	24.5609	28.8134
P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	26.6667	28.4524
Q09028	Histone-binding protein RBBP4	RBBP4	24.7224	28.6102
A0A7P0TA85	Basigin	BSG	25.5653	27.895
P54727	UV excision repair protein RAD23 homolog B	RAD23B	22.5212	27.8261

Q96I99	Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial	SUCLG2	24.9562	28.7329
A0A140T930	HLA class I histocompatibility antigen, C alpha chain	HLA-C	27.008	27.6214
O75381	Peroxisomal membrane protein PEX14	PEX14	26.4808	27.184
A0A7P0TAE9	Calnexin	CANX	23.0493	23.3899
G8JLB6	Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	20.6605	27.9216
Q9Y266	Nuclear migration protein nudC	NUDC	27.9028	26.3783
Q92485	Acid sphingomyelinase-like phosphodiesterase 3b	SMPDL3B	24.015	26.1346
Q6NZI2	Caveolae-associated protein 1	CAVIN1	23.8983	27.5732
P20645	Cation-dependent mannose-6-phosphate receptor	M6PR	24.1628	26.3331
Q86U42	Polyadenylate-binding protein 2	PABPN1	23.2376	27.18
P02765	Alpha-2-HS-glycoprotein	AHSG	25.5112	23.8117
Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4	22.7499	25.4521
O00592	Podocalyxin	PODXL	21.0321	25.5625
Q9H8Y8	Golgi reassembly-stacking protein 2	GORASP2	24.2069	22.7764
Q9BTT6	Leucine-rich repeat-containing protein 1	LRRC1	22.5617	23.4372
P31947	14-3-3 protein sigma	SFN	26.2506	24.9199
O60664	Perilipin-3	PLIN3	21.8348	26.5576
A0A7I2V428	Nucleolin	NCL	24.0436	23.7408
H3BT57	Protein PML	PML	23.2232	25.2924
A0A0A0MS07	Immunoglobulin heavy constant gamma 1 (Fragment)	IGHG1	23.8584	24.0678
Q12765	Secernin-1	SCRN1	21.8674	25.4256
P19256	Lymphocyte function-associated antigen 3	CD58	23.3411	24.3183
C9J0E4	Cystatin-A	CSTA	23.8386	23.4795
Q08380	Galectin-3-binding protein	LGALS3BP	20.0925	23.0636
P23229	Integrin alpha-6	ITGA6	22.7453	20.9965
S6AU73	HLA class I histocompatibility antigen	HLA-B	22.0979	21.5833
A0A7P0P2U7	Ras GTPase-activating protein-binding protein 1	G3BP1	22.2624	20.7617
P10599	Thioredoxin	TXN	22.5356	21.3227
A0A7P0MNE9	Trypsin-3	PRSS3	22.621	22.5405
H0YCI3	Transcriptional coactivator YAP1 (Fragment)	YAP1	23.0931	22.0548
P54687	Branched-chain-amino-acid aminotransferase, cytosolic	BCAT1	24.1378	22.6206
Q9UJZ1	Stomatin-like protein 2, mitochondrial	STOML2	22.1949	22.8159
I3L159	Heme oxygenase (biliverdin-producing) (Fragment)	HMOX2	21.6312	22.2859

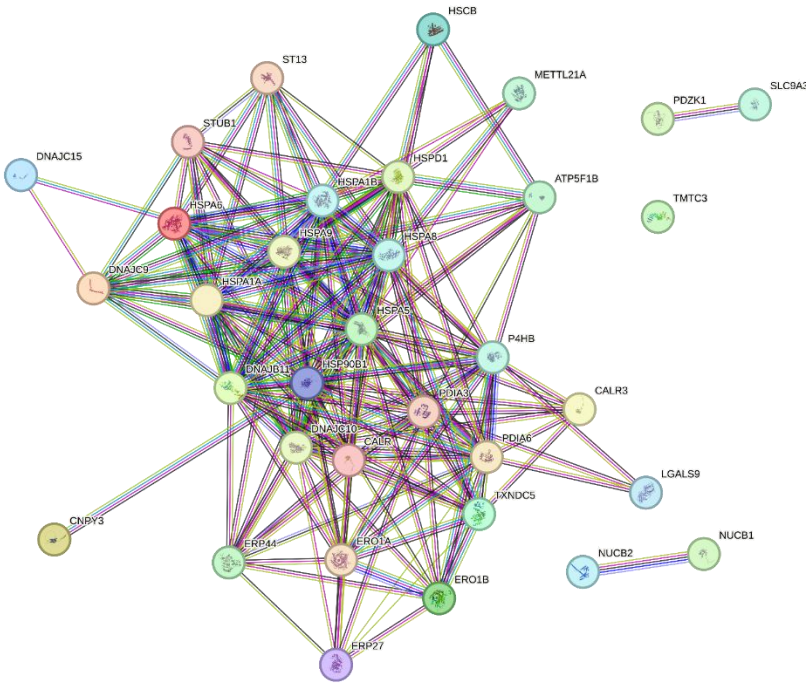

Figure 4B. Interactions between al the proteins with highest intensity in LC-MS analysis of the spots from the 2D blots. The analysis was done using the STRING software.

Table 4B. CFU counts from *P. aeruginosa* attachment to 16HBE14o$^-$ pretreated with different doses of the PDI inhibitor LOC14.

Experiment	DMSO	LOC14 3 µM	LOC14 6 µM	LOC14 20 µM
	2.04E+06	6.65E+05	1.20E+06	
1	1.68E+06	1.44E+06	1.07E+06	
	1.59E+06	6.65E+05	1.52E+06	
2	1.29E+06	1.44E+06	7.65E+05	
	8.13E+05	6.43E+05	4.08E+05	3.88E+05
	4.13E+05	1.10E+06	3.25E+05	5.40E+05
3	1.98E+06	5.78E+05	1.05E+06	4.58E+05
	9.58E+05			6.10E+05
	1.15E+06			4.55E+05
4	1.00E+06			6.33E+05
Average	1.29E+06	9.33E+05	9.05E+05	5.14E+05
(-) control		**3,58E+05**	**3,86E+05**	**7,77E+05**
Fold change		1,38	1,43	**2,51**

Table 5B. CFU counts from *P. aeruginosa* attachment to HEK293T cells transfected with a plasmid containing *pdia1* or *pdia3,* or an empty plasmid.

Experiment	EMPTY	PDIA3	P4HB
1	8,25E+05	1,96E+06	2,13E+06
	3,30E+05	1,78E+06	1,23E+06
	2,42E+05	9,73E+05	2,10E+06
2	1,98E+04	1,36E+06	1,30E+06
	1,08E+04	1,44E+06	1,00E+06
	5,85E+03	1,13E+06	1,60E+06
Average	2,39E+05	1,44E+06	1,56E+06
Change		**1,20E+06**	**1,32E+06**
Fold change		6,02	6,53

Table 6B. CFU counts from *P. aeruginosa* attachment to A549 cells and A549 *pdia3*$^{-/-}$.

Experiment	A549	A549 *pdia3* $^{-/-}$	A549 pdia3 $^{-/-}$ + PDIA3
1	2,64E+06	1,08E+06	1,87E+06
	1,77E+06	1,03E+06	1,51E+06
	1,45E+06	1,08E+06	1,10E+06
2	1,47E+06	1,61E+06	1,51E+06
	9,10E+05	1,16E+06	1,84E+06
	2,32E+06	7,60E+05	1,85E+06
Average	1,76E+06	1,12E+06	1,61E+06
Fold Change		1,57	1,44

Appendix C- Chapter 4

Table 1C. Examples of adjuvants used in *P. aeruginosa* vaccine candidates, the responses achieved against *P. aeruginosa* and their licensing status.

Adjuvant	Class	Mechanism of receptor	IR against PA	Use in humans	Ref
CpG ODN	IMS	TLR9	Ab, Th1, CD8+ T cells	Human HBV vaccine (HEPLISAV-B).	(367, 376-378)
Aluminium salts	B	NALP3, ITAM, Ag delivery	Ab, Th2	Several licensed vaccines (HAB, HBV, DTP, HiB).	(270, 366, 367, 369)
Curdlan	DS	Dectin-1; DC activation directs the differentiation of effector Th17 cells	Ab, Th17	FDA approved (1996) for use as a stabilizer or texturizer in foods. No evidence of any toxicity	(381, 458, 459)
PLGA	DS	Ag delivery	Ab, Th17	Registered safe by the U.S. FDA for clinical use.	(141, 460)
dmLT	B	Stimulates IL-17 secretion and increases the transport of secretory IgA (sIgA)	Ab, Th1, Th17	Phase I and II of ETEC and EVAX vaccines (no adverse events reported).	(140, 384)
AS04	B	TLR4	Ab, Th2	Used as part of an HBV vaccine (Fendrix, GSK) and an HPV vaccine (Cervarix, GSK).	(378)
MF59	DS	Immune cell recruitment	Ab, Th2	Currently licensed as part of a flu vaccine (Fluad™, Seqirus)	(369, 378, 379)
AdC7 vector	DS	Ag delivery to mucosal sites	Ab, Th1	Preclinical studies in mice against *P. aeruginosa*, Ebola virus and malaria in mice models.	(277, 460)
CIA06 (alum+ dLos)	IMS	TLR4 agonist derived	Ab, Th1, Th17, Th2	Preclinical studies in mice on the H1N1 pandemic influenza vaccine Greenflu-S®.	(390)
NLX +alum	IMS	Opioid receptor antagonists	Ab, Th1, Th17, Th2	Approved by the FDA as a prescription drug, used to reverse opioid-induced respiratory depression.	(269)
Live-attenuated (S. Typhimurium, *F. tularensis* LVS)	DS	Ag delivery to mucosal sites	Ab, Th2, Th1 (biased)	Retains toxicity in humans and animals but since 2008, oral live-attenuated Ty21a (*S.* Typhi) vaccines have been recommended for typhoid control.	(461, 462)

Abbreviations; Ab: Antibody, Ag: Antigen, B: Both, DS: Delivery system, IMS: Immunostimulatory, IR: immune response, PA: *Pseudomonas aeruginosa*

Table 2C. LPS quantification in rOprF sample

EU/mL	Absorbance		Average	Without blank			
	1	2					
1	1.055	1.028	1.042	0.698			
0.5	0.655	0.877	0.766	0.4225			
0.25	0.519	0.511	0.515	0.1715			
0.1	0.434	0.428	0.431	0.0875			
0	0.336	0.351	0.3435	0	**EU/mL**	***dil factor**	**EU/dose**
OprF 1:5	1.395	1.52	14.575	1.114	0.795	3,977	**0,20**

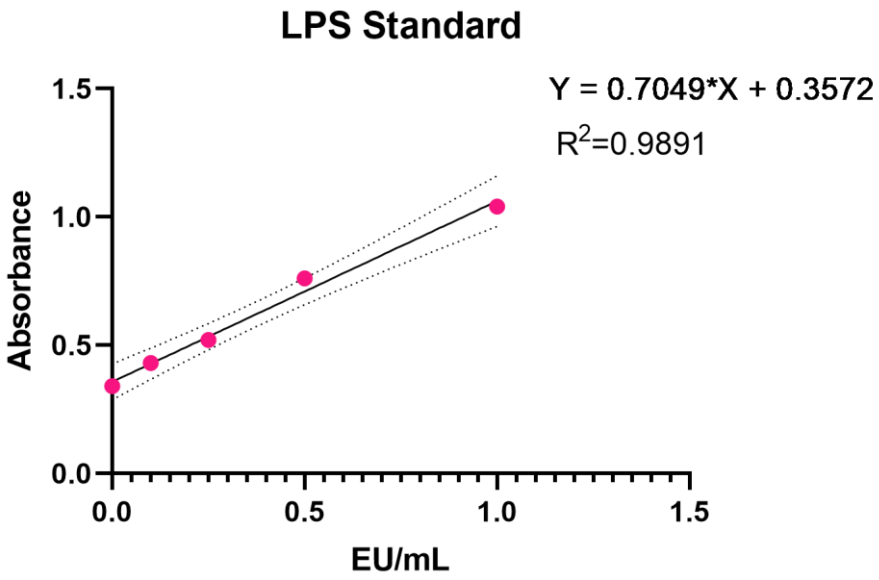

Figure 1C. Standard curve for LPS quantification in rOprF sample using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher).

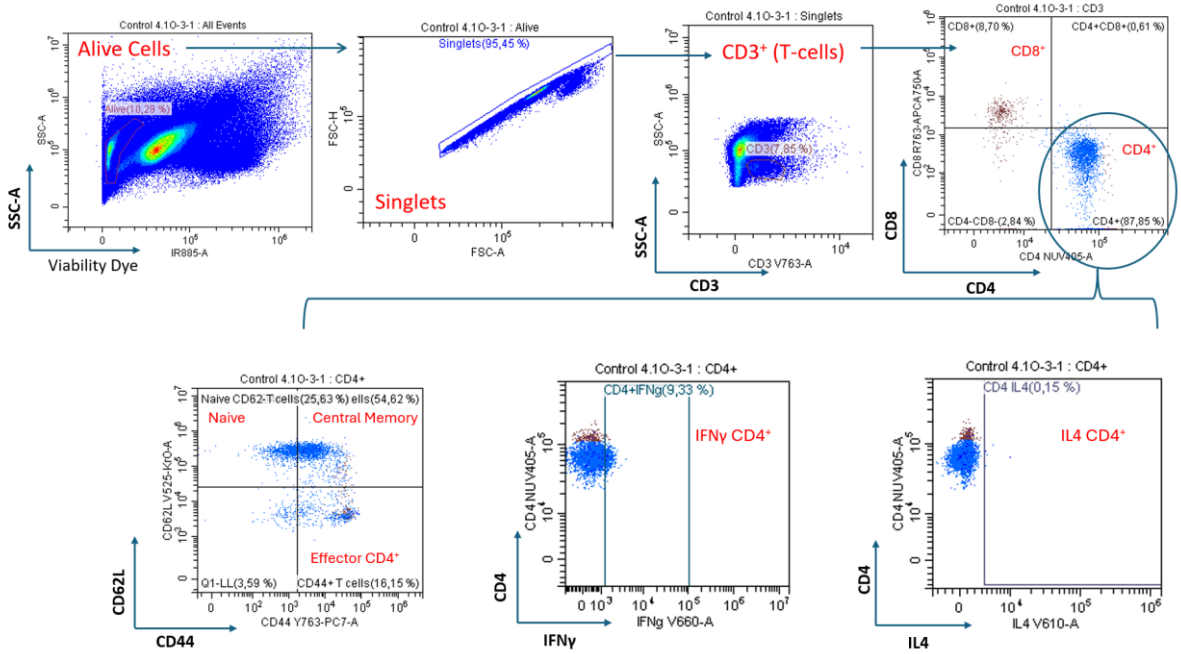

Figure 2C. Gating strategy of the flow cytometry for the analysis of cellular responses from restimulated splenocytes.

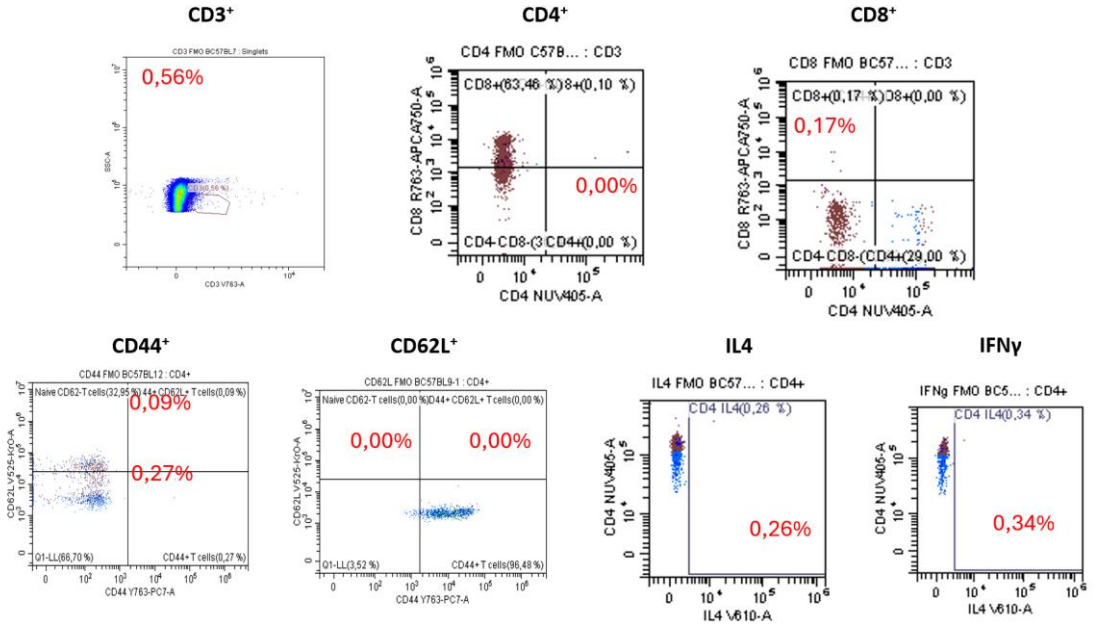

Figure 3C. Fluorescence Minus One (FMO) control for each of the markers used in the flow cytometry analysis.

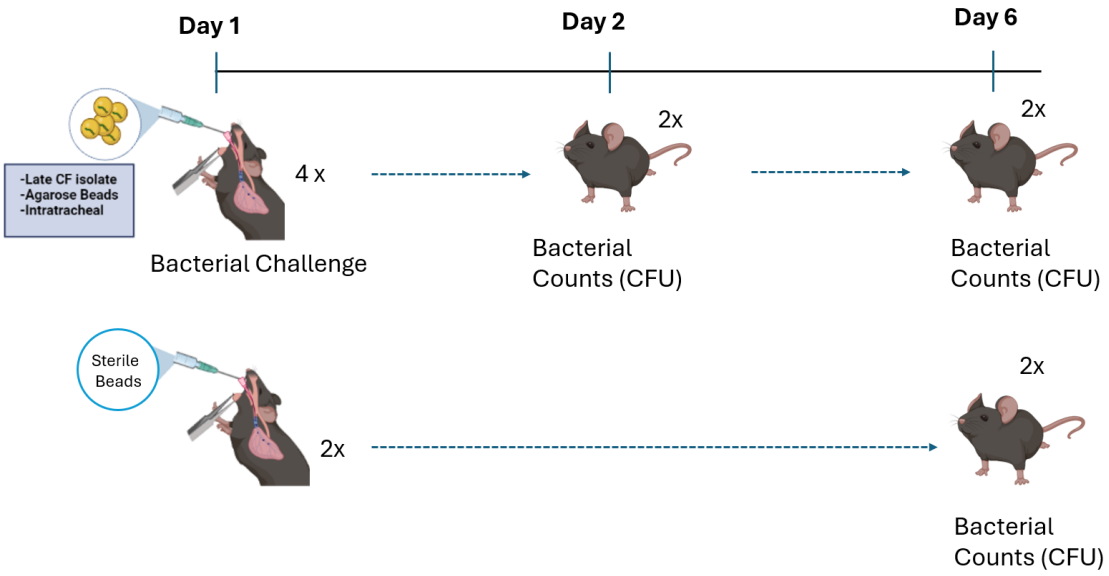

Figure 4C. Timeline for a pilot study of a long-term *P. aeruginosa* AA43 infection model in C57BL/6J mice.

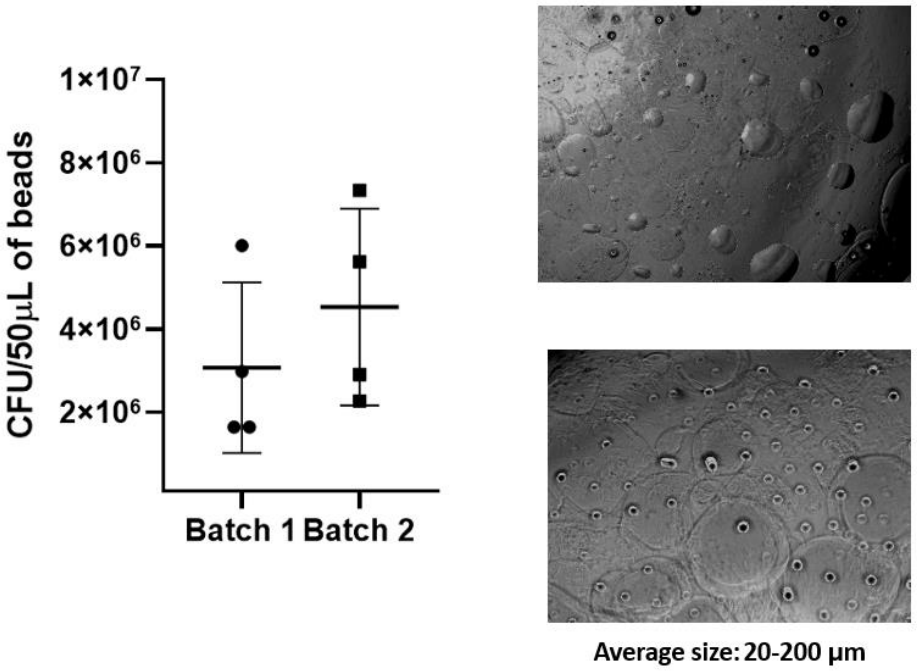

Figure 5C. Optimisation of *P. aeruginosa*- embedded into 2% agarose beads AA43. The average of *P. aeruginosa* CFU in the beads was almost always around $4x10^6$. The average size of the beads was between 20-200 µm.

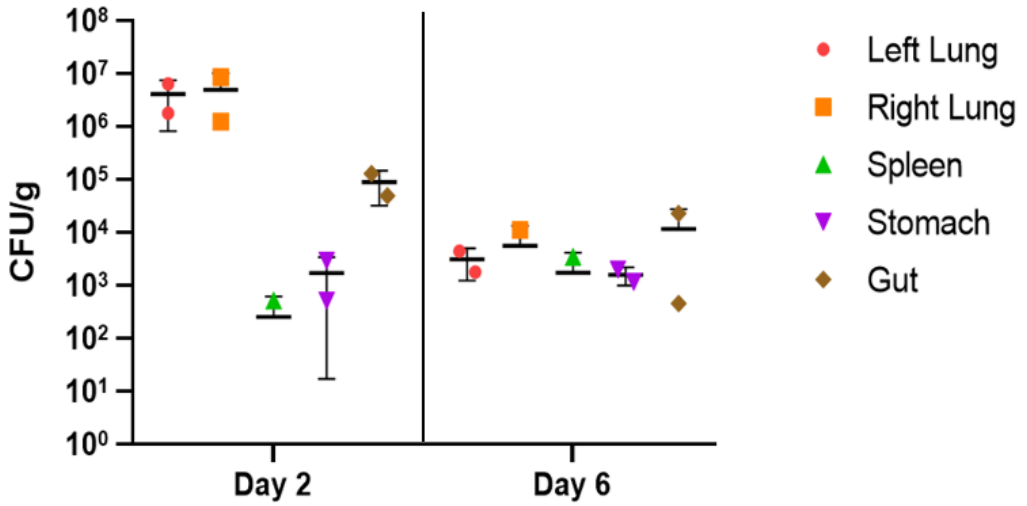

Figure 6C. *P. aeruginosa* AA43 colonisation of different mice organs (CFU/g) in an agarose-beads model of *P. aeruginosa* infection in C57BL/6J mice. The control (50 µL of sterile 2.1% agarose beads) is not shown in the graph as the two mice were free of *P. aeruginosa* in all the studied organs. Two mice were humanely killed after two days of infection and two mice 6 days after a challenge with 50µL of 2.1% agarose beads containing ~$5x10^6$ *P. aeruginosa* AA43 CFU. Each mouse is represented by a single dot in the graph.